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REVIEW

Deubiquitinating enzyme regulation of the p53 pathway: A lesson from Otub1

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Abstract

Deubiquitination has emerged as an important mechanism of p53 regulation. A number of deubiquitinating enzymes (DUBs) from the ubiquitin-specific protease family have been shown to regulate the p53-MDM2-MDMX networks. We recently reported that Otub1, a DUB from the OTU-domain containing protease family, is a novel p53 regulator. Interestingly, Otub1 abrogates p53 ubiquitination and stabilizes and activates p53 in cells independently of its deubiquitinating enzyme activity. Instead, it does so by inhibiting the MDM2 cognate ubiquitin-conjugating enzyme (E2) UbcH5. Otub1 also regulates other biological signaling through this non-canonical mechanism, suppression of E2, including the inhibition of DNA-damage-induced chromatin ubiquitination. Thus, Otub1 evolves as a unique DUB that mainly suppresses E2 to regulate substrates. Here we review the current progress made towards the understanding of the complex regulation of the p53 tumor suppressor pathway by DUBs, the biological function of Otub1 including its positive regulation of p53, and the mechanistic insights into how Otub1 suppresses E2.

Key words: p53; MDM2; Ubiquitination; Deubiquitinating enzymes; Otub1; Cell cycle; Apoptosis

Core tip: p53 is tightly regulated by dynamic ubiquitination and deubiquitination. A number of deubiquitinating enzymes (DUBs) have been shown to regulate p53 stability and activity by either directly deubiquitinating p53 or indirectly deubiquitinating its regulators. We recently discovered that Otub1, an OTU family DUB, stabilizes and activates p53 *via* distinct and non-canonical mechanism wherein it suppresses the MDM2 cognate ubiquitin-conjugating enzymes UbcH5. Here we review the current progress made towards the understanding of the Otub1 functions as a potent E2 inhibitor and the underlying mechanisms.

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MDM2 AND MDMX: KEEPING P53 UNDER CONTROL

The p53 tumor suppressor plays a central role in maintaining the genomic stability and preventing the organism from cancer[1-3]. Loss of p53 function, either through direct mutations in the *p53* gene or indirectly through alterations in the p53 regulatory networks, is associated with most, if not all, human cancers[4,5]. Germline mutations of *p53* result in the cancer-prone Li-Fraumeni

syndrome in human[6] and deletion of the *p53* gene leads to spontaneous tumors in mice[7,8]. p53 is a stress-induced transcription factor that activates or represses the expression of many target genes, thereby executing its anti-proliferative activity by inducing cell cycle arrest, apoptosis, or senescence[1,2,9-11]. Under normal circumstances, p53 is tightly controlled at low levels mainly by its negative regulator MDM2[12-14]. As a RING-finger-containing ubiquitin ligase (E3)[15,16] MDM2 mediates p53 ubiquitination and degradation through the proteasomal system[17,18]. MDM2 also directly suppresses p53 transactivation activity by binding and concealing the N-terminal transactivation domain of p53[19-21]. The centrality of the MDM2-mediated p53 suppression has been demonstrated by mouse genetic studies showing that deletion of the *mdm2* gene caused embryonic lethal phenotype, which is completely rescued by concomitant deletion of *p53*[22,23]. This essential function of MDM2 requires its E3 activity, as mice with homozygous knock-in of the E3 inactivation mutant, $MDM2^{C464A}$, are also embryonic lethal, which can be rescued by deleting p53 as well[24]. Consistently, MDM2 is overexpressed in a number of human cancers, most of which contain wild-type p53[25-29].

The MDM2 homolog MDMX has emerged as an equally important p53 regulator as MDM2[30]. MDMX shares high homology with MDM2 in their C-terminal RING-finger domain and the N-terminal p53-binding domain. Like MDM2, MDMX binds to the N-terminal transactivation domain of p53 and suppresses its activity. However, MDMX does not have appreciable ubiquitin ligase activity towards p53[31,32], yet it assists MDM2 to suppress p53 function. MDMX directly binds to MDM2 *via* their RING domains[33-35] and renders MDM2 sufficiently stable to ubiquitinate and degrade p53[33,36-38]. Also, MDMX suppresses p53 function by specifically promoting p53-induced MDM2 transcription following DNA damage[39]. MDM2, in turn, ubiquitinates and degrades MDMX in response to DNA damage[40-42]. Thus, the mutual regulation between MDM2 and MDMX ensures a proper cellular level and activity of p53. Supporting the indispensible role of MDMX towards p53, deleting the *p53* gene also rescues the lethal phenotype of knocking out the *mdmx* gene in mice[43-45]. Like MDM2, MDMX is also overexpressed or amplified in several types of human cancers that harbor wild-type p53[46-49]. Recent studies have provided further molecular insights into the non-redundant and indispensible role for MDMX in MDM2-mediated p53 degradation. First, like MDM2, the RING domain of MDMX and resulting MDM2-MDMX heterodimerization are required for the regulation of MDM2, as deletion of the RING-finger domain of MDMX or knock-in of the MDM2-binding defective MDMX mutant (C462A) resulted in embryonic lethal phenotype, which was completely rescued by deletion of *p53*[50,51]. Second, The extreme C-terminal short sequences outside of the RING domain of both MDM2 and MDMX contribute to the MDM2 E3 activity, owing to their role in the formation of MDM2-MDMX heterodimer and perhaps the E3 holoenzyme mediating p53 polyubiquitination[37,38,52]. Third, a recent *in vitro* study has shown that while MDM2 alone is sufficient to mediate multi-monoubiquitination of p53, the MDM2-MDMX complex is required for p53 polyubiquitination[53]. Thus, the stoichiometry of the p53-MDM2-MDMX complex is critical for the determination of whether targeting p53 for polyubiquitination or monoubiquitination.

The p53-MDM2-MDMX axis is among the most highly regulated pathways. Enormous molecules regulate the interplay among the three proteins in response to diverse stressors, leading to p53 stabilization and consequent activation. These include various post-translational modifications of all three proteins. Ubiquitination plays a key role in controlling the protein stability and activity of all three proteins. Under stress conditions, p53 ubiquitination mediated by MDM2/MDMX is crippled as a result of either dissociation of MDM2/MDMX from p53 or suppression of MDM2/MDMX activity towards p53. For example, DNA damage-mediated phosphorylation of both p53 and MDM2 disrupts their interaction, resulting in p53 stabilization[54-57]. DNA damage also triggers phosphorylation and degradation of MDMX, alleviating its suppressive effect on p53[58-63]. Oncogenic stress induces p53 *via* suppression of MDM2 by ARF[64-68], whereas ribosomal stress induces p53 *via* suppression of MDM2 by a number of ribosomal proteins[69-85]. Again, ARF also promotes MDM2-mediated MDMX degradation[40] and ribosomal stress-induced p53 activation requires MDM2-mediated MDMX degradation[86]. Thus, barricading the inhibition of p53 imposed by MDM2 and MDMX is centrally important for p53 activation in response to most, if not all, stressors. Indeed, both MDM2 and MDMX bind to p53 at its target gene promoters and suppress its transactivation activity[87-89]. Thus, p53 activation is thought to involve the release of such repression, called anti-repression under stress conditions, through diverse posttranslational modifications[90]. In addition, p53 is also ubiquitinated by a number of other ubiquitin ligases such as ARF-BP1[91], PIRH2[92], COP1[93], *etc.*[94,95]. For example, p53, under certain cellular levels, is thought no longer regulated by the MDM2/MDMX complex. Instead, the basal level of p53 is mainly regulated by ARF-BP1. Deletion of ARF-BP1 completely activates p53 in the presence of MDM2[91]. Adding to the complexity of the ubiquitination regulation of the p53 pathway, deubiquitination regulation has recently emerged as an equally important mechanism for p53 control.

REGULATION OF THE P53-MDM2-MDMX PATHWAY BY DEUBIQUITINATING ENZYMES

Like other posttranslational modifications, ubiquitination of p53, MDM2 and MDMX can be reversed through a process called deubiquitination, which is catalyzed by a different class of enzymes called deubiquitinating enzymes (DUBs). The human genome encodes approximately 95 predicted DUBs that are classified into

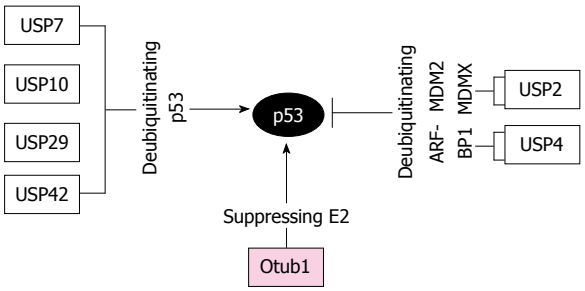

Figure 1 Diagram of the regulation of the p53 pathway by deubiquitinating enzymes. Arrows indicate activation and bars indicate inhibition. USP7, USP10, USP29, and USP42 deubiquitinate and activate p53, whereas USP2 destabilizes p53 by deubiquitinating MDM2 and MDMX and USP4 destabilizes p53 by deubiquitinating and stabilizing ARF-BP1. Otub1 stabilizes and activates p53 *via* non-canonical suppression of the MDM2 cognate E2 UbcH5, thereby inhibiting MDM2-mediated p53 ubiquitination and degradation. USP: Ubiquitin-specific protease

5 families: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor associated proteases (OTUs), Machado-Joseph disease (or Josephin domain) proteins (MJDs), and JAB1/MPN/MOV34 proteins (JAMMs). Except that the JAMMs are zinc metalloproteases, all other DUBs are cysteine proteases[96,97].

Recently, several DUBs from the USP family have been shown to regulate the p53-MDM2-MDMX loop (Figure 1). USP7, also called herpesvirus associated USP (HAUSP), is the first DUB reported to be a bona fide p53 deubiquitinase[98-100]. Overexpression of USP7 stabilizes and activates p53[99]. Intriguingly, MDM2 seems to be a better substrate of USP7 compared to p53 under physiological circumstances, as substantial knockdown of USP7 results in destabilization of MDM2 and activation of p53[98,101]. Further, USP7 also deubiquitinates MDMX in cells and *in vitro* and depletion of USP7 results in destabilization of the otherwise stable MDMX[100]. DNA damage triggers ATM-dependent phosphorylation of MDMX, which disrupts its binding to USP7 and leads to the consequent increase of ubiquitination and degradation of MDMX[100], whereas the interaction between p53 and USP7 is increased following DNA damage. Thus USP7 scrutinizes the homeostatic levels of p53, MDM2, and MDMX under both normal and stress conditions. The second p53 DUB, USP10, has also been shown to play a critical role in p53 activation following DNA damage[102]. Unlike USP7, USP10 is a cytoplasmic DUB and specifically deubiquitinates p53, but not MDM2 and MDMX[102], reversing MDM2-mediated ubiquitination, nuclear export, and cytoplasmic degradation of p53. Following DNA damage, ATM phosphorylates USP10 at Thr42 and Ser337, resulting in not only the stabilization of USP10, but also the translocation of a fraction of USP10 into the nucleus to deubiquitinate and activate p53. Consistent with its function in regulating p53, USP10 expression is down-regulated in high percentage of clear cell carcinomas[102]. Recently, USP42 was reported to be another DUB that positively regulates p53 stability and activity. Interestingly, USP42 deubiquitinates p53 only during the early stages of stress response, without significant effect on p53 regulation under unstressed conditions. Despite of this, it has been shown that USP42 is required for rapid p53 activation and cell cycle arrest in response to mild or transient DNA damage stress[103]. In addition, Liu *et al*[104] has shown that USP29 positively regulates p53 stability and function following oxidative stress. This is achieved by the increased transcription of USP29 induced by oxidative stress, which in turn cleaves polyubiquitinated p53, leading to p53-dependent apoptosis in cells.

In contrast to above USPs positively regulating p53, USP2a and USP4 were reported to destabilize p53 and suppress p53 function, albeit *via* targeting different p53 E3s. USP2a destabilizes p53 by deubiquitinating and stabilizing both MDM2[105] and MDMX[106], whereas USP4 destabilizes p53 by deubiquitinating and stabilizing ARF-BP1[107]. Consistently, USP2a is overexpressed in a subset of prostate cancers[108,109], whereas USP4 is overexpressed in a broad range of human cancers[107]. Thus, USP2a and USP4 are likely oncogenic DUBs.

Together, these studies demonstrate that deubiquitination plays a crucial role in finely tuning the normal homeostasis of the p53-MDM2-MDMX loop as well as its response to stress. They also imply that different DUBs could regulate the p53 pathway *via* different mechanisms within different cellular compartments following different stress. However, whether p53 is regulated by DUBs other than USP family members is previously unknown. We recently identified that the OTU domain-containing ubiquitin aldehyde-binding proteins 1 (Otubain 1, Otub1 thereafter), an OTU family DUB, controls p53 stability and activity *via* a novel non-canonical mechanism[110].

OTUB1: A UNIQUE MEMBER OF OTU DUB FAMILY

Otub1 was identified along with its close homolog Otub2 by affinity purification using the DUB-specific inhibitor, Ub aldehyde[111]. Subsequent studies, including our own, revealed that Otub1 possesses *in vitro* deubiquitinating enzyme activity preferentially towards K48-linked polyubiquitin chains[110,112,113]. Like other cysteine proteases, Otub1 contains a catalytic triad consisting of Cys (C) 91, His (H) 265, and Asp (D) 268[112]. However, crystal structure studies demonstrated that Otub1 possesses unique structure features wherein H265 is located distantly from the catalytic C91 and D268 and the access of C91 to ubiquitin is blocked by Glu (E) 214 residue, forming a conformation incompatible with catalysis by typical cysteine proteases[112], implying that the activity of Otub1 may be highly regulated in cells and its activation may be subjected to conformational change (See below). Otub1 is ubiquitously expressed in tested human tissues. A longer isoform called Otub1 ARF (alternative reading frame)-1, resulting from alternative splicing and start codon, is predominantly expressed in peripheral blood mononuclear cells, lymph nodes, spleen, and the tonsils[114]. The function of Otub1 ARF-1 is thought to antagonize the function of Otub1 in cells[114].

Functionally, Otub1 has been implicated in the regula-

tion of immune response, estrogen signaling, DNA damage response, as well as pathogen biology. Soares *et al*[114] first reported that Otub1 regulates $CD4^+$ T cell clonal anergy by enhancing degradation of the ubiquitin ligase called GRAIL (gene related to anergy in lymphocytes) and promoting interleukin 2 production following antigenic stimulation, whereas the Otub1 ARF-1 has an opposite effect. Interestingly, the effect of Otub1 does not depend on its catalytic activity. As a matter of fact, the role of Otub1 in degrading GRAIL is opposite to its predicted role as a DUB[114]. A possible explanation is that Otub1 forms a ternary complex with GRAIL and USP8, another USP family DUB, thereby suppressing the deubiquitination of GRAIL by USP8. In this case, Otub1 may act as an ubiquitin editing protease[114]. Li *et al*[115] reported that Otub1 (and Otub2) mediate virus-induced deubiquitination of TNF receptor-associated factor 3 (TRAF3) and TRAF6, two ubiquitin ligases required for virus-induced Interferon regulatory factor 3 (IRF3) and NF-kB activation, leading to the inhibition of viral-induced production of INFβ. However, whether this effect requires the DUB enzymatic activity of Otub1 is not clear[115]. Further, Otub1 has recently been shown to enhance TGFβ signaling by inhibiting ubiquitination and degradation of SMAD2/3[116]. Otub1 also plays a role in pathogen invasion of the host cells. The *Yersinia*-encoded virulence factor YpkA interacts with and phosphorylates Otub1[117] and recruits the small GTPase RhoA, leading to the stabilization of the active RhoA[118]. Consequently, overexpression of wild-type, but not the C91S mutant, Otub1 increased the susceptibility of host cells to the *Yersinia* evasion[118]. Otub1 has been shown to deubiquitinate and stabilize ERα in chromatin[119], albeit this stabilization results in the inhibition of ERα-mediated transcription. Adding to the complexity, the catalytic mutant Otub1, C91S in which the catalytic C91 is mutated to S, did not abolish Otub1-mediated suppression of ERα activity[119]. Otub1 has been shown to inhibit DNA-damage-induced chromatin ubiquitination, which is also independent of its DUB activity. Instead, Otub1 suppresses RNF168-dependent chromatin polyubiquitination by binding to and inhibiting the RNF168 cognate E2 enzyme UBC13[120]. Recently, Otub1 has been shown to regulate apoptosis by deubiquitinating the cellular inhibitor of apoptosis (c-IAP1)[121].

Together, Otub1 has been implicated in multiple biological processes. In most cases, the effects of Otub1 do not require its DUB activity, such as the regulation of DNA damage-induced chromatin ubiquitination[120], T-cell anergy[114], ERα[119], and SMAD2/3[116], implying a unique model of ubiquitination regulation by a DUB: suppression of the ubiquitin-conjugating enzyme (E2) (see below). Because of this and the fact that it is expressed in most tissues, Otub1 may have a broad function in cells.

OTUB1 IS A NOVEL POSITIVE P53 REGULATOR

We recently found that Otub1 positively regulates the stability and activity of p53[110]. Overexpression of Otub1, but not its close homolog Otub2, markedly stabilizes and activates p53 and induces p53-dependent apoptosis and cell growth inhibition. Interestingly, Otub1 regulation of p53 does not require its catalytic activity, as mutating C91 to either A or S did not abolish the activity of Otub1 to block MDM2-mediated p53 ubiquitination and degradation, to stabilize and activate p53, and to induce p53-dependent cell growth inhibition[110]. Mechanistically, Otub1 suppresses MDM2-mediated p53 ubiquitination by binding to and inhibiting the MDM2 cognate E2 enzyme UbcH5s[110]. This is consistent with the non-canonical role for Otub1 in suppressing DNA damage-induced chromatin ubiquitination by inhibiting UBC13[120]. Therefore, our study further supports that the suppression of substrate ubiquitination through inhibiting cognate E2s by Otub1 represents a unique noncanonical mode of DUB regulation compared to classical cysteine proteases and this may be a general mechanism for Otub1 to regulate the substrate protein ubiquitination and stability.

Consistent with the noncanonical mode of regulation, mutating C91 to either A or S did not abolish the activity of Otub1 to bind to and suppress UbcH5[110]. However, a point mutation of Asp 88 to Ala ($Otub1^{D88A}$) abolished the function of Otub1 to suppress p53 ubiquitination and degradation and this mutant interacts with p53 stronger than wild-type Otub1, indicating this mutation might create a dominant-negative effect. D88 is located closely to the donor ubiquitin-binding surface and thus its mutation would affect the binding of Otub1 to donor ubiquitin conjugated to UbcH5. Although D88 is not located directly in the E2 binding surface, our experimental data revealed that this mutation clearly disrupted the Otub1-E2 interaction in cells[110]. This might be due to the overall structure change after D88 mutation. Supporting this conformational change is that D88A mutant also results in the loss of Otub1's DUB activity.

Our functional studies of the endogenous Otub1 suggest that Otub1 plays an important role in p53 stabilization and activation following DNA damage induced by diverse agents. This is consistent, but not completely, with the observation that Otub1 suppresses DNA damage-induced chromatin ubiquitination, thereby suppressing DNA repair pathway[120]. One explanation is that upon DNA damage, Otub1 might target UbcH5-MDM2 to stabilize p53, while it may dissociate from the RNF168-Ubc13 complex, allowing RNF168 to catalyze K63-linked chromatin ubiquitination and subsequent DNA repair response. Whether DNA damage-induced posttranslational modification plays a role in this functional switch remains unclear. However, phosphorylation of Otub1 has been observed at several residues such as T134. Further, it has been shown that the phosphorylation mimicking Otub1 mutant T134E, but not T134A, failed to rescue the DNA damage response in Otub1-depleted cells[122]. Thus it is interesting to examine the signaling pathways involved in the phosphorylation of Otub1 and how this phosphorylation plays a role in regulating Otub1 function in

response to DNA damage stress.

MECHANISTIC INSIGHTS INTO THE NON-CANONICAL SUPPRESSION OF E2 BY OTUB1

Recent biochemical and structural studies have shed a light on how Otub1 suppresses E2s[122-124]. It has been shown that Otub1 preferentially binds to ubiquitin-charged E2[120,122]. Otub1 contains two ubiquitin-binding motifs: a distal site that binds to free ubiquitin and a proximal site that binds to donor ubiquitin conjugated to the active site of an E2 (*e.g.,* Ubc13 or UbcH5). The structure of two ubiquitin binding to Otub1 is reminiscent of that of K48-linked di-ubiquitin[122]. Interestingly, the binding of a free ubiquitin to the distal site allosterically causes the conformational change of Otub1, allowing the formation of a N-terminal ubiquitin-binding helix where the E2-charged donor ubiquitin then binds[122,124]. Consequently, this binding limits the donor ubiquitin interaction with the backside of another E2 and the attack on the thioester bond by an acceptor ubiquitin, a step important for ubiquitin transfer[122,124]. On the other hand, Otub1 also makes contacts with E2 and the Otub1-binding surface in E2 (UbcH5 and Ubc13) overlaps with the E3-binding surface. Thus this Otub1-E2 interaction may also attenuate the E2-E3 engagement[122,124]. Collectively, Otub1 is a potential inhibitor of the E2 enzymes. Further supporting this notion, Otub1 has recently been shown to be a major DUB that interacts with the D and E classes of E2 as well as UbcE2N[125]. Thus disruption of the Otub1-E2 interaction or donor ubiquitin-Otub1 interaction would theoretically abolish Otub1's activity to suppress E2. This could distinguish Otub1's E2 suppressing activity from its DUB enzyme activity. Indeed, several mutants involved in the E2-contacting surface of the Otub1, such as F133A, T134R, F138A, have been shown to lack the E2-suppressing activity but retain the DUB activity[122,124]. Therefore, it is interesting to examine whether these mutants could fail to stabilize and activate p53 in cells. On another note, we recently found that Otub1 can be monoubiquitinated by UbcH5 and this monoubiquitination in turn plays a critical role in the Otub1's E2 suppressing activity. We further found that UbcH5 preferentially binds to monoubiquitinated Otub1, through the ubiquitin interaction with the backside ubiquitin-interacting surface of E2[126]. This binding could potentially disrupt the formation of self-assembled ubiquitin-charged UbcH5 (UbcH5-Ub) conjugates that is critical for ubiquitin transfer, polyubiquitin chain formation and efficient polyubiquitination of substrates[127,128], suggesting another novel mechanism of Otub1 suppression of E2.

CONCLUSION

Recent studies have convincingly demonstrated Otub1 as a unique DUB that executes diverse biology functions by non-canonically suppressing E2 enzymes. Therefore it is expected that Otub1 may play broad functions in cells. One question would be how these broad functions coordinate with each other in cells. We also do not know how Otub1's activity is regulated in cells. Interestingly, a recent observation showed that Otub1 DUB activity can be regulated by UbcH5, which stimulates the binding of the Lys48-linked polyubiquitin substrate by stabilizing the folding of the N-terminal ubiquitin-binding helix of Otub1, thereby promoting its deubiquitinating enzyme activity[129]. It is interesting to know how these mutually regulatory functions are controlled in cells. It is also important to test how Otub1's activity and levels are regulated in cells under physiologic and stress conditions. As Otub1 is a potent activator of p53[110] and plays a role in DNA damage repair[120], Otub1 may act as a tumor suppressor. Thus it is important to determine whether Otub1 is deregulated in human cancers. Gene targeting in mice could provide further information regarding the function of Otub1 and whether Otub1 indeed possesses tumor suppression function *in vivo*. Further characterization of mechanistic insights into the Otub1 suppression of E2 could also be useful for developing strategies that target the E2 enzymes for cancer therapy, *e.g.,* small molecule compounds that resemble Otub1 interaction with E2.

Together, p53 is ubiquitinated by MDM2/MDMX and several other E3s whereas it is deubiquitinated by a number of DUBs, including USP7, USP10, USP29 and USP42. One obvious question is how these multiple DUBs are coordinated to ensure the tight, precise, and dynamic control of p53 stability and activity. Different DUBs may regulate the p53 pathway in response to different cellular stress (*e.g.,* USP29 deubiquitinates p53 in response to oxidative stress[104] whereas USP10 deubiquitinates p53 following DNA damage[102]). Different DUBs may also regulate p53 in different cellular compartments (*e.g.,* USP7 regulates p53 in the nucleus whereas Otub1 regulates p53 in the cytoplasm[110] and USP10 relocates from the cytoplasm to the nucleus to regulate p53 in response to DNA damage[102]). It is interesting to examine whether different DUBs may cooperate with each other to synergistically regulate p53 stability and activity in future studies.

Nevertheless, efforts have been made towards targeting the ubiquitin-proteasome system (UPS) for reactivating p53 in cancer therapy. For example, compounds have been developed to target the p53-MDM2 interaction such as Nutlin-3s[130], the p53-MDMX interaction such as WK298[131], or both such as RO-2443[132]. Targeting DUBs has promising potential as well. For example, the cyano-indenopyrazine derivatives small molecule compounds HBX 41108, HBX 19818, and HBX 28258[133] and P22077[134] were discovered as USP7 inhibitors. For further details about targeting the UPS for cancer therapy, please refer our recent review[135]. Future directions will aim to discover more potent and specific DUB inhibitors that can be used for cancer treatment.

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REVIEW

Oxidation of KCNB1 K^+ channels in central nervous system and beyond

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Abstract

KCNB1, a voltage-gated potassium (K^+) channel that conducts a major delayed rectifier current in the brain, pancreas and cardiovascular system is a key player in apoptotic programs associated with oxidative stress. As a result, this protein represents a *bona fide* drug target for limiting the toxic effects of oxygen radicals. Until recently the consensus view was that reactive oxygen species trigger a pro-apoptotic surge in KCNB1 current *via* phosphorylation and SNARE-dependent incorporation of KCNB1 channels into the plasma membrane. However, new evidence shows that KCNB1 can be modified by oxidants and that oxidized KCNB1 channels can directly activate pro-apoptotic signaling pathways. Hence, a more articulated picture of the pro-apoptotic role of KCNB1 is emerging in which the protein induces cell's death through distinct molecular mechanisms and activation of multiple pathways. In this review article we discuss the diverse functional, toxic and protective roles that KCNB1 channels play in the major organs where they are expressed.

Key words: Apoptosis; Kv2.1; Aging; Reactive oxygen species; Alzheimer's disease

Core tip: KCNB1 is a K^+ channel that plays a key role in the brain, pancreas and cardiovascular system. KCNB1 is unique in that it induces apoptosis in association with oxidative stress. In this review article we discuss the diverse roles of this channel in the organs where it is expressed including recent advances in the molecular mechanisms through which KCNB1 causes cytotoxicity.

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KCNB1 IS A PROMINENT MEMBER OF THE SHAB-RELATED FAMILY OF VOLTAGE-GATED K^+ CHANNELS

KCNB1 (HUGO nomenclature), formerly DRK1 and Kv2.1, is a *Shab* delayed rectifier voltage-gated K^+ channel which was cloned by Frech *et al*[1] using size-fractionated mRNA extracted from rat brain. KCNB1 is expressed in the central nervous system, pancreas, pulmonary arteries, heart, auditory outer hair cells, stem cells and retina[2-21]. As in other voltage-gated K^+ channels, KCNB1 spatial and temporal expressions are both developmentally regulated. For example, three distinct (4.4, 9.0, 11.5 kb) mRNA transcripts are expressed in the rat brain, with the 4.4 kb transcript being predominant in embryos and the 11.5 kb transcript being predominant in adults[15]. Accordingly, multiple KCNB1 isoforms are detected which differ in

their developmental expression. Functional KCNB1 channels can result from the assembly of four identical pore-forming subunits along a symmetry axis[22]. However, this simple stoichiometry is not likely to be observed in nature. In order to serve the specific requisites of the tissues in which the channel is expressed, heterogeneity of KCNB1 current can be achieved by formation of heterometric complexes containing non-conducting, pore-forming subunits of the KCNG and KCNS families as well as by assembly with accessory subunits of the KCNAB and KCNE families[16,17,23-28]. KCNB1 exhibits an unusual large number of consensus sites for phosphorylation. Accordingly, the channel is a substrate for protein kinases of different families and is constitutively phosphorylated in native cells[29-32]. KCNB1 can also be SUMOylated and acetylated in nervous system and pancreas even though the physiological role of these regulations awaits elucidation[33-35]. Finally, mature KCNB1 channels are not glycosylated despite the presence of consensus sites in the N-terminus[36].

Because of the potential therapeutic implications, the pharmacology of KCNB1 to a variety of toxins and drugs has been extensively investigated. Thus, KCNB1 is blocked by tarantula toxins that belong to the same structural family of inhibitor cystine knot spider peptides reticulated by disulfide bridges. Hanatoxin from *G. spatulata*, was the first toxin to be shown to interact with KCNB1, followed in more recent years by heteroscordratoxin and stromatoxin 1 from *H. maculata* and *S. calceata* and jingzhaotoxin (JZTX- Ⅰ, -Ⅲ, and -Ⅴ) and guangxitoxin (GxTx-1E), isolated from the venoms of the Chinese tarantulas *C. jingzhao* and *P. guangxiensis*[37-40]. All these structurally related toxins exhibit variable affinities for the channel in the nanomolar to micromolar range and act to alter its gating by interacting with the voltage sensor[41,42]. KCNB1 is susceptible to inhibition by a number of compounds of different classes including classic K^+ channel blockers tetraethylammonium (TEA) and 4-aminopyridine and antipsychotic, anesthetic and antiarrhythmic compounds[43-53]. Of particular relevance to the topic of this review is the fact that acetylcholinesterase inhibitor Donepezil, a drug used in the treatment of Alzheimer's disease and vascular dementia, protects neurons from apoptosis by inhibiting KCNB1[54]. The exact mechanism awaits elucidation but recent findings showing that KCNB1 is subject to direct oxidative modification may suggest that the protective effect of the drug may stem from its ability to prevent the oxidation of KCNB1[55].

In summary, toxins and synthetic drugs have significantly contributed to the effort of dissecting native KCNB1 currents in various tissues and probing channel's structure and functional mechanisms of gating.

KCNB1 IS A CRITICAL MEDIATOR OF HIPPOCAMPAL AND CORTICAL EXCITABILITY

KCNB1 is broadly expressed in the brain and is a major contributor to the delayed rectifier somatodendritic K^+ current in hippocampal and cortical neurons[14,56]. In electrically quiescent neurons, KCNB1 is mostly localized in microdomains in the membranes of dendrites and cell bodies where it is constitutively phosphorylated and poorly conducting[20,21,29-32,57-60]. Upon the onset of neuronal activity, a series of cellular events are initiated that lead to de-phosphorylation of the channel. This transition is associated with two major changes in channel's status: (1) its threshold for voltage activation is lowered; and (2) KCNB1 is released from the microdomains and begins to diffuse in the membrane[30]. The net effect of these changes is that KCNB1 conducts a delayed rectifier K^+ current that acts to slow down and/or terminate periods of high frequency firing. Activity-dependent phosphorylation/de-phosphorylation of KCNB1 is partly mediated by cyclin-dependent kinase 5 and the phosphatase calcineurin. The latter is activated by a calcium influx driven by the electrical activity of the neuron[29-32]. Using mass spectrometry, Trimmer and colleagues identified 16 phosphorylation sites in KCNB1, of which roughly half provided a substrate for calcineurin[32]. This indicates that modulation of KCNB1 by protein kinases is graded to reflect dynamic regulation of neuron firing properties. However, KCNB1 can also terminate periods of neuronal activity by being directly phosphorylated. For example, AMP-activated protein kinase (AMPK, which is activated by ATP depletion) can phosphorylate KCNB1 at residue S440 and induce hyperpolarizing shifts in the current-voltage relationship for activation, shifts that make the channel more conductive at negative voltages[61].

KCNB1 PROMOTES APOPTOSIS IN RESPONSE TO OXIDATIVE STRESS

KCNB1 is a specific mediator of apoptosis in a variety of neuronal cell types including hippocampal, cortical and granule neurons[62-65]. For example, a study investigating the molecular correlate of the apoptotic K^+ current in hippocampal neurons found that among nine alpha and 3 beta Kv subunits screened, KCNB1 was the primary correlate[63]. Several groups have demonstrated that the key event triggering KCNB1-induced apoptosis is an increase in reactive oxygen species (ROS), either following acute oxidation, or as a consequence of cellular stresses such as serum deprivation and excitotoxicity[55,62-65]. It is currently accepted that dysregulated K^+ homeostasis causes apoptosis by inducing mitochondrial swelling and depolarization, ROS generation, deficient energy production and cell volume decrease[66]. Accordingly, augmented insertion of KCNB1 channels into the plasma membrane is observed in neurons subjected to oxidative challenges[67]. The accompanying increase in KCNB1 current is thought to be a key step in the apoptotic program. The execution of the latter requires phosphorylation of KCNB1 by multiple types of protein kinases a fact that should not surprise considering the primary role that phosphorylation plays for the function of KCNB1. Zhou *et al*[68] investigated apoptosis induced by lack of serum

in granular neurons and found that this was associated with upregulation of KCNB1 *via* the activation of a signaling pathway involving cAMP, protein kinase A and cAMP response element-binding protein (CREB). Aras *et al*[69] have identified several kinases including apoptosis signal-regulating kinase 1 (ASK1), p38 MAPK-dependent kinase, c-Src tyrosine kinase, and $Ca(2^+)$/calmodulin-dependent protein kinase Ⅱ (CaMKII) that interact with KCNB1 in response to oxidative stress[70-73]. Their studies have provided a model that predicts that oxygen radicals induce simultaneous increases in cytosolic levels of Zn^{2+} and Ca^{2+}. These increases activate the previously listed kinases and accelerate KCNB1 forward trafficking by modulating and facilitating its interaction with SNARE family protein syntaxin. This apoptotic program is tightly regulated: knock down of just a single phosphorylation site (S800 for p-38, Y124 for c-Src) is sufficient to suppress the pro-apoptotic influence of KCNB1[70]. However, Src tyrosine kinases and protein tyrosine phosphatase epsilon (PTP epsilon) also play a role in the physiological modulation of KCNB1. In the Schwann cells of mice, Src-mediated phosphorylation of Y124, (the same residue responsible for Zn^{2+}/Ca^{2+} induced apoptosis), causes specific augmentation of KCNB1 current which appears to be critical for Schwann cell proliferation and myelination[74,75]. In fact, de-phosphorylation of KCNB1 at Y124 by PTP epsilon reduces KCNB1 activity and stops KCNB1-induced myelination[76,77]. Accordingly, mice lacking PTP epsilon exhibit hypomyelination of sciatic nerve axons at an early post-natal age, an effect due to constitutive activation of KCNB1 by Src tyrosine kinases[78]. Moreover, a number of K^+ channels can cause apoptosis *via* dysregulated K^+ homeostasis in a variety of cell types[66]. Therefore, increased K^+ current may not be the key feature responsible for the specific ability of KCNB1 to promote apoptosis, but rather a consequence of it. Recent work from our laboratory may shed light on this issue. Cotella *et al*[55] showed that oxygen radicals directly modify KCNB1 channels, leading to the formation of oligomers held together by disulfide bridges[55]. A KCNB1 variant which does not form oligomers, obtained by mutating an N-terminal cysteine (C73A), fails to increase apoptosis in mammalian cells. Cotella *et al*[55] further showed that in inside-out patches, oxidants inhibit KCNB1 current. These findings imply that the formation of oligomers, rather than KCNB1 current, is the event that triggers an initial pro-apoptotic stimulus. To answer this question, Wu *et al*[79] have investigated the fate of the KCNB1 oligomers. They found that they accumulate in the plasma membrane as a result of defective internalization. Notably, accumulation is transient, and normal endocytosis/surface expression are mostly restored within one hour post-oxidation. The transient accumulation of KCNB1 oligomers is associated with activation of c-Src and JNK kinases coupled to a steady increase in the levels of free radicals. Thus, oligomer-induced activation of a "death pathway" appears to trigger the initial pro-apoptotic stimulus. As apoptosis progresses and ROS levels increase in the cell, the surge of KCNB1 current follows to further execute the apoptotic program (Figure 1).

INHIBITION OF KCNB1 MAY REPRESENT A VALID ANTI-APOPTOTIC STRATEGY

Pharmacological inhibition of KCNB1 current may represent a valid approach to preventing apoptosis. Accordingly, Peers and colleagues have shown that carbon monoxide (CO) can provide neuronal protection against an increase in KCNB1 current *via* regulating ROS and protein kinase G activity[80]. The same group has further proposed that the anti-apoptotic effect of CO may also be partially responsible for the etiology of cancer, as many oncogenic cells constitutively express heme oxygenase-1 (HO-1), which generates CO as a by product of its catalytic activity[81]. Chronic viruses, which establish a state of persistent infection by rendering infected cells resistant to apoptosis also appear to exploit inhibition of KCNB1 current. In human hepatocytes infected with hepatitis C virus (HCV), oxidative insults fail to initiate apoptosis because the HCV NS5A protein inhibits phosphorylation of KCNB1 by p38 MAPK and thus suppresses the current surge[82,83]. Furthermore, a neuronal NS5A isoform from HCV genotype 1b, NS5A1b, protects rat neurons against apoptosis by inhibiting KCNB1[73]. However, while NS5A acts on tyrosine kinase phosphorylation at residue Y124, NS5A1b inhibits p38-MAPK at residue S800 suggesting that the actions of these viral proteins are genotype-selective probably reflecting the characteristic of these viruses to target specific tissues.

VASOCONSTRICTION OF SMALL PULMONARY ARTERIES MAY PROCEED THROUGH DIRECT INHIBITION OF KCNB1 CURRENT BY ROS

Hypoxic pulmonary vasoconstriction is a physiological response to alveolar hypoxia, in which blood flow is redirected to better ventilated lobes *via* constriction of small pulmonary arteries. The mechanical force leading to vasoconstriction is exerted by pulmonary arteries smooth muscle cells (PASMCs). Hypoxia initially promotes PASMCs depolarization *via* inhibition of an oxygen-sensitive K^+ current. This leads to the activation of L-type Ca^{2+} channels, which elevate cytosolic calcium thereby triggering PASMCs contraction. Biochemical, pharmacological, electrophysiological and genetic evidence designates KCNB1 - alone or mixed with KCNS3 silent subunits-as one of the major molecular correlates of the oxygen-sensitive K^+ current in PASMCs[16-18,84,85]. Studies using the human ductus arteriosus as model system have provided a detailed picture of the cellular and molecular events leading to vasoconstriction during hypoxia[86-88]. Changes in O_2 levels are translated to the mitochondrial electron transport chain (KCNB1 is insensitive to O_2[89])

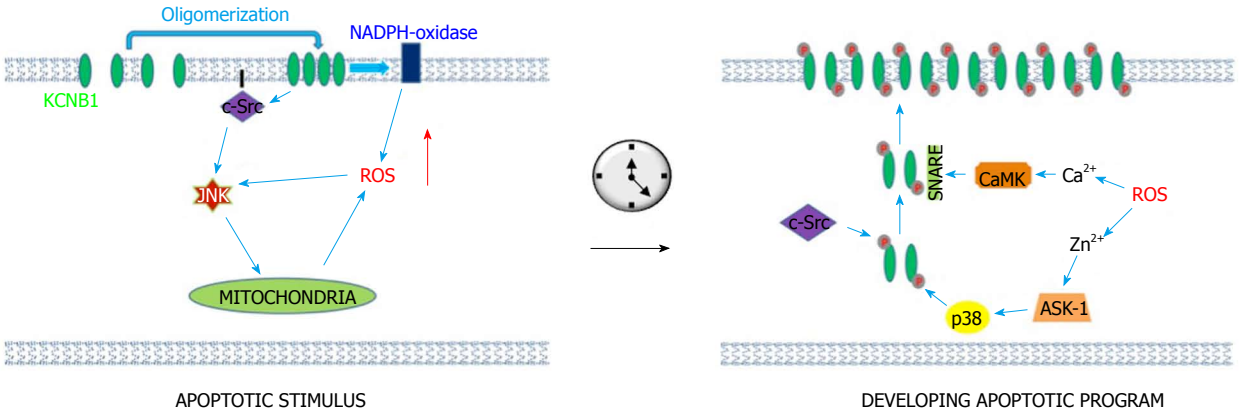

Figure 1 A two-step model for the pro-apoptotic actions of KCNB1. Upon exposure to oxidants, KCNB1 oligomers are formed. They accumulate in the plasma membrane thereby perturbing the organization of lipid rafts. This results in activation of an apoptotic stimulus mediated by c-Src and downstream, JNK kinases. As result of activation of c-Src and JNK kinases and in part of NADPH-oxidase (Xilong Wu, private communication) which is localized in the plasma membrane, ROS levels increase in the cell. ROS induce a raise in cytosolic Ca^{2+} and Zn^{2+} that initiate a phosphorylation-mediated surge of KCNB1 channels that further drives apoptosis. The signaling pathway activated by Zn^{2+} proceeds through activation of p38 by ASK-1 and independently, of c-Src tyrosine kinases (Zn^{2+} inhibits the activity of the tyrosine phosphatase PTP epsilon) which phosphorylate KCNB1 at S800 and Y124 thereby allowing interaction with SNARE family protein syntaxin. The Ca^{2+} signaling pathway results in activation of CaMKII kinase which in turn acts to modulate the interaction of KCNB1 with syntaxin. It is not known whether Src and p38 phosphorylation directly act to increase KCNB1 current. ROS: Reactive oxygen species.

which responds by speeding the synthesis of diffusible hydrogen peroxide (H_2O_2). H_2O_2 causes smooth muscle cell depolarization, *via* inhibition of K^+ current which further results in influx of calcium through L-type channels. The molecular details of the mechanism that links H_2O_2 to K^+ current inhibition were not known previously but the fact that KCNB1 can be directly oxidized by H_2O_2 and most importantly, that its current is suppressed by oxidants may now provide a natural explanation for this mechanism of inhibition. It is worth noticing that chronic hypoxia is characterized by depolarized resting potential and elevated cytosolic Ca^{2+}. Chronic depolarization is achieved by downregulation of KCNB1 protein[90-93] through mechanisms not completely understood, even though studies have implicated 15-lipoxygenase catalysis of arachidonic acid and hypoxia-inducible factor 1 in the mechanism[94,95]. Thus, different regulations of KCNB1 appear to mediate acute versus chronic conditions of hypoxia.

CONCLUSION

KCNB1 is a channel with a double-hedged sword nature: it is essential to the physiology of multiple organs, including the brain, pancreas and cardiovascular system and further acts as a mediator of apoptosis in response to oxidative stresses[2-21]. Dysregulated K^+ homeostasis is a well established mechanism through which K^+ channels contribute to an apoptotic program with a great deal of evidence implicating that KCNB1 do indeed work in this mechanism[55,62-65,67]. However recent findings have unveiled new ways through which KCNB1 mediates cell death: by giving rise to cytotoxic protein aggregates that result from direct oxidation of the protein[79]. The accumulation of these KCNB1 oligomers in the plasma membrane is transient but sufficient to trigger a pro-apoptotic signal *via* activation of a c-Src/JNK kinases pathway. As the apoptotic program progresses, a surge of KCNB1 current follows to induce mitochondrial destabilization, ROS generation, deficient energy production and cell volume decrease. Hence, KCNB1 plays a double role as both initiator and later executor of the apoptotic program.

Aging pathologies pose new challenges to health care, because even as advances in medicine are increasing lifespan, health problems become more prevalent as people age. A recent survey done by Harvard University School of Public Health and the Alzheimer's Europe Consortium suggests that senile dementia is the second leading health concern after cancer[96]. Aging is also the most important risk factor in neurodegenerative conditions such as Alzheimer's disease, the third most costly disease in the United Sates[97]. It is projected that the number of Western elders suffering from dementia and related neurodegenerative disease will increase by 350% by the midcentury[98,99]. Therefore, because of the impact of increasing lifespan on global human health issues, it is important to elucidate the cellular and molecular processes involved in aging. Oxidative modifications of KCNB1 are pervasive in the aging nervous system[55]. Hence, KCNB1 oxidation has the potential to impact all those conditions characterized by an imbalance in the redox status of the cell, from normal senescence to neuropathies such as Alzheimer's disease. Understanding how oxidation of KCNB1 influences the function of the brain during aging may provide the insight necessary to design better pharmacological strategies; these include targeting KCNB1 for the potential therapeutic use of antioxidants in neurological treatments or targeting other components of the signaling pathways activated by oxidation of KCNB1.

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REVIEW

Regulation of cell survival and death during *Flavivirus* infections

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Abstract

Flaviviruses, ss(+) RNA viruses, include many of mankind's most important pathogens. Their pathogenicity derives from their ability to infect many types of cells including neurons, to replicate, and eventually to kill the cells. *Flaviviruses* can activate tumor necrosis factor α and both intrinsic (Bax-mediated) and extrinsic pathways to apoptosis. Thus they can use many approaches for activating these pathways. Infection can lead to necrosis if viral load is extremely high or to other types of cell death if routes to apoptosis are blocked. Dengue and Japanese Encephalitis Virus can also activate autophagy. In this case the autophagy temporarily spares the infected cell, allowing a longer period of reproduction for the virus, and the autophagy further protects the cell against other stresses such as those caused by reactive oxygen species. Several of the viral proteins have been shown to induce apoptosis or autophagy on their own, independent of the presence of other viral proteins. Given the versatility of these viruses to adapt to and manipulate the metabolism, and thus to control the survival of, the infected cells, we need to understand much better how the specific viral proteins affect the pathways to apoptosis and autophagy. Only in this manner will we be able to minimize the pathology that they cause.

Key words: *Flavivirus*; Dengue virus; West Nile virus; Japanese encephalitis virus; Programmed cell death; Apoptosis; Extrinsic pathway; Intrinsic pathway; Autophagy; Necrosis

Core tip: The pathogenicity of *Flaviviruses* derives from their ability to infect many types of cells. They can activate both intrinsic and extrinsic pathways of apoptosis, by many means. Dengue and Japanese encephalitis virus can also activate autophagy, whereby autophagy temporarily spares the infected cell, allowing longer reproduction of virus and protecting the cell against other stresses. Given the versatility of these viruses, we need to understand much better how the specific viral proteins affect the pathways to apoptosis and autophagy. Only in this manner will we be able to minimize the pathology that they cause.

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INTRODUCTION

The aim of a virus is to infect and propagate and in doing so, affect the cell survival pathways. A wide range of viruses from different families (Poxviridae, Adenoviridae, Retroviridae, Picornoviridae, Flaviviridae, Orthomyxoviridae) have life cycles that intertwine with critical pathways involved in cell death and survival[1]. In this review we

focus our attention on *Flavivirus* (Flaviviridae).

Flaviviridae, a family of small and enveloped ss(+)-RNA virus, consists some of the worst pathogens known to mankind and mammals. The family is grouped into three genera, namely, *Flavivirus*, *Hepacivirus* and *Pestivirus* with each genus harboring potent killers, *viz*., dengue (DEN), hepatitis C (HCV) and classical swine fever virus (CSFV), respectively[2]. The largest and clinically the most relevant of three, *Flavivirus* contains almost 70 members, most of them transmitted to humans by mosquitos or ticks. Among the mosquito-borne are the most virulent viruses like dengue (DEN)[3,4], West Nile (WNV)[5], Japanese encephalitis (JEV) and Yellow fever (YFV)[6].

Although a few reviews address the role of cell death pathways during viral infection in general[1,7,8], there are none solely addressing *Flavivirus*. Here we summarize the most recent findings on survival and cell death pathways triggered by key members of *Flavivirus*. We focus on flaviviruses widely studied in relation to cell death - dengue, West Nile and Japanese encephalitis virus. We conclude that the viruses affect different parts of the apoptotic pathways in different cell types, and that dengue and JEV especially can protect cells by activating autophagy. Antiviral therapeutics will have to address these issues.

CELL DEATH AND ITS PATHWAYS

The ascendance of programmed cell death (PCD) as a theme of modern biology has followed an exciting trail from the mid-19th century until the present[9]. The idea of a cell programming its death had few takers during the early half of 20th century, though evidence was gathering since 1842, when Carl Vogt observed loss of notochord in amphibian metamorphosis[10]. Since then, evidence of programmed cell death has surfaced in various organisms as diverse as Dictyostelium[11], insects[12], and chicken[13]. Recognition of *apoptosis* as the primary form of programmed cell death, in the early 1970's[14] as well as recognition that apoptosis is conserved from *C. elegans* to humans)[15,16] has fueled interest among biologists. Moreover, association of apoptosis and other forms of cell death, notably the lysosomal (autophagic) cell death, with AIDS[17], cancer[18,19], Alzheimer's[20], and viral infection[1] has catapulted cell death to the forefront of biomedical research.

The importance of cell death was not fully appreciated until the late 1960's. This delay was partly due to the difficulty in documenting dying cells, as compared to dividing ones, as it was possible to monitor and finally trace a cell's duplication into daughter cells. While cells that have undergone mitosis can be traced considerably thereafter, an apoptotic cell in an organism is visible only up to 20 min after death[12].

Programmed cell death contributes to the sculpting of digits (prenatal disappearance of interdigital epidermis), removal of unnecessary tissues (involution of mammary glands during post-lactation) or irrelevant (wolffian/mullerian ducts after sex determination) organs, elimination of toxic and harmful cells (self-reactive thymocytes, UV-irradiated cells), and winnowing to only a properly integrated cell population (as in the case of differentiated neurons)[21,22]. A cell may trigger its own death (intrinsic/cell autonomous) or it may be brought upon by signals from the microenvironment (extrinsic). Deregulation of the cell death machinery can inflict upon the organism severe consequences like anomalous or stalled development, tumor formation, autoimmune disorder or neurological disorders (Huntington, Parkinson). In contrast, the vestiges of dead cells in some plants may serve important functions[22,23].

Most biologists make a clear distinction between "programmed" physiological (beneficial) and "accidental" (hazardous) cell death. The former denotes death of cells essential for physiological events (development, organogenesis, homeostasis, and defense) whereas the latter may be used for loss of cells during tissue damage. Apart from this functional distinction, cell death can also be classified based on morphology (apoptosis, autophagy, necrosis, and cornification) and enzyme involvement (proteases like calpains, caspases, and endonucleases). The Nomenclature Committee on Cell Death (NCCD) encourages researchers to clearly distinguish between "dying cells" and "dead cells", and by using the latter term, they should denote cells that have gone past the threshold "point-of-no-return" into a state of irreversibility. The NCCD has also revised the defining hallmarks for a dead cell: dissolution of the plasma membrane and complete fragmentation and engulfment by phagocytosis, since the traditional parameters like activation of caspases, mitochondrial trans membrane permeabilization and flipping of phosphatidylserine (PS) have been associated with non-lethal events[24].

APOPTOSIS

The most studied form of programmed cell death (PCD), *apoptosis* (Greek: falling of leaves), was first reported by Walter Flemming[10]. Kerr *et al*[14] characterized apoptosis (later described by Majno and Joris as PCD type I) and described it as a general process mistakenly previously identified as an arcane form of death called "shrinkage necrosis". While undergoing apoptosis, the cell separates from its neighboring cells, shrinks, undergoes chromatin condensation and DNA fragmentation, and is finally engulfed by a phagocyte (macrophage).

Apoptosis follows two distinct pathways, the extrinsic (death receptor) and intrinsic (mitochondrial) pathway[25]. The extrinsic branch of PCD is activated by external death signals. The cytotoxic effect is mediated by the binding of ligands [tumor necrosis factor-α (TNF-α), FasL, TRAIL] to the death receptors (TNF RI, Fas/CD95, DR3, TRAIL R1/DR4, or TRAIL R2/DR5) on the cell surface[26-28]. This binding leads to the trimerization of the membrane receptor, followed by the downstream activation of the DISC protein complex. The multi-protein complex initiates cleavage and activation of caspase-8, which in turn cleaves downstream zymogens (caspase-7, 10) and this sets forth a chain of reactions fi-

nally leading to activation of caspase-3 and cell death[25,28]. The caspase proteins (*C*ysteine-dependent *Asp*artate-directed Prote*ases* = C-A-S-PASES) are central to the entire apoptotic machinery within the cell. They are also integral to the intrinsic pathway, are synthesized as inactive zymogens that are activated by cleavage.

Intrinsic apoptosis is activated proximately by damage to mitochondria, which releases cytochrome C and apoptosis-activating factor from mitochondria. These latter, together with pro-caspase-9, bind together into an apoptosome, in which caspase-9 is activated. By means of this complex, caspase-3 is activated and, as in extrinsically-activated apoptosis, caspases 3 and 7 destroy the substructure of the cell.

Like caspases, Bcl-2 family members are also essential for carrying out intrinsic apoptosis. Based on domain structure and function, the members are grouped into anti-apoptotic guardians (Bcl-2, Bcl-xL, MCL-1), pro-apoptotic effectors (Bax, Bak) and sensors (Bad/Bim/Bid/Noxa)[29-31]. The intrinsic pathway is initiated by intracellular stress signals like ER stress, oxidative stress, DNA damage, growth factor withdrawals, and loss of contact with the extracellular matrix. Once the decision to die is made, the effectors are set free from their negative interaction with guardians by the sensors. They insert into and disintegrate the mitochondrial membrane, a phenomenon known as the mitochondrial outer membrane permeabilization (MOMP). This releases pro-apoptotic factors (cytochrome C, Smac/Diablo, HTRA2/Omi, apoptosis-inducing factor, and endonuclease G) into the cytoplasm. Cytochrome C interacts with the APAF-1, recruiting pro-caspase-9 (zymogen) to form the *apoptosome,* where the latter is cleaved and activated. This event triggers cleavage and activation of downstream caspases (2, 3, 7, 8) and accomplishes the death of cell[32]. Certain cell death regulators like inhibitor of apoptosis (IAP) can bind and suppress the apoptotic function of caspases[33].

AUTOPHAGY

Autophagy or PCD type II, literally meaning "self-eating", is a highly conserved catabolic process that is thought to precede apoptosis in evolution[34]. It is a surveillance process that is involved in the recycling of basic biomolecules. It oversees the entire cell homeostasis, packaging degraded/misfolded proteins or organelles in specialized bilayer membranes (autophagosomes) which fuse with the lysosome for digestion. This process is induced under conditions of high stress like starvation, growth factor withdrawal, viral invasion and ER stress. Deregulation of the autophagy pathway has been observed in pathogenic conditions like cancer or Parkinson's[35].

The induction of autophagy involves a set of multiprotein complexes, some of which have ubiquitin-like properties. mTORC1, a versatile signaling complex, strictly inhibits induction of autophagy by imposing an inhibitory phosphorylation on Unc-51-like kinase (ULK1). Under stress conditions, this block is removed by several factors, such as PTEN, AMPK, and TSC2. Activation of ULK1, which forms a complex with ATG13/FIP200/ATG101, leads to the nucleation of the pre-autophagosomal structure (PAS). This involves the phosphatidylinositol-3-kinase class Ⅲ (PI3K Ⅲ)-Vps34-Beclin 1 (ATG6) complex[36,37]. The subsequent elongation of the autophagosome is dependent on two ubiquitin-like conjugation systems. E1-like enzyme autophagy related gene 7 (*ATG7*) and E2-like enzymes ATG3, ATG10 are involved in the conjugation of ATG12-ATG5 and LC3 (ATG8)-phosphatidylethanolamine (PE). ATG12-ATG5 acts like an E3-like protein for the LC3-PE conjugation system, and then forms a complex with ATG16. These coordinated and combined steps accomplish the formation of a mature autophagosome which then fuses with a lysosome through a canonical endocytic pathway[25,38-40].

NECROSIS

Some forms of necrosis are programmed and controlled through a specific set of signal transduction pathways and degradative mechanisms. Cell death by specific necrosis can also contribute to embryonic development and adult tissue homeostasis[41]. Necrosis can be triggered by the same death signals that induce apoptosis[42]. The difference between apoptosis and specific necrosis lies in the rapid cytoplasmic swelling and release of extracellular components, seen in specific necrosis, which is often due to extreme physiochemical stress, osmotic shock, mechanical stress and high concentration of hydrogen peroxide[43]. When a cell is under such conditions, which can be produced by physiological or developmental situations, cell death occurs accidentally and uncontrolled. Necrosis signaling complex forms by interaction of receptor interacting protein 1 (RIPK1) with the receptor interacting protein 3 (RIPK3). This signaling complex forms by introducing death receptors either by inhibiting caspases or genotoxic stress[43]. In this type of cell death, unlike apoptosis, death is accidental and not programmed. Necrosis does not depend on caspase activation. In a study done by Nikoletopoulou *et al*[42], two different cell lines were treated with a tumor necrosis factor-α. In one cell line, apoptosis was triggered, whereas in another cell line it induced necrosis. In addition, necrosis can be in the form of regulated and programmed form of cell death. This phenomenon is referred to as necroptosis. Various death receptors associated with apoptosis, such as FAS, TNFR2, TRAILR1 and TRAILR2, have been shown to induce necroptosis in different cell types. Furthermore, necroptosis can be instigated by the members of the pathogen recognition receptor that are responsible for sensing pathogen-associated molecular patterns.

FLAVIVIRUS-STRUCTURE, INFECTIVITY, REPLICATION AND CELL SURVIVAL

Flaviviridae is a medically important family of animal virus, with members responsible for serious pathological conditions in human and other important mammals. This

group IV family (positive sense RNA) consists of three genera: *Flavivirus*, *Hepacivirus* and *Pestivirus*. The largest of them, *Flavivirus* (with approximately 70 members), includes some of the deadliest arthropod-transmitted virus. They are icosahedral, enveloped (+)-ssRNA virus measuring approximately 500Å in diameter. The typical *Flavivirus* (Latin *flavus* - yellow, indicating Yellow Fever) virion is composed of the genetic material surrounded by the capsid protein and 180 copies of two glycoproteins. The average genome size of the *Flavivirus* is 11kb, coding for a single polyprotein. The amino terminal accounts for the structural proteins: capsid (C), membrane precursor (prM) and envelope (E), and the remaining genome gives rise to the non-structural proteins (NS1, 2A, 2B, 3, 4A, 4B, and 5) which form the viral replication complex (RC)[2,44].

Infection starts as virions bind to the cell membrane through receptor-mediated endocytosis, aided by primary receptors (DC-SIGN, Grp78/BiP, CD-14 associated molecules) and low-affinity co-receptors (heparin, glycosaminoglycan). Acidification of the vesicle triggers disassembly of virus, releasing the genetic material into the cytoplasm. The resultant polyprotein undergoes co- and post-translational processing by viral and host proteases to give rise to the individual proteins. The structural proteins then assemble on the ER surface along with the RNA which is replicated on intracellular membranes. The assembly of virus in the ER lumen is followed by the movement of these immature viral particles through the trans-Golgi network. These are cleaved by the host protease furin to form mature virions, and are subsequently released by exocytosis[45-51].

Dengue virus

Among the members of *Flavivirus* family, Dengue is transmitted to human (in urban areas) and primates (in forests) by the urban-adapted mosquito strain *Aedes aegypti* (primary vector) and the emerging *Aedes albopictus*[52]. Dengue has been declared endemic in approximately 100 countries with 40% of the global population susceptible to infection. Dengue infection has doubled over the last two decades, and current annual figures have risen to 50-100 million humans affected[53].

Dengue has a genome of 10.7 kb positive sense single strand RNA that contains a type I cap at its 5' terminus[54]. The enveloped icosahedral virion measures 50 nm in diameter. The RNA is translated by the host cell machinery into a 3391-amino acid polyprotein that undergoes co- and post-translational processing by viral (NS2B-3) and cellular proteases[55-57]. The first quarter of the viral genome from the 5' end codes for the structural proteins C (capsid), prM (membrane), and E (lipopolysaccharide envelope), thus leaving the rest to code for eight non-structural proteins (NS1, 2A, 2B, 3, 4A, 2K peptide, 4B, 5) which are expressed only inside the host cell[58].

Dengue from different regions of the globe show four antigenically distinct serotypes (DENV 1-4), each having multiple phenotypes[59]. The distribution of these serotypes has spread alarmingly throughout the globe since 1970, when only South Asia had all four[60]. This spread has added to the complexity of dengue-induced pathogenesis since very little cross-immunity has been recorded between these serotypes, leading to multiple sequential infections and overwhelmed immune response[61]. Outcomes of dengue infection may lead to diverse pathogenic conditions, ranging from the mild-flu like febrile syndrome (dengue fever) to the very serious conditions resulting from infection with a second serotype, the lethal hemorrhagic condition dengue hemorrhagic fever (DHF) or the dengue shock syndrome (DSS)[62]. Dengue fever, the most important arboviral disease in humans, features rapid onset of fever, accompanied by headache, retro-orbital pain, myalgia, gastrointestinal irritation[63,64]. DHF, which claims more lives (5% mortality) than any other hemorrhagic fever, is characterized by bleeding, thrombocytopenia, increased vascular permeability beyond the usual dengue fever symptoms[65]. An equally lethal condition DSS is also characterized by vascular leakage, which is more pronounced in young children, and very low blood pressure[66]. Autopsies conducted on patients (predominantly children) dying from DSS have revealed a broad range of dengue susceptible tissue as shown by virus infecting skin, liver, spleen, lymph node, kidney, bone marrow, lung, thymus and brain[67-70].

Cell death and survival after infection with dengue

Dengue has been shown to derive pathogenic effect from apoptotic cell death in several types of mammalian cells. The role of apoptosis in dengue infection has been seriously studied since the mid-1990s, along with the identification of Bcl-2 superfamily members. Dengue-induced apoptosis has been observed in cells from the nervous system (human and mice neuroblastoma, murine cortical and hippocampal neurons, human cerebral cells); liver (human hepatoma); immune system(human peripheral blood mononuclear cells like $CD8^+$-T lymphocytes, monocyte-derived macrophages, human mast cells like KU812, HMC-1, and primary murine macrophages); vascular system (human umbilical cord vein endothelial cells/EA.hy296, human microvascular endothelial cells, pulmonary microvascular endothelial cells/MECs) and, digestive tract (intestinal cells); and kidney cells (human embryonic kidney HEK 293, green monkey kidney Vero). Of the four antigenically distinct serotypes infection with variants of dengue 1 (human isolates of dengue type 1 virus FGA/89 and BR/90, neurovirulent variant FGA/NA d1d), 2 (strain NGC, 16681) and 3 (DENV3/5532) lead to cell death and apoptosis within 25-36 h post infection.

Apoptosis is triggered by live virus or dengue proteins through components of both extrinsic and intrinsic apoptotic signaling (Figure 1). Death ligands and receptors participate in dengue-induced apoptosis. Increased levels of pro-apoptotic proinflammatory cytokines (TNF-α and interleukin-10) and Apo2L/TRAIL are observed after infection, which the virus possibly induces in a TNF-α-fashion[71]. Profiling of genes reveal the activation of death receptors FAS/CD 95, TNFR superfamily member

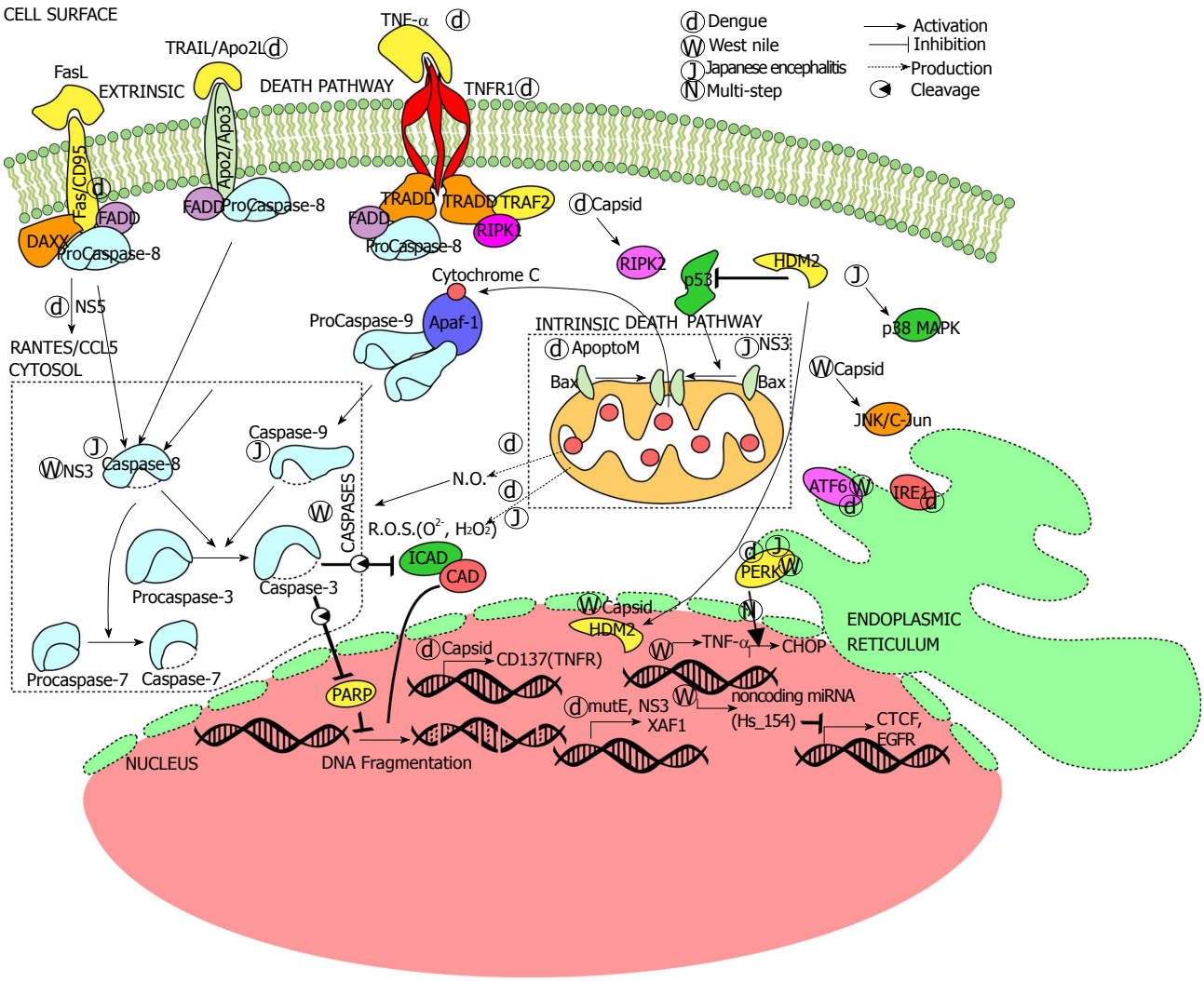

Figure 1 Flaviviruses target cell death and survival pathways. Extrinsic and intrinsic cell death pathways are activated during viral infection (d, w, and j are for dengue, west nile and japanese encephalitis live viruses respectively) or expression of specific viral proteins (d, w and j with viral protein). Expression of multiple genes including non-coding microRNAs (miRNA) also induced during flavivirus infections. FLaviviruses also activate ER stress signaling and increase metabolism related products (ROS and NO). TNF: Tumor necrosis factor; EGFR: Epidermal growth factor receptor.

9/CD 137, TNFRI/TNF-α (caspase-independent) and IL-1β/ NFκB (caspase-dependent) pathways[72,73].

Viral protein NS5 interacts with death protein 6 (Daxx), which among other functions interacts with death receptor FAS, to activate RANTES (CCL5), a cytokine closely associated with DHF[74,75]. Moreover, transfection with wild type capsid protein increased the expression of CD137, a member of the TNFR family. Receptor-interacting serine/threonine protein kinase 2 (RIPK2), a master regulator of stress pathways[76], is also necessary for capsid-induced apoptosis[76]. In addition to capsid protein modulation of death receptor expression, infection with live dengue virus leads to differential expression of several interferon-inducible genes, the most important being XAF1. XAF1 upregulates caspase 3 36 h after infection and mediates apoptosis[77]. The activation of caspases leads to the characteristic nuclear fragmentation and cytoplasmic blebbing of apoptosis.

Mitochondria-mediated or intrinsic apoptosis signaling also occurs after dengue infection. The reactive oxygen species (ROS) O_2 and H_2O_2, which are predominantly produced in the mitochondria, increase during infection. Toxic levels of ROS can activate calpains and lead to apoptosis. Secondary messenger oxides like nitric oxide (NO) also mediate in dengue-triggered apoptosis in a caspase dependent manner[78]. Other dengue structural proteins are also involved in apoptosis. Intracellular production of the M protein from all dengue strains activated the intrinsic pathway apoptosis in mouse neuroblastoma (Neuro2a) and human hepatoma (HepG2) cells. *ApoptoM*, a nine-residue sequence (M-32 to -40) from the M ectodomain (M-1 to -40), is instrumental in the cytopathic effect of the flavivirus[79].

The activation of apoptosis at different levels of the extrinsic and intrinsic pathways by several variants of dengue virus implies an important role in the life cycle of the virus. As infected cells undergo apoptosis by multiple means the extrinsic and intrinsic apoptotic pathways converge at the activation of phosphatidylserine (PS) for phagocytic clearance during secondary dengue infec-

tion[80].

Apoptosis, supposedly an innate immune response, is often manipulated by the viruses like dengue to act against the immune system itself, as shown by the more numerous apoptotic peripheral blood mononuclear cells (PBMC) in dengue infected children. The proportion of apoptosis and its mediators (CD95) in the circulating PBMCs was much higher in individuals progressing towards hemorrhage (DHF) than those developing febrile symptoms (Dengue Fever), indicating a higher viral load in the former. A fact that most of the apoptotic PBMCs were $CD8^+$-T lymphocytes bears testimony to the deranged immune machinery in infected individuals. The immune response to increased dengue-induced apoptosis does not curb virus proliferation. Apoptosis, in the context of dengue infection, fails to arrest viral reproduction and even correlates with increased virus production[72,73].

Unlike lytic viruses that indiscriminately trigger cell death, pro-apoptotic variants of dengue can lose their pathogenic ability in certain cells. For example the neurovirulent variant FGA/NA d1d, developed from the apoptosis inducing dengue 1 human isolate FGA/89, kills neuroblastoma but not hepatoma cells[81]. Apoptosis seen during infection of human umbilical cord vein endothelial cells (ECV304) and Swiss Webster primary macrophages by Dengue-2 virus strain 16681 is lost in MDCK, HeLa, HEK 293T, Vero and Swiss Webster primary mouse embryo fibroblasts (MEF) even after 144 h (6 d) post infection[82,83].

The differences in dengue outbreaks are partly explained by differences in cell killing by clinical isolates of virus from a fatal case (Paraguay 2007; DENV3/5532) had higher replication rate in monocyte-derived human dendritic cells (mdDCs) than isolates of virus from a non-fatal breakout (Brazil 2002; DENV3/290). The former also induced more proinflammatory cytokines associated with apoptosis[71]. Moreover, differences in cell toxicity among dengue variants have been attributed to mutations in the E and NS3[81]. Although adequate to explain certain differences in cell killing these mechanisms fail to explain the attenuated pathogenicity of immune/endothelial toxic dengue against other cells even in the presence of apoptotic agents like staurosporine, cycloheximide, camptothecin and influenza virus[83].

Involvement of autophagy in dengue infection is a relatively new finding, shown first in 2008. DENV2 caused ATG5-dependent autophagy in hepatic (Huh7) and fibroblast (MEF) cells. The virus' ability to induce autophagy correlated positively with viral replication without a direct role in infectivity, as its downregulation did not increase amounts of intracellular virus[84,85]. Denv2-mediated autophagy protects from toxic stimuli canine kidney epithelial (MDCK) and mouse embryo fibroblast (MEF) cells but not murine macrophages, where infection leads to apoptotic cell death. Expression of dengue NS4A protein, like infection with live virus, induces PI3K-mediated autophagy and protects these cells against death from toxins[83]. Specific inhibitors of autophagy like *spautin-1* have revealed the role autophagy plays in maturation of dengue virion. Blocking autophagy in Huh7.a.1, BHK21 cell lines and AG129 mice resulted in a heat-sensitive and non-infectious dengue virion[86].

West Nile virus

West Nile virus (WNV), first encountered in the New World in New York City (1999), has been the cause of three major arboviral neuroinvasive outbreaks in the United States[87]. It belongs to the same Flavivirus serocomplex as the Japanese encephalitis virus (JEV) and St. Louis encephalitis virus 15, following a bird-mosquito-bird transmission cycle. In the United States, *Culex pipiens* serves as the major arthropod vector. The human is a "dead-end host" for WNV due to low levels of serum viremia[88]. WNV consists of five phylogenetic lineages, of which 1, 2 have been associated with significant outbreaks. The primary targets are keratinocytes and dendritic cells, which upon infection migrate to visceral organs and the central nervous system. The neurovirulence of WNV is dependent on varying factors-its ability to cross the endothelium of blood-brain barrier (helped by cytokine mediated increased vascular permeability), import of infected macrophages into the CNS (Trojan horse mechanism) and viral retrograde transport from peripheral neurons to CNS[89-91]. Like dengue, outcome of infection varies from mild fever (WNV fever), accompanied by headache and diarrhea, to neurological symptoms (WNV neuroinvasive disease). While only 1% of infected individuals develop the latter, mild fever can be seen in 25%. However, neuroinvasive infections have a 10% fatality, which makes it extremely lethal. The serious pathological conditions (meningitis, encephalitis, acute flaccid paralysis) are also accompanied by chills, rash and visual disturbance. The severity is higher in elder patients, as is evident from the higher death rate (17%) in individuals aged at least 70 from those (0.8%) in their mid-40s[88,92,93]. Complete recovery following acute infection is extremely rare, and fatigue, cognitive difficulties, depression and muscle aches have been reported even after a year[94-97]. Diagnosis is dependent on detection of IgM levels in the cerebrospinal fluid by MAC-ELISA, although false positive results have been reported during infection with related Flavivirus[98,99]. To date, treatment has been supportive, relying on vector control, and no vaccine is licensed for human use. Human being the "dead-end host", future vaccinations will not prevent spreading of the virus in nature either[100-102]. It is extremely important that molecular mechanisms adopted by the virus, like manipulation of the cell survival pathway, be studied. This would help in developing an effective antiviral therapy.

Cell death and survival after infection with WNV

The relationship between WNV infectivity and cell survival pathways has been studied for more than a decade. WNV-mediated cell death and cytotoxicity depend on the severity of the initial infection. Vero cells infected with many virus particles (multiplicity of infection, *moi* > 10) showed signs of necrosis (leakage of HMGB1 and high LDH activity) within 8 h of infection. In contrast, cells infected with a lower load (*moi* < 10) showed signs

of apoptotic cell death at a later stage (32 *hpi*)[103]. Very similar to dengue, WNV induces apoptotic cell death in several cell types, such as, immune cells (human leukemic -K562), neuronal cells (mouse neuroblastoma - Neuro 2a, brain tumors), epithelial cells (Vero, A549), fibroblasts (MEF, BHK21), and embryonic cells (HEK293T)[104-106].

The upstream events leading to apoptotic death in WNV infected cells include endoplasmic reticulum (ER) stress pathways. Infection of human neuroblastoma (SK-N-MC) cells and primary rat hippocampal neurons led to activation of two branches of ER stress-mediated unfolded protein response (UPR). ATF6 and PERK pathways were induced during infection, resulting in CHOP activation and downstream apoptosis[107]. A different effect on the UPR pathways has been observed. The West Nile virus Kunjin strain (WNV$_{KUN}$) shuts off PERK pathway and interferon-mediated STAT phosphorylation in wild type MEFs. However, it activates the remaining two UPR (ATF6, IRE1) pathways. Studies with *ATF6*$^{-/-}$, *IRE1*$^{-/-}$ MEFs point to the synergetic role these pathways play in WNV$_{KUN}$ pathogenesis. They contribute to increased cell viability and viral load, by restricting apoptotic cell death[108].

WNV can regulate both extrinsic and intrinsic pathways to launch pathogenesis (Figure 1). The virus induces Bax-dependent intracellular apoptosis in human leukemic (K562) and mouse neuroblastoma (Neuro 2a) cells. Strains that did not possess the ability to induce apoptosis, due to UV-inactivation, could not establish infectivity in cells[104]. WNV encephalitis in CNS-derived mouse neurons was highly dependent on the activation of caspase-3, and infection in the permissive T98G (brain-derived tumor) cells involved both extrinsic and intrinsic apoptotic pathways[105,106]. Tetracyclines are well established antiviral compounds, and minocycline strongly inhibited WNV infection in three CNS-derived human cell types (HBN, HRPE, and T98G).The antibiotic blocked viral replication, apoptosis and the viral activation of JNK/c-jun pathway, establishing a link among them[109]. Kobayashi *et al*[110] proposed that the presence of ubiquitinated proteins had functional implication in apoptosis of WNV-infected mouse neuroblastoma (Neuro-2a) cells. Migration of CD8^{+}- T lymphocytes to drained lymph nodes (dLNs) was hindered in the CNS of *Cd22*$^{-/-}$ mice, which had a higher viral load than the wild type. This finding suggests a role for the B-cell marker, also an important component in cell survival, in modulating cellular immunity during infection[111].

Apoptosis often restricts viral replication and infection. Shrestha *et al*[112] showed the beneficial role of TNF-α related apoptosis inducing ligand (TRAIL), produced by CD8^{+}- T cells, in limiting WNV infection in mouse central nervous system. CD8^{+}- T cells in *TRAIL*$^{-/-}$ mice encountered difficulty in clearing the viral particles from the neurons. Zhang *et al*[113] demonstrated, using mouse neuron as an infection model, rise in the levels of TNF-α during infection. The rise served to downregulate the chemokine CXCR3, which would otherwise bind antiviral CXCL10 circulating in the central nervous system (CNS). This interaction results in calcium transients that lead to caspase-3 mediated apoptosis in the neurons, an adaptive mechanism to prevent cell death. Smith *et al*[114] showed an important aspect of WNV infection in human cell culture (HEK293, SK-N-MC) and mouse neuronal tissues - regulation of non-coding microRNAs (miRNAs). Among several miRNAs, Hs_154 is significantly up regulated in infection. Two of its targets, CCCTC-binding factor (CTCF) and epidermal growth factor receptor (EGFR), are associated with cell survival; this accounts for the role of Hs_154 in mediating apoptosis. While this activation has been found to lower viral replication, apoptotic cell death is also the basis for WNV pathogenesis.

As in dengue, both structural and non-structural proteins play a role in cellular survival after infection. WNV capsid (Cp) protein triggers a caspase-dependent apoptosis, leading to inflammation, in mouse brain and muscle[115]. WNV capsid is dependent upon p53 for its apoptotic effects. It has been shown to sequester HDM2, a negative regulator of p53, into the nucleolus. This results in a higher stability of p53, which can then target Bax to induce apoptosis in MEF cells[116]. Inhibitor-based studies on four types of mammalian cells (A549, HEK293T, Vero-76, BHK-21) suggest a role for WNV capsid (C) protein in the inhibition of apoptosis through Phosphatidylinositol-3-kinase (PI3K)- Akt prosurvival pathway[117]. The helicase and protease domains of NS3 protein are instrumental in inducing a caspase-8 dependent apoptosis in three types (Neuro 2a, HeLa, and Vero) of mammalian cells[118].

Our present knowledge does not suggest any significant role of autophagy in WNV pathogenesis, distinguishing it from dengue and Japanese encephalitis virus. Though infection induced autophagy in mice brain slice and several mammalian cells, it was actually PI3K that was involved in viral replication[119,120].

Japanese encephalitis virus

Japanese encephalitis virus (JEV) is extremely important as it is spreading throughout Asia, China, India, Australia, and Pakistan and is responsible for between 12500 to 17500 deaths reported annually. JEV is transmitted by a primary mosquito vector (*Culex tritaeniorhynchus)* and secondary mosquito vector (*Culex gelidus*, *Culex fuscocephala* and *Culex vishnui*) that primarily target domestic animals and human host[121]. Humans are "dead end host", since they cannot infect the feeding mosquitoes because of low viremia. Children are at higher risk for an infection with Japanese encephalitis than adults, especially in rural areas. They are also at higher risk for death due to their weaker immune system as compared to the adults. In addition, people who visit Asia and Indonesia are particularly prone to this viral infection since they lack the protective antibodies. Asymptomatic infection depends on host's age, immunity, general make-up and current health status. Symptoms include headache, fever, tremor, gadtrointestinal comfort as well as severe conditions of encephalitis

and Parkinson-like seizures[122].

The means of the entry of the virus entry into the system plays an important role on the progress of the infection. If the carrier, the mosquito, bites directly into the blood vessel, it is easier for the virus to spread directly to the central nervous system.

There have been efforts to make a vaccine against JEV, although its successful implementation has been impeded by frequent climate changes. The spread of Japanese encephalitis virus is assisted by wind-blown mosquitoes, bird migration and people traveling with infected virus, which further spread the disease. Programs in underdeveloped countries are established in order to prevent the increasing number of yearly deaths caused by Japanese encephalitis virus. These programs include mosquito control by using pesticide, mosquito nets, cattle segregation and vaccination of cattle as well as humans[121,123].

Cell death and survival after infection with japanese encephalitis virus

As shown in Figure 1, JEV-induced apoptotic cell death is reliant on endoplasmic reticulum (ER) stress and production of reactive oxygen species (ROS). ER stress-induced activation of UPR factors (CHOP-p38MAPK) is essential for triggering the apoptotic response in fibroblasts (BHK-21) and neuronal cells (N18, NT-2)[124]. Even replication-incompetent strains (UV-JEV), as shown by Lin *et al*[125], retain their ability to kill neuronal cells (N18, NT-2) by inducing ROS production and activating NF-κB. The structural E protein from JEV-YL induces apoptotic cytotoxicity in HepG2and Vero cells[126]. Earlier studies had pointed to a link between non-structural NS3 protein and induction of apoptosis. Transfection of pEGFP-NS3 1-619 plasmid (whole NS3 protein) into Vero cells caused apoptotic cell death. The same study also evaluated the role of caspases where it was found that NS3 only activates the intrinsic branch (casp -9,-3) of apoptosis[127,128].

Bcl-2 proteins can prevent apoptosis by controlling the release of cytochrome C. Overexpression of bcl-2, however, did not block viral replication and distribution in mouse neuroblastoma N18 cells, though it delayed cell death in BHK-21 cells. Moreover, in BHK-21 and CHO cells, bcl-2 overexpression established persistent infection by virtue of its antiapoptotic property. Thus, bcl-2 was not a fruitful target for preventing infection. It was due to the ability of this virus to activate complex pathways of caspase-dependent apoptosis in some cells. Though JEV induced classical intrinsic pathway in N18 neuroblastoma cells, it activated both caspase-8 (part of the extrinsic pathway) and caspase-9 in a predominantly mitochondria-dependent pathway in MCF cells[129-131].

Japanese Encephalitis virus causes autophagy to facilitate viral replication in certain cell types. Li *et al*[132] showed induction of autophagy by virulent (RP-9) and attenuated (RP-2ms) JEV strain in human NT-2 cells. They also showed the positive effect of rapamycin induced autophagy on viral infection, and the reversal of that effect on blocking autophagy. Infection with Japanese encephalitis virus triggers innate immune response (through RIG-1/IRF-3 and P13K/NF signaling pathway) and activates inflammatory cytokines, chemokines and IFN-inducible proteins[133]. JEV Infection also induces autophagy in human microglial (CHME-5) cell line, leading to pro-inflammatory cytokine response.

CONCLUSION

Dengue is the worst arboviral human disease and most lethal among all Flavivirus members. It is remarkable how it manipulates the cell survival pathway in many types of cells, ultimately increasing viral load. From the literature, it is evident that dengue triggers different responses in different mammalian cells. Most of the dengue proteins (NS2, NS3, NS5, C, and E) have been reported to trigger extrinsic apoptosis pathway in many cells, including neurons, hepatocytes, immune cells, and endothelial cells. TNF-α and interleukins (IL-1β, 10) play a key role in this mechanism. However, M protein domains induce intrinsic apoptosis in neurons and hepatocytes. The virus may have alternate strategies to kill the cell, in case one of the cell death pathways is nonfunctional. In some cases, the virus has been able to induce different kinds of stress (ER, ROS, NO) conditions that lead to apoptotic cell death (Figure 1). Recent discoveries have shown that dengue can also activate autophagy in epithelial cells, fibroblasts and hepatocytes. It even uses this pathway to increase energy production, which would facilitate viral replication. Nonstructural proteins (NS2, 3, 4) have been involved in this process. The ability of dengue to use cell death or protective autophagy for virus replication in specific cell types is crucial in dengue's versatility. Antivirals addressing the vast repertoire of the virus will contribute to counteracting dengue pathogenesis.

