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World Journal of Biological Chemistry
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

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E-mail: bpgoffice@wjgnet.com
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REVIEW

MicroRNA signature and function in retinal neovascularization

Saloni Agrawal, Brahim Chaqour

Saloni Agrawal, Department of Cell Biology, State University of New York (SUNY) Downstate Medical Center, Brooklyn, NY 11203, United States
Brahim Chaqour, Department of Cell Biology and Ophthalmology, and SUNY Eye Institute Downstate Medical Center, Brooklyn, NY 11203, United States
Author contributions: Agrawal S and Chaqour B conceived and co-wrote this review.
Supported by A Grant from the National Eye Institute of the National Institutes of Health EY022091-01 to Chaqour B
Correspondence to: Brahim Chaqour, Professor, Department of Cell Biology and Ophthalmology, and SUNY Eye Institute Downstate Medical Center, 450 Clarkson Avenue 5, Brooklyn, NY 11203, United States. bchaqour@downstate.edu
Telephone: +1-718-2708285 Fax: +1-718-2703732
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Abstract

Ischemic retinopathies are clinically well-defined chronic microvascular complications characterized by gradually progressive alterations in the retinal microvasculature and a compensatory aberrant neovascularization of the eye. The subsequent metabolic deficiencies result in structural and functional alterations in the retina which is highly susceptible to injurious stimuli such as diabetes, trauma, hyperoxia, inflammation, aging and dysplipidemia. Emerging evidence indicates that an effective therapy may require targeting multiple components of the angiogenic pathway. Conceptually, mircoRNA (miRNA)-based therapy provides the rationale basis for an effective antiangiogenic treatment. miRNAs are an evolutionarily conserved family of short RNAs, each regulating the expression of multiple protein-coding genes. The activity of specific miRNAs is important for vascular cell signaling and blood vessel formation and function. Recently, important progress has been made in mapping the miRNA-gene target network and miRNA-mediated gene expression control. Here we highlight the latest findings on angiogenic and antiangiogenic miRNAs and their targets as well as potential implications in ocular neovascular diseases. Emphasis is placed on how specific vascular-enriched miRNAs regulate cell responses to various cues by targeting several factors, receptors and/or signaling molecules in order to maintain either vascular function or dysfunction. Further improvement of our knowledge in not only miRNA specificity, turnover, and transport but also how miRNA sequences and functions can be altered will enhance the therapeutic utility of such molecules.

Key words: MircoRNA; Angiogenesis; Retinal neovascularization; Vascular endothelial growth factor; Ischemia; Endothelial cell

Core tip: This review examines the critical regulatory role of microRNAs (miRNAs) in the process of normal and pathological angiogenesis and the prospects that they provide for the development of new treatments. miRNAs are both upstream and downstream of multiple growth factors in regulating endothelial proliferation, migration, and vascular patterning, processes critical for normal and abnormal formation of blood vessels. Emphasis in this review is placed on how specific vascular-enriched miRNAs regulate cell responses to various cues by targeting several factors, receptors and/or signaling molecules in order to maintain either vascular function or dysfunction.

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INTRODUCTION

Angiogenesis is the generation of new blood vessels from pre-existing ones, a process initiated by branching "decisions" of endothelial cells (ECs) to undergo proliferation, guided migration, tubulogenesis, vessel fusion and pruning. Physiological angiogenesis is crucial in maintaining normal vascular growth and homeostasis from embryogenesis to postnatal life, especially in instances of fetal development, wound healing, transplantation, post-ischemic tissue repair and the menstrual cycle[1-4]. However, excessive angiogenesis is a commonly occurring pathogenic condition in more than 30 diseases, including eye diseases, cancer, rheumatoid arthritis, atherosclerosis, diabetic nephropathy, pathologic obesity, asthma, cystic fibrosis, inflammatory bowel disease, psoriasis, endometriosis, vasculitis and vascular malformations. In particular, the vascular beds supplying the retina often sustains injury as a result of underlying diseases such as diabetes, trauma, hyperoxia, aging, dyslipidemia, or the interaction of genetic predisposition, environmental insults and age. The high metabolic and oxygen demands make the retina highly susceptible to these injurious stimuli which lead to an arrest of vascular development, vaso-obliteration and/or vascular occlusion. The subsequent vascular pathological response observed, especially in intraocular vascular diseases, generates disorganized, leaky, and tortuous vessels that leak into the interface between the vitreous and the retinal tissue, attracting fibroglial elements causing severe hemorrhage, retinal detachment, and vision loss. These are the characteristic features of neovascular and fibrovascular diseases of the eye such as retinopathy of prematurity and proliferative diabetic retinopathy. The exudative or "wet" form of age-related macular degeneration (AMD) which largely affects choroidal vessels and cause blindness in elderly populations is characterized by the overgrowth of the choriocapillaris that invade the Bruch's membrane and grow into subretinal spaces[5,6].

GROWTH FACTOR EXPRESSION AS A DETERMINANT FACTOR OF NORMAL AND PATHOLOGICAL ANGIOGENESIS IN THE RETINA

The formation of an aberrant and dysfunctional vasculature is commonly initiated by the uncontrolled expression or, lack thereof of growth factors including vascular endothelial growth factor (VEGF), Notch and Wnt signaling components, bone morphogenic protein, thrombospondins and insulin-like growth factors (IGFs)[7-11]. In particular, VEGF, a highly specific mitogen for ECs, is a major determinant of normal and pathological formation of the retinal vasculature[12]. Loss of VEGF attenuates blood vessel formation in mice embryos leading to early embryonic lethality and causes defective vascularization in adults[13-16]. Conversely, high expression of VEGF is common in avascular peripheral hypoxic regions of the retina compared to already vascularized areas[17]. Under conditions of oxygen deprivation, hypoxia-inducible factor 1α (HIF-1α) is activated and binds to its responsive elements in the promoter region of VEGF and other hypoxia-responsive genes, causing their upregulation and subsequent abnormal vessel growth[18]. Anti-VEGF treatments have been useful in reducing neovascularization of the eye. However, not all patients have achieved an optimal response. Safety data from several studies identified ocular and systemic adverse events including subretinal fibrosis, endophthalmatis, traumatic cataract, non-ocular hemorrhage, *etc*. Additionally, the use of anti-VEGF treatments, in the case of AMD in diabetic patients, interfered with myocardial revascularization and, in some cases, worsened the pathology in the diabetic eyes as a result of VEGF-dependent loss of neurotrophic and vasculotropic factors[19].

There are numerous other factors that contribute to neovascular growth. The erythropoietin (Epo) and *VEGF* genes, for instance, exhibit a similar expression pattern during both physiological and pathological vessel growth and inhibition of Epo suppressed retinal neovascularization both *in vivo* and *in vitro*[20,21]. Other factors such as basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), transforming growth factor alpha, interleukin 8 (IL-8), connective tissue growth factor (CTGF), pigment epithelium-derived factor, IGF- I , and matrix metalloproteinase (MMP)-2 were similarly implicated in the neovascular response and are considered as potential therapeutic targets. In addition, inflammation-mediated cyclooxygenase-2 (COX-2) can modulate angiogenesis *via* its interaction with VEGF[22] and important pro-angiogenic and neovascular functions have been associated with the activation of the renin-angiotensin system, ephrins, tyrosine kinase receptors and ligands (*e.g.*, tie/angiopoietin receptors). Together, all these factors form a well-coordinated and functional network of molecules affecting the process of normal and pathological angiogenesis. Emerging evidence indicates that antiangiogenic therapy may require therapeutic approaches that target multiple components of the angiogenic pathway[23-26]. Conceptually, microRNA-based approaches may potentially provide the rationale basis for such approaches.

MICRORNA BIOGENESIS AND FUNCTION IN THE MODULATION OF GENE EXPRESSION

Key events in gene regulation depend on specific small non-coding RNA-guided posttranscriptional regulators, commonly referred to as miRNAs that target a "mixture" of diverse growth and differentiation factor mRNAs encoding networks[27]. MicroRNAs are a relatively abundant class of gene expression regulators that function as "micromanagers" of gene expression[28]. These are short non-coding RNAs (18-25 nucleotides) which work

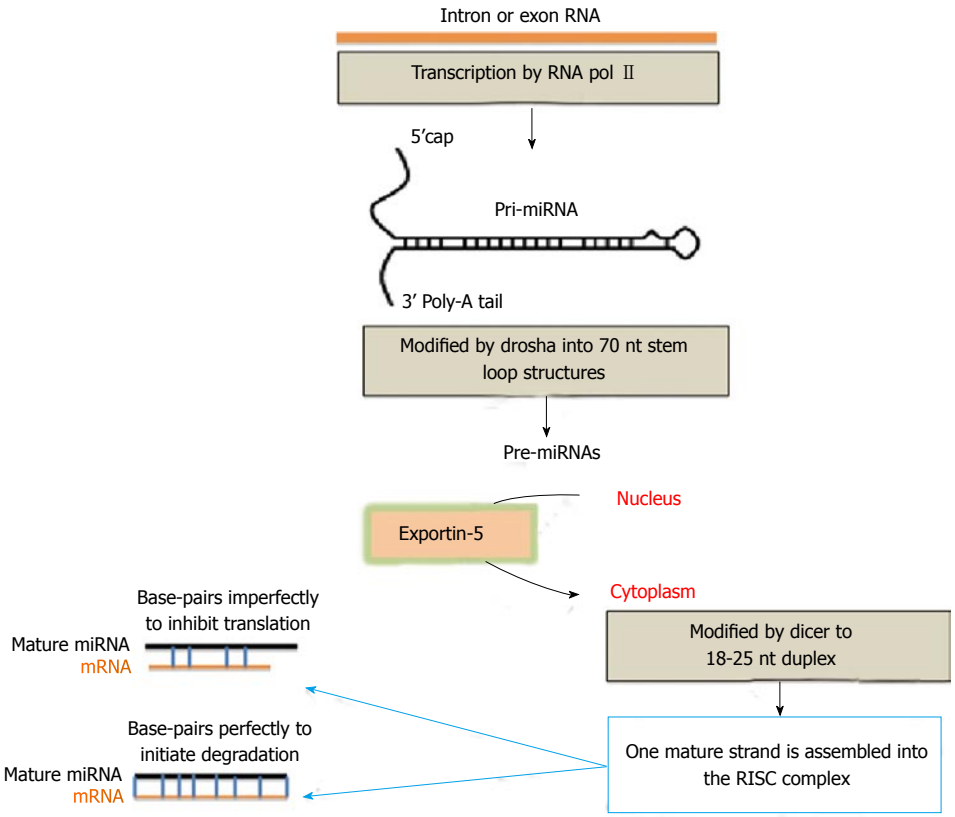

Figure 1 Schematic representation of microRNA (miRNA) biogenesis. *miRNA* genes are transcribed into large pre-miRNA (capital R) that are cleaved by a protein complex containing the endonuclease Drosha into shorter pre-miRNAs. The latter are then transported to the cytoplasm by exportin-5. A complex containing the endonuclease, Dicer, then cleaves the loop portion of the pre-miRNA (capital R) to form a short duplex molecule that is unwound, and the single-stranded mature mirNA is then passed to Argonaute to from a functional mature, approximately 22 nucleotide, miRNA that inhibit translation after base-pairing with the 3' UTR of the miRNA (capital R) target.

post-transcriptionally to negatively regulate gene expression through translational inhibition or degeneration of mRNAs. They might act as on-off switches to eliminate mRNAs that should not be expressed in a particular cell type or at a particular moment. MicroRNAs can also act to fine tune mRNA abundance and adjust the levels of their mRNA targets within a physiological range in response to environmental cues. A single miRNA has the capacity to target multiple target mRNAs, which can themselves be targeted by numerous other miRNAs. To date, 1186 mouse miRNA and 1872 human miRNA sequences have been noted on the miRBase database and may control at least 30% of all the protein-coding genes[29].

Since the discovery of miRNAs, their biogenesis has been thoroughly examined and it is now known that both miRNAs and small interfering (si) RNAs share the same cellular machinery[4,30]. Most *miRNA* genes are transcribed by RNA polymerase Ⅱ, which is usually responsible for the transcription of protein coding genes, to yield several kilobase-long primary miRNA (pri-miRNA) transcripts (Figure 1). Pri-miRNAs have characteristic loop stem (or hairpin) morphology and contain the mature miRNA sequence in the stem portion near the loop. The microprocessor, containing the endonuclease Drosha, cleaves the pri-miRNA into shorter pre-miRNAs that are transported to the cytoplasm by exportin-5. Once in the cytoplasmic compartment, pre-miRNAs undergo the final steps towards maturation. The first step involves "dicing" of the loop portion of the molecule by another endonuclease, Dicer and the transactivation response RNA binding protein (TRBP). A miRNA-miRNA duplex that is unwound is released together with the single-stranded mature miRNA. The latter is then passed to Argonaute to from a functionally mature, approximately 22 nucleotide miRNA. The 2-8-bp "seed" region in the 5' end of miRNAs binds to target 3'UTR of mRNA sequences and inhibits translation if base-pairing is imperfect or initiates mRNA cleavage if base-pairing is perfect.

REGULATION OF ANGIOGENESIS BY MICRORNAS

The first studies of the functional significance of the miRNA pathway in angiogenesis were performed using conditional deletion of Dicer alleles, as complete loss of Dicer resulted in a significant reduction of the mature miRNA profile and early embryonic lethality[31,32]. Yang *et al*[32] have shown that mice with Dicer gene deletion lack adequate blood vessel formation in embryos and yolk sacs and die between 12.5 and 14.5 d post-gestation, thus implicating Dicer-dependent miRNA genesis in the regulation of blood vessel formation. Defects in these mice were due to dysregulation of VEGF

and its receptors, KDR and FLT-1, along with Tie-1, an angiopoietin-2 receptor[32]. Similarly, silencing of Dicer or Drosha in (ECs) using siRNA significantly inhibited capillary sprouting and altered expression patterns of Tie-2, VEGF receptor 2 (VEGFR2/KDR), Tie-1, endothelial nitric oxide synthase (eNOS) and IL-8 *in vitro*[33,34]. Another study by Otsuka *et al*[35] showed that in female Dicer hypomorphic mice, infertility ensued from lack of angiogenesis in the ovaries. Further analysis revealed that impaired angiogenesis resulted from the absence of two pro-angiogenic miRNAs, miR17-5p and let-7b, which target anti-angiogenic factors[35]. Additionally, nude mice subcutaneously injected with siRNA-transfected ECs showed reduced angiogenic sprouting of transplanted cells[33]. In two EC-specific Dicer knock-out mouse models generated by Suarez *et al*[34], postnatal angiogenesis significantly decreased in response to multiple stimuli. In this study, transfection of cells with miR-18a, miR-17-5p, and miR-20a (collectively forming the miR-17-92 cluster) restored normal angiogenesis in Dicer knockout mice[34]. Taken together, these studies established a role of Dicer-dependent miRNA biogenesis in the control of angiogenesis *in vitro* and *in vivo*.

MICRORNA SIGNATURE IN NORMAL AND PATHOLOGICAL ANGIOGENESIS

Recent studies have examined miRNA expression profiles and patterns during retinal angiogenesis[8,36-38]. More than 250 miRNAs have been enumerated in the retina and new information on the regulation and mode of action of those miRNAs is progressively emerging[38]. Specific functions have been attributed to individual angiogenic miRNAs, although the challenge still remains in validating their protein targets[23,36,39-46]. Similarly, differential expression of miRNAs during retinal neovascularization has been studied in the mouse model of oxygen-induced retinopathy (OIR). In this model, seven miRNAs were upregulated, including miR-451, -424, -146, -214, -199a, -181 and -106a, when compared to control retinas, while miR-31, -150 and -184 were downregulated. However, this study provided only an exhaustive list of potentially key angiogenic miRNAs whose expression patterns, localization, and actual targets remain unclear.

Greater insights on angiogenic and antiangiogenic miRNA expression and function have been obtained from *in vitro* studies and other *in vivo* models of pathological angiogenesis. Poliseno *et al*[47] have performed the first large-scale analysis of miRNA expression in human umbilical vein endothelial cells using miRNA arrays. Twenty seven highly expressed miRNAs were identified, 15 of which were predicted to regulate the expression of receptors for angiogenic factors (*e.g.*, Flt-1, Nrp-2, FGF-R, c-Met, c-Kit). Additional studies from other groups have identified a total of 200 miRNAs that are expressed in ECs[4,24]. Overall 28 endothelial-specific miRNAs were highly expressed in 5 out of 8 of the profiling studies including miR-221/222, miR-21, the let-7 family, miR-126, miR-17-92 cluster, and the miR 23-27-24 cluster[4,24,25]. Angiogenic factors and receptors are putative targets of those miRNAs[1,7,48,49]. However, it should be noted that both abundantly expressed miRNAs as well as the rarely expressed ones play important regulatory roles and the exact *in vivo* relevance of all miRNAs expressed in ECs remains to be determined. Since angiogenesis involves complex and intertwined pathways, we have classified the currently known endothelial-specific miRNAs based on the context/conditions of their expression (Figure 2).

Hypoxia-sensitive miRNAs

Microarray-based expression profiling revealed that specific miRNAs are induced under hypoxic conditions and target angiogenic factors produced by ECs[50]. In particular, miR-15b, -16, -20a and -20b were shown to be upregulated under hypoxic conditions and target VEGF[50]. Additionally, miR-15b and miR-16 are predicted to be putative regulatory miRNAs of *uPAR*, *COX2*, and *c-MET*, which themselves are induced in response to hypoxic conditions[50]. Upregulation of these miRNAs is p53- and HIF-1α-dependent. Other microarray-based expression profiles have also revealed a set of hypoxia-induced miRNAs which are also over-expressed in tumors[51]. In particular, miR-210 is hypoxia-induced in all cell types tested[41,52]. In ECs subjected to hypoxia, miR-210 regulates the tyrosine kinase receptor ephrin-A3 that contributes to vascular remodeling. miR-210 promotes the formation of capillary-like structures in cultured ECs but, under hypoxic conditions, it decreases ECs tube formation and migration[52,53]. miR-100 is another hypoxia-sensitive miRNA that was shown to be significantly down-regulated after hind-limb ischemia[54]. Under these conditions, miR-100 repressed the expression of an angiogenic serine/threonine protein kinase targeted by rapamycin[55]. Furthermore, Shen *et al*[39] reported a dramatic increase in the expression of miR-106a, -146, -181, -199a, -214, -424 and -451 in a model of retinal ischemia suggesting their potential roles in the pathogenesis of neovascular diseases of the eye. Similarly, the hypoxia-induced miR-424 and miR-200 target the protein complex that stabilizes HIF-α and promote angiogenesis[56,57].

Growth factor-sensitive miRNAs

The effects of several angiogenic factors are mediated by miRNAs such as miR-155, -191, -21, -18a, -130a, -17-5p, -20a, -296, -101, -125b and -132[58]. In particular, serum, VEGF, and bFGF increased the expression of miR-130a, which enhances angiogenesis by downregulating the expression of anti-angiogenic homeobox proteins such as growth arrest-specific homeobox and Homeobox protein Hox-A5[52,59]. In the presence of VEGF or epidermal growth factor (EGF), the levels of miR-296 were significantly up-regulated in primary human brain microvascular ECs[60]. miR-296 was also found to be up-regulated in tumors and targets the hepatocyte growth factor-regulated tyrosine kinase substrate that inhibits degradation of key angiogenic growth factor recep-

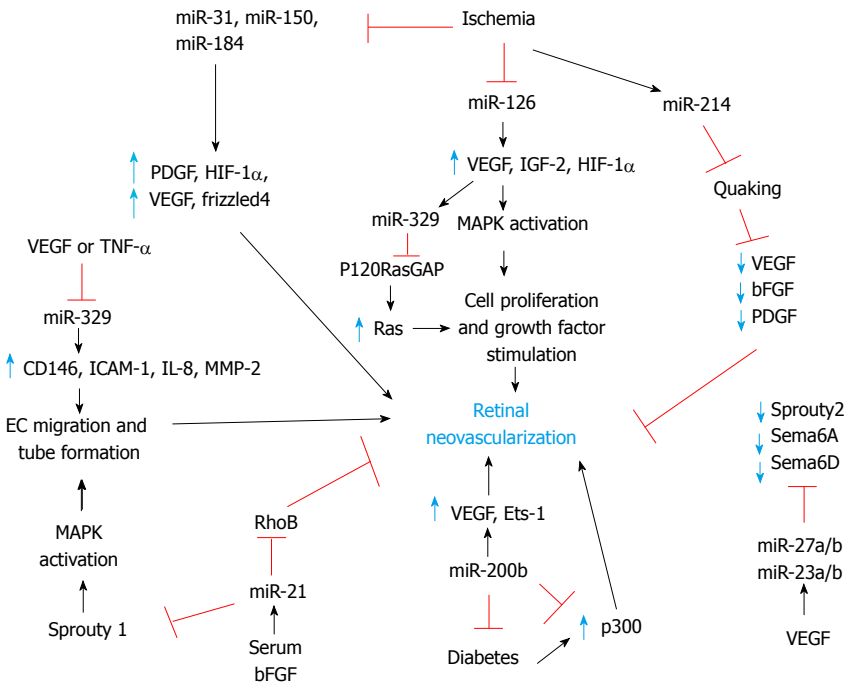

Figure 2 Overview of major angiogenic and antiangiogenic miRNAs and their targets in promoting or suppressing retinal neovascularization. VEGF: Vascular endothelial growth factor; IGFs: Insulin-like growth factors; HIF-1α: Hypoxia-inducible factor 1α; bFGF: Basic fibroblast growth factor; PDGF: Platelet derived growth factor; IL-8: Interleukin 8; MMP: Matrix metalloproteinase; ICAM-1: Intercellular adhesion molecule-1; TNF-α: Tumour necrosis factor-alpha; MAPK: Mitogen-activated protein kinase.

tors such as VEGF receptor 2 and PDGF receptor β[60]. Conversely, miR-101 was found to be down-regulated by VEGF which then allows the expression of histone-methyltransferase enhancer of zest homolog 2, increasing methylation of histone H3 and enhancing a pro-angiogenic response[61]. miR-125b was induced transiently by VEGF and negatively regulates vascular endothelial (VE)-cadherin to suppress tube formation *in vitro* and tumor growth *in vivo*[62]. A prolonged over-expression of miR-125b *in vivo* resulted in blood vessel regression. Transforming growth factor β (TGF-β) which is best known for its profibrotic activities, is a potent inducer of *VEGF* gene expression in retinal pigment epithelial cells[63]. However, numerous miRNAs have also been found to regulate and participate in TGF-β-induced VEGF expression[64]. Such effect is mediated by miR-29a which targets the phosphatase and tensin homolog (*PTEN*) gene, leading to the activation of the protein kinase B pathway, increased VEGF expression and angiogenesis[64]. Similarly, miR-132, which is undetectable in normal endothelium, was shown to be induced by VEGF and FGF in ECs and trigger neovascularization in the retina[65,66].