West Nile virus, though not as versatile as dengue, can trigger apoptosis in the central nervous system (CNS) to establish neuroinvasiveness. With a higher initial WNV dose, necrosis has been observed. An interesting aspect of infection with different strains lies in the differential regulation of ER stress-UPR pathways to achieve increased viral burden. The capsid protein positively interacts with p53 *in vivo*, activating the intrinsic pathway; however, in mammalian cells, it blocks apoptosis through PI3KI-Akt pathway. NS3 is involved in extrinsic apoptosis in neuroblastoma and cervical cancer cells. However, we need to know more about the effects of individual WNV proteins. A promising facet of WNV research is the attention focused on miRNA regulation, which needs to be extended to the other members of Flavivirus. This approach holds promise for antiviral therapy.

Japanese encephalitis virus, though pathogenetically similar to WNV, manipulates both intrinsic and extrinsic pathways to its advantage (Figure 1). JEV induces apoptosis in many neuronal cells by inducing upstream stress (UPR response, ROS production) events. JEV NS3, in contrast to DENV and WNV, induces the intrinsic pathway of apoptosis. There is also evidence that the virus

can infect and replicate even in the absence of caspase-3, as it can induce caspase-6 and activate caspase-8 and -9 in a mitochondria dependent pathway. Moreover, caspase inhibition does not block viral production. Thus this Flavivirus appears to rely more on mitochondrial apoptosis for its pathogenesis. To add to the severity, it also utilizes autophagy to mediate pro-inflammatory cytokine response in neuronal cells.

Under these circumstances, we postulate that the Flavivirus has the ability to manipulate cell survival and innate immune response. The aftermath of viral invasion is dependent on initial dose and cell type. It can also switch to different mechanisms to exert its pathogenic effect in different cells of our body. The current understanding of cell death and survival during Flavivirus infection has not addressed many critical and complicated issues like the role of apoptosis and autophagy in killing infected cells or helping them to survive. Future studies should be aimed at finding out the function of individual viral proteins and the regulation of non-coding RNAs in viral infection. More emphasis needs to be put on studying the signaling pathways by which viruses regulate the cell survival pathways.

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REVIEW

Review of application of mass spectrometry for analyses of anterior eye proteome

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Abstract

Proteins have important functional roles in the body, which can be altered in disease states. The eye is a complex organ rich in proteins; in particular, the anterior eye is very sophisticated in function and is most commonly involved in ophthalmic diseases. Proteomics, the large scale study of proteins, has greatly impacted our knowledge and understanding of gene function in the post-genomic period. The most significant breakthrough in proteomics has been mass spectrometric identification of proteins, which extends analysis far beyond the mere display of proteins that classical techniques provide. Mass spectrometry functions as a "mass analyzer" which simplifies the identification and quantification of proteins extracted from biological tissue. Mass spectrometric analysis of the anterior eye proteome provides a differential display for protein comparison of normal and diseased tissue. In this article we present the key proteomic findings in the recent literature related to the cornea, aqueous humor, trabecular meshwork, iris, ciliary body and lens. Through this we identified unique proteins specific to diseases related to the anterior eye.

Key words: Mass spectrometry; Proteomics; Ocular; Glaucoma

Core tip: Mass spectrometric based proteomics has been an indispensable tool for molecular and cellular biology. The ability of mass spectrometry to identify and precisely quantify thousands of proteins from complex samples has contributed greatly to biology and medicine. Through this we have studied protein-protein interactions *via* affinity-based isolations on a small and proteome-wide scale, the mapping of numerous organelles, and the generation of quantitative protein profiles from diverse species. The anterior segment of the eye is one of the most complicated parts of the human body with over 5000 proteins identified. Proteomic analyses of different parts of the eye, in particular the anterior eye structures, involve high throughput methods that help identify proteins and their posttranslational modifications. In this article we review the current state of advancement in the identification of anterior chamber proteins. We will present our findings in the following order: cornea, aqueous humor, trabecular meshwork, ciliary body, iris and lens.

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INTRODUCTION

Each organ in the human body has unique specialized

structures responsible for specific functions. Two investigational approaches are revealing the importance of the organization of molecular constituents in protein structure and function. The first approach focuses on one specific molecule at a time, the structure of the molecule, and the function the molecule is responsible for delivering. The second approach uses a high throughput analyses, capturing molecules in specific locations, performing experiments that enables us to determine their roles, and functions at these locations. The overarching goal of such high throughput experiments is a faster as well as greater understanding of composition, structure, and function. Proteomic analyses of different parts of the eye, in particular the anterior eye structures, involve high throughput methods that help identify proteins and their posttranslational modifications. Proteomics involves all methods that help identify proteins in the anterior eye chamber. The mass spectrometric methods to identify proteins in different locations in the anterior chamber use relatively older techniques and do not properly portray our current state of understanding. We aim to review the current state of advancement in identification of anterior chamber proteins, compared to the data gathered in the earliest era of proteomic mass spectrometry. We will present information on the following areas: cornea, aqueous humor, trabecular meshwork, ciliary body, iris, and lens. As each section of the anterior eye is uniquely different in proteins, functions and pathology, we have written the review specific to, what we believe, are the key relevant findings in the literature.

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE CORNEA

The human cornea is a transparent, avascular, and highly specialized connective tissue which reflects and absorbs light into the lens and retina, and contributes two thirds of the eye's refractive power. It is the most densely innervated tissue in the body and acts to protect the eye from infection as well as UV light[1]. The cornea also acts as a structural barrier providing the eye with biomechanical stability[2]. It is approximately 530 μm in thickness and is composed of five layers: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium[3]. The stroma contributes 90% of corneal volume[3]. Diseases of the cornea are commonly infectious, traumatic or genetic in nature and have a tendency to affect certain layers of the cornea[4]. Especially in developing countries, corneal disease often contributes to blindness. The most common etiologies of corneal blindness globally include infectious trachoma (C. trachomatis), oncherciasis (O. volvulus), leprosy (M. lepromatosis), and hypovitaminosis D (xerophthalmia)[5]. Keratoconus and Fuch's dystrophy, diseases of the stroma and endothelium respectively, are the most common causes of corneal disease resulting in blindness in developed countries[5].

In recent years, our understanding of the identities and functions of the various proteins involved in the cornea has grown immensely. In 2005 just over 140 proteins were identified in the cornea[3]. Since then, over 3000 proteins have been characterized[4]. We have chosen here to focus on a narrow set of 12 proteins that have been identified in multiple studies, and which have important cellular functions.

Transforming growth factor-beta-induced protein (TGFβIp) has been identified in multiple corneal proteome studies[3,6,7] and has been implicated in corneal disease[8]. Numerous isoforms of TGFβIp have been found in the human cornea with 29 isoforms being found in earlier mass spectrometric studies in the mid-2000s[3]. This protein group's most frequently described isoform, TGFβIp ig-h3, is 683 amino acids in length and has been described in several cellular compartments[7]. These include the membrane, Golgi apparatus, cytoplasm, endoplasmic reticulum, extracellular matrix/space, and the mitochondria[7,8]. Its molecular functions include catalysis, binding of nucleotides, signal transduction, regulation of enzyme activity, protein binding, and cell adhesion[7,8]. The relative abundance of this protein has been shown to be especially high in the stroma and endothelium[4]. In the stroma, it has been characterized as the second most abundant protein (17.6% abundance), and in the endothelium it has been described as the most abundant protein (36.8% abundance)[4]. As mentioned previously, this protein has been implicated in several disease states, including Fuch's endothelial corneal dystrophy[8]. Simply put, this disease involves the progressive loss of endothelial cells, which is associated with impaired vision[5]. Increased expression and accumulation of TGFβIp ig-h3 has also been associated with other corneal and lattice dystrophies[5]. Overall, more than 50 mutations of this protein have been noted to be involved in disease states[5].

Peroxiredoxins are a group of redox associated proteins[6] which play a role in oxidative stress response in the cornea[9]. These proteins decompose peroxide molecules[10]. It is thought that decreased expression of these and other antioxidant proteins may play a role in Fuch's dystrophy and keratoconus[8,9]. Peroxiredoxins 1, 2, and 6 have consistently been identified in corneal samples by mass spectrometry[3,6,7]. Peroxiredoxin 1 is 199 amino acids in length, and is found in the membrane, cytoplasm, nucleus, extracellular space, and mitochondria. It is involved in functions such as catalysis, DNA and protein binding, and inhibition of oxidation[7]. Peroxiredoxin 2 is 198 amino acids in length, found in the cytoplasm, nucleus, cytosol, mitochondria, organelle lumena, and chromosomes. It is also involved in catalysis, protein binding, and inhibition of oxidation, as well as metallic ion binding[7]. Peroxiredoxin 6 is 224 amino acids, and is found in similar cellular compartments as Peroxiredoxins 1 and 2, as well as in vacuoles; it also has similar cellular activities as its predecessors[7].

Transketolase is an enzyme involved in the pentose phosphate pathway and is involved in cell transparency[11]. It has been shown to be downregulated in keratoconus[12]. This protein is 623 amino acids in length and is found in the cytoplasm and cytosol. In addition to catalysis, it is

involved in protein and metallic ion binding[7].

Mitochondrial ATP synthase subunit alpha has also been found in multiple mass spectrometric corneal proteomic investigations. It is made up of 553 amino acids, and is found in the membrane, cytoplasm, extracellular space, mitochondria, and organelle lumena. In addition to its catalytic function, it also binds proteins, metals, and nucleotides and has transporter actions[7].

At a cellular level, L-lactate dehydrogenase is involved in fermentation of pyruvate to lactate. The protein is up-regulated in keratoconus[12]. The beta chain of this protein is 334 amino acids and is found in the cytoplasm, cytosol, nucleus, extracellular space, and mitochondri. It has been found in several corneal proteomics investigations, and in addition to its catalytic activity, it plays a role in transcription regulation, binding of nucleotides and metal ions, and transporter activity. It also regulates other enzymes[7].

F-actin-capping protein subunit alpha-1 is part of a protein which interacts with the fast-growing ends of actin filaments to prevent subunit exchange[13]. Its role in the cornea is not well characterized but it may play some role in colon cancer[14]. The protein is 286 amino acids in length and exists in a wide variety of cellular spaces. In addition to its catalytic activity, it is a structural protein, binds proteins and metals, regulates enzyme activity, and plays a role in redox reactions[7].

Vimentin is a class Ⅲ intermediate filament protein[15]. It is composed of 466 amino acids and is seen in the cytoskeleton, membrane, cytoplasm, cytosol, and extracellular space. It functions in catalysis, DNA and protein binding, motor and transportation activities, and is involved in structural activities[7]. It has been found to be increased in the epithelium of corneas with keratoconus. As this protein is generally found in mesenchymal cells, it is thought that epithelial to mesenchymal transformation may be a possible characteristic of keratoconus[15].

Annexin A5 is a blood/plasma protein[6] which is thought to be involved in cellular apoptosis and its expression is used to determine cytotoxicity[16]. This protein is found in the cytoplasm and extracellular space. It is 320 amino acids long, and functions in metal and protein binding, as well as in the regulation of enzymes[7].

Keratin, type Ⅱ cytoskeletal 4 is a protein found in the cytoskeleton. It is 534 amino acids in length, and functions in a wide array of cellular roles including catalysis, binding of nucleotides and proteins, and motor and structural molecular activities[7]. Epidermal fatty acid-binding protein is a small cytoplasmic protein of 135 amino acids, which is primarily involved in catalysis, protein binding, and transporter activity[7].

Understanding cornea proteomics has helped identify key proteins which in turn increased bimolecular understanding of disease and functions of proteins in wound healing[17,18].

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE AQUEOUS HUMOR

The aqueous humor plays a substantial role in maintaining homeostasis within the eye. The pigmented and non-pigmented ciliary epithelium is responsible for production of aqueous humor, which is secreted into the posterior chamber. From the posterior chamber a majority of the aqueous humor traverses the trabecular meshwork, (a filter like structure), and flows into the Schlemm's canal where it continues on to bathe the cornea. A small amount of the aqueous humor follows a less conventional pathway, the uveosceral pathway. The aqueous humor distributes through many sections of the anterior eye and is thus a key component in looking for proteomic biomarkers. A complication in the investigation of these biomarkers is that there is only 150-200 μL of aqueous humor in an average age individual and this amount decreases with age. There is also a low overall protein concentration present in the aqueous humor. These obstacles can make protein analysis in the aqueous humor challenging and with time, specialized techniques have evolved to provide more accurate analysis. Through the evolution of these specialized techniques, different groups have used specific techniques to analyze the protein make-up of the aqueous humor.

The aqueous humor is abundant in numerous proteins such as antioxidant proteins, immunoregulatory proteins, and anti-angiogenic proteins. These proteins were identified using Multidimensional Protein Identification Technology (MudPIT)[19]. protein composition of the aqueous humor is intricate as it is a key regulatory component of the eye. Up to 676 nonredundant proteins have been identified in the aqueous humor of patients with no disease. These proteins were identified using nanoflow liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC-ESI-MS/MS). An issue that complicates this type of identification is the high prevalence of albumin, a protein that which makes up 50% of the proteins in the aqueous humor. Its abundance result in the masking of less abundant proteins during analysis. In order to overcome this issue, immunodepletion of several aqueous humor samples of albumin, transferrin, antitrypsin, haploglobin, fibrinogen, IgG, and IgA is commonly performed[13]. The presence of complement regulatory molecules, specifically 23 complement proteins, demonstrates the importance of the aqueous humor in maintaining a healthy environment and protecting against autoimmune disease. Catalytic enzymes crucial for respiratory pathways are also present in the aqueous humor, specifically aldolase and ketolase. Angiogenin, and angiogenic inducer were present along with angiogenic inhibitors, specifically PEDF, type Ⅳ collagen, and vitamin D binding protein. Finally, members of the transforming growth factor β (TGFβ), tumor necrosis factor (TNF), fibroblast growth factor, interleukin, and growth differentiation families were also present in the aqueous humor[20]. Taken together, the numerous components present in the aqueous humor make it a powerful regulatory mechanism for maintaining homeostasis in the eye.

The identification of aqueous humor proteins in normal samples provided a baseline for further investigation

to take part in diseased counterparts. The study of protein levels in the aqueous humor in diseased individuals provides substantial information for potential biomarkers to possibly identify disease earlier. Analyzing these protein levels also assists in further profiling the protein composition of the aqueous humor. Glaucoma refers to a family of eye optic nerve disorders, some of which are associated with increased intraocular pressure (IOP). The most common form of glaucoma is primary open angle glaucoma. Research has been carried out to analyze alterations in the protein composition of the aqueous humor in patients with increased IOP. Endothelial leukocyte adhesion molecule 1 (ELAM 1) plays a key role in inflammation and is significantly increased in glaucomatous aqueous humor. Interestingly, apolipoprotein B and E are present in increased amounts. Typically, these proteins are responsible for in the delivery of cholesterol to cells. Another set of proteins present are responsible for muscle cell differentiation and function, specifically, myotrophin, myoblast determination protein 1, myogenin, vasodilator-stimulated phosphoprotein, and ankyrin-2. Presence of stress response proteins such as heat shock 60 kilodaltons (kDa) and 90 kDa proteins as well as ubiquitin fusion degradation 1-like are responsible for the removal of damaged protein. Finally, phospholipase C, β, and γ are shown to take part in signal transduction as well as neural development[21]. Similarly, in an investigation performed in patients with primary congenital glaucoma, a select set of proteins was shown to be upregulated and downregulated. Apolipoprotein A-Ⅳ (APOA-Ⅳ) is a plasma protein commonly involved in lipid absorption and transport. This specific protein is increased in glaucomatous samples. Albumin was also increased in these samples. This protein is crucial for maintenance of colloid osmotic pressure of plasma, antioxidant activity, regulation of normal microvascular permeability as well as fatty acid, and hormone transport. Another protein increased in glaucomatous aqueous humor is antithrombin 3 (ANT3 or SERPINC1), a protease inhibitor belonging to the serpin family. There were several proteins downregulated in glaucomatous samples including Transthyretin (TTR), Glutathione independent prostaglandin D synthase (PTGDS), opticin (OPT), and Retinol binding protein 3 IRBP. TTR is the main iodothyronine-binding protein that transfers T4 from the blood in the brain across the blood-choroid plexus barrier and tends to decrease in serum when acute inflammation is taking place. PTGDS is responsible for converting prostaglandin H2 (PGH2) to prostaglandin D2 (PGD2), common in smooth muscle contraction/relaxation as well as platelet aggregation inhibition. This protein has been demonstrated to bind to retinal and retinoic acid, key players in tissue development/maintenance. OPT, a member of the small leucine-rich repeats proteoglycan (SLRP) gene family is believed to be anti-angiogenic, is present in normal aqueous humor. IRBP is a glycoprotein synthesized by rods and cones. This protein binds to retinoids as well as fatty acids and may act as a retinoid transporter[22]. The presence of these proteins further supports the idea of necessary equilibrium between different elements in the eye that needs to take place in order to maintain a healthy environment.

The profiling of the proteins in the aqueous humor has given insight to its importance as a regulator in many aspects of the eye. Investigating these proteins in the normal state has been as important as investigating those in the diseased state. Overall, the investigations carried out in this area further supports underline the importance of maintaining specific protein levels in the aqueous humor.

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE TRABECULAR MESHWORK

The trabecular meshwork (TM) plays a fundamental role in the regulation of intraocular pressure (IOP) and is pathophysiologically involved in the development of glaucoma. The TM can be divided into the uveal, corneoscleral and juxtacanalicular meshworks. It consists of collagen beams, covered by endothelial cells and surrounded by extracellular matrix (ECM)[23,24]. Until recently, the pathogenesis of outflow resistance at the TM was largely unknown. Understanding the pathogenesis that contributes to outflow resistance has recently increased. We now know that TM cell gene expression alters with IOP and mechanical stress[25] which can induce changes in cell proteins. This can lead to altered cell behavior including the increased tendency of the TM to contract with raised IOP[26,27], alterations in metabolic processes, cell adhesion, signal transduction, regulation of transcription, increased stretch activated channels[28], and the remodeling of extracellular matrix of TM in POAG[29-32].

Proteomic analysis of the TM has played a major role in understanding the mechanisms involved in outflow obstruction. Over 850 proteins have been identified in the TM[32] and multiple studies have found alterations in the expression of proteins when IOP is raised[32-34]. Multiple proteins are altered in location and quantity with glaucoma. We previously discovered that cochlin, a protein of unknown function is present in conjunction with stretch activated channels, in glaucomatous TM in human eyes but absent in normal samples[35]. Cochlin was also uniquely found in DBA/2J mice with hypertensive IOP but absent in DBA2J with a normal IOP[36]. A study by Yu *et al*[32] used 2-DE protein-expression, combined gel-spot to identify proteins in the TM of human donors, some of which were cultured in dexamethasone. This study found 877 proteins in human TM, several of which were previously associated with glaucoma. Several proteins belonged to cytoskeletal protein families/extracellular matrix proteins, such as vimentin, lamin, actin, and annexin. The highest proportion of proteins found were involved in metabolic processes (13%), and similar percentages of proteins were involved in anti-apoptosis, motility, carbo-

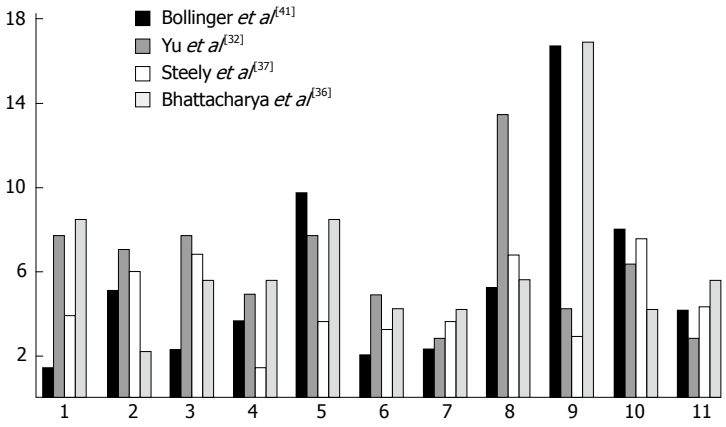

Figure 1 Comparing common protein functions in trabecular meshwork of eyes between fours students. 1: Anti-apoptosis; 2: Carbohydrate metabolic process; 3: Cell adhesion; 4: Cell cycle; 5: Cell motility; 6: Cell proliferation; 7: Lipid metabolic process; 8: Metabolic process; 9: Protein folding/metabolism; 10: Signal transduction; 11: Transport.

hydrate metabolism (10%-11%) (Figure 1). In contrast, few proteins were found to play roles in cell division and cell to cell signaling. Another study which grouped protein by their function found the largest number group were in protein folding (16.8%) which was significantly more than what we and Yu *et al*[32] found (2.9% and 4.2%).

Myocilin is a protein found in the TM; mutations in this protein have been associated with glaucoma[38-40]. Myocilin a prominent component of TM exosomes, suggesting that exosomes could contribute to aqueous humour outflow from the trabecular meshwork. As there are few studies which have examined TM exosomeoses proteomics and exosome protein mutation is involved in disease, this is an area of which deserves further investigation.

Transforming growth factor beta 2 (TGFβ2) is often elevated in the TM of patients with POAG. Bollinger *et al*[41] examined TGFβ2-induced proteomic changes from four donors who were treated with or without TGFβ2. Cellular proteins in the TM were then analyzed by liquid chromatography-mass spectrometry iTRAQ. This study found that TGFβ2 significantly altered 47 proteins. More than half of the elevated proteins induced extracellular matrix remodeling and cytoskeleton interaction. Thirty proteins were elevated and 17 decreased after TGFβ2 treatment. CD9 antigen and mitochondrial superoxide dismutase 2 (SOD2) were the most significantly reduced proteins 64% and 46%, respectively. Interestingly the proteins most greatly decreased were from the mitochondria (40%). Downregulation of mitochondrial proteins may result in mitochondrial dysfunction and reduced ATP production, which may lead to disruption of outflow dynamics.

Overall TM proteomic studies have identified multiple proteins alterations associated with hypertensive IOP. Modulated protein patterns in glaucomatous eyes have emerged through proteomic studies. Future studies may look further into the gene expression of these altered proteins for a better understanding of their occurrence.

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE CILIARY BODY

The ciliary body is a circumfirential layer of tissue behind the iris in the anterior chamber of the eye. Its epithelium serves as the main production center of aqueous humor. In recent years, literature regarding the proteome of the ciliary body has been sparse and had utilized immunohistochemistry, immunofluorescence, and Western blot technology, resulting in the characterization of fewer than 50 discrete proteins[42]. However, in 2013 Goel *et al*[42] profiled the ciliary body proteome utilizing MS/MS analysis on an LTQ-Orbitrap Velos ETD mass spectrometer. In this study, samples from the human ciliary body were processed and run on an SDS-PAGE. The bands were subsequently excised and digested with trypsin prior to LC-MS/MS analysis. MS data was then searched against the NCBI protein database, and 2815 proteins were characterized. Included in these data were proteins previously identified using the aforementioned techniques, including collagen type XVIII alpha 1 (COL18A1), cytochrome P450 family 1 subfamily B polypeptide 1 (CYP1B1), Opticin (OPTC), and aquaporin 1 (AQP1). Several of these proteins have possible implications in ocular disease. OPTC has been investigated as a possible target for primary open angle glaucoma. AQP1 is involved in the production of aqueous humor and its movement into the anterior chamber[42].

Goel *et al*[42] also identified a large number (> 2000) of proteins which were unknown to exist in the ciliary body. Some of these novel molecules include proteins involved in metabolism and energy pathways such as Neutrophil cytosol factor 2, Myosin-11, Pyruvate kinase isozymes M1/M2, and Alpha-1-antitrypsin. Other proteins such as ER lumen protein retaining receptor 2, Tubulin beta-2A chain, Exportin-1 are involved in transport mechanisms. Exportin-1 is overexpressed in cancer cells. Leukocyte surface antigen CD47 and complement C3 are part of the immune response mechanism. Desmin is an intermediate filament, which when defective is involved in several myopathies.

The Goel *et al*[42] group further investigated the proteins that were common and disparate between the ciliary body and plasma, and the ciliary body and aqueous humor. The majority of proteins found in the ciliary body (1895 of 2791) were also found in the plasma, which contained a total of 9393 proteins and therefore had 7498 unique proteins. In the comparison of the ciliary body and aqueous humor, 211 of the 2891 ciliary body

proteins were also found in the aqueous humor, leaving 321 unique aqueous humor proteins. These comparisons are important to know which proteins are natively found in the ciliary body, and which of them may have originated from elsewhere. In the future, work regarding ciliary body proteomics may explore the proteins now known to be unique in order to investigate further therapeutic targets.

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE IRIS

Mass spectrometric analyses of the human iris proteome have not been well-published. Other methods of proteomic analysis have been used on a small number of known iris proteins. One such example includes the immunohistochemical analysis of Opticin (OPTC)[43]. The is protein was identified using an antibody targeting its amino terminal[43,44]. OPTC is the ortholog of a cDNA sequence which has been shown to be expressed abundantly in the iris[42,44]. Mass spectrometric analyses of this and other iris proteins are required to better characterize the more complete human anterior chamber proteome.

Mass spectrometric proteomic analyses of the lens

The Human lens is responsible for the refractive properties of the eye. It is avascular and contains one layer of epithelium found in the anterior capsule and posterior capsule. The lens is mostly acellular, consisting mainly of crystalline proteins with some non-crystalline proteins also present[44]. Its main function is to change shape and thus allow for accommodation of vision. Another function of the lens is to maintain transparency. Loss of accommodation results in presbyopia and loss of transparency results in cataract. There are 3 main types of crystalline proteins in the human body, including type α, β, and γ. Type α-A is a heat shock and chaperone protein and is found mostly in the lens while α-B is ubiquitous throughout the human body. It was also known that the α-crystallines play a role as heat shock proteins and are chaperone proteins. Most recently protein analysis was performed in a mice mouse model in which the genes responsible for the α-crystallin wereas missing. This was carried out to determine what happens with the other proteins inside of the lens giving further insight into the development of cataracts[45]. Wild type and αA/αB knockout mice were compared using two-dimensional gel electrophoresis and mass spectrometry. There was a greater abundance of histones H2A,H4, and H2B fragment, and a low molecular weight β1-catenin in postnatal 2 d of the knockout mice. There was increased abundance of βB2-crystallin and vimentin in 30 d-old lenses of knockout mice. Gel permeation chromatography was able to demonstrate an aggregation of β-crystalline. Therefore, the absence of crystalline type αA and αB resulted in changes of protein expression indicating that lens proteins also result in interactive functions beyond just plain functions the. Aggregation of α crystalline was also found by recent Matrix-assisted laser desorption/ionization (MALDI) studies[4].

Type γ requires the use of post-translational modification in order to maintain its transparency. Given that crystallins are life-long proteins, post-translational modification may play a role in the development of cataracts[46]. Heat and deamidation (a chemical reaction in which an amide functional group is removed from an organic compound and damages the amide-containing side chains of asparagine and glutamine) may play a role in the change of the physical properties of the protein. This study used 2D LC-MS/MS to examine which major lens proteins undergoes deamidation and the exact sites of deamidation. It was found that all of the major proteins found in the lens were deamidated. Each crystallin protein differed in the sites and extents of deamidation. Many of the areas of deamidatation were characterized by the presence of a basic amino acid one residue from the glutamine and asparagine.

Although the lens consists mostly of crystalline proteins, the advent of new analytical techniques allowed for analysis of proteins involved in lens besides crystalline. One of the first complete proteomics studies to address the protein inside of the lens was in 2008[47]. The lens from fetal, cataract, and normal lenses were evaluated by 2D LC-MS/MS and PANTHER was used for protein classification. This study identified a total of 231 proteins across all of the lens samples. Fetal samples showed the highest amount of unique proteins compared to cataract and normal lenses. A 5-mm core of lens was used in the adult which some lacked epithelial and outer cortical fibers which play a role in the metabolic machinery of the lens. The fetal samples were all pooled together. While many studies have shown the crystallin class as the dominant protein, this study showed that many low abundance proteins existed in the lens.

A more recent study[48] identified using MALDI and concentrating on the major protein differences for identification was performed in order to determine the different between the proteins in age-related cataracts and normal lens nuclei . Observers graded cataracts and total solubilized proteins were compared using gel electrophoresis. MALDI was used to identify the proteins that had different abundances. LC-MS/MS analyses determined the compositions of > 200 kDa molecular weight aggregates found in age related nuclear cataract lens nuclei. It was identified that α, β-A3, βA4, βB1, and γD-crystallin were involving with the higher molecular weight aggregates. An uncharacterized protein found and this protein, along with αA, αB, and γ-D crystallin, were more found to be more prone to aggregation. Therefore, aggregation of crystallins may account for the development of cataracts. Also, some enzymes may play a role in the protein aggregation and possibly accelerate the process.

Membrane proteins were purified from young mouse lenses and shotgun proteomics was employed in order to analyze the membrane proteins of the mouse lens cells[49]. These same techniques were then applied to analyze the

human lens protein of the membrane[50]. HPLC-mass spectrometry with multidimensional protein identification technology (MudPIT) with and without phosphopeptide enrichment was applied for the study of the proteome of the lens membrane. There were 951 proteins that were identified in which 379 were membrane and membrane-associated proteins. Many of these proteins are responsible for carbohydrate metabolism, proteasome, cell-cell signaling and communication, glutathione metabolism and actin regulation.

LOXL-1 protein and apolipoprotein E, both found in the extracellular matrix, were abnormal in pseudoexfoliation syndrome, a disease of the anterior lens capsule[51]. This study performed mass spectrometry on isolated surgically removed anterior capsules in patients with pseudoexfoliation syndrome. Direct analysis showed LOXL-1 protein and apoliprotein E which shows that these extracellular matrix proteins play a role in pseudoexfoliation[52]. This study employed MALDI imaging on the anterior capsule which showed presence of LOXL-1 protein was more abundant in the iris region and apolipoprotein E in the pseudoexfoliation deposits in anterior capsule in the pupillary area. There could also be significant post-translational modification involved in promoting the aggregation of proteins.

The lens is unique in that it contains many fibers that are acellular and proteins that exist for the lifetime of the individual. The advantage of studying the proteomics of the lens is that it may provide a powerful model for the rest of the human body with regard to understanding the changes involved in proteins that are maintained throughout a lifetime. It is essential that the proteins maintain transparency, and aggregation may result in lack of solubility resulting in cataracts. Proteomics work has showed that α-crystallins play a role in preventing aggregation and serving as chaperone proteins. α-crystallins are present only in the lens while α-B crystallin is ubiquitous throughout the human body and dysfunction of the α-B protein has been implicated in many degenerative disorders. Post-translational modification also plays a role in the lens protein.

CONCLUSION

Identification of proteins in different regions of the anterior chamber including the: cornea, aqueous humor, trabecular meshwork, ciliary body, and lens has expanded in recent years. Among other proteomic methods, mass spectrometry has enabled rapid protein sequencing while simultaneously determining posttranslational modifications in the amino acid residues. Mass spectrometry has rapidly evolved since 1990, allowing improved identification of proteins. Although the advances in mass spectrometry ~~had~~ have been rapid, the identification of proteins from tissue or cell samples often remains unsatisfactory. Currently approximately 5000 proteins from each anterior eye segment tissue or fluid is identified against a theoretical prediction of 20000 proteins. Thus at best approximately 25% of actual proteins are captured compared to theoretical estimates. Part of the reason why protein identification is relatively poor compared to mRNA is due to differences in the chemistry of RNA and proteins. The identification of posttranslational modifications of proteins, remains another frontier in mass spectrometry (or any other suitable high throughput method) that is yet to be conquered. One important issue remaining to be elucidated is the process of natural aging. Several age-related changes that can be easily quantified occur in eyes such as prebyopia and the progressive ability to form sharp images. Several eye diseases are also age associated such as age-related macular degeneration and glaucoma. important insight into true age related changes, and the result of aging and disease on protein turnover. The Current methods do not allow the juxtaposition of mRNA and protein information together. Modern proteomic methods lack in their ability to juxtapose mRNA and protein information from inactive proteins, deactivated proteins, or proteins undergoing degradation. These are the avenues for future advancement which will expand our insight into how protein-drug interactions keeps proteins in their active states. We presented an account of current state of proteins in different regions of anterior eye chamber and what improvement has occurred compared to that in the previous decade. Further improvements will enable us to address the question of protein turnover in tissues and better enable us to distinguish active, inactive, partially degraded, and degraded states of proteins.

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REVIEW

Role of PRMTs in cancer: Could minor isoforms be leaving a mark?

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Abstract

Protein arginine methyltransferases (PRMTs) catalyze the methylation of a variety of protein substrates, many of which have been linked to the development, progression and aggressiveness of different types of cancer. Moreover, aberrant expression of PRMTs has been observed in several cancer types. While the link between PRMTs and cancer is a relatively new area of interest, the functional implications documented thus far warrant further investigations into its therapeutic potential. However, the expression of these enzymes and the regulation of their activity in cancer are still significantly understudied. Currently there are nine main members of the PRMT family. Further, the existence of alternatively spliced isoforms for several of these family members provides an additional layer of complexity. Specifically, PRMT1, PRMT2, CARM1 and PRMT7 have been shown to have alternative isoforms and others may be currently unrealized. Our knowledge with respect to the relative expression and the specific functions of these isoforms is largely lacking and needs attention. Here we present a review of the current knowledge of the known alternative PRMT isoforms and provide a rationale for how they may impact on cancer and represent potentially useful targets for the development of novel therapeutic strategies.

Key words: Protein arginine methyltransferase; Arginine methylation; Cancer, Alternative splicing; Isoforms

Core tip: This review focuses on the current knowledge regarding alternative protein arginine methyltransferases (PRMT) isoforms and evidence supporting their potential impact in cancer. Alternative PRMT isoforms have been identified for PRMT1, PRMT2, CARM1 and PRMT7 and more may exist for the other PRMT family members. The presence of these isoforms adds a layer of complexity to the functional roles PRMTs play in normal and disease contexts. These alternative isoforms have unique characteristics that may offer clarification to conflicting roles documented in the literature. Finally, understanding the specific functions of these isoforms is crucial for fully characterizing the therapeutic potential of PRMTs in cancer.

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INTRODUCTION

Cancer is a leading cause of death worldwide. As we improve our understanding of the complex biologic processes behind this devastating disease we are able to develop improved treatments and increase patient survival. The biology of human tumours has been characterized as

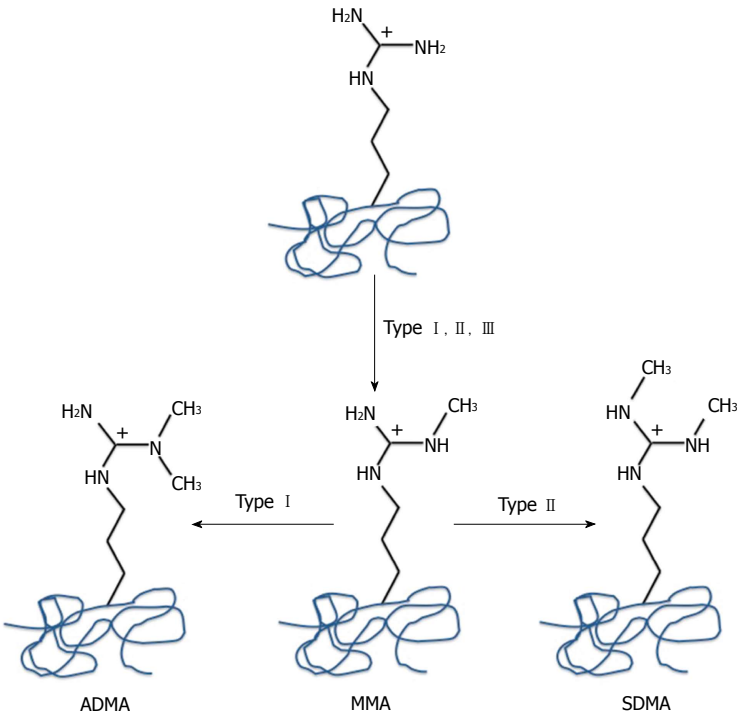

Figure 1 Arginine methylation reactions catalyzed by protein arginine methyltransferases. Type Ⅰ protein arginine methyltransferases catalyze the asymmetric dimethylation of arginine residues, Type Ⅱ symmetrically dimethylated arginine and Type Ⅲ monomethylated arginine residues.

having six key hallmarks: sustained proliferative capacity, evasion of growth suppressors, resisting death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis[1]. Each of these features is distinct, but they all cooperate to promote tumour development, growth and aggressiveness. Identifying key molecular regulators of one or more of these characteristics is essential in understanding cancer and potentially discovering new and better therapeutic strategies.

Arginine methylation is a common posttranslational modification that is known to have a role in several cellular processes, including signal transduction, DNA repair, transcription, protein subcellular localization and RNA processing[2,3]. Arginine methylation, in mammalian cells, is catalyzed by a family of enzymes called protein arginine methyltransferases (PRMTs). This family currently consists of nine characterized members in higher eukaryotes. These enzymes are subdivided into three categories based on the type of methyl mark produced on the arginine residue. These methylation reactions are depicted in Figure 1. Type Ⅰ [PRMT1, 3, 4 (CARM1), 6, and 8] generate ω-N^G,N^G-asymmetric dimethylarginine. Type Ⅱ (PRMT 5 and potentially PRMT9) generate ω-N^G,N^G-symmetric dimethylarginine. Finally, Type Ⅲ generate ω-N^G-monomethylarginine residues. Recently, it has been demonstrated that PRMT7 is the only bona fide type Ⅲ methyltransferase[4,5]. The majority of arginine methylation is catalyzed by PRMT1 (asymmetric) and PRMT5 (symmetric), and loss of expression of either of these enzymes is not compatible with life[6,7]. Currently, there is more that 120 known arginine methylated proteins, including histone and non-histone proteins[8,9]. The list of arginine methylated protein substrates is constantly growing, and along with it the discovery of new functional roles and involvement in numerous regulatory pathways[8,10,11].

Accumulating evidence convincingly shows that arginine methylation may represent a driving force behind the development, progression and aggressiveness of several cancer types. While the link between arginine methylation and cancer is a relatively new area of interest, the roles that the PRMTs have been shown to play in cancer thus far demonstrate their importance. These roles and the cancer types that have been studied are highlighted in Table 1. Dysregulated PRMT expression has been observed in a number of human tumours, including lung, breast, prostate, colorectal, bladder and leukemia[12-19]. For a comprehensive review summarizing the roles of each PRMT family member in cancer see Yang and Bedford's review article in Nature Reviews: Cancer entitled, Protein arginine methyltransferases and cancer[20]. The primary focus of this review is to specifically highlight the current knowledge regarding alternatively spliced PRMT family members and the potentially distinct roles that they play in cancer. While a survey within the *Ensembl* database predicts the existence of alternatively spliced isoforms for all the *PRMT* gene family members, only the expression of PRMT1, PRMT2, CARM1 and PRMT7 isoforms has been characterized and confirmed in mammalian cells[21-27].

Interestingly, the majority of these alternative isoforms were found in cancer cells, suggesting they may have specific roles in cancer. Characterization of several of these alternative PRMT isoforms has shown that they are differentially expressed in various cell types and they possess distinct functional characteristics. However, the individual roles that these alternative isoforms play in cells remains poorly understood and understudied. There-

Table 1 Protein arginine methyltransferases in cancer cells

PRMT	Cancer type	Role(s) in cancer	Ref.
PRMT1	Breast cancer, Lung cancer, Colon cancer, Bladder cancer, Acute myeloid leukemia, Mixed lineage leukemia	Cell proliferation and survival, Transformation, Resistance to DNA damaging agents, Invasion	[13,15-17,19, 21,36-38]
PRMT2	Breast cancer	Cell proliferation and invasion	[22,72]
PRMT3	Breast cancer	Cell survival	[101,102]
CARM1/PRMT4	Breast cancer, Prostate cancer, Colorectal cancer	Cell proliferation	[12,14,77-79,88]
PRMT5	Lung cancer, Leukemia, Lymphoma, Melanoma, Gastric cancer, Colorectal cancer	Cell proliferation, Transformation, Invasion, Resistance to DNA damaging agents	[18,103-109]
PRMT6	Lung cancer, Bladder cancer	Cell proliferation	[17,110]
PRMT7	Breast cancer	Resistance to DNA damaging agents	[27,91,92,94]
PRMT8	ND	ND	
PRMT9	ND	ND	

ND: Not determined; PRMT: Protein arginine methyltransferase.

fore, more attention needs to be given to their individual functions under normal biological conditions, as well as their contribution to diseases such as cancer. PRMTs are thought to be potentially useful therapeutic targets for the treatment of diseases such as cancer[28]. Moreover, these alternative PRMT isoforms must be taken into account when designing and evaluating potential candidate therapeutic strategies or compounds. This is essential so there is a clear understanding of the precise mechanism of action. Although our knowledge of the specific roles of these isoforms is limited, there is evidence in the literature strongly suggesting that they are not redundant. While they may share some similar functions, they also have clearly distinct roles.

PRMT ISOFORMS AND CANCER

PRMT1

PRMT1 is a Type I arginine methyltransferase and is responsible for generating upwards of 85% of the asymmetrically dimethylated proteins within cells[29]. PRMT1 is the most well characterized protein within this family of enzymes. While the PRMT1 protein is mainly described in the literature as a single entity, it has been identified, that at least seven distinct PRMT1 isoforms are generated by complex alternative splicing in the 5' region of its pre-mRNA[21,30,31]. The exon structure for the identified PRMT1 isoforms is summarized in Figure 2 and detailed in Goulet *et al*[21] 2007. Each of these isoforms, named PRMT1v1-v7, has distinct characteristics in terms of expression. PRMT1v1 is the most abundantly expressed isoform and likely represents the isoform that is described as PRMT1 in most reports. The expression levels of PRMT1v1, v2 and v3 have all been shown to be ubiquitous across tissues[21,30,31]. Interestingly, a higher level of PRMT1v1 mRNA expression is observed in the kidney, liver, lung, skeletal muscle and spleen[21]. PRMT1v2 mRNA was found to be elevated in the kidney, liver and pancreas, while, PRMT1v3 mRNA expression was observed at similar levels in all tissues examined (brain, heart, kidney, liver, lung, pancreas, skeletal muscle and spleen), however at low levels compared to PRMT1v1 and PRMT1v2. The mRNA expression levels of PRMT1v4 to v7 showed a more tissue specific profile, with v4 being detected only in the heart, v5 mainly in the pancreas, and v7 observed in the heart and skeletal muscle. PRMT1v6 mRNA was not detected in any normal tissues examined[21]. Further studies would need to be performed to determine if this differential expression has any correlation with the development of cancer from a particular tissue of origin.

While tissue specific expression of PRMT1 isoforms is observed, at the cellular level there are also differences in their subcellular localization (Table 2). PRMT1v3, v4, v5 and v6 all show an equal distribution of nuclear and cytoplasmic expression[21]. In contrast, PRMT1v1, v2 and v7 display a more compartmentalized expression profile within cells. PRMT1v1 and v7 display a more intense nuclear expression, while PRMT1v2 is expressed predominantly in the cytoplasm, however this may vary depending on cell type and methylation status of substrates as it was clarified by the Fackelmayer lab[32,33]. The cytoplasmic expression of PRMT1v2 is due to the retention of exon 2 within the N-terminal coding sequence. This short exon contains a leucine-rich nuclear export sequence (NES). Careful analysis showed that this NES does in fact control the nuclear export of PRMT1v2 and that its export is dependent on the nuclear export receptor CRM1[21].

A comparison of the PRMT1 isoforms revealed they have dstinct enzymatic activity and substrate specificity profiles[21]. Additionally, stable isotope labeling by amino acids in cell culture (SILAC[34,35]) followed by immunopurification of PRMT1v1 and PRMT1v2 from cells has been used to identify their isoform-specific protein binding partners and/or substrates (Figure 3). In Figure 3 we show the full data set from this analysis comparing the SILAC ratios of PRMT1v1 and PRMT1v2 binding proteins (unpublished data). Each point represents an identified interacting protein. This clearly shows that there is a potential set of PRMT1v1-specific interacting proteins (lower right quadrant) and PRMT1v2-specific interacting proteins (upper left quadrant). Also, there are some com-

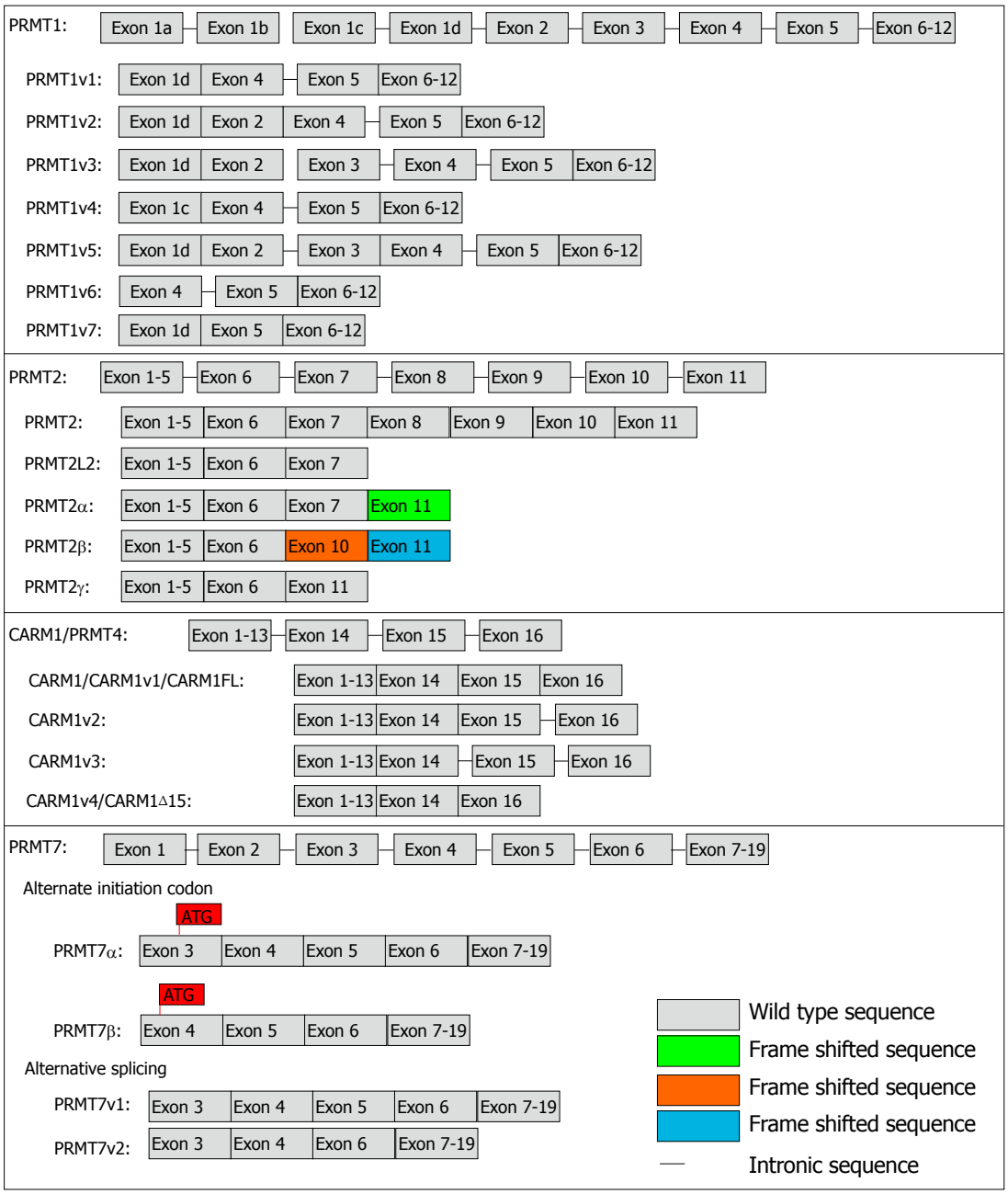

Figure 2 Protein arginine methyltransferase variant isoforms. Schematic representation of the identified variant isoforms of protein arginine methyltransferase (PRMT) 1, PRMT2, CARM1/PRMT4 and PRMT7. The PRMT1 sequence has 12 exons. Exon organization of the seven identified PRMT1 isoforms are shown. The intronic sequences (-) that have been shown to be included in several of these alternative PRMT1 isoform transcripts are due to the splicing sites[21]. PRMT2 is made up of 11 exons. The PRMT2L2 transcript is produced as a result of alternative polyadenylation[72]. This silences the 5' splice site on exon 7 and results in a transcript retains a significant portion of intron 7 and a premature termination codon. PRMT2α has a deletion of exons 8-10 with a frame shift that produces 12 new amino acids at the C-terminus (ν). The PRMT2β isoform has a deletion of exons 7, 8, 9 resulting in a frame shift that generates 83 alternate amino acids at the C-terminus (νν). PRMT2γ has an in frame deletion of exons 7 to 10. The full-length CARM1 gene, CARM1/CARM1v1/CARM1FL, consists of 16 exons. CARM1v2 is generated through retention of the intron 15 sequence; CARM1v3 is produced through the retention of introns 15 (-) and 16 (-). CARM1v4/CARM1Δ15 results from the skipping of exon 15[23,24]. The PRMT7 sequence consists of 19 exons. In Hamster cells, these two PRMT7 isoforms (α and β) are thought to be generated by the use of distinct 5' translation initiation codons within the primary transcript. The PRMT7β isoform sequence contains 37 extra amino acids at the N-terminus. Alternatively, at least 2 alternatively spliced PRMT7 isoforms can be produced from the human PRMT7 gene. These two isoforms have the same N- and C-terminal regions but variant 2 (PRMT7v2) has an in frame deletion of exon 5.

mon binding partners (upper left quadrant). This emphasizes the importance of understanding their individual functions. Conservation of these alternatively spliced isoforms of PRMT1 through evolution suggests they are likely to each have their own function(s) within cells and tissues.

Deregulated PRMT1 expression has been observed in a number of tumour types, which include those of the lung, breast, colon, bladder and leukemia[13,15-17,19,21,36-38]. The question is then, "What are the functions of these

Table 2 Protein arginine methyltransferase isoform specific subcellular localization and current cancer cell types in which they have been shown to be expressed

PRMT isoform	Molecular weight (kDa)	Subcellular localization	Cancer cell type	Ref.
PRMT1v1	40.5	Predominantly nuclear	Breast cancer cell lines and tumour samples, cervical cancers cells	[21]
PRMT1v2	42.5	Predominantly cytoplasmic	Breast cancer cell lines and tumour samples, cervical cancers cells	[21,39]
PRMT1v3	39.9	Cytoplasmic and nuclear	Breast cancer cell lines and tumour samples	[21]
PRMT1v4	40.1	Cytoplasmic and nuclear	Breast cancer cell lines	[21]
PRMT1v5	39.4	Cytoplasmic and nuclear	Breast cancer cell lines	[21]
PRMT1v6	37.7	Cytoplasmic and nuclear	Breast cancer cell lines	[21]
PRMT1v7	36.7	Predominantly nuclear	Breast cancer cell lines	[21]
PRMT2	48.5	Predominantly nuclear, excluding nucleoli	Breast cancer cell lines and tumour samples	[22,72]
PRMT2L2	32	Predominantly cytoplasmic	Breast cancer cell lines and tumour samples	[72]
PRMT2α	32.6	Predominantly nuclear, excluding nucleoli	Breast cancer cell lines and tumour samples	[22]
PRMT2β	34	Cytoplasmic and nuclear, including nucleoli	Breast cancer cell lines and tumour samples	[22]
PRMT2γ	25.8	Predominantly nuclear, excluding nucleoli	Breast cancer cell lines and tumour samples	[22]
CARM1/CARM1v1/ CARM1FL	66	ND	Breast cancer cell lines	[23,24]
CARM1v2	71	ND	Breast cancer cell lines	[23,24]
CARM1v3	63	ND	Breast cancer cell lines	[23,24]
CARM1v4/CARM1Δ15	64	ND	Breast cancer cell lines	[23,24]
PRMT7α	78	Cytoplasmic and nuclear	ND	[27]
PRMT7β	82	Predominantly cytoplasmic	ND	[27]

ND: Not determined; PRMT: Protein arginine methyltransferase.

isoforms and do they have specific roles in cancer?" To date our knowledge is limited as to the specific functions of each of these PRMT1 isoforms. However, there is evidence showing potential individual roles for them in cancer. In breast cancer, both the mRNA and protein expression of several alternative PRMT1 isoforms is elevated (Table 2)[21,31]. This is observed not only in breast cancer cell lines, but also in breast tumours. Specifically, the mRNA expression of PRMT1v1, v2, v3 and v7 is elevated across several breast cancer cell lines compared to a non-transformed mammary epithelial cell line[21]. In contrast, PRMT1v5 and v6 were upregulated only in a subset of breast cancer cell lines. Furthermore, PRMT1v1, v2 and v3 mRNA expression was increased in breast cancer tumour tissue compared to normal tissue. Interestingly, while this study concluded an overall upregulation of PRMT1 alternative isoforms in breast cancer, the cytoplasmically localized PRMT1v2 isoform had the greatest increase in expression in breast cancer compared to PRMT1v1, the most abundantly expressed isoform. It is difficult to assess the protein expression of each of these individual isoforms due to the sequence similarities between them. However, in the case of PRMT1v2, exploitation of the exon 2 sequence has allowed for a more specific examination. Indeed, results have shown that PRMT1v2 protein expression is elevated in breast cancer cells[21]. A recent clinical assessment of PRMT1v1, v2 and v3 expression within breast cancer tissues has identified that high PRMT1v1 mRNA expression correlates with poor patient prognosis and a reduced disease-free survival[16]. An examination of PRMT1 protein expression within breast tumours *via* immunohistochemistry demonstrated a predominantly cytoplasmic expression and only in rare cases nuclear expression. We, and others have shown that PRMT1v2 is predominantly localized to the cytoplasm[21,32,39]. Therefore, one could speculate that PRMT1v2 could represent a significant proportion of the cytoplasmic PRMT1 detected in these breast tumour samples. This evidence shows that the expression of the PRMT1v2 isoform is elevated in breast tumours and it may have its own unique contributions to breast cancer progression. This also emphasizes the need to study these alternative isoform individually, in order to determine their specific functions and contribution to disease. While this has been mainly assessed in breast cancer thus far, it does not rule out that these PRMT1 isoforms may be expressed in other cancer types as well and this should be explored further.

The involvement of PRMT1 in cancer is supported by evidence showing its involvement in pivotal oncogenic processes. PRMT1 plays an active role in MLL-mediated transformation of primary myeloid progenitor cells[13]. PRMT1 has also been shown to have a significant role in cell proliferation/viability and cell cycle progression. Depletion of PRMT1 resulted in a significant decrease in the proliferation of osteosarcoma, breast, bladder and lung cancer cell lines[6,17,37]. This reduction in cell proliferation was associated with cell cycle arrest at the G_0/G_1 phase. Additionally, breast cancer cells showed a loss of cyclin D1 and increase in $p21^{cip1}$ expression, indicative of a cell cycle arrest at this phase[37]. While these studies examined PRMT1 as a whole, PRMT1 isoform-specific contributions have also been investigated. The specific depletion of the PRMT1v2 isoform using RNA interference in breast cancer cells resulted in a significant reduction in cell viability and growth[40]. This decreased cell viability was attributed, at least in part, to an induction of apoptosis occurring with the suppression of PRMT1v2

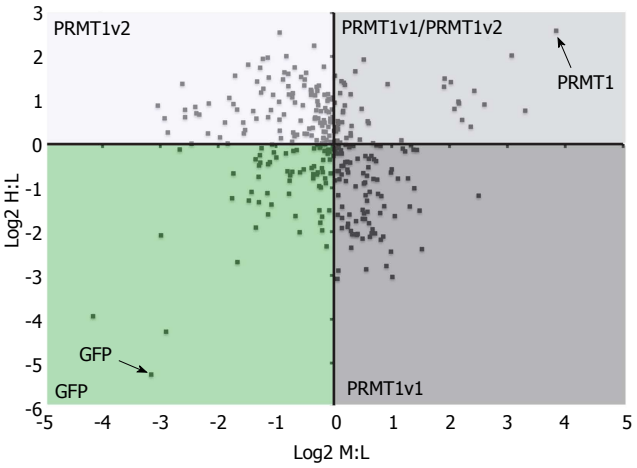

Figure 3 Protein arginine methyltransferase 1v1 and protein arginine methyltransferase 1v2 have potentially different interacting protein profiles. Stable isotope labeling by amino acids in cell culture (SILAC) and mass spectrometry was used to identify protein arginine methyltransferase (PRMT) 1v1 protein binding partners and PRMT1v2 protein binding partners. Cells stably expressing GFP alone, GFP-tagged PRMT1v1 or GFP-tagged PRMT1v2 were grown independently in media containing light (L), medium (M) and heavy (H) isotopes of arginine and lysine residues, respectively. Protein lysates were collected, immunoprecipitated for GFP (isolation of PRMT1v1 and PRMT1v2 interacting protein), and subjected to mass spectrometry for peptide identification. The Log2 of the SILAC ratios for the peptides identified from this experiment are plotted on the scatter plot. The x-axis is the Log2 of the H:L SILAC ratio or PRMT1v2 interacting proteins. The y-axis is the Log2 of the M:L SILAC ratio or PRMT1v1 interacting proteins. Each data point represents a single protein that was identified in this experiment. The greater this ratio is for a protein, the higher the probability of the interaction being real. This revealed a protein interacting profile identifying PRMT1v1-specific interacting proteins (PRMT1v1 quadrant), PRMT1v2-specific interacting proteins (PRMT1v2 quadrant) and common interacting proteins (PRMT1v1/PRMT1v2 quadrant; unpublished data). These results require further validation.

expression. Additionally, breast cancer cells overexpressing PRMT1v2 showed an increased growth rate, which was not observed upon PRMT1v1 overexpression and points to isoform specific effects. This evidence suggests that in these breast cancers cells PRMT1v2 may represent a key cell survival-promoting factor. Overall, this evidence links PRMT1 to the self-sustaining proliferative signaling acquired by cancer cells, enabling them to grow and survive.

The impact that PRMT1 has on the survival and aggressiveness of cancer cells is becoming increasingly evident with the identification of new intracellular substrates. It has been demonstrated that the asymmetric dimethylation of histone H4R3 is associated with active transcription and increased tumour grade in prostate cancer[41-43]. However, the downstream consequences of this methylation event are poorly understood in most cases[44]. Many of the recently identified PRMT1 substrates are key regulators of cancer cell growth, survival and invasion signaling. PRMT1 has been shown to influence receptor activation at the cell surface through direct methylation of the receptor or indirect methylation of a receptor associated protein. PRMT1 was shown to directly methylate the estrogen receptor α (ERα) at arginine (R) 260 and affects its downstream signaling[37,45]. This results in cytoplasmic retention of ERα and the interaction of ERα with Src, focal adhesion kinase (FAK) and the regulatory subunit of PI-3 kinase (p85). All three of which are involved in oncogenic intracellular signaling that promotes cancer cell survival and invasiveness[46-50]. Furthermore, loss of this methylation site on ERα, by point mutation, impaired downstream signaling, as evidenced by a loss of PKB/Akt phosphorylation. Recently, it was shown that PRMT1 is involved in the induction of transforming growth factor (TGF) β signaling in response to bone morphogenetic protein (BMP) binding its TGFβ receptors, R Ⅰ and R Ⅱ[51]. Activation of this receptor is achieved through the ligation and dimerization of the R Ⅰ and R Ⅱ receptors[52]. The R Ⅰ receptor is held in an inactive state by its association with Smad 6. Upon BMP ligation and dimerization of R Ⅰ and R Ⅱ, PRMT1 methylates Smad 6, causing its dissociation from RI and activation, thereby inducing BMP signaling which has a role in cancer stem cell proliferation and cancer cell invasion[53]. PRMT1 has also been shown to interact with PRMT8[54]. PRMT8 harbours a unique property, as it is tethered to the plasma membrane *via* an N-terminal myristoylation motif. Additionally, PRMT8 is specifically only expressed in brain tissue. This PRMT1-PRMT8 interaction effectively localizes PRMT1 activity at the plasma membrane and could potentially be affecting a distinct set of substrates. A specific role for PRMT8 in cancer has not been examined. These functions of PRMT1 occur in the cytoplasm of cells, and the RNA interference method used in these studies targeted all PRMT1 isoforms. Therefore, it would be of interest to assess whether specific PRMT1 isoforms might differentially contribute to the above-mentioned regulatory pathways. This would offer not only more functional understanding, but therapeutic insight as well.

PRMT1 has been shown to methylate key cytoplasmic proteins that are linked to apoptotic signaling pathways. Intriguingly, there have been conflicting roles presented for PRMT1 in apoptotic signal regulation. One study demonstrated that PRMT1 methylates apoptosis signal-regulating kinase 1 (ASK1) and this inhibits its activity[55]. This methylation promotes the interaction of ASK1 with its negative regulator, thioredoxin. As a consequence breast cancer cells were shown to be more resistant to treatment with paclitaxel. In contrast, the BCL-2 antagonist of cell death (BAD) has also been identified as a PRMT1 substrate in breast cancer cells[56]. This methylation prevents PKB/Akt mediated phosphorylation of BAD, thus preventing its inactivation, resulting in enhanced BAD-induced apoptosis. These conflicting roles highlight the complex role that methylation plays within cellular signaling pathways. These observations were seen in two distinct breast cancer cells, MDA-MB-231 and MCF7 respectively. Therefore, it is unknown whether these observations are due to cell specific behaviors or more interestingly the genetic differences between these two distinct breast cancer cells. Furthermore, they may also be influenced by differential expression of alternative PRMT1 isoforms, potentially reflecting differences in

function and substrate specificities within cancer cells.

A recent study identified Axin, a mainly cytoplasmic protein, as a PRMT1 substrate[57]. Importantly, it was shown that Axin could be methylated by two PRMT1 isoforms, PRMT1v1 and PRMT1v2 *in vitro*. However, this methylation analysis was not conducted within cells and would have been a very informative experiment, considering both Axin and PRMT1v2 share a cytoplasmic localization. Axin is a critical scaffolding protein that complexes with adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β), forming a degradation complex. This complex negatively regulates Wnt signaling and impacts actin cytoskeletal dynamics through the degradation of β-catenin[57,58]. Methylation of Axin by PRMT1 increases Axin protein stability, resulting in decreased β-catenin protein levels. Interestingly, isoform specific overexpression of PRMT1v1 or PRMT1v2 in a weakly invasive breast cancer cell line (MCF7) resulted in an increase in cell motility[40]. However, only the overexpression of the PRMT1v2 isoform increased cell invasion through a Matrigel barrier. Additionally, specific depletion of PRMT1v2 in an invasive breast cancer cell line, MDA-MB-231, resulted in decreased invasion through a Matrigel barrier. PRMT1v2 overexpression caused a decrease in β-catenin protein expression, which was not seen with the overexpression of PRMT1v1. This loss in β-catenin protein expression was directly linked to the PRMT1v2-induced invasion observed in breast cancer cells. Furthermore, PRMT1v2 enzymatic activities as well as proper subcellular localization were required for its ability to promote invasion. Therefore, it is conceivable that within cells Axin is preferentially methylated by PRMT1v2, thereby regulating β-catenin protein levels. This evidence has shown for the first time direct functional differences between PRMT1 isoforms in cancer, and identified a specific role for PRMT1v2 in promoting breast cancer cell invasion.

PRMT1 methylates several proteins within the nucleus that are involved in transcription, telomere stability and DNA repair. Similarly to the methylation of BAD, PRMT1 methylates the forkhead box protein 1 (FOXO1) at R248 and R250 blocking PKB/Akt-mediated phosphorylation of S253[59]. This methylation results in nuclear retention of FOXO1, increased transcriptional activity and increased oxidative-stress induced cell death. This evidence again supports a role for PRMT1 promoting cell death. PRMT1 also affects telomere length and stability, which impacts the replicative capacity of cancer cells[1,60]. PRMT1 methylates the telomeric repeat binding factor 2 (TRF2), thereby regulating its association with telomeres. TRF2 is a component of the sheltering complex that binds telomeric DNA and functions to protect telomeres and maintain their length. Depletion of PRMT1 in cancer cells increased the association of TRF2 with telomeres and promoted shortening. This supports a role for PRMT1 in dysregulated cancer cell replication. Additionally, PRMT1 is linked to the DNA damage response and DNA repair pathways through the methylation of MRE11 and p53 binding protein 1 (53BP1). PRMT1 has been shown to methylate MRE11 and 53BP1 within their GAR motif[61-63]. Methylation of MRE11 regulates its DNA exonuclease activity in response to DNA damage[61]. Similarly, methylation of 53BP1 is necessary for its DNA binding activity and localization to sites of DNA damage[63]. Mutation of this methylation motif in both MRE11 and 53BP1 disrupts the functions of these two key proteins in the DNA damage pathway. Finally, PRMT1 was shown to methylate the tumour suppressor gene BRCA1[36]. Methylation of BRCA1 had a significant impact on its ability to bind to different gene promoters, adding a level of complexity to the transcriptional regulating function of PRMT1. It would be interesting to determine if these effects are isoform specific, as it has been shown that the PRMT1v1 isoform is predominantly localized to the nucleus.

These studies demonstrate that PRMT1 has a significant impact on the vital processes and signaling that are involved in the development, progression and aggressiveness of cancer cells. The majority of these studies have examined PRMT1 as one single enzyme, however the existence of the distinct PRMT1 isoforms adds a level of complexity that requires further study and clarification. This evidence suggests that PRMT1 may be a potentially valuable therapeutic target for the treatment of several cancer types, however our knowledge of this target is limited due to our lack of understanding of the precise roles of the alternative isoforms that are present.

PRMT2

PRMT2, also known as HRMT1L1, was discovered through its sequence homology with the catalytic domain of PRMT1 (approximately 50%)[30]. Interestingly, within its sequence it contains an Src homology 3 (SH3) binding domain, which potentially links it to many intracellular processes. Initially, it had no characterized methyltransferease activity. However, more recent evidence has shown that it possesses Type I arginine methyltransferase activity, albeit much lower than that of PRMT1[64]. There is limited knowledge with regards to PRMT2 methyl substrates. Evidence has shown PRMT2 is recruited by β-catenin to histone H3 where it deposits an asymmetric dimethyl mark on R8 of target gene promoters[65]. However, further experiments are required in order to generate a more complete substrate repertoire for PRMT2. Nevertheless, it has been demonstrated that PRMT2 can affect the activation of several key receptors *via* a co-activator function within cells. PRMT2 has been shown to interact with and enhance the transactivation of ERα, progesterone receptor (PR), androgen receptor (AR), peroxisome proliferator-activated receptor γ (PPARγ) and the retinoic acid receptor α (RARα) in a ligand independent fashion[66]. Interestingly, the activation of these receptors within cells has both distinct and in some cases opposing effects. Activation of ERα, PR and AR has been implicated in tumour cell growth and progression, while PPARγ and RARα activation results in growth ar-

rest and apoptosis[67-71]. This suggests that the functional role PRMT2 plays within cells is quite diverse.

Recently, in two separate papers by Zhong *et al*[22,72], four alternatively spliced PRMT2 isoforms (PRMT2L2, PRMT2α, β, and γ) in addition to the original PRMT2 isoform were identified. The *PRMT2* gene consists of 11 exons and these alternative isoforms are generated through alterations in sequence that occur from exon 7 to exon 10 (Figure 2). The first report identified a novel PRMT2L2 transcript that is produced as a result of alternative polyadenylation[72]. This polyadenylation silences the 5' splice site on exon 7 and results in a transcript that retains a significant portion of intron 7 and a premature termination codon. Subsequently, they identified PRMT2α, β and γ and showed that these isoforms are generated through splicing events occurring in the 3' C-terminal region of the *PRMT2* pre-mRNA leading to exon exclusion[22]. PRMT2α has a deletion of exons 8-10 with a frame shift that produces 12 new amino acids at the C-terminus. The PRMT2β isoform has a deletion of exons 7, 8, 9 resulting in a frame shift that generates 83 alternate amino acids at the C-terminus, while PRMT2γ has an in frame deletion of exons 7 to 10. All of these deletions in the alternatively spliced isoforms result in the loss of conserved protein arginine methyltransferase motifs. They have each lost domain Ⅲ and the THW loop. The THW loop has been shown to form part to the AdoMet-binding pocket with domains I and post I [73], therefore these variant isoforms may lack arginine methylation activity. Methylation activity of these isoforms has not yet been examined. An examination of the subcellular localization of GFP tagged PRMT2 isoforms showed that PRMT2, PRMT2α and PRMT2γ have a predominantly nuclear localization, excluding the nucleolus (Table 2)[22]. The PRMT2β isoform showed a relatively even distribution throughout the nucleus, including the nucleolus, and also localized to the cytoplasm within cells. The PRMT2L2 had a predominantly cytoplasmic localization with concentrated perinuclear staining observed[72]. It is thought that the 3' sequence may impact the localization of these isoforms.

Characterization of these alternative isoforms showed differential expression across a panel of breast cancer cell lines (Table 2). Interestingly, mRNA and protein expression of all PRMT2 isoforms are elevated in ER, PR-positive cell lines (MCF7, T47D, BT474 and ZR-75-1) compare to double negative cell lines (MDA-MB-231, MDA-MB-453 and SK-BR-3)[22,72]. Furthermore, in breast tumour samples, the mRNA expression of all PRMT2 isoforms was shown to be significantly increased in breast tumour tissues compared to normal adjacent breast samples. Additionally, the expression of each isoform was shown to be slightly higher in ER-positive compared to ER-negative tumours. Moreover, an immunohistochemical analysis, which did not differentiate between isoforms, showed that PRMT2 protein expression is elevated in breast tumour samples compared to normal breast tissue[22]. Additionally, similar to the mRNA, PRMT2 protein expression was elevated to a greater extent in ER-positive tumours compared to ER-negative tumours.

A functional assessment of the PRMT2 isoforms showed that they are able to directly bind and enhance estrogen-mediated transactivation of ERα, and also enhance the promoter activity of the downstream target gene, snail[22,72]. Increased snail transcriptional activity is associated with an increased cancer cell invasive potential[74]. Interestingly, all the isoforms had a lower transcriptional activity compared to PRMT2. Additionally, PRMT2β also had the lowest estrogen stimulated transcriptional activity and showed the lowest interaction affinity for ERα. This demonstrates that these isoform may perform different functions within cells. This interaction with ERα occurs *via* the N-terminus of the PRMT2 isoforms. Each PRMT2 isoform was also shown to directly bind to the AR. Intriguingly, it was revealed that PRMT2 negatively impacts the proliferation of ERα positive breast cancer cells in response to estrogen stimulation[22]. Depletion of the PRMT2 isoforms caused an increase in estrogen-induced proliferation and an enhancement in E2F expression and downstream activity. This is consistent with results showing that PRMT2 can bind to retinoblastoma protein (RB), and this interaction causes repression of E2F transcriptional activity[75]. It should be highlighted that the increase in proliferation may be specific to the original PRMT2 isoform, as depletion of this specific isoform caused a result similar in magnitude to the depletion of all four isoforms (PRMT2, PRMT2α, PRMT2β, PRMT2γ) simultaneously. Therefore, the contribution of the PRMT2α, PRMT2β, PRMT2γ isoforms to this proliferation phenotype is unclear. Similar to PRMT1, further research is required into the specific functions of these newly identified PRMT2 isoforms in order to determine their exact contributions to cancer development and progression. Nevertheless, these results demonstrate that the expression of PRMT2 and its alternative isoforms are clearly positively correlated with ERα status in breast cancers, consistent with a regulatory role in this pathway.

PRMT4/CARM1

PRMT4, more commonly known as Co-activator-associated arginine methyltransferase 1 (CARM1), was originally identified through its binding to GRIP1, the p160 steroid receptor co-activator[76]. It is involved in the regulation of a number of cellular processes including, transcription, pre-mRNA splicing, cell cycle progression and the DNA damage response. CARM1 is a type I arginine methyltransferase. In contrast to other type I PRMTs, which generally recognize substrate GAR motifs, it has no known substrate methylation motif[8,44]. CARM1 is most well characterized for its co-activator role in transcription which it performs through its interaction and methylation of a diverse substrate repertoire, including both histone and non-histone proteins[77-81]. The activity of CARM1 has also been shown to be influenced by posttranslational modifications. Specifically, CARM1 can be phosphorylat-

ed at several sites that can inhibit both dimerization (S229) and AdoMet binding (S217)[82,83]. Alternatively, phosphorylation at another site (S448) facilitates association with the ERα and stimulates ligand-independent activation of ERα[84]. Recently, it was identified that CARM1 is also regulated by auto-methylation[85]. The auto-methylation site was mapped to R551 in exon 15 of the mouse homolog of CARM1. This site is conserved in all vertebrate CARM1 proteins. Mutation of this auto-methylation site did not affect the enzymatic activity of CARM1, however it significantly impaired both CARM1-activated ERα mediated transcription and CARM1 regulated pre-mRNA splicing. Furthermore, it has been shown that essentially 100% of CARM1 is auto-methylated at R551 in cells[24]. Therefore, the regulation of CARM1 activity appears to be complex.

The expression of CARM1 has been shown to be dysregulated in colorectal, prostate and breast cancer[12,14,15]. CARM1 was found to be overexpressed in a significant number of colorectal tumours[14]. In prostate cancer, CARM1 was found to be overexpressed not only in tumours, but also in prostatic intraepithelial neoplasia (PIN). PINs are thought to be a precursor to the development of prostate cancer[12,14]. Finally, CARM1 expression was also found to be upregulated in breast cancer[14,86]. Interestingly, in the study conducted by Kim *et al*[14], for both prostate and breast cancers the expression level of CARM1 was lower. In a more recent study by Cheng *et al*[86], CARM1 expression was observed to be increased in invasive breast cancer, correlating with high tumour grade and to a greater extent with HER2, p53 and Ki-67 expression. CARM1 expression showed a lower correlative rate with ER and PR expression. The results from these studies are surprising given the role that CARM1 plays in the association and co-activation of ERα and AR[87,88]. They suggest that CARM1 has a multifaceted contribution to the development and progression of cancers. Furthermore, it shows that CARM1 may be an informative prognostic marker for breast cancer.

Within tumour cells, CARM1 plays a role in regulating cell proliferation and survival through its interaction and cooperation with several critical cancer related proteins. CARM1 is recruited to the promoter of the *cyclin E1* gene, where it acts as a transcriptional co-activator in regulating cyclin E1 protein expression. Furthermore, both CARM1 and cyclin E1 were shown to be co-overexpressed and correlated with grade 3 breast tumours[78]. CARM1 has also been shown to be necessary for estrogen-stimulated proliferation of breast cancer cells[77]. This occurs *via* estrogen-stimulated methylation of H3R17 by CARM1, resulting in expression of the cell cycle regulator E2F1. Moreover, CARM1 is involved in the regulation of both the stability and activity of AIB1, a transcriptional co-activator that is often overexpressed in breast tumours. Additionally, it has been recently shown that CARM1 can promote breast cancer cell migration and metastasis through the methylation of BAF155, a component of the chromatin-remodeling complex[89].

While these studies define a role for CARM1 in promoting cancer progression, a study by Al-Dhaheri *et al*[79] showed some conflicting effects. Overexpression of CARM1 in MCF7 breast cancer cells, an ER+ cell line, inhibited estrogen-stimulated cell growth, while overexpression or depletion of CARM1 in MDA-MB-231 (ER-) breast cancer cells had no effect on their growth. Interestingly, the inhibited cell growth observed in MCF7 cells with CARM1 overexpression was accompanied by increased expression of cell cycle inhibitors, $p21^{cip1}$ and $p27^{kip1}$ and a change in cell morphology reminiscent of a more differentiated phenotype. Additionally, CARM1 was shown to repress the expression of approximately 16% of estrogen-activated target genes. An expression analysis in a set of ER+ tumours showed that CARM1 expression positively correlates with ERα expression. However, it inversely correlated with tumour grade. It should also be noted that a recent report suggested that only small proportion of endogenous CARM1 protein expression is required in order to perform its biological functions in cells[89]. Therefore, suppression of 100% of CARM1 protein expression is required in experimentation because it is thought that only a very small amount of CARM1 protein is necessary for its normal functioning. These reports suggest that a further understanding of CARM1 regulation and function is required in order to clarify its role and potential marker/therapeutic value in cancer.

A plausible explanation for these opposing results in breast cancer cells is the existence of alternatively spliced isoforms of CARM1. In the literature there are two papers that describe the presence of distinct alternatively spliced CARM1 isoforms. The first by Ohkura *et al*[23] describes, that in normal rat tissue, four isoforms are transcribed from the *CARM1* gene; the primary isoform CARM1 (CARM1v1) and three alternative isoforms, v2, v3 and v4 (Figure 2). All four contain the arginine methyltransferase domain and the GRIP1-binding domain. The primary *CARM1* isoform, CARM1v1, consists of 16 exons. CARM1v2 is generated through retention of the intron 15 sequence, CARM1v3 is produced through the retention of introns 15 and 16 and CARM1v4 results from the skipping of exon 15[23]. Each of these enzymes showed a distinct mRNA expression profile when examined across a panel of normal rat tissues. Functionally, the CARM1v3 isoform was shown to alter the splicing pattern of both E1A and CD44 reporters. This was not observed with the other isoforms suggesting they may have different functions. The splicing activity demonstrated for CARM1v3 was shown to be independent of the CARM1v3 methylation activity. In contrast to this, Cheng *et al*[90] showed that CARM1 enzymatic activity is required for its effect on alternative splicing of the CD44 pre-mRNA, which is thought to occur co-transcriptionally. They also suggest that while CARM1v3 is an alternative isoform, it may represent a very rare form not playing a major role in cells. Hence the precise biological roles of these CARM1 isoforms remains unclear.

Alternatively, in the second paper, Wang *et al*[24]

showed that in human cells and tissues, two CARM1 isoforms are present. These are designated CARM1 full length (CARM1FL) and CARM1Δ15 (Figure 2). The CARM1Δ15 is a transcript in which exon 15 is excluded by alternative splicing. This alternative isoform represents the CARM1v4 isoform described previously. The other two isoforms were not detected in human cells or tissues. Importantly, exclusion of exon 15 removes the auto-methylation site that can functionally regulate CARM1, however it does not impact the methylation activity. An examination of mRNA expression across a panel of normal human tissues revealed that the CARM1Δ15 isoform is the major isoform expressed, with the exception of the brain, heart, skeletal muscle and testis. The CARM1FL isoform is expressed highest in these tissues. Additionally, the CARM1FL isoform is predominantly auto-methylated in cells.

In breast cancer cells, the CARM1Δ15 was shown to be the predominant isoform expressed (Table 2)[24]. However, only a limited number of cancer cell lines were assessed. It would be interesting to know the expression profile in other cancer types as well. Specifically, an assessment of CARM1 isoform expression in a panel of breast cancer cell lines showed a greater percentage of the CARM1Δ15 isoform compared to the CARM1FL isoform. This is surprising due to the fact that the CARM1Δ15 isoform has impaired ERα co-activator activity and failed to stimulate ERα transcriptional activity. However, it may have distinct roles with respect to activity and functions within cells. The existence of these two isoforms may shed light on some of the conflicting reports in the literature with respect to the biological functions of CARM1 and potential roles in cancer. Further study of these isoforms is required to establish if they are responsible for the methylation of distinct substrates and their individual functions.

PRMT7

PRMT7 was originally identified from a screen of genetic suppressor elements (GSE) aimed at identifying genes conferring resistance to cytotoxic agents performed in Chinese Hamster cells[91]. This screen identified a gene that encoded two proteins, p77 and p82, that were highly homologous to the PRMT family and later designated PRMT7α and β, respectively[27,91]. In Hamster cells, these two isoforms are thought to be generated by the use of distinct 5' translation initiation codons within the primary transcript (Figure 2). The PRMT7β isoform sequence contains an extra 37 amino acids at the N-terminus. Both isoforms were shown to be active and have slightly different methylation profiles[27], though further analysis is required to clarify these differences between the isoforms. Each isoform has a distinct subcellular localization patterns (Table 2). PRMT7α localizes to the cytoplasm and nucleus, whereas PRMT7β is exclusively cytoplasmic[27]. In human tissues, only a single PRMT7 transcript is detected (approximately 3.6 kb) and in two human cell cancer cell lines, HeLa and HuH7, one protein at 78 kDa was detected. This transcript was shown to share the greatest homology to the PRMT7α isoform[25-27]. However, the limited subset of cell lines used cannot completely rule out the existence of PRMT7β isoform expression in human cells and a more comprehensive examination of expression in cells is required. Moreover, a survey within both NCBI and *Ensembl* databases predicts the existence of at least 2 alternatively spliced PRMT7 isoforms that can be produced from the human *PRMT7* gene (Figure 2). These two isoforms have the same N- and C-terminal regions but variant 2 (PRMT7v2) has an in frame deletion of exon 5. Importantly, this may affect methyltransferase activity because it removes the post I domain. Functionally, PRMT7 was initially characterized as a Type Ⅱ methyltransferase[26], but it has recently been deemed a Type Ⅲ and is thus the only PRMT enzyme known to catalyze predominantly this reaction in mammalian cells[4,5]. The generation of monomethylarginine is thought to represent a reaction intermediate for the other PRMTs.

There is limited knowledge into the precise biological functions of PRMT7, however evidence has shown it is linked to cancer. A gene expression analysis of independent data sets of more than 1200 breast tumours identified increased expression in the chromosomal region where the PRMT7 gene is located (16q22)[92]. Importantly, this was also correlated with an increased metastatic potential of breast cancer. The PRMT7 gene locus was also identified in an unbiased genome-wide study to confer resistance to etoposide-induced cytotoxicity in patients[93]. As previously mentioned, PRMT7 was originally identified by a screen for GSEs conferring resistance to cytotoxic agents (etoposide and 9-OH-E)[91]. This study showed that GSE-mediated repression of PRMT7 conferred resistance to topoisomerase Ⅱ inhibitors and also cisplatin. In contrast, in this same study, repression of PRMT7 caused increased sensitivity to other DNA-damaging agents, such as the topoisomerase I inhibitor, camptothecin, as well as UV-irradiation. Increased sensitivity to camptothecin was also observed when PRMT7 was depleted from HeLa cells[94]. Intriguingly, depletion of PRMT7 from NIH 3T3 cells conferred resistance to cisplatin, mytomycin C and chlorambucil[95]. Additionally, one of its only identified interacting protein partners, CTCFL, is a proposed proto-oncogene[96,97]. Further studies are required to identify additional PRMT7 substrates to better understand its role in cells. While these results strongly suggest that PRMT7 may play a key role in several cancer related processes, the opposing functions of PRMT7 in response to cytotoxic agents requires some attention. The reason for these differential effects is unclear; perhaps PRMT7 has distinct functions in different cell types. More interestingly, they could be the result of PRMT7 isoforms specific expression and function within cells.

CONCLUSION

The importance of PRMTs in cancer is only beginning to

be examined. There have been many key discoveries thus far that have demonstrated the potential impact that the PRMTs have in regulating critical effectors and pathways involved in the development and progression of cancer. In fact, the PRMTs have the potential of impacting the majority of the described hallmarks of cancer proposed by Hanahan *et al*[1,98]. Current research efforts aim to identify and characterize the precise mechanistic roles that these PRMTs play in cancer. Importantly, the functional contribution of PRMTs to different cancer types, as well as subtypes within the same cancer, requires further investigation. The significance of this requirement is highlighted by several of the conflicting findings describe in this review.

Here we have highlighted the existence of alternatively spliced PRMT isoforms that have been identified for PRMT1, PRMT2, CARM1 and PRMT7. While not currently realized, more PRMT isoforms for these and other PRMT family members may be present in cells. The presence of distinct PRMT alternative isoforms adds a further level of complexity to this family of enzymes. Additionally, the isoforms identified for PRMT1, PRMT2, CARM1 and PRMT7 have mainly been assessed in breast cancer cells and tissues as indicated in Table 2. A more extensive analysis of their expression in other tumour types has not been performed and could uncover more interesting results with respect to these PRMT isoforms. This fact requires more attention as it may provide possible explanations for the opposing functions identified within cells. Furthermore, while these isoforms may have overlapping functions, it is clear from the data presented here that they also possess distinct functions. Interestingly, while dysregulated PRMT expression has been observed in cancer, no genetic abnormalities have been identified, with one exception being PRMT8[99,100]. While there may be no obvious change at the genome level, a shift in the expression from one alternative PRMT isoform to another may be a crucial event that occurs in cancer cells, thereby affecting development, progression and aggressiveness. Interestingly, a particular PRMT isoform may not be expressed or is expressed at lower levels in normal tissues and as a consequence of the tumourigenic process cancer cells may preferentially upregulate a specific isoform due to its advantageous functions. Understanding both the shared and distinct functions of these alternative PRMT isoforms will not only improve our knowledge of their biological significance but also provide insight into their specific contributions to diseases, such as cancer.