Inflammation and cytokines

Inflammation typically has beneficial effects on an acute basis, but it produces undesirable effects if persisting chronically. Angiogenesis sustains inflammation by providing oxygen and nutrients for inflammatory cells which, in turn, stimulates pathological angiogenesis[67]. The increased expression of many inflammatory proteins such as IL-1, IL-3 and tumour necrosis factor-alpha (TNF-α), is regulated at the level of gene transcription through the activation of proinflammatory transcription factors, including nuclear factor-kappa-B (NF-κB). In retinal ECs of diabetic rats, the expression of miR-146, -155, -132 and -21 up-regulates *NF-κB* gene expression and activity[67]. In contrast, miR-146 negatively regulates IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6 which are themselves induced following NF-κB activation[52,67]. Thus, targeting miR-146 may have an anti-inflammatory potential.

Meanwhile, T cell derived cytokine IL-3, a pro-inflammatory and a pro-angiogenic cytokine, was reported to down-regulate the expression of miR-296, miR-126, and -miR-221/222 in ECs[68]. The miR-222 exhibited anti-angiogenic effects by negatively regulating STAT5A in a mouse model of retinal neovascularization[68]. miR-126 has been portrayed as an anti-inflammatory molecule because it suppresses TNF-α mediated vascular cell adhesion molecule 1 (VCAM-1) expression and leukocyte interactions with ECs[26,31,52].

Reactive oxygen species as inducers of EC senescence

There is considerable evidence that increased production of reactive oxygen species (ROS) in the retina affects retinal vessel formation, although the mechanisms by which this occurs are not fully understood[69]. ROS such as superoxide anions such as H_2O_2 inhibit EC growth and increased cell death which are commonly associated with vaso-obliteration preceding ischemia[70]. Over-expression of miR-23a from the miR-23-27-24 cluster inhibits H_2O_2-induced apoptosis in retinal pigment epithelial cells from AMD patients *via* the repression of Fas, an activator of the apoptotic pathway[71]. Similarly, the

miR-200c is up-regulated in ECs by oxidative stress and affects EC proliferation and death by inhibiting ZEB1[72].

The miRNA profiling of aging human primacy ECs revealed that miR-17,-21,-216,-217,-31b, and-181a/b are highly expressed[73]. In particular, miR-217 is progressively expressed in response to EC stimulation by ROS and targets Sirt1 (silent information regulator 1) that regulates angiogenic gene expression *via* deacetylation of histones[31,73]. Inhibition of miR-217 in ECs reduced senescence and enhanced angiogenesis[73]. Likewise, miR-34a targets Sirt1 and impairs angiogenesis which leads to the onset of senescence[31,74].

OTHER MIRNAS WITH POTENTIALLY IMPORTANT ANGIOGENIC FUNCTIONS IN THE RETINA

Other miRNAs with potentially important angiogenic functions in the retina were shown in Figure 2.

miR-221/222

miR-221 and miR-222 are two paralogue miRNAs located in close proximity to one another on Xp11.3 chromosome[26,47]. Over-expression of miR-221/222 reduced EC growth *in vitro* by targeting the c-Kit receptor, a tyrosine kinase receptor for stem cell factor which regulates EC migration, and survival as well as tube formation[47,52]. EC transfection with miR-221/222 inhibits tube formation, migration, and wound healing[47,52]. Conversely, miR-221/222 positively regulates proliferation and migration of cultured vascular smooth muscle cells, suggesting a cell type-specific function[68,75]. The proangiogenic effects of miR-221/222 in smooth muscle cells are p27 and p57-dependent. A recent study in zebra fish showed that miR-221 deficiency resulted in drastic developmental vascular defects which underscore an important function of miR-221/222 in angiogenesis[11]. In the latter study, miR-221 acts autonomously on the VEGF-C/Flt4 signaling pathway, altering endothelial tip and stalk cell phenotypes[11]. miR-221 promotes tip cell migration and proliferation by negatively regulating cyclin dependent kinase inhibitor 1b and phosphoinositide-3-kinase regulatory subunit 1[11]. The discrepancy between the *in vitro* and *in vivo* activities of miR-221/222 may be due to a differential effect on the mature and non-mature circulatory system. Further studies are needed to ascertain the regulation and function of miR-221/222 in developmental and pathological angiogenesis in the retina.

miR-17-92 cluster

The miR-17-92 cluster is a polycistronic *miRNA* gene located in intron 3 of chromosome 13 in humans, and contains six mature miRNAs, miR-17, -18a, -19a, -19b-1, -20a and -92a[3,4,26]. This cluster is highly expressed in ECs and tumor cells and is strongly up-regulated by ischemia[28,31,52,76]. Ectopic expression of the miR-17-92 cluster partially rescued the angiogenic phenotype of Dicer-deficient ECs[58]. Similarly, restoration of miR-17 in combination with let-7b in Dicer knockout mice also partially normalized corpus luteum angiogenesis by targeting the tissue inhibitor metalloproteinase-1, an anti-angiogenic factor[35]. The pro-angiogenic function of this cluster is due to the inhibition of the anti-angiogenic molecules thrombospondin-1 and CTGF by miR-18 and miR-19, respectively[58]. However, the function of this miRNA cluster in retinal angiogenesis remains to be elucidated.

miR-126

miR-126 is the best characterized EC-specific miRNA and is known to be highly conserved among species[1,26]. It is encoded by intron 7 of the EGF-like domain 7. miR-126 enhanced VEGF signaling by directly targeting the 3'UTR of Sprouty-related EVH1 domain containing protein-1 and phosphoinositol-3-kinase regulatory subunit 2[1,7,26,31,49]. Thus, miR-126 promotes angiogenesis by targeting negative regulators of the angiogenic pathway. miR-126 affects cell migration, reorganization of the cytoskeleton, capillary network stability, and cell survival *in vitro*[7]. It also altered developmental angiogenesis and vascular integrity. Fifty percent of miR-126 null mice died as a result of severe systemic edema, ruptured blood vessels and multifocal hemorrhages[49]. Vascularization of the retina was shown to be severely impaired in mice that survived the miR-126 deletion[49]. An intravitreal injection of miR-126 in the retina reduced the levels of VEGF, IGF-2, and HIF-1α[77]. Additionally, miR-126 exhibited tumor suppressor functions in lung cancer cells by negatively regulating VEGF both *in vivo* and *in vitro*[26,78]. Hence, strategies to modulate miR-126 levels may hold a great therapeutic value against retinal neovascular diseases.

miR-200b

The miR-200 family is up-regulated by stimuli such as TGF-β1 and PDGF and suppresses growth of human microvascular ECs[57]. Hypoxia inhibits miR-200b expression, prompting an elevated *Ets-1* gene expression and its downstream target genes such as *MMP1* and *VEGFR2*[57]. Intravitreal injection of miR-200b mimicked reduced elevated levels of VEGF and prevented angiogenesis in a model of diabetic retinopathy[79]. Thus, the regulation of miR-200b in retinal neovascular diseases may prevent the aberrant expression of critical factors associated with pathological angiogenesis.

miR-214

miR-214 is located on a non-coding intronic Dynamin-3 gene sequence and its expression is controlled by the transcription factor Twist-1. HIF-1α mediates Twist-1 transcription, which then allows miR-214 expression[80]. Concordantly, miR-214 was shown to be up-regulated in ischemic conditions when HIF-1α was stabilized[80]. A recent study has shown that miR-214 directly targets Quaking (QKI) and regulates the expression and secretion of angiogenic growth factors such as VEGF, bFGF

and PDGF[81]. Quaking plays an essential role in vascular development[82]. *In vivo* silencing of miR-214 enhanced the formation of blood vessels on Matrigel plugs and increased the secretion of pro-angiogenic growth factors[81]. Additionally, miR-214 is substantially increased in the mouse model of OIR[39]. Inhibition of miR-214 enhanced normalization of the vascularization of the retina through the expression of QKI, suggesting that miR-214 may function directly to either block pathological neovascularization or prevent hyperoxia-induced vasoobliteration[81].

miR-329

miR-329 targets the important pro-angiogenic gene, CD146, and inhibits angiogenesis *in vitro* and *in vivo*[83]. CD146 is an adhesion molecule and an endothelial biomarker which actively participates in the angiogenic process[83,84]. CD146 functions as a co-receptor for VEGFR2 and activates the p38/IκB kinase/NF-κB signaling pathway leading to increased EC migration and tube formation. A study by Wang *et al*[83] has shown that exposure of ECs to VEGF represses endogenous miR-329 expression, resulting in the simultaneous up-regulation of CD146 and treatment with miR-329 significantly reduced retinal neovascularization. miR-329 is thought to inhibit the expression of many downstream pro-angiogenic genes including intercellular adhesion molecule-1 (ICAM-1), IL-8, and MMP-2, among others. Thus, miR-329 serves as a potential therapeutic target in pathological retinal angiogenesis.

miR-21

miR-21 is located on chromosome 17q23.2 within the protein-coding region of the transmembrane protein 49[85]. miR-21 promotes angiogenesis by inhibiting phosphate and tensin homolog deleted on chromosome 10 (PTEN), a potent negative regulator of the phosphatidyl inositol-3 kinase/AKT signaling pathway. By blocking Akt signaling, PTEN decreases both eNOS activity and VCAM-1 expression[31,86,87]. In tumor cells, overexpression of miR-21 significantly increased the levels of HIF-α and VEGF. In primary bovine retinal microvascular ECs, inhibition of miR-21 drastically reduced proliferation, migration, and tube-forming capacity reinforcing the important pro-angiogenic role of miR-21 in the retinal microvasculature[88].

miR-23-27-24 cluster

The miR-23-27-24 cluster is highly enriched in ECs and is well conserved between rodent and humans[40]. There are two paralogs of the clusters: an intergenic miR-23a-27a-24-2 cluster and an intronic miR-23b-27b-24-1 cluster on vertebrate chromosomes 8 and 13 respectively[40]. miR-27a/b and miR-23a/b mediate proper capillary formation in response to VEGF *in vitro*[40]. miR-27a/b and miR-23a/b repress anti-angiogenic gene expression such as SPROUTY2, SEMA6A and SEMA6D[40]. These anti-angiogenic genes inhibit the mitogen-activated protein kinase pathway and VEGF pathway[40]. Additionally, miR-23a/b and miR-27a/b also promote choroidal neovascularization (CNV)[40]. Silencing of miR-23a/b and miR-27a/b suppressed CNV in mice[40]. Thus, targeting the miR-23-27-24 cluster may have beneficial therapeutic applications in the treatment of AMD.

miR-132

miR-132 is highly up-regulated in human embryonic stem cells and tumors whereas it was undetectable in a normal endothelium[26,65]. However, stimulation of ECs by growth factors increased the levels of miR-132 which then activates quiescent endothelium by suppressing p120RasGAP[26,65,66]. Suppression of p120RasGAP led to the activation of Ras which then increases VEGF-mediated phosphorylation of mitogen-activated protein kinase extracellular related protein kinase kinase-1[65]. Ectopic expression of miR-132 was sufficient to induce EC proliferation *in vitro* and its inhibition significantly reduced growth factor-mediated angiogenesis *in vivo* and *in vitro*[65]. Additionally, inhibition of miR-132 also greatly decreased retinal neovascularization in mice[65]. Thus, early detection and modulation of this miRNA may inhibit the onset of neovascularization.