The roles that the PRMTs play in cancer make them an attractive target for the development of drugs that could be used in treatment strategies. This increases the importance of gaining more knowledge about the alternative PRMT isoforms, so that there is a complete understanding of the therapeutic mechanism. This will enable the development of an optimal therapeutic strategy and an improved understanding of the resulting outcomes when targeting PRMT enzymes as a treatment in cancer.

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REVIEW

What have we learned about the kallikrein-kinin and renin-angiotensin systems in neurological disorders?

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Abstract

The kallikrein-kinin system (KKS) is an intricate endogenous pathway involved in several physiological and pathological cascades in the brain. Due to the pathological effects of kinins in blood vessels and tissues, their formation and degradation are tightly controlled. Their components have been related to several central nervous system diseases such as stroke, Alzheimer's disease, Parkinson's disease, multiple sclerosis, epilepsy and others. Bradykinin and its receptors (B1R and B2R) may have a role in the pathophysiology of certain central nervous system diseases. It has been suggested that kinin B1R is up-regulated in pathological conditions and has a neurodegenerative pattern, while kinin B2R is constitutive and can act as a neuroprotective factor in many neurological conditions. The renin angiotensin system (RAS) is an important blood pressure regulator and controls both sodium and water intake. Ang Ⅱ is a potent vasoconstrictor molecule and angiotensin converting enzyme is the major enzyme responsible for its release. Ang Ⅱ acts mainly on the AT1 receptor, with involvement in several systemic and neurological disorders. Brain RAS has been associated with physiological pathways, but is also associated with brain disorders. This review describes topics relating to the involvement of both systems in several forms of brain dysfunction and indicates components of the KKS and RAS that have been used as targets in several pharmacological approaches.

Key words: Kallikrein-kinin system; Renin-angiotensin system; Neurological disorders; Alzheimer's disease; Epilepsy; Parkinson's disease

Core tip: This review is a description of the involvement of the kallikrein-kinin and renin-angiotensin systems in neurological disorders. We describe all components of both systems, relating them to several brain diseases such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, blood brain barrier disruption, stroke and inflammation, including the involvement of each molecule, their receptor and specific enzymes in individual pathologies. We also show that brain homeostasis depends on a dynamic balance between the kallikrein-kinin and renin-angiotensin systems.

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KALLIKREIN-KININ SYSTEM IN NEUROLOGICAL DISORDERS

Components of the kallikrein-kinin system

The kallikrein-kinin system (KKS) is an intricate endogenous pathway involved in blood pressure regulation, inflammation, cardiovascular homeostasis, analgesic responses, pain-transmitting mechanisms, cytokines release, prostacyclin, nitric oxide and cell proliferation[1,2].

Initial studies on the importance of the KKS in mammals were performed at the beginning of the last century, when Abelous *et al*[3] verified that human urine injected into dogs induced a reduction in blood pressure. After that, several authors identified a great number of molecules, with biological activity, involved in this bioactive cascade[4-8]. Thus, since 1900 to date, all components of the KKS were sequentially identified in plasma and/or in tissue as part of a complex enzymatic process linked to several biological and pathological events.

Due to the effects of kinins in blood vessels and tissues, their formation and degradation are tightly controlled. In plasma, the coagulation factor XII (Hageman factor XII) is activated to XIIa by the negative surface and is then able to cleave prekallikrein into the active form of kallikrein. This latter enzyme hydrolyzes high molecular weight kininogen and releases bradykinin (BK) into the circulation, which is an important vasoactive nonapeptide (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9). After C-terminal arginine removal, by circulating and/or tissue kininases, BK is converted into Des-Arg^9BK, another potent peptide or to inactive peptides. BK has high affinity for the constitutive kinin B2 receptors (B2R), while Des-Arg^9BK shows preference for binding to inductive kinin B1 receptors (B1R)[8].

In tissues, prekallikrein is also converted into kallikrein, which hydrolyzes the low molecular weight kininogen, releasing Lys-BK, also known as kallidin. After the action of tissue kininases, Lys-Bk is converted into BK or Des-Arg^{10}-Lys-BK, which also have high affinity for B1R, while its precursor (kallidin) shows more affinity for B2R (Figure 1). All these enzymes involved in the KKS are serine-proteases. Plasma kallikrein and tissue kallikrein 1 (KK1) are the main enzymes involved in kinin release in blood and tissue, respectively.

KKS in the central nervous system

All components of the KKS have been localized in the cerebral cortex, brain stem, cerebellum, hypothalamus, hippocampus, and pineal gland, among others. They are found surrounding blood vessels, in neurons and glial cells[9-12]. Kinins are able to stimulate the production and release of inflammatory mediators such as eicosanoids, cytokines, nitric oxide (NO) and free radicals. Kinins also induce the release of excitatory amino acids, increasing intracellular (Ca^{2+})i levels and inducing brain excitotoxicity. These peptides are also involved in disruption of the blood-brain-barrier (BBB) and dilation of the parenchyma of cerebral arteries causing edema[13-15]. The mitogen-activated protein kinase pathway, which culminates in the transcription of many genes involved in later responses[16] is also activated by B1R. Stimulation of both B1R and B2R leads to classical G-protein activation with the generation of different second messengers (Figure 1).

In addition, plasma and tissue enzymes, other serinoproteases, similar to chymo/trypsin-like proteases, have been described and they are also known as kallikreins (KK1 to KK15). According to Sotiropoulou *et al*[17], this family of 15 enzymes has been related to diseases such as hypertension, renal dysfunction, inflammation, neurodegeneration and several types of cancer[18].

The KKS influences multiple players in the immune system acting on targets such as macrophages, dendritic cells, T and B lymphocytes modulating the activation, proliferation, migration and the effector function of these cells[19]. Thus, kallikreins have been associated with several pathologies, supporting new insights related to the KKS, which could be useful as targets in the treatment of pathological conditions.

KKS in inflammation

In neurodegenerative disorders, inflammation is considering a primary response to injury or to infection, repairing and healing the injured tissue[20]. Vascular permeability and blood flow increases in the first stage of inflammation and substances produced by mast cells and by platelets such as histamine, BK, leukotrienes, prostaglandins and serotonin are released during the initial inflammation process[20]. Blood vessel walls change their permeability allowing the entry of proteins and small molecules, which are important to the recruitment of defense cells. At this stage, leukocytes, adhesion molecules, cytokines and chemotactic factors are recruited to the injured site. Indeed, the release of BK may participate in this process and several authors have studied KKS targets to improve the delivery of drugs through the blood-tumor barrier[21-23].

KKS and cerebrovascular alterations

According to Kung *et al*[24], patients with traumatic brain injury, subarachnoid hemorrhage, intracerebral hemorrhage and ischemic stroke have increased BK levels in CSF and these high levels correlate with the intensity of edema formation. In addition, patients with aneurysmal subarachnoid hemorrhage have low levels of serum KK6 and KK6 levels in blood could predict early complications of this disease. Thus, Martinez-Morillo *et al*[25] suggested that KK6 could be a useful prognostic marker in this pathological condition. Similarly, cerebral hematoma expansion induced by hyperglycemia is mediated by plasma KK[26].

Kininogen-deficient mice show less severe BBB damage, edema and inflammation formation after thrombosis and ischemic stroke. According to some authors, kininogen deficiency is able to reduce thrombosis after stroke, without increasing the risk of intracerebral hemorrhage. In the absence of kininogen, mice are completely unable to produce BK. This lack of kininogen underlies

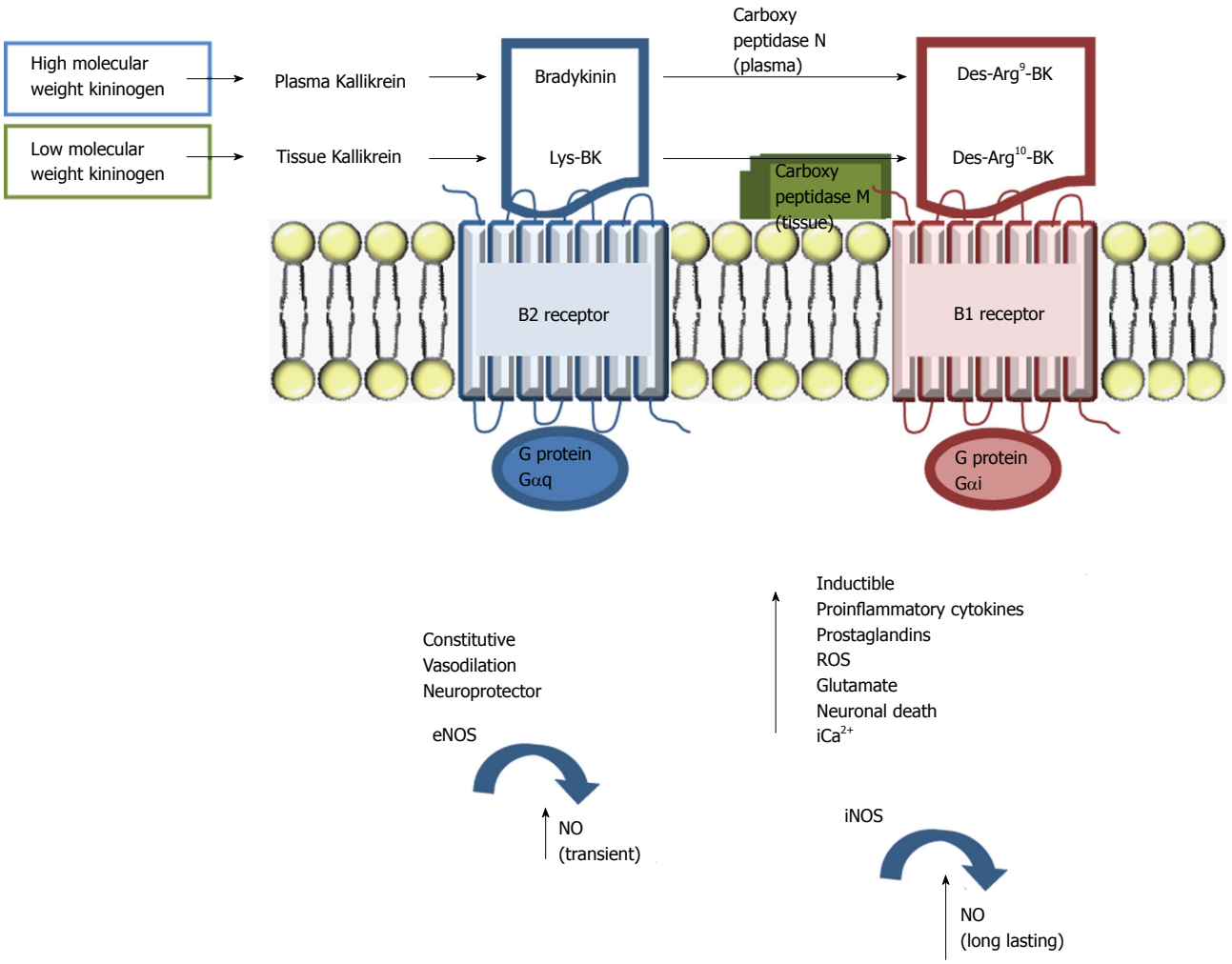

Figure 1 Schematic representation of the kallikrein-kinin system. Bradykinin and Lys-bradykinin (BK), generated by the action of plasma or tissue kallikrein on the precursor (high or low molecular weight kininogen) are the main bradykinin and its receptor (B2R) agonists. These peptides can be converted to B1R agonists after removal of C-terminal-Arg. Both peptidases, membrane-bound carboxypeptidase M, linked to B1R at the C-terminal domain or the soluble carboxypeptidase N are able to remove Arg from the C-terminal portion of BK. B2R is constitutively expressed, showing physiological effects such as vasodilation, transient nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS), whereas B1R expression is induced by injury or inflammatory conditions, with long-lasting NO production, resulting in a neurotoxic environment with reactive oxygen species (ROS) production and increased release of glutamate with excitoxicity-induced neuronal death.

the strong anti-inflammatory phenotype observed in the context of brain ischemia in these animals[27]. Moreover, genetic depletion of B1R improves functional outcome after focal head injury in mice. This effect is similar to that obtained by a pharmacological approach, using a selective B1R antagonist[8]. Thus, mice with B1R depletion show minor axonal damage, reduced apoptosis, astrocyte activation and less inflammation. In contrast, blockage of B2R had no effect on brain protection.

KKS and dementias

Decreased cerebral flow and BBB disruption are also features of Alzheimer's disease (AD)[28,29]. BK activity affects cerebrovascular tone and BBB permeability, both of which are abnormal in AD[30]. According to Farrall *et al*[30], the frontal cortex of patients with AD, the frontal and temporal cortex of patients with vascular dementia showed high levels of plasma kallikrein as well as its mRNA. In addition, this enzyme also had high activity showing that kinin production could influence cerebral blood flow and vascular permeability related to AD. Other types of KK are also modified in the CSF of patients with AD and with frontotemporal dementia. KK6, KK7 and KK10 were decreased in the CSF of patients with frontotemporal dementia, while KK10 increased in the CSF of subjects with AD. These differences could be useful in the diagnosis of both diseases[31]. Increased expression of KK6 was also observed in CSF, plasma and whole blood of patients with AD[32], showing a strong relationship between the KKS and brain degeneration. Furthermore, mice expressing human amyloid precursor protein (APP), carrying familial AD gene mutations, showed increased expression of B1R in astrocytes of the hippocampal formation. Similarly, blockage of this receptor, using specific antagonists, decreased amyloidosis plaque deposits in the somatosensory/cingulate cortex and dorsal hippocampus[33]. These authors also showed improvements in learning and memory after B1R block-

age in APP mice. Thus, according to Lemos *et al*[34] during the aging process, B1R could be involved in memory degeneration, while B2R could act as a neuroprotective factor.

Kallikrein 8 also known as neuropsin participates in extracellular proteolysis involved in long-term potentiation (LTP), necessary for the establishment of memory acquisition in the hippocampus[35]. According to these authors, KK8 knockout mice were impaired, failed memory tasks and showed the involvement of this enzyme in phosphorylation of the GluR1 subunit of AMPA receptors, linked with LTP and with memory acquisition. Taken together, these data show that the KKS participates in these degenerative diseases.

KKS and neuromuscular diseases

Kallikreins are also associated with secondary progressive multiple sclerosis and promote neurodegeneration[36]. According to these authors, high levels of KK1 and KK6 may serve as biomarkers of multiple sclerosis progression. KK1 levels correlate positively with expanded disability status scale (EDSS) scores and KK6 with future prognostic and worsening of the EDSS scale, in relapsing remitting patients. These authors also showed that exposure to kallikrein promoted neurite retraction and neuronal death in murine cortical neurons[36].

Recent work showed that deletion of the *KK6* gene affected the number of oligodendrocytes and the amount of myelin in the developing spinal cord, in particular the myelin basic protein[37]. These data suggest that KK6 has an important function in promoting oligodendrocyte development in the spinal cord as well as in damaged spinal cords. In addition, KK6 has also been associated with hypertrophic astrocytes in human pathological conditions, promoting astrocyte stellation, stimulating inflammatory cytokine (IL-6) secretion and suppressing GFAP mRNA expression[38]. Undoubtedly, KK6 seems to be very important for the homeostasis of CNS cells, participating in several events during physiological and pathological conditions.

KKS and epilepsy

It is already known that the brain inflammatory process is able to initiate seizures[39] and this event is accompanied by an immune-mediated leakage in the BBB. The first evidence linking the KKS with epilepsies was demonstrated by several authors around the 1970s[40,41]. Since then, a large number of studies have emerged localizing more specific targets in the KKS cascade that could help in understanding epilepsy physiopathology. In 1999, Bregola *et al*[42] showed changes in hippocampal and cortical B1R in two experimental models of epilepsy. These authors reported that Lys-des-Arg^{9}BK, an agonist of B1R, increased the overflow of glutamate after electrical stimulation, in hippocampal and cortical slices of rats submitted to kindling. This effect was also visualized in rats submitted to the kainate model of epilepsy, but to a lesser extent. The authors associated B1R with the condition of latent epileptic hyperexcitability[42]. These data were confirmed by Mazzuferi *et al*[43] when they showed the increased release of glutamate after B1R stimulation, induced by Lys-des-Arg^9-BK in kindled animals.

When studying the expression of B1R and B2R in the hippocampus of rats submitted to the pilocarpine model of epilepsy, our group[44] found increased expression of both receptors in the hippocampus. We also found[45] these alterations in knockout mice (B1KO and B2KO) in the pilocarpine model. This means that the absence of B1R (B1KO) decreases pyramidal cell death, decreases mossy fiber sprouting and decreases the number of spontaneous recurrent seizures, during the chronic phase, showing that B1R is proconvulsant. These data were confirmed by Silva *et al*[46]. However, using the model of audiogenic kindling with limbic recruitment, Pereira *et al*[47] found increased expression of B1R and B2R in the hippocampus of rats, but reported that this increase did not correlate with inflammatory levels as IL1β, COX2 and TNFα were not modified in this tissue.

We also showed[45] that B2R was linked to neuroprotection, as its absence is associated with decreased pyramidal cell survival and increased mossy fiber sprouting. Confirming these data, other authors have shown that BK triggers a neuroprotective cascade *via* B2R activation, which conferred protection against NMDA-induced excitotoxicity[48]. However, different data were recently reported concerning the role of B2R in epileptogenesis. Rodi *et al*[49] found that B2R was overexpressed in limbic areas and that slices prepared from B1R knockout mice (B1K0) were more excitable than those from wild-type mice. This effect was abolished using B2R antagonists. Due to this result, the authors concluded that this excitatory phenomenon was B2R dependent. In addition, these authors also demonstrated that kainic acid-induced seizures are attenuated by a B2R antagonist, supporting the hypothesis that B2R is involved in an early event that leads a normal brain to epileptic conditions.

When studying patients with temporal lobe epilepsy (TLE) and hippocampal sclerosis we also showed increased levels of B1R and B2R in the hippocampus[50], when compared with autopsy-control tissues. These receptors were visualized in pyramidal neurons of the hilus and in CA1 and CA3 regions of the hippocampal formation. The hippocampus of these patients also showed overexpression of KK1 by astrocytes, which were co-localized with GFAP protein, confirming participation of the KKS[51].

Together, these data show effective participation of the KKS system in TLE and Figure 2 shows our suggestion concerning a possible cross-talk between hippocampal neurons and astrocytes in the KKS in epileptic diseases.

RENIN-ANGIOTENSIN SYSTEM AND NEUROLOGICAL DISORDERS

Components of the renin-angiotensin system

The renin-angiotensin system (RAS) was initially consid-

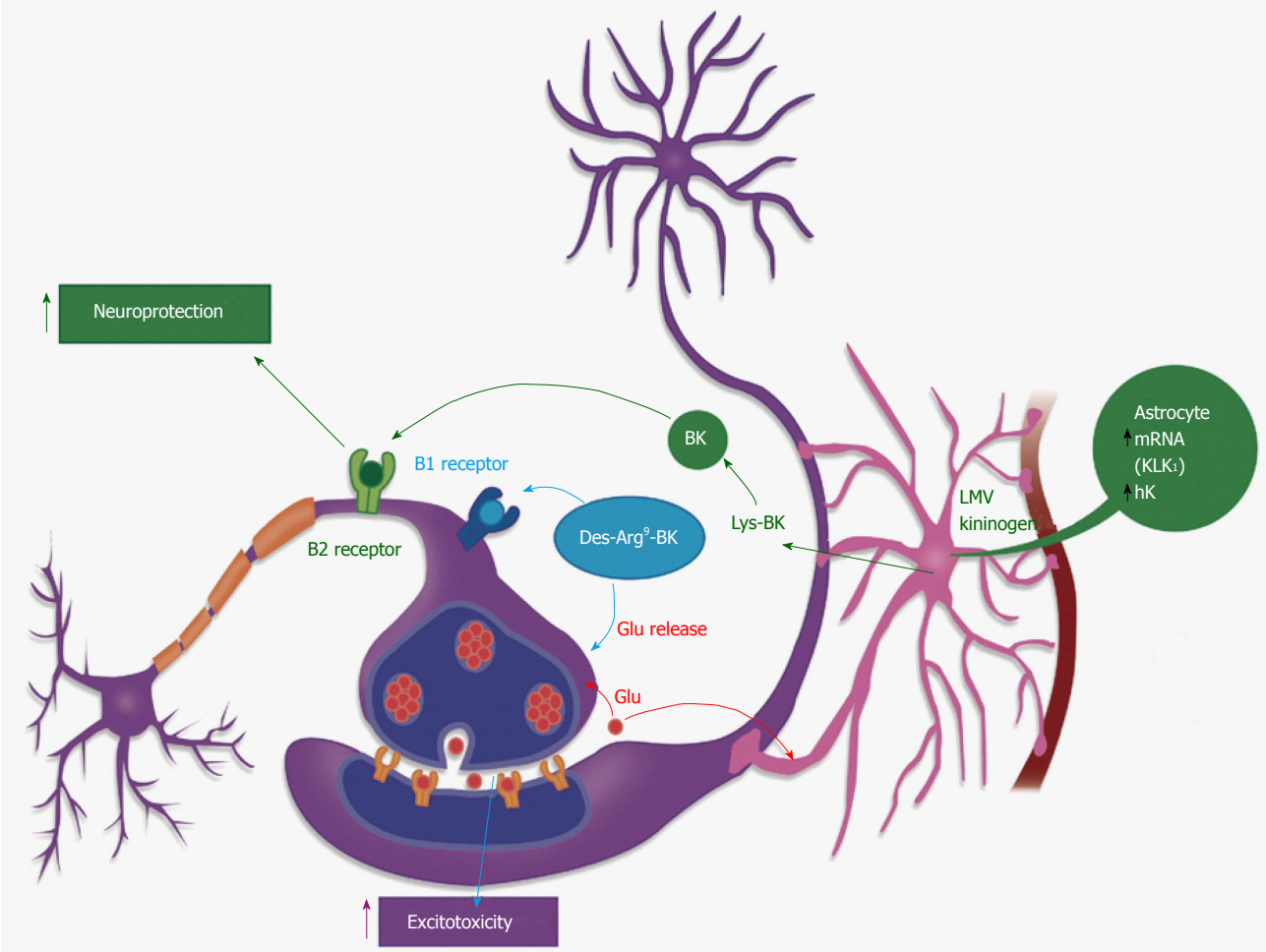

Figure 2 Cross-talk between glial and neural cells related to the kallikrein-kinin system. An adaptation based on the image found at the following site: http://learn.genetics.utah.edu/units/addiction/reward/images/neuronsAstrocyte.jpg. Kallikrein 1 (KK1) in the hippocampus, acts on its main substrate, the low molecular weight kininogen, to release Lys-bradykinin (BK) which can be hydrolyzed to BK, Des-Arg9BK or des-Arg10-Lys-BK by kininases, localized in astrocytes or at the extracellular matrix. These short-living peptides will act on the neuronal surface: binding to kinin B1R they will induce an increase in glutamate release, thus increasing neuronal excitability. Acting on kinin B2R these peptides will produce neuroprotection[42-45].

ered to be a circulating humoral system, involved in blood pressure regulation and the control of both sodium and water intake. Molecules formed by this system are associated with vasoconstriction and the release of aldosterone from the adrenal cortex and antidiuretic hormone from the neurohypophysis. RAS components act in the vasculature to promote vasoconstriction and at sites within the central nervous system to stimulate sympathetic outflow, impair the baroreflex sensitivity for heart rate control, promote release of catecholamines and aldosterone, and sodium retention, which have an important role in the development and maintenance of hypertension and insulin resistance during aging[52].

Renin is the rate-limiting enzyme of the RAS and acting on its precursor, angiotensinogen, releases angiotensin I , Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (Ang I). After dipeptide His-Leu removal by angiotensin converting enzyme (ACE), Ang Ⅱ is produced (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Ang Ⅱ is the main effector peptide in this system. Binding to Ang Ⅱ type 1 receptor (AT1R), Ang Ⅱ stimulates vasoconstriction, aldosterone and steroid hormones release, which are involved in sodium reabsorption and water retention. AT1R activity is also related to hypertension, heart dysfunction, brain ischemia, abnormal stress responses, BBB breakdown and inflammation in several species[53]. The second receptor involved in Ang Ⅱ activity is AT2R. However, the function of AT2R is more elusive and controversial. AT2R is expressed during fetal development, decreasing after birth and remaining at a low concentration during adulthood. It has been linked to cell proliferation, differentiation, apoptosis and regeneration of several tissues[54] (Figure 3).

RAS in CNS

In addition to the well-known humoral RAS, in the last decades a tissue RAS has been described, particularly in the CNS. Thus, all components of the RAS have been found in the brain. However, as this tissue has a low level of renin, it remains controversial as to how Ang I is generated by this system. Recently[55], the presence of a prorenin receptor (PRR) was reported, which has a high

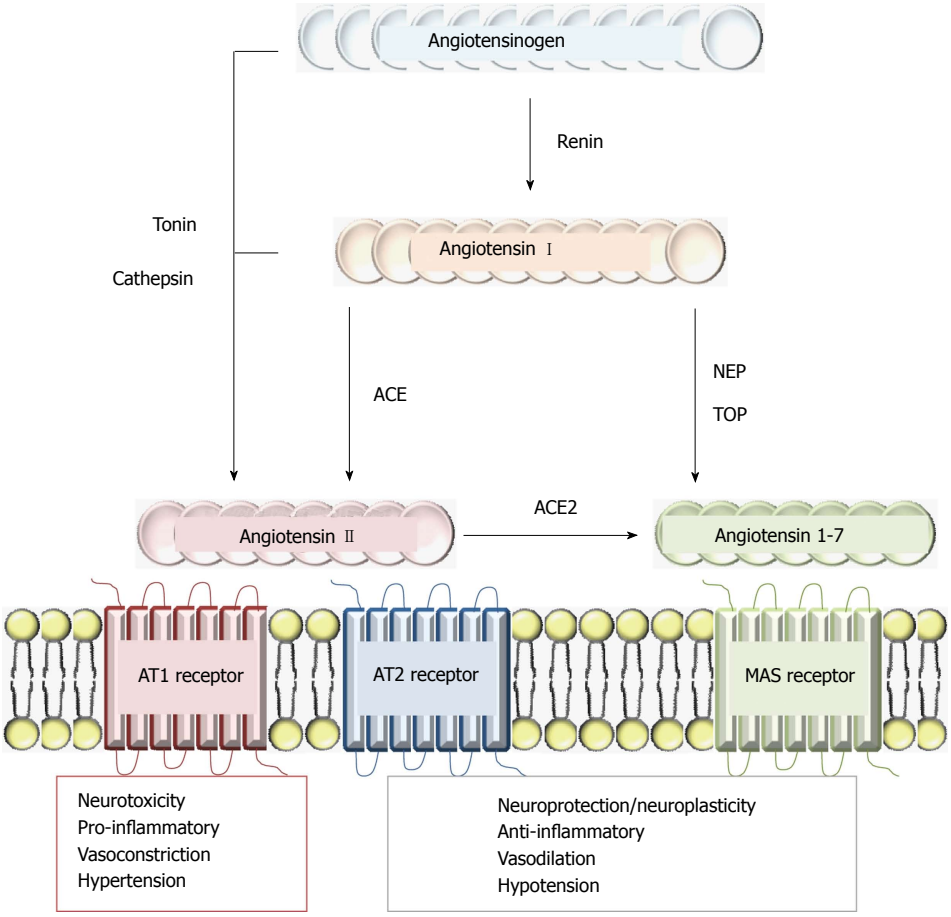

Figure 3 Schematic representation of the renin-angiotensin system and its physiopathological effects. Ang Ⅱ may be generated in the brain *via* the classical pathway, through renin and angiotensin converting enzyme (ACE) action (through Ang Ⅰ cleavage) or can be directly released from angiotensinogen by cathepsin G or tonin actions. Ang1-7 is active in several organs including the brain and several endopeptidases such as thimet oligopeptidase (TOP) or neutral endopeptidases (NEP) may metabolize Ang Ⅰ, generating Ang1-7. Ang Ⅱ may also be hydrolyzed by ACE2 to generate Ang1-7. Binding to Ang Ⅱ type 1 receptor (AT1R), Ang Ⅱ stimulates vasoconstriction, aldosterone and steroid hormones release, which are involved in sodium reabsorption and water retention. AT1R activity is also related to hypertension, heart dysfunction, brain ischemia, abnormal stress responses, blood-brain barrier breakdown and inflammation. The second receptor involved in Ang Ⅱ activity is AT2R and is expressed during fetal development, decreasing after birth and remaining at a low concentration during adulthood. It has been linked to cell proliferation, differentiation, apoptosis and the regeneration of several tissues. Ang1-7 is a Mas receptor agonist, which is related to neuronal plasticity and changes in cellular phenotype that are produced by neuronal activity such as synaptic rearrangements and mossy fiber sprouting in the hippocampus.

level of expression in the brain by neurons and astrocytes. Prorenin binds to its receptors without proteolytic activation and this binding initiates the rate-limiting step in angiotensin formation in the CNS. PRR also acts as an accessory protein for vesicular ATPase, linked to vesicular acidification.

Further to ACE, some homologue components of the RAS have been described such as ACE2 and chymase. Furthermore, peptides such as angiotensin 1-7 (Ang1-7), angiotensin Ⅲ (Ang Ⅲ) and Ang Ⅳ are involved in RAS function. Ang Ⅳ acts at AT4R and Ang1-7 at the Mas receptor. Another enzyme involved in Ang Ⅱ generation is Tonin, which is able to hydrolyze angiotensinogen releasing Ang Ⅱ in tissue, without ACE intervention (Figure 3).

Connection between the KKS and RAS

There is a connection between the KKS and RAS (Figure 4), which is produced by ACE linking both of these important systems. ACE is considered to be the most potent kininase in the blood and in several tissues, such as lung and liver. This enzyme, removes the dipeptide His-Leu from Ang Ⅰ, generates Ang Ⅱ, removes Phe-Arg dipeptide from BK, and inactivates this hypotensor peptide. This is a very important link and it is through the balance between RAS and KKS, that blood pressure can be controlled. This balance is also very important in the brain due to control of BBB permeability.

RAS and inflammation

Despite its action in important physiological processes, RAS has also been associated with pathological conditions. In a recent review[53], authors showed a relationship between the RAS and inflammatory brain disorders, focusing attention on the actions of AT1R in diseases such as stress-induced disorders, anxiety and depression, stroke, brain inflammation, traumatic brain injury and DA. These authors reported that AT1R activation up-regulates common pro-inflammatory mechanisms, activating transcription factors such as NF-κB, triggering an inflammatory cascade with the production of adhe-

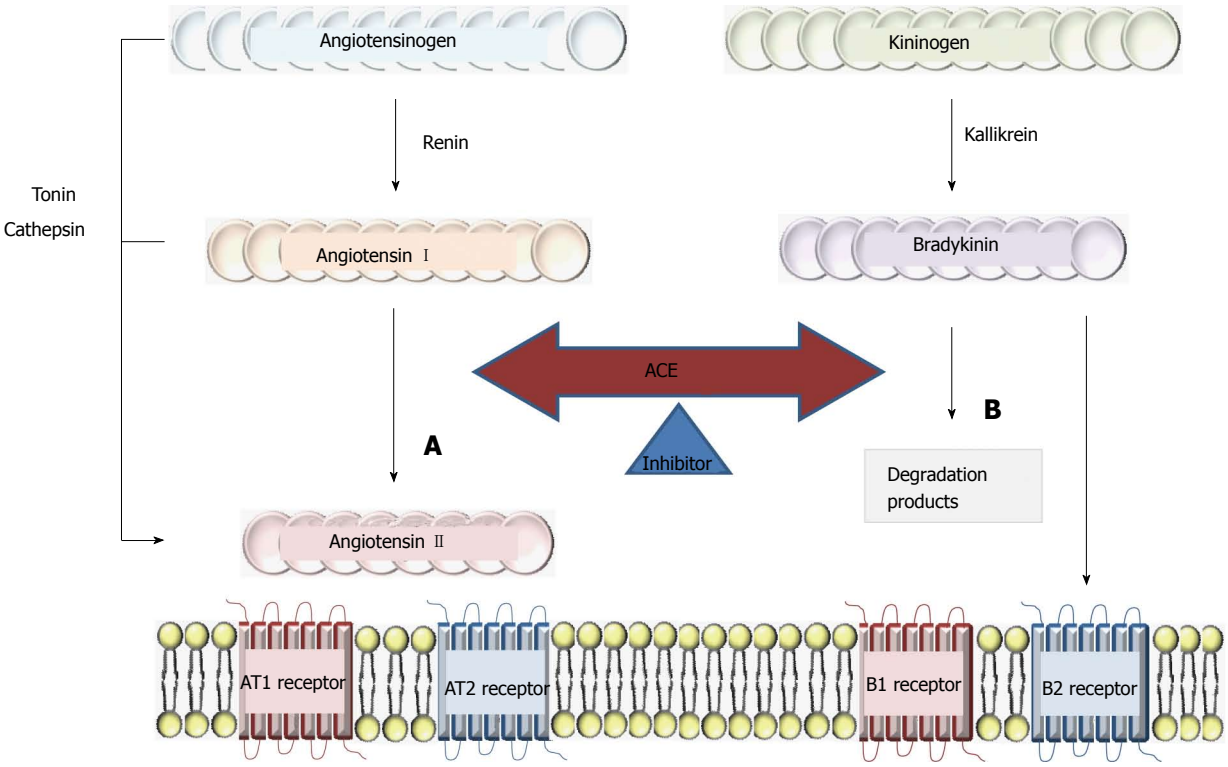

Figure 4 Schematic representation of the role of angiotensin converting enzyme in the renin-angiotensin and kallikrein-kinin systems. A: Conversion of Ang I into Ang II by angiotensin converting enzyme (ACE); B: Bradykinin (BK) degradation by ACE. Physiological effects on the renin-angiotensin system mediated by Ang II type 1 receptor (AT1R) include: vasoconstriction, neuroinflammation, and increased sympathetic nerve activity. Those mediated by Ang II type 2 receptor (AT2R) include cell differentiation and vasodilation. The effects on the kallikrein-kinin system, mediated by kinins, bradykinin and their receptor (B2R) also include vasodilation and hypotension, *via* the release of nitric oxide (NO), prostacyclins and endothelium-derived hyperpolarizing factor (EDHF). It is important to emphasize that in human pathological conditions, the use of ACE inhibitors results in downregulation of Ang II production. In this sense, the kallikrein-kinin system is upregulated and the physiological effects of kinins are potentiated, as all kinin-related peptides are less hydrolyzed by ACE inhibition.

sion molecules, cytokines, reactive oxygen species (ROS), prostaglandins and NO. It was also proposed that circulating Ang II stimulates brain vascular endothelial target cells, producing BBB breakdown, allowing macrophage infiltration into brain parenchyma, increasing microglia and astrocytes activation[53]. Ang II also induces C-reactive protein production by vascular cells as well as by macrophages in culture[56].

RAS and cerebrovascular alteration

Several authors have shown that captopril (ACE inhibitor) improves cerebrovascular structure and function in old hypertensive rats, attenuating eutrophic and hypertrophic inward, remodeling cerebral arterioles. In contrast, Tanahashi *et al*[57] showed that Ang II is related to stroke protection, mediated by AT2R, AT4R and Ang1-7/Mas receptor. However, these authors also indicated that recent clinical trials demonstrated that blockade of the RAS has a potential role in stroke prevention. These data show that the RAS may have dual function in the brain, depending on the action of different peptides and their receptors.

RAS in extrapyramidal diseases

RAS has been identified in the nigrostriatal system and, according to several authors, dopaminergic neurons have an intracellular/intracrine RAS[58,59]. As already mentioned, Ang II acts on the inflammatory cascade, *via* AT1R, producing high levels of ROS by activating the NADPH oxidase complex[60], which are the early processes leading to dopaminergic cell death, in the nigrostriatal system, in Parkinson's disease[61]. These data showed that AT1R blockage reduces dopaminergic neuron loss as well as lipid peroxidation in the Parkinson model (injection of 6-OHDA in rats). These authors also concluded that the RAS is present in dopaminergic neurons with high vulnerability in the nigrostriatal system. The interaction of dopamine/Ang II may be a major factor in age-related dopaminergic vulnerability, that could be the result of increased AT1R expression, decreased AT2R expression, enhanced levels of inflammatory mediators and ROS in dopaminergic pathways[61]. Thus, manipulation of RAS using AT1R antagonists or ACE inhibitors could be helpful in the treatment of Parkinson's disease. In addition, other authors[53,62] also advocate the use of AT1R blockers in the treatment of several inflammatory brain disorders.

RAS and dementias

Other brain pathologies such as AD have also been linked to the RAS. Longitudinal studies have suggested an association between high blood pressure and demen-

tia, showing that hypertension is a risk factor for the development of AD during aging. Patients treated with perindopril (ACE inhibitor) with previous stroke and/or ischemic events were followed for 4 years and dementia and/or cognitive decline were reduced in the treated group, showing a connection between these dual pathologies[63]. Captopril (ACE inhibitor) improves cerebrovascular structure in hypertensive subjects. Indeed, benefit was found when an ACE inhibitor was able to cross the BBB, showing that peripheral action is important, but the effect on cognition is not exclusively due to blood pressure control, but is related to the central action of these drugs[64]. Yamada *et al*[65] showed that perindopril ameliorated cognitive performance in rats submitted to AD models, through inhibition of brain ACE.

In contrast, other authors showed that ACE converts Aβ1-42 (amyloidogenic form) to Aβ1-40 (soluble form), decreasing the Aβ1-42/Aβ1-40 ratio. According to these authors, ACE is also able to degrade Aβ1-42 and Aβ1-40, thus reducing the risk of AD development. They also suggested that treatment with captopril promotes predominant Aβ1-42 deposit in the brain, increasing neuronal vulnerability and death, contradicting the data obtained in patients with hypertension and dementia, treated with this ACE inhibitor. These authors suggest that new strategies could be implemented to improve ACE activity, as novel targets in the treatment of AD[66].

RAS and epilepsy

Other ACE inhibitors such as fosinopril, zofenopril, enalapril and captopril have been associated with the potentiation of antiepileptic drugs[67]. These authors showed that the combination of carbamazepine, lamotrigine, topiramate and valproate with ACE inhibitors decrease audiogenic seizures. Captopril also potentiates the effect of carbamazepine and lamotrigine against electroshock seizures[68]. These data were confirmed in other models of epilepsy. According to Pereira *et al*[69], ACE inhibitor and/or AT1R antagonist were able to reduce the severity of audiogenic seizures. These data link the RAS with generalized seizures and with other types of epilepsies.

In 2008 our group showed, for the first time, an upregulation of AT1R as well as its messenger expression in the cortex and hippocampus of patients with temporal lobe epilepsy, associated with temporal mesial sclerosis[70]. Increased expression of AT2R was also found in the hippocampus showing that the RAS is inwardly associated with this brain disorder. AT1Rs were colocalized with NeuN protein, labeling pyramidal neurons in more vulnerable areas. We also found that a common mutation, which increases ACE activity, occurs in high frequency in the blood cells of patients with TLE and mesial sclerosis. Interestingly, in the hippocampus of these patients, ACE activity was down regulated. Investigating this contradictory data we found that carbamazepine, used to treat seizures was able to inhibit hippocampal ACE activity in these patients. The inhibition of ACE by carbamazepine occurred *in vitro* and *in vivo*, confirming a strong link between TLE and RAS. Patients not treated with carbamazepine showed increased ACE activity[71].

In trying to understand the alteration of RAS components in the epileptogenic process we studied Ang I, Ang II and Ang1-7 levels in the hippocampus of rats submitted to pilocarpine-induced TLE. We found decreased levels of Ang I in acute (status epilepticus), silent (seizure-free period) and chronic (spontaneous recurrent seizures) phases. In contrast, Ang II was increased in the chronic phase, while Ang1-7 was increased in acute and silent periods. These data showed that during the epileptogenic process Ang I was converted into Ang II or Ang1-7. However, ACE expression was decreased in all phases, showing that other enzymes in the RAS may participate in this event such as NEP and Tonin. Indeed, both enzymes were upregulated in the hippocampus of these rats[72]. Our results also showed an upregulation of AT1R during the spontaneous seizure period (chronic phase)[71], in accordance with data found in patients with TLE[70], supporting the involvement of this receptor in seizure generation. The silent phase was characterized by an increase in Ang1-7 levels as well as its Mas receptor. Interestingly, during the silent phase of this model, intense hippocampal reorganization occurs, which has been related to Ang1-7/Mas-induced plasticity.

CONCLUSION

In conclusion, peptides generated by the RAS or KKS are deeply involved in several neurological diseases and an improvement in the knowledge of their function and release in tissues and blood could be useful in the development of new targets and drugs to treat these pathologies.

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REVIEW

"Stop Ne(c)king around": How interactomics contributes to functionally characterize Nek family kinases

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Abstract

Aside from Polo and Aurora, a third but less studied kinase family involved in mitosis regulation is the never in mitosis-gene A (NIMA)-related kinases (Neks). The founding member of this family is the sole member NIMA of Aspergillus nidulans, which is crucial for the initiation of mitosis in that organism. All 11 human Neks have been functionally assigned to one of the three core functions established for this family in mammals: (1) centrioles/mitosis; (2) primary ciliary function/ciliopathies; and (3) DNA damage response (DDR). Recent findings, especially on Nek 1 and 8, showed however, that several Neks participate in parallel in at least two of these contexts: primary ciliary function and DDR. In the core section of this in-depth review, we report the current detailed functional knowledge on each of the 11 Neks. In the discussion, we return to the cross-connections among Neks and point out how our and other groups' functional and interactomics studies revealed that most Neks interact with protein partners associated with two if not all three of the functional contexts. We then raise the hypothesis that Neks may be the connecting regulatory elements that allow the cell to fine tune and synchronize the cellular events associated with these three core functions. The new and exciting findings on the Nek family open new perspectives and should allow the Neks to finally claim the attention they deserve in the field of kinases and cell cycle biology.

Key words: Cell cycle; Mitosis; DNA damage response; Protein interactions; Kinases

Core tip: Never in mitosis-gene A (NIMA)-related kinases (Neks) are a family of 11 human kinases involved in cell cycle regulation. This article represents an in-depth review of the current knowledge on the function of each of the 11 human Nek kinases. Furthermore, we present arguments in the discussion of how systems biology, especially interactomics, helped to uncover that the majority of Neks are involved in more than one of

the three Neks core functions: (1) centrioles/mitosis; (2) primary ciliary function/ciliopathies; and (3) the DNA damage response. Possibly, the Neks act on a higher regulatory level which may control the core functions.

Meirelles GV, Perez AM, de Souza EE, Basei FL, Papa PF, Melo Hanchuk TD, Cardoso VB, Kobarg J. "Stop Ne(c)king around": How interactomics contributes to functionally characterize Nek family kinases. *World J Biol Chem* 2014; 5(2): 141-160 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i2/141.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i2.141

INTRODUCTION

The never in mitosis-gene A (NIMA)-related kinases (Neks) represent, aside from the Polo and Aurora kinase families, a third family of mitotic kinases, but remain the least studied to date and hence least understood family of kinases involved in the regulation of the cell cycle. The founding member of this family of kinases is the *Aspergillus nidulans* NIMA, which exists as a single member in this fungus, is functionally involved in the initiation of mitosis and promotes the chromosome condensation by phosphorylation of histone H3[1]. Humans have 11 members of the Nek family which show highly conserved kinase domains but differ significantly in the composition and length of their N- and especially C-terminal regulatory and docking domains (Figure 1).

Although some protein interaction partners have been described for the majority of the human Neks (Figure 2), the domain of interaction at the side of Neks has been mapped only for a smaller subset of interacting proteins (Figure 1). As we can see, most interactors are assigned to specific regions in the regulatory domains, which represent in most cases classical protein-protein interaction modules, such as coiled coil regions. Identification of interaction with the kinases domains have been scarce due to the transient and weak nature of these interactions and therefore the discovery and characterization of true *bona fide in vivo* substrates of Nek kinases remain one of the main challenges in the field. Among the interacting proteins identified by our[2,3] and other groups, through both yeast two-hybrid screens and mass spectrometry analyses, there were hopefully not only those that regulate the Neks but maybe also candidate substrate proteins. The binding of these substrate proteins possibly contributes to "opening up" the Neks or to the activation of these kinases and then, as a consequence, these proteins may be phosphorylated by the Neks.

There has been a series of very good and concise reviews on NIMA and Neks in the past years[4-8]. However, due to scarce or absent knowledge on several family members, including Nek5, 10 and 11 for instance, most reviews opted to focus on a subset of Neks or grouped them according to phylogenetic or functional relatedness. Here, we try to discuss all 11 human Neks in some depth and to include all recent novelty on the least studied Neks as well as our own group's published and unpublished findings, with a special emphasis on the characterization of the functional context based on the identification of interacting proteins (interactomics). A point we would like to stress here is that most Neks interact with proteins of several of the classical functional contexts reported initially for a subset of specific Neks. In other words, we may characterize the following three areas as the main functional contexts of Neks: (1) centriolar function and mitosis regulation (Nek2, 6, 7 and 9); (2) primary ciliary function, ciliopathies and microtubule dynamics in general (Nek1, 4 and 8); and more recently (3) DDR and G_2/M checkpoint (Nek1, 4, 6, 8, 10 and 11)[8,9].

However, published interactome data (Figure 2), as well as our group's efforts to identify new interacting proteins for all Neks, showed some surprising cross-connections and novelties, which we would like to point out here. Most of the above mentioned Neks seem to interact with proteins that are functionally linked to two or even all three of the above mentioned areas, thereby raising the possibility that these are somehow connected on a higher regulatory level and that the Neks may be key elements to understand how the regulation of these functional contexts is performed. A typical recently published example is the role of Nek8 in both primary ciliary function and DNA repair mechanisms[10]. Our own studies revealed that Nek6, a kinase primarily associated with mitotic regulatory events[11,12], also interacts with proteins involved in the DNA damage response, such as putative DNA repair and recombination protein RAD26-like (RAD26L) and PHD finger protein 1 (PHF1) (Figure 2)[3]. In fact, for the majority of Neks we found interacting partners of the DDR or effector proteins of different DNA repair pathways, which clearly suggests a larger than initially imagined involvement of Neks in these biological processes. Other insights came from the identification of interacting proteins from the apoptosis regulatory pathways with several Neks (*e.g.*, Nek 1[13] and 5). This suggests that, aside the well established mitotic context, we must be open minded about additional new roles for Neks (Table 1). Before we go into details of new cross-connections and suggested additional functional contexts in the final discussion, we will present each of the 11 human Neks in detail in the following section of this review.

NEK1

Although Nek1 is only the third most studied Nek family member after Nek2 and aside from Nek6, it is in many ways a representative member of this family of protein kinases. Along this line, Nek1 started to draw the attention of the kinase and signaling research communities, not only to itself but to the Nek family after the publication of the seminal article of Upadhya *et al* in 2000[14]. It reported that deletion mutations in the Nek1 gene in mice caused polycystic kidney disease (PKD) among other pleiotropic effects, ranging from facial dysmorphism,

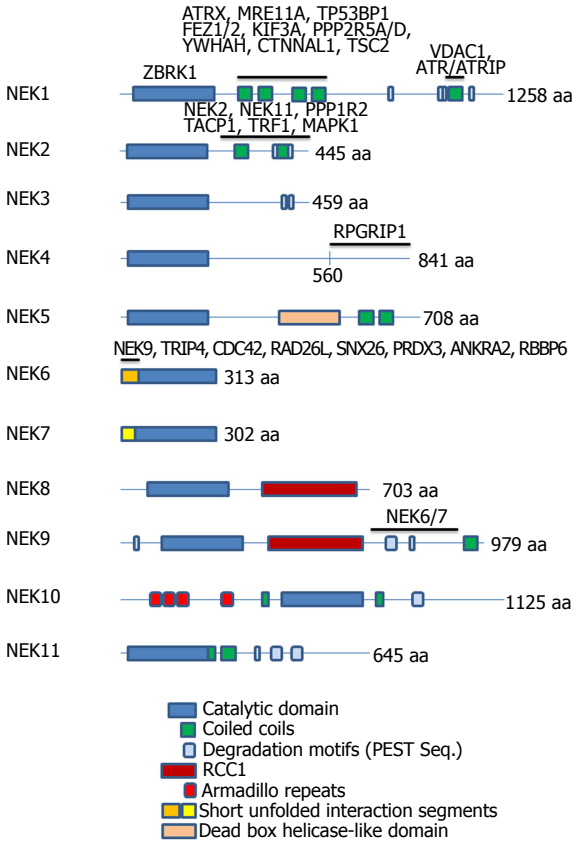

Figure 1 Representation of the domain organization of the eleven human Neks depicting the domain regions for selected protein interactions. The gene symbols corresponding to interacting proteins are shown above the Neks primary structure regions with which they have been found to interact. The list of interactors is not intended to be complete but is necessarily shorter than the list of all proteins known in the literature to interact with Neks (*e.g.*, see Figure 2), since, for the majority of interactors, the location of interaction in the Neks has not been reported. Different repeated domains have been indicated by the color code at the bottom of the figure. The lengths of the full proteins are indicated by number of amino acids (aa) at the C-terminal of the proteins. At least two isoforms of Nek1, 2, 3 and three of Nek4 and 11, all generated by alternative splicing, have been reported and known functional distinctions have been briefly discussed in the text, where feasible. References for the proteins and their mapped interactors: Nek1[2,13,25]; Nek2[116,121-124]; Nek4[53]; Nek6[3]; Nek9[66]. Nek: Never in mitosis-gene A-related kinases.

dwarfing, male sterility, anemia and cystic choroid plexus. The pleiotropic nature of these phenotypes suggested a role of Nek1 early on in basic cellular functions, possibly involved in signaling pathways associated with polycystin-1 and 2, whose mutations also cause PKD and signaling initiates at the renal epithelial cell's primary cilia[15].

Recently, another set of insertion, non-sense and splice site mutations in the Nek1 gene were reported in Majewski type short-rib polydactyl syndrome (SRPS), an autosomal-recessive familiar ciliopathy[16,17]. Ciliopathies have been associated with a series of defects of proteins involved in intra-flagellar transport (IFT), as well as cilia, basal body and centrosome maintenance, and in the case of Nek1, SRPS also presents a broad phenotypic spectrum, including reduced cilia number and cell cycle associated cilia morphogenesis. This results ultimately in severe or lethal embryonic malformations and especially osteochondrodysplasia, shortened ribs and tibias, polysyndactyly, fused kidneys, heart defects and mouth clefts, among others[17].

In terms of molecular functions, a first breakthrough came from a protein interactome study that shed light on the involvement of Nek1 in several pathways related to the above diseases, but also opened new avenues in the context of cell cycle regulation and DNA damage responses[2]. These findings were later not only confirmed by functional studies but also extended to other Nek family members, including Nek4, 6, 10 and 11[3,8,9,18]. The interactome study was a yeast two-hybrid assay using Nek1 as bait and a human fetal brain cDNA library as prey. Nek1 is a rather large, 1258 amino acids containing protein and interacts with these proteins mainly through the two N-terminals of its four coiled coil regions, which are located at the C-terminal of its kinase domain (Figure 1). Among the Nek1 interacting proteins were the kinesin-like protein KIF3A, tuberin and alpha-catulin, mutation in all three of these genes also have been reported to cause PKD. This suggests the existence of a multicomponent signaling or regulatory pathway, which regulates the kidney cell's proliferation and when affected by mutations may lead to PKD[19-21]. Evidence in support for a major role of Nek1 in primary ciliary function also came from other model organisms, including *Chlamydomonas*[22].

Surprising at that time was the discovery of interactions with several cell cycle regulatory proteins, 14-3-3 protein η (*eta*, YWHAH), tumor suppressor p53-binding protein 1 (TP53BP1), serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit alpha/delta isoform (PPP2R5A/D) and especially with proteins involved in the DNA damage response, such as the double-strand break repair protein MRE11A (MRE11A) and the transcriptional regulator ATRX (ATRX)[2]. Soon, additional experiments with the irradiation of wild-type and Nek1-/- cells revealed that Nek1 is overexpressed and activated in response to ionizing radiation (IR) and co-localizes to γ-H2AX positive DNA repair foci in the nucleus[23]. Cells without Nek1 died in response to sub-lethal doses of IR and knockdown of Nek1 also diminished their capacity to clear DNA damage caused by chemical genotoxic agents, such as cisplatin and methyl-metanesulfonate (MMS)[24]. This line of experiments culminated recently in a paper where the authors showed that Nek1 kinase is not only physically associated with ATR-ATRIP, but also required for ATR priming to allow an efficient DNA damage signaling[25]. Furthermore, Nek1 has been indicated to act in apoptosis signaling, especially by phosphorylation of key mitochondrial proteins such as the voltage-dependent anion-selective channel protein 1 (VDAC1)[13]. This is a pore complex that functions both as a voltage dependent anion channel and permeability pore that regulates cytochrome c leakage to the cytoplasm, which upon exit initiates apoptotic events[13]. Nek1's activity to maintain cells in homeostasis is mediated through phosphorylation of a specific external VDAC1 Ser residue. Upon apoptotic stimuli, Nek1 is degraded and the lack of

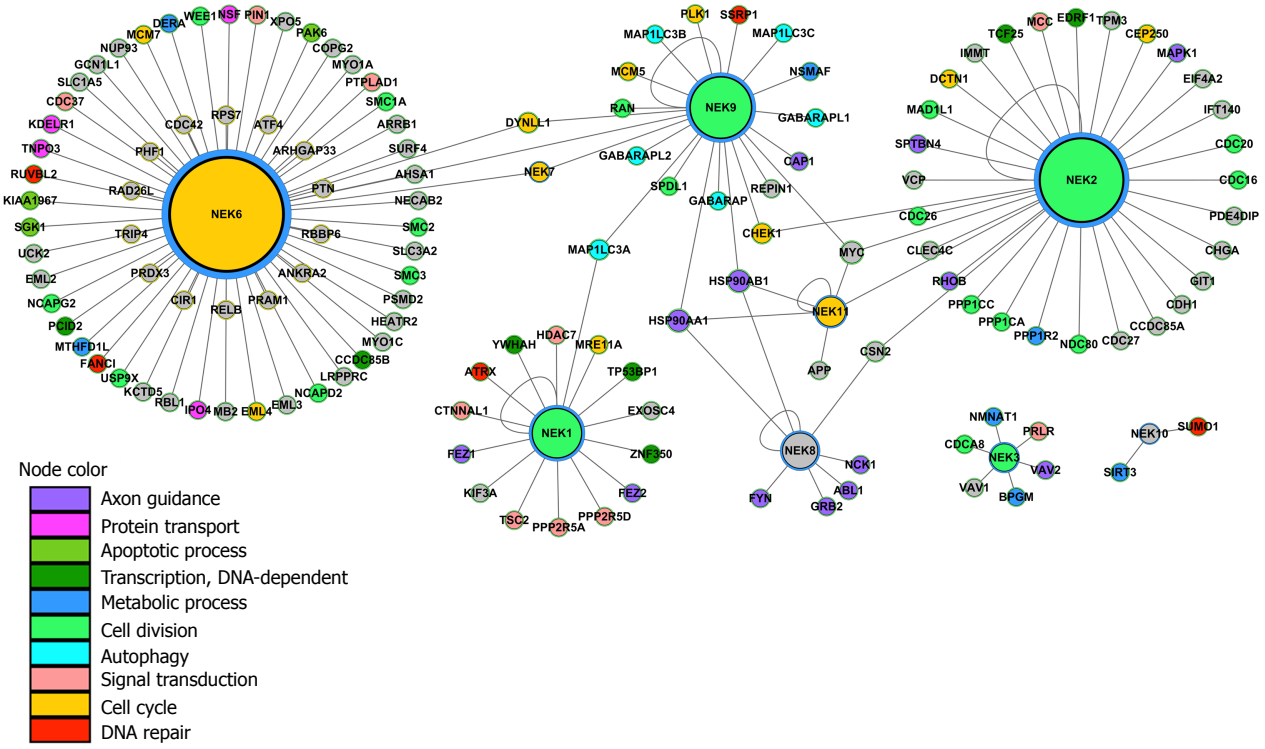

Figure 2 Global interactome of Nek1-11, involving their published interactors. The proteins color code refers to their main biological function given by the top enriched Gene Ontology[125] biological processes ($P \leqslant 0.05$). Common interactors establish crosslinks between Neks, thereby emphasizing their common functional contexts. The protein sizes are depicted proportional to their connectivity degree. The protein-protein interaction network was built for the first neighbors of Neks using the Integrated Interactome System (IIS) platform, developed at National Laboratory of Biosciences, Brazil (http://www.lge.ibi.unicamp.br/lnbio/IIS/) and visualized using the Cytoscape software[126]. Nek: Never in mitosis-gene A-related kinases.

VDAC1 phosphorylation causes opening of the channel, loss of the membrane potential and leakage of cytochrome c to the cytoplasm.

Finally, Nek1 has been implicated in gametogenesis due to its high expression levels in meiotic tissues[26]. In another interactome study, this time using a testicular tissue cDNA library, the protein Nurit was found to be an interactor of Nek1[27]. Nurit is expressed in the late phase of spermatogenesis, has structural resemblance with leucin zippers and contains additional super helix domains, possibly involved in its homo-multimerization. Furthermore, the structural maintenance of chromosomes protein 3 (SMC3) was found to interact with Nek1, further implying important functions in meiotic events such as spindle assembly checkpoints[28].

In summary, Nek1 has been functionally implied in three major functional contexts and their sub-functions: ciliogenesis (PKD, SRPS), DNA damage response in a wider sense, also including cell cycle checkpoints and centrosome functions and, finally, gametogenesis. Unpublished recent mass spectrometry studies of the Nek1 interactome after challenging cells with genotoxic drugs identified a number of nuclear proteins, the majority of which were associated with DNA repair, replication and transcription regulation. This, together with a very recent article which reports on Nek1 interaction with NHEJ (Non homologous end joining) repair protein Ku80, clearly establishes Nek1 as a key player in DDR signaling[29].

NEK2

Nek2 is the most studied and most well understood of the human Neks. In fact, it will be difficult to cover all of its aspects in the context of this review. Therefore, we focused on the most important features of Nek2 and would like to apologize to the many researchers whose work could not be covered here due to space restrictions.

Nek2 shares the highest sequence similarity with NIMA in its kinase domain and many biochemical, structural and functional features. This has led many researchers to believe that it may be the prototype NIMA among all vertebrate Neks and that Nek2 may maintain the primordial functions of NIMA in mitosis progression. For this reason, Nek2 became the most studied Nek family member in mammals[6]. However, care must be taken with such an interpretation since Nek2 cannot rescue NIMA defective mutants and Nek1 also shares many NIMA characteristics[30].

Nek2 expression varies during the cell cycle, being maximal between the S and G_2 phase, during which it localizes predominantly to the centrosome[31,32]. Nek2 is a component of the MTOC (microtubule organization center) at mitosis entry and a core component of the centrosome, where it phosphorylates the centrosomal key components C-Nap1 and rootletin, which form the intercentriolar linker that holds the pair of centrioles physically together. This event in turn promotes centro-

Table 1 Subcellular localization, established and possible additional functions of human and mammalian Neks

Nek	Gene/ protein synonyms	Subcellular localization	Established function	Possible additional functions (under investigation)
1	NY-REN-55 SRPS2, SRPS2A, KIAA1901	Cytoplasm, cilia, centrosome, γH2X positive DNA damage sites in nucleus	Stability and function of the primary cilium/polycystic kidney disease[14], DNA damage response to IR and chemical mutagens[2,23-25]	Meiosis[26-28], apoptosis mediated by mitochondria[13]
2	NEK2A, NLK1, RP67, HsPK21, SRPS2A	Centrosome	Regulation and promotion of centrosome segregation[33-35]	DNA damage response[127]
3	HSPK36, RP11-248G5.5	Cytoplasm	Regulation of prolactin response[41], microtubule deacetylation in neurons[47]	?
4	STK2, NRK2, pp12301	Cilia/basal bodies	Microtubule stability (silencing alters sensitivity to vincristine/taxol)[54]	DNA damage response[9], replicative senescence[9], primary cilia function[53]
5	-	?	Skeletal muscle differentiation[60], caspase-3 substrate/ apoptosis[60]	?
6	SID6-1512, RP11-101K10.6	Citotic spindle, centrosome	Mitotic spindle formation[11-12], centrosome separation[69-70]	DNA damage response[18], NF-kappa B signaling?[3,71]
7	-	Spindle poles	Mitotic spindle formation[12,88], centrosome separation[69-70]	DNA damage response?[1]
8	JCK, NEK12A, NPHP9, RHPD2	Centrosome, cilia, γH2X positive DNA damage sites in nucleus	Stability and function of the primary cilium/polycystic kidney disease[95], DNA damage response[10]	Integration of primary cilia function and DNA damage response[10]
9	NERCC, NERCC1, KIAA1995, (NEK8)	Spindle poles, centrosome, cytoplasm	Mitotic spindle formation[106], centrosome separation[100]	?
10	-	Possible centrosome/pericentriolar localization (?)	DNA damage response after UV induced damage[74]	Centrosome function?
11	-	Nucleus, nucleoli	DNA damage response induced by IR[73]	?

[1]Souza *et al*, unpublished observation.

some separation itself[33,34]. During the interphase, Nek2 is maintained in an inactive state by association with the protein kinase MST-2 and the phosphatase PP1, which keeps Nek2 dephosphorylated. After mitosis onset, polo-like kinase 1 (PLK1) phosphorylates MST-2, disrupting the trimeric complex and resulting in Nek2´s activation through auto-phosphorylation. In addition, the centrosomal proteins Nlp (ninein-like protein) and centrobin contain coiled coils and are dislocated from the centrosomes in Nek2 overexpression conditions. In contrast, the Nek2 knockdown or inhibition of its catalytic activity results in the inhibition of the centrosome separation[35].

A second important functional context for Nek2 is at the spindle assembly checkpoint, where through its interaction with the major kinetochore proteins Mad1/2 and the phosphorylation of the kinetochore core protein Hec1, Nek2 may be involved in the identification of unaligned sister chromatids[36]. Failure at this checkpoint may lead to aneuploidy and other chromosomal abnormalities and knockdown or knockout of other Neks, including Nek7, has been reported to cause aneuploidy, pointing to a potential major involvement of the Nek family in the spindle assembly checkpoint[37].

Another functional context for Nek2 is in the gametogenesis, where Nek2 acts in chromatin condensation reminiscent of the role of NIMA in *Aspergillus nidulans*. In spermatocytes, the architectural chromatin protein Hmga2 is under control through phosphorylation by mitogen-activated protein kinase (MAPK) and possibly also by Nek2[38].

Finally, in *Drosophila*, Nek2 was detected at the midbody in the late mitosis and overexpression of Nek2 led to actin and actin-binding protein dislocation and cytokinesis failure, among other phenotypic effects[39].

NEK3

Nek3 is a 506 amino acid serine/threonine kinase[40] and localizes both to the nucleus and cytoplasm[41,42]. It is highly expressed in testis, prostate, ovary and brain, and shows moderate to low expression in lung and liver[40]. Its gene localizes to chromosome 13q14.2 and its mRNA is expressed in tumor, normal prostate and blood control cell lines. Insertion/deletion polymorphisms were described, in which a stretch of adenines at the end of exon 9 leads to the introduction of a premature stop codon, resulting in a truncated protein that encodes only 298 or 299 of the protein amino acids. Interestingly, this polymorphism around 13q14 is a mutational hotspot for several cancer types[43-45]. Moreover, human Nek3 has an N-terminal catalytic domain and a C-terminal regulatory domain and shares high amino acid sequence identities with mouse Nek3 (56%), but not with other NIMA-related kinases due to the absence of coiled coil regions (Figure 1)[46]. This suggests that Nek3 and its orthologs constitute a separated sub-family of the Neks[40].

Nek3 is involved in the invasion and motility of T47D cells (a human ductal breast epithelial tumor cell

line) through interaction with the guanine nucleotide exchange factor VAV2, which promotes both p21-Rac1 and transforming protein RhoA activation. These interactions are mediated by prolactin-induced association of Nek3 with the human prolactin receptor (PRLR). The signaling pathway resulting from prolactin's binding to its receptor promotes phosphorylation of paxillin, a cell adhesion mediator, and is dependent on Nek3's association with VAV2[41,42].

In its C-terminal domain, Nek3 contains a PEST motif which contains Thr475, a residue that is phosphorylated upon activation. The Thr475 and the PEST domains are phylogenetically conserved, suggesting that they are important for Nek's regulation. Expression of mutants without the Thr475 or the PEST domain cause changes in cellular morphology and polarity of both epithelial and neuronal cells. Thus, Nek3 may also be crucial to the regulation of neuronal microtubules and in disorders which involve axonal degeneration, possibly through modification of its acetylation status[47].

Another functional involvement of Nek3 with cytoskeleton components is mediated through its interaction with the EH domain-containing protein 2 (EHD2). EHD2 interacts with plasma membrane phospholipids, associates with VAV1, and forms the complex VAV1-NEK3-EHD2, which modulates p21-Rac1 activity, causing actin reorganization close to the plasma membrane at the initial stages of endocytosis[48]. In summary, Nek3 plays a role in cytoskeleton organization and dynamics through actin re-organization and may be involved in the regulation of neuronal development, endocytosis, cell motility and invasiveness of breast cancer tumor cells.

NEK4

Nek4 was initially described as serine/threonine-protein kinase 2 (STK2) by Cance *et al*[49]. In a study of a kinase specific cDNA library in human breast cancer tumors or cell lines, they identified STK2 that showed homology to *Aspergillus nidulans* NIMA and expression levels that varied widely in human breast tumors. Later, Levedakou *et al*[50] showed that STK2 is highly expressed in the heart and that its mRNA level does not vary along the cell cycle. After studies characterizing the murine STK2 the nomenclature changed to Nek4[51,52].

The human Nek4 gene is located on chromosome 3p21.1 and is transcribed into about 4kb mRNA, which encodes an 841 amino acid residue protein[50]. It is constituted by a N-terminal kinase domain and a C-terminal regulatory domain (Figure 1). Hayashi *et al*(1999)[51] described a short and a long isoform for murine Nek4. The long mNek4 isoform differs from hNek4 due to the absence of a small fragment in the regulatory domain that corresponds to an *Alu* sequence[51,52]. To date, three isoforms have been described for human Nek4. The longest canonical sequence (isoform 1: UniProtAccession P51957-1, NCBI RefSeq NM_003157) was identified by the Cance and Levedakou groups[49,50] and used to compare it to mNek4. The isoform 2 (UniProt database (UniProt Accession P51957-2 , KJ592714), is identical to mNek4 and lacks the *Alu* sequence. The isoform 3 (UniProtAccession P51957-3 and NCBI RefSeq NM_001193533) is the shortest one, with a smaller alternative N-terminal region.

Hayashi *et al*[51], (1999) showed that two isoforms of mNek4 are expressed in most tissues, except in the liver and heart where only a short isoform is expressed[50]. Recently, hNek4 expression was also observed in ciliated tissues, such as the retina, kidney tubules, brain (specifically the ventricles), heart and testis[53]. Expression in testis suggests a role in meiosis, as has been already reported for mNek4[52]. Furthermore, these new functional studies demonstrated that hNek4 depletion does not alter the cell cycle[53,54]. Therefore, as shown for other Nek family members, roles other than the regulation of the cell cycle can be attributed to Nek4, including microtubule stabilization, primary cilium assembly and, more recently, replicative senescence entry and DNA damage response[9,53,54].

Interestingly, Nek4 activity is evidenced mainly in the presence of chemotherapeutic agents. For example, in lymphoma cells, a simple Nek4 knockdown is not enough to change cell cycle or microtubule dynamics, but Nek4 knockdown triggers taxol resistance and promotes sensibility to vincristine in these cells[54]. These results indicate that Nek4 has an effect on microtubule stability in the presence of these drugs and suggests that this particularity could be explored in therapies, depending on the patient's specific levels of Nek4 protein in the tumor cells.

Besides the direct role in microtubule polymerization, Nek4 is also important for primary cilium stabilization, as was already described for Nek1 and Nek8[14,55,56]. Nek4 interacts with RPGR-interacting protein 1 (RPGRIP1) and RPGRIP1-like protein (RPGRIP1L)[53], both associated with ciliopathies. Both the eye-restricted disease "Leber Congenital Amaurosis" and the "Joubert and Meckel syndrome", which affects multiple organs, are at the severe end of the ciliopathy spectrum. After Nek4 knockdown, the number of ciliated cells decreases, but this effect is apparently not related to RPGRIP1 and RPGRIP1L phosphorylation status. This suggests that Nek4 may act as a scaffold for other cilia signaling proteins[53] and, together with Nek1 and Nek8, may be important to other ciliopathies such as PKD[14,55,56].

More recently, the role of Nek4 was also connected to the DDR because Nek4 depleted cells were found to be resistant to DNA damaging agents, such as etoposide or bleomycin, and to γ-irradiation. Besides, Nek4 interacted with DNA-PKcs, Ku70 and Ku80, proteins that have important roles in the NHEJ (non-homologous end joining) repair pathway. Nek4 depleted cells also show a decrease of histone γ-H2AX activation, probably as a result of an impairment of the DNA-PKcs recruitment[9].

NEK5

Among all members of the Nek family, Nek5 is the kinase with the least amount of information. Although identified in different organisms such as *Homo sapiens,*

Mus musculus, Arabidopsis thaliana, among others, there is little information about its function and localization. In humans, Nek5 is a protein of 708 amino acids, whose kinase domain is located at its N-terminus[4,8]. According to Moniz *et al*[7], Nek5 is the only member of the Nek family that has a dead box domain (Figure 1). This domain is involved in cellular processes such as pre-mRNA processing, rearrangement of ribonucleoprotein (RNP) complexes and gene expression[57]. In *Arabidopsis thaliana*, during epidermal cell expansion, Nek5 interacts with Nek4 and 6 and these interactions are important to regulate microtubule organization, probably through the phosphorylation of beta-tubulins[58]. Therefore, Nek5 may be associated with the already established cascade consisting of Nek9, 6 and 7 (see details below). However, care must be taken because the evolutionary gap between mammals and flower-plants is too large to deduce direct conclusions and the functional information on Neks in plants is even scarcer than in mammals[59]. In human cells, Nek5 is able to interact with caspase-3 and this interaction is important for skeletal muscle differentiation[60]. Caspase-3 is a protease involved in mechanisms such as apoptosis and cell differentiation. It was proposed by Larsen *et al*[61] that caspase-3 activates caspase-activated DNase to promote and regulate DNA strand breaks introduced into promoter regions of genes encoding effector proteins such as p21 and that this process may represent a more general mechanism of genome alterations that occur during cell differentiation. Since Nek5 interacts with caspase-3 during cell differentiation, other members of this kinase family may also be involved in differentiation associated molecular events and this possibility should be explored in future experiments.

NEK6

Unlike the other Neks, Nek6 and Nek7 are the smallest and structurally the simplest Neks, consisting only of the catalytic domain with a relatively short N-terminal extension[8]. Although they share significant similarity with each other, being about 86% identical within their catalytic domains, their N-terminal extensions are not conserved and it has been suggested that they may play a role in the differential regulation of these kinases[3,62]. SAXS experiments, together with SEC-MALS and comparative molecular modeling performed by our group revealed that hNek6 is a monomeric kinase, slightly elongated, with a flexible and disordered N-terminal domain[63].

Nek6 was initially identified in a classic biochemical screen for kinases capable of phosphorylating the hydrophobic regulatory site of the p70 ribosomal S6 kinase (S6K). Nek6 phosphorylated the Thr412 residue of S6K and other sites, *in vitro* and *in vivo*, suggesting it to be a possible regulator of this kinase[64]. Subsequently, Nek6 was described as not seeming to be responsible for the physiological phosphorylation of S6K, SGK or PKB since it was characterized as having a high preference for a Leu three residues N-terminal to the phosphorylation site of the substrate[65], and more recent evidence supports a NIMA-like mitotic role for Nek6.

Both Nek6 and Nek7 co-purify with Nek9 as a result of specific interactions and strong binding to a region located between the RCC1 domain and coiled coil motif of Nek9[66] (Figure 1). The endogenous Nek6 is activated during mitosis, concomitant with an increase in its level of expression, but this requires phosphorylation at the Ser206 residue, which is mediated through Nek9. Nek7 too is phosphorylated by Nek9 at Ser195 and both phosphorylation sites are found in the activation loops of these kinases[67]. This information led to the construction of a model in which Neks 6, 7 and 9 act as partners of the same signaling cascade[67], with Nek6/7 being substrates of Nek9. However, Nek9 remains inactive during the interphase but is activated during mitosis, phosphorylating and activating Nek6/7, which, in turn, coordinates the organization and maintenance of the mitotic spindle[66].

Overexpression of a catalytically inactive mutant of Nek6 generates cells displaying high mitotic index, defects in mitotic spindle, nuclear abnormalities and apoptosis[11]. These phenotypes are also observed from the depletion of Nek6/7 in HeLa cells using siRNA, which causes retention of cells in metaphase, with a normal chromatin condensation and alignment, but an inability to complete the segregation of chromosomes. The activity of Nek6 and also 7, therefore, seems necessary for the progression of anaphase, where the cells are either retained at the spindle assembly checkpoint (SAC), or undergo apoptosis or complete mitosis, but with an elevated risk of acquiring chromosomal abnormalities during the process[11,12]. Moreover, treatment of these depleted cells with an Aurora B inhibitor to bypass the SAC led to a reduction in the frequency of metaphase arrest, concomitant with an increase in the frequency of cells blocked in cytokinesis. Cells expressing the hypoactive mutants, even in the absence of the SAC inhibitor, also accumulated in cytokinesis. Therefore, Nek6 and Nek7 seem to have independent, non-redundant roles in mitotic spindle formation and cytokinesis: one at metaphase that requires a certain level of kinase activity and one in late mitosis that requires a higher level of activity[12].

Intriguingly, using phospho specific antibodies that detect activated Nek6, Rapley *et al*[68] showed that Nek6 activity increased 2 h after release from a nocodazole arrest, when cells would be progressing through cytokinesis. In this same study, the kinesin-related motor protein Eg5, required for spindle bipolarity, has also been described as a substrate of Nek6. It phosphorylates Eg5 kinesin *in vitro* at several residues, including Ser1033, which is also phosphorylated *in vivo* during mitosis at the spindle poles[68]. A signaling cascade seems to occur where Nek2 first phosphorylates proteins at the intercentrosomal linker in G_2 phase, resulting in their dissociation, followed by activation of Nek9 by the cyclin-dependent kinase 1 (CDK1) and the polo-like kinase 1 (PLK1) in early mitosis and subsequent activation of Nek6 and Nek7. These

kinases, in turn, phosphorylate Eg5 (previously phosphorylated by CDK1), promoting the separation of the centrosomes by the motor activity of Eg5 accumulated in the centrosomes[69,70].

Apart from roles in mitosis, human Nek6 was recently reported by our group to have a broad set of protein partners involved in diverse biological processes[3]. The hNek6 interactome showed that it is a high confidence hub kinase possibly involved in several known and novel cellular pathways, through interactions with and phosphorylation of diverse proteins. Figure 3 depicts some of the main cellular pathways identified for hNek6 based on the interacting proteins retrieved by our screenings. The novel putative pathways shown are the non-canonical Wnt signaling, Notch signaling and the actin cytoskeleton regulation, whereas the other pathways were already suggested by other studies: the nuclear factor kappa B (NF-κB) signaling[71] and the DNA damage response[18]. In regard to the DNA damage response category identified in our work, many studies show its importance among the tasks triggered by Neks[2, 8-10,18,23-25,72-74].

On the other hand, Nek6 phosphorylates the transcription factor Oct-1 (POU2F1), a potent regulator of metabolism and tumorigenicity, at S335 in the DNA binding domain during mitosis, causing Oct-1 to dissociate from the chromatin and concentrate in the centrosomes, spindle poles, kinetochores and midbody[75]. Furthermore, Nek6 phosphorylates histones H1 and H3 *in vitro*, possibly contributing to mitotic chromatin condensation[76]. Nek6 finally also binds the BTB/POZ domain-containing protein KCTD5, which appears to have a role in cytokinesis[77] and apoptosis[78].

As the other human Neks, hNek6 was recently found to be linked to carcinogenesis. It shows an increased expression and activity in gastric cancer according to the progression of the disease[79] and up-regulation of Nek6 mRNA correlates with the Peptidyl-prolyl cis-trans isomerase Pin1 up-regulation in 70% of hepatic cell carcinomas[80]. The overexpression of a catalytically inactive Nek6 promotes cell cycle arrest in human breast cancer in metaphase and leads to apoptosis[11], while its knockdown induces senescence and also apoptosis[81]. In a large-scale screening of serine/threonine kinases on different types of human tumors, Nek6 was shown to be up-regulated in non-Hodgkin's lymphoma, breast, colorectal and lung tumors[82]. Moreover, NEK6 gene, besides AURKA, has its expression increased in esophagitis and esophageal adenocarcinoma, representing a promising candidate marker of these diseases[83]. Recently, it was demonstrated that transcript, protein and kinase activity levels of Nek6 were highly elevated in malignant tumors and human cancer cell lines compared with normal tissue and fibroblast cells, indicating an important role for Nek6 in tumorigenesis[84]. Its phosphorylation at Thr210 and Ser206 is critical for the phosphorylation of STAT3 (signal transducer and activator of transcription 3) at Ser727[85]. Furthermore, its overexpression suppresses p53-induced senescence in cancer cells: it inhibits the cell cycle arrest at both G_1 and G_2/M transition, the reduction in the Cdc2 and cyclin B levels and the increase in ROS levels induced by p53[86]. Its overexpression also makes cancer cells resistant to premature senescence induced by the anti-cancer drugs camptothecin and doxorubicin[87]. The inhibition of the Nek6 function sensitizes human tumor cells to premature senescence after anti-cancer drug treatment or serum depletion[81], suggesting Nek6 to be a potential therapeutic target for various types of human cancers.

NEK7

Human Nek7 was originally described as a possible regulator of the p70 ribosomal S6 kinase[64] and of important events in the mitotic progression[12,6,67,88] (see above for Nek6). These findings have led to studies on the regulatory effects of hNek7 in key functions of the cell cycle and in cancer. The siRNA-mediated down-regulation of hNek7 and expression of kinase inactive mutants reduced centrosomal γ-tubulin levels in interphase cells and caused prometaphase arrest with defects in mitotic spindles[6,88]. Nek7 overexpression in culture cells, on the other hand, resulted in multinucleated cells and a higher proportion of apoptotic cells[89]. In the same line, the Nek7 depletion also decreased microtubule stability, while its ectopic overexpression rescued this phenotype[90]. Furthermore, hNek7 deficient mice die early in development and, on a cellular level, lack of Nek7 led to decreased chromosome numbers, increased centrosome numbers, binucleation, micronuclei formation, cytokinesis failure, growth retardation or cell death[37]. The PCM (centrosomal pericentriolar material) proteins do not accumulate at the centrosome in Nek7-depleted cells in the G_1/S and G_2/M transitions[91], indicating that Nek7 is required for centriole duplication, centrosome maturation and mitotic spindle formation[88].

The direct interaction of Nek7 with the non-catalytic domain of Nek9 allosterically activates Nek7 by interruption of its autoinhibitory conformation[92]. Consistent with these findings, recent studies demonstrated that PLK1 and CDK1 control the centrosome separation through phosphorylation and activation of Nek9 during mitosis. This leads to the Nek6/7-dependent phosphorylation of kinesin Eg5, a key event for centrosome separation and mitosis[69]. Thus, as in the case of Nek6, it is not surprising that cancer cells express elevated levels of Nek7, suggesting a role in tumor progression. Higher expression levels of Nek7 were found in larynx, breast, colorectal[82] and gall bladder cancers[93]. Taken together, these findings suggest Nek7 as a potentially important regulator of the cell cycle and reveal it as an essential component for growth and survival of mammalian cells. Furthermore, the linkage with a failure in centrosome biogenesis, chromosomal stability and ploidy, as well as the observed disturbance of microtubule dynamics connects Nek7 to hallmark features of oncogenesis.

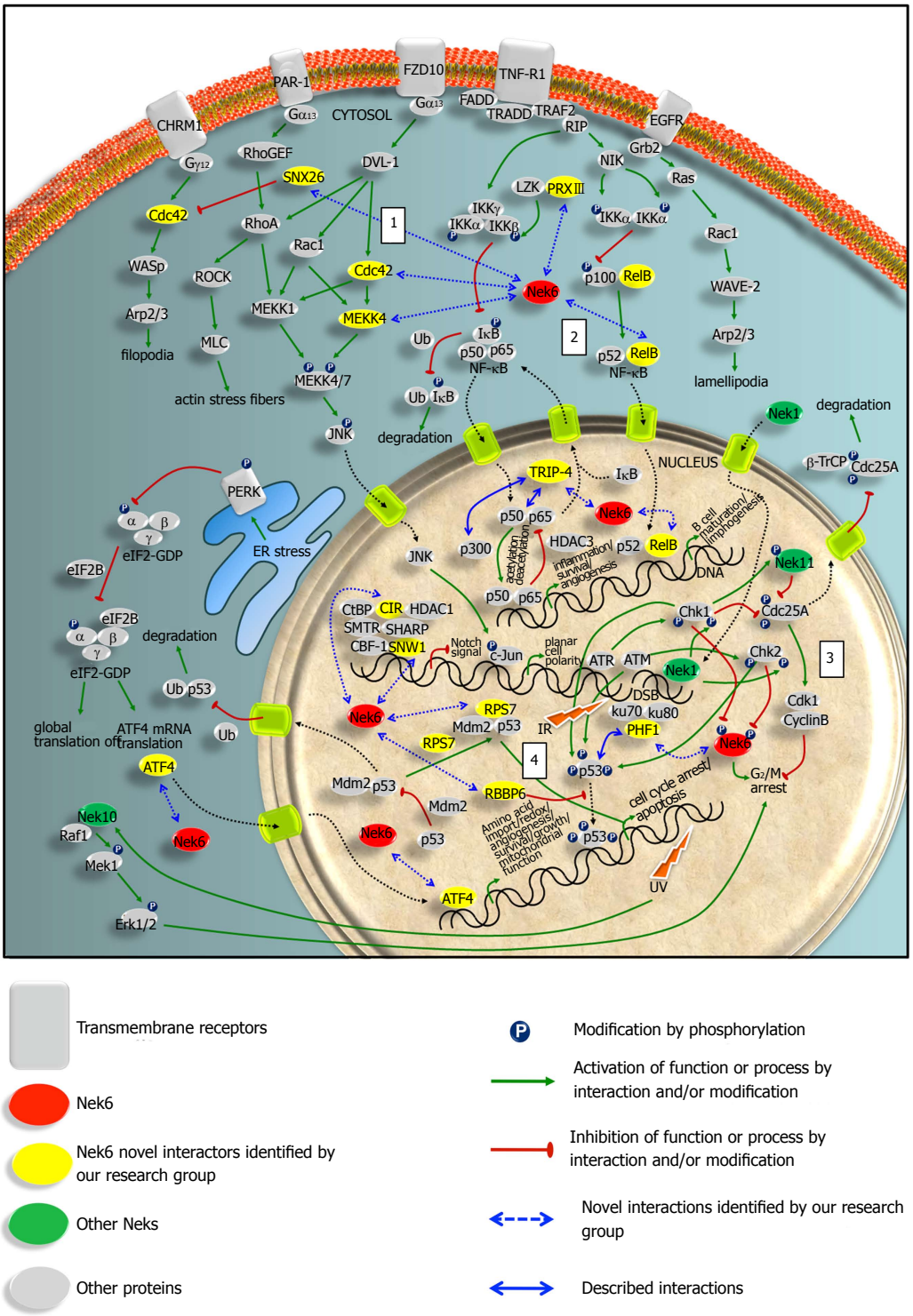

Figure 3 Nek6 interactome and the cellular functional contexts based on its interacting proteins. The four major pathways discussed in the text are: (1) actin cytoskeleton organization; (2) nuclear factor-κB signaling; (3) DNA damage response; (4) p53 signaling (according to Meirelles *et al*). See detailed legend for symbols at the bottom of the figure. IR: Ionizing radiation.