CONCLUSION

Treatment and management of neovascular diseases rely mainly on pharmacotherapy and/or surgical procedures. However, these treatments are seldom efficacious and they often are plagued by unwanted side effects and/or insurmountable complications. The use of miRNAs that specifically target a set of angiogenic genes appears to be a viable alternative approach. Currently, there are numerous ongoing clinical trials designed to test the efficacy and effectiveness of such approach in the treatment of various disorders (*e.g.*, atherosclerosis, cancer, inflammatory diseases) and the preliminary results are promising[89-91]. Neovascular diseases including those of the eye will likely test/use such approach in a near future as our understanding of miRNA regulation and the molecular mechanisms underpinning their functions increases every day.

MicroRNAs are also increasingly considered as potential diagnostic markers of disease stages. Indeed, miRNAs have been discovered in a wide variety of extracellular body fluids such as saliva, serum, plasma, milk, and urine as nuclease resistant entities[24,31,92]. These extracellular circulating miRNAs enable cell-to-cell communication and also provide insight into the physiological states or progression of pathological diseases within the secreting cells[92-94]. miRNAs are thought to be secreted from cells in three possible ways: (1) *via* passive leakage from cells resulting from injury, inflammation, apoptosis or necrosis; (2) *via* an active secretion method in membrane-bound vesicles such as exosomes, shedding vesicles and apoptotic bodies; and (3) *via* an active secretion method of protein-miRNA complexes[92]. Exosomes are 30 nm-100 nm ves-

icles, arising from multivesicular bodies and their release is mediated by the enzyme sphingomyelinase-2[31,92-95]. Shedding vesicles, arising from the plasma membrane, is facilitated *via* a ligand-receptor method. Further insight into the exosomal miRNA formation and circulation may not only validate their prognostic potential in the slowly developing neovascular diseases of the eye but, will also help design optimal delivery systems of miRNAs *in vivo*.

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REVIEW

Systems biology unravels interferon responses to respiratory virus infections

Andrea L Kroeker, Kevin M Coombs

Andrea L Kroeker, Department of Physiology, University of Manitoba, Winnipeg, MB R3E 0J9, Canada
Kevin M Coombs, Department of Microbiology, University of Manitoba, Winnipeg, MB R3E 0J9, Canada
Author contributions: All authors contributed equally to this work.
Correspondence to: Kevin M Coombs, Professor, Department of Microbiology, University of Manitoba, 730 William Avenue Winnipeg, Winnipeg, MB R3E 0J9, Canada. kevin.coombs@med.umanitoba.ca
Telephone: +1-204-7893976 Fax: +1-204-7893926
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Abstract

Interferon production is an important defence against viral replication and its activation is an attractive therapeutic target. However, it has long been known that viruses perpetually evolve a multitude of strategies to evade these host immune responses. In recent years there has been an explosion of information on virus-induced alterations of the host immune response that have resulted from data-rich omics technologies. Unravelling how these systems interact and determining the overall outcome of the host response to viral infection will play an important role in future treatment and vaccine development. In this review we focus primarily on the interferon pathway and its regulation as well as mechanisms by which respiratory RNA viruses interfere with its signalling capacity.

Key words: Respiratory virus; Interferon; Systems biology; Proteomics; Genomics; Innate immunity

Core tip: Many novel regulators of innate immune signalling pathways, such as the interferon signalling pathway, have been discovered recently. These advances may be in part attributed to high-throughput systems biology techniques including genomic, proteomic, miRNA and siRNA screens, as well as through various confirmatory methods using quantitative polymerase chain reaction, microscopy, and animal models. Collectively, these studies have provided insights into novel drug targets that could boost host innate immunity or could potentially serve as broad-spectrum anti-virals against RNA respiratory viruses.

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INTRODUCTION TO SYSTEMS BIOLOGY AND INTERFERONS

Virus-host studies of a wide range of viruses have identified many host changes that occur upon infection, including the induction of a variety of anti-viral pathways. For example, these include autophagy, apoptosis, endoplasmic-reticular stress, nuclear-factor kappa B (NF-κB) and proteasomal degradation pathways as well as the topic of this review, interferon signalling. Some of these studies have utilized global genomic, transcriptomics and proteomic technologies and have led to the characterizations of "infectomes", "interactomes" and "interferomes". One of the great advantages to systems biology tools is that they can provide a relatively unbiased "bottom-up" discovery approach such as with global transcriptome and siRNA screens. These have proven useful in the characterization of innate immune responses. Biological tools for detection of specific subsets of the cell are also continually being developed, including probes for specific

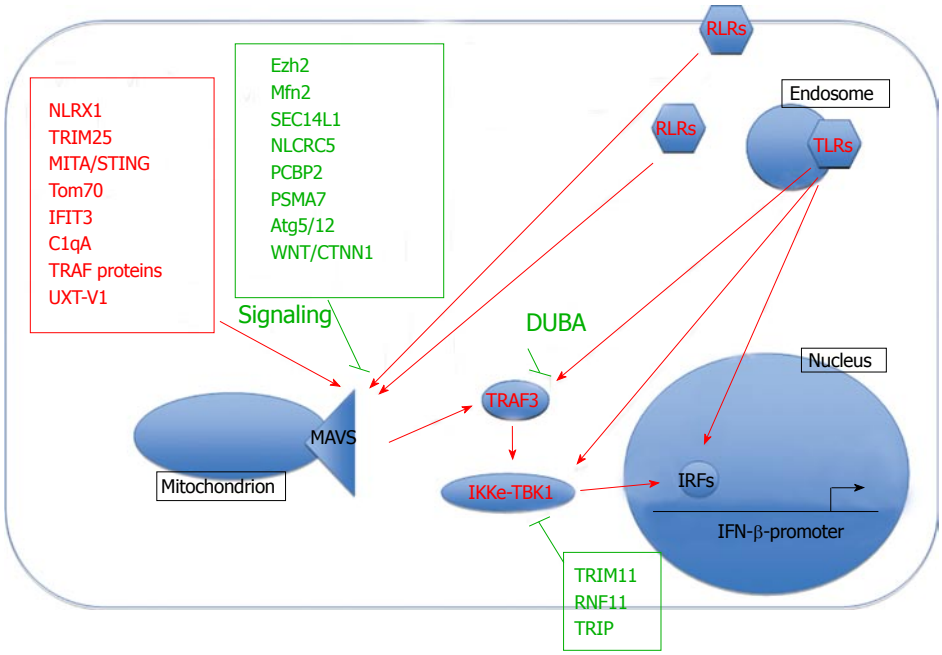

Figure 1 Interferon activation. NLRX1 : Nucleotide-binding oligomerization domain, leucine rich repeat containing X1; TRIM25: Tripartite motif-containing 25; MITA/STING: Mediator of IRF3 activation/stimulator of interferon genes protein; TRIM11: Tripartite motif containing 11; RNF11: Ring finger protein 11; TRIP: Thyroid receptor-interacting protein; DUBA: Deubiquitinating enzyme A; RLRs: RIG-like receptors; TLRs: Toll-like receptors; IFIT3: Interferon-inducible transmembrane protein 3; MAVS: Mitochondrial antiviral signaling protein; NLRC5: Nod-like receptor C5; PCBP2: Poly(rC)-binding protein 2; PSMA7: Proteasome subunit alpha type-7; TBK1: Tank binding kinase; IKKe: Inhibitor of nuclear factor kappa-B kinase; IRFs: Interferon regulatory factors; WNT/CTNNB1: Wnt/beta-catenin.

classes of enzymes, methods to detect different protein post-translational and epigenetic modifications, and subcellular fractionation techniques. As will be discussed below, many studies have begun to characterize gene transcription programs in response to viruses, have identified novel anti-viral proteins and regulators of interferon production and have experimented with novel approaches to treatment of viral infection.

The study of interferons (IFN) is one of the oldest known family of proteins with anti-viral properties. They are produced and released in response to pathogens, such as viruses and bacteria, and function in establishing an anti-viral state in host cells and activating immune cells (for review see[1]). Type I interferons in humans include IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω and are classified as such by their ability to bind the IFNAR1-IFNAR2 interferon receptor complex[2]. IFN-γ is a type Ⅱ interferon and signals through the IFNGR1-IFNGR2 receptor complex. A third class of interferons, type Ⅲ, has been proposed and would likely contain IFN-λ1, -λ2 and -λ3, which are also known as interleukin-29 (IL-29), IL-28A and IL-28B, respectively, and bind IFNLR1 (also known as IL-28 receptor-α, IL-28Rα) and IL-10Rβ[3]. Effects of interferons are numerous and depend on downstream signaling pathways. The canonical activation of Janus-Kinase-Signal Transduction Activator (JAK-STAT) signalling[4], for example, induces a variety of interferon-stimulated genes (ISGs) of which some have known anti-viral activities. Activation of mitogen-activated protein kinases[5] has also been shown to have anti-viral as well as anti-proliferative effects. In contrast, phosphatidylinositol 3-kinase activation[6] induces cell proliferation and increased protein synthesis (for review see[7]). Autophagy has also been described as an inducer of interferon[8,9] as well as being induced by interferons[10,11]. The interactions and cross-regulation of these pathways are complex and are not well defined but overall, the ability of the host to mount an effective interferon response typically plays a significant protective role against viral pathogenicity.

Regulation of the interferon signalling pathway is influenced by multiple cellular regulatory systems including phosphorylation, ubiquitination and miRNA silencing. In addition, viral components such as viral proteins and viral RNA can also significantly impact interferon production by the infected host cell. Systems biology approaches have substantially contributed to understanding the interactions of these various regulatory networks, the overall outcome of their actions, and their impact on respiratory virus replication. For example, it is becoming increasingly popular to combine various omics technologies such as transcriptome and proteomic screens with functional validation using techniques such as siRNA screens, pPCR and microscopy imaging.

REGULATION OF INTERFERON INDUCTION

Activation of viral pattern recognition receptors

Innate immune responses are initially triggered in response to viral infection through the recognition of highly conserved pathogen association molecular patterns (PAMPs). In terms of RNA viruses this typically involves activation of RIG-like (RLR), Toll-like (TLR) and Nod-like receptors (NLR) in the cytoplasm and at membranous surfaces such as the plasma membrane, endosomes and endoplasmic reticulum. A major outcome of RLR and TLR activation is the production of interferons. This induction, and its regulation, will be the focus of this review (summarized in Figure 1).

Coordination of antiviral responses at the mitochondrial outer membrane

An important event following RLR activation consists

of the formation of mitochondrion-centric anti-viral signalling complexes that regulate interferon and NF-κB signalling cascades and subsequent immune responses. The mitochondrial anti-viral signaling protein (MAVS)/virus-induced signaling adaptor/interferon-beta promoter stimulator protein 1/Cardif protein is central to this process. Located at the outer mitochondrial membrane, it acts as a scaffolding protein that interacts with a variety of different host proteins that regulate downstream signalling pathways. There are many activators and facilitators of MAVS-mediated signalling and some of the most recently discovered ones include retinoic-acid inducible gene I (RIG-I), nucleotide-binding oligomerization domain, leucine rich repeat containing X1 (NLRX1), MITA/Stimulator of interferon genes protein[12], Tom70[13], interferon-induced protein with tetratricopeptide repeats 3 (IFIT3)[14], C1qA[15], tumor necrosis factor receptor associated factor (TRAF) proteins[16] and UXT-V1[17]. The formation of MAVS-mediated complexes can subsequently lead to the recruitment of tank binding kinase (TBK1) and inhibitor of nuclear factor kappa-B kinase (IKKe). However, this process is also carefully controlled through recruitment of negative regulators such as Ezh2[18], Mfn2[19], SEC14L1[20] and Wnt/beta-catenin (WNT/CTNNB1) signalling[21]. MAVS has also been described to associate with the endoplasmic reticulum[12, 22-24], peroxisomes[22], and autophagosomes[25], although the outcome of these events are beyond the scope of this review. For further details we direct readers to a review by Belgnaoui[26]. Overall, MAVS-interacting partners influence the extent of activation or inhibition of downstream interferon and NF-κB anti-viral pathways.

Activation of interferon regulatory factors

RLR and TLR activation culminate in the phosphorylation, activation and nuclear translocation of various IRF transcription factors. Two well-known factors are IRF3 and IRF7, which can be activated by kinases TBK1, IKKi, TAK1, and interleukin-1 receptor-associated kinase. This activation is carefully controlled through ubiquitin-mediated degradation of TBK1, which can be negatively regulated by tripartite motif containing 11 (TRIM11)[27], ring finger protein 11 (RNF11)[28] and thyroid receptor-interacting protein[29]. Interaction with other molecules such as TRAF3, DDX3 [(DEAD (Asp-Glu-Ala-Asp) box polypeptide 3][30] and nef-associated protein 1[31] can also modulate downstream signalling. Interestingly, a recent study using triple IRF3/IRF5/IRF7 knockout mice[32] demonstrated a formerly unappreciated role of IRF5 in interferon induction in myeloid dendritic cells. Genome-wide IRF1 binding sites have also been characterized in primary monocytes[33]. Overall, the IRF family members are essential mediators of interferon signalling in response to RNA viral infection.

Other regulators of interferon production

Numerous other proteins have been described in regulating interferon production including activators Gab1[34] and suppressors protein tyrosine phosphatase 1[35], forkhead box protein O3[36], and toll/interleukin-1 receptor domain containing adaptor molecule 2 (TRIF) degradation[37]. Several E3 ligases promote interferon signalling such as Pellino1[38], TRIM25[39], TRIM32[40] and Riplet[41]. Other E3 ligases have been characterized with a negative regulatory role in interferon production, such as Smurf1[42], RNF125[43], disintegrin and metalloproteinase domain-containing protein 15[44], TRIM38[37], TRIM11[27] and TRIM21[45]. Finally, several deubiquitinases appear to negatively regulate interferon responses, for example OTUB1[46] and UCHL1[47]. In addition, miRNAs are emerging as important regulators of interferon-mediated anti-viral responses such as miR-155[48], miR-21[49,50], miR-146[51] and miR-466l[52].

JAK-STAT signalling

Secreted type I interferons bind to interferon receptors at the cell-membrane and induce the janus activated kinase-signal transducer and activator (JAK-STAT) pathway. The bound receptor activates self-catalyzed kinase activity and causes phosphorylation, dimerization and nuclear translocation of STAT proteins. Ubiquitination has also been demonstrated to negatively regulate this pathway, for example, by ubiquitinating JAK1[53] and STAT1[54,55] as well as through binding of suppressor of cytokine signaling and protein inhibitor of activated STAT proteins, which recruit E3 ligases[56]. In addition, mir-19a has been identified as a JAK-STAT regulator[57].

ISG-induced gene transcription

There are many different interferon transcriptional programs that depend on factors such as the receptor and JAK isoforms, as well as the type of STAT dimer[58] that are induced. These in turn are dependent on the stimulus, species, cell type, and co-stimuli. Because of this complexity, the study of interferon-stimulated gene (ISG) transcription patterns has benefited greatly from omics studies and has begun to provide powerful insights into the effects of interferons on host transcription. The response to interferon-gamma, for example, has been a source of recent interest and has been demonstrated to regulate ISGs at both the mRNA[59] and miRNA level[59,60]. A few specific miRNAs that have been identified as interferon regulators include miR-203[61] and miR-9[62]. Genome-wide DNA-binding sites for STAT1 have also been characterized using ChIP-Seq[63]. Many quantitative proteomic studies have also identified altered expression patterns of interferon-induced proteins upon various stimuli, especially after viral infection; some of these genes have also been found to be dependent upon NF-κB signalling[64].

Microarrays and quantitative proteomics: Identifying global viral-induced alterations to the host response

A variety of models have been used to study the induction of innate immune pathways following virus infection, including epithelial cells, productive and abortive

Table 1 For example references

	Cell type	Proteomics	Genomics
Respiratory syncytial virus	Epithelial cells	[95-98]	[99]
	Macrophages	-	[65,100]
	Cord blood	-	[101]
Coronavirus	Epithelial cells	[79,80,102-104]	[76-78]
	Pro-monocytes		-
Influenza	Macrophages	[70,73,105,106]	[110]
	Epithelial cells	[71, 72, 107]	[111]
	Mice	[108]	[112,113]
	Ferrets	-	[114,115]
	Macaques	[109]	[112,116]
Reovirus	Epithelial cells	[117-120]	[64]
	Myocytes	[119]	-
	Mice	-	[121]
Rhinovirus	Epithelial cells	-	[74,122,123]
	Dendritic cells	[66]	-
	Human nasal cells	-	[75]

infections in macrophages[65], dendritic cells[66] and animal models (see Table 1). Microarrays have been particularly popular for these studies due to its ability to provide a comprehensive analysis of the entire cellular genome with relatively sensitive quantification of gene expression (see[67] for review of microarray technologies). Quantitative proteomic studies have also been important in validating these findings at the protein level and have been useful, for example, in the search for biomarkers. Many respiratory viruses, such as influenza[68-73], reovirus, and rhinovirus[74,75], demonstrate a robust activation of antiviral pathways and pro-inflammatory cytokines. Both genomic and proteomic analyses have demonstrated hubs of gene and protein induction that are induced by key transcriptional factors such as IRFs, STAT proteins, NF-κB and JNK. On the other hand, genomic profiling of respiratory syncytial virus[65] and pathogenic coronaviruses such as severe acute respiratory syndrome (SARS) and EMC strains have been reported to elicit weaker innate immune responses[76-78]. The absence of interferon signalling has also been recapitulated in several proteomic viral-host studies[79-81].

Analyses of microRNA expression during influenza have recently begun to emerge in a variety of models including respiratory epithelial cells[82-85], human blood[86], immune cells[87-89] and lung tissue in animal models[90,91]. Collectively these have identified roles for miR-18a[86,92] and miR-223[86,93] in negative regulation of STAT3, mir-29 in IFN-γ1 production[89], and miR-449b as a positive regulator of IFN-β production[85]. miR-23b has also been identified as a novel anti-viral molecule that is induced through RLR signaling during rhinovirus infection[94].

Strain differences: One of the fundamental questions of virology revolves around deciphering factors of pathogenesis. Hence, some studies have attempted to identify pathways that are differentially altered by pathogenic viral strains compared to less pathogenic strains. Influenza has been particularly well studied in this respect and several host factors have been identified that are unique to the replication of strains such as the pathogenic avian H5N1, the p2009 swine flu and the 1918 strain[69,70,108,124]. However, rather than inducing radically different cell responses, many different influenza strains have been found to activate surprisingly similar immune signatures (reviewed in[125]). It was, instead, the degree and timing of activation and resolution[125] of these pathways that was found to significantly impact the severity of disease[126]. Dysregulation of the host inflammatory response in particular is a major determinant of influenza pathogenicity and is influenced by both viral and host factors[127]. Different rhinovirus strains, for example type 14 and 1B[128,129], have also been demonstrated to have different abilities to attenuate interferon production and secretion from epithelial cells. This effect has been attributed to the inhibition of IRF3 dimerization[74,129] but the viral mechanism leading to this is unknown.

Cell type differences: Cell types have also been demonstrated to express different basal levels of interferon and hence, have different innate susceptibilities to viral infection[130,131]. For example, a direct comparison of interferon signaling between primary bronchial lung epithelial cells and the A549 continuous alveolar epithelial cell line suggested differences between either primary and cancer cell lines and/or epithelial cells of different origins in the lung[72]. Additionally, different cell types have been shown to influence the degree of interferon activation after reovirus infection[132].

Correlation of interferon signaling with pathogenesis: Generally interferon production is considered protective against viral infections. It has been shown numerous times that cells that produce less interferon, such as Vero cells, are more susceptible to viral infection and produce high titers of the virus[133]. The extent of interferon inhibition by the influenza non-structural (NS)-1 protein[134] and RSV NS1 and NS2 proteins[135,136] has also been extensively studied and correlates negatively with pathogenicity[137,138]. Similarly, models in which interferon signaling has been disrupted, such as by deleting IFNR, can produce high viral titers[139] and display increased lung tissue pathology[140]. Conversely, type I interferon signaling has also been shown to contribute to secondary bacterial infections[141,142]. In some studies the degree of interferon induction correlated positively with the degree of pathogenicity. For example, the reovirus T3D strain is considered more pathogenic the T1L strain, but the T3D strain was found to induce higher levels of innate immunity proteins[64,117,118]. The role of interferons in these situations is not currently understood.

Altered innate immune responses in chronic lung diseases: Many studies with rhinovirus have investigated differences in the immune response between healthy and non-healthy donor cells. In one study, infection of chronic obstructive pulmonary disorder (COPD) epithelial cells induced higher transcription levels of cytokines, chemokines, RNA helicases, interferons and increased apoptosis compared to infection of healthy control cells. In addi-

tion, basal levels of several antiviral signalling pathways were altered in COPD patients[128]. Similarly, asthma-derived epithelial cells also showed altered expression of several immunity genes both at basal levels and during rhinovirus infection[122,143]. Modulation of rhinovirus-induced host responses has also been investigated in the presence of Echinacea extracts and cigarette smoke[123].

Core innate immune responses shared by multiple respiratory viruses: While many studies that have been discussed in this review have focused on identifying global host responses towards a single virus, a few studies have directly compared viruses from multiple families. For example, Smith *el al*[144]. identified common gene networks that were activated in response to seven respiratory viruses: influenza A virus, respiratory syncytial virus, rhinovirus, SARS-coronavirus, metapneumonia virus, coxsackievirus and cytomegalovirus[144]. Among those responses were pathways associated with a general immune response including interferon signalling[144]. A second study also identified core immune responses to four respiratory viruses including apoptosis induction, endoplasmic reticulum stress and interferon signalling[98]. In addition several host interferon-induced proteins have been tested against multiple families and strains of viruses. For example, IFIT1[145], Interferon-inducible transmembrane (IFITM) proteins[146], ISG15[147,148] and Viperin[149-152] protect against multiple virus families.

Overall, microarrays and quantitative proteomics have allowed sensitive and comprehensive analyses of the host genome, and have contributed substantially to understanding the types and kinetics of signaling pathways that are activated upon viral infections.

Identification of host-virus interactions and novel restriction factors

Interactomes, viral-mediated antagonism of interferon signaling: As many viruses encode interferon-antagonizing proteins, there has been significant interest in defining their interacting partners in the host cell. Several studies have also been undertaken to identify host proteins that recognize dsRNA and 5'pppRNA. This has, for example, led to the discovery and characterization of the IFIT family[145] and their role anti-viral innate immunity.

Influenza: The influenza NS1 protein is a well-known antagonist of interferon signalling and is able to interfere with multiple anti-viral pathways. Viral-host studies have identified additional host proteins that interact with the influenza NS1 protein, using either plasmid-based expression of NS1[153-155] or during whole virus infection[153,156]. Collectively, the integration of multiple interactome studies has allowed networks such as Flu-Pol to be established which provide the basis for comparing differences and commonalities between influenza strains and cell types and are useful for targeted drug design.