NEK8

Nek8 was first described as the mutated gene in murine autosomal recessive juvenile cystic kidney (*jck*) mice[55]. As observed for Nek1, these mutational changes found in Nek8 C-terminal domain can cause genetic kidney diseases, including polycystic kidney disease (PKD)[55]. PKD is one of the most frequent genetic kidney diseases and has a highly variable pathology, involving aberrant cell proliferation in the kidney and pleiotropic effects in multiple other organ systems, including the liver and the pancreas. Evidence that renal cyst formation is caused by defects in ciliogenesis or ciliary function is substantial[56]. In mouse cells, Nek8 localizes to the proximal region of

the primary cilium and is not observed in dividing cells[56]. In humans, Nek8 is overexpressed in primary breast tumors[94] and localizes to centrosomes and the proximal region of cilia in dividing and ciliated cells, respectively. The localization of Nek8 to centrosomes and cilia is dependent on both the kinase activity and the C-terminal non-catalytic domain homologous to RCC 1 (regulator of chromosome condensation). It is capable of autophosphorylation in the non-catalytic C-terminal region to regulate its localization or activation. Its activity is not cell cycle regulated but, in the same way as observed for Nek3, activity levels are higher in G_0-arrested cells. The kinase domain alone, although catalytically active, does not localize correctly, while a fragment containing only the RCC1 domain shows correct localization and can also be phosphorylated by Nek8[95].

Nek8 carries the causal mutations of two of the eight established mouse models of polycystic kidneys (*jck*). In these models, an abnormal interaction between Nek8 and the polycystin complex may give rise to PKD by disturbing microtubule dynamics, the mitotic spindle checkpoint and the cytoskeleton. Nek8 mutations cause overexpression of galectin-1, sorcin and vimentin and accumulation of the MUP (major urinary protein) in renal cysts of *jck* mice[96].

The role of the RCC1 domain in Nek8 is yet unknown. However, a single G448V substitution is responsible for the *jck* phenotype[55]. Overexpression of mutant forms of Nek8 (including G448V) in tissue culture cells leads to the formation of enlarged multinucleated cells and reduced numbers of actin stress fibers, although tubule cells in *jck* mice are not multinucleated, suggesting that the cellular role of Nek8 may be related to the regulation of the cytoskeleton[55].

Co-immunoprecipitation experiments demonstrated that Nek8 interacts with polycystin-2 (PKD2), a mechanosensing receptor protein, involved in the regulation of the cilium length. However, the *jck* mutation of Nek8 did not apparently affect this interaction directly. These data suggest that Nek8 interferes with the polycystic signal transduction pathways and/or the control of the targeting process of these ciliary proteins. Dysfunction of Nek8 may lead to cystogenesis by altering the structure and function of cilia in cells located at the distal nephron[97].

Recent results suggest that Nek8 has a function in the maintenance of genomic stability[10]. Loss of Nek8 leads to spontaneous DNA damage and a defect in the response of cells to replication stress. Furthermore, Nek8 interacts physically and functionally with components of the ATR-mediated DDR. The disease-related *jck* mutant of Nek8 fails to both interact with the ATR pathway proteins and to rescue the genome maintenance defects associated with Nek8 knockdown. Thus, Nek8 is a critical component of the DDR that links replication stress with primary ciliary functions and the related cystic kidney disorders[10].

NEK9

Nek9, also called Nercc1, is one of the largest Neks with 979 amino acids, with an extensive C-terminal regulatory domain, which contains seven RanGEF homology repeats, an RCC1 domain, a segment rich in Ser/Thr/Pro residues and, like in Nek2, a coiled coil dimerization motif (Figure 1)[66,98].

Nek9 was first described as Nek8 and isolated with a catalytic activity against beta-casein in rabbit lung extracts treated with IL-1, revealing the co-chromatography of a second protein homologous to the Drosophila bicaudal D protein, Bicd2, which is *in vitro* phosphorylated by Nek9 and resembles a cytoskeleton structure[99]. Moreover, Nek9 immunoprecipitation of *Xenopus laevis* egg extracts showed γ-tubulin and other members of the γ-tubulin ring complex (γ-TuRC), which are essential for the microtubule nucleating activity of the centrosome[98]. Centrosomal γ-tubulin recruitment depends on the adaptor protein NEDD1 and is controlled by PLK1. In a recent study by Sdelci *et al*[100], it was reported that PLK1 activates Nek9, which phosphorylates the Ser377 in NEDD1, promoting its recruitment together with γ-tubulin to the centrosomes of dividing cells (independently of Nek6/7). Furthermore, the microinjection of anti-Nek9 in human cells during prophase, after the chromosomes condensation, interferes in the organization of the spindles and the proper segregation of chromosomes, resulting in cell cycle arrest in prometaphase or aneuploidy[66].

Nek9 expression remains constant in different cell cycle phases (G_1/S, G_2, M, G_1); however, as observed for NIMA, there is a specific increase in its catalytic activity during mitosis, which was found to be triggered by *in vitro* and *in vivo* phosphorylation events[66]. The recombinant wild-type Nek9 shows reduced activity when extracted from exponentially growing cells, but its pre-incubation with ATP and Mg^{2+} induces its autophosphorylation at its activation loop Thr210 residue and its activation, whereas mutants lacking the coiled coil dimerization motif show significantly reduced activity[66,98]. Interestingly, the deletion of the RCC1 region leads to a catalytic hyperactivity, indicating that this region may be required for Nek9 autoinhibition[66]. Moreover, Nek9 binds to dynein light chain 1, cytoplasmic (DYNLL1), a highly conserved protein originally described as a component of the dynein complex, *via* its C-terminal (K/R) XTQT motif adjacent to Nek9 C-terminal coiled coil motif, resulting in Nek9 oligomerization, an increase in its autoactivation rate and a reduction in its binding to Nek6[101].

It is possible that Nek9 activation in mitosis involves a very small percentage (< 5%) of the total expressed protein, and in contrast with the vast majority of inactive protein, the active Nek9 (Thr210P) is first evident during prophase, concentrated at the centrosome, where it can be phosphorylated by CDK1/cyclin-B[102], until metaphase is reached. During the transition to anaphase, the immunoreactivity of Nek9 (Thr210P) decreases at the centrosomes and becomes detectable at the chromo-

somes, which is evident until telophase. Before disappearing, the active Nek9 is detected at the midbody as two points flanking the cleavage furrow during cytokinesis[98].

Due to its possible roles in the mitotic spindle organization and chromosome segregation through its activation during mitosis and interaction with Nek6/7, it is possible that most of the phenotypes observed with the microinjection of anti-Nek9 antibodies in human cells are caused by interference with Nek6/7 function[66]. Taken together, the data suggest that Nek9 is a positive upstream regulator of Nek6/7.

Among other kinases, Nek9 was recently identified by quantitative chemical proteomics as a possible marker for the diagnosis and therapy of head and neck tumors[103]. Moreover, Nek9 shows, along with other kinases implicated in cancer, its activity inhibited by the drug quercetin[104]. Its expression is increased in chronic myeloid leukemia cells resistant to imatinib[105], indicating that its up-regulation could be involved in chemotherapy resistance mechanisms. Depletion of Nek9 in glioblastoma (U1242) and renal carcinoma (Caki2) cells results in failures in cytokinesis and cell death in Caki2 cells, after overriding mitosis, and incorrect alignment of chromosomes and micronuclei formation. Therefore, it is suggested that inhibition of Nek9 is a potential anti-cancer therapeutic strategy by induction of mitotic catastrophe *via* reduced dynamics of the spindle, cytokinesis and mitotic checkpoint control[106].

NEK10

One of the most intriguing but less studied members of the Nek family is Nek10 since it has its catalytic domain flanked by two regulatory domains (Figure 1). Each of these two regulatory domains has their own peculiarities. As NIMA and Neks 1, 2, 5, 9 and 11, Nek10 also has coiled coil regions closely located to the kinase domains[8]. Furthermore, four repetitions of an armadillo repeat motif in its N-terminal regulatory domain may serve as an important region for protein-protein interactions, as has been reported for other proteins[107]. In the case of its C-terminus, a PEST region may be important to the proteolytic regulation of the protein's abundance. There are some contradictions and a debate about Nek10's full length since several different cDNAs have been deposited that differ in the C-terminal domain length.

Mutations in the Nek10 gene locus have been linked to breast cancer in different studies that were trying to find new polymorphisms in carriers of mutations in BRCA1/2 (breast cancer type 1/2 susceptibility protein)[108-110]. Moniz *et al*[74] have shown an important role for Nek10, comparing normal and tumor mammary gland cell lines. They found that Nek10 affects the ERK1/2 (extracellular signal-regulated kinase 1/2) signaling pathway, after activation with UV radiation. Nek10 has been shown to form a functional complex with RAF1 and MEK1 (dual specificity mitogen-activated protein kinase kinase 1). In this sense, cell cycle arrest in G_2/M was observed and Nek10 caused both MEK1 activation and the ERK1/2 phosphorylation. However, these preliminary data suggest a possible involvement of Nek10 in the DDR, as already demonstrated for Nek1, 4, 6, 8 and 11[2,8-10,18,23-25,72-73]. Moreover, like BRCA1 and BRCA2, Nek10 may be a therapeutic target in breast cancer.

NEK11

Nek11 is one of the least studied Nek family members and has the highest sequence similarity to Nek4. Its gene is present on the same chromosome as that of Nek4 but on the long arm (3q22-1). Nek11 was first identified by Noguchi *et al* (2002)[111] and shows a high sequence similarity with Nek4 and 3 in its kinase domain, but is more similar to Nek2 in its regulatory region (Figure 1). Interestingly, Noguchi *et al*[111] have not found Nek4/11-related kinases in *C. elegans* or *D. melanogaster*, suggesting that the Nek11-containing subfamily may have only appeared through gene or genome duplication after separation of the deuterostome branch in the animal kingdom[111].

Noguchi *et al*[111] (2002) described two isoforms for Nek11. The longer isoform (Nek11L) is composed of 645 residues, while the shorter one (Nek11S) contains only 470 residues. Nek11 shows a N-terminal kinase domain and a C-terminal regulatory domain with a coiled coil and three PEST sequences, suggesting a proteolytic, cell cycle specific regulation of its expression. Nek11, different from Nek1, 2 and 4, is not present in a higher quantity in the testis or ovary, but its mRNA is found in the brain's cerebellum, trachea, lung, appendix and uterus[111]. Another important difference to Nek4 is that Nek11 shows a timely cell cycle related expression pattern, relating it closer to Nek2, with both showing an expression peak at the G_2/M transition.

The first indication that Nek11 could be important in the regulation of cell cycle checkpoints was the identification of histones H1, H2A and H3 as Nek11 phosphorylation substrates. Furthermore, in the presence of genotoxic agents, Nek11 showed both an increased expression and activity at the G_2/M transition. Although this is decreased by caffeine, suggesting that Nek11 DDR may be associated with the ATM/ATR pathways, which also showed the same inhibition by caffeine[111].

Another common point between Nek11 and Nek2 is their localization to the nucleolus. In the study of Noguchi *et al*[112] (2004), it was observed that in U2OS cells Nek11L is present in the nucleolus during interphase and telophase and that it probably interacts with Nek2A in the nucleolus. Moreover, Noguchi *et al*[112]speculated that Nek2A could phosphorylate Nek11L C-terminal and, in this way, antagonize its auto-inhibitory function, which would cause Nek11 activation in G_1/S arrested cells[112].

Recently, some of Noguchi's results were followed up by Melixetian *et al*[73]. This study points to Nek11 as an important player in cancer development. Melixetian *et al*[73] observed that Nek11 depleted U2OS cells lose an important G_2/M checkpoint after IR. In this way, it was

verified that after IR Chk1 phosphorylates both M-phase inducer phosphatase 1 (CDC25A) and Nek11. Nek11 in turn also phosphorylates CDC25A, leading to its proteasomal degradation and subsequent inhibition of cyclins followed by a cell cycle arrest at the G2/M transition.

The studies involving Nek11 so far point to it as an important protein for the cell cycle regulation in the context of the DDR. However, more interactome studies are required to clarify other possible functions of Nek11 in the cell.

DISCUSSION

After knowing sufficient details on all of the eleven individual Neks, we will now return to a more general and integrative approach and try to find common functional contexts for the family as a whole in human cells. As pointed out in the introduction, Neks may be assigned to three major functional contexts: (1) centrioles and mitotic spindle functions; (2) primary ciliary function; and (3) G2/M phase associated DDR. Although most individual Neks have been associated with one main context, recent functional data as well as the identification of interaction partners for several Neks from two or even all three contexts may suggest that Neks have a broader function, possibly on a regulatory level, that consequently affects the three main functions. A first way of looking at this is by comparing the interaction profiles and functional contexts of the published interacting partners, summarized in Figure 2, which shows the Neks global interaction profile and the possible new biological processes in which they are involved due to their interaction with multiple proteins.

Several protein interactors with violet color interact with Nek1, 2, 3, 8, 9 and 11 and can be described as associated with the "axon guidance"/transport processes. They include, for example, fasciculation and elongation protein zeta (FEZ)-1 and 2 that interact with Nek1[2,113,114].

Several proteins associated with apoptotic processes interact with Nek6: serine/threonine-protein kinase PAK 6 (PAK6), serine/threonine-protein kinase Sgk1 (SGK1) and DBIRD complex subunit KIAA1967 (KIAA1967) (darker green color).

Nek9 interacts with several proteins from the autophagy-related protein 8 family (GABARAP, GABARAPL1, GABARAPL2, MAP1LC3A, MAP1LC3B and MAP1LC3C) (light blue).

Several proteins from DNA repair processes interact with either Nek1, 6, 9 or 10: RuvB-like 2 (RUVBL2), Fanconi anemia group I protein (FANCI), transcriptional regulator ATRX (ATRX), FACT complex subunit SSRP1 (SSRP1) and SUMO-1 (SUMO1) (red). The putative DNA repair and recombination protein RAD26-like (RAD26L), the PHD finger protein 1 (PHF1), and also the double-strand-break repair protein rad21 homolog (RAD21, not shown in Figure 2), all identified as Nek6 interactors in our yeast two-hybrid screens[3], are also possibly involved in the DDR[115,116].

In order to demonstrate the potential discovery of additional functional contexts through interactomics studies, we will now have a closer look at the Nek6 interactome as described by our group[3] (Figure 3). Novel Nek6 interacting partners are indicated by yellow ellipses and suggest the following new functional contexts: (1) Nek6 is possibly involved in actin cytoskeleton organization through its interaction with cell division control protein 42 homolog (CDC42) and sorting nexin-26 (SNX26)[3]. Since SNX26 has a negative regulatory role on CDC42 and Nek6 interacts with both of them, the final output of Nek6 must be addressed by future experiments. However, these findings are supported by the fact that for Nek3 a clear involvement in related processes has been reported (see Nek3 section above); (2) Nek6 may be involved in the activation of the NF-κB signaling on multiple layers, since it interacts with the transcription factor RelB, Prx-Ⅲ and/or TRIP-4[3,71]. Matsuda *et al*[71] found Nek6 as an activating protein in a siRNA knockdown screen to identify proteins that participate in the regulation of cellular survival transcription factor NF-κB[71]. The regulation may occur on several levels: through direct phosphorylation, interaction or regulation of the nuclear translocation of key components of the NF-κB complex, like RelB, or even on the transcriptional level. The latter seems likely, since Nek6 also interacts with SNW domain-containing protein 1 (SNW1) and a PHF domain containing protein (PHF1)[3], both of which have been recently identified as key components involved in the complex, multiprotein machinery involved in the transcriptional activation of the NF-κB gene[117]. Again, Nek6 regulatory role here may be mediated through interaction and/or phosphorylation; (3) the IR-induced DNA damage response is mediated by Nek1, 6 and 11, leading to cell cycle arrest[18,23,25,72,73]. The UV-induced DNA damage response is mediated by Nek10, also leading to cell cycle arrest[74]. This may suggest that different Neks may have specialized to mediate different forms of DNA damage responses; and (4) it is known that Nek6 can counteract p53 induced senescence[86]. As we can observe in Figure 3, this may occur indirectly through Nek6 modulation of p53 interactors 40S ribosomal protein S7 (RPS7) and/or E3 ubiquitin-protein ligase RBBP6 (RBBP6). It is worth noting here that Nek4 has the opposite effect of Nek6. Nek4 seems to be required for the cell to enter in senescence[9].

Another important point is the finding that certain functions first only described for isolated specific Neks have been later confirmed for most if not all other Neks. Nek1 was the first family member to be associated with DDR signaling events[23]. In our yeast two-hybrid screen to identify Nek1 interacting proteins, we identified proteins involved in the repair process itself (MRE11A) and in different signaling pathways associated with it (ATRX, PPP2R5 A/D, YWHAH, TP53BP1) (Figure 4).

Nek4, 6, 8, 10 and 11 have also been reported to physically interact with key members of DDR pathways or to interfere functionally in signaling cascades in a

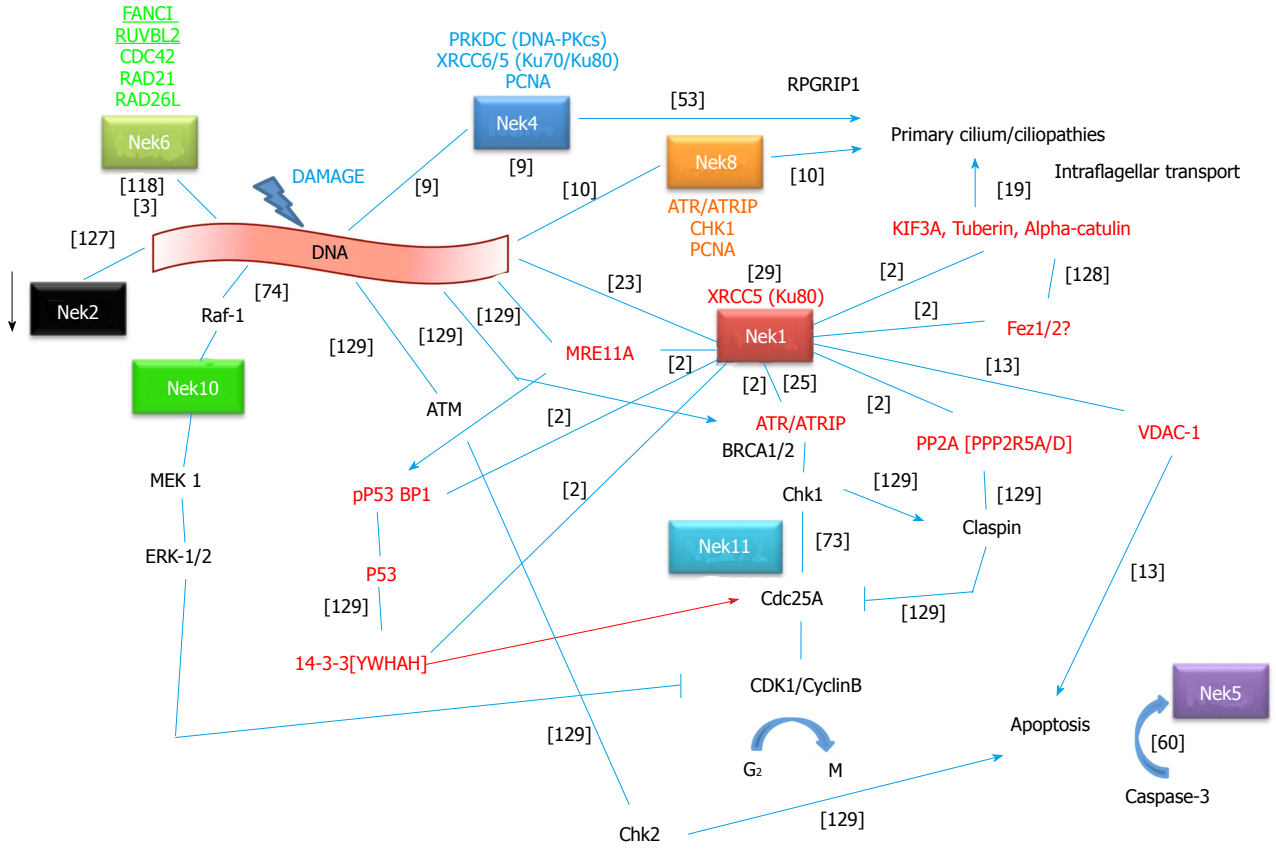

Figure 4 Nek1 interactome and crosstalk with other Neks and protein interactors in the context of the DNA damage response pathways. Interactions between proteins are depicted as simple lines, activation is depicted as an arrow and inhibition as an arrow with a line as arrowhead. A red arrow for 14-3-3 means that it causes activation by the transport of CDC25 to the nucleus. Nek1 interacted with a specific 14-3-3 isoform called YWHAH[2] (gene symbols inside brackets correspond to the isoforms of those proteins which were described to interact with Nek1). Not necessarily the same specific 14-3-3 protein promotes the indicated functions. Rather, a family characteristic is intended to be assigned. Nek2 kinase activity is inhibited after DNA damage (arrow)[127]. The red protein names are those that have been identified to directly interact with Nek1 as identified by the yeast two-hybrid system[2] or other as indicated in the figure. Gene symbols above/under protein names represent other interactors of those proteins. Nek4 interactors have been identified by mass spectrometry[9]. As can be seen, all but three Neks (Nek3, 7 and 9) seem to be directly linked to the DNA damage response. Most strikingly, we can see a direct connection for Nek8, 4 and 1 between DDR and primary cilium function and ciliopathies. New connections to apoptosis have been recently pointed out for Nek1 and 5. References for interactions are depicted in brackets: Nek6[3,118]; Nek1[2,13,25]; Nek4[9,53]; Nek8[10]; Nek11[73]; Nek10[74]; Nek2[127]; Nek5[60]; KIF3A[19]; Fez1/2[128], various known interactions[129].

broader context of the G2/M transition[8-10,18,73-74]. As described above for Nek6, the interactors RAD26L, PHF1, RAD21[3], FANCI and RUVBL2[118] are all associated with the DDR. Together with the relatively recent work by Lee *et al* (2008)[18], this suggests Nek6 may also interfere in DDR. However, the stimuli that activate such possible pathways *via* Nek6 are still unknown. In further yeast two-hybrid screens and mass spectrometry interactomics studies we found other DDR members interacting with Nek3, 4, 5, 7, 8, and 10 (unpublished data). Recent publications clearly confirmed part of those findings or went beyond them by characterizing this new involvement not only functionally, but also establishing possible cross-connections between primary cilia signaling and DDR in the case of Nek8[10]. For Nek4, an involvement in senescence signaling was established and in mass spectrometry experiments, several DDR proteins such as DNA-PKcs (PRKDC), Ku70/Ku80 (XRCC6/5) and PCNA were identified as Nek4 interacting proteins (Figure 4)[9]. Furthermore, Nek4 has been reported to interact with RPGRIP at the primary cilium[53], thereby establishing another link between DDR and primary cilium function.

A new role for Nek5 in differentiation and apoptosis signaling has been identified and characterized through its interaction with and proteolytic processing by caspase-3[60]. Evidently, apoptosis signaling is closely related to DDR and the G2/M checkpoint because cells unable to repair major DNA damage must either halt in the cycle or be dispatched by apoptosis. The link between Neks, DDR and apoptosis is not new as Chen *et al*[13] had also already reported an interaction of Nek1 with mitochondrial VDAC1. Nek1 phosphorylates VDAC1 and prevents apoptosis by avoiding VDAC1 opening and leakage of cytochrome c, which would activate apoptotic caspases. The down-regulation of Nek1 protein level or kinase activity through apoptosis signaling decreases VDAC1 phosphorylation and results in its opening and leakage of cytochrome c, thereby activating the apoptosis program.

For Nek1, the coexistence of functional roles in both DDR and ciliopathies and primary cilia function has been long established (Figure 4). Nek1 interacts with several proteins involved in the primary cilia function

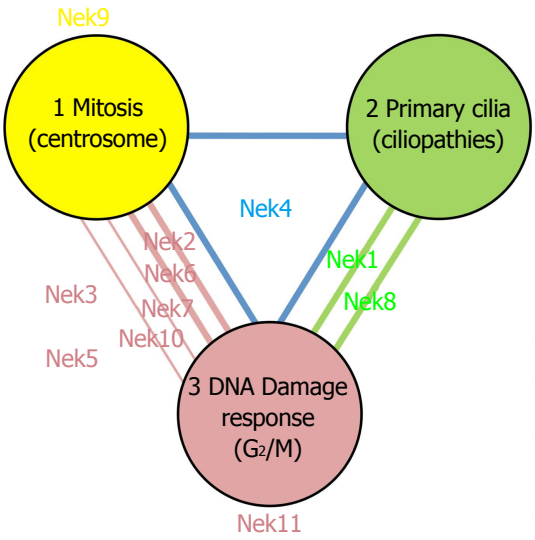

Figure 5 Functional overlap in the human Nek kinase family: seven of eleven Neks participate in two and one Nek in all three of the main core functions of the Nek family (centrosome-related mitosis, primary cilia and DNA damage response). The three corners of the triangle represent each a key concept function for the Nek family, *e.g.*, Nek9 and 11 sole involvement in mitosis[66,67] and DDR[73] respectively, has been well documented. The Nek names and bold lines represent cases where accumulated experimental evidence strongly suggests a regulatory role for that Nek in that context or in both of the contexts the line connects: Nek1[2,22,23]; Nek2[123]; Nek4[9,53] (Basei *et al* unpublished); Nek6[3]; Nek7[67]; Nek8[8,10]; Nek10[74]. The thinner lines represent our own group's preliminary or unpublished interaction data (both from yeast two-hybrid system and immunoprecipitation coupled to mass spectrometry analysis data), suggestive of a participation of that Nek in both connected functions (Nek7: Souza *et al*, unpublished).

and especially in kidney duct mechanosensing (KIF3A, tuberin, alpha-catulin, polycystin 1/2). Mutations in the genes that encode all of these proteins like those that cause expression of truncated non-functional Nek1 itself, cause PKD[14]. Since Nek8 is functionally and evolutionary most closely related to Nek1 among the Nek family, it came as no surprise that Nek8 mutations were also found to cause ciliopathies and cystic kidney disease. Moreover, Nek8 interacts with some key DDR proteins, including ATR, Chk1 and PCNA, just like Nek1[10]. What is new in these milestone discoveries, however, is the possibility that somehow these two pathways are causative or coincidentally connected. Choi *et al*[10] made the observation that mice cells with diminished Nek8 kinase activity, simulating a kidney ciliopathy, already show a constitutive activation of DDR pathways in the embryonic phase, as evidenced by repair foci in their kidney cells nuclei. This raises a couple of possibilities to consider: either the cilia have some function in the sensing of DNA damage or in transmitting downstream events, or otherwise, the cilia defects somehow transduce (*via* Nek8) to a possible lack of repair of replication defects. Of course a simpler explanation could be that both phenomena are affected simply because Nek8 participates in both of them simultaneously. However, an additional possibility is that Nek8 acts on a higher regulatory level that coordinates both pathways based on the necessity of the cell to coordinate these events closely during the course of the cell cycle. Clearly, further studies are necessary to evaluate these new possibilities. However, it seems to be clear now that the three central functions controlled by Neks, mitosis, primary cilia and DDR, are more connected than previously expected and that several if not all Neks participate in more than one of them.

A possibility exists that the Neks *per se* are the key regulatory elements that may connect these three functions. The seemingly functional redundancy may in fact rather represent connecting elements between hitherto non-connected regulatory circuits (Figure 5), *e.g.*, between primary ciliary function and DDR for Nek8[10] and Nek1[2,23,14]. Furthermore, these circuits may cooperate in a concerted one or two-directional fashion (Nek8).

Most interestingly, from a cilium perspective, recent evidence also indicates a strong link between cilia, stress responses and DNA damage repair processes. A recent study showed that environmental stresses, including UV and IR, result in altering the protein composition of centriolar satellites, thereby promoting de novo ciliogenesis[119]. Together with the recent findings that ciliopathy-associated mutations in DNA damage key regulators (*e.g.*, Mre, Znf423) also connect cilia and DDR[120-124], it is tempting to speculate that cilia may act as platforms for cell cycle checkpoints or the DDR.

CONCLUSION

Clearly, the past 10 years have provided new and exciting insights into the multifaceted functions of this interesting protein kinase family and the future promises to hold more surprises and the discovery of new functional connections. An exciting time has come to the field of Nek research and the Neks are ready to step out of the shade and take a main role along the other important cell cycle regulatory kinases: Polo-like kinases, Aurora kinases and Cyclin-dependent kinases. It is time to stop Ne(c)king around with them and allow them to enter the spot light in the field of cell cycle biology.

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REVIEW

Value of a newly sequenced bacterial genome

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Abstract

Next-generation sequencing (NGS) technologies have made high-throughput sequencing available to medium- and small-size laboratories, culminating in a tidal wave of genomic information. The quantity of sequenced bacterial genomes has not only brought excitement to the field of genomics but also heightened expectations that NGS would boost antibacterial discovery and vaccine development. Although many possible drug and vaccine targets have been discovered, the success rate of genome-based analysis has remained below expectations. Furthermore, NGS has had consequences for genome quality, resulting in an exponential increase in draft (partial data) genome deposits in public databases. If no further interests are expressed for a particular bacterial genome, it is more likely that the sequencing of its genome will be limited to a draft stage, and the painstaking tasks of completing the sequencing of its genome and annotation will not be undertaken. It is important to know what is lost when we settle for a draft genome and to determine the "scientific value" of a newly sequenced genome. This review addresses the expected impact of newly sequenced genomes on antibacterial discovery and vaccinology. Also, it discusses the factors that could be leading to the increase in the number of draft deposits and the consequent loss of relevant biological information.

Key words: Next-generation sequencing; Drafts; Prokaryotic genomes; Computational tools; *Omics*

Core tip: Next-generation sequencing (NGS) technologies have made high-throughput sequencing available to medium- and small-size laboratories, culminating in a tidal wave of genomic information. The quantity of bacterial genomes has not only brought excitement to the field of genomics, it has also heightened expectations that NGS would boost antibacterial discovery and vaccine development. Although many possible drug and vaccine targets have been discovered, the success rate of genome-based analysis has remained below expectations. Furthermore, NGS has consequences for genome quality, resulting in an exponential increase in draft genome deposits in public databases. This review will address the expected impact of newly sequenced genomes on antibacterial discovery and vaccinology, as well as the impact of NGS on draft bacterial genomes.

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INTRODUCTION

Since its release in 2005, next-generation sequencing (NGS) has been responsible for a drastic reduction in the price of genome sequencing and for a tidal wave of genetic information[1]. NGS technologies have made high-throughput sequencing available to medium- and small-size laboratories. The new possibility of generating a large number of sequenced bacterial genomes not only brought excitement to the field of genomics but also heightened expectations that the development of vaccines and the search for new antibacterial targets would be boosted. Nevertheless, these expectations were shown to be naïve. The complexity of host-bacteria interactions and the large diversity of bacterial genetic products have been shown to play greater roles in vaccine development and antibacterial discovery[2-4].

Additionally, as with any methodology, NGS presents its own drawbacks. Among the new sequencing technologies the most consolidated in the market are the 454 GS FLX platform (Roche), Illumina (Genome Analyzer) and SOLiD (Life Technologies)[5,6]. These devices are capable of generating millions of reads, providing high coverage genomic but with a drawback, reads are considerably smaller than the ones produced by Sanger methodology[7,8]. While Sanger methodology produces reads ranging from 800 to 1000 bases, NGS platforms produces reads ranging from 50 (SOLiD V3) to 2 × 150 bases (Illumina)[9]. The small amount of information contained in each read makes it difficult to completely assemble a genome using exclusively computational tools[10,11]. Therefore small reads made the genome assembly process a quite more laborious task.

In recent years, approaches that use hybrid assemblies were developed to facilitate the assembly process. They take advantage of high read quality of second generation sequencers, *i.e.,* Illumina (Genome Analyzer), and longer read lengths from third generation sequencers, *i.e.*, SMRT sequencers (Pacific Biosciences) and Ion Torrent PGM[12,13]. Although empirically logical, this kind of approach wasn't facilitated due to the lack of integration between sequencers.

In order to improving and verifying quality genome is essential to know which combination of sequencing data, computer algorithms, and parameters can produce the highest quality assembly[14,15]. Also, it is necessary to know the more likely type of error data a sequencer platform will present. For instance, Illumina and SOLiD are more likely to present nucleotide substitution, while 454 GS FLX and Ion Torrent are more likely to present indels[16]. Nearly none bioinformatic system has been developed to integrate reads from different sequencers into a single assembly[12,17]. This new developed approaches aim to reduce the manual intervention in finishing genomes, since repetitive regions may be solved using an hybrid approach.

Although NGS is directly responsible for considerable growth in the size of genomic databases, it has also been indirectly responsible for a decrease in genome quality[1,10]. The number of draft genome (partial data) deposits in public databases has grown exponentially since 2005 (Figure 1). In general, if no further studies will be developed using a particular organism's genome, it is more likely to be deposited as a draft genome. Otherwise, the painstaking tasks of improving and finishing the genome (complete data) must be undertaken[18].

This review will address the "scientific value" of a newly sequenced genome and the amount of insight it can provide. We will address the factors that could be leading to the increase in the number of draft deposits and the consequent loss of relevant biological information. Additionally, we will summarize the expectations created by NGS technologies regarding vaccine development and antibacterial discovery.

OVERVIEW OF SEQUENCING AND ASSEMBLY

For 30 years, sequencing technologies based on Sanger chemistry dominated the market. Although sequencing had undergone numerous improvements over the years, gene cloning techniques were still necessary to obtain genomic DNA sequences. Therefore, the time and cost required to obtain a complete genome sequence remained high. Moreover, the capacity of parallel sequencing was quite limited[19-21]. NGS platforms made it possible to sequence complete prokaryotic genomes using massively parallel sequencing more rapidly and at a lower cost[20,22].

Although NGS has facilitated sequencing processes, its relatively smaller reads make the assembly process a computational challenge[10,11]. The main limitation of short-read assembly methods is their inability to resolve repetitive regions of the genome without paired libraries[11]. The assembly of repetitive regions was an important issue even before the introduction of NGS platforms; shorter reads only made the problem worse.

In 2001, Kececioglu *et al*[23] argued about the impossibility of correctly assembling regions of the genome that contain identical copies of a sequence. Usually, long DNA repeats are not exact copies. They contain small differences that could, in principle, permit their correct assembly. Nevertheless, a major difficulty arises from sequencing errors. Assembly software must accept imperfect sequencing alignments to avoid missing genuine connections between sequences[22]. With the small amount of information within each read adding to the inherent sequencing error, it is difficult to separate true differences within repeated sequences from sequencing errors.

A study by Phillippy *et al*[24] revealed that the majority of contig ends in draft genomes were associated with repeated regions. They concluded that it was possible to

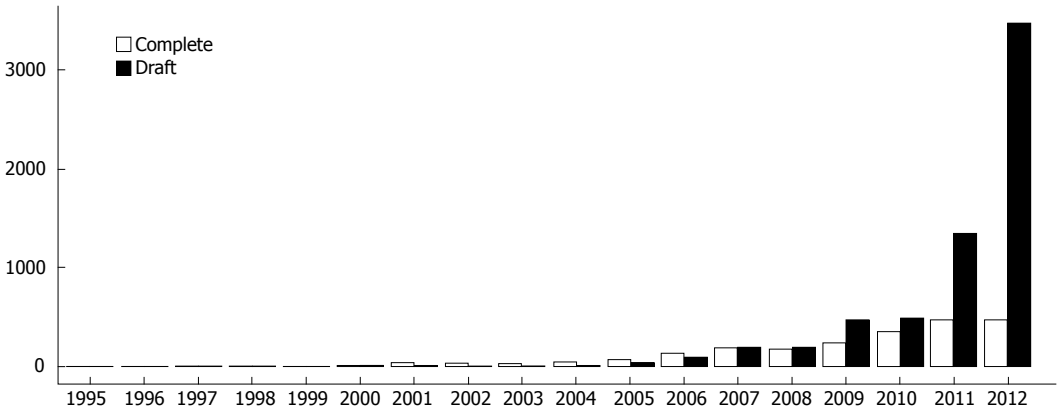

Figure 1 Number of complete genome and draft genome (partial data) deposits in public databases.

categorize the majority of mis-assembly events into two general classes: (1) repeat collapse or expansion; and (2) sequence rearrangement and inversion. Each of these classes exhibits specific mis-assembly signatures: the first class is the result of incorrect assembly in repetitive regions, including fewer or additional copies; the second class is the result of the rearrangement of multiple repeated copies, which is caused by the insertion of a read between them. The second class may be considered more influential because, if not fixed, it might be interpreted as a real biological rearrangement event[25,26]. If the assembler cannot resolve the region between two genomic fragments, a gap is formed. Gaps may occur due to: (1) an intrinsic characteristic of the sequencing platform that leads to incomplete or incorrect information; or (2) the inability of an assembly algorithm to handle regions of low complexity or repeated DNA[18,27,28]. The process of identifying and closing these gaps is quite laborious and requires additional manual intervention.

Gap closure processes usually involve the design of primers flanking the gap region to perform semi-automated sequencing of the unrepresented parts of the genome[28]. Several bioinformatics methodologies have been developed to facilitate gap closure. IMAGE is a tool that uses de Bruijn methodology to fill gaps with short reads that are aligned with flanking regions of the gap and were not used in the assembly[28]. In 2011, Cerdeira *et al*[29] generated a similar strategy by using CLC Genomics Workbench for the recursive alignment of unused short reads from the SOLiD platform. GapFiller is another tool that uses local alignment; its main advantage is the use of paired reads to estimate gap size and allows define the type of paired library: reverse-reverse, forward-forward, reverse-forward and forward-reverse[30].

From a purely practical standpoint, assembly tools are not required to produce a perfectly finished genome as an output. Their main function is to reduce the sequencing reads to a manageable number of contigs[26]. The process of finishing a genome, ensuring that gaps are closed and the gene order is correct, requires human decision-making. Therefore, the lack of fully automated processes constitutes a bottleneck in generating complete genomes.

"SCIENTIFIC VALUE" OF A NEWLY SEQUENCED GENOME

The value of a newly sequenced genome can be assessed using many different metrics. If publications are considered the main "currency" within the scientific community, there has been a considerable decrease in the value of new sequences over the last four decades.

The introduction of Sanger methodology in 1977 was one of the main landmarks in the early stages of the genomic era[31]. During the first years of using Sanger sequencing, a sequence of no more than 1000 nucleotides was sufficient for a work to be accepted in a journal such as Cell (current impact factor: 32.40) or Nature (current impact factor: 36.28)[32-34]. In 1980, the shotgun DNA sequencing methodology was introduced, enabling the sequencing of longer DNA fragments[35]. Complete bacterial operons were sequenced and published in journals such as Molecular Microbiology (current impact factor: 5.01) and Proceedings of the National Academy of Sciences (PNAS - current impact factor: 9.68)[36-38].

A combination of DNA sequencing improvements and the newly developed TIGR Assembler[39] culminated in the publication of the first complete bacterial genomes in 1995. Papers containing the complete nucleotide sequences of *Haemophilus influenzae* Rd (1830137 base pairs) and *Mycoplasma genitalium* (580070 base pairs) were both published in Science (current impact factor: 31.20)[40,41]. Almost 20 years later, a paper containing the sequence of a prokaryotic genome alone may be published in the Genome Announcement section of the Journal of Bacteriology (current impact factor: 3.82) or in Standards in Genomic Sciences (SIGS - has not been published sufficiently long to receive an impact factor). A recent article by Smith even refers to the not-so-distant "death" of the "genome paper", noting that the space for genome publication may come to an end soon[42].

The publication impact of newly sequenced genomes decreased following DNA sequencing improvements, and the reason is no mystery. High-impact journals only publish groundbreaking original scientific research or

results of outstanding scientific importance. To produce a higher-impact publication, more information must be extracted from genomes. For instance, several genomes may be examined in a comparative genomic analysis or pangenomic study[43,44], or an analysis may focus on the presence or absence of specific markers or on small differences between DNA sequences[26,45]. In this context, the genome becomes a stepping stone to the main goal, the comparative analysis. As the basis of the analysis, the genome sequence remains important. Nevertheless, it may not be of sufficient importance for one to undertake the painstaking task of completing the genome sequence.

WHAT IS LOST WHEN WE OPT FOR A DRAFT GENOME?

Over the years, arguments have been presented in favor both of complete genomes[41,46] and of the superior "tradeoff" that a draft genome represents[47]. The discussion has been centered around two main points: (1) to provide the greatest amount of useful data, sequences must be as complete as possible; and (2) draft genomes (partial data) are sufficient for most scientific contexts. The issue at stake is the extra money and manpower necessary to finish a genome. Is the additional information contained in a finished genome worth the investment? To answer this question, one must identify the information that is lost from a draft and analyze the quality of data that is generated using drafts. Furthermore, it is necessary to understand the limits of draft genome use.

The first issue to consider is whether it is possible to properly identify all of an organism's genes in a draft genome. Gene characterization consists of the following: (1) gene prediction with the identification of an open reading frame (ORF); and (2) the functional annotation of the gene product. The main gene identification problems in drafts are associated with the partial or complete loss of ORFs[10]. Such errors may lead either to over-annotation, due to the annotation of multiple fragments originating from the same ORF, or to under-annotation, possibly due to the absence of partial or entire domains from the ORF[10]. These problems affect genomic analyses, causing errors due to missing ORFs that are not annotated or due to multiple fragments that belong to the same ORF but are annotated separately. In other words, the mere absence of a gene from a draft cannot be considered definitive proof of its absence from the organism's genome[10,41].

The pangenomic approach is one type of analysis that may be impaired by reliance on draft genomes, because many genes in a draft may be misidentified due to fragmentation. Pangenomic projects attempt to characterize the gene pool of a bacterial species as the genes that are present in all strains (the "core genome") and the genes that are present in only a few species (the "dispensable genome")[43]. Horizontal gene transfer (HGT) analysis is another approach that cannot be performed using drafts. HGT is one of the main sources of variability among bacteria because it allows the acquisition of several new genes[36,37]. There is evidence that most gaps in genomic sequences are associated with transposases, insertion sequences and integrases, structures that usually flank a genomic island[48]. Another approach that may be impaired by reliance on drafts is phylogenomics, which aims to reconstruct both the vertical and lateral gene transfer processes of a bacterial species using a whole-genome analysis[49].

Although not strictly related to drafts, the functional annotation of genes is another feature that is usually neglected when we opt for a draft genome (Figure 2). Complete genomes may also present this problem because the quality of functional annotation is related to the amount of effort dedicated to a genome. DNA sequence is being generated much more rapidly than it can be analyzed; thus, a large proportion of the sequence information in databases has been annotated solely by automatic algorithms[50]. It is disturbing that although automatic annotation algorithms have improved over the years, misannotation has increased over time[50]. The misannotation of a reference strain is particularly harmful because the error will likely be propagated to other genomes. In our attempts to exploit the full potential of NGS, we risk having databases filled with incomplete and/or incorrect genomic data.

Because the purpose of many sequencing projects is to identify a small number of differences between a newly sequenced genome and the sequence of a closely related species, a large number of genomes are left as drafts[26]. Considering the constant evolution of organisms, a sequenced genome represents a snapshot in the biological history of a species. Therefore, a single finished genome might be useful for decades of future studies. By opting for draft genomes, we may be shutting down the full gamut of future scientific analysis.

VACCINE DEVELOPMENT

Genomic information was expected to boost vaccine discovery. In an attempt to measure the impact of genomic information on this field, Prachi *et al*[2] analyzed all the patent applications that contained genomic information. They observed that there was an enormous increase in such applications shortly after the first complete genomes were released, but since 2002, there has been a continuous decrease. The authors attributed this decrease to more stringent legal requirements, which call for empirical evidence to complement in silico data.

The initial increase in patent applications containing genomic information was related to the development of a new paradigm in vaccine development. In 2000, Rappuoli[51] described the "reverse vaccinology" (RV) concept, in which he proposed inverting the traditional process of antigen identification. Instead of identifying the antigenic components of a pathogenic organism using serological or biochemical methods, RV uses the organism's genome to predict all of its protein antigens. RV approaches mainly focus on secreted proteins because they are more likely to induce immune responses. Secreted proteins are involved in several processes that modulate

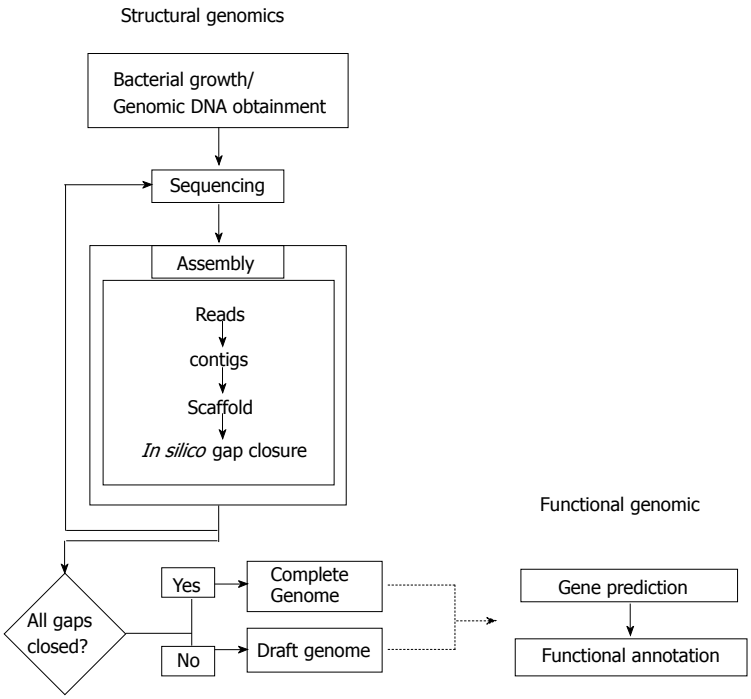

Figure 2 General workflow during sequencing process a bacterial genome.

the host-pathogen relationship, such as cell adhesion and invasion, as well as resistance to stress conditions[52-54]. Over the years, several methodologies have been developed to predict secreted proteins and to evaluate their potential immunological properties.

In 2010, Vaxign was released as the first vaccine design tool with a web interface (http://www.violinet.org/vaxign/). Vaxign allows users to submit their own sequences to perform vaccine target predictions. The Vaxign predictions have been consistent with existing reports for organisms such as *Mycobacterium tuberculosis* and *Neisseria meningitides*[55]. Another vaccine design tool is MED (Mature Epitope Density - http://med.mmci.uni-saarland.de/). MED attempts to select the more promising vaccine targets by identifying proteins with higher concentrations of epitopes[56]. There are also tools exclusively for protein epitope prediction, such as Immune Epitope Analysis (http://tools.immuneepitope.org/main/) and Vaxitope (http://www.violinet.org/vaxign/vaxitop/index.php).

Because a large number of bacterial genomes are already available, reverse vaccinology is quite accessible and inexpensive. Nevertheless, as has been previously discussed[57,58], the expectations for reverse vaccinology techniques do not correspond to reality, given the small number of vaccines have been developed using the bacterial genome sequences available[59]. This occurs because there are also several factors that are involved in the host response during infection, for example, the production of antibodies by the immune system.

ANTIBACTERIAL DISCOVERY

The period between the 1930s and the 1960s is known as the "golden age" of antibiotic discovery[11,60]. During this period, most of the known classes of antibiotics were discovered. These discoveries involved screening natural products regardless of their mechanisms of action. After most of the low-hanging fruits were harvested, the rate of antibacterial discovery decreased, culminating in a slowdown beginning in the 1990s[61].

Hopes for turning this void into a rapid acceleration accompanied the completion of the first bacterial genome sequences. The goal was to use comparative genomic analysis to identify potential targets present in a desirable spectrum (*e.g.*, the bacteria responsible for upper respiratory tract infections)[3,4,62]. It was naive to assume that having the genome sequences would be sufficient for this level of discovery; a possible drug target must undergo numerous stages, from discovery through human clinical tests, and it is not possible to develop drugs for all potential targets[3,62]. Nevertheless, the prospect of exploring hundreds of potential targets revived the interest of pharmaceutical companies.

After some years of trials, several companies ended their target-based programs because of a lack of productivity. Despite reports of multi-resistant bacterial strains, the efforts to discover new antibacterial targets were again reduced[63,64]. Although genomics has not been able to reverse the lack of new antibiotic development, it has significantly improved screening methodologies. Genomics has facilitated high-throughput drug campaigns, which are being used to determine the mechanisms of action of antibacterial compounds and bacterial resistance mechanisms[4].

CONCLUSION

Several next-generation platforms have been developed

in recent decades, as well as bioinformatics programs to an enhancement of performance and optimization omics techniques. Is not yet possible to integrate reads from different sequencers into a single assembly[17,23]. This newly developed approach aims to reduce the amount of manual intervention needed to complete a genome sequence by using a hybrid approach to resolve repetitive regions.

Improvements are expected not only in sequencing platforms but also in assemblers. Recently, two groups assessed the quality of the currently available assemblers. The 2011 Assemblathon was the first competition among assemblers[65]. For this competition, simulated data were generated and groups of assemblers were asked to blindly assemble it. The use of simulated data poses a problem in determining the applicability of the results to other data sets. The 2012 GAGE (Genome Assembly Gold-Standard Evaluations) competition for assembling real data resulted in the following conclusions: (1) the data quality has a greater influence on the final outcome than the assembler itself; and (2) the results do not support the current measures of correctness (related to contiguity)[26].

There is a large gap between the availability of genomic sequences in databases and the commercial production of vaccines and antibiotics in recent years, especially in the fields of investment and success ("expected return"). Drug development for all potential targets and effective vaccines has produced limited success. In contrast, there has been an acceleration in the discovery of new targets due to the refinement of bioinformatics tools for this purpose, such as epitope mapping and searching for secreted proteins. However, the major problems facing vaccine and antibiotic development, such as resistance mechanisms and host immune responses, remain unsolved.

Genome analysis constitutes a strategy for the expansion and diversification of the pharmacology and vaccinology sectors. This methodology can be used to explore a large number of targets and to reduce the costs of molecular and immunological tests. Finally, to improve the production of antibiotics and vaccines, it is necessary to know more about bacterial regulatory pathways. New interactome and microbiome studies must be implemented to assist this search.

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REVIEW

Activated protein C: A regulator of human skin epidermal keratinocyte function

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Abstract

Activated protein C (APC) is a physiological anticoagulant, derived from its precursor protein C (PC). Independent of its anticoagulation, APC possesses strong anti-inflammatory, anti-apoptotic and barrier protective properties which appear to be protective in a number of disorders including chronic wound healing. The epidermis is the outermost skin layer and provides the first line of defence against the external environment. Keratinocytes are the most predominant cells in the epidermis and play a critical role in maintaining epidermal barrier function. PC/APC and its receptor, endothelial protein C receptor (EPCR), once thought to be restricted to the endothelium, are abundantly expressed by skin epidermal keratinocytes. These cells respond to APC by upregulating proliferation, migration and matrix metalloproteinase-2 activity and inhibiting apoptosis/inflammation leading to a wound healing phenotype. APC also increases barrier function of keratinocyte monolayers by promoting the expression of tight junction proteins and re-distributing them to cell-cell contacts. These cytoprotective properties of APC are mediated through EPCR, protease-activated receptors, epidermal growth factor receptor or Tie2. Future preventive and therapeutic uses of APC in skin disorders associated with disruption of barrier function and inflammation look promising. This review will focus on APC's function in skin epidermis/keratinocytes and its therapeutical potential in skin inflammatory conditions.

Key words: Activated protein C; Endothelial protein C receptor; Protease-activated receptor; Keratinocyte; Proliferation; Junction protein; Barrier function

Core tip: The anti-inflammatory, barrier stabilisation and anti-apoptotic properties of APC make it an appealing treatment for skin conditions associated with inflammation, barrier disruption and keratinocyte dysfunction.

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INTRODUCTION

Protein C (PC) is a vitamin-K dependent glycoprotein that circulates in blood plasma in its zymogenic and activated forms [activated PC (APC)]. PC/APC was first characterised for its role in blood coagulation, but has a range of cytoprotective functions including anti-inflammation, anti-apoptosis and barrier stabilisation. Although originally thought to be synthesised almost exclusively by the liver and vascular endothelial cells, PC/APC has been found to be synthesised by skin epidermal keratinocytes. Keratinocytes are the major cell type in

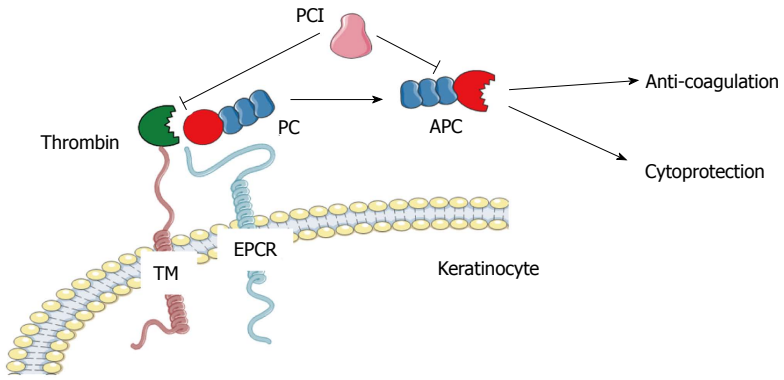

Figure 1 Schematic representation of protein C/activated protein C activation and cellular effects. APC: Activated protein C; EPCR: Endothelial protein C receptor; PC: Protein C; PCI: Protein C inhibitor; TM: Thrombomodulin. Figure was produced using Servier Medical Art - www.servier.com.

the skin epidermis, the most outer layer of human skin that provides a semi-impermeable barrier against injury from the external environment, including ultraviolet radiation, heat, water loss and infectious pathogens. On keratinocytes, PC/APC promotes cell proliferation, survival, migration, and the barrier function. This review will focus on the actions of APC on skin epidermis/keratinocytes and its therapeutical potential in the treatment of skin inflammatory conditions.

PC and APC

The PC pathway plays a key role in the regulation of blood coagulation. As a vitamin K-dependent zymogen, PC is activated to APC when thrombin binds to thrombomodulin and cleaves the activation peptide (Figure 1). This conversion is augmented by its specific receptor, endothelial cell protein C receptor (EPCR)[1]. In human plasma APC is present at relatively low levels approximation 40 pmol/L and has a short physiological half-life of approximation 20 min compared to PC at 70 nmol/L and approximation 10 h[2,3]. Thrombin is the only endogenous activator of PC. The importance of APC as an anticoagulant is reflected by findings that deficiencies in PC result in severe familial disorders of thrombosis[4]. Replenishment of PC in patients with systemic or local hypercoagulation can reverse the abnormality.

Independent of its effect on anti-coagulation, APC possesses strong anti-inflammatory and anti-apoptotic properties, as well as enhancing endothelial and epithelial barrier integrity (Figure 1).

Inhibiting inflammation: The anti-inflammatory effects of APC are associated with a decrease in pro-inflammatory cytokines and a reduction in leukocyte recruitment. APC inhibits neutrophil, monocyte and lymphocyte chemotaxis[5] and directly suppresses expression and activation of nuclear factor (NF)-κB[6]; a pathway that controls the expression of a wide range of inflammatory genes including tumour necrosis factor (TNF)-α and cell adhesion molecules. Acute inflammation is exacerbated in mice genetically predisposed to a severe PC deficiency[7]. *In vitro*, APC suppresses the activation of NF-κB and production of TNF-α, upregulates matrix metalloproteinase (MMP)-2 activity yet inhibits MMP-9 in rheumatoid synovial fibroblasts and monocytes[8]. In addition to the degradation of extracellular matrix, these MMPs can regulate inflammation by processing cytokines/chemokines with MMP-9 having stimulatory and MMP-2 having inhibitory effects on inflammation both *in vitro* and *in vivo*[9-11].

Promoting cell proliferation and inhibiting cell apoptosis: APC promotes cell proliferation in cultured human umbilical vein endothelial cells[12], smooth muscle cells[13], keratinocytes[14], neural stem and progenitor cells[15,16], neuroblasts[17], osteoblasts[18] and ovine tenocytes[19]. Consistent with the stimulatory effects on cell growth, APC displays strong anti-apoptotic properties in keratinocytes, endothelial cells and podocytes[14,20-22]. APC-dependent anti-apoptotic activity shows improved survival in human and various animal models of sepsis[23-28]. APC inhibits spontaneous monocyte apoptosis leading to increased lifespan and phagocytosis *in vivo*[29] and protects murine cortical neurons from N-methyl-D-aspartate and staurosporine excitotoxicity-induced apoptosis[30].

Stabilising endothelial and epithelial barrier: Endothelial cells normally form a dynamically regulated stable barrier at the blood-tissue interface. Breakdown of this barrier is a key pathogenic factor in inflammatory disorders. APC enhances endothelial barrier integrity by stabilising the cytoskeleton and reducing endothelial permeability[20,31-33]. Recently, APC has been shown to promote epithelial barrier function in human skin epidermal keratinocytes[34] and mouse intestine[35].

APC's signalling pathway: Many of the anti-inflammatory properties of APC are mediated through EPCR, which itself is anti-inflammatory[36]. APC bound to EPCR can activate protease-activated receptor (PAR)-1 and promote the anti-inflammatory actions of APC[37]. Cytoprotective effects of APC are also mediated by the other PAR receptors. Akin to PAR-1, APC can bind to PAR-2 and activate the Akt signaling pathway to promote keratinocyte proliferation[37]. Independent of EPCR, APC can inhibit podocyte apoptosis by activating PAR-3[38]. APC-mediated arrest of lymphocyte chemotaxis is dependent on epidermal growth factor receptor (EGFR)[39]. In addition, EGFR transactivation by APC/EPCR/PAR-1 supports cell motility and invasiveness of endothelial cells

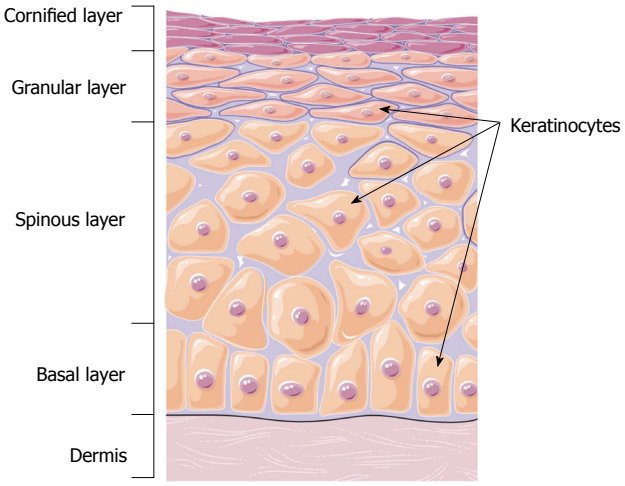

Figure 2 Schematic representation of the structure of skin showing the epidermal layers. Figure was produced using Servier Medical Art - www.servier.com.

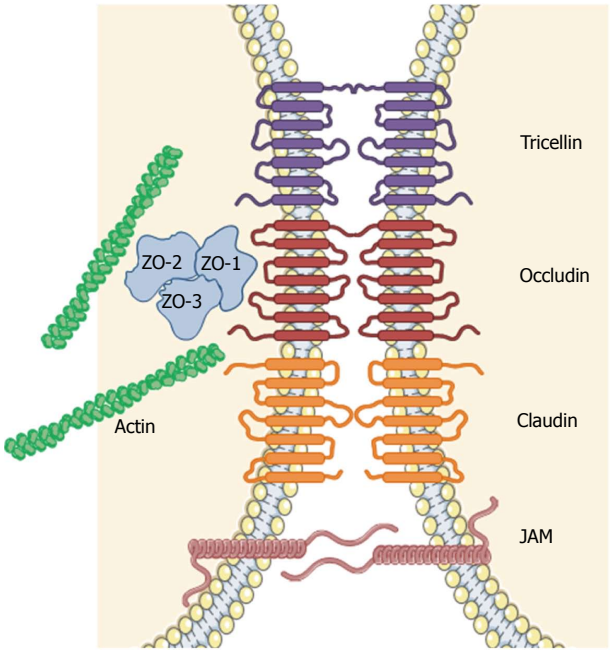

Figure 3 Schematic representation of epidermal tight junction complex. JAM: Junctional adhesion molecule; ZO: Zona occludin. Figure was produced using Servier Medical Art - www.servier.com.

and breast cancer cells[40]. APC utilises the angiopoietin/Tie2 axis to promote endothelial barrier function[33]. In addition other receptors such as integrins[41] and apolipoprotein E receptor-2[42] also mediate the effects of APC.

Skin function and keratinocytes

The skin forms an effective barrier between the human body and outside environment and protects the body from mechanical trauma, pathogens, radiation, dehydration, and dangerous temperature fluctuations[43]. Skin consists of two main layers, the outermost epidermis layer and the underlying dermis (Figure 2). The epidermis is a stratified epithelium composed of proliferating basal and differentiated suprabasal keratinocytes. The dermis provides the epidermis with mechanical support and nutrients. The barrier function of skin is provided by the epidermis. Defective epidermal barrier is responsible for many inflammatory and blistering skin disorders[43,44].

Keratinocytes are the most abundant cell type in the epidermis and are responsible for maintaining structure and homeostasis of the epidermal barrier. The epidermal barrier is generated by a sophisticated differentiation program[44] comprising stratified epithelium composed of basal, spinous, granular, and cornified layers (Figure 2)[45]. The basal layer consists of proliferating keratinocytes, that maintain the epidermis and post-mitotic basal keratinocytes which migrate out of the basal layer. This migration marks the start of epidermal differentiation that ends with the formation of the cornified layer, where keratinocytes end their lives and are sloughed off. The epidermis has complete self-renewal capacity with an estimated turnover time of approximately 40 d in humans[46].

The physical barrier of the epidermis is localised primarily in the upper layers of the epidermis (granular and cornified layers). The barrier properties of nucleated keratinocytes in the granular layer are largely dependent on the function and integrity of the tight junctions [involving the proteins tricellin, occludin, claudins and junctional adhesion molecule (JAM)] and their corresponding intracellular proteins, such as zona occludin (ZO)-1[44], which seal the intercellular space between neighbouring keratinocytes and control the pathway of molecules and liquid (Figure 3)[46].

Deregulation of these junction proteins perturbs this barrier[43] and is characteristic of many inflammatory skin diseases[47,48]. Psoriatic skin, characterised by small scaly plaques, has an over-expression of occludin and ZO-1, while claudin-1 and 3 are down-regulated[49,50]. Keratinocyte cytoskeletal elements are also important for maintaining the epidermal barrier. Among the genetic mutations in atopic dermatitis is the filaggrin gene (*FLG*)[51,52], which encodes a protein in the corneal epidermal layer and aids terminal differentiation of keratinocytes, water retention and barrier stabilisation[53]. Loss or mutation of this gene contribute to the red, dry, itchy skin that is hallmark of this condition.

In addition, keratinocytes provide an immunological barrier in response to injury or infection. Keratinocytes are a potent source of cytokines and chemokines[54]; freshly isolated and cultured keratinocytes express toll-like receptors[55] and inflammasomes[56]. This allows keratinocytes to elicit innate immune responses to microbial components when the epidermal barrier is breached, particularly through secretion of interleukin (IL)-1β and activation of leukocytes.

Upon activation, keratinocytes express a plethora of cytokines, chemokines and accessory molecules, which can transmit both positive and negative signals to cells of the innate and adaptive immune system. Dysregulation of the immune response of keratinocytes is implicated in the pathogenesis of chronic inflammatory skin diseases.

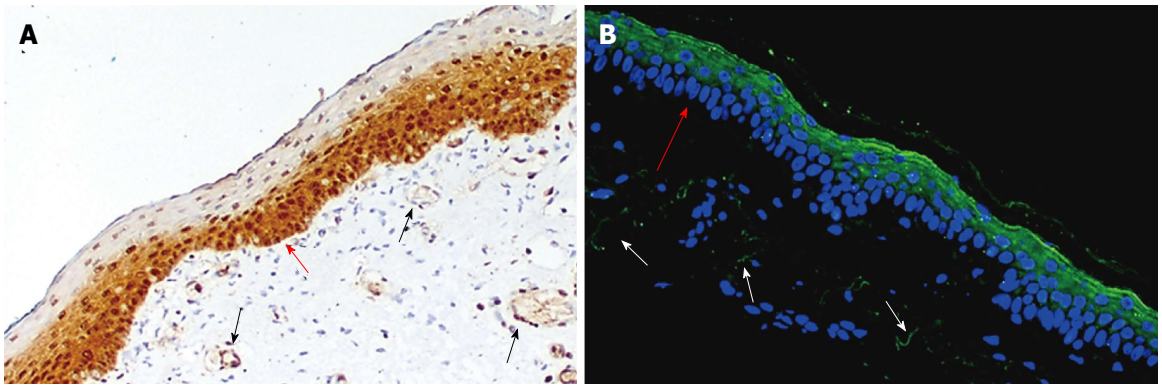

Figure 4 Immunostaining of protein C/activated protein C in human neonatal and adult skin epidermis. A: Neonatal; B: Adult. PC/APC indicated by brown and green staining in the epidermis (red arrow) and dermal blood vessels (arrow). APC: Activated protein C; PC: Protein C.

PC system on keratinocytes

Keratinocytes in the epidermis express all the components of the PC/APC pathway, including EPCR[57], thrombomodulin[58], thrombin and PC inhibitor[59], PAR-1, EGFR[60], and Tie2[34] which can regulate the activation of PC to APC and mediate the functions of APC on keratinocytes in skin epidermis.

PC/APC and its activation on keratinocytes

PC/APC: Since its discovery in 1960[61], PC has been characterised as the vitamin-K dependent protein precursor for the anticoagulant APC[62]. Thought to be exclusively synthesised by the liver and vascular endothelial cells, recent evidence shows that keratinocytes can also synthesise PC[60]. Cultured keratinocytes express PC mRNA and protein, and APC activity is presented on these cells[60]. In neonatal foreskin, PC is strongly expressed in the basal and suprabasal layers of the epidermis, with weaker expression in the outer cornified layer[60]. In the adult skin, however, the PC/APC is strongly stained in the upper layer of epidermis (Figure 4)

Thrombin: Thrombin is the only endogenous activator of PC. Keratinocytes express mRNA for the thrombin precursor, pro-thrombin[63]. Pro-thrombin and thrombin are expressed at low levels in normal epidermis, with thrombin markedly upregulated in scar tissue[63]. Thrombin activity is regulated by keratinocyte thrombomodulin at sites of cutaneous injury[64].

Thrombomodulin: Upon binding to thrombomodulin on surface of vascular endothelial cells, thrombin cleaves PC at the activation peptide between Arg^{211} and Leu^{212} and converts it to APC. Cultured human keratinocytes constitutively express thrombomodulin on their cell surface[58,64]. In normal epidermis thrombomodulin is present in spinous layer and on the outer root sheath of hair follicles[58,64].

PC inhibitor: PC inhibitor is a non-specific serpin that inhibits a variety of serine proteases, including PC and thrombin[65]. This inhibitor can inhibit the activation of PC to APC by inactivating thrombin and/or preventing thrombin binding to thrombomodulin[66,67]. It can also inactivate APC. PC inhibitor mRNA and protein is constitutively expressed by immortalised human keratinocytes (HaCaT) and epidermoid carcinoma cells (A431) in culture[59]. Normal skin from the trunk of adults show strong staining for PC inhibitor antigen throughout the epidermal layers[59].

In summary, epidermal keratinocytes express all aspects of the PC system to not only activate PC to APC, but regulate this activation process and APC activity (Figure 4).

PC/APC function and regulation

EPCR: EPCR is a type I transmembrane protein which exhibits significant homology with the major histocompatibility class 1/CD1 family of proteins. EPCR is the main receptor to regulate the function of PC/APC. Although first described as being restricted to the endothelium, EPCR is abundantly expressed by cultured human keratinocytes and is strongly expressed in the basal and suprabasal layers of the epidermis of neonatal foreskin[57].

EPCR has similar affinity for both PC and APC[1]. After binding to EPCR, APC cleaves PAR-1 to promote its cytoprotective functions in keratinocytes[57]. In addition PAR-1, EGFR and Tie2 are shown to mediate keratinocyte proliferation, migration and barrier stabilisation. In addition, EPCR enhances the rate of PC/APC activation by thrombin/thrombomodulin 3-4 fold[68]. Inhibition of EPCR reduces the level of circulating APC by more than 80% following thrombin infusion[69].

PAR-1: PARs are a family of G-protein coupled receptors which utilise G-protein and non-G-protein signaling pathways to mediate their cellular responses[70]. They are expressed by a wide range of cell types in the skin, including keratinocytes[57]. PARs are activated by a range of proteases through cleavage of an activation peptide. The most common endogenous activator is thrombin which activates PAR-1, PAR-3 and PAR-4, but not PAR-2. Other serine proteases including trypsin, mast cell tryptase

and factor Xa activate PAR-2. In keratinocytes, PAR-1 mediates APC's induction of cell proliferation, anti-inflammatory and barrier protective effects[34,57].

Cytoprotective effects of APC are also mediated by the other PAR receptors. APC can bind to PAR-2[37] and activate the Akt signaling pathway to promote keratinocyte proliferation[71]. Though only PAR-2 activity appears to be required for APC-mediated wound healing in a murine model[71].

EGFR: EGFR is a crucial receptor for autocrine growth of healthy epidermis. Its activation suppresses terminal differentiation, promotes cell proliferation and survival, and regulates cell migration during epidermal morphogenesis and wound healing[72]. Following tissue injury, EGFR is upregulated to promote re-epithelialisation of the wound by encouraging keratinocyte proliferation and migration. EGFR regulates cell adhesion, extracellular matrix degrading enzymes, and cell migration to contribute to the migratory and invasive potential of keratinocytes[72]. In human skin, EGFR and EPCR are expressed in the basal and suprabasal layers of the epidermis, consistent with the localisation of PC/APC[60]. Expression of EGFR by keratinocytes appears to be synchronised with the PC pathway. APC treatment increases EGFR expression while silencing of PC decreases EGFR levels[60].

Tie2: Tie2 is a protein-tyrosine kinase receptor expressed by endothelial and epithelial cells. Its major ligands are angiopoeitin 1 and 2 which bind with similar affinity[73,74]. Both Tie2 and its activated form phosphorylated (P)-Tie2 are present on neonatal foreskin and adult skin keratinocytes[34]. However, adult skin keratinocytes show less intensive staining for Tie-2 and P-Tie2 when compared with neonatal foreskin keratinocytes. Foreskin epidermis exhibits faint staining of Tie2 but strong staining for P-Tie2, which is mainly located in the uppermost layers of the epidermis (Figure 4). Similarly, P-Tie2 is expressed by normal adult skin epidermis, although the staining intensity is considerably lower than neonatal foreskin.

Functions of PC/APC in keratinocytes

APC promotes proliferation and inhibits apoptosis in keratinocytes: APC promotes cell proliferation in cultured human skin keratinocytes[14]. The replicative capacity of keratinocytes is mediated by EGFR, and acts to inhibit terminal differentiation and apoptosis. APC increases keratinocyte proliferation, while gene silencing of PC increases apoptosis in keratinocytes 3-fold[60]. Proliferation is mediated by APC's regulation of mitogen activated protein (MAP) kinase activity[12,14-16,18]. This family of highly conserved serine/threonine protein kinases enhances DNA synthesis, and regulates cell survival/apoptosis and differentiation[13]. In human skin keratinocytes, PC/APC-induced proliferation is mediated by EPCR, PAR-2, EGFR, activation of ERK1/2 and PI3K/Src/Akt signalling and suppression of p38[34,60,71].

Consistent with the stimulatory effects on cell growth, APC displays strong anti-apoptotic properties. APC prevents apoptosis of keratinocytes[14]. The molecular mechanism of APC's ability to protect cells from apoptosis is multi-faceted. APC regulates caspase activation, DNA degradation and the induction of anti-apoptotic mediators[25-28]. PC regulates the activation of apoptosis marker caspase-3, of which the inactive form is expressed in a wide range of tissues, including the epidermis[75]. In normal oral epithelium, cleaved caspase-3 distinguishes apoptotic keratinocytes from cells that are terminally differentiated[76]. Recent findings indicate that caspase-14, not caspase-3, is activated during normal keratinocyte differentiation[77]. Therefore caspase-3 activation appears to be restricted to keratinocytes undergoing apoptosis, and is increased by blocking PC by siRNA consistent with a role for PC in preventing keratinocyte apoptosis[60].

While additional anti-apoptotic pathways for APC have not yet been demonstrated in keratinocytes, in hypoxic retinal epithelia and photoreceptor cells APC reduces caspase-8 and 9[78]; decreases p21 and p53 proteins in murine model of sepsis-induced apoptosis[79]; and prevents glucose-induced apoptosis in endothelial cells and podocytes by reducing Bax induction and Bcl-2 suppression[21].

APC promotes migration of keratinocytes: Keratinocyte migration is a crucial step in stratification of the epidermis to form a protective barrier, and during re-epithelialisation of a wound site. EGF is a chemotactic factor for keratinocytes, as shown by phagokinetic track analysis[80]. In human skin, EGFR localises with PC/APC and EPCR in the basal and suprabasal layers of the epidermis[60]. Recombinant human (rh) APC treatment of keratinocytes increases EGFR activation and keratinocyte migration[57,60]. APC promotes keratinocyte migration at concentrations 5 μg/mL but had an inhibitory effect at 20 μg/mL[14]. At 5 μg/mL APC, the migration of keratinocytes was equivalent to that induced by 50 ng/mL EGF[14]. Gene silencing of PC inhibits EGFR expression and reduces keratinocyte migration by 20% using an *in vitro* scratch wounding assay[60].

MMP secretion appears to be are required for keratinocyte migration, as blockade of MMP's using GM6001, a broad spectrum MMP inhibitor, eliminated cell migration in a dose-dependent manner and delayed *in vitro* wound healing[60]. Full-thickness rat excisional skin wound healing model, a single topical application of rhAPC enhances wound healing compared to saline by stimulating re-epithelialisation[71,81]. This is also observed in human skin wound healing. In humans, topical application of 200 μg/mL rhAPC to chronic wounds of varying aetiology reduced wound area by 52%-95% over 16 wk[82]. A follow-up study of venous and diabetic ulcers treated with 400 μg/mL rhAPC showed a significant reduction in wound area and volume compared to baseline at 20 wk[83].

APC reduces inflammation of keratinocytes: APC regulates the expression of serine protease MMP-2. MMPs degrade tissue components and are commonly as-

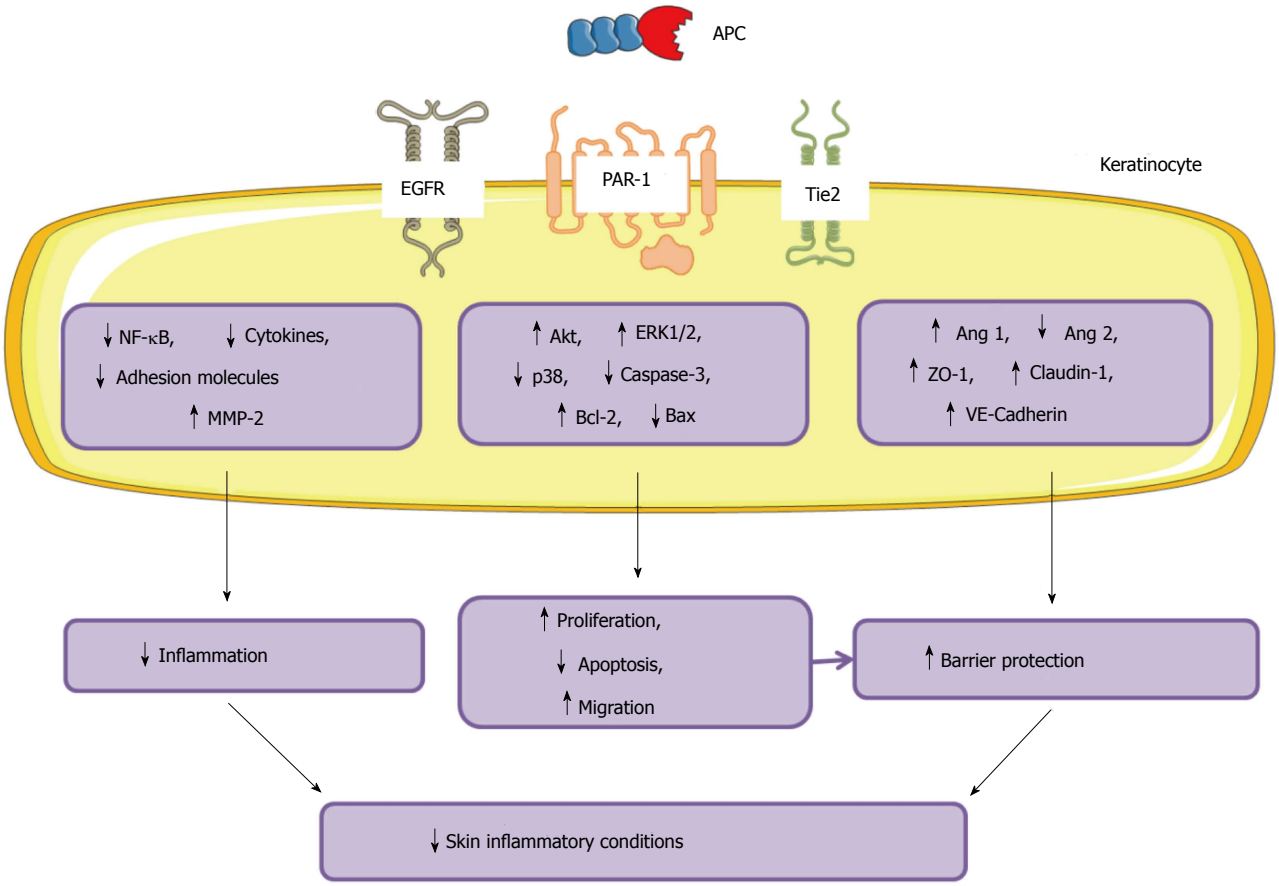

Figure 5 Schematic representation of protein C/activated protein C effects on skin epidermal keratinocyte function. APC: Activated protein C; EGFR: Epidermal growth factor receptor; PAR-1: Protease-activated receptor 1. Figure was produced using Servier Medical Art - www.servier.com.

sociated with skin inflammatory conditions[84]. In cultured human keratinocytes, APC enhances MMP-2 activity[14] which has anti-inflammatory properties[11,85] and plays a vital role in the tissue repair process by remodelling the extracellular matrix[86]. In contrast, MMP-9, which exhibits pro-inflammatory actions[11,87-89], is suppressed by APC[8,90].

Other indirect effects APC may have on suppressing cytokine production and activation is *via* inhibition of NF-κB subunits p50 and p52[28]. APC inhibits calcium- and lipopolysaccharide-stimulated activation of NF-κB in keratinocytes[14]. The NF-κB pathway is important for the expression of a wide variety of inflammatory genes including TNF-α and cell adhesion molecules, intercellular adhesion molecule-1, vascular cell adhesion molecule-1and E-selectin.

APC promotes barrier function of keratinocyte monolayers: The barrier protective effect of APC is relevant to skin epidermal keratinocytes[34]. Keratinocytes play a critical role in maintaining epidermal barrier function *via* tight junctions[43,91,92]. Dysregulation of tight junction proteins such as occludins, claudins and JAMs perturbs this barrier[43,91] and contributes to many skin inflammatory conditions[93].

APC enhances the barrier function of cultured human keratinocyte monolayers in a dose-dependent manner by up-regulating tight junction protein and redistributing them to cell-cell contacts *via* regulation of Tie2 and subsequent activation of Akt[34]. In response to APC treatment, Tie2 is activated within 30 min on keratinocyte monolayers, and relocates to cell-cell contacts where it impedes barrier permeability[34]. Expression of ZO-1, claudin-1 and vascular endothelial cadherin are subsequently increased. Interestingly, APC does not activate Tie2 through its major ligand, angiopoeitin-1, but binds directly to EPCR, cleaves PAR-1, and transactivates EGFR, then Tie2 which activates PI3K/Akt signalling to increase stabilisation of the keratinocyte barrier[34].

Prospective therapeutic potential of PC/APC

The skin, the body's largest organ, provides an epidermal barrier to protect the body from external insults, maintain temperature and control evaporation. Breaches of this barrier are common events. However, the inability to restore this barrier function can result in health problems, including inflammatory skin diseases, which are very common and have high morbidity. This group of diseases includes: acne, which affects 50% of teenagers (5% have severe acne); rosacea which affects 10% of the adult population; atopic dermatitis which affects up to 20% population; psoriasis which affects 2%-3% population[94,95]; chronic wounds which affect < 1% population and the devastating, often fatal, toxic epidermal necrolysis[96,97].

These diseases can be controlled to a certain extent, but no cure exists and they have high morbidity[98,99].

Management of most skin inflammatory conditions involves the use of emollients, phototherapy, topical corticosteroids, antibiotics, retinoids, immunomodulators (tacrolimus, pimecrolimus), or systemic treatments (ciclosporin, azathioprine). While targeted immunosuppressive drugs have been developed, including TNF-α inhibitors, antibodies and receptor blockers, in most studies they do not show improved outcome and their use for skin inflammatory conditions remains controversial[97]. For other conditions such as Stevens-Johnson syndrome and toxic epidermal necrolysis, to date no treatment has been identified to be capable of halting the progression of skin detachment[96].

APC is emerging as a critical regulator of keratinocyte and epidermal function. APC protects the epidermis by promoting keratinocyte proliferation, survival, reducing inflammation and maintaining barrier function. These keratinocyte cytoprotective functions are dependent on APC's interaction with EPCR, PARs, EGFR and Tie2.

Topical administration of rhAPC has shown promising results in the field of skin wound healing. Single or multiple topical applications of rhAPC to excisional wound sites reduced oedema and leukocyte infiltration, in addition to promoting angiogenesis and re-epithelialisation of wounds in rat models of skin wound healing[71,81]. These same APC-mediated benefits have been demonstrated in humans chronic wounds of venous and diabetic origin[82,83], as well as recalcitrant orthopaedic wounds[100].

The anti-inflammatory, barrier stabilisation and anti-apoptotic properties of APC make it an appealing treatment for skin diseases associated with inflammation, barrier disruption and keratinocyte dysfunction. A summary of the actions of APC on keratinocytes and skin inflammatory disorders is shown in Figure 5.

In late 2011, rhAPC (Xigris; drotrecogin alfa [activated]; Eli Lily) was withdrawn from the market after failure to significantly improve patient outcome in a clinical trial of septic shock[101], in an attempt to replicate earlier favourable results[102]. One concern was the observation of serious bleeding in patients, although there was no significant difference between patients treated with rhAPC and placebo[101,102]. Most *in vivo* studies, including our own, show that systemic rhAPC does not induce any bleeding side-effects[71,82,100,103-105]. Bleeding has occurred in a subset of near-death sepsis patients with recent surgery and although APC efficacy and safety is controversial in treatment of sepsis patients, it is beneficial and safe in clinical trials for chronic wound healing[82,100], acute lung injury[106,107], and solid organ transplantation[108]. Recently APC mutants (3K3A-APC and APC-2Cys) with minimal anticoagulant activity, but normal cytoprotective activity have been generated[109,110] and shown pre-clinically to be safe[12,111-116]. Although both variants are yet to be assessed in the field of skin inflammatory diseases. The notion that rhAPC may increase bleeding during wound healing could be circumvented by use of APC variants lacking anticoagulant activity.

Nevertheless, the future for utilising exogenous APC as a topical treatment for skin inflammatory conditions remains a novel and exciting avenue of investigation.

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REVIEW

Endoglin in liver fibrogenesis: Bridging basic science and clinical practice

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Abstract

Endoglin, also known as cluster of differentiation CD105, was originally identified 25 years ago as a novel marker of endothelial cells. Later it was shown that endoglin is also expressed in pro-fibrogenic cells including mesangial cells, cardiac and scleroderma fibroblasts, and hepatic stellate cells. It is an integral membrane-bound disulfide-linked 180 kDa homodimeric receptor that acts as a transforming growth factor-β (TGF-β) auxiliary co-receptor. In humans, several hundreds of mutations of the endoglin gene are known that give rise to an autosomal dominant bleeding disorder that is characterized by localized angiodysplasia and arteriovenous malformation. This disease is termed hereditary hemorrhagic telangiectasia type Ⅰ and induces various vascular lesions, mainly on the face, lips, hands and gastrointestinal mucosa. Two variants of endoglin (*i.e.*, S- and L-endoglin) are formed by alternative splicing that distinguishes from each other in the length of their cytoplasmic tails. Moreover, a soluble form of endoglin, *i.e.*, sol-Eng, is shedded by the matrix metalloprotease-14 that cleaves within the extracellular juxtamembrane region. Endoglin interacts with the TGF-β signaling receptors and influences Smad-dependent and -independent effects. Recent work has demonstrated that endoglin is a crucial mediator during liver fibrogenesis that critically controls the activity of the different Smad branches. In the present review, we summarize the present knowledge of endoglin expression and function, its involvement in fibrogenic Smad signaling, current models to investigate endoglin function, and the diagnostic value of endoglin in liver disease.