RSV: RSV proteins NS1 and NS2 strongly inhibit IFN α/β by preventing the phosphorylation of the IFN regulatory factor-3[157,158] as well as activation of NLRX1 and RIG-I[35]. Additionally, the RSV NS1 protein interferes with interferon signaling through interaction with an elonginC-cullin2 E3 ligase complex that ubiquitinates and degrades STAT2[97,159]. RSV NS1 and NS2 have also been shown to alter miRNA expression, which can contribute to antagonism of interferon and NF-κB responses[160].

Coronavirus: In studies with coronaviruses, it has been previously proposed that the viral deubiquitinase, PLpro, plays a major role in suppressing interferon-alpha induction. In support of this idea, Li *et al*[161] recently demonstrated that PLpro overexpression mediated the down-regulation of mitogen-activated protein kinase and up-regulation of the ubiquitinase Ubiquitin ligase (UBC E2-25k). The open reading frame 6 protein has also been shown to attenuate antiviral responses by sequestering host nuclear impact factors including STAT1[162], vitamin D receptor, cyclic AMP-responsive element-binding protein 1, mothers against decapentaplegic homolog 4, p53, Epas I and Oct3/4[163].

Rhinovirus: Despite induction of interferon gene transcription, rhinovirus (type 14) infection can strongly attenuate interferon secretion from epithelial cells. This effect has been attributed to the inhibition of IRF3 dimerization[74,129] but the viral mechanism leading to this is unknown. In contrast, rhinovirus 1B readily stimulated interferon production in bronchial smooth muscle cells[164], suggesting different interferon regulation between strains and/or cell types.

Reovirus: The degree of IFN-α/β induction after reovirus infection has been attributed to both host and viral factors but is not well understood. However, repression of interferon signaling has been mapped to the *M1*, *L2* and *S2*[132,165] genes.

Knockdown/Knockout studies: siRNA technology has been important in testing functional effects of interferon-induced proteins. Both whole genome siRNA screens, and individual knockdown experiments have discovered and validated anti-viral effects of many including interferon-induced proteins such as the IFITM1-3 proteins[166], IRF3 and IRF2 (Shapira), ISG15[147] and Viperin[167]. In contrast, several interferon pathway members have been assigned pro-viral functions such as MxB[168] and IFIT2[156,168].

Knock-out animals have also underscored the protective effects of interferon signaling during respiratory virus infections, for example, ISG15$^{-/-}$[147,169], IFNAR$^{-/-}$[170], and MxA$^{-/-}$[171]. In addition to its role in innate immunity, interferons have also been demonstrated to have profound effects on the adaptive immune system, for example, by priming CD^{+} T-cells during influenza infection[172] and inhibiting neurotropism of reovirus infection[173,174]. Although discussion of the effects of interferon on whole host immunity is beyond the scope of this review, further discussion can be found in several comprehensive reviews[175,176].

Collectively, these studies have provided fundamental insights into how cells respond to RNA virus infection and have highlighted the importance of interferon induction in restricting virus replication and activating an appropriate host immune response. Many new and unex-

pected regulators of interferon signalling have been discovered and have demonstrated how multiple anti-viral networks interact such as ubiquitin-mediated regulation of interferon signalling molecules. As large omics studies move forward, it will become possible to compare and draw connections between anti-viral networks that are induced by different viruses.

FUTURE DIRECTIONS: INTERFERON SIGNALING AS A BROAD-SPECTRUM ANTI-VIRAL PATHWAY?

Using interferons therapeutically has been most extensively studied in models of hepatitis. However, it has also shown some promise in protecting against a variety of other virus families, including the respiratory viruses discussed in this review. For example, exogenous IFN-alpha treatment has proven effective against influenza[177-179], rhinovirus[128,180] and coronavirus[181-183]. Interferons are also important in protecting against reovirus infections[184]. The role of type Ⅲ interferons is generally not as well understood as type I but may also afford protection against respiratory viruses[185].

Interferons can also be endogenously elicited through a variety of RLR and TLR agonists. 5'pppRNA, for example, is a well-known and potent RIG-I agonist and has been demonstrated to protect against both RNA and DNA viruses, including Dengue virus, influenza, hepatitis C and human immunodeficiency virus-1[186]. Similarly, TLR agonists such as dsRNA[187,188] or inosine-containing ssRNA[189] have been shown to protect against coronavirus, influenza, and respiratory syncytial virus infections in mice. A commercial compound, Arbidol, has also had some success in neutralizing various respiratory viruses such as influenza, rhinovirus, adenovirus, coxsackie virus and RSV[190]. Additional small molecules that induce type I interferons have recently been identified using high-throughput screens[191,192]. Alternatively, inhibiting antagonists of interferon signaling can also boost the production of interferon. As discussed above, these antagonists can either be host molecules or viral proteins, and inhibitors to each have been described[193]. Interestingly, ribavirin treatment of RSV-infected epithelial cells was shown to enhance interferon-stimulated gene expression[194] and treating RSV-infected macrophages with lovastatin was shown to blunt pro-inflammatory cytokine gene expression[100]. These therapies may have potential for broad-spectrum anti-viral properties.

Despite successfully treating some viral infection with interferon, it has also been noted that interferon stimulation can increase lung inflammation. Many gene array studies have also positively correlated pathogenicity or cytopathology with the induction of interferon and/or inflammatory genes. For example, the severe pathology of the 1918 influenza pandemic and of H5N1 (bird flu) viruses has been attributed to a "cytokine storm" (reviewed by[125]). It is therefore important to identify the mechanisms behind interferon-dependent protection against viruses. Numerous studies, for example, have suggested that MxA is a major effector of INF-α pre-treatment against influenza[195-197]; other newly identified interferon-induced anti-viral proteins include IFITM proteins[146,198], ISG15[147] and Viperin[149-152]. It may also be useful to combine interferon treatment with anti-inflammatory compounds such as curcumin [199-201], resveratrol[202], S1P agonists[203,204], COX-2 inhibitors[205,206] and statins[100,207].

CONCLUSION

The study of immune responses to viral infection has benefited greatly from viral proteomic studies. However, knowledge of proteomic subsets is still limited and future studies could provide more detailed insight into the dynamics of protein localization, activity and regulation through post-translational modifications during virus infection. Based on current technologies and identified networks, it may be beneficial to also investigate alterations of the phosphoproteome, ubiquitome, and the activity of proteasomes after viral infection. The development of broad-spectrum anti-virals has also shown some potential and could benefit from comparative analyses of multiple viruses.

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REVIEW

Cystic fibrosis transmembrane conductance regulator chloride channel blockers: Pharmacological, biophysical and physiological relevance

Paul Linsdell

Paul Linsdell, Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada
Author contributions: Linsdell P contributed to the manuscript.
Correspondence to: Paul Linsdell, PhD, Department of Physiology and Biophysics, Dalhousie University, PO Box 15000, Halifax, Nova Scotia B3H 4R2, Canada. paul.linsdell@dal.ca
Telephone: +1-902-4942265 Fax: +1-902-4941685
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Abstract

Dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel causes cystic fibrosis, while inappropriate activity of this channel occurs in secretory diarrhea and polycystic kidney disease. Drugs that interact directly with CFTR are therefore of interest in the treatment of a number of disease states. This review focuses on one class of small molecules that interacts directly with CFTR, namely inhibitors that act by directly blocking chloride movement through the open channel pore. In theory such compounds could be of use in the treatment of diarrhea and polycystic kidney disease, however in practice all known substances acting by this mechanism to inhibit CFTR function lack either the potency or specificity for *in vivo* use. Nevertheless, this theoretical pharmacological usefulness set the scene for the development of more potent, specific CFTR inhibitors. Biophysically, open channel blockers have proven most useful as experimental probes of the structure and function of the CFTR chloride channel pore. Most importantly, the use of these blockers has been fundamental in developing a functional model of the pore that includes a wide inner vestibule that uses positively charged amino acid side chains to attract both permeant and blocking anions from the cell cytoplasm. CFTR channels are also subject to this kind of blocking action by endogenous anions present in the cell cytoplasm, and recently this blocking effect has been suggested to play a role in the physiological control of CFTR channel function, in particular as a novel mechanism linking CFTR function dynamically to the composition of epithelial cell secretions. It has also been suggested that future drugs could target this same pathway as a way of pharmacologically increasing CFTR activity in cystic fibrosis. Studying open channel blockers and their mechanisms of action has resulted in significant advances in our understanding of CFTR as a pharmacological target in disease states, of CFTR channel structure and function, and of how CFTR activity is controlled by its local environment.

Key words: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; Chloride channel; Open channel block; Channel pore; Permeation; Anion secretion; Potentiators

Core Tip: This review summarizes our understanding of small molecules that inhibit the cystic fibrosis transmembrane conductance regulator (CFTR) by blocking the channel pore. It describes how such inhibitors could be used in the treatment of diarrhea and hereditary kidney disease; how studying these inhibitors' mechanisms of action has led to advances in our understanding of CFTR channel structure and function; and how substances acting *via* this mechanism could contribute to the physiological control of CFTR function in epithelial cells. Ironically, studying channel inhibitors has recently led to the discovery of a new class of CFTR potentiators that could be used to treat cystic fibrosis.

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INTRODUCTION

Cystic fibrosis (CF) is the most common fatal autosomal recessive disease affecting Caucasians, with around 80000 CF sufferers in the world today. CF is caused by mutations that cause loss of function of the CF transmembrane conductance regulator (CFTR) protein[1]. Over 1900 different mutations that affect the transcription, synthesis, trafficking, turnover, or function of CFTR have been shown to cause CF. CFTR is expressed in the apical membrane of many different epithelial tissues, where it plays a central role in epithelial Cl^-, HCO_3^-, and fluid transport[2]. As a consequence, CF is associated with respiratory, pancreatic, gastrointestinal, and reproductive disease that results from deficient salt and fluid secretion in these epithelia[1,3]. Conversely, inappropriately elevated CFTR function results in excessive intestinal fluid secretion in secretory diarrhoeas such as that associated with cholera[4]. CFTR-mediated Cl^- transport by renal epithelial cells also underlies fluid accumulation and growth of renal cysts in autosomal dominant polycystic kidney disease (ADPKD), the most common hereditary kidney disease[5]. The involvement of CFTR in such common and serious disease states makes it an attractive target for therapeutic intervention. Many different small molecules interact directly with the CFTR protein, and these have proven useful experimental tools. The therapeutic potential of drugs that act directly with CFTR is also receiving increasing interest. This review focuses on one class of small molecules interacting with CFTR-those that directly block Cl^- movement through the open channel pore.

OVERVIEW OF CFTR ARCHITECTURE

CFTR is a member of a large family of membrane proteins, the adenosine triphosphate (ATP)-binding cassette (ABC) proteins, most members of which function as active transport ATPases[6,7]. CFTR appears to be unique within the ABC family in functioning instead as an ATP-dependent Cl^- channel[8]. The structure and function of CFTR has been reviewed in detail recently[8-12] and will be described only briefly here. In common with other ABC proteins, CFTR has a modular architecture, consisting of two membrane-spanning domains (MSDs) each comprising six transmembrane α-helices (TMs) (Figure 1). Each MSD is followed by a cytoplasmic nucleotide binding domain (NBD), and the two MSD-NBD modules are joined by a cytoplasmic regulatory domain (R domain) that is unique to CFTR. The modular architecture of CFTR also corresponds with its defining functional features. The R domain contains multiple consensus phosphorylation sites for protein kinase A and protein kinase C, allowing the channel to be regulated physiologically by hormones that act through these protein kinases. Phosphorylation of the R domain is a prerequisite for channel activity. Following R domain phosphorylation, CFTR channel gating (opening and closing) is controlled by ATP binding and hydrolysis at a dimer of the two NBDs. The NBDs also make physical contact with the long intracellular loops (ICLs) that join individual TMs (Figure 1). The channel pore that forms the transmembrane pathway for the movement of Cl^- ions is formed by a pseudo-symmetrical arrangement of the two MSDs. Recent evidence suggests that the ICLs form a functional link that allows a conformational rearrangement initiated by ATP interaction with the NBDs to be transmitted to the TMs, controlling the opening and closing the channel pore.