Key words: Telangiectasia; Signalling; Transforming growth factor-β; Disease; Bleeding disorders

Core tip: Endoglin is an accessory receptor for transforming growth factor-β impacting various aspects of its signaling and biological functions. Endoglin mutations are inherited as autosomal dominant disorders and may cause severe defects in different organs, including brain, lung and liver. In the present review, we will highlight the pathogenesis of several of these disorders and give an overview about the important role of endoglin dysfunction in the pathology of liver fibrosis.

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INTRODUCTION

Endoglin (OMIM 131195) was originally identified 25 years ago by immunofluorescence staining of vascular endothelium with a monoclonal antibody (mAb 44G4) that

was produced against a human pre-B leukemia cell line[1]. It is composed as a homodimer of two subunits with an apparent molecular weight of 95000 kDa that are linked by disulfide bonds[1]. Two years later, cDNA clones were isolated from an endothelial cell λgt11 expression library using a rabbit antibody prepared against endoglin purified from placenta[2]. Subsequent screening with an endoglin-specific cDNA probe resulted in the isolation of a different splice variant in which the encoded cytoplasmic tail contains only 14 amino acids (aa) as opposed to the stretch of 47 residues that was published previously[3]. The *ENG* gene was mapped to the long arm of human chromosome 9 (9q34→qter) by Southern blot analysis of DNA isolated from human-hamster somatic cell hybrids and by fluorescent *in situ* hybridization coupled with DAPI banding on human chromosomes[4]. The detailed chromosomal assignment was subsequently predicted from the fact the mouse homolog is located on chromosome 2 directly in the close proximity of the adenylate kinase-1 gene that is syntenic to human chromosome subband 9q34.1[5,6].

Mutations within endoglin were first brought into context of hereditary hemorrhagic telangiectasia type I (HHT-1) in three affected individuals in whom nucleotide substitutions or deletions gave rise to premature termination codons[7]. Since that, several hundred independent mutations or variations have been identified in the *ENG* gene that most often show regional distribution[8-12]. The different mutations show different phenotype-genotype correlation with the severity of HHT-1[13]. Moreover, it has been shown that soluble endoglin (sol-Eng) is an anti-angiogenesis factor that contributes to the pathogenesis of pre-eclampsia that is associated with hypertension, proteinuria, premature labor, hemolysis, liver abnormalities, thrombocytopenia, seizures and death[14,15]. Increased levels of sol-Eng in vascular surgical specimens were also brought into context with the pathogenesis of arteriovenous malformations (AVM) of the brain and aberrant cerebral vascular remodelling[16]. Other reports propose sol-Eng as a marker in diabetic patients[17] for estimating progression or treatment efficacy of the atherosclerotic process[18,19], systemic lupus erythematosus[20], non-small cell lung cancer patients[21], hypertension[22], disturbed angiogenesis in systemic sclerosis[23], Alzheimer's disease[24], breast cancer[25], premalignant lesions of the colon mucosa[26], outcome of biliary atresia[27] and cystic fibrosis associated liver disease[28], unexplained fetal death[29], malaria pathogenesis[30], prostate cancer[31] and many other diseases. In addition, endoglin expression was found to be related to tumor size, aggressiveness and metastatic potential in patients with gastroenteropancreatic neuroendocrine tumors[32].

A similar phenotype, *i.e.,* HHT type 2, is observed when the activin-like kinase (ALK)-1 receptor is functionally altered[33]. Likewise, mutations in the gene encoding Smad4 (MADH4) can cause a syndrome called Juvenile Polyposis/Hereditary Hemorrhagic Telangiectasia Syndrome (JPHT), consisting of both juvenile polyposis and hereditary hemorrhagic telangiectasia phenotypes[34]. Also, the mutations of other yet unidentified genes on the long arm of chromosome 5[35] and on the short arm of chromosome 7[36] were linked to the formation of other HHT types.

Endoglin expression and dysregulation has been shown in a number of cell types, including mesangial cells, cardiac and scleroderma fibroblasts, and hepatic stellate cells (HSC), suggesting some important function in cell and organ homeostasis and disease formation[37-40]. In particular, many independent findings demonstrate that endoglin is a critical factor that orchestrates transforming growth factor-β (TGF-β) signaling in wound healing in the pathogenesis of fibrosis. In regard to hepatic fibrogenesis, it was shown that endoglin is expressed in HSC[41] representing the most pro-fibrogenic cell type within the liver. Interestingly, endoglin expression is upregulated during liver damage and transiently induced in HSC by TGF-β1[40]. In this hepatic subpopulation, endoglin binds to the TGF-β type Ⅱ receptor (TβRⅡ), becomes phosphorylated by the activity of the TβRⅡ, and shows highest expression during maximal cell activation with a transdifferentiation-dependent cellular localisation and ligand affinity[40]. Interestingly, transient overexpression of endoglin results in a stronger activation of the Smad1/Smad5 signaling cascade and a prominent increase of α-smooth muscle actin expression, thereby promoting cellular activation and transdifferentiation[40], while contrarily the activity of the TGF-β1/Smad3 pathway is inhibited[42]. All these findings demonstrate that endoglin is one of the central switches controlling fibrotic and anti-fibrotic activities by producing different variant forms, adjusting ligand affinity, amending expression levels, and interacting with a versatile receptor network, thereby modulating the specific outcome of TGF-β-dependent and -independent pathways.

In the present review, we will summarize the actual knowledge of endoglin function and discuss the impact of this receptor on disease formation, hepatic fibrogenesis and its diagnostic value in initiation, progression and prognosis of various liver diseases.

MOLECULAR AND BIOCHEMICAL CHARACTERISTICS OF ENDOGLIN

The human endoglin gene contains 15 exons numbered 1 to 14, where exon 9 is split into 9a and 9b (Figure 1)[7]. Beside the full length endoglin (FL-Eng), a splice variant has been identified, *i.e.*, short-endoglin (S-Eng), that is characterized by the retention of intron 14 in the mature mRNA[3,40,43]. The expression of S-Eng is increased in senescent endothelial cells and alternative splicing is most likely performed by the alternative splicing factor or splicing factor-2 (ASF/SF2)[44,45]. However, the orthologous S-Eng mRNAs of men and mice give rise to different proteins (Figure 1) with either shortened and in part alternate C-termini[2,43] or a full length endoglin with a peptide insertion in rat[40]. Although the C-terminal domain of FL-Eng does not possess catalytic activity, it

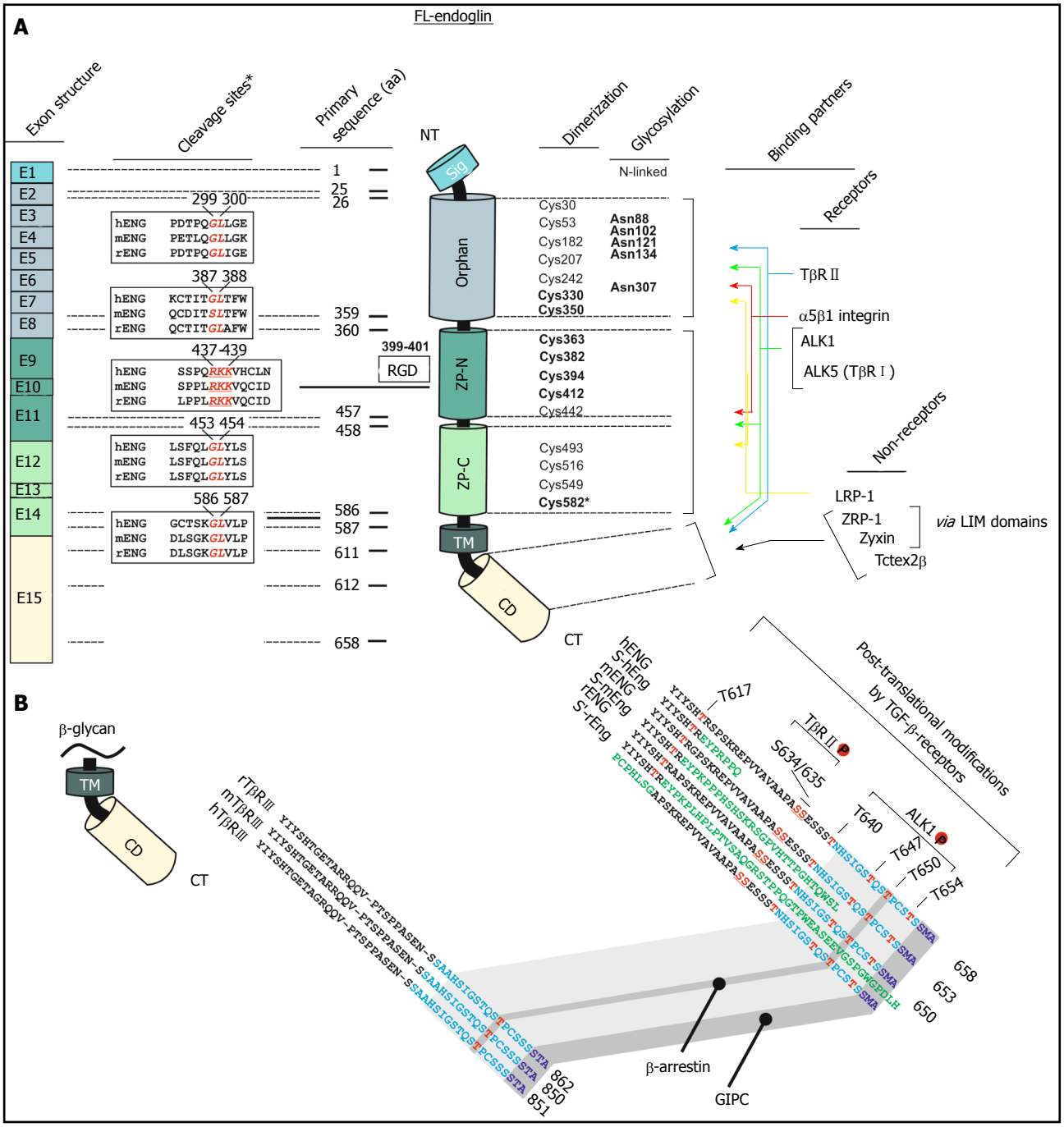

Figure 1 Schematic representation of the structural and functional modules of human endoglin. A: Left, first panel, Exon structure: Structure of the endoglin gene and assignment of respective protein modules. Left, second panel, cleavage sites: Predicted consensus proteolytical cleavage sites deduced from the primary sequence and experimentally confirmed matrix metalloproteinases-14 cleavage site between aa positions 586 and 587[59]. Middle panel, primary sequence positions: aa boundaries of the structural domains of human endoglin and location of the Arginine-Glycin-Aspartic acid (RGD) sequence that is only present in human endoglin. Right, first panel, dimerization: The cysteine residues of the extracellular domain of endoglin are depicted including the 8 highly conserved residues within the ZP-domain[47]. Cysteines that are involved in dimerization are shown in bold. Cysteine 582 that is involved in human endoglin dimerization is not present in the mouse and rat homologues[56]. Right, second panel, glycosylation: This figure part displays verified N-linked glycosylation sites within human endoglin. There is experimental evidence for O-linked glycosylation, but respective sites are not shown[1]. Right, third panel, binding partners: Depicted are interaction partners of endoglin as either receptor proteins (upper) or cytosolic non receptors proteins (lower). Colored arrows indicate interacting domains of endoglin with its binding partners. Zyxin and ZRP-1 bind to endoglin *via* their LIM-domains; B: Displayed is an aa alignment of betaglycan (left) and endoglin (right) of human, mouse and rat. Receptor kinase substrates (serine and threonines) are shown in red. Threonine 650 is essential for binding to β-arrestin2[66]. The C-termini of betaglycan and endoglin that are highly conserved are indicated in light blue. The PDZ-I domain which binds to GIPC is depicted in dark blue[67]. The alternative C-termini which results from differential splicing are shown in green.

is substrate for different kinases and comprises several protein-protein interaction domains (Figure 1)[46]. Therefore, structural alterations imposed by differential splicing of the mRNA that encodes the intracellular domain results in functional consequences for Eng in signaling (see below). In addition to splicing, Northern blot analy-

sis of mouse and rat transcripts revealed two mRNA species differing in molecular weight more than the size of the retained intron 14 of S-Eng. Analysis of the corresponding cDNA with 3'-RACE and inspection of the rat genomic DNA sequence confirmed a variation in the non-coding region of the mRNA and the presence of a second polyadenylation signal in the genomic DNA[40]. Whether this differential polyadenylation modulates mRNA stability or other features of the mRNA is currently not known. Since it has been realized that endoglin mutations are causative for HHT-1[7], a wealth of different mutations in the endoglin gene which lead to altered expression or formation of aberrant protein products has been identified (see below). Nevertheless, mutations are not spread randomly in the genomic sequence. A bias for mutations is found in the orphan domain and the N-terminal zona pellucida (ZP-N) subdomain in which three highly conserved cysteines (Cys363, Cys382 and Cys412) are exceptionally prone to mutations[47].

Biochemical characteristics

Endoglin, a type I transmembrane glycoprotein, is expressed as a disulfide-bound dimer at the cell surface[48]. Endoglin belongs structurally to the zona pellucida (ZP) family of sperm receptors sharing a ZP domain of approximately 260 aa in their extracellular part[49,50]. This domain is localized between Lys362-Asp561 (Figure 1) and contains eight highly conserved cysteine residues[47]. Common characteristics of ZP domain proteins are that they are: (1) shed to generate a soluble form; (2) membrane proteins with a hydrophobic region at their C-termini; (3) strongly glycosylated; and (4) finally highly expressed in the corresponding tissues in which they occur[50].

Among TGF-β-family receptors, endoglin and betaglycan constitute the TGF-β type Ⅲ receptor family. Both receptors share a high degree of similarity, especially in their intracellular domain (Figure 2) that is also the most conserved region between endoglin from different species (Figure 3), implying that this region has an important function, although lacking enzymatic activity[40].

In line, the signaling specificity of endoglin compared to betaglycan is at least for some specific functions determined by the extracellular domain (ECD)[51]. Since both of these receptors possess no enzymatic activity in their short C-terminal domain and are not obligatory for general signaling, they have been assigned an accessory/modulating function in signaling[52]. The primary sequence of FL-Eng comprises 658 aa in human[2,3], 650 aa in rat[41], and 653 aa in mouse (Figure 3)[53]. The ECD of human Eng harbors a Arginine-Glycin-Aspartic acid (RGD) peptide representing a potent binding site for integrins which is not present in the rat and mouse homologues[2,41,53]. Along with the FL-Eng, a splice variant designated S-Eng has been identified. The longer mRNA is due to the retained intron 14 (see above) and codes for a protein with a shortened C-terminus of 14 aa in human and 35 aa in mouse because of an in-frame stop codon present in the intron which is not found in rat resulting in a protein that contains a 49 aa insertion[3,41,53]. As outlined below, the shortening of the C-terminal domain of the splice variant in human and mouse have structural and functional consequences because specific modules are missing. The mentioned insertion in rat FL-Eng only causes minor effects which may be due to sterical alterations in the C-terminal domain[40]. In addition to these splice variants, two transcripts in mouse and rat occur which differ in the 3'-non-coding part and which arise from differential polyadenylation[40]. With respect to post-translational modifications, the primary Eng sequence contains several potential N- and O-dependent glycosylation sites. Initial enzymatic de-glycosylation studies confirmed the usage of both N- and O-dependent glycosylation consensus motives[48]. In a more detailed study, single N-dependent glycosylation sites (Asn88, Asn102, Asn121, Asn134 and Asn307) have been identified by mutational analysis[54]. Although the corresponding N-glycosylation sites seem to influence the stability of the corresponding domain, *e.g.,* Asn102 and Asn307[54], the removal of carbohydrates by peptide N-glycosidase F (PNGase F) was shown to be exiguous for function of the ECD[55].

In general, FL-Eng has a tripartide structure comprising a short intracellular region (47 aa), a single transmembranal portion (25 aa), a large ECD (561 aa) and a predicted signal peptide (25 aa)[3]. Preceding the ZP domain there is an orphan domain (Glu26-Ile359), sharing no similarity to other protein families/domains[47]. The ZP domain (Gln360-Gly586) is further subdivided in a ZP-N (Gln360-Ser457) and ZP-C (Pro458-Gly586) subdomain (Figure 1). Deletion and substitution studies revealed that at least Cys582 in human FL-Eng in the ECD is involved in intermolecular disulfide binding[56]. Additional work revealed that the six cysteines between Cys330 and Cys412 are necessary to mediate receptor dimerization[57], allowing the receptor to be expressed as a dimer at the cell surface, or in case of the soluble form as a secreted dimer[58]. A high resolution structure established for the ECD of endoglin revealed information about the sterical arrangement of the 3-dimensional protein fold[47]. These studies confirmed the three-modular-structure (orphan domain, ZP-N and ZP-C domain) and further raised the hypothesis of the occurrence of a putative cleavage site for a sheddase with specificity for the linker region between the folded domains of ZP-N and ZP-C at position Arg437-Lys438-Lys439 (RKK)[47]. However, the biochemical elucidation showed that the cleavage site is located closer to the membrane at position Gly586-Leu587. The executing enzyme was shown to be matrix metalloprotease-14 (MMP-14 or MT1-MMP)[59,60] promoting a shedding process that is similar to that described for betaglycan before[61]. On a functional level, endoglin is able to interact with the TGF-β signaling receptors (cf. Figure 1, Figure 4)[62] as well as other regulatory proteins[63-67]. These interactions are mediated by the different subdomains (or combinations). In general, FL-Eng is able to interact with ALK5 and TβR Ⅱ independent of ligand and the activation state of the signaling receptors[56]. In more detail it was

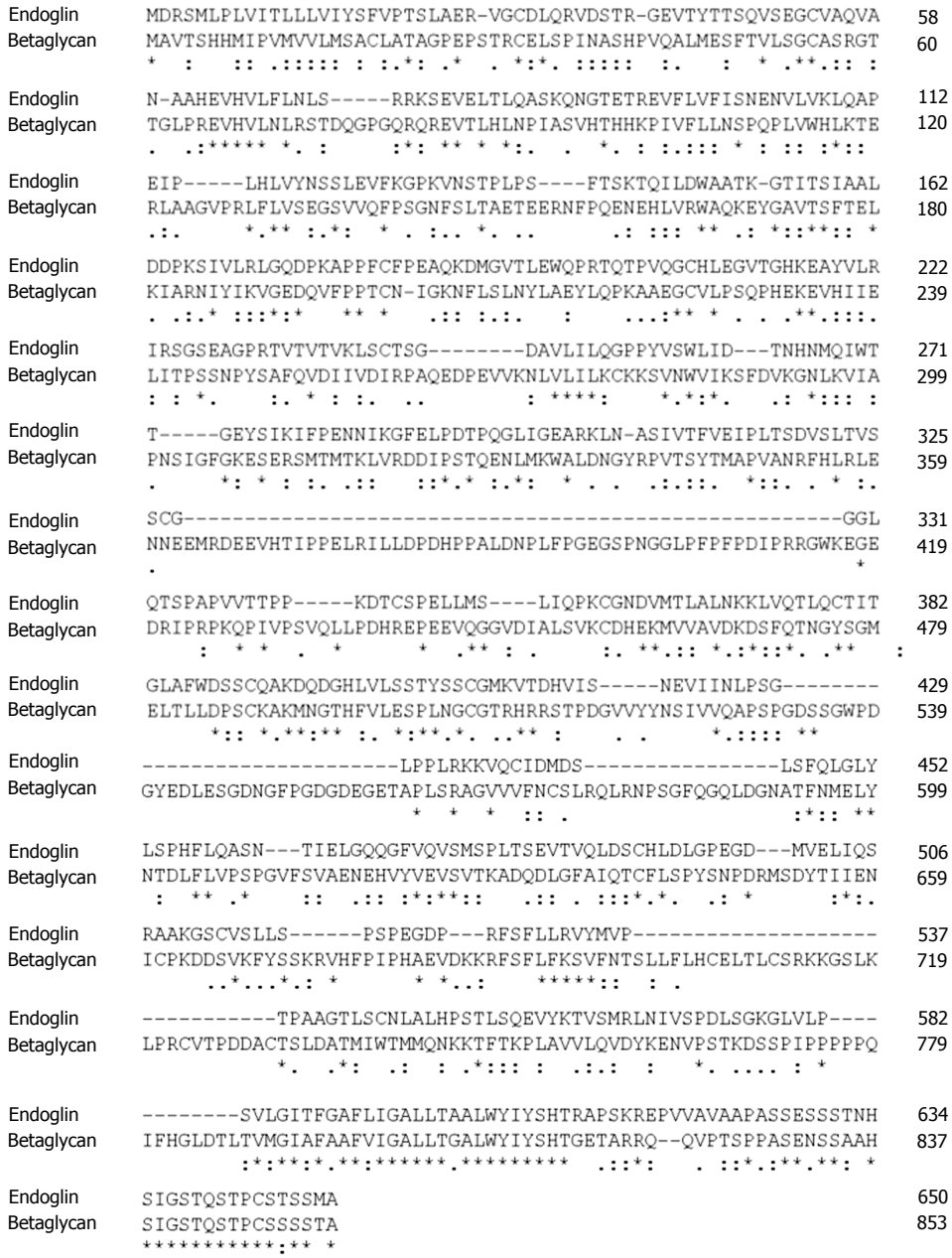

Figure 2 Sequence alignment of rat endoglin and betaglycan. The protein sequences of rat endoglin and betaglycan were aligned using the ClustalW2 algorithm. Respective sequences of rat endoglin (AAS67893) and betalycan (AAA42236.1) were taken from the GenBank. Please note the high degree of similarity of both proteins at their C-termini. Fully conserved aa in endoglin are marked by asterisk (*), positions that carry aa with strongly similar properties by a colon (:) and positions with weakly similar properties by a period (.), respectively. Please note that the highest degree of homology is found at the C-terminal regions that encompass the cytosolic part of endoglin

shown that TβR Ⅱ interacts with the region 437-558 (mainly ZP-C domain) of the endoglin ECD. In contrast to TβR Ⅱ, ALK5 contacts two regions, spanning aa 26-437 and aa 437-558 (orphan and ZP-C domains)[56]. Similarly, the second type I receptor ALK1 was shown to interact with the region Glu26-Gly586 of the ECD of endoglin[68].

Since the soluble variant of endoglin comprises all these ECD, it should in principal also be capable of mediating the same receptor interactions. Nevertheless, the binding of the soluble ECD to membrane bound endoglin could not be shown[57].

Whereas the binding to the ECD of FL-endoglin is independent of the signaling receptor activity, interaction of TβR Ⅱ, ALK5 and ALK1 with the intracellular domain of endoglin is regulated by the activation state of the signaling receptors since binding of the constitutive active ALK5/ALK1 could not be detected, while the binding of kinase dead and wild type ALK5/ALK1 could be demonstrated[56,68]. In line, the association of endoglin with the inactive form (kinase dead) of TβR Ⅱ was reported to be stronger when compared to wild type TβR Ⅱ[56].

It is known that FL-Eng is phosphorylated at serine and threonine residues[69,70] and both ALK5 and TβR Ⅱ use the C-terminus of endoglin as a substrate[56,70,71]. In

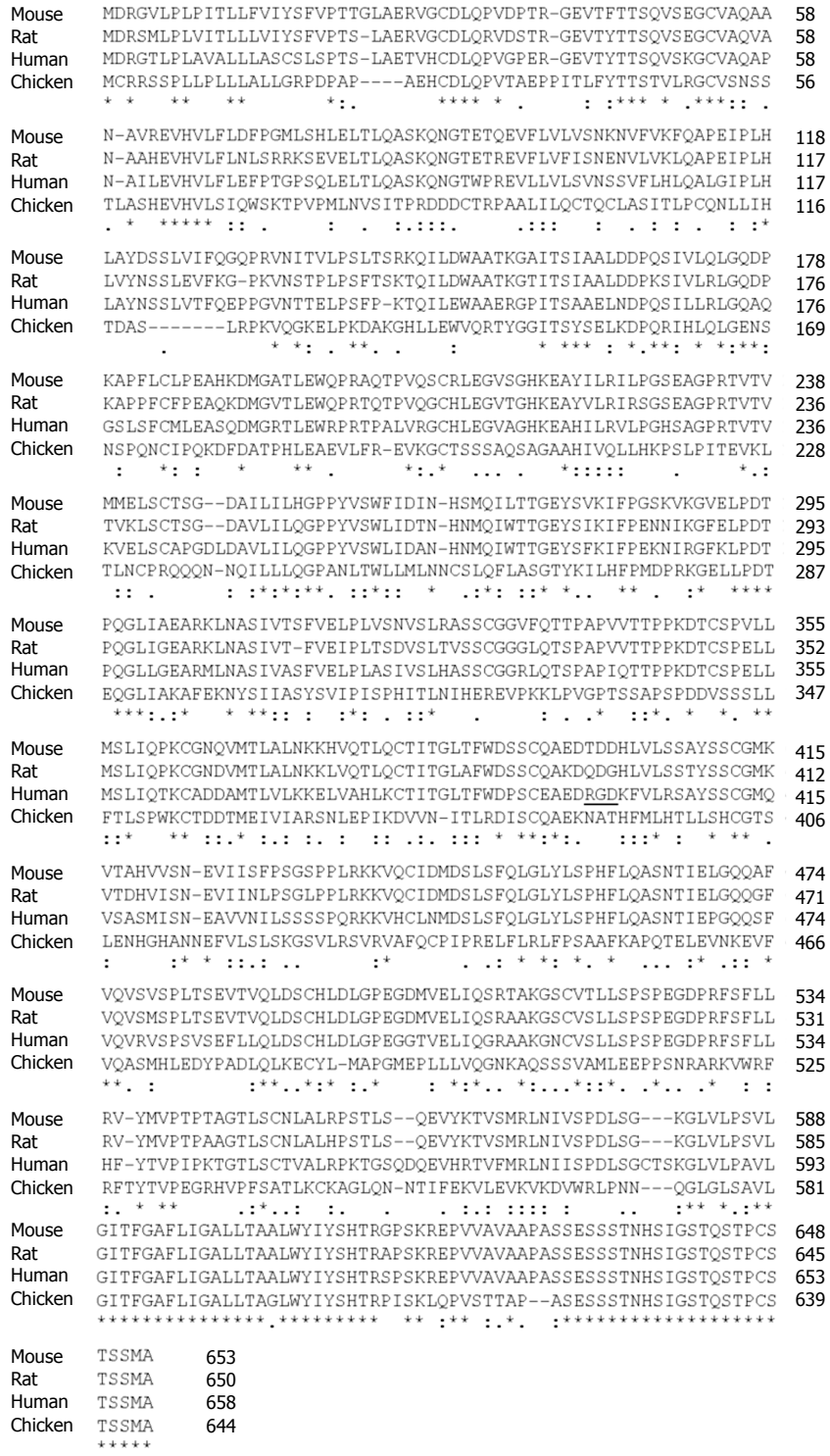

Figure 3 Sequence alignment of endoglin from different species. The protein sequences of rat, mouse, human, and chicken endoglin were aligned using the ClustalW2 tool (http://www.expasy.org/genomics/sequence_alignment). Sequences of mouse (NP_031958), rat (AAS67893), human (NP_001108225) and chicken (AAT84715) were taken from the GenBank (http://www.ncbi.nlm.nih.gov/). The Arginine-Glycin-Aspartic acid sequence in human endoglin (aa 399-aa 401) is underlined. Fully conserved aa in endoglin are marked by asterisk (*), positions that carry aa with strongly similar properties by a colon (:) and positions with weakly similar properties by a period (.), respectively. Please note that the highest degree of homology is found at the C-terminal regions that encompass the cytosolic part of endoglin.

turn, FL-Eng inhibits autophosphorylation of TβR Ⅱ but enhances phosphorylation of ALK5 by TβR Ⅱ leading to a stronger Smad2 transcriptional activity (see below)[56]. Aside from ALK5, ALK1 is also able to phosphorylate the FL-Eng C-terminus, but in contrast to ALK5, primarily on threonine residues[70]. Threonine phosphorylation by ALK1 (Thr654) necessitates serine phosphorylation by TβR Ⅱ which is enforced by removal of the C-terminal PDZ domain[70]. Moreover, ALK1 phosphorylation and binding of endoglin was observed only in the presence of TGF-β1 and this phosphorylation leads to loss of FL-Eng from focal adhesions (see below)[70]. This modulates

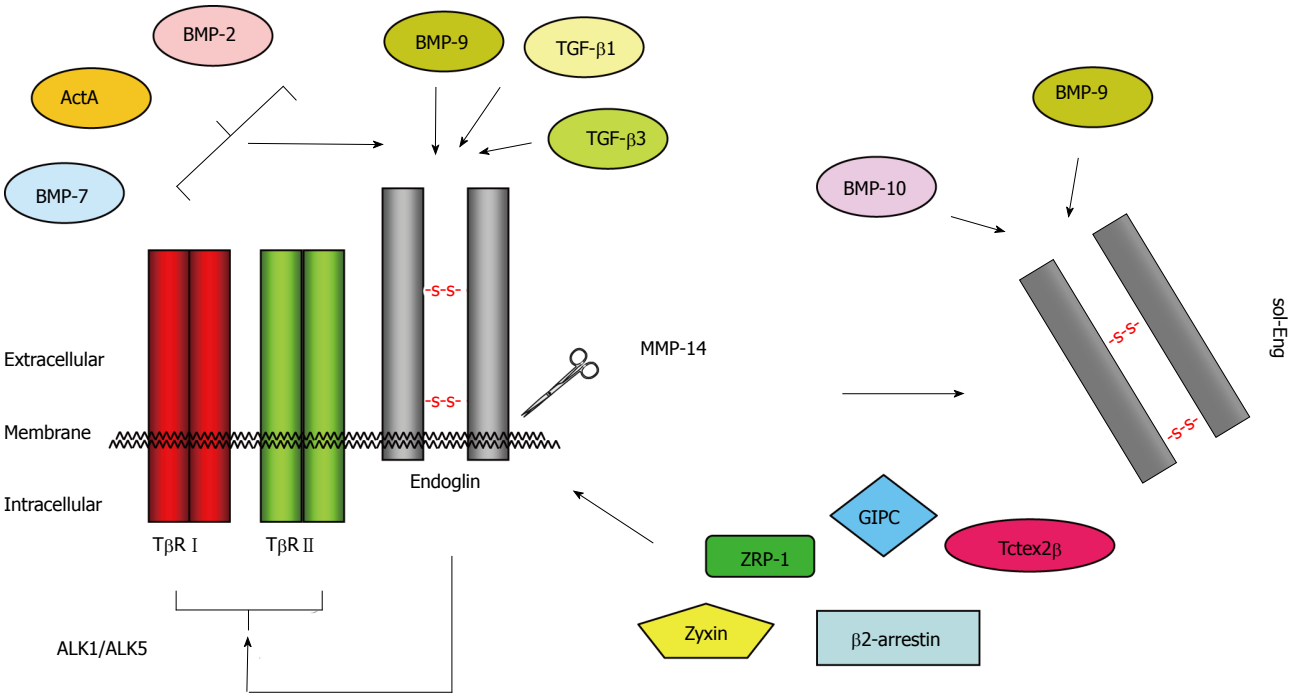

Figure 4 Binding partners of endoglin. Endoglin physically interacts *via* its extracellular domain with TGF-β1, TGF-β3 and BMP-9[110]. The short cytoplasmic domain has affinity for ZRP-1[63], Zyxin[64], GIPC[67], β-arrestin-2[66], and Tctex2β[65]. In conjunction with TβR Ⅰ and TβR Ⅱ, the binding spectrum is extended to BMP-2, BMP-7 and ActA[111]. After proteolytic cleavage (shedding) by MMP-14 (also known as membrane-type matrix metalloproteinase MT1-MMP), the soluble form of endoglin (sol-Eng) is released[59]. This form has capacity to bind BMP-9 and BMP-10[113]. TGF: Transforming growth factor; BMP: Bone morphogenetic protein.

proliferative and adhesive properties of endothelial cells. In more detail, it was shown that the exponentiation activity of endoglin on ALK1 signaling and Smad1 activity is located between residues 26-558 within the ECD of endoglin[68]. Another interaction with the ECD of endoglin is mediated by integrin α5β1, which contacts not only the RGD-peptide but several parts of the ECD of endoglin. Clustering of α5β1/endoglin/ALK1 leads to an enhancement of TGF-β1-mediated Smad1/Smad5 activation and signaling[72]. Recently, leucine-rich α_2-glycoprotein 1 (Lrg1) has been further shown to interact with the ECD of endoglin. This protein is a regulator of endothelial functions during angiogenesis. In addition to endoglin, it interacts with ALK5 and TβR Ⅱ directly and facilitates recruitment of ALK1 into the receptor complex thereby promoting Smad1/Smad5-signaling[73].

In contrast to the signaling type Ⅰ and type Ⅱ receptors, the type Ⅲ receptors betaglycan and endoglin do not possess a kinase activity in their short intracellular domains[3,53,41]. Nevertheless, respective domains have important functional implications for the interaction with the signaling receptors as described above. Although the C-termini of betaglycan and endoglin are very homologous to each other (Figure 2), several residues used as substrates by the signaling receptors are unique to endoglin[70].

Phosphorylation by a respective receptor serves as a switch to regulate the interaction with a certain receptor. Besides receptor interactions, other regulatory proteins have been identified which specifically bind to the C-terminal domain of FL-Eng. Using the two hybrid method, zyxin and zyxin-related protein-1 (ZRP-1) were found to specifically and exclusively, with respect to type Ⅲ receptors, interact with FL-Eng[63,64]. Association with FL-Eng redirects these proteins from focal adhesions to actin stress fibers and leads to endoglin dependent inhibition of cell migration[63,64]. Another protein identified in the yeast system is the dynein light chain member Tctex2β. In addition to FL-Eng, Tctex2β also interacts with TβR Ⅱ and betaglycan and it inhibits TGF-β signaling[65].

However, it has to be mentioned here that all these interaction screens have been solely performed using protein baits of the endoglin intracellular domain which have not been posttranslationally modified, *e.g.*, phosphorylated. The interaction of at least zyxin with endoglin is stronger with the so called -ΔSMA deletion mutant that lacks the 3'-carboxyl-terminal protein part harbouring the PDZ-domain[64]. In line, removal of this domain causes an increase in endoglin phosphorylation[70] implying that this modification (phosphorylation) most likely modulates/regulates protein-protein interaction with the carboxyterminal domain (CD) of endoglin. Therefore, it is most likely that the group of proteins able to interact with endoglin is currently somewhat underestimated.

Based upon the high homology of the CD of endoglin and betaglycan, it is not surprising that both β-arrestin2 and GIPC were found to associate with both proteins[66,67,74,75]. The interaction of β-arrestin2 and endoglin is lost in the absence of threonine 650 and increases when co-expressed with TβR Ⅱ and ALK1[66].

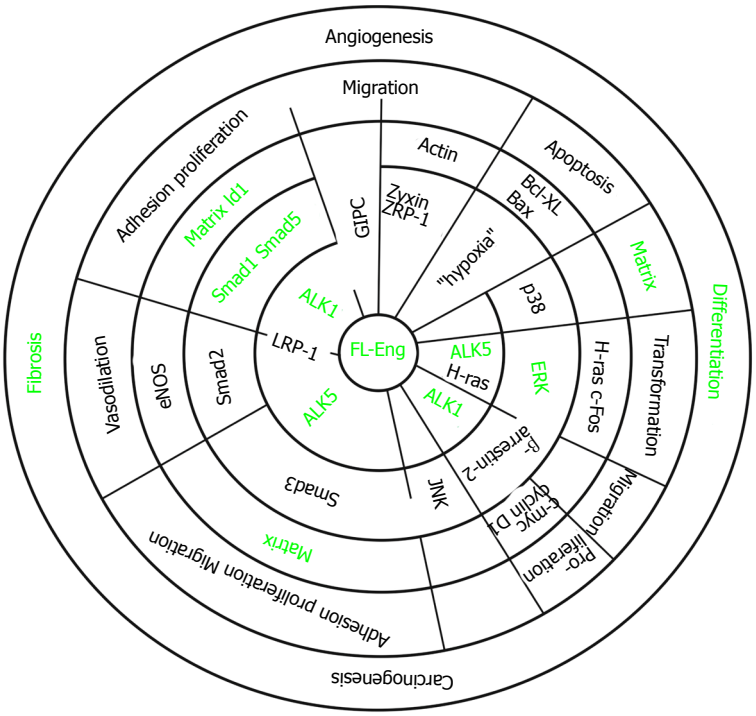

Figure 5 Association of endoglin with different signaling cascades. The concentrical circles display the hierarchy of signaling. Signaling starts at the membrane with receptors and adaptors (inner two circles). The next circle represents activated intermediates and adaptors. Thereafter, target genes are indicated. These are involved in shaping a cellular response which is part of a complex process (last circle). Partially open radial lines indicate that the corresponding molecules interact or interaction of molecules is mediated by the protein displayed on the radial line (LRP-1). The green font indicates items addressed in liver cells which are modulated by endoglin.

Whether the latter receptor regulates this interaction *via* phosphorylation is unclear since Thr650 is not a prominent ALK1 substrate[70]. On a functional level, β-arrestin2 causes endocytosis of the receptor complex, including endoglin, TβR Ⅱ and ALK1, and impacts MAPK-signaling in an endoglin-dependent manner[66]. In contrast to β-arrestin-2, the C terminus of the G alpha interacting protein (GAIP)-interacting protein (GIPC) binds to the C terminus of endoglin in a manner that is restricted to the endoglin class I PDZ-motif. This leads to a stabilization of endoglin at the plasma membrane and changes in Smad1/Smad5 activation and endothelial cell migration (see below)[67]. Moreover, GIPC mediates the interaction of endoglin and phosphatidylinositol 3-kinase in a TGF-β1 dependent manner to regulate endothelial cell sprouting and capillary tube stability[76].

ENDOGLIN FUNCTION AND IMPACT ON TGF-β SIGNAL TRANSDUCTION

Endoglin is an accessory receptor for TGF-β impacting various aspects of its signaling and biological functions. Special features for the full length, soluble and short forms of endoglin have been reported. In the following, we provide a brief overview about TGF-β signaling and the impact of the different endoglin protein variants. Functional aspects of FL-endoglin are summarized in Figure 5.

Brief overview of TGF-β signaling

Signaling by ligands of the TGF-β superfamily is initiated by binding of the ligand to a heterooligomeric membrane receptor complex. Binding of TGF-β1 is mediated by a homodimer of the TGF-β type Ⅱ receptor which in turn recruits and phosphorylates a type I receptor (ALK5 or ALK1) homodimer into the complex. After ligand binding, the receptor complexes are internalized in general *via* two different pathways. Endocytosis mediated by clathrin-coated vesicles, enriched for Smad anchor for receptor activation (SARA), leads to active signaling. Depending on the type I receptor involved, the signal is propagated to two different Smad protein subfamilies, with the specificities of ALK5 phosphorylating Smad2/Smad3 or ALK1 in triggering phosphorylation of Smad1/Smad5. Phosphorylated Smads bind to the common Smad4, translocate into the nucleus and regulate transcription of target genes. Of these, the I-Smads, *i.e.*, Smad6 and Smad7, are important regulators since they are direct target genes and shut off the signaling cascade at diverse points in a negative feedback loop. If internalization occurs *via* the lipid-rafts-caveolae-1, the receptors are bound to I-Smad/Smurf complexes targeting the receptor for ubiquitination and degradation[77].

Since this simple "core" of TGF-β signaling is involved in the regulation of a wide array of different target genes and control of diverse cellular responses, cells are endowed with a plethora of switches to adjust this cascade for their needs. Such cell type specific regulators for example are the type Ⅲ receptors, *i.e.*, betaglycan and endoglin, which are engaged in TGF-β receptor-complex formation and modulation of downstream signaling.

In the liver and especially in HSC, it has been assumed that the key operating TGF-β1 pathway is the ALK5/Smad3 branche that regulates proliferation, activation and profibrogenic responses of these cells. However, it has been anticipated that other signaling modalities like the ALK1/ALK5/Smad1/Smad5/Id1 axis is also engaged by TGF-β1 in regulating HSC physiology under normal and pathological conditions[40,78,79].

Impact of full length endoglin on TGF-β1-signaling

Analysis regarding the role of endoglin in signaling was primarily based on TGF-β-signaling and Smad-activation in monocytes and myoblasts[51,80]. Since it is known that endoglin is the candidate gene affected in HHT-1, detailed experimental work has been done using different endothelial cells[7,81]. So far the functional data regarding the involvement of endoglin in HSC are rather sparse. Endoglin is expressed in quiescent HSC and transdifferentiated myofibroblasts (MFB) and is transiently upregulated during cellular activation[40,41]. Upregulation of endoglin during activation/differentiation of cells is also seen in endothelial cells and monocytes[82,83]. Similar to other cell types, endoglin is not only affecting TGF-β1-signaling but is itself regulated by this ligand on the transcriptional level, most likely involving the Sp1 transcription factor[40,84-86]. As a mutual prerequisite, endoglin is membrane localized and interacts with and is phosphorylated by TβR Ⅱ in HSC[41,40]. Overexpression of endoglin causes an increased phosphorylation of Smad1/Smad5 in HSC of rat and mouse origin[40,79]. In line with the HSC data, it was previously found that endoglin enhances ALK1/Smad1/5 signaling in endothelial cells and other cell types[87], leading to increased proliferation and migration (characteristics of the activation phase of angiogenesis), responses which are negatively affected upon endoglin reduction[72,88,89]. However, other laboratories claimed that endoglin causes reduced activation of ALK1/Smad1/5 as well as reduced migration and proliferation[90] or even having no impact on Smad-signaling at all[66]. These differences might be explained in part by the experimental set up (method used to modulate the endoglin expression, *i.e.*, siRNA *vs.* knockout, concentration of the ligand, time scale of stimulation, cell type analyzed) and by the expression level of the two corresponding type I receptors, *i.e.*, ALK1 and ALK5, both of which are expressed in HSC[78]. On the other hand, ALK5/Smad3 signaling that inhibits proliferation and migration (characteristics of the resolution phase of angiogenesis) is blocked by endoglin[67,88,91]. Interestingly, in contrast to ALK5/Smad3 which is downregulated, the signaling *via* ALK5/Smad2 leading to increased eNOS expression/activity is promoted in endothelial cells[56,92]. This effect is in part due to a stabilization of the Smad2 protein[92].

Although collagen type I expression is reduced, the overexpression of endoglin has no significant impact on ALK5/Smad3/Smad2 activation in mouse and rat HSC cell lines[40,79]. An inhibitory role of endoglin in collagen type I expression has been well documented in diverse kinds of cells, including mesangial cells, fibroblast of different origins and myoblasts[87,93-95] and was attributed to a reduced Smad3 activation[87,94]. A contribution of MAPK in the endoglin dependent modulation of collagen expression and Smad3 phosphorylation was postulated for JNK1 and ERK1/2[94,96].

In HSC, endoglin causes an increase in TGF-β1 dependent ERK1/2 activation[79]. A positive effect of endoglin on ERK1/2 activation was also observed in human T cells upon crosslinking of endoglin[97]. In line with an enhancement of ERK1/2 phosphorylation, TGF-β1 mediated expression of the connective tissue growth factor (CTGF) is promoted by endoglin in HSC[79]. There are several other reports showing an ERK1/2 dependent expression of CTGF, once more underscoring these results[98,99]. Nevertheless, the activation of ERK1/2 and increased expression of CTGF by endoglin is most likely cell type specific. In endothelial cells and epidermal cells it was shown that endoglin, in association with β-arrestin2, leads to suppression of ERK1/2 activation and a change in the cellular distribution[66,100]. On the contrary, in myoblasts in which TGF-β1 and endoglin have only a minor effect on ERK1/2 activation, CTGF is reduced in the presence of endoglin[87,95]. A negative impact of endoglin on CTGF expression was also found in scleroderma fibroblasts by some groups[39,101]. However, in a subset of scleroderma fibroblasts it was shown that the TGF-β1/ALK1/Smad1 pathway mediates fibrogenic responses, *e.g.,* collagen I and CTGF expression, and that endoglin promotes this ALK1 pathway[102,103]. Finally, it was shown that ERK1/2 and Smad1 activation are functionally linked[102]. If endoglin-dependent up-regulation of ERK1/2 phosphorylation in HSC is directly linked to Smad1 activation and CTGF expression, and if ALK1 is involved in these responses is currently under investigation. Moreover, if the co-expressed betaglycan is involved in the up-regulation of CTGF is actually only speculative[101]. In addition, the basis of the forced expression of α-smooth muscle actin (α-SMA) in endoglin overexpressing cells needs to be analyzed[40,79]. One comprehensible option is a direct promoting effect on TGF-β1 signaling mediating α-SMA expression, which was shown to rely not exclusively on Smad3[104], or alternatively endoglin may cause a general shift in the transdifferentiation process leading finally to up-regulation of α-SMA.

Role of short (S-) endoglin on TGF-β1-signaling

Similar to FL-Eng, the S-Eng splice variant, although missing a large part of the C-terminal tail, binds to TGF-β1[3] and interacts with the signaling type Ⅱ receptor[40] and both type I receptors (ALK5 and ALK1)[44]. FL-Eng was shown to be phosphorylated at serine residues by TβR Ⅱ receptor[70] that fortuitously can be detected by a phospho-specific NF-κB antibody[105]. TβR Ⅱ-mediated phosphorylation of both isoforms of rat endoglin could be detected in HSC using this antibody[40], implying a functional association of endoglin and the TGF-β-signaling receptors in HSC. Both splice variants are co-expressed in endothelial cells and HSC and can form heteromeric L-/S-Endoglin dimers[40,43]. Nevertheless, S-Eng is unable to substitute for FL-Eng since animals that carry an S-Eng transgene on an Eng null background are not viable, implying that S-Eng alone is inappropriate to rescue the lethal phenotype[43]. Using the afore mentioned S-Eng overexpressing animals in a model for tumor angiogenesis and metastatic infiltration by injecting Lewis lung carcinoma (3LL) cells, it was

found that tumor growth is retarded when compared to control mice[43]. Even more, in a model of chemically induced skin tumors, overexpression of S-Eng in the vascular endothelium reduces benign tumor formation[43]. Nevertheless, functional data obtained in the rat system for the specific S-Eng variant yielded similar results when compared to FL-Eng[40]. Whether these results can be transferred to the mouse or human system is questionable due to the completely different C-termini.

Soluble endoglin: more than just a disease marker

As described above, endoglin can be shedded by MT1-MMP (MMP-14) from the cell surface to generate a soluble extracellular domain (sol-Eng) which reduces spontaneous and VEGF-induced endothelial sprouting[59]. In addition, the occurrence of sol-Eng has been observed in the serum/plasma of patients suffering from diverse tumors[106]. In pre-eclamptic women, the elevation sol-Eng precedes the onset of the disease, correlates with the severity of the disease and therefore its detection is of prognostic value[107]. Increased serum levels of sol-Eng have been found in cystic fibrosis associated liver disease (CFLD) patients, with the highest levels in patients suffering from HCV coupled with cirrhosis[28]. Significantly elevated sol-Eng levels are also observed in patients with hepatocellular carcinoma [Hepatocellular carcinoma (HCC)] combined with cirrhosis[108]. However, the role of sol-Eng in TGF-β1 signaling is presently controversial. Initially it was shown that the soluble domain is able to reduce TGF-β1-mediated reporter-gene activity and eNOS activation in endothelial cells[14]. In line with a ligand sequestering function, complexes of sol-Eng and TGF-β1 have been detected in serum of breast cancer patients using ELISA and co-immunoprecipitation[58]. Nevertheless, although part of the TGF-β1 ligand binding complex, a direct binding of TGF-β1 to endoglin is questionable[109,110]. If the signaling receptor type I and type Ⅱ are present/co-expressed, endoglin can be precipitated together with labelled ligand. If endoglin on the other hand is overexpressed in cells lacking type I and type Ⅱ receptor, there is no binding of TGF-β1 to endoglin[110]. The increase of the sol-Eng concentration in pre-eclamptic women and a few studies with a focus on sol-Eng function, using overexpression systems and luciferase assays, suggest that sol-Eng indeed has a functional role in TGF-β1 signaling[14,59]. In addition, we could show by co-immunoprecipitation that heterologous expressed sol-Eng is able to bind to TGF-β1 directly (SKM unpublished data) but experimental data suggest that it is unlikely for soluble endoglin to simply interfere with TGF-β1 signaling by competing with membrane bound type Ⅱ receptor for TGF-β1. Using a BIACore facility, the measured dissociation constants are 5 pM for TβR Ⅱ/TGF-β1[111] and in the micromolar range for sol-Eng/TGF-β1[112], underscoring the higher affinity of TβR Ⅱ for TGF-β1 compared to the soluble endoglin counterpart. On the other hand, Van Le *et al* found that CHO-overexpressed and purified soluble endoglin increased TGF-β1 mediated p3TP-lux activity in U937 monocytic cells[55] in which L-endoglin was shown to antagonize several TGF-β1-responses[80]. Nevertheless, direct ligand binding and functional mechanisms used by sol-Eng to affect cellular responses have to be analyzed in more detail in the future. There are currently no data focussing on functional aspects of sol-Eng, especially in the liver.

ENDOGLIN IN DISEASE

As outlined above, mutations that affect human endoglin function are inherited as autosomal dominant disorders and may cause AVM in different organs, including brain, lung and liver (Figure 6). In the following paragraphs we will highlight the pathogenesis of several of these disorders and associated diseases and give an overview about the important role of endoglin dysfunction in the pathology of liver fibrosis.

Hereditary hemorrhagic telangiectasia

Hereditary hemorrhagic telangiectasia (HHT, Osler-Weber-Rendu syndrome) is an autosomal dominant inherited vascular disorder with a variety of clinical manifestations. Common symptoms of this disease occur due to the forming of AVM in small and large blood vessels. This leads to epistaxis, gastrointestinal bleeding and microcytic anemia due to iron deficiency, along with characteristic mucocutaneous telangiectasia[113]. AVM are found in pulmonary, hepatic and cerebral vascular tissue (Figure 6). The diagnosis of HHT is based on these clinical features, which are summarized in consensus criteria known as the "Curaçao criteria"[114]. Rupture of AVM contributes to significant morbidity.

Mutations in at least five genes result in manifestation of hereditary hemorrhagic telangiectasia. However, about 85% of the cases develop due to mutations of the *ENG* gene (coding for endoglin) and *ACVRL1* (activin A receptor type Ⅱ-like 1 kinase 1, ALK1)[115]. This disease is usually autosomal dominantly inherited, varying in penetrance and expression. Juvenile Polyposis/Hereditary Hemorrhagic Telangiectasia (JPHT) is a rare juvenile form of HHT which is associated with polyposis and occurs due to mutations in the MADH4 gene coding for Smad4[116]. In gene linkage analyses, two other loci have been shown to be in a disequilibrium with HHT symptoms; one on chromosome 5, defining HHT-3[35], the other on chromosome 7[13], defining HHT-4. However specific genes on these chromosomes involved in disease formation remain to be identified. Mice deficient for endoglin or ALK1 expression show clinical features of HHT[117]. Eng knockout (null) mice are embryonically lethal, dying at day 10.5 p.c. due to impaired extraembryonic vascular development and several cardiac defects (see below). Heterozygous animals show clinical symptoms of HHT-1 with variable penetrance. Human patients with HHT-1 exhibit less endoglin expression in peripheral blood monocytes and newborn umbilical vein endothelial cells[118].

To prevent fatal clinical events like stroke, high-output heart failure, pulmonary hypertension and hemorrhage, the embolization of visceral AVM is a valuable course

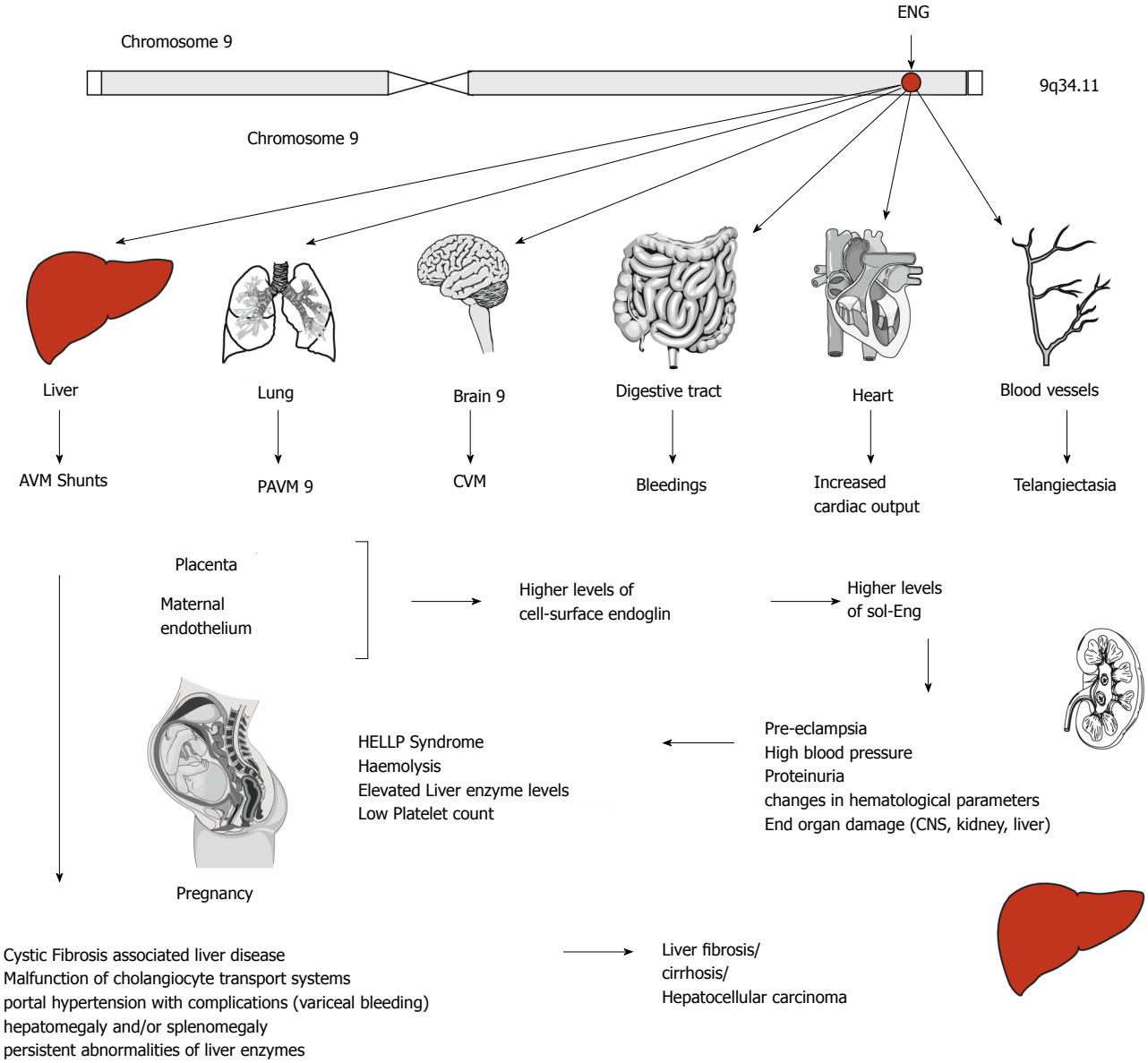

Figure 6 Endoglin and disease. The human endoglin gene (ENG) is located on the long arm of human chromosome 9. Mutations are inherited in an autosomal dominant manner and affect several organs. In liver, abnormal connection formed between blood vessels, arteriovenous malformations (AVM), malfunction of the cholangiocyte transport system gives rise to liver damage indicated by portal hypertension, persistent abnormalities of liver enzymes, hepatomegaly and/or splenomegaly, fibrosis, cirrhosis or even hepatocellular carcinoma. Intrahepatic connection between arteries and veins results in a large amount of blood bypasses for which the heart compensates by increasing the cardiac output resulting on long term in heart insufficiency. Similar arteriovenous (pulmonary AVM, cerebral AMV) are found in lung and brain. In the digestive tract bleedings occur and telangiectasias of blood vessels are found on the skin of the hands, face and mouth. During pregnancy, the placenta and the maternal endothelium produce higher levels of cell-surface endoglin that is shedded and leads to higher systemic concentration of soluble endoglin (sol-Eng) that leads to an imbalance of the antiangiogenic factors resulting in life-threatening obstetric complication (*e.g.*, pre-eclampsia, HELLP syndrome).

of treatment. Furthermore, symptomatical treatment approaches with antiangiogenic or antihormonal agents have been investigated. In some patients, the use of antiangiogenic therapies known from cancer therapy, such as thalidomide[119], lenalidomide[120] and bevacizumab[121], reduces the incidence of nasal and gastrointestinal bleeding. The β-receptor blocker propanolol, usually used for prophylaxis of esophageal variceal bleeding in patients with liver cirrhosis or the treatment in infantile haemangiomas, was able to decrease cellular migration and tube formation, concomitantly with reduced RNA and protein levels of ENG and ALK1 in cell culture[122]. Other studies showed that tamoxifen, an estrogen receptor antagonist, and the selective estrogen receptor modulator, raloxifene, can reduce episodes of epistaxis and transfusion requirements in patients suffering from nasal vascular malformations[123,124]. However, limited controlled studies, severe side effects of those drugs and the need for life long treatment limits the applicability for most patients.

Pre-eclampsia

Pre-eclampsia is a disease of high incidence (about 3%) in pregnant women with an onset after 20 wk of gestation. It complicates pregnancy and can lead to death of

mother and baby. The disease is characterized by new-onset hypertension (140 mmHg or diastolic blood pressure 90 mmHg) and proteinuria (excess of protein in the urine of at least 0.3 g of protein/d)[125]. Eclampsia is characterized by additionally occurring grand mal seizures[126]. Typical complications for the pregnant woman are the involvement of the central nervous system, acute renal or liver failure, and changes in hematological parameters. Women with pre-eclampsia are prone to higher lifetime cardiovascular morbidity, including hypertension and ischemic heart disease. Effects on the fetus can be severe and include prematurity, fetal growth restriction, oligohydramnios and placental abruption. A family history of pre-eclamsia, advanced maternal age, obesity or pregestational diabetes increases the mothers risk to develop this condition[127].

The pathophysiology of pre-eclampsia is still poorly understood. Prior to the development of clinical symptoms, cells migrating to the placenta lack the expression of endothelial surface adhesion markers. This leads to incomplete invasion of maternal arteries by the developing trophoblast, resulting in placental ischemia and the release of antiangiogenic factors, including sol-Eng and soluble fms-like tyrosine kinase (sFlt1)[128]. Vascular endothelial growth factor (VEGF) and placental growth factor are antagonized by soluble fms-like tyrosine kinase-1 (sFlt1 or sVEGFR-1) and sol-Eng antagonizes TGF-β1 and TGF-β3 activity[129]. These effects on vascular homeostasis promote changes in placental circulation. Numerous studies show the effect of VEGF and TGF-β signaling pathways on circulation and angiogenesis. These pathways directly influence the development of pre-eclampsia. By regulating endothelial cell proliferation, migration, vascular permeability and secretion, VEGF-A is an important ligand for angiogenesis. It binds to two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). The soluble receptor VEGFR-1 (sFlt1) acts as an endogenous VEGF inhibitor. In patients with pre-eclampsia, sFlt1 is overexpressed in the maternal circulation[130], which corresponds to a decrease of VEGF and placental growth factor expression in the placenta of pre-eclampsia patients[131]. This leads to the development of major symptoms of the disease due to abnormal trapping of VEGFs. The role of sFlt1 is underlined by studies in which pregnant rats were treated with exogenous sFlt1, inducing severe pre-eclampsia. Immunoprecipitation of sFlt1 in cells derived from placental villous explants normalized their angiogenic responses[129].

In addition, the VEGF-signaling changes in the TGF-β signal transduction pathway promotes the development of pre-eclampsia. Placentas of pre-eclamptic women show increased levels of membrane-bound Eng and sol-Eng[14]. Hypoxia and oxidative stress seem to be important triggers for the release of sol-Eng, as shown in a study where oxysterol activation promoted MMP-14-mediated cleavage of sol-Eng in cells of trophoblast origin[132]. sol-Eng antagonizes TGF-β1 induced vasodilatation, leading to vascular hypertension[133-135]. The increase of systemic sol-Eng in pregnant women is a factor that prequels the onset of pre-eclampsia[106,136,137]. Modulating the TGF-β pathway, endoglin can, alone or together with sFlt1, induce pre-eclamsia symptoms in pregnant rats[14].

The pathogenesis of pre-eclampsia is defined by the imbalance of the anti-angiogenic factors, sFlt1 and sol-Eng, and the proangiogenic factors, placental growth factor, TGF-β and VEGF[138]. Current treatment concepts therefore include the use of antibodies and small molecules to sequester or limit synthesis of anti-angiogenic molecules. Improvement in blood pressure and renal function could be achieved after administration of exogenous VEGF in a preclinical model of pre-eclampsia, modulating the balance of angio- and anti-angiogenic factors[139]. Recently, a study using a dextran sulfate column to remove sFlt1 from the maternal circulation by extracorporeal apheresis showed a potential therapeutic approach for the treatment of pre-eclampsia[140]. Other studies using induction of hemoxygenase-1 with cobalt protoporphyrin in pre-eclamptic rats[141] and prevention of the release of sol-Eng by direct inhibition of MMP-14 showed promising results[142]. As mentioned before, any therapeutic approach must be safe for mother and fetus and should be evaluated by controlled studies. Currently these problems still limit any effective therapy.

HELLP Syndrome

The HELLP syndrome is a complex of maternal symptoms in pregnancy, including hemolysis, elevated liver enzymes and low platelet count. HELLP syndrome occurs in 0.2%-0.8% of pregnancies and is a serious threat for mother and child. 70%-80% of women expressing HELLP symptoms also suffer from pre-eclampsia[143]. As in pre-eclampsia, a previous HELLP pregnancy increases the risk of HELLP as well as pre-eclampsia in subsequent pregnancies, suggesting related pathogenetics. Anti-angiogenic factors play an important role in both symptom complexes. In comparison to pre-eclampsia, maternal blood levels of anti-angiogenic sFlt1 are similar, but HELLP shows higher sol-Eng levels[144]. The pathogenesis of symptoms defining HELLP is driven by those angiopathogenic mechanisms. Activated vascular endothelium leads to an inflammatory response, including coagulation and complement activation, increased white blood count and elevated levels of inflammatory cytokines such as TNF-α and von Willebrand factor, leading to clinical symptoms of disseminated coagulation in microvessels[144,145]. Activation of these inflammatory signaling cascades leads to hemolysis in response to microangiopathy, reduced liver blood flow with elevated liver enzymes and low platelet counts due to consumption of platelets by microvessel thrombosis (= HELLP).

Cystic fibrosis associated liver disease

Cystic fibrosis (CF, mucoviscidosis) is an autosomal recessive genetic disorder affecting lungs, pancreas, liver and intestine. A mutation in the gene for the protein

cystic fibrosis transmembrane conductance regulator (CFTR) causes an abnormal transport of chloride and sodium across an epithelium, resulting in viscous secretions[146]. The most severe symptoms affect the lungs, often causing lung transplantation or death in those patients. Gastrointestinal symptoms due to thick mucus are common[147] and cystic fibrosis associated liver disease (CFLD) is often (30%) diagnosed, accounting for 2.5% of overall mortality, representing the third most common cause of death in these patients[148].

Rath *et al*[28] showed in a recent study that patients suffering of CFLD show elevated serum levels of TIMP-4 and endoglin. Expression levels correlate with hepatic staging, therefore allowing, together with transient elastography, to increase the sensitivity for the non-invasive diagnosis of CFLD in patients suffering from CF. High endoglin levels showed a significant association with the severity of liver injury, suggesting an active role for endoglin in the pathology of liver fibrosis.

Endoglin in liver fibrosis and HCC

Liver fibrosis and cirrhosis is the outcome of most types of chronic liver injury. The excessive accumulation of extracellular matrix (ECM) proteins promotes hepatic scarring and eventually leads to organ failure[149]. In the pathogenesis of liver fibrosis, TGF-β is the most potent fibrogenic cytokine. It induces fibrosis through multiple mechanisms, including direct activation of HSC, stimulation of ECM production, as well as prompting the synthesis of tissue inhibitors of matrixmetalloproteases (TIMPs) and thereby inhibiting ECM degradation[150]. Knock-out mice with deletions in components of the TGF-β signaling cascade (TGF-β1, SMAD3 and MMP13) develop less severe fibrosis[151]. TGF-β ligands and receptors form a complex signaling network, which can be modulated by endoglin and betaglycan (TGF-β type Ⅲ receptor). By inhibiting ALK5-Smad2/3 and promoting ALK1-Smad1/5 signaling, endoglin can shift TGF-β downstream signals to pro-fibrogenic effects[40]. Presently, there is not much knowledge how the expression of the different endoglin isoforms and sol-Eng is regulated in diverse liver cell subpopulations but it was reported that the concentration of sol-Eng increases during hepatic fibrogenesis (see below). In previous studies, we could show that endoglin expression is increased in activated HSC *in vitro* and in murine models of liver injury (carbon tetrachloride application and bile duct ligation) *in vivo*[41]. HSC are the major source for ECM production in liver fibrosis. Endoglin overexpression leads to enhanced TGF-β-driven Smad1/5 phosphorylation and α-smooth muscle actin expression without affecting Smad2/3 signaling in these cells. By shifting TGF-β signaling from ALK5-Smad2/3 to ALK1-Smad1/5 pathway, endoglin exceeds a central role in TGF-β signal modulation and the development of liver fibrosis.

HCC develops most often (80%) in cirrhotic livers. Angiogenesis and irregular capillary distribution are a key feature for malignant lesions[152]. Blood vessels are needed to supply nutrients and oxygen to the growing tumors. Most malignant tumors as well as HCCs have developed efficient strategies to promote fast vessel growth. Angiogenesis is a highly regulated, complex process modulated by many intersecting pathways, including vascular endothelial growth factor (VEGF), TGF-β and endoglin[26], angiopoietins[153], Notch[154] and integrins[155]. Usually, pro-angiogenic and anti-angiogenic factors are tightly balanced. In contrast to physiological angiogenesis (*i.e.,* in wound healing), tumor angiogenesis is not controlled by normal physiological inhibition, resulting in an imbalance of pro-angiogenic and anti-angiogenic factors. By modulating TGF-β signaling, endoglin plays a crucial role in angiogenesis and tumor growth and could be linked to HCC[108], as well as esophageal cancer[156], breast carcinoma[157], colorectal cancer[158] and tumor angiogenesis[44].

EXPRESSION OF ENDOGLIN IN ISOLATED LIVER CELLS AND LIVER TISSUE

Endoglin expression has been studied in many different tissues and diseases. It is highly expressed on proliferating vascular endothelial cells[159,160]. However, Meurer *et al*[40,41] showed that endoglin is expressed on HSC and activated MFB as well. By molecular cloning of endoglin cDNA, surface labeling, immunoprecipitation and immunocytochemistry experiments, it could be shown that endoglin plays a significant role in liver injury and fibrosis development[40,41]. Endoglin expression is differentially regulated at the plasma membrane of HSCs and in activated myofibroblasts (MFB)[40,41]. Endoglin expression is increased in transdifferentiating HSC and in two models of liver fibrosis but not in hepatocytes. Furthermore, endoglin is expressed in cultured portal fibroblasts, representing another important fibrogenic cell type in biliary types of liver disease. Transient overexpression of endoglin leads to significantly increased TGF-β1-driven Smad1/5 phosphorylation and α-smooth muscle actin expression, while Smad2 phosphorylation is not changed[40]. These results are in line with a study by Lebrin *et al*[88] which showed endoglin promoting TGF-β1/ALK1-Smad1/5 signaling in endothelial cells.

To further investigate the influence of endoglin on TGF-β signal transduction, we recently established and characterized a new mouse HSC line expressing collagen 1(I) promoter/enhancer driven green fluorescent protein (GFP). These cells, originating from quiescent HSC, show an activated MFB phenotype in culture and express low endogenous endoglin concentrations. By selective overexpression of endoglin in these cells, stimulation with TGF-β and PDGF, and specific inhibition of endoglin/ALK signaling with antagonists, the differential effect of endoglin on downstream Smad-signaling could be shown[77].

Because of the complexity of endoglin and TGF-β signaling pathways, it is important to investigate the modulation of TGF-β signal transduction in cells of different origin. For example, Velasco *et al*[87] showed the differ-

ential effects of endoglin isoforms in L6E9 myoblasts[85]. Because these cells have no endogenous endoglin expression, this cell line is an ideal tool to selectively express specific isoforms of endoglin and show a different and sometimes opposing effect of L- and S-Eng isoforms on downstream regulation of TGF-β-induced responses. While endoglin expression is well investigated in vascular endothelial cells, HHT and tumor angiogenesis, the role of endoglin in liver disease is poorly understood. Liver cell lines overexpressing endoglin or single members of the TGF-β pathway, as well as cells with low endogenous endoglin expression and specifically induced endoglin expression are needed to further dissect the functional roles of endoglin in liver injury and fibrosis.

ANIMAL MODELS IN UNDERSTANDING ENDOGLIN FUNCTION

Endoglin deficiency in humans has a strong phenotype and is responsible for many diseases, such as HHT, preeclampsia liver fibrosis and cancer. To study its impact on the pathogenesis of those diseases, murine endoglin knockout models were needed. Because a complete homozygous endoglin knockout is embryonically lethal, several alternative strategies were established. Endoglin plays an important role in angiogenesis; a complete endoglin deficiency has fatal consequences in the development of heart and major vessels. To study the role of endoglin *in vivo* and its impact on HHT-1, Arthur *et al*[161] established a mouse carrying a targeted nonsense mutation (deletion of exons 9-11) in the endoglin gene. These mice already showed that endoglin expression is critical for early vascular development. Embryos with two mutated endoglin genes die at day 10 - 10.5 post coitum (dpc) due to cardiac malformations and a failure to form mature blood vessels in the yolk sac. Homozygous endoglin knockout embryos generated by a deletion of 609 bp including exon 1 show a similar phenotype as mice lacking TGF-β1 and the TGF-β receptor Ⅱ, suggesting that endoglin plays a crucial role in TGF-β signaling in early vascular development[162,163]. Li and co-workers reported that mice lacking functionally active endoglin by replacing the first two exons die from defective vascular development but do not show defective vasculogenesis, which is observed in mice lacking TGF-β1[163]. Loss of endoglin caused poor vascular smooth muscle cell (vSMC) development and arrested endothelial remodelling. Therefore, endoglin is required for the differential growth and sprouting of endothelial tubes and recruitment and differentiation of mesenchymal cells into vSMC and pericytes[164]. Both studies show slight differences in vascular embryonic development. Eng deficient mice generated by Li *et al*[163] die at day 11.5 dpc. While Arthur *et al*[161] used embryonic stem cells of 129/Ola origin, Li *et al*[163] generated endoglin knockout mice by targeting embryonic stem cells from 129/SVJ background. Those different approaches already suggest a strong impact of genetic background on murine models of Eng deficiency.

To overcome the problems of embryonic lethality and to study the effect of endoglin in disease, several groups have used alternative approaches to generate endoglin deficient mice. Allinson *et al*[164] for example generated a mouse in which the endoglin gene is flanked by loxP sites at exons 5 and 6. These mice show a normal phenotype comparable to wild type littermates. Using the *Cre*-loxP genetic recombination system and an appropriate *Cre* expressing mouse line, specific endoglin knockout mice can be created. To generate a null allele of the endoglin gene, the floxed construct was designed to allow a conditional deletion of exons 5 and 6, which would also lead to frameshift mutation in exon 7 before reaching a stop codon, resulting in a functional inactive endoglin[164].

Using this approach, two mouse models were generated expressing Cre in smooth muscle (SM22αcre) and endothelial cells (Tie2cre) to evaluate the role of endoglin in vascular smooth muscle and endothelial cells during angiogenesis[165]. In this study, endoglin null embryos show ectopic arterial expression of the venous specific marker COUPTF Ⅱ (chicken ovalbumin upstream promoter transcription factor Ⅱ). Normal expression of COUPTF Ⅱ was restored after endoglin re-expression in endothelial cells. COUPTF Ⅱ plays an important role in vascular development, including heart, blood vessels and smooth muscle cell differentiation. Endoglin induces changes in COUPTF Ⅱ expression patterns and therefore can influence vSMC recruitment and differentiation in angiogenesis.

Other groups used heterozygous endoglin knockout mice to investigate the function of endoglin and avoid embryonic lethality. Bourdeau *et al*[166] developed a mouse model with a single copy of the endoglin gene and another mouse line with a homozygous deletion of the endoglin gene. As already observed by Arthur *et al*[161], mice lacking any functional endoglin die at day 10.0-10.5 dpc due to defects in vessel and heart development. Embryos show a normal angiogenesis and vessel formation until hemorrhage occurs in the yolk sac around 9.0-10.5 dpc. Heart development stopped at day 9.0 and the atrioventricular canal endocardium did not undergo mesenchymal transformation and cushion-tissue formation. Similar to the study published by Arthur *et al*[161], Bourdeau *et al*[166] used 129/Ola origin on C57BL/6 background. The heterozygous mouse displays a multiorgan vascular phenotype similar to the human HHT, which is often caused by endoglin haploinsufficiency. To evaluate the impact of the genetic background on endoglin deficiency, different Eng/null mouse strains were generated. The 129/Ola strain developed HHT symptoms at an earlier age and with greater severity than C57BL/6 mice. The F2 strain intercrosses between both strains showed an intermediate phenotype. As in humans, Eng deficiency shows variable penetrance. Of 171 mice observed in this study over a 12 mo period, 50 developed clinical signs of HHT. Disease prevalence was high in the 129/Ola strain (72%), intermediate in the intercrosses (36%), and low in C57BL/6 backcrosses (7%)[166].

Using the heterozygous Eng null mouse generated by Bordeau *et al*[166], another study showed that endoglin is

required for paracrine TGF-β signaling between endothelial cells and adjacent smooth muscle cells to promote smooth muscle cell differentiation[167].

In primary cultures of endothelial cells generated from mice carrying only one functional Eng allele, a significantly reduced migration and proliferation along with increased collagen production, vascular endothelial growth factor (VEGF) secretion and decreased NO synthase expression was observed[168]. This again highlights the important role of endoglin in vascular pathology.

As outlined above, endoglin modulates both the ALK1 and ALK5 pathways. Park *et al*[169] generated an ALK1 conditional knockout mouse line. The specific deletion of ALK1 in vascular endothelial cells by an endothelial specific Cre was lethal through massive hemorrhage in the lungs. ALK1 deficient mice showed heavy pulmonary vascular malformations mimicking all pathological features of HHT-2, such as dilation of vessel lumen, thinning of vascular walls, loss of capillaries, development of excessive tortuous vessels, and AVM[169].

Dolinsec *et al*[170] used another approach to investigate endoglin deficiency in murine models without affecting embryonical vascular development[170]. By applying siRNA against endoglin to human and murine endothelial cells (HMEC-1, 2H11) *in vitro* and in TS/A mammary adenocarcinoma growing in BALB/c mice, they evaluated the therapeutic potential of siRNA in cancer treatment. *In vitro*, the transfection resulted in reduced levels of endoglin mRNA and protein, leading to a 60% decrease of endothelial cell proliferation. *In vivo* silencing of endoglin expression showed lower endoglin mRNA levels and a decreased number of tumor blood vessels resulting in significantly reduced TS/A tumor growth. The study demonstrated that siRNA molecules against endoglin have a good anti-angiogenic therapeutic potential[171].

The endoglin gene gives rise to two different isoforms resulting from differential splicing, *i.e.,* S- and L-Eng (for details see above). Pérez-Gómez *et al*[43] investigated the role of S-Eng *in vivo* using a mouse with ICAM-2 driven overexpression of human S-Eng on the vascular endothelium. Interestingly, breeding these mice to endoglin deficient mice did not rescue the embryogenic lethal phenotype. Furthermore, this study investigates the impact of S-Eng on carcinogenesis. Therefore, Lewis lung carcinoma cells were transplanted into mice expressing S-Eng. Carcinoma cells in these mice showed reduced tumor growth and less neovascularization. Additionally, benign papilloma formation was reduced significantly in respective S-Eng positive mice. These results show that S-Eng has anti-angiogenic properties in cancer development, showing new potential approaches for tumor therapy[43].

DIAGNOSTIC VALUE OF ENDOGLIN IN LIVER-ASSOCIATED DISEASES

Genetic testing

HHT is phenotypically heterogeneous both between affected families and amongst members of the same family in regard to penetrance and age of disease onset. There are hundreds of different mutations in the human *ENG* gene known that affect proper gene function. Although HHT is most common in Caucasians, disease causing mutations with ethnic-related differences also occur in Asians, Africans and Middle Eastern[171]. The overall incidence of HHT in North America is more frequent than initially estimated and ranges between 1:5000 and 1:10000[172], while the frequency in Europe varies between 1:2500 to 1:40000[173-175]. In a cohort of the northern part of Japan, the prevalence of HHT in the population was estimated to be 1:8000[176], demonstrating that HHT is more common among Asians than often assumed.

HHT is a dominantly inherited autosomal disorder and genetic testing of individuals with a known family history is generally performed for disease confirmation (Figure 7). In addition, pre-symptomatic screening of relatives of patients with a positive molecular diagnosis and in patients with suggestive (but not confirmatory) clinical features of HHT is well established[177].

At the molecular level, there is a large spectrum of different gene mutations that influence the expression, integrity and stability of the endoglin protein. Missense (nonsynonymous) mutations introducing different aa, nonsense mutations introducing premature stop codons, splice-site mutations that affect consensus splice donor sites and provoke exon skipping, frame shift and in frame deletions resulting in proteins with markedly different sizes, and several intronic mutations are rather common and show an ethnic and regional distribution[7-10,178-180]. However, the penetrance of the different mutations and gene variations are rather different and subtle genotype-phenotype correlations in HHT-1 have been reported, revealing that truncating mutations in *ENG* are associated with more affected organs and more severe hemorrhage than *ENG* missense mutations[13]. Pulse-chase experimentation and overexpression studies have further shown that several endoglin gene mutations form proteins that are only barely detectable, do not form heterodimers with normal endoglin, and are further unable to interfere with endoglin trafficking to the cell surface and remain intracellular as a precursor form[12,181]. On the contrary, another study that investigated six different missense and two truncation mutations have shown that not all mutants are unable to dimerize with normal endoglin, suggesting that haploinsufficiency and dominant-negative protein interactions both can cause HHT-1[12,182]. No homozygotes that carry two abnormal copies of the *ENG* gene have been reported so far, suggesting that this constellation is not compatible with life[183]. Likewise, mice lacking both copies of the *ENG* gene die at gestational day 10.0-10.5 due to defects in vessel and heart development[161].

However, there are four other genetic types of HHT identified that are not associated with alterations in the *ENG* gene. It is essential to know that there are likely to be differences in the normal requirements for the individual disease-causing genes in different vascular beds and cell types that, when affected by mutation, result in somewhat diverse clinical features and symptoms[183]. The onset of epistaxis for example was found to have

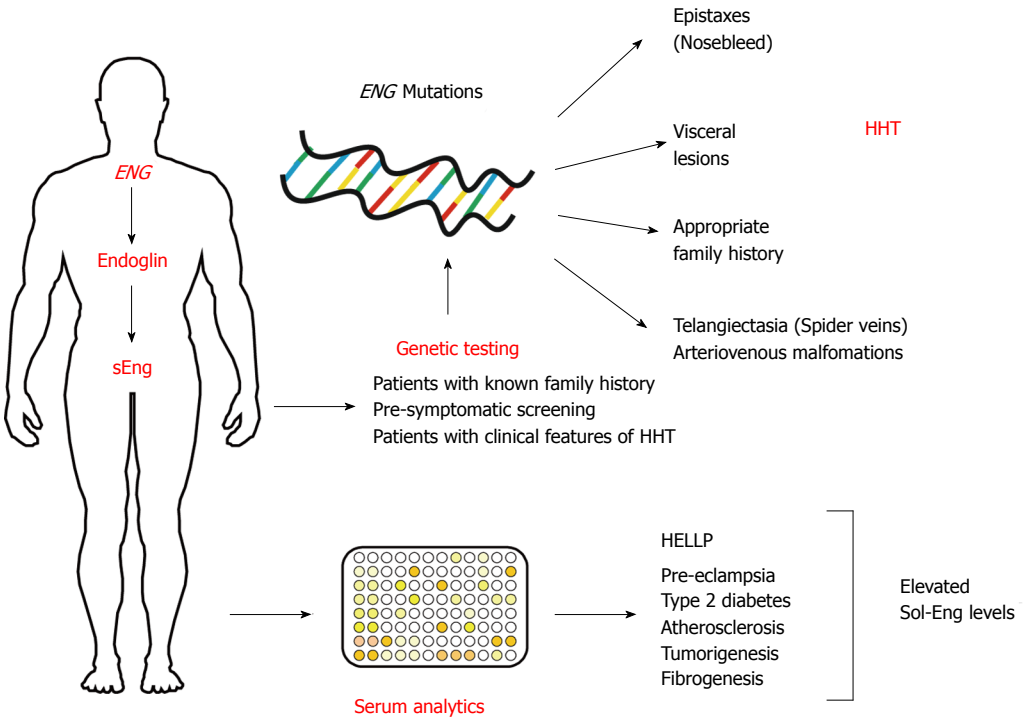

Figure 7 Endoglin in diagnostics. Several distinct mutations in the endoglin gene (ENG) give rise to hereditary hemorrhagic telangiectasia (HHT) that is mainly characterized by epistaxes (nosebleed), various visceral lesions, telangiectasia (spider veins) and arteriovenous malformations. Patients often show an appropriate family history. The clinical diagnosis "HHT" is made if three of the four classical signs (*i.e.*, epistaxes, visceral lesions, telangiectasia and family history) occur. Elevated levels of soluble endoglin have been reported in patients suffering from hemolysis, elevated liver enzymes and low platelets syndrome (HELLP), pre-eclampsia, type 2 diabetes, atherosclerosis, tumorgenesis in several organs, and fibrogenesis.

an earlier onset in patients with HHT-1 than those with HHT-2 and AVM of the brain and lungs were more common in respective patients, while hepatic and spinal AVM were noticed at a lower frequency in patients with HHT-2[13,178,184,185]. Based on all these findings, several guidelines were proposed in which the *ENG* gene should be first targeted for mutational screening when large visceral AVM in the lungs in patients younger than 45 years occur[185]. However, based on the fact that all 15 exons and their non-coding introns can be easily sequenced, it is self-evident that these molecular diagnostic tests have refined and supplemented the criteria that were first proposed for clinical diagnosis of HHT[114].

Serum measurements

Based on the finding that the serum or plasma concentration of sol-Eng is increased dramatically in several disease conditions, its predictive value for the outcome of various diseases is presently intensively discussed and a large variety of commercially available ELISA test systems that allow reliable and accurate detection of endoglin in biological fluids have been established by many companies. It was shown that serum sol-Eng that plays a major role as an anti-angiogenic factor increases two- and three-fold in preterm and term pregnancy compared to non-pregnant controls and further dramatically increases two to three months before the onset of pre-eclampsia and in patients with HELLP syndrome, suggesting that sol-Eng alone or in combination with other variables is usable as a biomarker with a high predictive value in pregnancy complications[14,106,186,187]. Other studies demonstrated that plasma sol-Eng levels are significant higher in patients with diabetes than in healthy control subjects and that the duration of diabetes is an independent predictor of plasma sol-Eng increase[17]. The measurement of sol-Eng also has predictive value for the progression of the atherosclerotic process and correlates well with the expression of eNOS in endothelium, repair of the vessel wall, plaque neoangiogenesis, production of collagen and stabilization of atherosclerotic lesions[19]. As an indicator of endothelial dysfunction, the measurement of sol-Eng was proposed to monitor the therapy efficacy during extracorporeal LDL-cholesterol elimination therapy for familial hypercholesterolemia[18]. Since endoglin expression was shown to be extremely relevant for cancer formation[159], it is not surprising that sol-Eng is a potential angiogenic marker to indicate and predict diseases associated with metastases[32,188-190]. Patients suffering from Alzheimer's disease were also found to have elevated levels of sol-Eng combined with decreased levels of TGF-β, possibly indicating impairment of cerebral circulation that is associated with this neurodegenerative process[24]. Of course, the wide expression pattern of endoglin that encompasses endothelial cells, subsets of bone marrow cells, activated macrophages, fibroblasts, chondrocytes, smooth muscle cells and pro-fibrogenic cells (*e.g.*, HSC) as well as its linkage with the TGF-β signaling pathways has further offered several new avenues in which sol-Eng

measurements might be beneficial. In regards to liver, it is well established that intrahepatic and circulating levels of endoglin are elevated in patients suffering from chronic hepatitis C infection, liver cirrhosis and carcinoma. In addition, there is a correlation of histological and serum markers of hepatic fibrosis and endoglin is abundantly expressed in hepatic sinusoidal endothelium of non-tumor tissues with cirrhosis[108,191,192]. Increased endoglin expression was recently also documented by proteomic profiling in patients suffering from cystic fibrosis associated liver disease[28]. Likewise, high circulating endoglin concentrations are correlated with a poor outcome for biliary atresia that represents a chronic progressive disorder of the extrahepatic and intrahepatic biliary system[27]. Therefore, there is no doubt that these measurements enrich the panel of available diagnostic options to identify proliferative disorders, including organ diseases that are associated with fibrogenesis.

CONCLUSION

Endoglin is found on many cell surfaces and plays a crucial role in TGF-β signaling. It forms homodimers and consists of a large extracellular domain, a hydrophobic transmembrane domain and a short cytoplasmic tail. This receptor binds to a large variety of extra- and intracellular binding partners and modulates numerous cellular properties, including morphology, migration, endocytic vesicular transport, microtubular structures and functionality of focal adhesion proteins. Several hundred independent *ENG* gene mutations result in HHT that is associated with various vascular lesions, mainly on the face, lips, hands and gastrointestinal mucosa. Recent work has demonstrated that endoglin expression is also altered during ongoing hepatic fibrogenesis. The unravelling of the underlying pathways that are associated with alterations in endoglin expression will be of fundamental interest, not only for establishment of potential new therapeutic options for HHT treatment, but might allow re-establishing the activities of Smad2/3 and Smad1/5/8 that are both part of TGF-β homeostasis and pathologically altered in ongoing and established organ fibrosis.

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REVIEW

Ceruloplasmin-ferroportin system of iron traffic in vertebrates

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Core tip: The ceruloplasmin-ferroportin system represents the main pathway for cellular iron egress in vertebrates and it is responsible for physiological regulation of cellular iron levels. This review focuses on the structural and functional features of the two proteins, with special emphasis on their coordinate regulation at the transcriptional and post-transcriptional levels.

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Abstract

Safe trafficking of iron across the cell membrane is a delicate process that requires specific protein carriers. While many proteins involved in iron uptake by cells are known, only one cellular iron export protein has been identified in mammals: ferroportin (SLC40A1). Ceruloplasmin is a multicopper enzyme endowed with ferroxidase activity that is found as a soluble isoform in plasma or as a membrane-associated isoform in specific cell types. According to the currently accepted view, ferrous iron transported out of the cell by ferroportin would be safely oxidized by ceruloplasmin to facilitate loading on transferrin. Therefore, the ceruloplasmin-ferroportin system represents the main pathway for cellular iron egress and it is responsible for physiological regulation of cellular iron levels. The most recent findings regarding the structural and functional features of ceruloplasmin and ferroportin and their relationship will be described in this review.

INTRODUCTION

The importance of iron for all eukaryotes, and particularly for humans, is well established. Iron is fundamental for the transport, storage and activation of oxygen, for electron transport and for many other important metabolic processes. It is therefore not surprising that any genetic defect leading to iron imbalance can have severe consequences on our health. The loss of regulation of iron metabolism can lead to development of iron overload as seen in hereditary hemochromatosis, a common inherited disorder which may lead to progressive organ dysfunction. Conversely, iron deficiency is typical of many pathological states, such as the anemia of chronic disease or anemia associated with inflammation. In the last fifteen years, several new genes and proteins involved in iron disorders in animal models and in humans have been identified, which has greatly improved our understanding of the molecular mechanisms of iron absorption, the regulation of iron transport and general iron homeostasis in mammals[1-3].

Table 1 List of the most relevant papers

Topic	Ref.
Fpn identification and structure	[4,6,8,9,12,14,16]
Cp structure and function	[29,34,35,39,43,45,46,49,50,52,82]
Cp/Fpn connection	[40,42,52,53]
Transcriptional regulation of Cp/Fpn	[56-58,61,62,64,68,71]
Post-transcriptional regulation of Fpn	[21,24,73]
Aceruloplasminemia	[75-78,81,85]
Fpn disease	[10,88,89,91,92,94]

Cp: Ceruloplasmin; Fpn: Ferroportin.

Serum transferrin and the almost ubiquitously expressed transferrin receptor-1 (TfR1) represent the most important system for distribution and delivery of iron to the different organs of the body. Iron delivery to the bloodstream for transferrin-dependent transport is mediated by enterocytes, which release iron absorbed from the diet, and mostly by macrophages, which recycle iron from damaged and senescent erythrocytes. These specialized cells export iron through the recently identified protein ferroportin (SLC40A1, initially also named Ireg-1 or MTP-1), the only known mammalian iron exporter[4-6]. A group of enzymes that convert Fe^{2+} to Fe^{3+} collaborates with ferroportin, facilitating iron loading onto transferrin, which binds only Fe^{3+}. These enzymes belong to the family of the blue multicopper oxidases and possess ferroxidase activity; members of this family include ceruloplasmin, hephaestin and zyklopen in mammals.

In this review the most recent findings regarding the structural and functional features of ceruloplasmin and ferroportin and their relationships will be described. A list of the most relevant papers in the field is presented in Table 1.

FERROPORTIN, STRUCTURE AND FUNCTION

Human ferroportin (Fpn) is constituted by 571 amino acids, the corresponding SLC40A1 gene is located on chromosome 2 (2q32), it spans about 20 kb and has 8 exons. Fpn has been identified in many organisms and its amino acid sequences can be easily retrieved from annotated genome projects. The protein is well conserved, with over 60% identity between distantly related proteins such as human and zebrafish Fpn, indicating a wide distribution and a critical role for Fpn. This assumption is supported by the finding that inactivation of the Fpn gene in mice is embryonically lethal[7].

Fpn is a polytopic membrane protein with a predicted 9-12 transmembrane topology. A model proposed by Liu *et al*[8] suggested that Fpn has 12 transmembrane domains. A number of studies have indicated that the N-terminus of Fpn is cytosolic[8-11]. On the other hand, the location of the C-terminus is unclear, with studies based on epitope-tagged proteins supporting the hypothesis of a cytosolic localization[8,12] and other studies claiming that the C-terminus is extracellular. In particular, Yeh *et al*[13] suggested that the presence of the epitope might affect the topology of Fpn. It should be noted, however, that epitope-tagged Fpn is fully functional with respect to transport activity and regulation.

Putative structure of human ferroportin

Most questions regarding the structure and mechanism of action of Fpn could be answered by an experimentally determined three-dimensional structure of the protein. Unfortunately, such a structure will probably not be available in the near future due to the difficulties of obtaining crystals of membrane proteins. Therefore, functional studies of Fpn mostly rely on theoretical modeling to provide a framework for analysis of Fpn wild type and mutants.

Recently, two molecular models of human Fpn based on different approaches have been reported[9,14]. Both models predict that Fpn belongs to the major facilitator superfamily (MFS) of membrane transporters. Wallace and coworkers based their model on the topology proposed by Liu *et al*[8], and confirmed the intracellular localization of both N- and C-termini. They used the structure of the glycerol-3-phosphate transporter from *E. coli* as template for building a three-dimensional model of Fpn. Using the model, they showed that all reported loss-of-function Fpn mutations localize at the membrane/cytoplasm interface, while gain-of-function mutations are largely associated with the inner channel running down the axis of Fpn (see below for details on Fpn mutations and "ferroportin disease"). They concluded that the phenotypic variability of "ferroportin disease" likely arises from the different functional consequences of the various mutations.

On the other hand, using sensitive profile-profile alignment methods, Le Gac *et al*[14] provided an alignment of Fpn with MFS proteins. Along with the crystal structure of the *E. coli* EmrD antiporter, this alignment served as a basis for the homology modeling of the three-dimensional structure of Fpn. The authors focused their attention on key functional amino acids and disease-causing mutations, and showed that their model of Fpn could be used to identify critical amino acids. In particular, they proved the involvement of a specific tryptophan residue in both the iron export function and the mechanism of inhibition by hepcidin.

Neither model gives any clue about the localization of iron binding site(s) inside Fpn. We are currently building a different structural model of human Fpn using two MFS *E. coli* proteins (manuscript in preparation). A preliminary analysis shows that the model allows to postulate the presence of a potential iron binding site in the central cavity of the protein, whose relevance can be tested through measurement of the iron export ability of wild type and mutated Fpn. A depiction of our preliminary Fpn model and of the iron binding site is shown in Figure 1.

Oligomeric state of ferroportin

The multimeric structure of Fpn is still the subject of

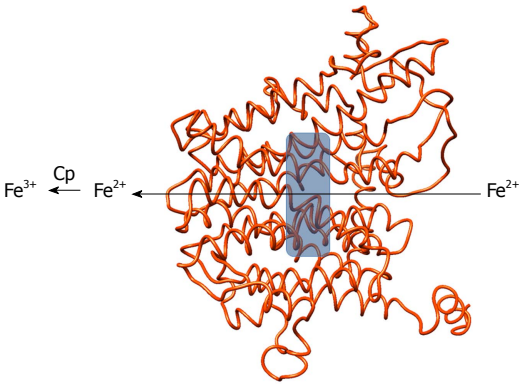

Figure 1 Structural model of human ferroportin viewed along the membrane plane. The gray box indicates the location of a putative iron-binding site, ferrous iron flows through the protein from the cell interior and is then oxidized by ceruloplasmin at the extracellular side. The figure was produced with Chimera[96].

much debate, with reports demonstrating that the protein is dimeric[10,12,15] while other studies have suggested that it is a monomer[11,16-19]. Most of the studies addressing the oligomeric state of Fpn have relied on the use of recombinant Fpn tagged with different epitopes. The techniques employed are mainly (but not only) co-immunoprecipitation, gel-filtration chromatography and cross-linking. Evaluation of the effect of co-transfection of wild type and mutant Fpn on iron export function and subcellular localization has also been taken into consideration[10,15,16,18,19]. Conflicting results on the multimeric structure of Fpn obtained by the methods outlined above can have many explanations: the efficiency of co-immunoprecipitation can depend on the tags (and antibodies) or the experimental conditions imposed on the cell lysates. For instance, different groups have reported that it is possible to co-immunoprecipitate Fpn-GFP and Fpn-flag while co-precipitation of Fpn-flag and Fpn-myc was less reproducible. Also, high expression levels of recombinant Fpn could be in part responsible for reported discrepancies. Some negative results obtained with different cross-linkers might be explained by the chemical features of the reagent (*i.e.*, group reactivity and spacer arm length), which can be suboptimal. Similarly, negative results obtained by fusion of Fpn to fluorescent/luminescent protein tags to exploit FRET or BRET do not necessarily imply the lack of Fpn dimers because these techniques are highly dependent on close spatial proximity of the probes. The most convincing evidence that Fpn is dimeric comes from cross-linking of endogenous Fpn in rat glioma C6 cells and bone marrow-derived macrophages, which resulted in doubling of the molecular mass of the protein[12]. This experimental set-up circumvents the possibility of artifacts due to the presence of the tags and/or overexpression of Fpn. In any case, the strength of the interaction between monomers appears to be quite low because differently tagged Fpn expressed separately and mixed after detergent-extraction from the lipid bilayer do not co-immunoprecipitate[10,12]. Multimerization of Fpn is particularly attractive to explain the dominant inheritance of "ferroportin disease" (see below).

Ferroportin and hepcidin

Fpn is the receptor for hepcidin, a peptide of 25 amino acids forming a bent β-hairpin stabilized by four disulfide bonds. Inflammatory states and/or increased iron stores trigger the hepatic synthesis of the peptide[20]. Binding of hepcidin to Fpn leads to the internalization and degradation of Fpn, resulting in impaired iron export[21].

Conflicting reports have been published on the molecular mechanism of hepcidin-induced Fpn degradation. In particular, there is no agreement on the possible phosphorylation by JAK2 kinase of two tyrosine residues on Fpn in hepcidin-triggered internalization of the protein[22,23]. On the other hand, Fpn is certainly ubiquitinated on lysine residues before degradation[23,24]. The hepcidin binding site has been identified on the extracellular loop of Fpn containing cysteine in position 326[25]. Cells expressing the C326S mutant Fpn export iron normally but do not bind the peptide and export iron even in the presence of hepcidin[26]. Modeling of the hepcidin-Fpn interaction suggested that Cys326 is involved in a thiol-dependent interaction with hepcidin, perhaps involving the disulfide framework of hepcidin, while Phe324 and Tyr333 may form crucial contacts with two phenylalanine residues on the hepcidin moiety[27].

CERULOPLASMIN, STRUCTURE AND FUNCTION

Structure of ceruloplasmin and of its copper binding sites

Ceruloplasmin (Cp) is an enzyme, ubiquitous among vertebrates, that belongs to the family of the multicopper oxidases. Members of this family posse multiple copper sites that can be classified, on the basis of their spectroscopic properties, in type 1, type 2 and type 3 sites[28]. Human Cp is constituted by 1046 amino acids; the *Cp* gene maps on chromosome 3 (3q23-q24), it spans about 65 kb and it is organized in 20 exons. Determination of the three-dimensional structure of Cp[29,30] has shown that this enzyme is made up of six domains arranged in a ternary symmetry. Domains 1 and 2, 3 and 4, and 5 and 6 interact with each other through extensive, highly packed hydrophobic interfaces, while polar interactions and loosely packed interfaces are observed between domains 2 and 3 and 4 and 5. Three of the six domains (domains 2, 4 and 6) bind a type 1 blue copper coordinated by nitrogen and sulphur ligands, supplied by histidine and cysteine residues arranged in tetrahedral geometry with an axial methionine ligand, which is absent in the type 1 site of domain 2.

Three more copper ions are coordinated by eight histidine ligands at the interface between domain 1 and 6. The latter copper ions represent the trinuclear cluster formed by two antiferromagnetically coupled type 3 and one type 2 copper ions. The oxidation of substrates is coupled to the reduction of oxygen to water in a mecha-

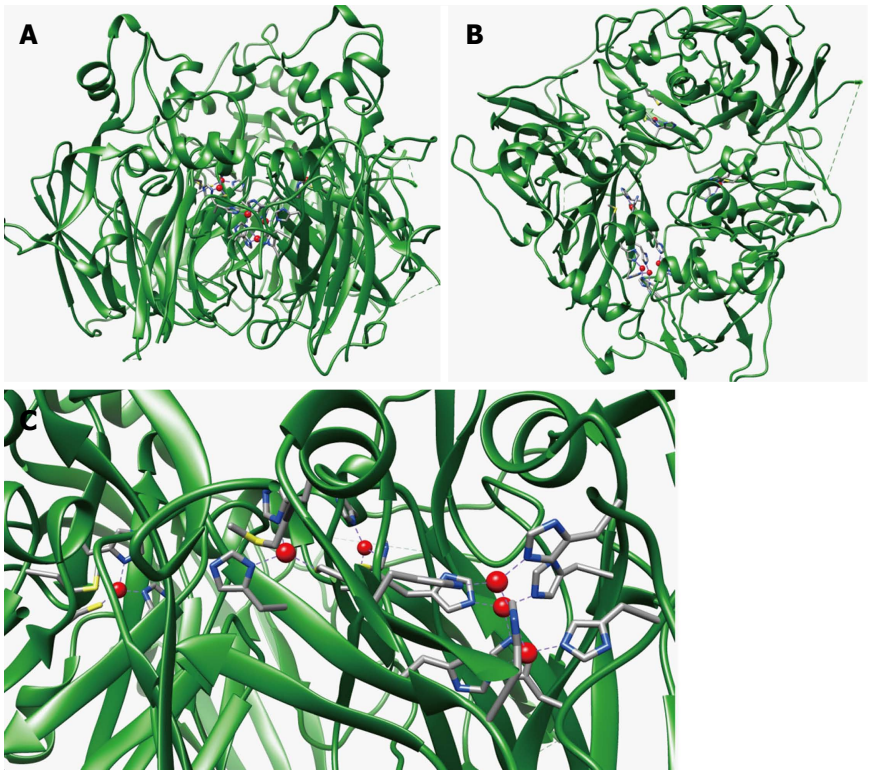

Figure 2 Structure of human ceruloplasmin. Overall structure of the protein (PDB 1KCW) in two orientations (A: Side view and B: Bottom view), the copper atoms are shown as red spheres, the side chains of the copper ligands are represented as sticks; C: Close-up view of the type 1 and trinuclear cluster catalytic copper binding sites. The figure was produced with Chimera[96].

nism involving electron transfer from the type 1 copper sites, the primary sites of substrate oxidation, to the trinuclear cluster, where oxygen binds and is reduced in a controlled way, *i.e.,* without release of potentially toxic intermediates (O_2^-, H_2O_2). While electron entrance at type 1 copper sites in domains 4 and 6 is established, the role of the blue copper ion in domain 2 is less clear. In fact, there is no experimental evidence from crystallographic data that reducing substrates can bind in domain 2. Moreover, site-directed mutagenesis at this copper site failed to modify either the spectroscopic or catalytic properties of the protein[31]. Thus, the blue copper ion in domain 2 could be an "evolutionary relic" or, alternatively, it could serve for still unknown other functions. Figure 2 reports the structure of human Cp and the localization of its copper sites.

Beside copper, other metals have been proposed to bind to Cp. In particular, refined crystallographic data showed an extra metal-binding site in domain 1, likely filled with a calcium ion. The finding of a calcium binding site is consistent with a previous study from our laboratory showing that human and sheep Cp bind divalent ions, and that this could be exploited in a one-step purification protocol based on the affinity of the protein for calcium ions[32].

Physiological role of ceruloplasmin

Cp is mainly synthesized by hepatocytes, where the P-type ATPase ATP7B incorporates copper into apo-Cp during transit through the trans-Golgi network[33], and secreted into the plasma where it is found at micromolar concentration. The molecular mechanism of copper loading of Cp by ATP7B is still unknown. Inspection of the structure of Cp shows that large solvent exposed loops connect the six domains of Cp. Despite a low degree of sequence homology, all these loops start with a C-X-R/K motif, with the cysteine residue stabilizing the loop by forming a disulfide bridge. Our recent work indicates that the basic residues of the five loops connecting the six domains of Cp, and the disulfide bridges that stabilize the loops, are required for proper copper loading by ATP7B[34].

A GPI-anchored form of Cp was initially identified on the plasma membrane of astrocytes[35] and leptomeningeal cells[36] in the CNS, in Sertoli cells[37] and in the retina[38]. Synthesis of this isoform is *via* alternative splicing of exons 19 and 20 where the last 5 amino acids are replaced by 30 alternative residues leading to addition of the GPI anchor[39]. More recently Cp-GPI has been detected also in macrophages[40], immune cells and hepatocytes[41] and in many other tissues[42], indicating a wider than anticipated distribution of this isoform.

Despite the knowledge of the details of the three-dimensional structure, the true biological function of Cp has been the subject of much debate mainly because Cp is a rather promiscuous enzyme, as regards the multitude of substrates it can act on and the possibility that copper bound to sites other than the active site can give rise to accessory activities. In fact, several functions have been attributed to Cp, ranging from copper transport to ferrous iron and biological amines oxidation, as well as antioxidant activity *via* prevention of the formation of free radicals in serum[43]. Conversely, pro-oxidant activity leading to LDL oxidation has also been attributed to Cp due to the presence of a seventh copper atom that is bound to a site unrelated to the active site[44]. However, among

various substrates, the enzyme displays the highest affinity for ferrous ions and a role for Cp in iron metabolism had been proposed as early as in 1966[45]. The study of the ferroxidase activity of Cp evidenced two K_m values which differ by approximately two orders of magnitude (K_{m1} 0.6 μmol/L and K_{m2} 50 μmol/L) and binding of Fe^{2+} in the vicinity of type 1 copper sites has been demonstrated by X-ray diffraction studies, soaking crystals of Cp with Fe^{2+}[46]. Cp is thought to promote iron release from cells, facilitating loading of the metal onto transferrin, which only binds Fe^{3+}. An important point regarding the ferroxidase activity of Cp is that Fe^{2+} readily oxidizes, at physiological pH, even in the absence of a protein catalyst. However, spontaneous oxidation of Fe^{2+} is potentially dangerous as it triggers the formation of oxygen radicals *via* Fenton chemistry. Thus ferroxidation by Cp would prevent iron-induced oxidative stress.

An increasing body of evidence supports earlier work[47,48] and points to an essential role for Cp in iron metabolism (and specifically in iron efflux from cells) *via* its ferroxidase activity. Stimulation of iron release from macrophages by Cp in the presence of apotransferrin and hypoxia has been demonstrated[49]. Targeted Cp gene disruption in mouse evidenced a striking impairment in the movement of iron out of reticuloendothelial cells and hepatocytes[50]. Moreover, increased deposition of iron in several regions of the CNS was noted in $Cp^{-/-}$ mice[51], and Cp-GPI was found to be required for iron efflux from astrocytes[52]. In addition, individuals carrying a defective gene coding for Cp, thus suffering from aceruloplasminemia, show normal copper homeostasis but present a severely impaired iron metabolism.

CERULOPLASMIN-FERROPORTIN

Ceruloplasmin is essential for ferroportin stability

The essential role of the ferroxidase activity of Cp in iron release from cells was attributed to facilitation of loading of the metal onto transferrin, which only binds Fe^{3+}. However, a new molecular connection between Cp and Fpn has been established by the finding that ferroxidase activity is required to stabilize Fpn at the cell surface in cells expressing Cp-GPI[40]. Thus, Cp can be considered as a second determinant of Fpn stability after hepcidin (Figure 3). As described in detail below, ferroxidase active Cp stabilizes Fpn at the plasma membrane supporting iron export (Figure 3A); on the other hand, absence of Cp or presence of an inactive Cp lead to degradation of Fpn in specific cell types (Figures 3B, C); hepcidin induces internalization and degradation of Fpn also if Cp is present (Figure 3D), unless hepcidin levels are very low. It is worth noting that removal of Fpn from the plasma membrane appears to be the only means to 'turn off' iron export from the cell because no inhibitor of Fpn is known.

The starting point was the observation that loss of Cp-GPI either by gene silencing or by incubation of rat C6 glioma cells and bone marrow macrophages with the copper chelator BCS led to disappearance of Fpn from the cell surface. Fpn was rapidly internalized and degraded in the absence of Cp-GPI. Addition of exogenous Cp or of the yeast ferroxidase Fet3p or of an iron chelator such as BPS or DFO, restored Fpn at the cell surface in cells silenced for Cp-GPI. The activity of the ferroxidase or the presence of the iron chelator were essential to lower the concentration of extracellular Fe^{2+} establishing an iron gradient and promoting removal of the metal from Fpn. In the absence of Cp-GPI, radioactive ^{59}Fe remained associated with Fpn and the protein was found to be ubiquitinated on Lys253. It can be hypothesized that a conformational state of Fpn with bound iron is recognized by a specific ubiquitin ligase, triggering degradation of the transporter. The requirement for a ferroxidase to maintain iron transport appears specific to cells that express Cp-GPI, because transfected Fpn is stable in many cell lines that do not express this isoform of Cp. In this respect, this new function of Cp is particularly relevant for brain iron metabolism because any factor affecting the ferroxidase activity of Cp-GPI cannot be compensated by circulating plasma Cp, which is unable to cross the blood-brain barrier. Iron uptake by endothelial cells of the blood-brain barrier takes place through the Tf-TfR1 system, how the metal is then moved out of these cells and taken up by CNS cells is still unclear. Recent data indicate that iron efflux from brain microvasculature endothelial cells is mediated by Fpn and requires the action of a ferroxidase, which can be either endogenous hephaestin or extracellular Cp[53]. These findings highlight once again the importance of ferroxidases for correct cellular iron management. Astrocytes are in close contact with the abluminal surface of capillary endothelial cells and therefore are ideally positioned to control the transport of metabolites between the blood and the neuropil. Since astrocytes are able to take up and release iron, they have been proposed to be largely responsible for distributing iron in the brain[54]. Therefore, Fpn and Cp-GPI would represent the central system for release of iron from astrocytes to meet the requirements of neurons and other brain cells.

A physical interaction between Cp and Fpn has not been evidenced despite many efforts; however, it has been reported that Cp is able to partially prevent hepcidin-induced internalization of Fpn when cells are treated with 0.15 μmol/L hepcidin[42]. This finding could be taken as an indication that Cp can compete with hepcidin for binding to Fpn, suggesting that probably such interaction exists but it is transient and/or too weak to be detected. A direct consequence of this hypothesis is that the Cp-binding site on Fpn would partially overlap with the hepcidin-binding site. An alternative explanation would be that Cp interacts with hepcidin, making the peptide unavailable for binding to Fpn.

Transcriptional regulation of the ceruloplasmin-ferroportin system

The Cp-Fpn functional connection is strengthened also by the finding that expression of the two proteins can be coordinately regulated in specific cell types.

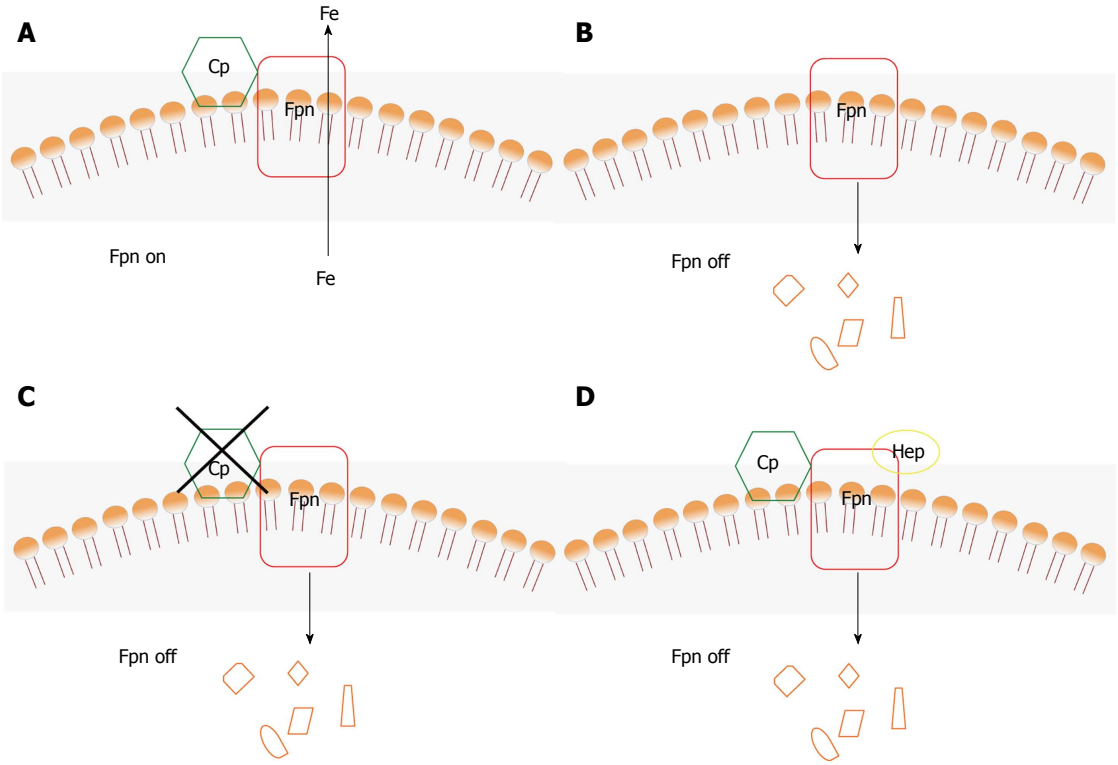

Figure 3 Scheme of the role of ceruloplasmin and hepcidin in the degradation of ferroportin. A: In the presence of Cp, Fpn is stable and exports iron; B: In the absence of Cp, Fpn is degraded; C: In the presence of inactive Cp, Fpn is degraded; D: In the presence of Cp and hepcidin, Fpn is degraded. Cp: Ceruloplasmin; Fpn: Ferroportin.

Cp was recognized to be an acute phase protein many years ago, and it is known to be induced in response to pro-inflammatory stimuli, such as IL-1β[55-57], INF-γ[58] and IL-6[59]. Recent data demonstrate that IL-6 mediates induction of Cp *via* the transcription factor FOXO1[60]. Metal-dependent regulation of Cp has not been conclusively assessed, although indirect effects of iron deficiency mediated by hypoxia-inducible factor-1 (HIF-1) have been reported[61].

Expression of Fpn is regulated by different stimuli: iron and transition metals, heme, hypoxia and inflammation among others. Many studies have highlighted a tissue-specific regulation of expression of Fpn and point to Fpn regulation by systemic rather than local signals of iron status. Actually, two layers of regulation are active to control Fpn: one at the level of mRNA (transcriptional and post-transcriptional) and one at the level of the protein (hepcidin-dependent and hepcidin-independent internalization and degradation). Moreover, any factor affecting hepcidin synthesis in turn will affect Fpn protein levels.

The Fpn promoter contains different response elements sensitive to hypoxia, heme/oxidative stress and metals. The presence of HIF-Responsive-Elements was evidenced using Fpn reporter constructs and HIF2α was demonstrated to be a direct activator of Fpn transcription[62]. It is worth noting that HIF2α expression has recently been shown to depend on IRP1[63], strengthening the link between iron and hypoxia. Metal-Responsive-Element induction of Fpn mediated by the transcription factor MTF-1 in response to zinc was recently demonstrated[64]. Antioxidant-Responsive-Elements enable up-regulation of Fpn transcription in response to heme *via* activation of the redox-sensitive transcription factor Nrf2 in mouse and human macrophages[65,66]. Other studies indicated that heme-induced Fpn transcription required the release of iron from heme[67]. Ultimately, these results link transcriptional control of Fpn synthesis directly and indirectly to iron levels: *i.e.*, iron is crucial for HIF2α stability and IRP1-mediated expression, iron mediates oxidative stress and activation of Nrf2.

Fpn is down-regulated by pro-inflammatory cytokines in reticuloendothelial cells, as demonstrated by the finding that treatment with IFN-γ and LPS reduced Fpn mRNA and iron release from monocytes[68,69]. Fpn mRNA and protein levels were also found to decrease significantly in astrocytes treated with LPS but not with IL-6 or TNF-α[70]. Interestingly, we have found that in rat C6 glioma cells Cp and Fpn are up-regulated by IL-1β, suggesting that the response of Fpn to cytokines might be tissue-specific[57]. The expression of Cp and Fpn in response to IL-1β requires the activation of MAP kinase pathways as a consequence of IL-1β receptor stimulation. Moreover, we have observed that IL-1β regulates the expression of Cp and Fpn genes through (1) p38 MAPK-mediated activation of C/EBP transcription factor; (2) ERK1/2-, JNK1- and partially p38 MAPK-dependent activation of AP-1; and (3) activation of NF-κB partially mediated by p38 MAPK[71]. A similar pathway was found to activate Fpn expression in response to the isoflavone

genistein[72]. In this case, p38 MAPK activation was found to be triggered by activation of the estrogen receptor β.

Post-transcriptional regulation of ferroportin

At the post-transcriptional level, Fpn expression is regulated by iron-responsive sequences both at the 5' UTR and at the 3' UTR. Repression of Fpn mRNA translation in conditions of iron deficiency was shown to be mediated by the well-characterized IRE/IRP system, due to the presence of an IRE sequence at the 5' UTR. Also the 3' UTR of Fpn plays a role in post-transcriptional regulation of expression through a recently discovered miRNA-dependent mechanism. microRNAs are small non-coding RNAs that bind the 3' UTR of target mRNAs driving translational repression or mRNA degradation. In particular, it has been demonstrated that miR-485-3p is induced during iron deficiency and it targets the 3' UTR of Fpn to reduce iron export in several cell lines and primary macrophages[73]. In duodenal and erythroid precursor cells alternative splicing produces an isoform of Fpn lacking the 5' IRE indicating that these cells can evade IRE/IRP-dependent translational repression[74] becoming sensitive to systemic rather than local (intracellular) cues. It would be interesting to evaluate whether miR-485-3p is expressed in these cell types and this isoform of Fpn is subject to miRNA-mediated control.

CERULOPLASMIN-FERROPORTIN SYSTEM AND PATHOLOGY

The importance of the ceruloplasmin-ferroportin system is highlighted by the fact that mutations in the Cp and Fpn genes lead to severe consequences. Impairment of the Cp-Fpn system is common to aceruloplasminemia and "ferroportin disease", two genetic diseases that share a common phenotype of iron overload.

Aceruloplasminemia

Aceruloplasminemia is a rare autosomal disease caused by mutations in the *Cp* gene[75,76]. Approximately forty mutations of the *Cp* gene have been so far described, including frameshift, nonsense and missense mutations[77,78]. Heterozygous individuals have partial Cp deficiency with normal iron metabolism and no clinical symptoms, with some exceptions. Homozygotes present iron overload mainly in the brain, but also in liver, pancreas and retina. Patients develop retinal degeneration, diabetes mellitus and neurological symptoms, which include ataxia, involuntary movements and dementia. Onset of clinical manifestations usually occurs in adulthood. Laboratory findings include absence of serum Cp ferroxidase activity (although low levels of Cp protein were reported in some cases), low transferrin saturation, high serum ferritin and moderate anemia; magnetic resonance imaging of the brain shows iron deposits in the basal ganglia, striatum, thalamus and dentate nucleus. These features place aceruloplasminemia in the group of disorders known as NBIA (neurodegeneration with brain iron accumulation), clearly distinguishing it from hereditary hemochromatosis (serum iron is high and the brain is usually not affected) and from disorders of copper metabolism, Menkes and Wilson disease, that are also characterized by low/absent serum Cp ferroxidase activity because of impaired functioning of copper ATPases ATP7A and ATP7B, respectively[33].

Iron-mediated oxidative stress has been shown to contribute to tissue injury and neuronal cell death in aceruloplasminemia. In particular, it has been suggested that astrocytes, which are the most affected cell type, accumulate iron and die from iron toxicity, while neuronal loss would be secondary to loss of metabolic support provided by astrocytes[79,80].

The ferroxidase activity of Cp-GPI plays a critical role in the targeting of Fpn to the plasma membrane in astrocytes and bone marrow-derived macrophages[40]. Thus, brain iron overload and low serum iron levels observed in aceruloplasminemia patients can be explained by impaired iron export from these cell types due to lack of active Cp. On the other hand, the origin of iron overload in liver and pancreas, which is observed in aceruloplasminemia patients has still to be clarified.

Actually, the situation is even more complicated. In fact, while it is obvious that frameshift and nonsense mutations produce a truncated non-functional Cp, *in vitro* characterization of missense mutants yielded some unexpected findings. The first mutants to be studied invariably lacked ferroxidase activity either due to retention in the endoplasmic reticulum (P177R) or to production as apo-Cp lacking copper (D58H, G631R Q692K and G969S), due to structural or folding defects[81-84]. Indeed, residue Pro177 is found in a hydrophobic pocket, while residues Gly631, Gln692 and Gly969 are close to type 1 copper sites, suggesting that substitutions in these positions can affect folding and copper binding. Residue Asp58 is located on the protein surface and it has been suggested that substitution with histidine could cause aberrant incorporation of copper. However, another set of mutants (I9F, Q146E, F198S, W264S, A331D, G606E, G876A) that we characterized based on their ability to stabilize Fpn on the plasma membrane of rat C6 glioma cells silenced for endogenous Cp-GPI, revealed that they were partly or fully functional[85]. Also other studies showed that some mutants (Y356H, G876A) appeared to partly retain ferroxidase activity, but were less efficient than wild type Cp in protecting Fpn from hepcidin[42]. In these cases, inspection of the structure of Cp suggests that the position of the mutations is such that the protein can retain ferroxidase activity.

A quite different scenario was apparent for mutant R701W, which has been found in a very young heterozygous patient with severe extrapyramidal movement coordination deficit[86]. Both isoforms of Cp R701W (secreted and GPI-anchored) were inactive due to lack of copper, and dominant over wild type Cp in glioma cells. Moreover, they induced dispersal of the Golgi apparatus and "functional silencing" of ATP7B[85]. Of note, Cp R701W

could load copper in appropriate conditions, in particular when Ccc2p, the yeast homologue of ATP7B, was co-expressed. The resulting holo-Cp R701W was fully functional with respect to stabilization of Fpn[85]. It was reported that Cp R701W expressed in HeLa cells retained some oxidase activity but it was unable to stabilize Fpn at the cell surface[42], raising the possibility that a threshold level of activity might be required to observe this stabilizing effect. Further investigations have demonstrated that Cp R701W caused massive production of reactive oxygen (ROS) species in the cell. Scavenging ROS production with different antioxidants, such as N-acetyl-cysteine, glutathione and zinc, restored Golgi morphology and rescued Fpn on the cell membrane[87]. Whether ROS are produced directly by Cp R701W or by other cellular systems such as NOX, remains to be established. Residue Arg701 is found in the surface-exposed loop connecting domains 4 and 5 of Cp and it is difficult to understand why replacement with tryptophan should cause such a dramatic phenotype.

Ferroportin disease

Hemochromatosis is the most common genetic iron overload disease, it is inherited recessively and it is caused by defects of genes (*HFE, TfR2, HJV, HAMP*) that ultimately lead to inefficient synthesis of hepcidin. Fpn missense mutations are responsible for a different form of hemochromatosis which exhibits autosomal dominant inheritance with rather heterogeneous phenotypes, the so-called "ferroportin disease"[88]. Decreased function of Fpn appears to be limiting for macrophage iron export but not for intestinal iron export, due to the very different amounts of the metal mobilized by enterocytes (1-2 mg/d) compared to reticuloendothelial cells (20-30 mg/d). Fpn missense mutants can give rise to two different phenotypes: iron overload in macrophages and low serum transferrin saturation due to mutants that are transport incompetent or are not correctly targeted to the plasma membrane (loss-of-function mutants); hepatocyte iron overload and high serum transferrin saturation due to mutants that are unable to respond to hepcidin (gain-of-function mutants)[89,90]. Most of the mutations identified so far appear to lead to loss-of-function of Fpn, affecting plasma membrane localization of the protein and (less commonly) iron export function.

Many studies on the molecular features of the Fpn mutants have attempted to correlate mutation with phenotype. However, such analyses are complicated by difficulties in establishing a satisfactory experimental model. In most cases, recombinant Fpn mutants have been overexpressed in HEK293T or polarized MDCK cells. Subcellular localization is determined by employing Fpn-GFP fusions, Fpn function is investigated by analyzing hepcidin-induced internalization and by assessing intracellular iron levels. Conflicting results have been reported for some Fpn mutants, possibly due to the different experimental systems and conditions employed. For example, expression of Fpn in polarized MDCK cells resulted primarily in plasma membrane localization for all 16 mutants examined[11], compared to nonpolarized HeLa or HEK293T cells where some intracellular staining was apparent but could be eliminated by treatment with cycloheximide. Discrepancies in hepcidin resistance can probably be attributed to differences in hepcidin concentration and time of incubation, such that partial resistance at low (0.4-0.7 μmol/L) hepcidin concentration[9,10,91,92] can become sensitivity at high (2 μmol/L) hepcidin concentration[11]. Also, if a mutant is found to be predominantly intracellular, impaired iron export or hepcidin-resistance would simply reflect unavailability of Fpn at the plasma membrane and not a true property of the mutant protein.

Resistance to hepcidin can derive from different mechanisms: mutation of residues belonging to the hepcidin-binding site (C326Y/S and S338R) or impairment of the mechanism of internalization of Fpn (Y64N, N144H/D/T)[26]. Mutation of other residues (G204S, Y501C, H507R) has been reported to result in hepcidin resistance[93-95], suggesting that the hepcidin-binding site is probably formed by residues belonging to more that one extracellular loop of Fpn.

Other mutations impact the iron transport function of Fpn for as yet unidentified reasons (I152F). In summary, it is evident that the difficulties of working *in vitro* with Fpn make it tricky to unequivocally link patient phenotype to molecular defects of Fpn. This is further complicated by phenotypic heterogeneity among patients carrying the same Fpn mutation[93], suggesting that modifier genes might influence the penetrance of the disease.

CONCLUSION

Less than fifteen years have passed from the initial discovery of Fpn and a huge amount of information has been gained on this elusive protein. However, many questions still require an answer regarding our understanding of the structure and function of Fpn and the full implications of the connection between Fpn and Cp. Fpn is predicted to belong to the MFS transporters that function with an alternate "inward open-outward open" mechanism, involving extensive conformational changes to translocate their substrate across the membrane. The molecular details of how Fpn works are still a mystery, it is also unknown if transport of iron is coupled to other ions (either as symport or antiport). Why does Cp stabilize Fpn only in specific cell types is not clear.

Future studies should be aimed at addressing these and many other questions, in order to gain a better understanding of how Fpn and Cp collaborate for correct iron handling by cells.

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REVIEW

FBW7-mediated ubiquitination and degradation of KLF5

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Abstract

Krüppel-like factor (KLF) family proteins are transcription factors that regulate numerous cellular functions, such as cell proliferation, differentiation, and cell death. Posttranslational modification of KLF proteins is important for their transcriptional activities and biological functions. One KLF family member with important roles in cell proliferation and tumorigenesis is KLF5. The function of KLF5 is tightly controlled by post-translational modifications, including SUMOylation, phosphorylation, and ubiquitination. Recent studies from our lab and others' have demonstrated that the tumor suppressor FBW7 is an essential E3 ubiquitin ligase that targets KLF5 for ubiquitination and degradation. KLF5 contains functional Cdc4 phospho-degrons (CPDs), which are required for its interaction with FBW7. Mutation of CPDs in KLF5 blocks the ubiquitination and degradation of KLF5 by FBW7. The protein kinase Glycogen synthase kinase 3β is involved in the phosphorylation of KLF5 CPDs. In both cancer cell lines and mouse models, it has been shown that FBW7 regulates the expression of KLF5 target genes through the modulation of KLF5 stability. In this review, we summarize the current progress on delineating FBW7-mediated KLF5 ubiquitination and degradation.

Key words: Krüppel-like factor 5; FBW7; Ubiquitin proteasome system; Degradation; Krüppel-like factor family

Core tip: The protein levels of Krüppel-like factor (KLF)5 are tightly controlled in cell. Ubiquitination and destruction of KLF5 *via* FBW7, a famous tumor suppressor, has proved to have important roles in multiple cellular progresses by different studies. Here, we summarize these studies and show the physiological and pathological significance of FBW7-mediated degradation of KLF5.

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INTRODUCTION

Krüppel-like factor (KLF) family proteins are important transcription factors that regulate numerous cellular processes[1]. KLF5 is a member of the KLF family that has been well-studied and shown to play a key role in mediating multiple cellular activities, such as proliferation and differentiation, in both normal and tumor cells[2]. Post-translational modifications of KLF5, including ubiquitination, SUMOylation, acetylation, and phosphorylation, can impact both the stability and activity of KLF5, thus affecting its downstream cellular functions[3-8].

FBW7 is the mammalian homolog of CDC4 in *Saccharomyces cerevisiae* and SEL10 in C. elegans. It is a component of the SCF (SKP1-CUL1-F-box protein) ubiquitin

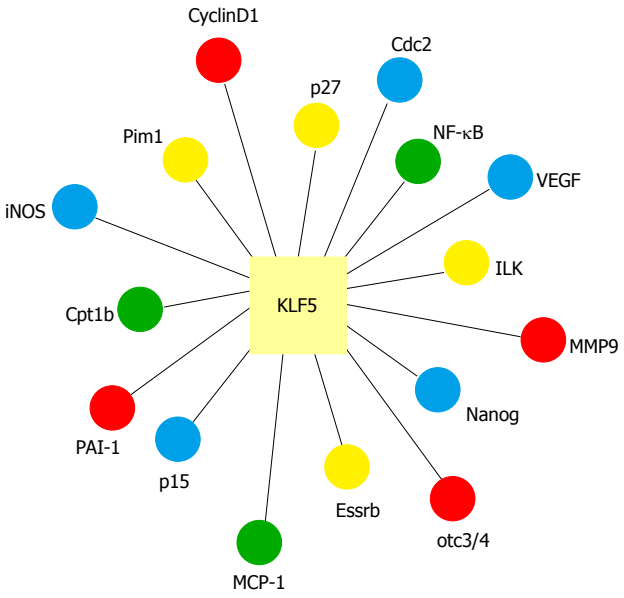

Figure 1 Regulation of gene expression by Krüppel-like factor 5. VEGF: Vascular endothelial growth factor; MCP-1: Monocyte chemoattractant protein-1; NK-κB: natural killer κB; MMP-9: Matrix metalloproteinase-9; PAI-1: Plasminogen activator inhibitor-1; iNOS: Inducible nitric oxide synthase.

ligase complex. FBW7 is thought to have an important role in tumor biology by serving as a critical regulator of several oncoproteins, and mutations of FBW7 are found in a rapidly expanding number of human neoplasms[9].

In this review, we summarize the progress of research on FBW7-mediated KLF5 degradation and ubiquitination and show the physiological and pathological significance of KLF5 regulation by FBW7.

KRÜPPEL-LIKE FACTOR FAMILY AND KLF5

KLFs are a family of transcription factors with homologies to the Krüppel protein and the transcription factor Sp1 in Drosophila melanogaster and mammals, respectively[1]. To date, 17 mammalian KLFs have been identified, all of which contain three zinc finger motifs at the carboxyl-terminals, which are responsible for binding to GC-rich DNA sequences[10,11]. The KLFs have been demonstrated to play essential roles in development, immunity and cancer[1,10-15].

KLF5, also known as BTEB2 and IKLF, is an important KLF factor. KLF5 is widely expressed in various tissues, including lung, colon, intestine, and pancreas[2,16-19]. KLF5 is located at chromosomal position 13q22.1 in the human genome. It is involved in the regulation of diverse cellular functions, including cell cycle, proliferation, apoptosis, differentiation and stem cell self-renewal, by regulating the expression of numerous genes (Figure 1)[2,20-23]. Previous studies have shown that KLF5 plays a pivotal role in regulating cardiovascular remodeling[24-26]. Heterozygous KLF5-knockout mice showed reduced responses to cardiac injury, angiogenesis, hypertrophy and fibrosis[24,25]. In addition, KLF5 activity is regulated by other transcriptional regulators and nuclear receptors that are also involved in cardiovascular remodeling and injury response[24,25]. In tumor biology, KLF5 also has context-dependent proliferative or anti-proliferative activities in cancer cells and may function as either a tumor suppressor or an oncoprotein[27-29].

The functions of KLF5 are tightly controlled by post-translational modifications, including ubiquitination, SUMOylation, acetylation and phosphorylation[3-8,21,30,31]. For example, the SUMOylation of Lys151 and Lys202 regulates KLF5 nuclear localization[3]. Phosphorylation of KLF5 by PKC may enhance the transcriptional activities of KLF5 by promoting its interaction with CREB-binding protein[21]. In addition, KLF5 activity is also regulated by its acetylation status[4]. Moreover, KLF5 is a short-lived protein in cells and its protein level is tightly controlled by the ubiquitin-proteasome system[5-8,31,32]. Several E3 ubiquitin ligases, such as Smurf2, WWP1 and EFP, have been shown to degrade KLF5[7,31,32]. In 2010, Dr. Chen C's group and our laboratory both reported that KLF5 is targeted for ubiquitination and degradation by the E3 ubiquitin ligase FBW7[6,8]. In the past three years, several studies from different groups have also provided evidence strongly supporting KLF5 as an essential FBW7 substrate under both physiological and pathological conditions[6-8,31-34].

UBIQUITIN-PROTEASOME SYSTEM AND FBW7

Cellular protein levels are tightly controlled by protein degradation. The ubiquitin-proteasome system (UPS) is the major pathway for the degradation of approximately 90% of all proteins in cells[35-37]. The UPS acts by promoting protein ubiquitination and delivering the ubiquitinated proteins to the 26S proteasome for degradation[36]. The UPS is an enzymatic cascade containing three enzymes: enzyme-1 (E1), the ubiquitin-activating enzyme; E2, the ubiquitin carrier protein (ubiquitin-conjugating enzyme); and E3, the ubiquitin-protein ligase. E3 determines the specificity of protein degradation[35]. To date, more than 600 E3s have been identified in mammals and categorized into either the RING or HECT family of E3 ubiquitin ligases[38-40].

FBW7 (F-box and WD repeat domain-containing 7, also named CDC4, SEL10, or AGO) is the substrate recognition subunit of the E3 ubiquitin ligase complex SCF^{FBW7} (Skp1-Cullin-FBW7), which can target various proteins that are involved in cell proliferation for degradation[9]. Many substrates of FBW7 have been identified, including c-Myc, Cyclin E, Notch, TGIF, c-Jun, Mcl-1, p100 and so on (Table 1)[41-56]. There are three known isoforms of FBW7 with different subcellular localizations, including FBW7α, FBW7β and FBW7γ[9,57]. FBW7α is mainly localized to the nucleoplasm. FBW7β contains a transmembrane domain and is localized to the cytosol. FBW7γ is localized to the nucleolus *via* a nucleolar localization signal at its N terminus[9]. Each FBW7 isoform

Table 1 Sequences of Cdc4 phospho-degrons in FBW7 substrates

Substrate	Cdc4 phospho-degron	Phospho-site
CyclinE	LLTPPQSG	T380 S384
Myc	LPTPPLSP	T58 S62
JUN	GETPPLSP	T239 S243
NOTCH1	FLTPSPE	T2512
TGIF	FNTPPPTP	T235 T239
SRC3	VHSPMASS	S505 S509
mTOR	LLTPSIHL	T631
MCL1	DGSLPSTP	S159 T163 S121
KLF5	LNTPDLDM/PPSPPSSE/ NLTPPPSY	T244 S303 T324
KLF2	PDTPPLSPD/LLTPPSSP	T171 S175 T243 S247
SREBP	TLTPPPSDAGSP	T426 S430 S434
SV40 large T antigen	PPTPPPEP	T701
MED13/MED13L	SSVTLTPPTS	T326
NF-κB2	LPSPPTSDSDSD	S707 S711
C/EBP	HPTPPPTP	T222 T226
C/EBP	QPTPPQSP	T157 S161
HIF1a	DQTPSPSDGSTRQSS	T497 S451
AuroraA	LSYCHSK/NSSKPSN	S245 S387
C-Myb	LMTPVSED	T572 S556 S528
NRF1	LFSPEVE	S350
PGC1	PLTPESPN/GLTPPTTP	T263 T295

NK-κB: natural killer κB; KLF: Krüppel-like factor.

contains a F-box domain and WD40 repeats. The F-box domain contains approximately 40 amino acids that are involved in recruiting the SCF complex through direct interaction with SKP1. WD40 repeats are thought to form multiple contacts with various substrates[57-62].

FBW7 recognizes its substrates through a conserved phospho-epitope known as the Cdc4 phospho-degron (CPD), in which a central phospho-threonine/serine is embedded within hydrophobic residues in a I/L-I/L/P-pT-P-<K/R>4 (where K and R are unfavorable residues at positions 2 to 5) motif[9]. Most of the FBW7 substrates contain at least one conserved CPD, and the phosphorylation of the central Ser/Thr is usually mediated by the protein kinase Glycogen synthase kinase 3 (GSK-3)β[61,63,64].

Numerous studies have demonstrated that FBW7 functions as a tumor suppressor in various cancers. Mutant FBW7 is frequently found in human tumors. For example, amino acid substitutions such as Q264R, H460R, and R465C have been found in breast cancer, cholangiocarcinoma and colon cancer, respectively[52,65-67].

FBW7 INTERACTS WITH KLF5 *IN VIVO* AND *IN VITRO*

KLF5 contains several potential CPDs[6]. Data from Dr. Chen's group and our laboratory have indicated that all three isoforms of FBW7 can bind to KLF5 *in vivo*[6,8]. Mass spectrometry data have also shown that endogenous KLF5 can be co-purified with FBW7 in different cell types[46]. The interaction of KLF5 with FBW7 is dependent on the KLF5 CPD(s). Mutations within the KLF5 CPDs were shown to abolish the interaction. In addition, FBW7 binds to KLF5 *via* the WD40 repeats on FBW7. This interaction is also dependent on the phosphorylation of KLF5 CPDs by GSK3β, and inhibition of GSK3β activity can reduce FBW7 binding to KLF5. GSK3β activity is regulated by various extracellular stimuli such as Wnt and growth factors[68,69], but it is still unclear whether the interaction between KLF5 and FBW7 is also regulated by extracellular signals.

FBW7 TARGETS KLF5 FOR UBIQUITINATION AND DEGRADATION

As a component of the SCF E3 ubiquitin ligase complex, co-expression of FBW7α or FBW7γ was shown to markedly promote the degradation of co-expressed KLF5, which could be blocked by the proteasome inhibitor MG132. In contrast, other F-box-containing proteins such as β-TrCP1, FBXW2, FBXW5 and FBXW8 had little effect on KLF5 stability. FBW7 with its F-box domain deleted or the WD40 domain of FBW7 alone failed to mediate KLF5 degradation, suggesting that FBW7-mediated KLF5 degradation requires the recruitment of other components of SCF E3 ligase. R338 residue in FBW7 is considered as a key residue in regulating the interaction of FBW7 with its substrates. Mutation of R338 to lysine blocks FBW7 mediated KLF5 degradation (Figure 2). Depletion of endogenous FBW7 significantly increased the amount of endogenous KLF5 protein without affecting the KLF5 mRNA level. KLF5 protein level was also upregulated in FBW7-deficient DLD1 cells and the half-life of endogenous KLF5 was dramatically extended in these cells compared with the WT DLD1 cells.

Moreover, FBW7 also promotes KLF5 ubiquitination *in vitro* and *in vivo*. The ubiquitination of KLF5 by FBW7 is dependent on the phosphorylation of KLF5 CPDs. Mutation of KLF5 CPDs dramatically blocked FBW7-induced KLF5 ubiquitination.

In addition to FBW7, WWP1, EFP and Smurf2 were also identified as E3 ligases that can target KLF5 for degradation[7,31,32]. Both WWP1 and Smurf2 belong to the HECT E3 ubiquitin ligase family[70,71]. Unlike FBW7, WWP1 and Smurf2 degrade KLF5 in a phosphorylation-independent manner. Interestingly, FBW7 and WWP1 appear to degrade KLF5 in a compensatory manner because knockdown of WWP1 was shown to cause an increase in FBW7 expression, and vice versa[8]. Degradation of KLF5 by multiple E3 ubiquitin ligases signifies the importance of the regulation of KLF5 protein stability under various physiological and pathological conditions[5-8,31-34].

KLF5 CONTAINS CPDS THAT ARE REQUIRED FOR ITS DEGRADATION THROUGH FBW7

FBW7 targets a substrate for degradation through the CPD consensus sites on the substrate[63]. KLF5 contains three potential CPDs: 242-LNTPDLDM, 301-PPSPPSSE and 322-NLTPPPSY (Table 1). Mutations of individual

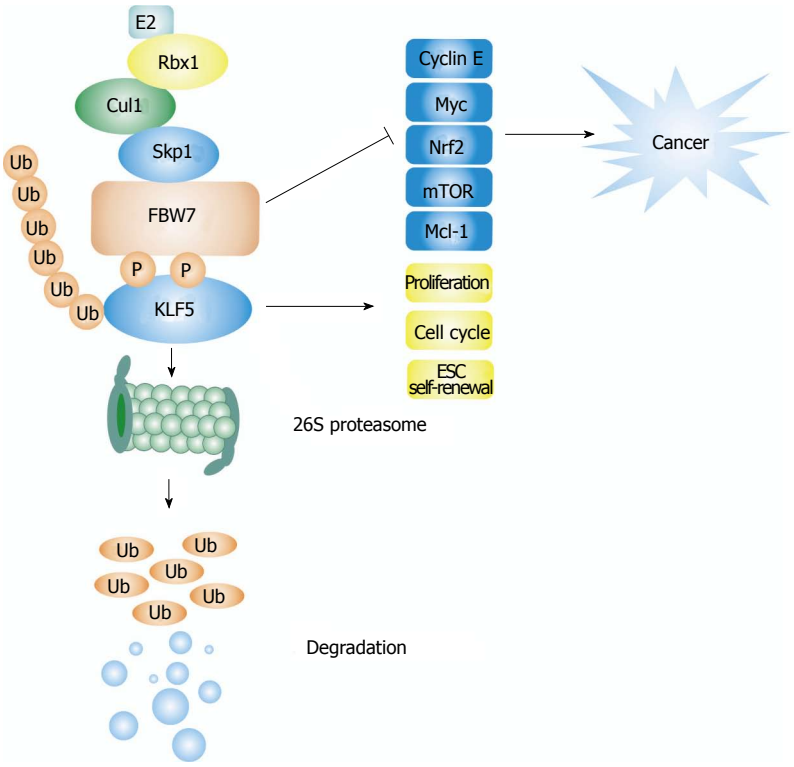

Figure 2 A model for FBW7 mediated Krüppel-like factor 5 degradation. SCFFBW7 recognizes KLF5 *via* conserved Cdc4 phospho-degron (CPD) in KLF5, GSK3 phosphorylates the threonine of the CPD, which facilitates the degradation of KLF5. FBW7 plays an important role in tumor suppression *via* targeting numerous oncoproteins for degradation, such as Myc, cyclin E, mammalian target of rapamycin (Mtor), Mcl-1, and so on. KLF5 has an important role in regulating cellular functions, including promoting cell proliferation, cell cycle, and embryonic stem cell (ESC) self-renewal. FBW7 promotes KLF5 ubiquitination and degradation through 26S proteasome. KLF: Krüppel-like factor.

CPDs in mouse KLF5 were shown to have a minor effect on FBW7-mediated degradation. However, simultaneous mutations of two CPDs markedly blocked KLF5 interaction with FBW7 and KLF5 degradation. Mutations of all three CPDs completely abolished FBW7-induced KLF5 ubiquitination and degradation. Although KLF5 contains three CPDs, both Dr. Chen's group and ours have found that phosphorylation of Ser303 in 301-PPSPPSSE is especially essential for FBW7-mediated degradation. In addition, Dr. Vincent W Yang's group also found that P301 in KLF5 CPD is important for interaction between FBW7 and KLF5 and FBW7-mediated degradation of KLF5. P301S KLF5, a somatic mutation in KLF5 found in human colorectal cancer tissues, has a higher transcriptional activity than WT KLF5 and is resistant to FBW7α-mediated degradation, suggesting that P301S KLF5 mutant play an oncogenic role in colorectal cancer[72].

GSK3α IS A KEY PROTEIN KINASE FOR KLF5 PHOSPHORYLATION AND DEGRADATION

GSK-3 is a serine/threonine protein kinase[73] that phosphorylates the central serine/threonine residues in the CPDs of numerous FBW7 substrates[9], including KLF5. Co-expression of KLF5 with GSK3β was shown to promote KLF5 phosphorylation and KLF5 interaction with FBW7. Data from *in vitro* phosphorylation assays indicated that phosphorylation of wild-type KLF5 by GSK3β was much greater than that of a CPD-deficient KLF peptide, indicating that the KLF5 CPDs are phosphorylation targets of GSK3β. Inhibition of GSK3β by LiCl was shown to block FBW7-mediated KLF5 degradation. Conversely, KLF5 degradation was enhanced in the presence of the constitutively active GSK3β-S9A. Dr. Chen's group reported similar results, and together these data indicate that GSK3β is required for FBW7-mediated degradation of KLF5.

Protein phosphorylation by GSK3β requires the phosphorylation of the priming phosphate group on a Ser/Thr residue that is located at the +4 position of a target residue[63]. For example, phosphorylation of c-Myc at T58 by GSK3β requires prior mitogen-activated protein kinase-dependent phosphorylation at serine S62[74-77]. Two of the KLF5 CPDs, 301-PPSPPSSE and 322-NLTPPPSY, contain a Ser at the +4 position. The protein kinase(s) that is involved in the phosphorylation of priming sites on KLF5 CPDs is still unknown.

REGULATION OF CANCER CELL PROLIFERATION BY FBW7-MEDIATED KLF5 DEGRADATION

We have previously shown that FBW7 negatively regulates the biological activity of KLF5[6]. An earlier study has also shown that KLF5 promotes the growth and proliferation of colorectal cancer cells[78]. Co-expression of FBW7 with KLF5 significantly inhibited the wild-type KLF5-mediated cell proliferation but had little effect on the proliferation of cells containing a CPD-mutant KLF5[6]. FBW7 can also inhibit the expression of KLF5 target genes, such as survivin, which regulates mitosis and caspase activity[79]. A high level of KLF5 has also been correlated with low survival in breast cancer patients[28].

Dr. Chen and his colleagues have determined the expression of FBW7 and KLF5 in multiple cancer cell lines, including HeLa, MCF10A, and 184B5 cells. Interestingly, they found that degradation of KLF5 by FBW7 is dependent on both the cell type and the FBW7 isoform[8]. For example, in 184B5 mammary gland cells, knockdown of FBW7α but not of the FBW7β and FBW7γ isoforms, upregulated the expression of KLF5 and its downstream target FGF-BP, which is a known promoter of breast cancer cell proliferation[8,80], suggesting that the different isoforms of FBW7 specifically regulate KLF5 stability and activity in breast cells.

REGULATION OF KLF5 BY FBW7 IN MOUSE MODELS

Recently, several lines of evidence from mouse models indicate that KLF5 stability can be regulated by FBW7 *in vivo*[33,34,81]. As mentioned above, mutations of FBW7 occur frequently in multiple cancers, including those of the lung, colorectum, stomach, blood, pancreas, and endometrium. FBW7 R482Q is one of the loss-of-function mutants that have been identified in various cancers. A mouse model harboring the R482Q mutation was generated in Dr. Ian Tomlinson's laboratory. Interestingly, the protein levels of KLF5 and TGIF1 were upregulated in the lungs of the heterozygous mutant mice, but the mRNA levels of these two genes remained the same between the mutant and the wild type mice[33,34]. Further investigation revealed that the levels of KLF5 and TGIF1 were also upregulated in normal intestine and adenomas of FBW7-deficient or FBW7-mutant mice. These data serve as strong *in vivo* evidences for KLF5 regulation by FBW7.

Regulation of KLF5 target gene expression by FBW7 has also been demonstrated in a mouse model[81]. Kumadaki *et al*[81] showed that *in vivo* knockdown of FBW7 significantly increased the hepatic expression of PPARγ2 as well as its targeted genes. More importantly, the degradation of KLF5 by FBW7 was associated with the inhibition of PPARγ2 expression. Thus, these findings suggested that degradation of KLF5 by FBW7 contributes to hepatic lipid metabolism.

CONCLUSION

In summary, FBW7 is an E3 ubiquitin ligase for KLF5. KLF5 contains functional CPDs that are phosphorylated by GSK3β, thus promoting the interaction between KLF5 and the WD40 domain of FBW7. This interaction subsequently leads to KLF5 ubiquitination and degradation by the ubiquitin-proteasome system. Mutation or deletion of FBW7 in cancer cells results in increased level of the KLF5 protein due to impaired degradation of KLF5, which in turn causes increased expression of KLF5 target genes, many of which can promote cell proliferation. Moreover, the KLF5 protein level is tightly controlled by FBW7 under normal physiological conditions, thus affecting many developmental and metabolic processes. In summary, the FBW7-KLF5 axis is important for both normal cellular activities, such as lipid metabolism, and cancer cell proliferation. This pathway may therefore serve as a novel target for cancer therapy

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Extracellular *O*-linked β-*N*-acetylglucosamine: Its biology and relationship to human disease

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Abstract

The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc)ylation of cytoplasmic and nuclear proteins regulates basic cellular functions and is involved in the etiology of neurodegeneration and diabetes. Intracellular *O*-GlcNAcylation is catalyzed by a single *O*-GlcNAc transferase, *O*-GlcNAc transferase (OGT). Recently, an atypical *O*-GlcNAc transferase, extracellular *O*-linked β-*N*-acetylglucosamine (EOGT), which is responsible for the modification of extracellular *O*-GlcNAc, was identified. Although both OGT and EOGT are regulated through the common hexosamine biosynthesis pathway, EOGT localizes to the lumen of the endoplasmic reticulum and transfers GlcNAc to epidermal growth factor-like domains in an OGT-independent manner. In *Drosophila*, loss of *Eogt* gives phenotypes similar to those caused by defects in the apical extracellular matrix. Dumpy, a membrane-anchored apical extracellular matrix protein, was identified as a major *O*-GlcNAcylated protein, and EOGT mediates Dumpy-dependent cell adhesion. In mammals, extracellular *O*-GlcNAc was detected on extracellular proteins including heparan sulfate proteoglycan 2, Nell1, laminin subunit alpha-5, Pamr1, and transmembrane proteins, including Notch receptors. Although the physiological function of *O*-GlcNAc in mammals has not yet been elucidated, exome sequencing identified homozygous *EOGT* mutations in patients with Adams-Oliver syndrome, a rare congenital disorder characterized by aplasia cutis congenita and terminal transverse limb defects. This review summarizes the current knowledge of extracellular *O*-GlcNAc and its implications in the pathological processes in Adams-Oliver syndrome.

Key words: Extracellular *O*-linked β-*N*-acetylglucosamine; Notch; Adams-Oliver syndrome

Core tip: The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) on extracellular protein domains is the most recently identified *O*-glycosylation of epidermal growth factor repeat-containing proteins such as Notch receptors. This *O*-GlcNAc modification occurs in the secretory pathway by an endoplasmic reticulum-resident *O*-GlcNAc transferase, extracellular *O*-linked β-*N*-acetylglucosamine (*EOGT*). In *Drosophila*, Dumpy, a membrane-tethered cuticle protein, was identified as a major *O*-GlcNAcylated protein that mediates the interaction between epithelial cells and the extracellular matrix. In mammals, extracellular *O*-GlcNAc was detected on Hspg2, Nell1, Lama5, Pamr1, and Notch receptors, although the physiological function of *O*-GlcNAc in mammals has not yet been elucidated. However, the recent finding that *EOGT* is a causative gene for Adams-Oliver syndrome provided important insights into the significance of extracellular *O*-GlcNAc in mammals. This review summarizes the current knowledge of extracellular *O*-GlcNAc and its implications in the pathological processes in Adams-Oliver syndrome.

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INTRODUCTION

O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) was first identified in 1984 as a cell-surface saccharide moiety on intact lymphocytes[1]. Later studies, however, revealed that *O*-GlcNAc is present on nuclear, cytosolic, and mitochondrial proteins. This modification is prevalent in multicellular organisms, where more than 1000 *O*-GlcNAcylated proteins have been identified[2]. Intracellular *O*-GlcNAcylation is reversible, and its cycling is dynamically regulated by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase[3-5]. A large number of studies have indicated that *O*-GlcNAcylation is involved in various cellular functions, including transcription, epigenesis, cellular signaling, cell differentiation, and glucose sensing[6-9]. It had long been believed that *O*-GlcNAc is a unique intracellular modification and that OGT is the sole enzyme catalyzing the *O*-GlcNAc transfer reaction. However, extracellular *O*-GlcNAc was recently discovered on the extracellular domains of Notch receptors (Figure 1A). In this minireview, we will focus on extracellular *O*-GlcNAc and its relevance to human disease.

EXTRACELLULAR *O*-GLCNAC ON EGF DOMAINS

The first example of the *O*-GlcNAc modification of extracellular protein domains was the 20th EGF domain (EGF20) of *Drosophila* Notch expressed in S2 cells. Biochemical analyses revealed that *O*-GlcNAcylation occurs on the threonine located between the fifth and sixth cysteine[10]. Moreover, *in vivo* studies revealed that *O*-GlcNAc is abundantly expressed in the *Drosophila* cuticle[11]. Among cuticle proteins, Dumpy, a giant 2.5-MDa membrane-anchored cuticle protein containing a very large number of EGF-like domains (308 EGF-like repeats), was identified as a major *O*-GlcNAcylated protein[11]. In addition to Notch and Dumpy, Delta and Serrate, ligands for Notch receptors, have been shown to be *O*-GlcNAcylated by extracellular *O*-linked β-*N*-acetylglucosamine (EOGT)[10,12] (Figure 1B) in *Drosophila* S2 cells.

Similar to intracellular *O*-GlcNAc, extracellular *O*-GlcNAc is conserved in mammals but can be subjected to subsequent modification. The co-expression of Notch1 with EOGT in HEK293T cells suggests that the *O*-GlcNAc moiety is further modified with galactose to form *O*-linked *N*-acetyl-lactosamine (*O*-LacNAc)[13]. Recently, five extracellular *O*-GlcNAcylated proteins [Hspg2(Perlecan), Nell1, Lama5, Pamr1, and Notch2] were identified by a modified chemical/enzymatic photochemical cleavage approach for enriching *O*-GlcNAcylated peptides from mouse cerebrocortical brain tissue[14]. Another carbohydrate analysis revealed that *O*-GlcNAcylation occurs in the native thrombospondin-1 (TSP1) purified from platelets as well as in the recombinant TSP1 fragments expressed in insect High Five cells[15] (Figure 1B). The sequence alignment of *O*-GlcNAcylated proteins suggests that the predictive consensus sequence for the modification is C^5XXGX(T/S)GXXC^6, where C^5 and C^6 are the fifth and sixth conserved cysteines of the EGF domain, respectively. It should be noted, however, that no experimental data are available to indicate whether the C^5XXGX(T/S)GXXC^6 sequence is necessary or sufficient for the modification[10].

EOGT IS RESPONSIBLE FOR EXTRACELLULAR *O*-GLCNAC

In contrast to the OGT-catalyzed intracellular modification, the addition of *O*-GlcNAc onto extracellular proteins is mediated by a distinct *O*-GlcNAc transferase, the EGF-domain specific *O*-GlcNAc transferase (EOGT)[11,13]. *Eogt* is evolutionarily conserved from *Caenorhabditis elegans* to humans. EOGT contains a hydrophobic region corresponding to a signal peptide and a KDEL-like ER-retrieval sequence at the carboxyl terminus (Figure 2A)[11]. EOGT exhibits no similarity to OGT, but it is phylogenetically related to plant xylosyltransferases. EOGT possesses a putative UDP-GlcNAc-binding DXD motif[12]. EOGT specifically utilizes uridine diphosphate (UDP)-GlcNAc as a sugar donor, and its *in vitro* enzyme activity is enhanced in the presence of divalent cations, especially Mn^{2+}[11,13].

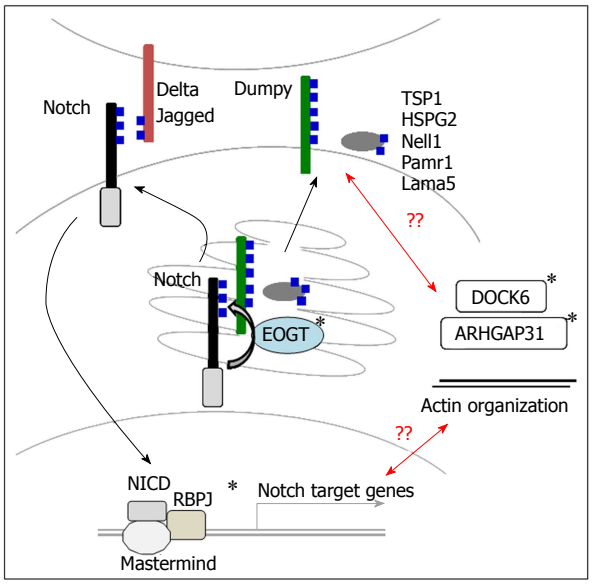
Because the levels of *O*-GlcNAcylation on Notch are increased by treatment with glucosamine or GlcNAc[8], it is suggested that the hexosamine biosynthesis pathway (HBP) is upstream of extracellular *O*-GlcNAc modification. The end product of the HBP is UDP-GlcNAc, which is utilized by EOGT as a donor substrate to modify proteins with *O*-GlcNAc in the ER. The transport of UDP-GlcNAc across the ER or Golgi membrane is mediated by nucleotide-sugar transporters[16-19]. However, it remains unclear which UDP-GlcNAc transporters are required for *O*-GlcNAcylation by EOGT.

Although *EOGT* expression has been detected in all adult mouse tissues, its expression is highest in the lung and lowest in the skeletal muscles[13]. During mouse development, high expression was detected in the growing edge of the limb buds; the expression was localized to the digits of the four limbs at later stages[20].

BIOLOGICAL FUNCTION OF EXTRACELLULAR *O*-GLCNAC IN *DROSOPHILA*

The biological function of extracellular *O*-GlcNAc was first suggested by the phenotype of the *Eogt* mutant in

A



B

***O*-GlcNAcylated proteins**	***O*-GlcNAc site**	**Protein function**	**Species**	**Ref.**
Notch1	LNAFSCQCMPGYTGQKC	Receptor for Notch signaling	*Drosophila*	[10]
Dumpy	Unspecified	Apical extracellular matrix protein	*Drosophila*	[11,12]
Hspg2 (Perlecan)	ACAPGYTGRRCES	heparan sulfate proteoglycan/extracellular matrix component	Mouse	[14]
Nell1	VCPSGFTGSHCEK	Glycoprotein involved in bone physiology	Mouse	[14]
Lama5	TCPPGLSGERCDT	One of the vertebrate laminin alpha chains	Mouse	[14]
Pamr1	ACLAGYTGQRCEN	Regeneration-associated Muscle protease homolog	Mouse	[14]
Notch2	VCSPGFTGQRCNI (?)	Receptor for Notch signaling	Mouse	[14]
thrombospondin-1	CGACPPGYSGNGIQCTLELVPR	Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions	Human	[15]
Delta	Unspecified	Ligand for Notch signaling	Drosophila	[11,12]
Serrate	Unspecified	Ligand for Notch signaling	Drosophila	[12]

Figure 1 Extracellular *O*-linked β-*N*-acetylglucosamine. A: The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc)ylation of extracellular protein domains is a newly identified translational modification of epidermal growth factor (EGF) domains, including Notch, HSPG2, Pamr1, and Lama5. Extracellular *O*-GlcNAc is mediated by EOGT in the endoplasmic reticulum (ER). Mutations in EOGT were recently identified in patients with Adams-Oliver syndrome (AOS). The role of EOGT in the pathogenesis of AOS is currently unknown. Given that RBPJ, a transcriptional factor for Notch signaling, is a causative gene for AOS, *O*-GlcNAcylation of Notch receptors by EOGT might regulate Notch receptor trafficking or Notch-ligand interactions. ARHGAP31 or DOCK6, another causative gene for AOS, affects the actin cytoskeleton by regulating Cdc42 and Rac1 activity. Thus, another possibility is that the *O*-GlcNAcylation of unidentified cell adhesion molecules by EOGT affects actin dynamics. It should be noted, however, that Dumpy homologues are not present in mammals. The *O*-GlcNAcylation of Notch ligands was reported in Drosophila. The causative genes for AOS are shown by asterisks; B: Summary of proteins with extracellular *O*-GlcNAc identified to date.

Drosophila[11]. Although the *Eogt* mutant does not exhibit the classical Notch phenotype, it shows defects in the wings, notum, and cuticle (*i.e.*, wing blistering, vortex, and cuticle detachment), similar to the *dumpy* mutant[11,12]. As mentioned above, Dumpy is a membrane-tethered protein that represents a major *O*-GlcNAcylated protein in the cuticle[11]. Moreover, the genetic interaction and phenotypic similarity between *Eogt* and *dumpy* suggests that EOGT is required for Dumpy-dependent epithelial cell-matrix interactions.

Previous studies using *Eogt* mutant embryos suggested that *O*-GlcNAc is required for the correct targeting of Dumpy into the chitinous matrix, possibly by mediating interactions with other components in the extracellular matrix (ECM)[11]. Currently, the molecular mechanisms by which Dumpy mediates cell adhesion are unknown, and thus the precise mechanism by which *O*-GlcNAc mediates cell adhesion must await the functional characterization of Dumpy. However, it is intriguing to speculate that multiple *O*-GlcNAc moieties arranged regularly along the EGF repeats of Dumpy have the ability to associate with unidentified chitin (a polymer of GlcNAc)-binding lectins in the ECM, thereby enabling the cuticle assembly/maintenance required for epidermis adhesion.

Interestingly, comprehensive genetic interaction studies revealed an interaction between *Eogt* and pyrimidine metabolism in the wing blister phenotype[12]. Thus, an alternative possibility is that loss of *Eogt* directs the increased UDP-GlcNAc pool in the cytoplasm. This will lead to elevated pyrimidine synthesis, such as uracil, that is likely to promote wing blistering[12]. If this is the case, EOGT might regulate pyrimidine metabolism by *O*-GlcNAcylating Dumpy. The contribution of pyrimidine metabolism to the *Eogt* phenotype was also suggested by the genetic interaction between *Eogt* and the Notch signaling genes,

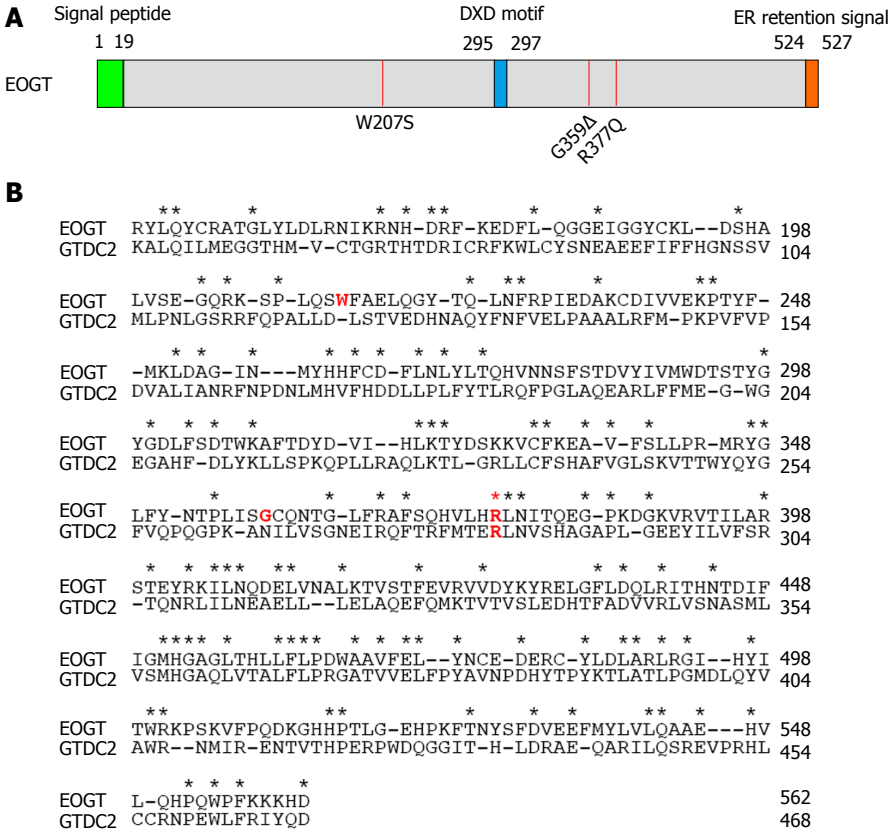


Figure 2 Extracellular *O*-linked β-*N*-acetylglucosamine mutations found in Adams-Oliver syndrome. A: A schematic representation of the primary structure of EOGT. The amino-terminal signal peptide is shown in yellow and the carboxyl-terminal Lys-Asp-Glu-Leu-like endoplasmic reticulum (ER) retrieval signal is in orange. The putative DXD motif involved in binding the nucleotide sugar is shown in blue. The position of each mutation is indicated by a red line; B: The amino acid sequence alignment of mouse EOGT (NP_780522, 149-562 aa) and mouse GTDC2/EOGT-L (Q8BW41, 55-468 aa). Identical amino acid residues are indicated by asterisks. Amino acid residues corresponding to the mutations in patients with Adams-Oliver syndrome are highlighted by red letters. EOGT: Extracellular *O*-linked β-*N*-acetylglucosamine.

which are involved in pyrimidine synthesis regulation[12].

EXTRACELLULAR *O*-GLCNAC AND ITS RELATIONSHIP TO ADAMS-OLIVER SYNDROME

The significance of the *O*-GlcNAcylated proteins was only tested in the context of Dumpy function, and the physiological roles of *O*-GlcNAc in mammals have not been investigated. However, exome sequencing in Adams-Oliver syndrome (AOS) patients provided important insights into the significance of extracellular *O*-GlcNAc in mammals. AOS is a rare congenital disorder characterized by vertex scalp defects [aplasia cutis congenital (ACC)] and terminal transverse limb defects (TTLDs)[21]. Recently, homozygous mutations in *EOGT* were identified in some patients with AOS[20,22]. These mutations include missense mutations (W207S and R377Q) and a frame shift mutation that creates a premature stop codon (G359Dfs*28) (Figure 2A). Currently, the blood levels of extracellular *O*-GlcNAc, the sugar moiety and its metabolites in the patients have not yet been investigated. However, the frame shift mutation in *EOGT* likely abolishes the enzyme activity because the truncated form of EOGT lacks the putative catalytic region containing the sequences conserved between EOGT and GTDC2, another ER-resident GlcNAc transferase modifying α-dystroglycan[23-25] (Figure 2B). The biochemical properties of the W207S and R377Q mutations have not yet been addressed. However, the R377 residue of EOGT is conserved in GTDC2. Thus, it is likely that the R377 residue may be important for GlcNAc transferase activity in EOGT and GTDC2 and that the R377Q mutation impairs the *O*-GlcNAc transferase activity of EOGT.

AOS is genetically heterogeneous, and its molecular pathology appears complex. In addition to *EOGT*, homozygous mutations of *DOCK6,* gain-of-function mutations of *ARHGAP31*, and heterozygous mutations for *RBPJ* were reported in AOS[26-28] (Figure 1A). *ARHGAP31* and *DOCK6* encode proteins that regulate the activity of key regulators of the actin cytoskeleton, RAC1 and CDC42. Accordingly, patient fibroblasts harboring disease-causing *ARHGAP31* or *DOCK6* mutations exhibited disorganized cytoskeletons and morphologies[27,28]. By contrast, *EOGT* mutant fibroblasts showed a typical spindle appearance comparable to that of control fibroblasts[22]. Therefore, it appears that EOGT does not directly affect the actin cytoskeleton, although the pos-

sibility remains that EOGT affects actin dynamism in restricted cell-types other than fibroblasts.

EXTRACELLULAR *O*-GLCNAC AND NOTCH SIGNALING

Another intriguing possibility for the role of EOGT in the pathogenesis of AOS involves Notch regulation because *RBPJ* encodes the transcriptional factor for Notch signaling. It has been reported that disease-causing *RBPJ* mutations decrease binding to the Notch target promoter, *HES1*[26]. Therefore, if EOGT and RBPJ act through a common signaling pathway in AOS, EOGT might positively regulate Notch signaling by the *O*-GlcNAcylation of Notch receptors. It should be noted, however, that no experimental data are available to support this hypothesis.

In *Drosophila*, *O*-GlcNAcylated EGF domains could be simultaneously modified with other *O*-glycosylations, namely *O*-fucose and *O*-glucose. *O*-fucosylation and *O*-glucosylation are catalyzed by ER-resident glycosyltransferases, POFUT1/Ofut1[29] and POGLUT1/Rumi[30]. These enzymes play indispensable roles for Notch signaling by affecting the trafficking, processing, and ligand-binding ability of Notch receptors[30-37]. In contrast, *O*-GlcNAc is dispensable for the majority of Notch receptor functions because *Eogt* mutants failed to exhibit apparent defects in most Notch-dependent biological processes, including embryonic neurogenesis, wing margin formation, and wing vein specification[11]. Given that the mutation of *Ofut1* or *rumi* does not produce Dumpy-like phenotypes, *O*-GlcNAcylation and *O*-fucosylation/*O*-glucosylation appears to be significant for the separate protein functions and distinct developmental processes in *Drosophila*. Nonetheless, there remains the possibility that these *O*-glycosylations may have partially redundant roles for Notch function, which would be revealed by genetic interaction studies between *Eogt* and *rumi*/*Poglut1* or *Eogt* and *Ofut1*/*Pofut1*.

Currently, no animal models for AOS have been established, and no AOS-related phenotypes were reported in *RBPJ* heterozygous mice[38]. In this regard, it would be interesting to investigate whether *EOGT* mutant mice would serve as a disease model for AOS.