The channel pore itself has been studied using a combination of structural[10,13], functional[8,14], substituted cysteine accessibility[8,15,16] and molecular modeling[17-21] approaches. A simple model of the proposed overall functional architecture of the pore is shown in Figure 1C. The pore is thought to have a relatively narrow region over which discrimination between different anions is predominantly determined. This region is flanked by outer and inner vestibules, with functional evidence suggesting that the inner vestibule is both deeper and wider. Of the 12 TMs (Figure 1), TM6 appears to play a dominant role in determining functional interactions between the narrow pore region and permeating anions[15,22]. TM1, TM6, TM11 and TM12 all contribute to the inner vestibule[15,23-29], while TM1, TM6, TM11, TM12, and the extracellular loops (ECLs) adjacent to these TMs contribute to the outer vestibule[16,30-33]. As described in detail below (see "*Biophysical Relevance*"), residues from TM1 (K95), TM5 (R303), TM6 (S341) and TM12 (S1141) have all been proposed to interact with CFTR open channel blockers (Figure 1D and E).

CFTR CHANNEL BLOCKERS

The first kinds of CFTR inhibitors to be identified were those that act as open channel blockers[34,35] (Figure 2). These are substances that enter into the open channel pore and physically occlude it, temporarily preventing the flow of Cl^- ions until the blocker molecule dissociates from the pore. Many diverse substances share this mechanism of CFTR channel block, the best known (and best studied) of which are sulfonylureas such as glibenclamide[36-42] and related substances[36,42-44], arylaminobenzoates such as 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and diphenylamine-2-carboxylate[23,45-48], and disulfonic stilbenes such as 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid and 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS)[49]. Detailed biophysical analysis of the blocking effects of these groups of negatively charged substances reveal a number of common features that may reflect a common mechanism of action. In each case the blocker enters the pore only from its cytoplasmic end to reach its binding site inside the channel pore (Figure 2); block is voltage-dependent, being stronger at more hyperpolarized voltages that favour entry of negatively charged substances into the pore from its cytoplasmic

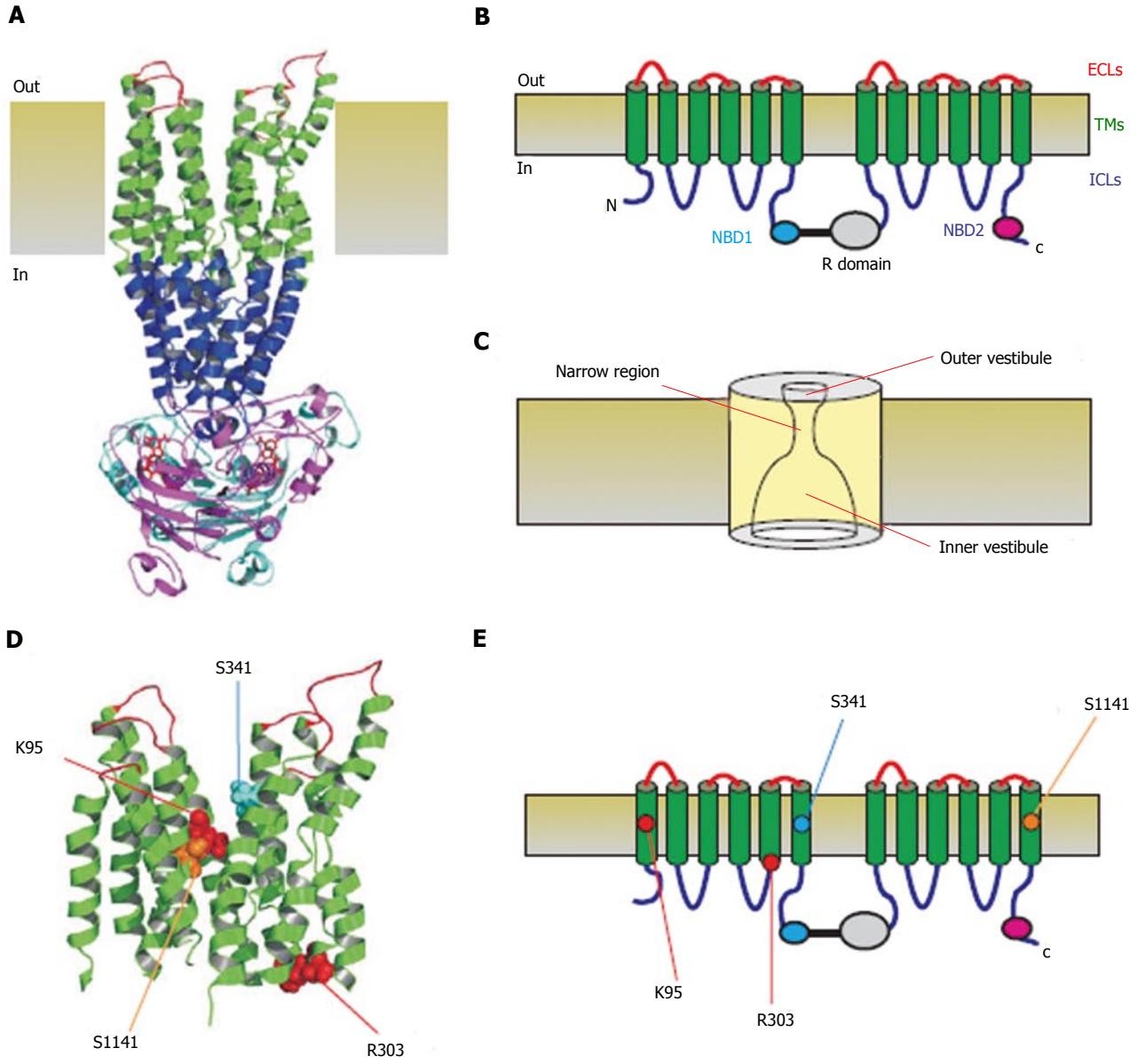

Figure 1 Three-dimensional and two-dimensional representations of cystic fibrosis transmembrane conductance regulator structure. A: Atomic homology model of cystic fibrosis transmembrane conductance regulator in a so-called "channel like" conformation[20]. Different colours are used to illustrate the approximate extent of the extracellular loops (ECLs, red), transmembrane domains (TMs, green), intracellular loops (ICLs, blue), and two nucleotide binding domains (NBD1, cyan; NBD2, magenta). The cytoplasmic R domain is not included in this homology model; B: Schematic representation of these different domains (and the R domain), using the same colour scheme; C: Functional model of pore architecture. As described in the text, experimental evidence suggests that the pore has a narrow region that is connected to the cytoplasmic and extracellular solutions by a wide inner vestibule and a narrower outer vestibule, respectively; D: Location of putative blocker-interacting residues in the TMs (K95-TM1; R303-TM5; S341-TM6; S1141-TM12) within the same homology model shown in A. E: Location of these same residues in the same schematic model shown in B.

end; and block is sensitive to the extracellular Cl^- concentration, being stronger at low Cl^- and weaker at higher Cl^-. Each of these defining features tells us something about the mechanism of inhibition and the location of the blocker binding site. Inhibition from the cytoplasmic side of the membrane was originally used to suggest that the open CFTR channel pore is structurally asymmetric, with a wide inner vestibule that is easily accessible from the cytoplasm[35,49], and a narrower extracellular entrance that prevents the entry of large substances from the extracellular solution (Figure 2). Voltage-dependent block suggests that the blocker binding site is located within the transmembrane electric field, such that the blocker apparently experiences at least part (generally about 20%-50%) of this electric field as it moves between the cytoplasm and its binding site inside the pore. While the relationship between distance across the transmembrane electric field and physical distance across the membrane itself is neither direct nor straightforward, this voltage-dependence is consistent with the blocker moving into the membrane-spanning parts of CFTR to access the blocker binding site. Finally, sensitivity of block to the extracellular Cl^- concentration is usually ascribed to repulsive electrostatic interactions between Cl^- and the negatively

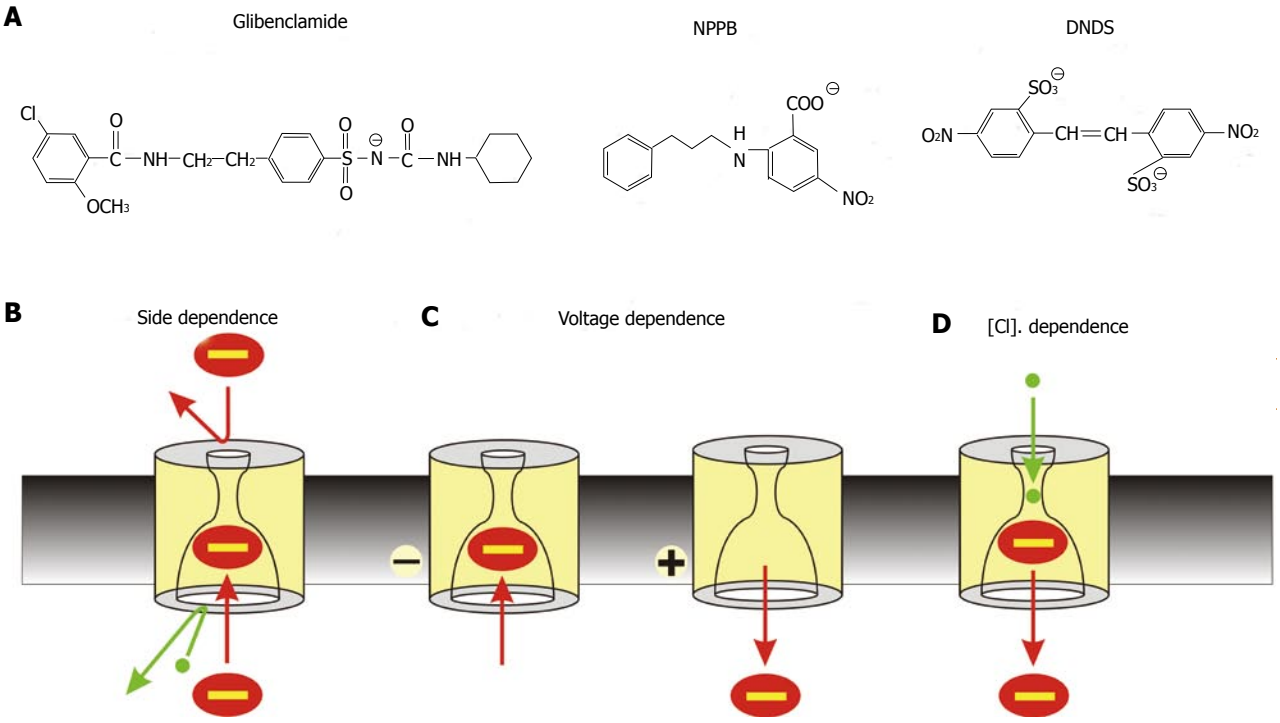

Figure 2 Mechanism of open channel blocker action. A: Chemical structures of three well-known voltage-dependent cystic fibrosis transmembrane conductance regulator (CFTR) channel blockers: the sulfonylurea glibenclamide, the aryl amino benzoate 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and the disulfonic stilbene 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS). B-D: Characteristic functional properties of block shared by these and other CFTR open channel blockers: block is side-dependent, voltage-dependent, and sensitive to extracellular Cl^- concentration; B: Blockers enter the pore only from its cytoplasmic end, likely because the extracellular entrance to the pore is too small and/or the narrow pore region prevents them from accessing their binding site in the wide inner vestibule; C: Block is relatively strengthened at hyperpolarized membrane potentials that favour entry of negative substances into the pore from the cytoplasm, and weakened at depolarized membrane potentials that favour anion retention in the cytoplasm; D: Block is weakened at higher extracellular Cl^- concentrations; this is usually ascribed to a "knock-off" mechanism whereby Cl^- entering the pore from its extracellular end electrostatically repels negatively charged blockers back into the cytoplasm, destabilizing blocker binding inside the pore.

charged blocker molecule that take place when both are bound simultaneously within the open channel pore.