CONCLUSION

The *O*-GlcNAc on extracellular protein domains is the most recently identified *O*-glycosylation of EGF repeat-containing proteins such as Notch receptors. This *O*-GlcNAc modification occurs in the secretory pathway by EOGT in the ER. In *Drosophila*, Dumpy was identified as a major *O*-GlcNAcylated protein that contributes to the interaction between epithelial cells and cuticles. Recent reports revealed that the mutations in *EOGT* cause AOS. However, the significance of the *O*-GlcNAcylated proteins was only tested in the context of Dumpy function in *Drosophila*, and the roles of *O*-GlcNAc in mammals have not been elucidated. In mammals, extracellular *O*-GlcNAc was detected on the TSP1, Hspg2, Nell1, Lama5, Pamr1, and Notch receptors[14,15]. Considering that a number of extracellular and transmembrane proteins are potentially *O*-GlcNAcylated by EOGT, additional studies will be required to address the roles of extracellular *O*-GlcNAc in Notch-dependent and independent biological processes in mammals as well as the molecular pathogenesis of human disease.

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MINIREVIEWS

Regulation and function of signal transducer and activator of transcription 3

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Key words: Signal transducer and activator of transcription 3; Phosphorylation; Acetylation; Signal pathway; Tumor

Core tip: The differential subcellular localization of signal transducer and activator of transcription 3 makes it play distinct functions in transcriptional regulation, cell proliferation and cellular respiration, thus contributing to development, reproduction and tumorigenesis in physiological and pathological conditions.

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Abstract

Signal transducer and activator of transcription 3 (STAT3), a member of the STAT family, is a key regulator of many physiological and pathological processes. Significant progress has been made in understanding the transcriptional control, posttranslational modification, cellular localization and functional regulation of STAT3. STAT3 can translocate into the nucleus and bind to specific promoter sequences, thereby exerting transcriptional regulation. Recent studies have shown that STAT3 can also translocate into mitochondria, participating in aerobic respiration and apoptosis. In addition, STAT3 plays an important role in inflammation and tumorigenesis by regulating cell proliferation, differentiation and metabolism. Conditional knockout mouse models make it possible to study the physiological function of STAT3 in specific tissues and organs. This review summarizes the latest advances in the understanding of the expression, regulation and function of STAT3 in physiological and tumorigenic processes.

INTRODUCTION

Signal transducer and activator of transcription factors (STATs) are a family of transcription factors that regulate cell growth, survival, differentiation, and motility. Structural studies identified that STAT proteins consist of an N-terminal domain, a coiled-coil domain, a DNA-binding domain, a Src homology 2 (SH2) domain and a transactivation domain, of which the DNA-binding domain is required for the recognition of specific binding sequences. Until now, seven members of the STAT family have been identified and characterized, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Despite the difference from canonical oncogenes, STAT3 has been recognized as a critical regulator in tumor cells since its identification[1]. STAT3 is over-expressed or activated by various carcinogenic agents, and can induce cell proliferation, differentiation and anti-apoptosis by activating the target genes, including STAT3, c-Myc and p53[2]. STAT3 exists in two main isoforms, full-length STAT3α

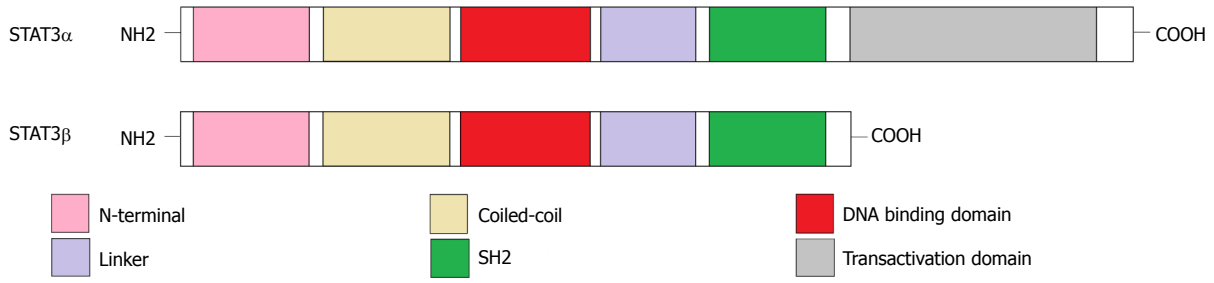

Figure 1 Domain structure of signal transducer and activator of transcription (3α and 3β). The signal transducer and activator of transcription 3α (STAT3α) protein is composed of N-terminal, coiled-coil, DNA binding, linker, SH2, and transactivation domains. However, the transactivation domain is absent in the alternative splicing variant, STAT3β.

and truncated STAT3β generated by alternative splicing. Under normal circumstances, STAT3α is the main isoform expressed in cells. STAT3β can competitively bind to the promoter of STAT3α target genes and inhibit the transactivation function of STAT3α. Additionally, STAT3β has its own specific target genes that differ from those of STAT3α[3].

STAT3 protein exists in a latent or inactive form in the cytoplasm. STAT3 can be activated by receptor-associated kinases and phosphorylated at various phosphorylation sites, particularly at Tyr-705 and Ser-727. Previous studies suggested that only phosphorylated STAT3 (p-STAT3) can translocate into the nucleus. However, recent data indicated that the nuclear translocation and transcriptional activity are partially independent of phosphorylation pathways[4]. Furthermore, STAT3 may translocate into mitochondria to control cell metabolism independent of its transcriptional regulatory activity[5]. Here we review the emerging biochemical and biological data on STAT3 and discuss its comprehensive roles in animal development and etiopathology of various diseases.

TRANSCRIPTIONAL REGULATION OF STAT3

STAT3 protein is expressed at a basal level in cells but rapidly increases once activated by specific cytokines. STAT3 is a critical factor in interleukin-6 (IL-6) induced gene regulation. STAT3 can be phosphorylated by IL-6 signal pathway, whereas IL-6 can also activate STAT3 at the transcriptional level. The level of STAT3 mRNA increases 1 h after IL-6 treatment and reaches to the maximum value at 3 h. There is an IL-6 response element (IL-6RE) in the promoter of STAT3 which contains a low affinity STAT3-binding element and a cAMP-responsive element (CRE). STAT3 executes its regulation in cooperation with this CRE-binding protein through self-activation[6].

In diabetic mice, estrogen administration can increase the level of STAT3 mRNA. There is a binding site of estrogen receptor α (ERα) in STAT3 promoter. Estrogen treatment induces the accumulation of ERα on STAT3 promoter and regulates the expression of STAT3[7]. STAT3 overexpression in tumor cells is related to the cytoplasmic/nuclear accumulation of β-catenin and the activation of β-catenin/T-cell factors (TCF) pathway. β-catenin is a key mediator in cell adhesion and signal transduction. Overexpression of β-catenin enhances both STAT3 mRNA and protein levels. There is a functional TCF binding element in STAT3 promoter, indicating that β-catenin/TCF may participate in the regulation of STAT3 expression[8].

The suppressors of cytokine signaling (SOCS) family consists of eight members, including SOCS1 to SOCS7 and cytokine-inducible SH2 domain proteins (CIS)[9]. SOCS proteins exist at low levels in resting cells and dramatically increase after STAT activation. SOCS proteins serve as classic negative regulatory factors of STAT activation[10]. Among them, SOCS3, a target gene of STAT3, contributes to negative feedback regulation of the JAK/STAT3 signal pathway, and inhibits the self-activation of STAT3[11]. Bone marrow SOCS3 deficient mice exhibit overexpression of STAT3 and continuous activation of the JAK/STAT3 signal pathway, suggesting that STAT3 expression is negatively regulated by SOCS3[12].

POST-TRANSCRIPTIONAL REGULATION OF STAT3 EXPRESSION

Human *STAT3* gene is located on the long (q) arm of chromosome 17 at position 21.31. The encoding product of the STAT3 gene is an 89 kDa protein[13]. Further study identified a cDNA clone encoding a variant of STAT3 (named STAT3β), which is different from classic STAT3 (named STAT3α). Compared to STAT3α, STAT3β is the truncated form and lacks the internal domain of 50 base pairs located near the C-terminus (Figure 1). The encoding product of STAT3β is an 80 kDa protein. Under normal conditions, STAT3β exists in various cells, such as monocytes, lymphocytes and neutrophil granulocytes. In COS cells, STAT3β is phosphorylated at tyrosine sites by IL-5R treatment and binds to the palindromic IL-6/interferon-γ response element (pIRE) located in the promoter of intercellular adhesion molecule-1 (ICAM-1). However, this phosphorylated STAT3β exhibits a negative transcriptional regulation through inhibiting the transactivation potential of STAT3α, suggesting that STAT3β may be a dominant-negative regulator of transcription and

promotes apoptosis[14].

Depending on context, truncated STAT3β can be phosphorylated at tyrosine 705 and bind to DNA sequence that is equal to that bound by STAT3α with negative transcriptional regulation. Overexpression of STAT3β can induce apoptosis and inhibit tumor growth[15,16]. However, alternative splicing regulation by antisense oligodeoxynucleotides targeting STAT3 can specifically shift the expression from STAT3α to STAT3β. High expression of endogenous STAT3β promotes cell apoptosis and leads to cell cycle arrest. This apoptosis-promoting effect of STAT3β is independent on the inhibition of STAT3α target genes. Several genes that differ from classic STAT3α target genes are specifically decreased by STAT3β knockdown, including lens epithelium-derived growth factor, p300/CBP-associated factor, Cyclin C, peroxisomal biogenesis factor 1 and STAT1β[3], indicating that STAT3β may promote cell apoptosis through regulating its own specific target genes in addition to negative transcriptional regulation of STAT3α.

POST-TRANSLATIONAL MODIFICATION OF STAT3

STAT3 phosphorylation

STAT3 protein exists in the cytoplasm as an inactive form until phosphorylation by receptor-associated kinases. Activated JAK kinases phosphorylate STAT3 through binding of the SH2 domain to a phosphorylated tyrosine residue, by which the C-terminus of p-STAT3 triggers its release from receptor, and form a homo- or hetero-dimerization of p-STAT3. Dimerized STAT3 translocates to the nucleus and binds to the promoters bearing cognate DNA-binding sequences[17]. STAT3 can be also phosphorylated by other tyrosine kinases, such as the Src family. However, such Src-induced STAT3 phosphorylation does not always result in STAT3 activation[18]. Tyrosine phosphorylation is necessary for STAT3 activity. In addition, serine phosphorylation at residue 727 of STAT3 also leads to the up-regulation of the transcriptional activity. STAT3 phosphorylation at Ser-727 is mediated by MAPK, P38 and c-Jun N-terminal kinase (JNK) pathways, and involved in transcriptional regulation of the target genes of STAT3[19]. Ser-727 mutant STAT3 knock-in mice display impaired development and survival process[20]. Recently, several articles reported that un-phosphorylated STAT3 can interact with nuclear factor-κB (NF-κB). Un-phosphorylated STAT3 (U-STAT3)/NF-κB complex translocates into the nucleus and activates the expression of NF-κB target genes[21].

STAT3 acetylation

Protein acetylation is a crucial post-translational modification of gene expression and involved in extensive physiological and pathological processes[22]. Investigation on protein acetylation is focused on the alteration of chromatin structure and activation of transcription factors. The inhibition of histone deacetylases (HDACs) can induce the acetylation of STAT3 at Lys-685, and acetylated STAT3 (Ac-STAT3) regulates the function of dendritic cells through activating the transcription of indoleamine 2,3-dioxygenase[23].

The significant increase in STAT3 acetylation at Lys-685 is detected in tumor tissues. CD44, a transmembrane glycoprotein, has been recognized as a marker for tumor cells. Activated CD44 can bind STAT3 and p300 in the nucleus and acetylate STAT3 at Lys-685. CD44/Ac-STAT3 complex activates cyclinD1 expression by binding to its promoter, leading to cell proliferation[24]. Additionally, Ac-STAT3 may be the major determinant for promoter methylation of tumor suppressor genes. DNA methyltransferase 1 (DNMT1) is primarily involved in the maintenance of methylation. Ac-STAT3/DNMT1 complex can induce gene silencing through binding to target genes, leading to increased CpG island methylation. STAT3 mutant at Lys685 exhibits impaired STAT3 acetylation and tumor growth. Acetylation inhibitors and HDAC activators can inhibit STAT3 acetylation with demethylation and reactivation of several tumor-suppressor genes, including cyclin-dependent kinase inhibitor 2A (CDKN2A), deleted in lung and esophageal cancer 1 (DLEC1) and STAT1. In triple-negative breast cancer cells and melanoma, Ac-STAT3 is related to the methylation of the ERα gene. Therefore, inhibition of Ac-STAT3 is favorable for hormone therapy through reactivating ERα expression[25].

Other post-translational modification of STAT3

Except for phosphorylation and acetylation, STAT1 and STAT3 are also subjected to SUMOylation through binding to small ubiquitin-like modifier (SUMO). STAT3 SUMOylation suppresses the transcriptional activity of STAT3 by affecting STAT3 phosphorylation and dimerization[26].

STAT3 LOCALIZATION AND FUNCTION

Nucleo-cytoplasmic shuttling of p-STAT3

Since protein synthesis and modification are processed in the cytoplasm, most transcription factors need to pass through the nuclear pore complex and enter into the nucleus to exert their transcriptional activity. In general, proteins that have a molecular weight greater than 50 kDa require specific structural domain named nuclear localization sequence (NLS) and nuclear export sequence (NES). Both NLS- and NES-containing proteins can recognize and combine with specific soluble carriers to mediate the nucleo-cytoplasmic trafficking[27]. Most NLS can recognize importin α and co-regulate the shuttling of proteins through interacting with importin β1[28].

The transcriptional regulatory activity of STAT3 is dependent on nuclear translocation. The distinction between STAT3 and other STAT members is that activated STAT3 can shuttle between the cytoplasm and nucleus, and accumulate in the nucleus to play the role in tran-

scriptional activation. In the canonical nuclear translocation, p-STAT3 is released from the receptor, forms a homo- or hetero-dimer, and translocates into the nucleus. Importin α3 can specifically recognize the coiled-coil domain and mediate the nucleo-cytoplasmic shuttling of STAT3 protein[29].

Nucleo-cytoplasmic shuttling of U-STAT3

Previous studies showed that STAT3 protein acquires its DNA binding activity only in a phosphorylated form. However, recent studies indicated that the transcriptional activation of STAT3 in the nucleus is also independent of phosphorylation[21]. Both phosphorylated and unphosphorylated STAT3 proteins exist in the nucleus and regulate different target genes. Data from fluorescently-labeled STAT3 mutants in STAT3 deficient cells show that U-STAT3 can shuttle constitutively between the cytoplasm and nucleus under the condition of NLS and NES mutation, indicating that the nuclear accumulation of U-STAT3 is independent of the binding of NLS or NES and importins. Both native gel electrophoresis and dual-focus fluorescence correlation spectroscopy identify that the N-terminal domain is essential for dimer formation and nuclear accumulation of U-STAT3. The monomeric N-terminal deletion mutant can be phosphorylated and dimerized in response to IL-6 treatment without nuclear accumulation. Therefore, the N-terminal domain has an important role in nucleo-cytoplasmic trafficking of U-STAT3[30].

STAT3 in mitochondria

Except for the classic transcriptional regulation during cell proliferation and differentiation through nuclear translocation, STAT3 translocation in different organelles may regulate cell metabolism and be involved in a broad range of biological functions independent of transcriptional activity. For instance, phosphorylated STAT3 at Serine 727 (P-Ser-STAT3) is localized to the mitochondria of hepatocytes and myocardial cells. STAT3 deficient cells exhibit a low activity of complex I and Ⅱ[31], suggesting that STAT3 regulates mitochondrial respiration *via* electron transport chain. Data from co-immunoprecipitation indicate that the translocation of STAT3 to mitochondria is mediated by the presequence receptor Tom20[32]. However, the mechanism that STAT3 alters mitochondrial respiration is controversial. There is an unfavorable ratio of complexes Ⅰ / Ⅱ and STAT3 in cardiac tissue, which implied the existence of an additional mechanism of STAT3 regulation of ATP production *in vivo*[33]. The sirtuin 1 (SIRT1), a NAD-dependent deacetylase, is located in the nucleus and known as a key factor regulating and controlling the mitochondrial bioenergetics by means of activating gene expression through deacetylating some important signal molecules, such as STAT3. In Sirt1-null cells, there is a significantly higher serine-phosphorylated STAT3 level in mitochondria with an increase in the mitochondrial bioenergetics and ATP formation[34].

In eukaryotes, the primary function of mitochondria is aerobic respiration and energy production, in which the reactive oxygen species (ROS) is the inevitable by-products. During the process of ischemia-reperfusion injury in the myocardium, the opening of mitochondrial permeability transition pore (MPTP) is a major response to cardiomyocyte death, while the ROS from respiratory chain is the primary endogenous reason for MPTP opening. Mitochondria play a major role in cardio-protection, most likely by preventing MPTP opening, while mitochondrial STAT3 has an impact on inhibiting MPTP opening and cardio-protection. In calcium-induced MPTP opening model, STAT3-KO mitochondria tolerate less induction of MPTP opening. The function of STAT3 in MPTP stability may be carried out through binding to cyclophilin D[32]. Another study found that GRIM-19-induced mitochondrial STAT3 location may involve in TNF-mediated necroptosis[35].

It is identified that cancer cells have the feature of metabolic turnover in aerobic glycolysis - the Warburg effect[36], in which STAT3 acts as a central mediator of cell metabolism through both HIF-1α-dependent and -independent mechanisms. Oncogenic signals activate STAT3 phosphorylation and induce STAT3 translocation into the nucleus where it regulates HIF-1α expression. Mitochondrial STAT3 displays Serine 727 phosphorylation, while tyrosine phosphorylation or DNA binding activity is not detected, unlike canonical transcriptional activation. p-Ser-STAT3 located in mitochondria shows many metabolic functions and induces malignant transformation mediated by oncogenic Ras[37]. Fibroblast growth factor receptor 4-R388 (FGFR4-R388), a known single nucleotide polymorphism which promotes breast cancer cell motility and invasiveness, can promote mitochondrial cytochrome c activity and induce pituitary tumor cell growth through STAT3 serine phosphorylation. Therefore, serine phosphorylation of STAT3 and mitochondrial translocation may contribute to tumor cell transformation and tumorigenesis[38].

FUNCTION OF STAT3 IN PATHOPHYSIOLOGY AND DEVELOPMENT

STAT3 in stem cells

Mouse embryonic stem cells (ES cells) are pluripotent cells derived from the inner cell mass of blastocysts. The self-renewal and pluripotency of ES cells depend on leukemia inhibitory factor (LIF) and bone morphogenetic protein 2 (BMP2) during *in vitro* culture[39]. Based on chromatin immunoprecipitation-deep sequencing (ChIP-seq), 13 specific transcriptional factors (Nanog, Oct4, STAT3, Smad1, Sox2, Zfx, c-Myc, n-Myc, Klf4, Esrrb, Tcfcp2l1, E2f1, and CTCF) and 2 transcription regulators (p300 and Suz12) are identified in the regulatory network of ES cells, and these factors are involved in LIF and BMP signaling pathways, and play important roles in self-

renewal, reprogramming and pluripotency of ES cells[40].

LIF activates STAT3 through the Janus kinase (JAK) signal pathway. p-STAT3 is functionally associated with the transcriptional regulation of target genes for the self-renewal of ES cells, including Kruppel-like factors (Klf4 and Klf5)[41]. Furthermore, persistently activated STAT3 can maintain the self-renewal process without LIF[42]. Transcriptional factors Nanog and STAT3 are the molecular markers of ES cells. Nanog and STAT3 co-regulate the transcriptional activation of STAT3 target genes through binding to their promoters, such as α2M and Nanog promoters. This activation is abrogated by eliminating LIF, indicating that the function of Nanog and STAT3 is dependent on the LIF signal pathway[43]. Overexpression of STAT3 target genes, such as Klf4 and Klf5[41], has been shown to promote self-renewal of ES cells, while knockdown of these genes has no impact on the self-renewal in the presence of LIF or STAT3[44]. Gastrulation brain homeobox 2 (Gbx2), a LIF/STAT3 target gene, can facilitate the pluripotency of ES cells when over-expressed without LIF and STAT3[45]. These results illustrated that LIF/STAT3 may act upstream to trigger the maintenance of ES cells through activating a range of downstream target genes.

STAT3 in proliferation and apoptosis

P-STAT3 can activate proliferation-related genes to promote cell proliferation. Moreover, U-STAT3 can bind to the promoters of pro-apoptotic genes and inhibit their expression in tumor cells, but not in normal cells. Inhibitors of STAT3 phosphorylation or dominant-negative STAT3 mutants facilitate the expression of pro-apoptosis factors, suggesting that STAT3 plays a dominant role in regulating cell proliferation and anti-apoptosis[46]. STAT3 knockout mice exhibit complete embryonic lethality. STAT3 deficient embryos show a rapid degeneration on day 7 of pregnancy, highlighting the important role of STAT3 in embryo development[47]. Conditional ablation of STAT3 in myocardial cells leads to higher susceptibility to drug-induced heart failure[48]. In addition, ischemic preconditioning can induce the phosphorylation of STAT3 at Tyr-705 and Ser-727 in myocardial cells. However, the expression of cardio-protective factor (COX-2 and HO-1) and anti-apoptotic proteins [Mcl-1, Bcl-x (L) and c-FLIP (S)] is elevated in normal cells 24 h later, but not in STAT3 deficient cells[49]. These results illustrated the function of STAT3 in anti-inflammation and anti-apoptosis.

Mammary gland involution initiates at the ending of lactation, involving extensive apoptosis of the secretory alveolar epithelium and inflammatory response. Although STAT3 is expressed in the mammary gland throughout the whole reproductive cycle, it is only activated by LIF on the day of delivery and at 6-12 h after weaning[50]. STAT3 has an important role in mammary gland involution. Conditional ablation of STAT3 in mammary cells causes delayed involution of the mammary gland[51]. STAT3 is involved in the apoptotic process of mammary epithelial cells and tissue remodeling through inducing the expression of pro-apoptotic factors and regulating the balance of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP)[52]. Mammary STAT3 deficient mice have impaired accumulation of inflammatory factors, macrophages and mastocytes in the mammary gland[53]. In addition, p-STAT3 in mammary epithelial cells is also involved in lysosomal-mediated cell death pathway through up-regulating the expression of lysosomal proteases cathepsin B and L[54]. Therefore, STAT3 expression in the mammary gland may participate in apoptosis under physiological conditions.

STAT3 in tumorigenesis and cancer-related inflammation

As a key transcriptional factor, p-STAT3 can translocate into the nucleus and bind to specific DNA sequences to activate the expression of target genes, including c-Myc and FGFR2, consequently regulating the proliferation, differentiation and anti-apoptosis of tumor cells[55,56]. Furthermore, acetylated STAT3 can induce the down-regulation of tumor suppressor genes through promoter methylation and facilitate tumorigenesis. MicroRNAs are short non-coding RNAs (ncRNAs) mediating post-transcriptional down-regulation of target genes and functioning in cell proliferation and apoptosis. MicroRNA-21 (miR-21) is an oncogene that contributes to anti-apoptosis in most tumor cells. There are two strictly conserved STAT3 binding sites in the enhancer of miR-21. MiR-21 induction by IL-6 is STAT3-dependent. ChIP results also confirm the accumulation of STAT3 in the upstream enhancer of miR-21[57], indicating that IL-6/STAT3 pathway contributes to miR-21 induction.

Chronic infection and inflammation contribute to about 15% of human cancers. The inflammatory response can induce necrotic cell death accompanied with activation of numerous cytokines, growth factors and chemokines which facilitate cell proliferation and survival[58]. The STAT3 signal pathway is the major intrinsic pathway for inflammation in tumor cells. STAT3 activates many inflammatory-related genes including BCL-XL, intercellular adhesion molecule 1 and vascular endothelial growth factor, and is involved in the maintenance of inflammatory environment[59]. NF-κB has the ability to induce the expression of inflammatory mediators, and is the major pathway functioning in inflammation-induced carcinogenesis and anti-tumor immunity. The signaling pathways of STATs, especially STAT3, are closely related with NF-κB signaling[60]. The inflammatory factor IL-6, the target gene of NF-κB, is the important STAT3 activator. In tumor cells, STAT3 directly interacts with NF-κB, translocates into the nucleus and contributes to the constitutive NF-κB activation in cancer. In addition, STAT3 binding to NF-κB also regulates numerous oncogenic and inflammatory genes[61].

Targeting the STAT3 pathway should be a promising and novel form of treatment for human cancers. Blocking STAT3 by siRNAs, antisense oligonucleotides, dominant-negative mutants, and specific inhibitors of

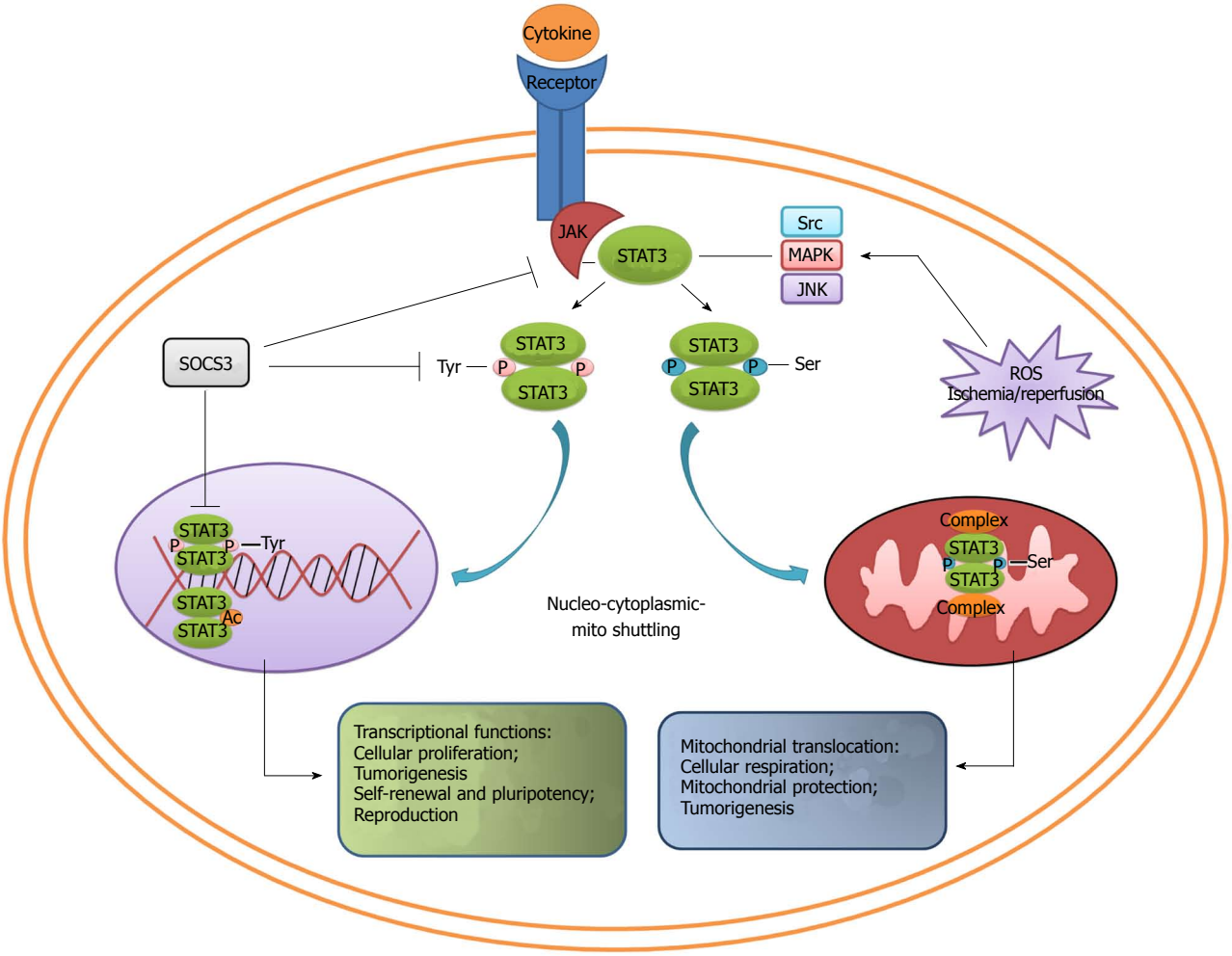

Figure 2 Converging roles of signal transducer and activator of transcription 3. Different signals can selectively trigger signal transducer and activator of transcription 3 phosphorylation. Tyr-phosphorylated STAT3 translocates into the nucleus and regulates gene expression, thus playing an important role in cell proliferation, tumorigenesis, self-renewal and pluripotency. On the other hand, Ser-phosphorylated STAT3 translocates into mitochondria, binds with the complexes in respiratory chain, and ultimately maintains the cellular respiration and mitochondrial protection. MAPK: Mitogen-activated protein kinase; ROS: Reactive oxygen species; JNK: c-Jun N-terminal kinase; JAK: Janus kinase.

STAT3 in combination with chemotherapeutics can synergistically inhibit the growth, invasion and metastasis of carcinoma cells[62-64]. Therefore, inhibiting STAT3 signals are a promising therapeutic target for most types of human cancers with constitutively activated STAT3.

STAT3 in reproduction

In mammals, the uterus is receptive to blastocyst during a restricted time termed as "implantation window". LIF is expressed at a high level during implantation window in humans and mice. LIF deficient mice display embryo implantation failure[65]. In mouse uterus, STAT3 protein is expressed and phosphorylated in the luminal epithelium on day 4 of pregnancy. LIF treatment induces the STAT3 phosphorylation in mouse uterine luminal epithelium isolated from day 4 of pregnancy but not for days 3 and 5[66]. LIF antagonist (LA, truncated LIF protein) injection led to the failure of mouse embryo implantation through inhibiting STAT3 phosphorylation[67]. In humans, LIF and STAT3 are expressed in decidual tissues during early pregnancy. LIF can activate STAT3 phosphorylation in both non-decidualized and decidualized human endometrial stromal cells *in vitro*[68], indicating that LIF/STAT3 signaling is involved in human embryo implantation and decidualization.

To investigate the function of STAT3 during embryo implantation, a cell-permeable STAT3 peptide inhibitor is injected into mouse uterine lumen before implantation, which significantly reduces embryo implantation by 70%. STAT3 phosphorylation in uterine luminal epithelium activated by LIF and some LIF targeted genes, such as Irg1, is significantly inhibited by STAT3 inhibitors both *in vivo* and *in vitro*[69]. Meanwhile, the injection of STAT3 decoy into uterine lumen during implantation also causes implantation failure[70]. Co-immunoprecipitation assay showed that STAT3 can bind to progesterone receptor A (PR-A) and co-regulate the embryo implantation and decidualization in mice. Conditional ablation of STAT3 only in PR-positive cells ($PR^{cre/+}Stat3^{f/f}$; $Stat3^{d/d}$) is used to investigate the role of STAT3 in reproduction. Conditional ablation of STAT3 in the uterus ($Stat3^{d/d}$) results in embryo implantation failure. Furthermore, $Stat3^{d/d}$ mice

are also defective in hormonally induced decidual reaction[71], suggesting that the interaction between STAT3 and PR is essential for successful implantation.

CONCLUSION

STAT3 is a key transcription factor and regulates a multitude of genes important for proliferation, differentiation, apoptosis, inflammation and tumorigenesis. STAT3 expression and activity are regulated through alternative splicing, post-translational modification and subcellular localization. STAT3β, the new isoform of STAT3, participates in apoptosis and plays a role distinct from STAT3α. Despite the different mechanism, STAT3 activation through phosphorylation or acetylation can facilitate tumorigenesis synergistically. STAT3 shuttles among the cytoplasm, nucleus, mitochondria and some other possible organelles, and exerts its diverse functions in transcriptional regulation, cellular respiration, proliferation and apoptosis. A variety of animal models reveal that STAT3 is essential for embryo development, pluripotency maintenance of stem cells, embryo implantation and decidualization. Increasing evidence confirms that STAT3 is a key modulator of cancer and inflammation (Figure 2). Hence, further clarification of the biological function of STAT3 will validate its promising application prospect for gene therapy in multi-directions.

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ORIGINAL ARTICLE

Functional analysis of human Na^+/K^+-ATPase familial or sporadic hemiplegic migraine mutations expressed in *Xenopus* oocytes

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Abstract

AIM: Functional characterization of ATP1A2 mutations that are related to familial or sporadic hemiplegic migraine (FHM2, SHM).

METHODS: cRNA of human Na^+/K^+-ATPase α_2- and β_1-subunits were injected in *Xenopus laevis* oocytes. FHM2 or SHM mutations of residues located in putative α/β interaction sites or in the α_2-subunit's C-terminal region were investigated. Mutants were analyzed by the two-electrode voltage-clamp (TEVC) technique on *Xenopus* oocytes. Stationary K^+-induced Na^+/K^+ pump currents were measured, and the voltage dependence of apparent K^+ affinity was investigated. Transient currents were recorded as ouabain-sensitive currents in Na^+ buffers to analyze kinetics and voltage-dependent pre-steady state charge translocations. The expression of constructs was verified by preparation of plasma membrane and total membrane fractions of cRNA-injected oocytes.

RESULTS: Compared to the wild-type enzyme, the mutants G900R and E902K showed no significant differences in the voltage dependence of K^+-induced currents, and analysis of the transient currents indicated that the extracellular Na^+ affinity was not affected. Mutant G855R showed no pump activity detectable by TEVC. Also for L994del and Y1009X, pump currents could not be recorded. Analysis of the plasma and total membrane fractions showed that the expressed proteins were not or only minimally targeted to the plasma membrane. Whereas the mutation K1003E had no impact on K^+ interaction, D999H affected the voltage dependence of K^+-induced currents. Furthermore, kinetics of the transient currents was altered compared to the wild-type enzyme, and the apparent affinity for extracellular Na^+ was reduced.

CONCLUSION: The investigated FHM2/SHM mutations influence protein function differently depending on the structural impact of the mutated residue.

Key words: Na^+/K^+-ATPase; Electrophysiology; Voltage dependence; Familial hemiplegic migraine; C-terminus; β-subunit

Core tip: Mutations of the human *ATP1A2* gene, which encodes the Na^+/K^+-ATPase α_2-subunit, are associated with familial hemiplegic migraine (FHM2) that is inherited in an autosomal dominant fashion. We studied seven ATP1A2 mutations related to FHM2 or sporadic hemiplegic migraine by electrophysiological and biochemical methods to characterize functional impairments. The mutations G855R, G900R, E902K, L994del, D999H, K1003E and Y1009X were selected according to their structural importance: in putative interaction sites between α- and β-subunit and in the α-subunit's C-terminal region. Some of these mutations showed a severe loss of function, and we discuss the functional and physiological consequences in order to better un-

derstand the molecular basis for neurological impairments.

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INTRODUCTION

Migraine is a common neurological disease, and the different forms are defined by the International Headache Society criteria[1]. Familial hemiplegic migraine (FHM) and sporadic hemiplegic migraine (SHM) are rare autosomal-dominant subforms of migraine with aura. These syndromes are associated with some degree of motor weakness (hemiparesis) and other neurological symptoms during the aura phase. FHM is inherited in an autosomal dominant fashion and genetically heterogeneous. There are a number of mutations related to FHM in three different genes: the *CACNA1A* gene (FHM1) coding for the neuronal $Ca_V2.1$ calcium channel[2,3], the *ATP1A2* gene (FHM2) encoding the α_2-subunit of the Na^+/K^+-ATPase[4], and the *SCN1A* gene (FHM3) encoding the neuronal $Na_V1.1$ sodium channel[5]. The clinical symptoms of SHM are identical to those of FHM but without affected family members.

The Na^+/K^+-ATPase is a transmembrane protein which transports two K^+ ions in and three Na^+ ions out of the cell upon hydrolysis of ATP (Figure 1A). This electrogenic P-type ATPase assumes two principal conformational changes during its reaction cycle. Upon binding of three intracellular Na^+ ions in the ATP-bound E_1 conformation, the phosphorylated intermediate with occluded Na^+, $E_1P(3Na^+)$, is formed, followed by a change to the phosphorylated $E_2P(3Na^+)$ conformation, from which Na^+ ions are released to the extracellular medium. Because of the increased affinity for K^+ in this configuration, two K^+ ions bind subsequently, which triggers the dephosphorylation, and binding of intracellular ATP accelerates the conformational change from E_2 to E_1. At last, the K^+ ions dissociate to the cytoplasm. The sequential translocation of Na^+ and K^+ ions requires strict cation specificity of the phosphorylation and dephosphorylation reactions. According to the $3Na^+/2K^+$ stoichiometry of transport, electrogenic turnover activity of the Na^+/K^+-ATPase corresponds to outward movement of one positive charge per reaction cycle, and the major electrogenic event has been shown to take place during extracellular release or reverse binding of Na^+[6-8]. This has been suggested to arise from passage of Na^+ ions through a narrow, high-field access channel or 'ion well'[9,10].

The Na^+/K^+-ATPase consists of at least two mandatory subunits (Figure 1B). The large catalytic α-subunit is composed of ten transmembrane domains (M1-M10), which are linked by five extracellular and four intracellular loops. The smaller regulatory β-subunit is a single-span transmembrane protein (βM) with an ectodomain exhibiting several glycosylation sites. Several isoforms of both subunits are expressed in human cells in a tissue-specific manner. In human brain, the α_2-subunit is mainly expressed in glial cells (astrocytes), and loss-of-function of the Na^+/K^+-ATPase can result in neuronal hyperexcitability, which is commonly explained as follows. The Na^+/K^+-ATPase maintains the gradients for K^+ and Na^+ ions, which are essential for the accurate function of secondary active transporters or ion channels, whose activities depend on these gradients. On one hand, changes of the Na^+ gradient influence, first, the activity of the Na^+/Ca^{2+} exchanger (NCX) which is crucial for, *e.g.*, Ca^{2+} signaling. Second, the ability of the glial Na^+/glutamate symporter to remove the neurotransmitter glutamate from the synaptic cleft is affected. On the other hand, an altered K^+ gradient impairs the repolarizing activity of neuronal K^+ channels, which is critical for setting the threshold of action potential generation. Hyperkalemia is known to trigger the phenomenon of cortical spreading depression (CSD), the putatively causal mechanism of the aura phase during a migraine attack[11].

Up to now, far more than 50 mutations of the *ATP1A2* gene, which are associated with SHM or FHM2, have been described in literature[12,13]. Yet, most of these mutations have not been studied by electrophysiological techniques, which is a prerequisite for a better understanding of the functional consequences on enzyme activity.

In continuation of previous works[14,15], we studied seven FHM2 or SHM mutations, which are located in regions that are putatively critical for transport properties of the human Na^+/K^+-ATPase α_2-subunit, (Figure 1B), with the two-electrode voltage-clamp technique (TEVC) and biochemical methods to analyze protein expression. Since mutations in the α_2-subunit's C-terminal region were shown to have complex effects on enzyme activity, cation affinities and voltage dependence[16-19], we analyzed four mutations in the transmembrane segment αM10 and in the C-terminus (L994del, K1003E[13], D999H[20] and Y1009X[21]) to further understand structure-function relationships in the C-terminal region. Furthermore, interactions between the α- and β-subunit are not satisfactorily clarified so far. Especially, the highly conserved SYGQ motif in the αM7/M8-loop is believed to interact with the β-ectodomain[22,23]. The FHM2 mutations G900R[24] and E902K[25] are located within this motif and were functionally analyzed in this work. In addition, Gly852 (αM7) has previously been shown to interact with two tyrosines of the βM[26]. In this work, we show that the FHM2 mutation G855R[27] which is located near this interaction site, has severe consequences on the mutant protein's plasma membrane expression.

MATERIALS AND METHODS

Mutagenesis

As described before[14,19], human Na^+/K^+-ATPase α_2- and β_1-subunit cDNAs were subcloned into a modified pCD-

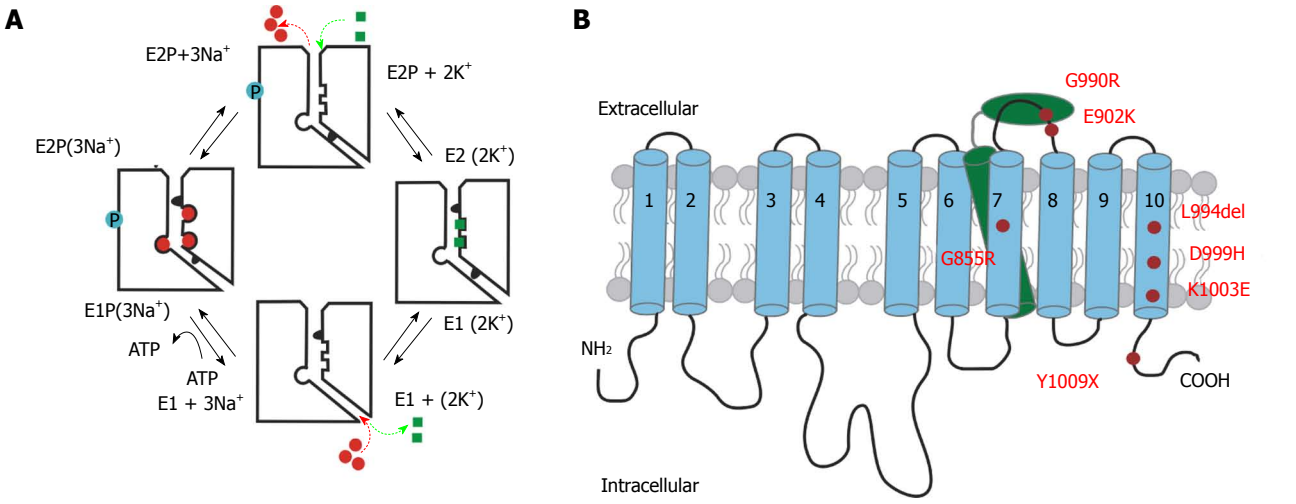

Figure 1 Reaction mechanism and structural detail of the Na^+/K^+-ATPase. A: Schematic reaction cycle of one Na^+/K^+-ATPase pump molecule. The cytosolic side is shown at the bottom of each molecule depicted with an ion pathway to the right, whereas the extracellular side is set at the top. Na^+ ions are shown as red circles, and K^+ ion are shown as green squares. Blue circles depict the phosphorylated state; B: Simplified structure of the Na^+/K^+-ATPase indicating FHM2/SHM mutation positions studied in this work. The α-subunit is composed of ten transmembrane domains (blue). The N- and C-terminus are located intracellularly. The β-subunit comprises only one transmembrane domain (green) and a large ectodomain with several glycosylation sites. FHM2/SHM mutations are marked in red.

NA3.1 vector. To distinguish the activity of the heterologously expressed constructs from the endogenous *Xenopus* Na^+/K^+-ATPase, the mutations Q116R and N127D were introduced in the human α_2-subunit to reduce the ouabain sensitivity (IC_{50} in a mmol/L range)[28]. This construct is herein referred to as "RD-WT". Mutants were designed by introducing mutations into the RD-WT α_2-construct by site-directed mutagenesis (Quikchange® kit, Stratagene). All PCR-derived fragments were verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Two-electrode voltage-clamp

cRNA synthesis was carried out with the T7 mMessage mMachine kit (Ambion, Austin, TX). 25 ng of α_2- and 2.5 ng of β_1-subunit cRNAs were coinjected into oocytes of *Xenopus laevis*. After three days incubation in ORI buffer (contents in mmol/L: 110 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, pH 7.4, and 50 mg/L gentamycin) at 18 ℃, oocytes were subjected to a Na^+ loading procedure preceding experiments to elevate $[Na^+]_{in}$. For this purpose, oocytes were incubated for 45 min in Na^+ loading solution (contents in mmol/L: 110 NaCl, 2.5 sodium citrate, 5 MOPS, 5 TRIS, pH 7.4) and stored subsequently in Na^+ buffer (in mmol/L: 100 NaCl, 1 $CaCl_2$, 5 $BaCl_2$, 2 $MgCl_2$ and 2.5 MOPS, 2.5 TRIS, pH 7.4) for at least 30 min.

Currents were recorded at room temperature (21 ℃-23 ℃) using a Turbotec 10CX amplifier (NPI instruments, Tamm, Germany) and pClamp 10 software (Axon Inst., Union City, CA). Solutions used for measurements were: Na^+ buffer (in mmol/L: 100 NaCl, 1 $CaCl_2$, 5 $BaCl_2$, 2 $MgCl_2$, 2.5 MOPS, 2.5 TRIS, 0.01 ouabain, pH 7.4), and K^+ buffers with distinct K^+ concentrations, which were prepared by adding appropriate amounts of KCl to Na^+ buffer.

Stationary currents

K^+-induced currents were determined as the difference of currents measured in a distinct K^+ buffer and currents measured in Na^+ buffer. Oocytes were subjected to the following voltage pulse protocol: from -30 mV holding potential, cells were clamped to potentials between +60 mV and -140 mV (in -20 mV decrements) for 200 ms, followed by a pulse back to -30 mV. All currents within one experiment were normalized to the pump current amplitude at 10 mmol/L K^+ and 0 mV. To determine the apparent affinity for extracellular K^+, voltage-dependent $K_{0.5}(K^+_{ex})$ values were determined using fits of a Hill equation $I = \frac{I_{max}}{1+\left(\frac{K_{0.5}}{[K^+]}\right)^{n_H}}$ to the normalized K^+-induced currents at a given membrane potential ($K_{0.5}$ is the concentration at half-maximal current, and n_H is the Hill coefficient). n_H values from the fits were between 1 and 1.5.

Analysis of transient currents

To obtain kinetic information about extracellular Na^+ binding/release and the voltage-dependent distribution of pump molecules between E_1P and E_2P states, pre-steady state currents under Na^+/Na^+ exchange conditions were recorded. These ouabain-sensitive transient currents were calculated as the difference between currents measured in Na^+ buffer with 10 μmol/L ouabain (blocking only the endogenous Na^+ pump) and in the presence of 10 mmol/L ouabain (to inhibit the RD-mutated enzyme). Data were fitted by using a monoexponential function, excluding the first 3-5 ms to eliminate capacitive artifacts, yielding time constants τ and amplitudes A. The translocated charge Q was determined from the product $A \times \tau$. The resulting Q(V) curves were approximated by a

Boltzmann function:

$$Q(V) = Q_{min} + \frac{Q_{max}-Q_{min}}{1 + \exp\left(\frac{z_q \times F(V-V_{0.5})}{RT}\right)}$$

where Q_{max} and Q_{min} are the saturation values of Q(V), $V_{0.5}$ is the half-maximal voltage at which equal distribution of E_1P and E_2P states is achieved, z_q the fractional charge, F the Faraday constant, R the molar gas constant, T the temperature, and V the membrane potential. After fitting, the translocated charge values were normalized to saturating values (Q_{max} - Q_{min}) after subtracting Q_{min}.

Isolation of membrane fractions from oocytes

To assess impairments in plasma membrane targeting or expression of mutant proteins that showed no pump current activity in TEVC experiments, plasma membrane (PM) and total membrane (TM) fractions were isolated from oocytes injected with cRNA of the constructs as described before[14,29]. All obtained samples were dissolved in SDS-PAGE sample buffer, and the amount of protein corresponding to the equivalent of two oocytes was separated by 10%SDS-PAGE and blotted on nitrocellulose membranes. Since oocytes are homogenous in size, the procedure of loading the equivalent of a certain number of cells provides an internal loading standard, as shown previously[15]. The α_2-subunits of Na^+/K^+-ATPase were detected with the specific polyclonal antibody AB9094 (Chemicon, Temecula, CA). Afterwards, blots were incubated with a HRP-conjugated secondary antibody (Dako, Glostrup, Denmark). Proteins were visualized by an enhanced chemiluminescence reaction (Roche, Mannheim, Germany).

Structural examinations and figures

Structural inspections of the Na^+/K^+-ATPase (PDB structure entry 3B8E) were carried out with Swiss PDB viewer 3.7. Figures were prepared with PyMOL 1.0r1 (http://www.pymol.org). Data analysis and figure presentation were carried out with Origin 7.0 (OriginLab Corp., Northampton, MA).

Statistical analysis

Statistical analyses were carried out based on the Student's *t*-test for independent samples. The significance level $P < 0.05$ is indicated in the figures by an "a" above the data points reaching this significance level.

RESULTS

Stationary K^+-induced pump currents and apparent K^+_{ex} affinity

From the investigated ATP1A2 mutants, only G900R, E902K, D999H and K1003E showed K^+-induced currents with amplitudes that were sufficiently large for electrophysiological analysis (> 10 nA, Figure 2), whereas no measurable pump activity could be detected for the mutants G855R, L994del and Y1009X. For G900R, E902K and K1003E, the bell-shaped I(V) curves at different $[K^+]_{ex}$ did not differ significantly from those of the RD-WT enzyme. This voltage dependence of currents is due to the extracellular competition between K^+ and Na^+ ions for the two "shared" cation binding sites. With proceeding hyperpolarization of the membrane, reverse binding of extracellular Na^+ is stimulated and K^+ pump activity inhibited[30,31].

For D999H, in contrast, the voltage dependence of K^+-induced currents apparently deviated from RD-WT behavior (Figure 2C). In general, at negative potentials, the current amplitudes of the mutant were small compared to RD-WT amplitudes (data not shown), but at +60 mV, they were in the same range as RD-WT amplitudes (100-200 nA). We suppose that the activity of the D999H construct was similar to the RD-WT enzyme at positive potentials. In contrast to the RD-WT enzyme, the I(V) curves of D999H at high K^+ concentrations (2, 5, 10 mmol/L) were nearly constant at potentials between -100 to -40 mV and even increased at hyperpolarization below -100 mV, indicating that the inhibition of K^+ pump activity by reverse binding of extracellular Na^+ is not as efficient as in the RD-WT enzyme. At potentials more positive than -20 mV, the K^+-induced currents started to rise steeply, which shows that positive membrane potentials had a stronger effect on enzyme activity of the D999H mutant compared to the RD-WT enzyme in this voltage range.

As for the apparent K^+ affinity in Na^+ containing buffers, $K_{0.5}(K^+)$ values were determined from K^+-induced currents at different $[K^+]_{ex}$ and plotted as a function of the membrane potential (Figure 3). For G900R, E902K, K1003E and RD-WT, the voltage dependence of $K_{0.5}(K^+)$ values can be approximated by a parabolic function. The minimal $K_{0.5}(K^+)$ values were similar, with values between 1.09-1.25 mmol/L (Table 1). For the RD-WT enzyme, the apparent K^+ affinity decreases at negative potentials because the reverse binding of extracellular Na^+ is stimulated. In contrast, the $K_{0.5}(K^+)$ values determined for mutant D999H did not increase at hyperpolarization, but were nearly voltage-independent between -140 mV and -40 mV (Figure 3C). The minimal $K_{0.5}(K^+)$ value was 0.67 mmol/L and shifted to negative potentials. Apparently for D999H, extracellular Na^+ does not compete as efficiently with K^+ as for the RD-WT enzyme, which indicates a reduced affinity of the mutant for extracellular Na^+ (or destabilization of the Na^+-bound E_2 state). To further investigate this question, the electrogenic Na^+/Na^+ exchange mode was examined.

Electrogenic Na^+/Na^+ exchange

To investigate changes in apparent Na^+_{ex} affinity, we measured transient currents under Na^+/Na^+ exchange conditions (ouabain-sensitive currents, 0 mmol/L K^+). Representative transient currents of the RD-WT enzyme are shown as inset in Figure 4E, and the reciprocal time constants of the charge translocation are shown in Figure 5. Basically, the voltage dependence of the reciprocal time constants determined for mutants G900R, E902K and K1003E conformed to that of the RD-WT protein.

Table 1 Minimal $K_{0.5}$ values from $[K^+]_{ex}$ dependence of pump currents and parameters of Boltzmann fits to Q(V) curves derived from transient currents (means ± SE)

	Minimal $K_{0.5}$ (K^+)/mmol/L	Membrane potential at minimum/mV	$V_{0.5}$/mV	zq
RD-WT	1.12 ± 0.01	-6.2 ± 1.5	0.9 ± 1.3	0.77 ± 0.02
G900R	1.09 ± 0.04	0.2 ± 4.7	0.3 ± 3.1	0.76 ± 0.02
E902K	1.25 ± 0.03	-15.2 ± 2.3	-2.1 ± 2.1	0.81 ± 0.02
D999H	0.67 ± 0.08	-97.6 ± 4.4	-67 ± 14	0.33 ± 0.11
K1003E	1.10 ± 0.03	6.6 ± 3.3	-11.3 ± 4.3	0.75 ± 0.06

However, kinetics of charge translocation was slightly faster for these mutants compared to the RD-WT enzyme. Especially for G900R and E902K, the rise of the reciprocal time constants (τ^{-1}) at hyperpolarizing potentials was enhanced.

The voltage dependence of charge translocation is shown in Figure 4 and provides information about the distribution of pump molecules between E_1P and E_2P states[32]. For the mutants G900R and E902K, the Q(V) curves are similar to that of the RD-WT protein, and the $V_{0.5}$ values in particular did not differ (Table 1). The $V_{0.5}$ value of mutant K1003E was shifted by -5 to -15 mV. This hints at a slightly reduced apparent Na^+_{ex} affinity of this mutant[10,33], which, however, does not seem to impair function in terms of the voltage dependence and the amplitudes of K^+-induced currents (Figure 2D).

The D999H mutation had more severe consequences on Na^+/Na^+ exchange. In general, the transient current signals were fast and small compared to the RD-WT enzyme (data not shown). In addition, the Q(V) curve of translocated charge was linearly dependent on membrane potential, and saturating values were not clearly detectable within the investigated voltage range (Figure 4C). Hence, the approximation with a Boltzmann function and determination of $V_{0.5}$ proved to be difficult. For fitting, the z_q value (Table 1) was reduced until the fitted function superposed the Q values. For this reason, the determined z_q can only be regarded as an upper limit, and with a value of 0.33, z_q was very small compared to the RD-WT enzyme (0.77). Since $V_{0.5}$ also directly depends on the quality of the fit, it is likely that the shift of $V_{0.5}$ by about -70 mV is only a rough estimate for the lower limit of the actual shift. Nonetheless, this strong negative shift shows that D999H has a considerably reduced affinity for extracellular Na^+ since very strong hyperpolarization is required to force Na^+ ions into the binding sites and to enable the subsequent conformational change to E_1P[10,33]. This is in good agreement with the simultaneously reduced $K_{0.5}(K^+)$ values at negative potentials. Furthermore, kinetics of the D999H transient currents was less voltage-dependent than for the RD-WT protein (Figure 5C). τ^{-1} values varied between 200 and 300 s^{-1} at potentials below 0 mV and increased up to 400 s^{-1} at depolarization. These results show that the apparent affinities for Na^+ and K^+ (or stabilization of the cation-occluded state) as well as charge translocation and kinetics of the Na^+/Na^+ exchange reaction were significantly affected by this mutation.

Plasma membrane protein expression

Since the constructs G855R, L994del and Y1009X did not yield measurable Na^+/K^+ pump currents in TEVC experiments, it was necessary to examine whether or not these proteins were expressed in oocytes and properly targeted to the plasma membrane. For this purpose, plasma membrane (PM) and total intracellular membrane (TM) fractions were prepared using oocytes that had been injected with cRNA of these constructs. Representative Western blots with TM and PM fractions of G855R, L994del (Figure 6C) and Y1009X (Figure 6B) are shown in Figure 6. Densitometric analysis of four Western blots prepared from independent cell batches indicated a disturbed expression pattern of these mutants (Figure 6C). By trend, larger amounts of mutant proteins could be detected in the TM fraction than for the RD-WT protein, which in turn was highly concentrated in the PM fraction. However, analysis of the PM fractions showed that the mutants were not or only minimally expressed in the plasma membrane. The band intensities of PM fractions were only 10%-20% of RD-WT values. Thus, G855R, L994del and Y1009X accumulate in cytoplasmic membranes, and targeting to the plasma membrane was disturbed by these mutations.

DISCUSSION

α/β-interactions

Several studies have shown that the C-terminal ectodomain of the β-subunit is important for modulation of cation transport by the Na^+/K^+-ATPase[34-36]. A motif of eight amino acids (Asp897-Tyr905, amino acid sequence DSYGQEWTY) in the αM7/M8-loop seems to be of special importance. Interactions of the β-subunit with this sequence element that encompasses a highly conserved SYGQ motif were identified as crucial for correct folding of newly synthesized α-subunits in the endoplasmic reticulum, and furthermore, it is suspected that an hypothetical sequence motif for proteolytic degradation is masked by these interactions[22,37,38]. Four FHM2/SHM-associated mutations have been identified in the extracellular αM7/M8-loop so far: W887R, G900R, E902K and R908Q[4,24,25,39]. W887R and R908Q, which are not directly located in the SYGQ motif, have already been analyzed[26,40].

The W887R construct was found to be correctly targeted to the plasma membrane of *Xenopus* oocytes[40],

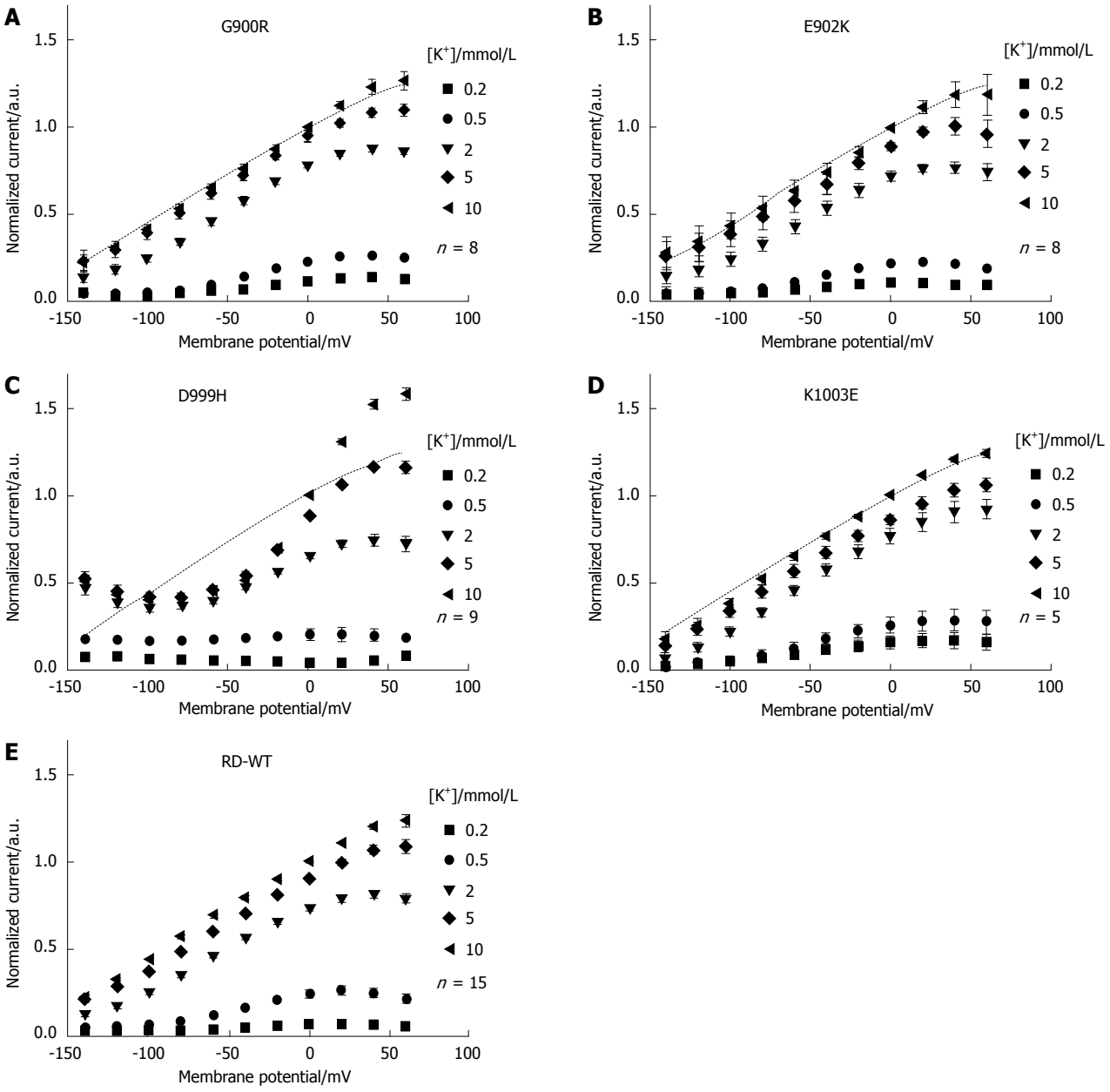

Figure 2 Voltage and $[K^+]_{ex}$ dependence of stationary currents for ATP1A2 RD-WT, G900R, K902E, D999H and K1003E. A-E: Dependence of K^+-induced stationary currents of the RD-WT enzyme (E) and the mutants G900R (A), E902K (B), D999H (C) and K1003E (D) on the extracellular K^+ concentration and on membrane potential. $[K^+]_{ex}$-dependent currents were calculated as the difference between currents induced by voltage steps first in presence of different $[K^+]_{ex}$ and then at $[K^+]_{ex}$ = 0. The amplitudes at $[K^+]_{ex}$ = 10 mmol/L and 0 mV were used for normalization. Different $[K^+]_{ex}$ are indicated by symbols. The RD-WT curve at 10 mmol/L K^+ is superimposed as dotted line for comparison. Data are means ± SE obtained from 5-15 cells of at least three batches.

but this mutation caused a complete loss-of-function and a strongly reduced ouabain affinity. Koenderink *et al*[29] argued that Trp887 might rather have an influence on Arg880, which is critical for ouabain sensitivity, than on targeting-relevant interactions between α- and β-subunits. However, the loss of catalytic function might be due to disturbed α/β-interactions during ion transport. The R908Q mutation, which is very close to the SYGQ motif, indeed affected targeting, since plasma membrane expression in *Xenopus* oocytes was reduced compared to the RD-WT protein, which easily explains the diminished pump currents[26]. The highly conserved residues Gly900 and Glu902 are located directly in the SYGQ motif and are presumably important for interactions with the β-ectodomain. It was expected that the mutations G900R, which substitutes the small unpolar glycine with a large positively charged arginine, and E902K, where the negatively charged glutamic acid is replaced by a positively charged lysine, would have a strong effect on function. However, both constructs showed no differences compared to the RD-WT enzyme, neither regarding pump activity (Figure 2A, B) nor the apparent affinities for extracellular K^+ ($K_{0.5}(K^+)$ values in Figure 3A, B) and for extracellular Na^+ (Q(V) curves and $V_{0.5}$ values in Figure 4A, B). Presumably, either these amino acids are not directly interacting with the β-subunit, or the positively

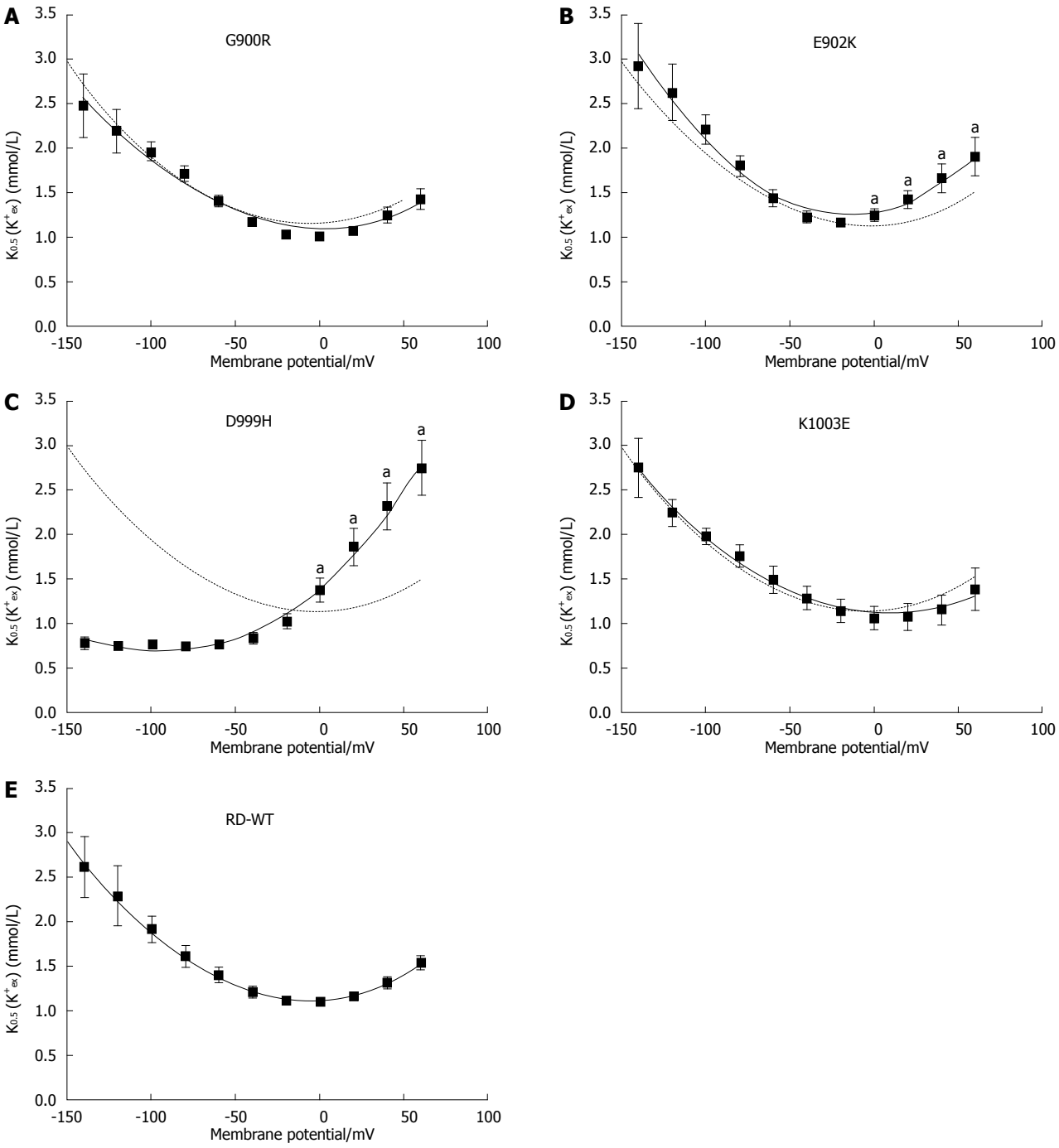

Figure 3 Apparent K^+ affinity. A-E: $K_{0.5}$ values for the $[K^+]_{ex}$ dependence of stationary currents at different membrane potentials for the RD-WT enzyme (E) and the mutants G900R (A), E902K (B), D999H (C) and K1003E (D), as calculated from fits of a Hill function to the data in Figure 2, respectively. Data were approximated with polynomial functions of second or third (D999H) grade to determine the minimum. The curve derived from RD-WT data is superimposed as dotted line for comparison. An "a" indicates that the data point was significantly different from the RD-WT data ($^aP < 0.05$ *vs* RD-WT, Student's *t*-test). Data are means ± SE obtained from 5-15 cells of at least three batches.

charged side chains of arginine and lysine do not interfere with α/β-interactions, at least under the conditions of our study.

According to the crystal structure of the Na^+/K^+-ATPase[16,23], Tyr39 and Tyr43 of βM can directly interact with residues at positions 848-856 in αM7 (Figure 7A). Especially, interactions between Gly852 (M7) and both aforementioned tyrosines of the β-subunit seem to stabilize the E_2 conformation, and, as confirmed by mutagenesis studies[26,41], not only are hydrogen bonds involved, but also the aromatic ring system of the tyrosines. The β-subunit stabilizes the orientation of αM7 and, consequently, also the position of αM5 because Tyr851 (αM7) can interact with Asn780 in αM5. These interactions are relevant for conformational stabilization during K^+ transport[26].

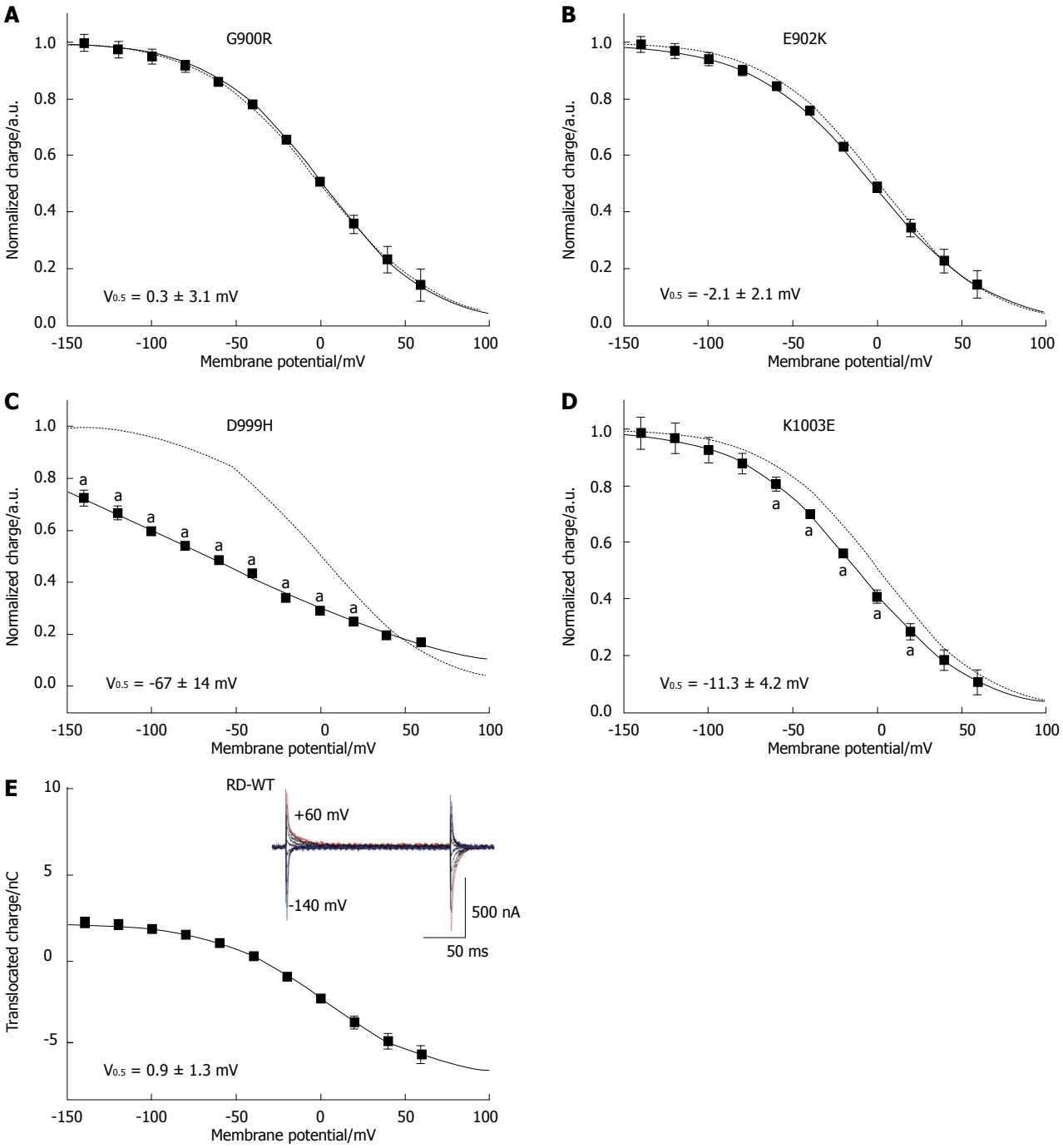

Figure 4 Voltage dependence of translocated transient charge. A-E: Normalized Q(V) curves from ouabain-sensitive transient currents for the RD-WT enzyme (E), and for the mutants G900R (A), E902K (B), D999H (C) and K1003E (D). Fits of a Boltzmann function to the data are superimposed. Q_{min} and Q_{max} determined by the fit were used for normalization. The Boltzmann curve of the RD-WT enzyme is shown as a dotted line for comparison. Transient current signals are shown in a box for the RD-WT enzyme in panel (E). An "a" indicates that the data point was significantly different from the RD-WT data ($^aP < 0.05$ *vs* RD-WT, Student's *t*-test).

Gly855 is separated by three positions from Gly852, but due to the α-helical structure, it is oriented towards αM5 rather than to βM (Figure 7A). Two mutations at this position have been identified in patients with hemiplegic migraine forms: G855R (FHM2)[27] and G855V (SHM)[13], with G855R presumably having a stronger effect on Na^+/K^+-ATPase function. Our study indeed shows that the G855R mutant protein is not correctly targeted to the plasma membrane of *Xenopus* oocytes (Figure 6A, C) although it could well be detected in the total intracellular membrane fraction. However, disruption of α/β-interactions would cause degradation of the protein already in the ER. It is conceivable that the long side chain of the introduced arginine might disturb the structure in a way that transmembrane domains (especially αM7 and αM5) are not correctly positioned. Here, we cannot clarify if the integration in the plasma membrane of G855R is affected because of deficient α/β-interactions or because

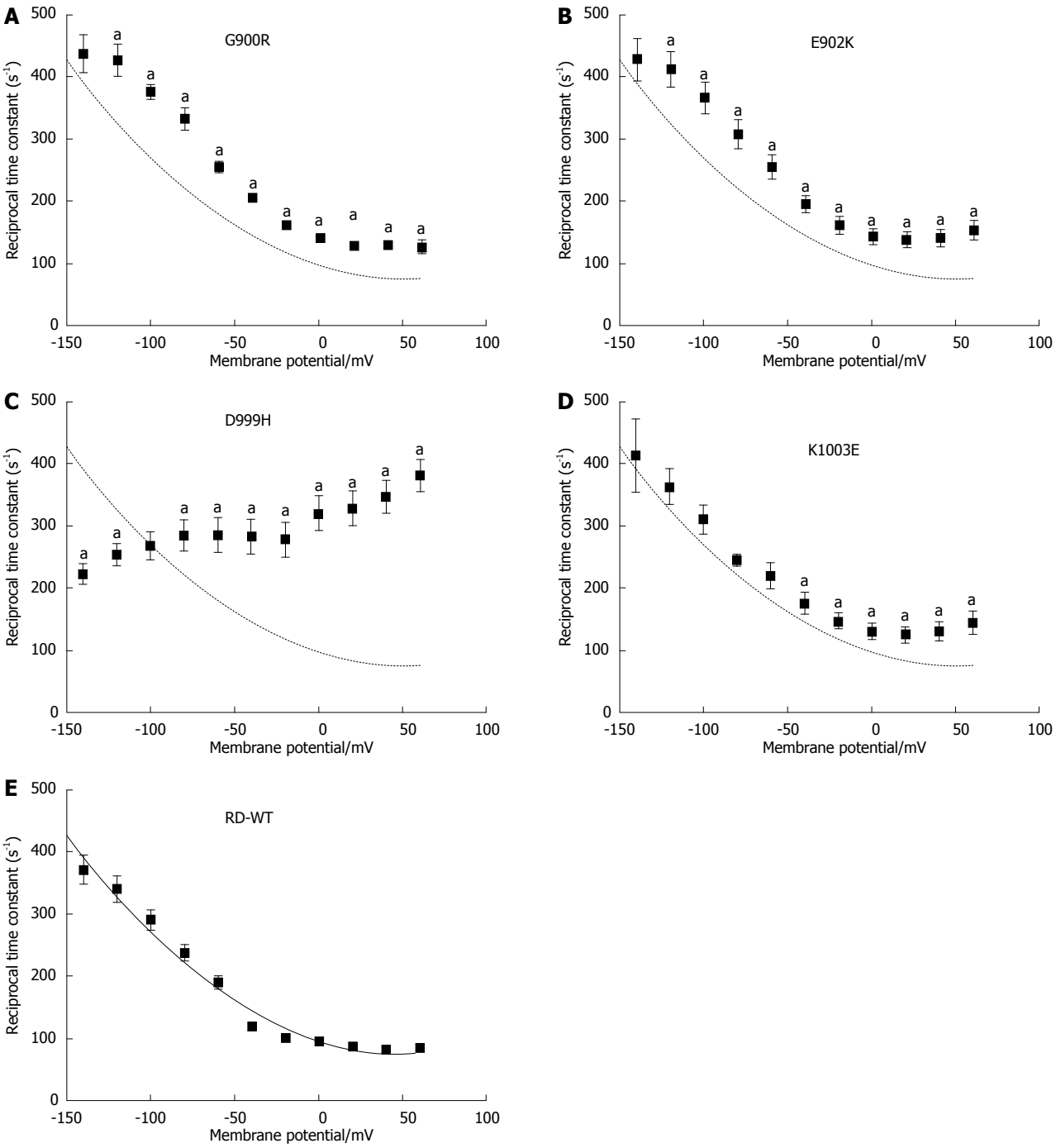

Figure 5 Reciprocal time constants of transient currents. A-E: Voltage dependence of reciprocal time constants τ-1 from ouabain-sensitive transient currents of RD-WT enzyme (E) and the mutants G900R (A), E902K (B), D999H (C) and K1003E (D) under K^+-free Na^+/Na^+ exchange conditions. The fit of a polynomial function to RD-WT values is superimposed as a dotted line. An "a" indicates that the data point was significantly different from the RD-WT data ($^aP < 0.05$ *vs* RD-WT, Student's *t*-test). Data are means ± SE from 5-21 oocytes of at least three batches.

of misfolding, but Gly855 seems to be a critical position.

In this context, the effect of Y1009X and L994del, which are not targeted to the plasma membrane either but are present in the TM fraction (Figure 6), might be of interest. As shown in Figure 7A, the flexible C-terminus (orange) of the α-subunit is oriented towards a region between βM and αM7, in interaction distance to Lys770 in αM5 (Figure 7B). It was suggested that Tyr998 in αM10 directly interacts with βM[23]. The Y1009X mutant protein lacks the 11 C-terminal residues, and in L994del, the 25 C-terminal amino acid residues are shifted N-terminally by one position. These modifications in the C-terminus might affect the orientation of αM7 and αM5 and thereby, correct protein folding. To what extent α/β-interactions are influenced cannot be clarified in this study.

C-terminal region

A number of functional studies imply that the C-terminus

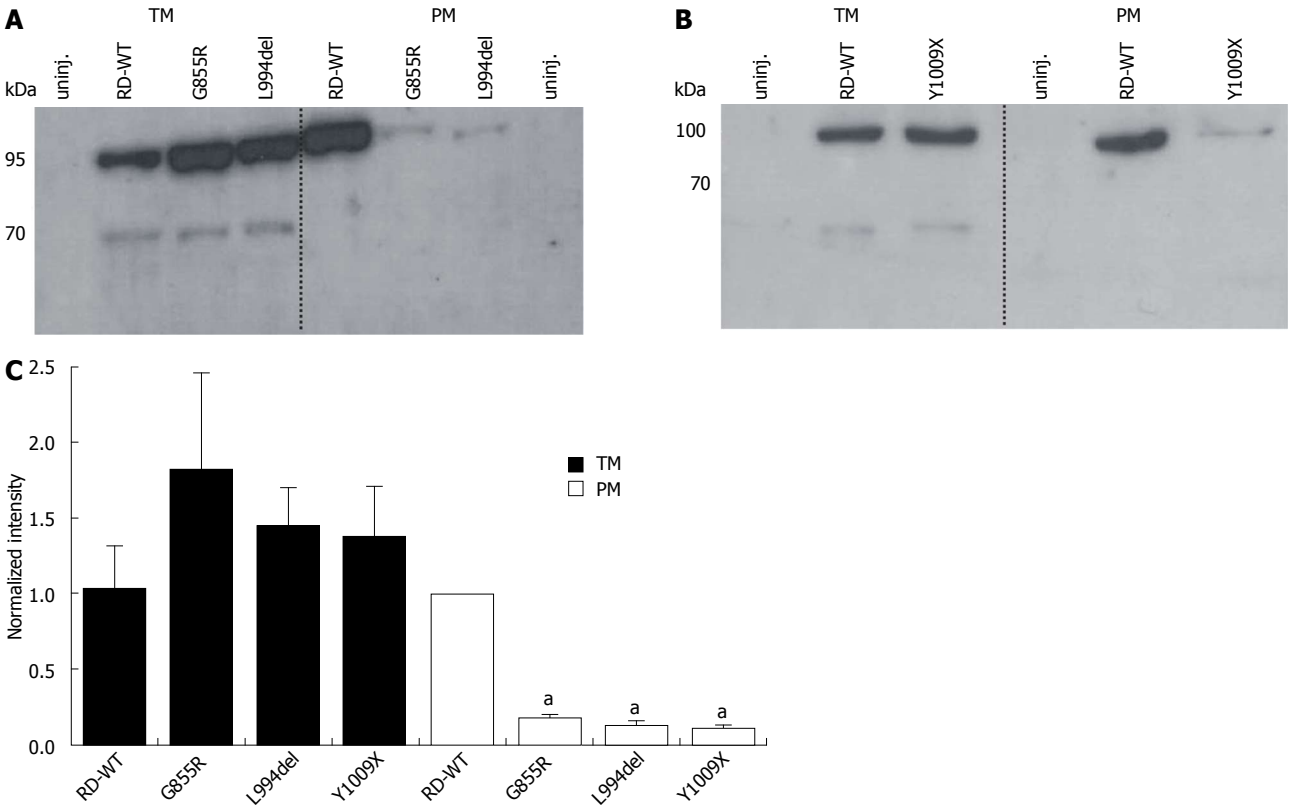

Figure 6 Protein expression of the constructs G855R, L994del and Y1009X. A, B: Representative Western blots for the constructs G855R, L994del (A) and Y1009X (B) compared to the RD-WT enzyme and non-injected cells. Samples of total intracellular membrane (TM, left) and plasma membrane (PM, right) fractions corresponding to the protein amount of two oocytes were loaded in each lane (the number of cell equivalents serves as internal loading standard); C: Densitometric analysis of band intensities from at least four Western blots of G855R, L994del, Y1009X and RD-WT. The program ImageJ 1.44o (Wayne Rasband, United States) was used for analysis. In each experiment, signals were normalized to the intensity of the RD-WT signal from PM samples. An "a" indicates that the data point was significantly different from the RD-WT data ($^aP < 0.05$ *vs* RD-WT, Student's *t*-test). Data are means ± SD.

is intimately involved in the stabilization of the third Na^+ binding site[16,19,42,43], including analyses of mutations which are suspected to trigger neurological diseases. Elongation of the C-terminus provoked different functional abnormalities. Investigations on a mutation found in a patient with rapid-onset dystonia parkinsonism, where the α_3-subunit's C-terminus is extended by one tyrosine, implied a direct participation of the C-terminus in Na^+ binding[43]. Another C-terminal mutation X1021R (mutation of the stop codon resulting in an elongation of the C-terminus by 28 amino acids) was analyzed electrophysiologically in *Xenopus* oocytes[14]. Interestingly, this mutation affected the apparent Na^+_{ex} affinity of the enzyme in a similar way as the D999H mutation. The Q(V) curve of transient currents of X1021R was comparably shallow, as for D999H (Figure 4C), and linear in the tested potential range. The z_q value was reduced to 0.3 for both mutations, which implies that Na^+ release and rebinding is less voltage-dependent. Furthermore, the reciprocal time constants of transient currents showed inverse voltage dependence compared to the RD-WT enzyme (kinetics accelerated with increasing potentials, Figure 5C). Similar curves were also detected for other C-terminally mutated enzymes like ΔYY or ΔKE(S/T)YY (deletion of the last two or five amino acids, depending on species isoform)[17-19]. The transient currents correlate with the movement of the third Na^+ ion through a substantial fraction of the membrane dielectric, which reaches its bindings site through a high-field access channel[10,33]. The $\tau^{-1}(V)$ curve measured for D999H (Figure 5C) or ΔYY[19] corresponds to a voltage dependence that is predicted by Vasilyev *et al*[19,44] for a reaction cycle in which the intra- and extracellular access for Na^+ to its binding sites is facilitated. In conclusion, the C-terminus stabilizes the Na^+-occluded state. This argumentation is also shared by Vedovato and Gadsby, who argued that the C-terminally deleted mutations increase the free energy for $E_1P(3Na^+)$[18]. This destabilization manifests in a faster conformational change or in a faster access/release of intracellular Na^+ ions, which means that the function of the $E_1P(3Na^+)$ state is impaired and correct closure of an intracellular occlusion gate for Na^+ ions is not assured.

Not only are the two terminal tyrosines involved in this stabilization, but also the residues Arg937 (αM8/M9-loop), Asp999 (M10) and Arg1002 (M10) are part of a network of interactions with these tyrosines (Figure 7B). The FHM2/SHM mutations R937P, R1002Q[42] and D999H, as well as the ΔYY or ΔKE(S/T)YY sequence variants have similar effects on transient currents (kinetics and Q(V) distribution). The functional studies all show that the C-terminus not only regulates the apparent Na^+_{ex} affinity in the E_2P conformation, but also the Na^+_{in} affin-

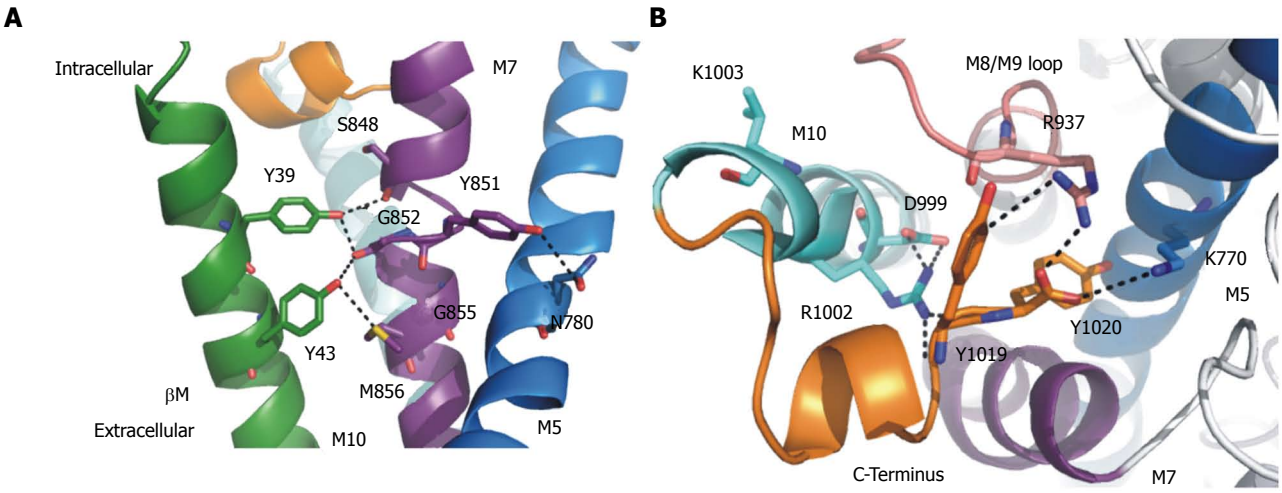

Figure 7 Structural details of the C-terminal region and α/β-interactions. A: Structural details (PDB structure entry 3B8E) of putative interactions between the α- and β-subunit. Interacting residues are shown as sticks. Tyr39 and Tyr43 in the β-transmembrane domain (green) can interact with αM7 (purple). The α-helix is unwound at residue Gly952 (αM7). Tyr851 can form hydrogen bonds to Asn780 in αM5 (marine), which is part of the K^+ binding site Ⅰ and Ⅱ. Also shown is αM10 (light blue) with the C-terminus (orange) of the α-subunit; B: Structural details of the C-terminal region viewed from the intracellular side. Possibly interacting residues are shown in sticks. The C-terminal Tyr1019 and Tyr1020 (orange) can interact with Arg1002 in αM10 (light blue), Arg937 (αM8/M9-loop in purple) and Lys770 in αM5 (marine). Asp999 (αM10) can form hydrogen bonds to Arg1002. Lys1003 (αM10) is not involved in the C-terminal network.

ity in the E_1 conformation[17,19,43]. Based on molecular dynamics simulations of the wild-type enzyme and C-terminally mutated α_2-subunits, it was proposed that the amino acids Arg937, Asp999, Arg1002 and Tyr1019/1020 form an intracellular ion pathway with Asp930 at its end, which controls the access to the third Na^+ binding site depending on the protonation state of Asp930[42]. Our study confirms that Asp999 is at least indirectly involved in the stabilization of Na^+ binding because its substitution by a histidine affected electrogenicity and kinetics of Na^+ charge translocation in a similar fashion. In contrast, the overall electrophysiological data of K1003E did not show severe functional abnormalities, and with regard to the crystal structure of the Na^+/K^+-ATPase, we conclude that Lys1003 (αM10) is not directly involved in the C-terminal network (Figure 7B).

Functional consequences

Dysfunction of the Na^+/K^+-ATPase affects excitatory processes in the CNS, especially in patients suffering from hemiplegic migraine. How do the mutations studied in this work affect the physiological processes in neuronal signaling cascades, since the α_2-isoform in human brain is mainly expressed in astrocytes and not in neurons? The CSD phenomenon is discussed as pathophysiological mechanism of the migraine aura. It is promoted by hyperexcitability caused by insufficient removal of K^+ and neurotransmitters such as glutamate from the synaptic cleft, which is the primary function of astrocytes. The glial Na^+/K^+-ATPase is directly involved in K^+ transport, and it indirectly influences glutamate and Ca^{2+} transport by regulating the Na^+ gradient, which is the energy source of the glutamate transporter (EAAT) and the Na^+/Ca^{2+}-exchanger (NCX).

G900R, E902K and K1003E did not show significant functional abnormalities compared to the RD-WT enzyme, at least under the conditions tested here. It is possible that these mutations impair the enzymatic function in human cells *e.g.* due to different temperature conditions (37 ℃ as opposed to oocytes, which need to be kept at room temperature), as shown previously for another FHM2 mutation P979L[15]. Furthermore, the constructs G855R, L994del and Y1009X exhibited strongly reduced expression in the plasma membrane (Figure 6). This hints at an incomplete or improper folding of the protein so that these mutants could not be correctly targeted to the plasma membrane. In patients with such mutations, the pump enzyme is seriously damaged, and cannot contribute to the maintenance of ion gradients or to the removal of K^+. As a consequence, hyperexcitability is probable.

Compared to all other mutants in this study, which gave rise to measurable Na^+/K^+ pump currents, the D999H mutation had the largest impact on pump function. The voltage dependence of Na^+/K^+ pump activity was shifted to positive potentials compared to the RD-WT enzyme (Figure 2C). We suppose that K^+ transport of this construct is only effective at around zero or positive membrane potentials. Since the α_2-isoform is dominant in astrocytes with resting potentials at -85 to -90 mV, this mutant exhibits a severe loss-of-function. K^+ cannot be removed efficiently from the synaptic cleft at negative potentials, which lowers the excitation threshold and may trigger CSD. Furthermore, regarding the negative shift of the Q(V) curve (Na^+/Na^+ exchange conditions, Figure 4C) and the low $K_{0.5}(K^+)$ values at hyperpolarization (Figure 3C), we conclude that the apparent affinity for extracellular Na^+ is reduced in the D999H mutant. As explained above, Asp999 is part of the C-terminal interaction network which plays a role in Na^+ binding (especially concerning stabilization of the third Na^+ binding site, Figure 7B). Mutations at positions Arg937 and Tyr1019/Tyr1020, which are also part of this network, affected the

affinity for both, intra- and extracellular Na^+[17,19,43]. The ATP1A2 α_2-isoform (expressed in non-excitable cells of the CNS) has a slightly increased Na^+_{in} affinity compared to the α_3-isoform[45,46], which is expressed in neurons. This is advantageous because enzyme activity in astrocytes presumably depends mainly on the increase of the intracellular Na^+ concentration. In other words, $[Na^+]_{in}$ is the important factor determining the sensitivity of the Na^+/K^+-ATPase towards increasing extracellular K^+[47]. For instance, the intracellular Na^+ concentration increases upon glutamate uptake by EAAT, and this stimulates pump activity and K^+ transport. Accordingly, a reduced Na^+_{in} affinity would constrain forward pumping with serious consequences for the recovery of the neuronal resting potential.

In effect, K^+ and glutamate removal from the synaptic cleft not only depends on Na^+/K^+-ATPase activity, but other transporting enzymes are also involved. Furthermore, the penetrance of ATP1A2 mutations can be low or heterogenous because of a large diversity of phenotypic expression depending on genetic and environmental conditions[48-50]. In consequence, physiological impacts of α_2-mutations vary and provoke clinical symptoms of different severity.

In conclusion, this study shows that the investigated FHM2/SHM mutations influence protein function differently depending on the structural impacts of the mutated residue, and thereby, the spectrum of molecular phenotypes of ATP1A2 mutations is widened. We have identified at least two positions that are critical for correct protein function, with Asp999 being involved in Na^+-binding and with Gly855 being essential for plasma membrane targeting. The functional analysis of FHM2/SHM mutations are mandatory to elucidate structure-function relationships of the Na^+/K^+-ATPase and, furthermore, to identify biochemical linkage between impairments of protein function and neurological diseases. Our results may help to understand molecular mechanisms in order to develop a basic approach for future therapeutic strategies.

ACKNOWLEDGEMENTS

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COMMENTS

Background

The Na^+/K^+-ATPase is a very important transmembrane protein in the signaling cascade and it has been investigated for over 50 years. There are still open questions concerning details of the reaction mechanism and structure-function relationships. In patients suffering from a genetically inherited subform of migraine with aura (familial hemiplegic migraine), mutations of the *ATP1A2* gene, which codes for the α_2-subunit of the Na^+/K^+-ATPase, have been identified.

Research frontiers

To clarify structure-function relationships of the Na^+/K^+-ATPase, different methods have to be applied like molecular dynamics simulations, crystallography, mutagenesis studies together with biochemical assays or electrophysiology. Especially, interactions between the two mandatory enzyme subunits, the role of the α-subunit's C-terminus and the detailed mechanism of Na^+ binding remain unclear. This study analyzed ATP1A2 mutants functionally by electrophysiological and biochemical methods to clarify some of these questions.

Innovations and breakthroughs

More than 50 mutations of the ATP1A2 gene associated with familial hemiplegic migraine have been identified, but many of them have not been functionally analyzed. This study identifies critical structure elements of the Na^+/K^+-ATPase and discusses their impact on correct protein function. After publication of the first crystal structure, many efforts were made to clarify the role of the α-subunit's C-terminus and its structural interaction. The authors show in this study that Asp999 is indeed part of the C-terminal network and is critical for Na^+ binding. Furthermore, the authors have identified Gly855 to be a very critical position for correct protein function.

Applications

This study helps to elucidate structure-function relationships of the Na^+/K^+-ATPase and its correlation with neurological diseases. It is mandatory to understand the molecular basis of genotype-phenotype relations and to develop therapeutic approaches and future therapeutic strategies.

Terminology

The Na^+/K^+-ATPase is an ion pump. This transmembrane protein maintains the electrochemical gradients for sodium and potassium ions, which are necessary for the transmission of stimuli in neurons or muscle cells. The Na^+/K^+-ATPase can be inhibited by ouabain, a cardiac glycoside which was used for the treatment of heart diseases. *Xenopus* oocytes are the eggs of the African Clawed Frog. They are used for the expression of proteins like ion channels or ion pumps to study ion transport by electrophysiological methods. The two-electrode voltage-clamp technique is used to measure changes in conductivity and ion currents over the cell membrane. With this method, it is possible to control the membrane potential of the cell and to analyze current-voltage relationships of ion-transporting membrane proteins.

Peer review

This paper represent a very good piece of scientific information, it provides information on the consequences of mutations in the Na,K-ATPase alpha subunit, measured by voltage clamp.

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ORIGINAL ARTICLE

Binding of rhodopsin and rhodopsin analogues to transducin, rhodopsin kinase and arrestin-1

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Abstract

AIM: To investigate the interaction of reconstituted rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin with transducin, rhodopsin kinase and arrestin-1.

METHODS: Rod outer segments (ROS) were isolated from bovine retinas. Following bleaching of ROS membranes with hydroxylamine, rhodopsin and rhodopsin analogues were generated with the different retinal isomers and the concentration of the reconstituted pigments was calculated from their UV/visible absorption spectra. Transducin and arrestin-1 were purified to homogeneity by column chromatography, and an enriched-fraction of rhodopsin kinase was obtained by extracting freshly prepared ROS in the dark. The guanine nucleotide binding activity of transducin was determined by Millipore filtration using β,γ-imido-(^{3}H)-guanosine 5′-triphosphate. Recognition of the reconstituted pigments by rhodopsin kinase was determined by autoradiography following incubation of ROS membranes containing the various regenerated pigments with partially purified rhodopsin kinase in the presence of (γ-^{32}P) ATP. Binding of arrestin-1 to the various pigments in ROS membranes was determined by a sedimentation assay analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

RESULTS: Reconstituted rhodopsin and rhodopsin analogues containing 9-*cis*-retinal and 13-*cis*-retinal rendered an absorption spectrum showing a maximum peak at 498 nm, 486 nm and about 467 nm, respectively, in the dark; which was shifted to 380 nm, 404 nm and about 425 nm, respectively, after illumination. The percentage of reconstitution of rhodopsin and the rhodopsin analogues containing 9-*cis*-retinal and 13-*cis*-retinal was estimated to be 88%, 81% and 24%, respectively. Although only residual activation of transducin was observed in the dark when reconstituted rhodopsin and 9-*cis*-retinal-rhodopsin was used, the rhodopsin analogue containing the 13-*cis* isomer of retinal was capable of activating transducin independently of light. Moreover, only a basal amount of the reconstituted rhodopsin and 9-*cis*-retinal-rhodopsin was phosphorylated by rhodopsin kinase in the dark, whereas the pigment containing the 13-*cis*-retinal was highly phosphorylated by rhodopsin kinase even in the dark. In addition, arrestin-1 was incubated with rhodopsin, 9-*cis*-retinal-rhodopsin or 13-*cis*-retinal-rhodopsin. Experiments were performed using both phosphorylated and non-phosphorylated regenerated pigments. Basal amounts of arrestin-1 interacted with rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin under dark and light conditions. Residual arrestin-1 was also recognized by the phosphorylated rhodopsin and phosphorylated 9-*cis*-retinal-rhodopsin in the dark. However, arrestin-1 was recognized by phosphorylated 13-*cis*-

retinal-rhodopsin in the dark. As expected, all reformed pigments were capable of activating transducin and being phosphorylated by rhodopsin kinase in a light-dependent manner. Additionally, all reconstituted photolyzed and phosphorylated pigments were capable of interacting with arrestin-1.

CONCLUSION: In the dark, the rhodopsin analogue containing the 13-*cis* isomer of retinal appears to fold in a pseudo-active conformation that mimics the active photointermediate of rhodopsin.

Key words: Rhodopsin; Rhodopsin analogues; 9-*cis*-Retinal; 11-*cis*-Retinal; 13-*cis*-Retinal; Photointermediates; Transducin; Rhodopsin kinase; Arrestin-1; Visual process

Core tip: Rhodopsin is a specialized G protein-coupled receptors composed of a single polypeptide chain, opsin, and a covalently linked 11-*cis*-retinal. It is well known that rhodopsin uses the 11-*cis* form of retinal exclusively as the chromophore. Retinal analogues have long been used to probe the chromophore binding pocket and to study ligand-protein relationships to better understand the photochemical cis-trans isomerization of rhodopsin. However, little is known about the interactions of rhodopsin analogues with other proteins in the visual cascade. Here, we were able to reconstitute a rhodopsin analogue containing 13-*cis*-retinal. We compared the binding of reconstituted rhodopsin, 9-cis-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin to transducin, rhodopsin kinase and arrestin-1, both in the dark and under illumination. Interestingly, we found that in the dark the rhodopsin analogue containing the 13-*cis* isomer of retinal appears to fold in a pseudo-active conformation that mimics the active photointermediate of rhodopsin.

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INTRODUCTION

G protein-coupled receptors (GPCRs) activate signaling paths in response to a diverse number of stimuli such as photons, Ca^{2+}, organic odorants, amines, hormones, nucleotides, nucleosides, peptides, lipids and even large proteins[1]. All GPCRs share a conserved seven-transmembrane-helix structural bundle connected by six loops of varying lengths. Binding of specific ligands to the transmembrane or extracellular domains of members of the GPCR superfamily causes conformational changes that act as a switch to relay the signal to heterotrimeric G proteins that in turn evoke further intracellular responses[2].

The dim-light photoreceptor rhodopsin is a highly specialized GPCR composed of a single polypeptide chain of 348 amino acids that conforms the apoprotein opsin, and a covalently linked 11-*cis*-retinal chromophore that is tightly packed within the bundle of helices[3,4]. The chromophore is bound to the ε-amino group of Lys296, located in the seventh helix (TM7) *via* a protonated Schiff base linkage. In the ground state this charge is stabilized by the counter-ion Glu113 that is located in the third helix (TM3)[5]. Another important structural feature of the 11-*cis*-retinal chromophore in rhodopsin is its extended polyene structure, which accounts for its visible absorption properties and allows for resonance structures[6].

In rhodopsin, 11-*cis*-retinal serves both as the chromophore and as an inverse agonist that holds the visual pigment protein in an inactive conformation. Absorption of a photon by the 11-*cis*-retinal of rhodopsin causes its photoisomerization to the all-*trans* form[7], converting the ligand into an agonist, and leading to a conformational change of the protein moiety that triggers the signal transduction cascade *via* reactions of the G protein transducin. Following *cis-trans* isomerization of the chromophore, rhodopsin relaxes through a series of photoproducts, which have been identified by their characteristic absorption spectra. One of the photointermediates, metarhodopsin Ⅱ (meta Ⅱ), is the active conformation of rhodopsin responsible of binding transducin and initiating the signaling process. Transducin, which is arranged as two units, the α subunit and the βγ-complex, transmits the visual stimuli by activating a potent cGMP phosphodiesterase known as PDE6. The resulting decrease in the cytosolic concentration of cGMP causes the closure of cation-specific cGMP-gated channels located in the plasma membrane, leading to the hyperpolarization of the rod cell. Additional protein molecules participate in modulating the duration of the signal and the achievement of the appropriate response[8]. Particularly, the phosphorylation of photoactivated rhodopsin by rhodopsin kinase, also known as GPCR kinase 1 or GRK1, and its interaction with arrestin-1, are both involved in signal desensitization since the transducin activation phase is terminated by the interaction of meta Ⅱ with rhodopsin kinase and arrestin-1[9,10]. Subsequently, the retinal Schiff base is hydrolysed and the photolysed all-*trans*-retinal is released from its binding site. Regeneration of the light sensitive rhodopsin ground state requires the supply of new 11-*cis*-retinal through the so-called retinoid cycle[11,12].

It is well known that the rod visual pigment rhodopsin uses the 11-*cis* form of retinal exclusively as the chromophore, and the strict selection of this isomer appears to have occurred early in the evolution of visual function. Under certain pathological conditions, however, also the 9-*cis* configuration of retinal is observed, which generates a pigment known as isorhodopsin[13]. Retinal analogues have long been used to probe the chromophore binding pocket and to study ligand-protein relationships to better understand the photochemical *cis-trans* isomerization of rhodopsin[14] and cone opsins[15]. Yet, little is known about the interac-

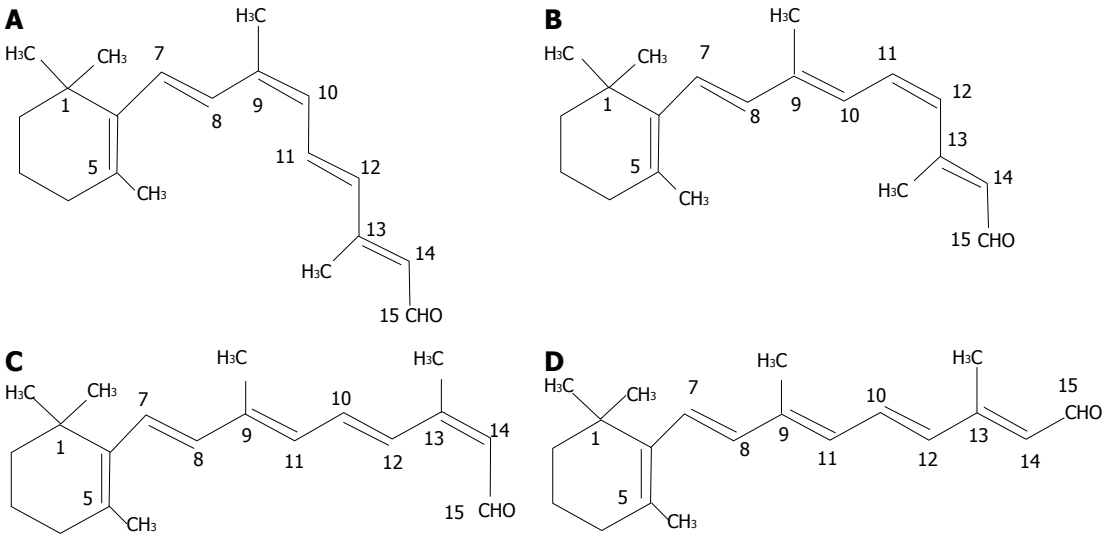

Figure 1 Structures of retinal analogues. A: 9-*cis*-retinal; B: 11-*cis*-retinal; C: 13-*cis*-retinal; D: all-*trans*-retinal.

tions of rhodopsin analogues with other proteins in the visual cascade. Although, it has been reported that the retinal binding site in the inactive state of rhodopsin can accommodate the 7-*cis*, 9-*cis* and 11-*cis* isomers of retinal, but not the longer all-*trans* or 13-*cis* isomers[16], in the present work we were able to reconstitute a rhodopsin analogue containing 13-*cis*-retinal, in addition to photoreceptor proteins containing 9-*cis*-retinal and 11-*cis*-retinal. We compared the binding of reconstituted rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin to transducin, rhodopsin kinase and arrestin-1, both in the dark and under illumination. The chemical structures of the geometrical retinal isomers used here and of all-*trans*-retinal are shown in Figure 1.

MATERIALS AND METHODS

Materials

Bovine eyes were obtained from the nearest abattoir (Beneficiadora Diagon, CA, Matadero Caracas, Venezuela). Retinae were extracted in the dark, under red light, and were maintained frozen at -80 ℃. Reagents were purchased from the following sources: β,γ-imido-(^{3}H)-guanosine 5'-triphosphate [(8-^{3}H) GMPpNp] (17.9 Ci/mmol) and (γ-^{32}P) ATP (3000 Ci/mmol), Amersham; 9-*cis*-retinal, 13-*cis*-retinal, bovine serum albumine (BSA), hydroxylamine, phytic acid or inositol hexakisphosphate (IP_6), n-dodecyl β-D-maltoside, and DEAE-cellulose, Sigma-Aldrich; ATP, heparine-sepharose and concanavalin A-Sepharose 4B, Pharmacia; molecular weight pre-stained protein markers, and Bradford reagent, Bio-Rad; anti-rabbit IgG antibodies conjugated to alkaline phsophatase, KPL; bromocloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT), and molecular weight protein standards, Promega; X-ray films, Kodak. The 11-*cis*-retinal was donated by Dr. Debra Thompson, University of Michigan, United States.

Preparation of rod outer segments and washed membranes

Rod outer segments (ROS) were isolated from frozen bovine retinas as described previously[17]. Dark depleted ROS membranes were prepared by washing ROS with 5 mmol/L Tris-HCl (pH = 7.4), 2 mmol/L EDTA, and 5 mmol/L β-mercaptoethanol until no significant amount of peripheral proteins was released with the wash buffer. ROS and dark-depleted ROS membranes were stored in the dark at -80 ℃. Rhodopsin concentration was calculated from its UV/visible absorption spectra, using its molar extinction coefficient (40700 $M^{-1}cm^{-1}$, at 500 nm)[18]. In addition, rhodopsin was identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using anti-bovine rhodopsin polyclonal antibodies raised in mice.

Purification of transducin

Transducin was obtained from ROS prepared under room light, at 4 ℃, following the affinity procedure described by Kühn[19]. GTP (100 μmol/L) was employed to elute transducin from the washed illuminated ROS, and transducin was further purified to homogeneity by anion exchange chromatography on a DEAE-cellulose column as described elsewhere[20]. Fractions containing transducin were identified by SDS-PAGE and Western blot using anti-bovine transducin polyclonal antibodies raised in mice.

Preparation of an Enriched fraction of Rhodopsin Kinase

Freshly prepared ROS were washed three times with an isotonic buffer containing 70 mmol/L potassium phosphate (pH = 6.8), 5 mmol/L magnesium acetate, 5 mmol/L β-mercaptoethanol, and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Following centrifugation, the washed ROS pellet was hypotonically extracted with 5 mmol/L Tris-HCl (pH = 7.4), 5 mmol/L magnesium acetate, 5 mmol/L β-mercaptoethanol, and 0.1 mmol/L PMSF[21]. Under these conditions, soluble proteins and proteins weakly associated with the membrane, including transducin, cGMP phosphodiesterase PDE6, arrestin-1, and rhodopsin kinase, appear in the supernatant gener-

ated after centrifugation. This supernatant was considered as the enriched fraction of rhodopsin kinase. The whole procedure was carried out at 4 ℃, in the dark under red light.

Purification of arrestin-1

Arrestin-1 was purified following the procedure described by Buczylko *et al*[22]. Frozen bovine retinas were homogenized with 10 mmol/L Hepes (pH = 7.5), 0.1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol, under dim red light, at 4 ℃. Following centrifugation at 70000 *g*, for 25 min, the supernatant containing the soluble proteins was chromatographed on a DEAE-cellulose column, previously equilibrated in the same buffer. The column was washed with 10 mmol/L Hepes (pH = 7.5), 15 mmol/L NaCl, 0.1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol (Buffer A) until the absorbance at 280 nm dropped below 0.1. Adsorbed proteins were eluted with a 0 to 150 mmol/L linear gradient of NaCl in Buffer A, and the fractions containing arrestin-1 were identified by SDS-PAGE and Western blot using anti-bovine arrestin-1 polyclonal antibodies prepared in rabbits. These fractions were pooled and applied to a heparin-sepharose column, which was previously equilibrated with 10 mmol/L Hepes (pH = 7.5), 100 mmol/L NaCl, 0.1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol (Buffer B). Arrestin-1 was eluted using a gradient of 0 to 8 mmol/L phytic acid in Buffer B. The peak of arrestin-1 was pooled, dialyzed against Buffer A, applied to a second heparin-sepharose column, and eluted with 10 mmol/L Hepes (pH = 7.5), 400 mmol/L NaCl, 0,1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol.

Bleaching of rhodopsin in washed ROS membranes

Washed ROS membranes were incubated with 50 mmol/L hydroxylamine in 10 mmol/L Tris-HCl (pH = 7.4), at 4 ℃, for 15 min, under illumination with a tungsten 100 W lamp. Then, the mixture was centrifuged at 50000 *g* for 20 min, at 4 ℃. The supernatant was discarded and the pellet was washed twice with 5 mmol/L Tris-HCl (pH = 7.4), 5 mmol/L magnesium acetate, 5 mmol/L β-mercaptoethanol.

Regeneration of rhodopsin and rhodopsin analogues

Samples of bleached washed ROS membranes containing about 25 μmol/L of opsin were resuspended in 10 mmol/L Tris-HCl (pH = 7.4). Then, appropriate aliquots of stock solutions of 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal prepared in ethanol were added in the dark. A molar ratio of 3:1 retinal to opsin was used for the reconstitution of the pigment with the 9-*cis*-retinal and 11-*cis*-retinal isomers, whereas a ratio of 15:1 retinal to opsin was employed for the regeneration of the rhodopsin analogue containing the 13-*cis*-retinal isomer. Following an overnight incubation, at room temperature, all samples were centrifuged at 50000 *g*, for 20 min, at 4 ℃. The regeneration of the pigments was followed by UV-Vis spectroscopy using the extinction coefficient of rhodopsin[18]. The excess of 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal was eliminated by washing the membranes containing the reconstituted pigments with 2% BSA in 10 mmol/L Tris-HCl (pH = 7.4). BSA was then removed by successive washes with 5 mmol/L Tris-HCl (pH 7.4), 5 mmol/L magnesium acetate, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol. ROS membranes containing the reconstituted pigments were resuspended in 5 mmol/L Tris-HCl (pH = 7.4), 100 mmol/L NaCl, 1 mmol/L magnesium acetate, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol.

Binding of (8-^{3}H) GMPpNp to transducin

Guanine nucleotide binding was measured by Millipore filtration using (8-^{3}H) GMPpNp, a radioactive non-hydrolyzable analogue of GTP, as previously described[23].

Phosphorylation of reconstituted rhodopsin and rhodopsin analogues

ROS membranes containing the reconstituted pigments were incubated with a 50-μL aliquot of an enriched fraction of rhodopsin kinase, in the presence of 50 mmol/L Tris-HCl (pH = 7.5), 12 mmol/L $MgCl_2$, 20 mmol/L KF, 40 μmol/L [γ-^{32}P] ATP (specific activity about 4500 cpm/pmol), 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol. Following incubation for 1 h, at room temperature, under illumination with a 100 W tungsten lamp, the phosphorylated membranes were centrifuged at 100000 *g*, for 20 min, at 4 ℃. Identical control experiments were carried out in the dark. Samples were separated by SDS-PAGE and the phosphorylated bands were identified by autoradiography following staining and drying of the gels.

Regeneration of phosphorylated rhodopsin and phosphorylated rhodopsin analogues

ROS containing 1.9 mg of rhodopsin were sedimented by centrifugation at 100000 *g* for 20 min, and resuspended in 50 mmol/L Tris-HCl (pH = 7.5), 12 mmol/L $MgCl_2$, 20 mmol/L KF, 40 μmol/L ATP, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol, in the presence of a 50-μL aliquot of an enriched fraction of rhodopsin kinase. Following illumination for 1 h with a tungsten 100 W lamp, the mixture was centrifuged and the resulting pellet containing the phosphorylated protein was resuspended in 10 mmol/L Tris-HCl (pH = 7.4). Phosphorylated rhodopsin was bleached with 50 mmol/L hydroxylamine to obtain phosphorylated opsin. Samples of phosphorylated opsin were reconstituted with 9-*cis*-retinal, 11-*cis*-retinal and 13-*cis*-retinal as described above.

Interaction of reconstituted rhodopsin and rhodopsin analogues with arrestin-1

The binding of arrestin-1 to the pigments reconstituted in washed ROS membranes was determined according to Gurevich *et al*[24], with slight modifications. Briefly, samples of arrestin-1 (14 μg) were incubated with 12 μg of the regenerated pigments, for 1 h, at room temperature. Experiments were performed in 100 μL of 5 mmol/L Tris-HCl (pH = 7.4), 5 mmol/L magnesium acetate, 0.1

mmol/L PMSF, 5 mmol/L β-mercaptoethanol, both in the dark and under illumination, using phosphorylated and non-phosphorylated pigments (about molar ratio of 1:1 arrestin-1 to pigment). The original mixture, and the resulting supernatant and pellet after centrifugation at 100000 *g*, for 20 min, were separated by SDS-PAGE. The gels were colored by silver staining and the bands of arrestin-1 and rhodopsin or rhodopsin analogues were evaluated by densitometry.

Other procedures

Protein concentration was determined as reported by Bradford[25] using BSA as protein standard. SDS-PAGE was carried out on 1.5-mm thick slab gels containing 12% polyacrylamide as described by Laemmli[26]. Coomassie blue R-250 or silver staining was used for protein visualization. For Western blot analyses, the proteins were electrophoretically transferred from the gels to nitrocellulose sheets (0.45 μm pore size) as reported by Towbin *et al*[27]. Rhodopsin was purified to homogeneity by batchwise affinity chromatography on concanavalin A-Sepharose[28], using n-dodecyl β-D-maltoside instead of n-octyl β-D-glucopyranoside as the detergent. Polyclonal antibodies against rhodopsin and transducin were prepared in mice as described[29]. Purified arrestin-1 was used to raise polyclonal antibodies in rabbit serum following the procedure described by Harlow *et al*[30].

Statistical analysis

For statistical analysis, mean value comparisons were performed by using the Student *t*-test or Anova and Krunskal-Wallis test. *P*-values below 0.05 were considered significant. Data in all histograms are graphed as mean ± SD.

RESULTS

Analysis by SDS-PAGE showed that isolated ROS membranes contained all the proteins involved in the photoexcitation process (Figure 2A), including rhodopsin, transducin, cGMP phosphodiesterase PDE6, arrestin-1 and rhodopsin kinase[19]. As revealed by Western blot using anti-rhodopsin polyclonal antibodies, the major polypeptide band with an apparent molecular mass of approximately 35 kDa corresponded to rhodopsin (Figure 2B). Since rhodopsin has a tendency to oligomerize, higher order oligomers of rhodopsin, such as dimers, trimers, *etc*., were also detected by immunoblotting (Figure 2B). Rhodopsin polypeptide bands were observed in the original ROS sample and remained in the pellet following the washing procedure (Figure 2A and B). The presence of rhodopsin was also demonstrated by measuring the UV/visible absorption spectra of the samples and estimating the ratio of the absorbance at 280 nm to the absorbance at 500 nm[28,31]. Crude ROS showed a spectral ratio $A_{280\ nm}/A_{500\ nm}$ of 2.68, which decreased to 2.05 in dark-depleted ROS membranes after removal of the peripheral proteins (Figure 2C).

Rhodopsin was bleached by exposing washed ROS membranes to light in the presence of hydroxylamine. This treatment caused the complete detachment of the retinal chromophore. Rhodopsin and rhodopsin analogues containing the 9-*cis* and 13-*cis* isomers of retinal were reformed by incubating opsin with an excess of each retinal in the dark. The regeneration of rhodopsin is shown in Figure 2D as an example. As illustrated in Figure 2D, the 11-*cis*-retinal molecule possessed a broad absorption band at about 370 nm that overlapped with the absorption peak of the reconstituted rhodopsin pigment. Washes in the presence of BSA completely removed the residual retinal (Figure 2D).

As can be seen in Figure 3, pigments were reconstituted after the addition of the three retinal isomers to opsin in the dark. Reconstituted rhodopsin rendered the characteristic absorption spectrum of rhodopsin in the dark (Figure 3B), showing a maximum peak at 498 nm (about 500 nm). Following illumination, this band was shifted to 380 nm that corresponded to the meta Ⅱ photointermediate. In the dark, the reconstituted pigment analogue containing 9-*cis*-retinal (isorhodopsin) showed an absorbance peak at 486 nm (Figure 3A), which was slightly blue shifted in comparison to rhodopsin. Once photolyzed, the maximum of illuminated isorhodopsin was obtained at 404 nm which was slightly red shifted in comparison to meta Ⅱ. The absorption spectra of the rhodopsin analogue containing 13-*cis*-retinal showed broader bands than rhodopsin and isorhodopsin, under both, dark and light conditions (Figure 3C). In the dark, the absorption peak of 13-*cis*-retinal-rhodopsin was blue shifted showing its maximum at about 467 nm. After photolysis, the highest absorption peak of the illuminated 13-*cis*-retinal-rhodopsin was acquired at about 425 nm, more red shifted than meta Ⅱ and illuminated isorhodopsin. The percentage of reconstitution of the three pigments was estimated by comparing the absorption values at their maximum wavelength, using the extinction coefficient of rhodopsin as an approximate value[18], and the amount of total protein determined for each sample by the method of Bradford[25]. Our results showed that rhodopsin and isorhodopsin were reconstituted with a yield of 88% and 81%, respectively, whereas the rhodopsin analogue containing the 13-*cis* isomer of retinal was reformed with a yield of only 24%.

A partially purified transducin sample was initially obtained by GTP elution from illuminated ROS membranes. Then, transducin was purified to homogeneity by chromatography on a DEAE-cellulose column (Figure 4A). The elution of transducin was evaluated by measuring the rhodopsin- and light-dependent guanine nucleotide binding by a filtration assay using (8-^{3}H) GMPpNp. SDS-PAGE revealed that the same fractions comprising the GMPpNp binding activity also contained the polypeptide bands corresponding to the α-, β-, and γ-subunits of transducin (Figure 4A, Inset, top). In addition, anti-transducin polyclonal antibodies that preferentially detect

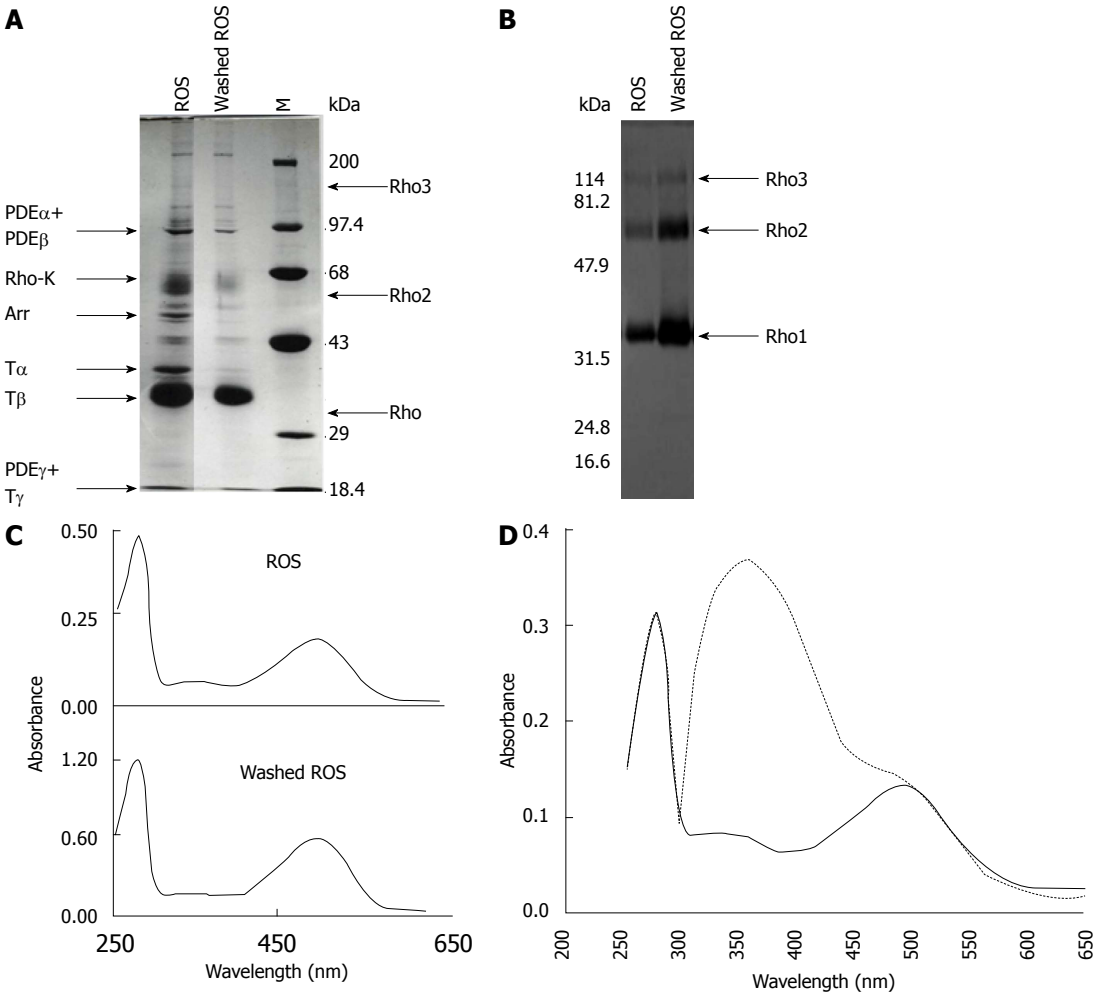

Figure 2 Isolation of rod outer segments, preparation of washed rod outer segments membranes, and reconstitution of rhodopsin. A: ROS were isolated from frozen bovine retinas and were hypotonically washed in the dark until no peripheral proteins were released. Arrows indicate the migration of rhodopsin (Rho), rhodopsin oligomers (Rho2 and Rho3), α-, β- and γ-subunits of the cGMP phosphodiesterase PDE6 (PDEα, PDEβ and PDEγ), α-, β- and γ-subunits of transducin (Tα, Tβ and Tγ), rhodopsin kinase (Rho-K), and arrestin-1 (Arr); B: ROS and dark-depleted ROS membranes were separated by SDS-PAGE, electrotransferred to a nitrocellulose filter and analyzed using polyclonal anti-rhodopsin antibodies. Arrows point out the migration of rhodopsin (Rho), rhodopsin dimers (Rho2), and rhodopsin trimers (Rho3). C: Absorption spectra of solubilized ROS and washed-ROS membranes in the dark; D: Regeneration of rhodopsin. A sample of depleted ROS membranes was bleached with hydroxylamine and incubated with an excess of 11-cis-retinal. Shown is the UV/visible spectra of rhodopsin in the dark, before (dashed line) and after (continuous line) removing the excess of 11-*cis*-retinal by washing with BSA. M: Molecular weight markers; ROS: Rod outer segments.

the α-subunit of transducin also recognized the α-subunit in the fractions containing the protein peak (Figure 4A, Inset, bottom).

Transducin binding to reconstituted rhodopsin and rhodopsin analogues was evaluated by measuring their capacity to induce the exchange of guanine nucleotides on transducin. The amount of reconstituted pigment, instead of the total amount of protein, was employed to normalize the reported values. As shown in Figure 5, all reformed pigments were capable of catalyzing the GMPpNp binding activity of transducin in a light-dependent manner. As expected, little activation of transducin (about 10%-15%) was observed in the dark when reconstituted rhodopsin and isorhodopsin were employed (Figure 5). Moreover, the apoprotein opsin was unable of inducing the exchange of GMPpNp on transducin (data not shown). In contrast and surprisingly, the rhodopsin analogue containing the 13-*cis* isomer of retinal was capable of activating transducin independently of light (about 40%) (Figure 5), suggesting that this pigment possesses a conformation in the dark that is similar to that of meta Ⅱ.

Figure 4B (left) shows the polypeptide composition of an aliquot of the enriched fraction of rhodopsin kinase, compared with samples of ROS and washed ROS membranes. This partially purified fraction of rhodopsin kinase contained polypeptide bands that corresponded to reported ROS peripheral proteins (transducin, cGMP phosphodiesterase PDE6, arrestin-1, rhodopsin kinase, *etc*.). As shown in Figure 4B (right) by autoradiography, intact ROS included active rhodopsin kinase given that rhodopsin was specifically phosphorylated in a light-dependent manner. Phosphorylated rhodopsin oligomers were also obtained in the crude ROS sample (Figure 4B, right). The enriched fraction of rhodopsin kinase was also capable of phosphorylating rhodopsin in washed-ROS membranes and under illumination (Figure 4B,

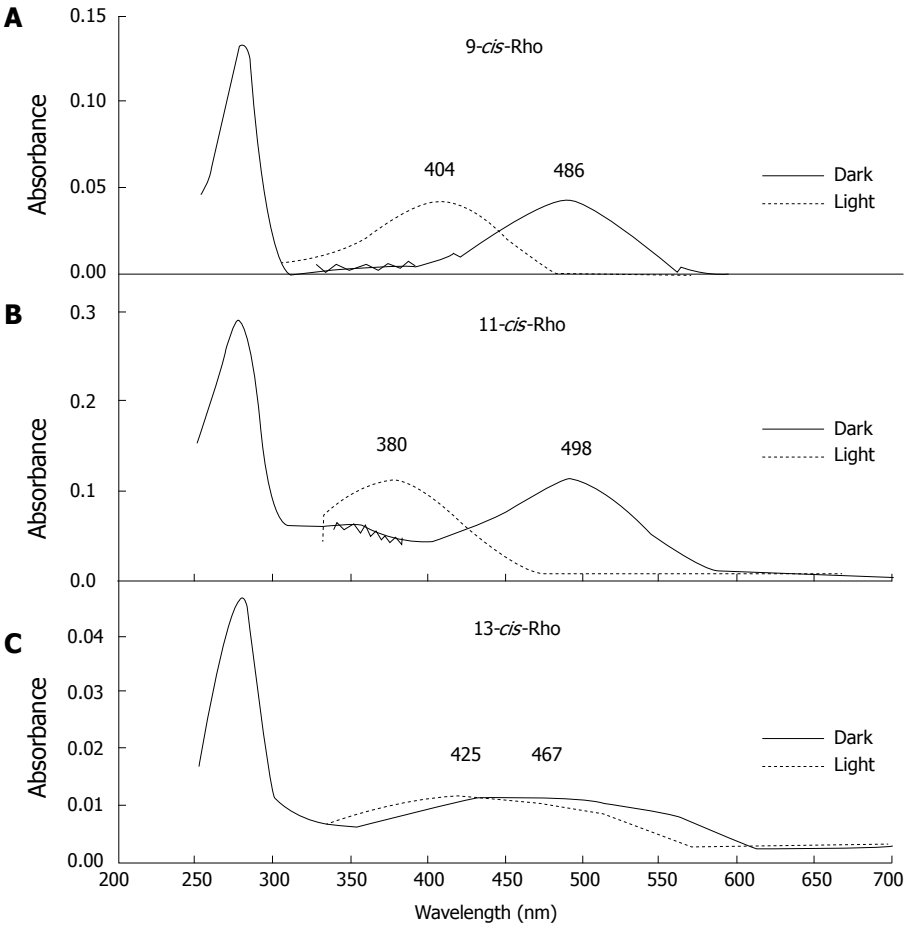

Figure 3 Absorption spectra of rhodopsin and rhodopsin analogues. Absorption spectrum of 9-*cis*-retinal-rhodopsin (9-*cis*-Rho) (A), rhodopsin (11-*cis*-Rho) (B) and 13-*cis*-retinal-rhodopsin (13-*cis*-Rho) (C) in the dark (continuous line) and under illumination (dashed line). Shown are the maximum wavelengths for each pigment.

right).

The ability of the reconstituted rhodopsin and rhodopsin analogues to serve as substrates for rhodopsin kinase was then measured by incubating each sample with an aliquot of the enriched fraction of rhodopsin kinase. As shown in Figure 6A by Coomassie blue staining, the same amount of each reconstituted protein was loaded in the gel lanes. Figure 6B illustrates by autoradiography that an enriched fraction of rhodopsin kinase was capable of phosphorylating all the reformed pigments in a light-dependent manner. Only basal amounts of the reconstituted rhodopsin and isorhodopsin samples were phosphorylated by rhodopsin kinase in the dark (Figure 6B). Opsin behaved similar to inactive rhodopsin given that the apoprotein was not phosphorylated by rhodopsin kinase (data not shown). However, the pigment containing the 13-*cis*-retinal was highly phosphorylated by rhodopsin kinase even in the dark (Figure 6B). Autoradiograms were quantified by densitometry in Figure 6C, corroborating the results qualitatively obtained in Figure 6B. The amount of regenerated pigment, instead of the total amount of protein, was used to normalize the reported values. These results suggest that 13-*cis*-retinal-rhodopsin, in its dark state, folds in a conformation that appears to be comparable to that of meta Ⅱ, given that it can be recognized by rhodopsin kinase even in the absence of light.

Arrestin-1 was purified to homogeneity by using three consecutive chromatography steps: (1) a DEAE-cellulose column; (2) a heparin-sepharose column that was eluted with a gradient of phytic acid; and (3) a second heparin-sepharose column that was eluted with 400 mmol/L NaCl[21]. Figure 4C shows the protein profile obtained after the last chromatography step. The elution of arrestin-1 was evaluated by SDS-PAGE analysis, which showed a polypeptide band with an apparent molecular mass of approximately 50 kDa (Figure 4C, Inset, top). This band was specifically recognized by anti-arrestin-1 polyclonal antibodies (Figure 4C, Inset, bottom). The ability of arrestin-1 to interact with the reconstituted rhodopsin and rhodopsin analogues was then evaluated by an affinity binding procedure. Arrestin-1 was incubated with rhodopsin, isorhodopsin or the 13-*cis*-retinal-rhodopsin, which were reconstituted using washed ROS membranes. Experiments were performed both in the dark and under illumination, and using phosphorylated and non-phosphorylated pigments. An experiment using opsin was also included as a control. After centrifugation, the resulting supernatants and pellets of all the samples were separated by SDS-PAGE. The interaction between arrestin-1 and the three pigments was determined qualitatively by

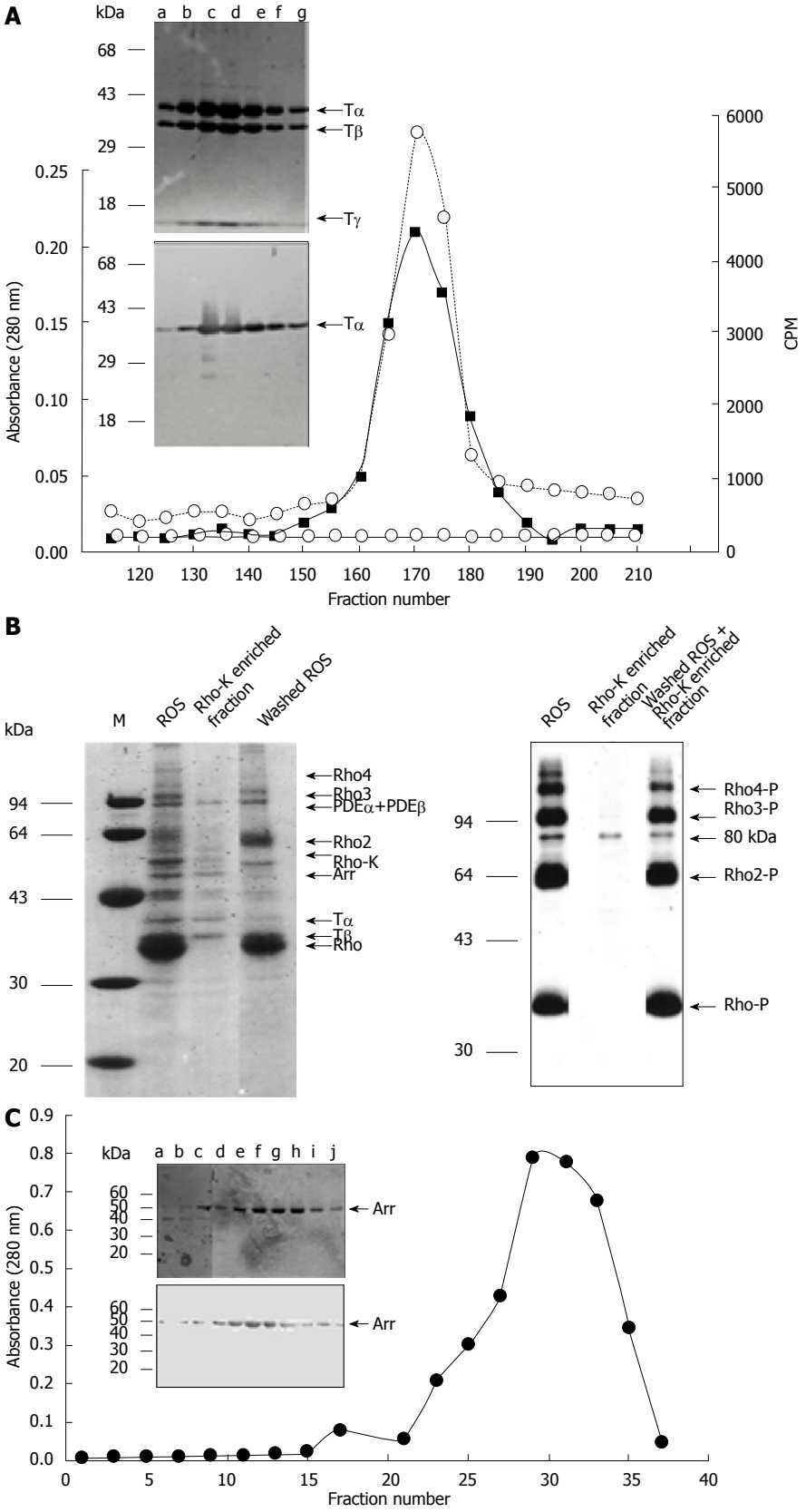

Figure 4 Purification of transducin and arrestin-1, and preparation of an enriched fraction of rhodopsin kinase. A: Transducin was purified to homogeneity on a DEAE-cellulose column. The elution profile was monitored at 280 nm (■). Fractions were analyzed for [8-^{3}H] GMPpNp binding activity (CPM) in the absence (○, continuous line) or presence (○, dashed line) of light-activated rhodopsin (as dark-depleted ROS membranes). Fractions were also examined by SDS-PAGE (Inset, top) and Western blot using anti-transducin polyclonal antibodies (Inset, bottom). Lanes a, b, c, d, e, f, and g correspond to column fractions Nº 155, 160, 165, 170, 175, 180 and 185, respectively. Arrows indicate the migration of α-, β- and γ-subunits of transducin (Tα, Tβ and Tγ); B: Autoradiography showing the light-induced *in vitro* phosphorylation of rhodopsin by rhodopsin kinase (Rho-K). Left, Coomassie blue staining; Right, Autoradiography. Samples of intact ROS membranes, a partially purified fraction of Rho-K, or a mixture of dark-depleted ROS membranes together with the enriched fraction of Rho-K were incubated with [γ-^{32}P] ATP under light conditions as described in Materials and Methods. Arrows indicate the migration of phosphorylated rhodopsin (Rho), rhodopsin dimers (Rho2), rhodopsin trimers (Rho3) and rhodopsin tetramers (Rho4). A polypeptide band of 80 kDa was phosphorylated in the Rho-K enriched fraction. M: Molecular weight markers; C: Arrestin-1 was purified to homogeneity after three consecutive chromatography steps, a DEAE-cellulose column, a heparin-sepharose column eluted with a gradient of phytic acid, and a second heparin-sepharose column eluted by increasing the salt concentration in the buffer. Shown is the elution profile of the last heparin-sepharose column, which was monitored at 280 nm (•). Fractions were inspected by SDS-PAGE (Inset, top) and Western blot using anti-arrestin-1 polyclonal antibodies (Inset, bottom). Lanes a, b, c, d, e, f, g, h, i, and j correspond to column fractions Nº 17, 21, 23, 25, 27, 29, 31, 33, 35, and 36. Arrows indicate the migration of arrestin-1 (Arr). ROS: Rod outer segments.

measuring the amount of arrestin-1 that was translocated from the initial mixture to the pellet. No arrestin-1 was bound to non-phosphorylated apoprotein opsin in the dark or light (Figure 7A and B, lane P). Moreover, as seen in the same figure (Figure 7A and B, lane P), no arrestin-1 interacted with phosphorylated opsin in the dark or light. Basal amounts of arrestin-1 interacted with rhodopsin, isorhodopsin and the 13-*cis*-retinal-rhodopsin complex, both in the dark and under illumination (Figure 7A and B, lane P), and as expected, all reformed photolyzed and

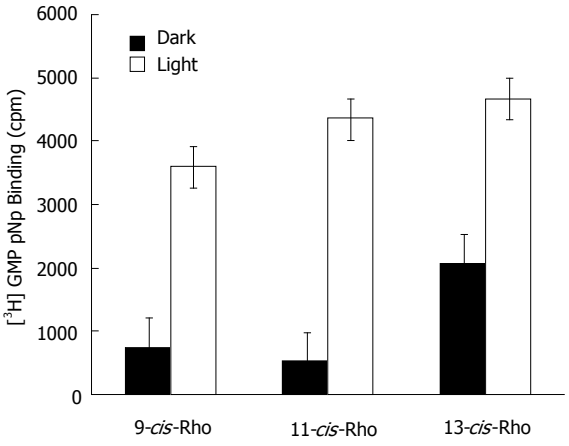

Figure 5 Activation of transducin by reconstituted rhodopsin and rhodopsin analogues. Binding of guanine nucleotides to transducin was evaluated by Millipore filtration using [8-^{3}H] GMPpNp. Dark and white bars correspond to experiments performed under dark and light conditions, respectively. Duplicate assays of three independent experiments were carried out. Mean ± SD are reported. Differences with *P*-values < 0.05 were considered significant.

phosphorylated pigments were capable of recognizing and binding arrestin-1 (Figure 7B, lane P). Although only residual arrestin-1 was bound to phosphorylated rhodopsin and phosphorylated isorhodopsin in the dark (Figure 7A, lane P), arrestin-1 was efficiently recognized by phosphorylated 13-*cis*-retinal-rhodopsin in the dark (Figure 7A, lane P). The silver stained gels shown in Figure 7A and B were quantified by densitometry (Figure 7C) and confirmed the results described above. The amount of reconstituted pigment, instead of the total amount of protein, was used to normalize the reported values shown in the histograms. The interaction of arrestin-1 with phosphorylated 13-*cis*-retinal-rhodopsin in the dark is consistent with our findings using transducin and rhodopsin kinase, that suggest that the rhodopsin analogue containing the 13-*cis* isomer of retinal exists as a pseudo-active state even without illumination.

DISCUSSION

To study ligand binding pockets in proteins, specific analogues with systematically altered chemical property in their structural moieties have usually been employed to establish structure-activity relationships with regard to their functional groups. Retinal has four C = C double bonds that give rise to the four mono-*cis* isomers, the 7-*cis*, 9-*cis*, 11-*cis* and 13-*cis* forms. These isomers undergo *cis*–*trans* isomerization upon photoexcitation. The chromophore of rhodopsin is 11-*cis*-retinal and, thus, in its absence, opsin is not photosensitive and no visual function exists. Here, the 9-*cis* and 13-*cis* retinal isomers have been used to probe the rhodopsin chromophore binding pocket and to study ligand-protein relationships to better understand the photochemical *cis-trans* isomerization of rhodopsin.

The production of 11-*cis*-retinal occurs in the retinal pigment epithelium. One of the more abundant proteins in this tissue is RPE65, which has been shown to be essential for the conversion of all-*trans*-retinyl ester to 11-*cis*-retinol[32]. Leber's congenital amaurosis, a childhood blinding disorder, results from disruption of a number of genes, but in many cases, the gene for RPE65 is defective[33-36]. When RPE65 is mutated or lacking, as in the RPE65 knockout mouse and Leber's congenital amaurosis, visual function is impaired[32]. However, in the RPE65 knockout mouse, where synthesis of 11-*cis*-retinal does not occur, a minimal visual response from rod photoreceptors is obtained, which is mediated by isorhodopsin, the rod pigment formed with 9-*cis*-retinal, rather

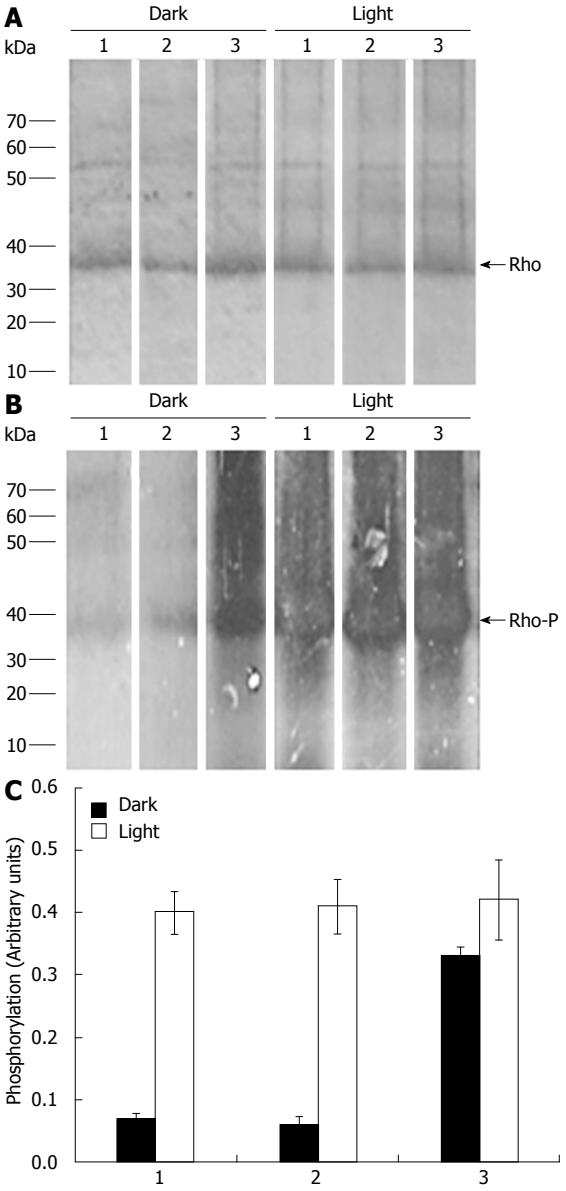

Figure 6 Ability of the reconstituted rhodopsin and rhodopsin analogues to serve as substrates for rhodopsin kinase. A: Coomassie blue staining; B: Autoradiography. Arrows indicate the migration of rhodopsin (Rho) and phosphorylated Rho (Rho-P); C: Densitometry of the autoradiograms shown in B. Dark and white bars correspond to experiments performed under dark and light conditions, respectively. Mean ± SD of three independent experiments are reported. Differences with P-values below 0.05 were considered significant.1: 9-*cis*-Rho; 2: 11-*cis*-Rho; 3: 13-*cis*-Rho.

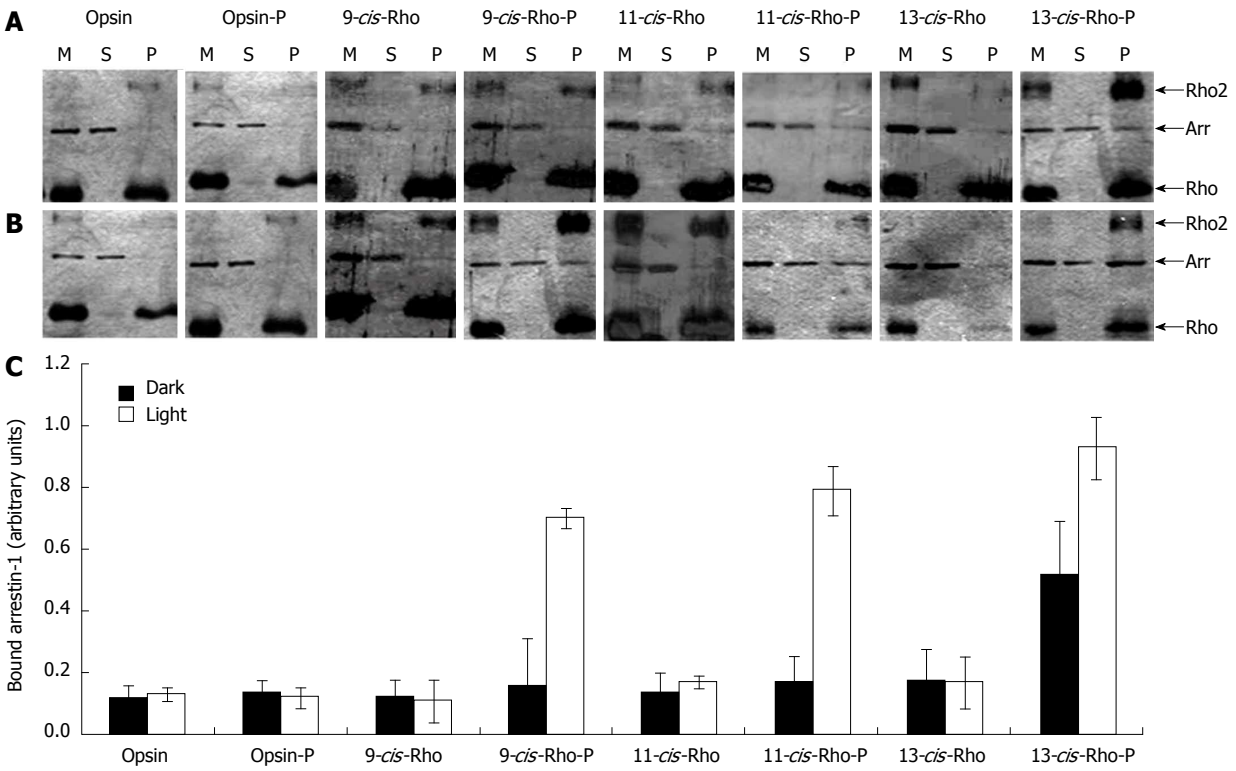

Figure 7 Ability of arrestin-1 to interact with the reconstituted rhodopsin and rhodopsin analogues. Arrestin-1 was combined with membranes containing opsin, phosphorylated opsin (opsin-P), isorhodopsin (9-*cis*-Rho), phosphorylated isorhodopsin (9-*cis*-Rho-P), rhodopsin (11-*cis*-Rho), phosphorylated rhodopsin (11-*cis*-Rho-P), 13-*cis*-retinal-rhodopsin (13-*cis*-Rho), and phosphorylated 13-*cis*-retinal-rhodopsin (13-*cis*-Rho-P), under dark (Panel A) and light (Panel B) conditions. The mixtures (M) were centrifuged and aliquots of each mixture and of the resulting supernatants (S) and pellets (P) were separated by SDS-PAGE. Gels were colored by silver staining. In Panel C, the amount of arrestin-1 that interacted with the phosphorylated pigments in the pellet fraction was quantified by densitometry. Mean ± SD of three independent experiments are reported. Differences with *P*-values < 0.05 were considered significant Arrows indicate the migration of rhodopsin (Rho), arrestin-1 (Arr), and rhodopsin dimers (Rho2).

than rhodopsin[13]. Isorhodopsin, is photosensitive and appears to be very similar to rhodopsin, as determined in numerous *in vitro* studies and experiments using intact retinae and isolated photoreceptors[37,38]. Then, although endogenous 9-*cis*-retinal has not been reported in the retina, the high expression of 9-*cis*-retinol dehydrogenase (RDH4/RDH5) in the retinal pigment epithelium[39,40] suggests that 9-*cis*-retinal could be generated in that tissue. Actually, 9-cis-retinoids do exist in many tissues, with highest concentrations in liver and kidney, and are essential for gene regulation, growth and development[41,42]. In contrast, the 13-*cis* configuration of retinal has never been observed in vision and as such is not physiologically relevant in the visual process. Nevertheless, all-*trans*-retinal is an essential component of type I , or microbial, opsins such as bacteriorhodopsins, channelrhodopsins, sensory rhodopsins and halorhodopsin. Type I opsin genes are found in prokaryotes, algae, and fungi, where they control diverse functions such as phototaxis, energy storage, development, and retinal biosynthesis[43]. Using microbial opsin genes, prokaryotes can transduce light to shift proton gradients, modulate chloride balance, or switch flagellar motor direction, whereas motile algae transduce light to change flagellar beating to direct locomotion toward environments optimally illuminated for their photosynthetic requirements. In these seven-trans-membrane-segment receptor proteins, light causes the all-*trans*-retinal to become 13-*cis*-retinal, which then cycles back to all-*trans*-retinal in the dark state. Unlike the situation with rhodopsin, in which the retinal-protein linkage is hydrolyzed after photoisomerization[44], the activated retinal molecule in type I opsins, 13-*cis*-retinal, does not dissociate from its opsin protein, but thermally reverts to the all-*trans* state while maintaining a covalent bond to its protein partner[45]. Accordingly, 13-*cis*-retinal is physiologically crucial in those organisms that possess type I opsins.

The regular instability of 11-*cis*-retinal limits its commercial availability. The standard procedure used to prepare 11-*cis*-retinal consist of an isomerization reaction of all-*trans*-retinal by irradiation under 436 nm[46-48], which generates a mixture of 9-*cis*-retinal, 11-*cis*-retinal and 13-*cis*-retinal that requires to be separated by chromatography techniques, such as alumina column chromatography, thin-layer chromatography, high-performance liquid chromatography (HPLC), or flash countercurrent chromatography (FCCC). Photochemical and enzymatic processing of retinoids in the eye is essential for perception of the light signal and for sustaining vision by regeneration of visual pigments[12]. Specifically, the photoisomerized all-*trans*-retinal is converted back to the 11-*cis*-retinal chromophore by an enzymatic pathway of chemical

reactions termed the retinoid cycle[11,12]. Why the 9-*cis*- and 13-*cis*-isomers of retinal are not formed in the eye in addition to 11-*cis*-retinal? The retinal G protein-coupled receptor (RGR) is a protein that structurally resembles visual pigments and other G protein-coupled receptors. RGR appears to play a role as a photoisomerase in the production of 11-*cis*-retinal. The proposed function of RGR, in a complex with 11-*cis*-retinol dehydrogenase (RDH5), is to regenerate 11-*cis*-retinal under light conditions[49]. Maeda *et al*[50] evaluated the role of RGR using RGR single knockout mice, and RGR and RDH5 double knockout mice, under various conditions. The most striking phenotype of RGR knockout mice after illumination included light-dependent formation of 9-*cis*- and 13-*cis*-retinoid isomers. These isomers were not formed in wild-type mice because either all-*trans*-retinal is bound to RGR and protected from isomerization to 9-*cis*- or 13-*cis*-retinal or because RGR is able to eliminate these isomers directly or indirectly. These results suggest that RGR and RDH5 are likely to function in the retinoid cycle.

In the present manuscript, we focused on comparing the interactions of rhodopsin and rhodopsin analogues containing 9-*cis*-retinal and 13-*cis*-retinal with other proteins of the visual cascade, such as transducin, rhodopsin kinase and arrestin-1. Under dark conditions, 13-*cis*-retinal-rhodopsin was capable of catalyzing transducin GDP/GTP exchange and was highly phosphorylated by rhodopsin kinase. Since 13-*cis*-retinal-rhodopsin behaves like active rhodopsin independently of light, and given that both transducin activation and phosphorylation by rhodopsin kinase require the generation by photolysis of the meta II intermediate of rhodopsin, we propose that the structure of dark 13-*cis*-retinal-rhodopsin adopts a tridimensional conformation that mimics the active photoproduct of rhodopsin. Moreover, arrestin-1 was also efficiently recognized by phosphorylated 13-*cis*-retinal-rhodopsin in the dark. As shown by Gurevich *et al*[24], arrestin-1 binds phosphorylated light-activated rhodopsin with remarkable selectivity. However, arrestin-1 binding to an equal amount of dark (inactive) phosphorylated rhodopsin or active unphosphorylated rhodopsin (light-activated rhodopsin) is 10-20 times lower, whereas its binding to inactive unphosphorylated rhodopsin is barely detectable[24]. Thus, rhodopsin activation or phosphorylation alone promotes relatively weak arrestin-1 interaction. In addition, arrestin-1 binding to phosphorylated light-activated rhodopsin is many times greater than the sum of dark phosphorylated rhodopsin and light-activated rhodopsin levels, suggesting that the binding mechanism is more sophisticated than a simple cooperative two-site interaction. Gurevich *et al*[24] proposed a model positing that arrestin-1 has two sensor sites, an activation sensor that binds receptor elements that change conformation upon activation, and a phosphate sensor that binds receptor attached phosphates. When the receptor is phosphorylated and active at the same time, both sensors bind. Simultaneous engagement of the two sensor sites allows arrestin-1 transition into the active high affinity receptor-binding state. Since the conformation of dark 13-*cis*-retinal-rhodopsin appears to mimic the structure of the meta II photointermediate, phosphorylated 13-*cis*-retinal-rhodopsin seems to be sufficient to be recognized by arrestin-1 even in the absence of light.

Since the 9-*cis*, 11-*cis*, and 13-*cis* isomers of retinal are not planar, changes at the *cis* configuration in the polyene structure may cause important non-planar distortions in the retinal molecule that in turn may affect its longitudinal size. Employing the molecular orbital program MOPAC (version 1.11), we determined the structures of minimal energy for the various retinal isomers used here. The distances from carbon C-2 to carbon C-15 were found to be 10.84 Å, 10.96 Å, and 11.54 Å for 11-*cis*-retinal, 9-*cis*-retinal, and 13-*cis*-retinal, respectively. The retinal molecule reaches its longest longitude in its all-*trans* configuration (13.02 Å). A clear relationship between the size of each isomer and its accessibility to the chromophore binding pocket in the apoprotein opsin can be established when these theoretical distances were taken in consideration and contrasted with the percentage of pigment that was regenerated with each retinal isomer. 11-*cis*-Retinal and 13-*cis*-retinal, which corresponded to the shortest and longest isomers, showed the highest and lowest percentage of pigment reconstitution, respectively. Thus, it is evident that some size restrictions exist within the prosthetic group binding site. In addition, structural differences may occur when the various retinal isomers are incorporated and accommodated into the apoprotein to reform the distinct pigments.

It is known that the spectral properties of 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal are very similar; all three compounds show absorption maxima at 365-370 nm. Interaction between 11-*cis*-retinal and opsin generates the ground state of rhodopsin with its characteristic peak at about 500 nm. The red shift of 11-*cis*-retinal in rhodopsin is a result of the protonated Schiff base linkage between the aldehyde and the ε-amino group of Lys296, which is stabilized by the Glu113 counter-ion. Moreover, the positive charge is delocalized through the polyene moiety of retinal. Rhodopsin is constrained in an inactive conformation because binding of 11-*cis*-retinal to Lys296 *via* the protonated Schiff base induces changes in rhodopsin' s helical transmembrane domain and cytoplasmic surface that prevent interaction with native transducin, rhodopsin kinase and arrestin-1. Upon photoisomerization of 11-*cis*-retinal to all-*trans*-retinal, the receptor undergoes major structural rearrangements that include displacement of the positively charged Schiff base from its interaction with negatively charged Glu113. Based on this mechanism of action, a bulky ligand might affect and modify the regular distance between the Glu113 counter-ion and the retinal attachment site in the protein, affecting in turn the spectroscopic properties of the regenerated pigment. Blue shifts in isorhodopsin (λ_{max} = 486 nm) and 13-*cis*-retinal-rhodopsin (λ_{max} - 467 nm) correlate well with the increase in longitudinal size of 9-*cis*-retinal and 13-*cis*-retinal compared to 11-*cis*-retinal. During the rho-

dopsin photocycle, the protein relaxes through a series of distinct photointermediates, each with characteristic UV/visible absorption maxima. Most of these intermediates can only be trapped by using ultra freezing temperatures. The metarhodopsin I photointermediate (meta I), which is the inactive precursor of meta II, possesses a characteristic peak at 478 nm. Interestingly, the spectroscopic properties of 13-*cis*-retinal-rhodopsin in the dark were comparable to those of meta I. The resemblance of meta I and the 13-*cis*-retinal-rhodopsin pigment might cause the pseudo-activation state seen for the latter even without illumination. Since meta I can only be generated following freeze-trapping at -40 ℃, and at temperatures below the phase transition temperature of the surrounding lipids, it was not viable for us to carry out a direct comparison between the properties of meta I and the 13-*cis*-retinal-rhodopsin analogue.

Rhodopsin pigment regeneration studies using available retinal isomers showed that stable isomeric pigments can be formed using a diversity of isomers such as 11-*cis*, 9-*cis*, 7-*cis*, 9,13-*dicis*, 7,13-*dicis*, 9,11-*dicis*, 7,11-*dicis*, 7,9-*dicis*, 7,9,11-*tricis*, 7,9,13-*tricis*, *etc*., with varying rates of pigment formation[51]. With the exception of 9-*cis*-retinal, all isomers required much longer times to give isomeric pigments at reduced yields[51]. By using the crystal structures of rhodopsin, Liu *et al*[52] reproduced the binding cavity of rhodopsin containing the 11-*cis*-retinal, and examined whether other isomers were capable of being accommodated within the pocket. When the 9-*cis* and 7-*cis* isomers of retinal were tested it was clear that all atoms of the two isomeric pigment analogs fitted well within the binding cavity. However, when the pigment was replaced with atoms of the 13-*cis* protonated Schiff base, it was clear that the 13-methyl group and partly C13 and C14 of the 13-*cis* chromophore was projected far beyond the binding pocket overlapping with atoms in the β-sheet of the loop that connects the TM4 and TM5 helices[52]. These results confirm that steric restrictions exist in the binding cavity and explain previous reports showing nonbinding of the 13-*cis* or all-*trans* isomers to the inactive state of the protein[16], as well as our results that showed a low percentage of regeneration of the 13-*cis*-retinal-rhodopsin analog. The much reduced rate for pigment formation for the 13-*cis* isomer and other retinal isomers is likely due to the altered ring conformations, the relocated 9-methyl groups, and shifts of the polyene chain.

The C-9 and C-13 methyl groups of the 11-*cis*-retinal appear to be pivotal elements in ligand-receptor communication. For instance, 9- and 13-demethylretinals yielded analogue pigments, but with an increase in constitutive activity and/or much reduced physiological activity[53,54]. Ebrey *et al*[55] observed that 13-demethyl-rhodopsin, which is opsin regenerated with 11-*cis*-13-demethyl-retinal, activated transducin as measured by cGMP-phosphodiesterase PDE6 activity in the dark. This finding was surprising, since 13-demethyl-retinal lacks only the methyl group in position 13. However, the 9-*cis* isomer of 13-demethylretinal like all the other activating pigments required light[55]. When, 11-*cis*-13-demethyl-retinal was preincubated with opsin in the dark, significant phosphorylation was observed[56]. The activity was increased when the all-*trans* isomer was used, but decreased with 9-*cis*-13-demethyl-retinal. The results obtained by Buczyłko *et al*[56] were consistent with the observations of Ebrey *et al*[55]. Deletion of methyl groups to form 9-demethyl and 13-demethyl analogues, as well as addition of a methyl group at C10 or C12, shifted the meta I/meta II equilibrium toward meta I, such that the retinal analogues behaved like partial agonists[54]. To examine the steric limits of the 9-methyl and 13-methyl binding pocket of opsin, deGrip *et al*[57] prepared cyclopropyl and isopropyl derivatives of 11-*cis*- and 9-*cis*-retinal, at C-9 and C-13, and of α-retinal at C-9. Most isopropyl analogues showed very poor binding, whereas most cyclopropyl derivatives exhibit intermediate binding activity. The data of deGrip *et al*[57] were in line with the growing body of evidence showing that the interplay between a receptor and its ligand is very finely tuned. Small modification of a ligand can already alter this interplay and thereby redirect the conformational space of a receptor, leading to a different activity profile. Here we have shown that 13-*cis*-retinal-rhodopsin behaves as a pseudo-active pigment in the dark. Similar to 11-*cis*-13-demethyl-retinal-rhodopsin, the structure of 13-*cis*-retinal-rhodopsin probably embraces a tridimensional conformational fold that mimics to some extent the active meta II photointermediate of rhodopsin. Consequently, 13-*cis*-retinal-rhodopsin is capable of interacting with transducin, rhodopsin kinase and arrestin-1 even without illumination. Palczewski *et al*[58] have also shown that active pseudo-photoproducts, which stimulate transducin activation and opsin phosphorylation by rhodopsin kinase, are formed with opsin and retinal analogues lacking the 13 methyl or the terminal two carbons of the polyene chain as well as with opsin and all-*trans*-retinal. Other reports have also shown that an activated receptor may be generated without illumination by addition of all-*trans*-retinal or its analogues to opsin[56,59-61]. Cohen *et al*[59] found that transducin activation by the all-*trans*-retinal-opsin complex was strongly pH-dependent with the most efficient catalysis at pH = 5-6. Hofmann *et al*[60] demonstrated that free all-*trans*-retinal can react with the apoprotein to form pseudo-photoproducts that are spectrally identical to the photoinduced metarhodopsin species (meta I/II/III). By measuring the increased phosphorylation of opsin by rhodopsin kinase, Buczyłko *et al*[56] showed that the potency of stimulation depended on the chemical and isomeric nature of the analogues and the length of the polyene chain. For example all-*trans*-C17 aldehyde was the most effective in stimulation of opsin phosphorylation, while longer (all-*trans*-retinal) and shorter analogues (all-*trans*-C15 aldehyde) were less potent. All-*trans*-C22 aldehyde was not effective suggesting that the length of this retinoid excluded it from the binding to opsin, while the shortest aldehyde, all-*trans*-C12 aldehyde, was only modestly effective. This specificity suggested a unique inter-

action of opsin with retinoids, rather than a nonspecific lipid-like effect or interaction with peripheral amines[56]. Ligand-free opsin is also capable of activating transducin, although at a much reduced level than light-activated rhodopsin[61,62], but this activity was enhanced by a factor of about 10 by the presence of all-*trans*-retinal. Interestingly, when the sizes of the various isomers of retinal used in the present work were compared, 13-*cis*-retinal was more alike to all-*trans*-retinal than 9-*cis*-retinal or 11-*cis*-retinal.

Various tridimensional conformations of the photoreceptor protein have been solved. Park *et al*[63] reported the X-ray crystal structure of ligand-free native opsin from bovine retinal rod cells. Compared to rhodopsin[64], opsin shows prominent structural changes in the conserved E(D)RY and NPxxY(x)$_{5,6}$F motifs and in the transmembrane fifth to transmembrane seventh regions (TM5-TM7). These structural changes reorganize the empty retinal-binding pocket to disclose two openings that may serve for the entry and exit of retinal. The lack of the interacting prosthetic group causes distinct structural alterations in the retinal-binding pocket. For example, part of the space occupied by the β-ionone ring of retinal is filled in opsin with the side chains of some aromatic residues[63]. In rhodopsin, retinal is held along the polyene chain by amino acids located in TM3, TM6 and loop E2[64]. In opsin, the extracellular part of TM3 and loop E2 are slightly moved away from helices TM5-TM7. Thereby, the retinal-binding pocket becomes wider towards the retinal attachment site in Lys296, and the ε-amino group of Lys296 does not seem to be involved in a salt bridge with Glu113, which corresponds to the retinal Schiff base counter-ion in the rhodopsin dark state, or with Glu181, which was proposed to be part of a complex counter-ion which forms in meta I [65]. Moreover, it has been shown that opsin can readily adopt inactive and active conformations *in vitro*, and low pH and a synthetic peptide derived from the C terminus of the α-subunit of transducin stabilized this active conformation of opsin[66]. Scheerer *et al*[67] reported the crystal structure of the complex between active opsin and the carboxy terminus peptide of the α-subunit of transducin, and clear conformational differences can be detected when the structures of inactive and active opsin are compared. More recently, Choe *et al*[68] used the low pH induced-active conformation of opsin to obtain crystals of meta Ⅱ, by soaking crystals of active opsin with all-*trans*-retinal. They presented the crystal structures of meta Ⅱ alone or in complex with a C-terminal fragment derived from the α-subunit of transducin. The binding site for all-*trans*-retinal appears to be preformed in the active conformation of opsin because the presence of retinal in the meta Ⅱ structures causes only a small adjustment of some amino acid side chains[68], while the Lys296 side chain, which is more flexible in ligand-free opsin[63], becomes ordered due to its linkage with retinal. From the crystal structures of rhodopsin, opsin, activated opsin and meta Ⅱ, it is clear that changes in the prosthetic group binding pocket occur in each of the different conformations of the protein, and receptor can make use of the conformational flexibility of the ligand and the variability of its interaction with the binding site.

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COMMENTS

Background

G protein-coupled receptors activate signaling paths in response to a diverse number of stimuli such as photons, Ca^{2+}, organic odorants, amines, hormones, nucleotides, nucleosides, peptides, lipids and even large proteins. The dim-light photoreceptor rhodopsin is a highly specialized G protein-coupled receptor composed of the apoprotein opsin, and a covalently linked 11-*cis*-retinal chromophore.

Research frontiers

Little is known about the interactions of rhodopsin analogues with other proteins in the visual cascade. Although, it has been reported that the retinal binding site in the inactive state of rhodopsin can accommodate the 7-*cis*, 9-*cis* and 11-*cis* isomers of retinal, but not the longer all-*trans* or 13-*cis* isomers[16], in the present work we were able to reconstitute a rhodopsin analogue containing 13-*cis*-retinal, in addition to photoreceptor proteins containing 9-*cis*-retinal and 11-*cis*-retinal.

Innovations and breakthroughs

This study compared the binding of reconstituted rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin to transducin, rhodopsin kinase and arrestin-1, both in the dark and under illumination.

Applications

The rhodopsin analogue containing the 13-*cis* isomer of retinal was capable of activating transducin and was highly phosphorylated by rhodopsin kinase independently of light. Arrestin-1 was also efficiently recognized by phosphorylated 13-*cis*-retinal-rhodopsin in the dark.

Peer review

This manuscript by Araujo *et al* is aimed to study if reconstituted rhodopsin, 9-cis-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin interact with transducin, rhodopsin kinase and arrestin-1. The authors isolated rod outer segments (ROS) from bovine retinas, generated rhodopsin and rhodopsin analogues with the different retinal isomers, purified transducin and arrestin-1 to homogeneity, and obtained an enriched-fraction of rhodopsin kinase by extracting freshly prepared ROS. The authors characterized the reconstituted rhodopsin and rhodopsin analogues through three sets of experiments: activation of transducin, ability to serve as substrates for rhodopsin kinase, and binding to arrestin-1. Different approaches including column chromatography, guanine nucleotide binding assay, *in vitro* phosphorylation, *etc.* were used. They found that rhodopsin analogue harboring the 13-*cis* isomer of retinal is capable of activating transducin in a light-independent way. They concluded that the rhodopsin analogue containing the 13-*cis* isomer of retinal seems to fold in a pseudo-active conformation that mimics the active photointermediate of rhodopsin.

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Format

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English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning

tool assembly. United States patent US 20020103498. 2002 Aug 1

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