Many or all of these features of the blocking reaction have been observed with other, unrelated blocking anions, including substrates of related ABC proteins[50,51] such as the conjugated bile salt taurolithocholate-3-sulfate (TLCS) and the conjugated steroid β-estradiol 17-(β-D-glucuronide), indazole compounds such as lonidamine[52,53], short-chain fatty acids butyrate and 4-phenylbutyrate[54], the fluorescein derivative phloxine B[55], and even commonly used experimental compounds such as the pH buffer 3-(4-morpholino) propane sulfonic acid[56] and the negatively charged cysteine-reactive reagent (2-sulfatoethyl) methanesulfonate (MTSES)[57]. Together these open channel blocking substances represent a large and structurally diverse group of organic anions, suggesting that entry of anions into the CFTR Cl^- channel pore from its cytoplasmic end is a process that shows little specificity or size discrimination. Furthermore, most of these blockers show relatively low potency (dissociation constants usually in the micromolar to millimolar range, depending on voltage). At the single channel level, these blockers may cause discrete "flickers" in the open channel current (due to resolved individual blocking events)[3,7,38,44,45,47,49,51,52], or an apparent reduction in single channel current amplitude where blocking and unblocking are too fast to be resolved at the bandwidth used for patch clamp recording[44,48,50,54,56-58]. These effects reflect kinetically "intermediate" and "fast" blocking and unblocking reactions, respectively, according to the scheme proposed by Hille[59]. The low apparent affinity of CFTR open channel blockers limits their potential for use *in vivo*. Furthermore, many of these substances also block other classes of Cl^- channels[34,60], perhaps reflecting some structural similarity amongst Cl^- channel pores that results in similar sensitivity to block by organic anions[14].

In more recent years, high-throughput screening technologies have been used to identify more potent CFTR inhibitors[61]. The thiazolidinone $CFTR_{inh}$-172 inhibits CFTR from the cytoplasmic side of the membrane at sub-micromolar concentrations[62], due to a voltage-independent effect on channel gating[63,64]. Glycine hydrazides such as GlyH-101 cause voltage-dependent block from the extracellular side of the membrane at low micromolar concentrations[65]. These substances appear to inhibit CFTR by different mechanisms than that described above and in Figure 2 for intracellular open channel blockers. $CFTR_{inh}$-172 has been shown to bind preferentially to open channels, perhaps triggering a conformational change to an "inactivated" nonconducting state[64]. GlyH-101 does appear to act as an open channel blocker, however acting from the extracellular side of the mem-

brane[65], perhaps becoming lodged close to the narrow pore region to occlude Cl^- permeation[66]. These two potent and relatively selective inhibitors have become drugs of choice for experimental inhibition of CFTR activity; because of their different sidedness of action, $CFTR_{inh}$-172 is preferred when applied to the intracellular side of the membrane, and GlyH-101 for extracellular application.

Finally, a 3.7 kDa peptide toxin isolated from scorpion venom and named GaTx1 inhibits CFTR channels from the cytoplasmic side of the membrane at sub-micromolar concentrations[67]. Although the molecular mechanism of GaTx1 inhibition is not well defined, this substance has been described as acting as a non-competitive inhibitor of channel gating[67,68], with no demonstrated open channel blocking action. Currently GaTx1 is the only known peptide inhibitor of CFTR.

PHARMACOLOGICAL RELEVANCE

Because of the inviolable relationship between loss of CFTR function and CF, there is tremendous current interest in the identification and development of small molecules that directly interact with the CFTR protein to increase its function (known as CFTR "potentiators")[1,69-72]. On the other hand, it has long been suggested that CFTR channel blockers could (at least in theory) be used in the treatment of secretory diarrhoea and ADPKD[35,73]. CFTR inhibitors have also been suggested as potential male contraceptives[53,74]. As described above, known intracellular-active open channel blockers lack either the potency or the specificity for *in vivo* use. However, the higher affintiy CFTR inhibitors $CFTR_{inh}$-172 and GlyH-101 have been shown to be effective in *in vitro* and *in vivo* models of secretory diarrhea[62,65,75]. Moreover, non-absorbable lectin conjugated forms of GlyH-101 were active against cholera-induced fluid secretion and mortality in mice when administered orally[76]. Similarly, $CFTR_{inh}$-172 and GlyH-101 (or closely related substances) have been shown to retard cyst growth in *in vitro*[77,78] and *in vivo*[78] models of ADPKD. The therapeutic potential of potent and specific CFTR inhibitors has been discussed in several recent reviews[61,79,80].

BIOPHYSICAL RELEVANCE

Since open channel blockers bind to specific sites within the channel pore with relatively high affinity (compared to Cl^- and other permeant anions), they have proven invaluable probes of the structure and function of the Cl^- permeation pathway. Mutations in TM6 and TM12 have been shown to alter the affinity of block by arylaminobenzoates[23,46], sulfonylureas[42,81] and lonidamine[52], consistent with functional evidence[25-28,82] and molecular models[17-21] that suggest that these two TMs make substantial contributions to the inner vestibule of the pore where the blocker binding site is thought to reside (Figures 1 and 2). Because open channel blockers are anions, and because positively charged amino acid side chains in the CFTR channel pore are known to play important roles in electrostatic attraction of Cl^- ions[24,30,31,82,83], much attention has also been placed on the role of such fixed positive charges in interactions with blockers. In particular, mutations that remove the positive charge at lysine residue K95 in TM1 (Figure 1D and E) dramatically reduce the channel blocking affinity of glibenclamide, DNDS, lonidamine, NPPB and TLCS[24,82]. This finding suggests that these structurally diverse open channel blockers share a common molecular mechanism of block - they are attracted into the wide inner vestibule by electrostatic attraction between the negative charge on the blocker molecule and the positive charge on the lysine side chain at K95, and once in the inner vestibule they bind tightly enough to occlude the pore and temporarily prevent Cl^- permeation (Figure 3). This model of blocker binding in the pore inner vestibule is also supported by a recent *in silico* investigation of blocker docking inside the pore of an atomic homology model of CFTR[20]. Neutralization of fixed positive charge in the inner vestibule by mutagenesis of K95 also decreases single channel Cl^- conductance by about 85%[22,29,82], suggesting that this positive charge also plays an important role in the normal Cl^- permeation mechanism, most likely due to electrostatic attraction of Cl^- ions. The functional importance of the positive charge on the side chain of K95 may explain the sensitivity of CFTR to broad range of intracellular anionic blockers: a positive charge in the inner vestibule is necessary to attract Cl^- ions and so maximize the rate of Cl^- permeation, however, this fixed positive charge also attracts all anions in the cytoplasm, many of which reside within the wide inner vestibule for long enough to temporarily block the passage of Cl^- ions beyond into the narrow pore region. Mutagenesis of all positively charged lysine and arginine residues within the TMs suggests that K95 plays a unique role within the pore inner vestibule in attracting permeant and blocking ions[31,83], although other positive charges may also play somewhat analogous roles in attracting cytoplasmic ions to more superficial parts of the pore close to its intracellular mouth.

If K95 does play a unique role in attracting anions into the pore inner vestibule - suggesting that it might be the only fixed positive charge located close to the blocker binding site within this vestibule[82] then what would be the effect of adding a second positive charge to the walls of this vestibule? This question has been addressed by using mutagenesis to introduce additional positively charged lysine residues at positions that have been shown to donate pore-lining side chains to the pore inner vestibule. Initially it was demonstrated that the unique, important role played by the positive charge at K95 could be "moved" from TM1 to TM12. Thus, while the charge-neutralizing K95S mutation dramatically decreased both Cl^- conductance and sensitivity to open channel blockers, the double mutant K95S/S1141K showed similar single channel conductance and open channel blocker binding properties as wild type CFTR[82]. This "rescue" of channel function suggests that these two amino acids play

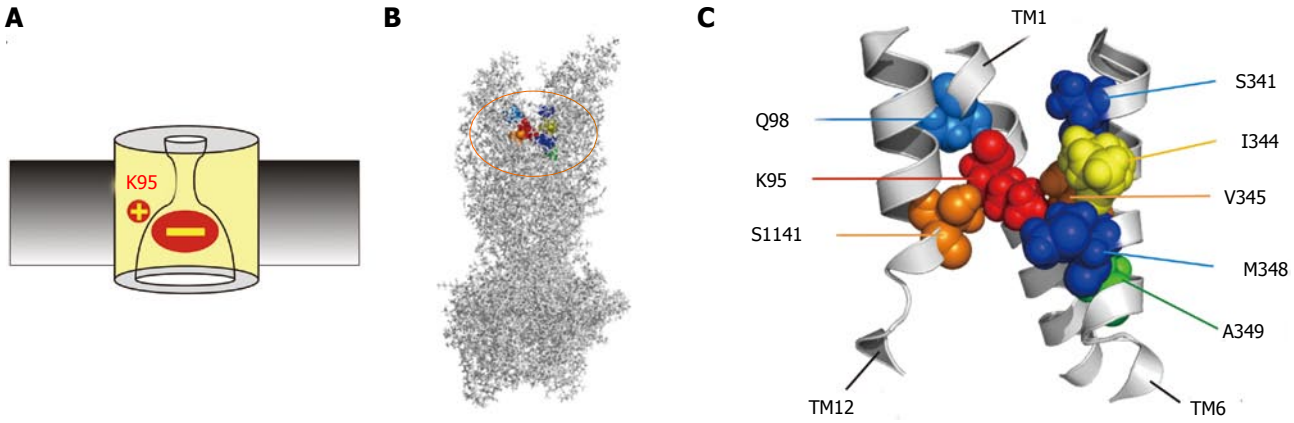

Figure 3 Location of amino acid residues key for blocker interactions in the pore inner vestibule. A: The positively charged side chain of lysine residue K95 is essential for block, due to electrostatic attraction between this positive charge and the negatively charged blocker. However, this important charge can also be supported by other amino acid side chains that line the pore inner vestibule. B, C: Sites that have been shown to host positive charge that can support block are shown in an atomic homology model of the whole cystic fibrosis transmembrane conductance regulator protein (B) and in a detailed view of the central portions of TMs 1, 6 and 12 (C) the area highlighted in (B). The endogenous positively charged side chain of K95 is shown in red; those residues that were deemed best able to support this functionally important positive charge in orange (V345, S1141) or yellow (I344); and those that were able to host this positive charge to a lesser extent in blue (Q98, S341, M348) or green (A349). The homology model used here is the "channel like" conformation presented by ref[20] and shown in Figure 1A; other models give similar relative positions of these pore-lining side chains.

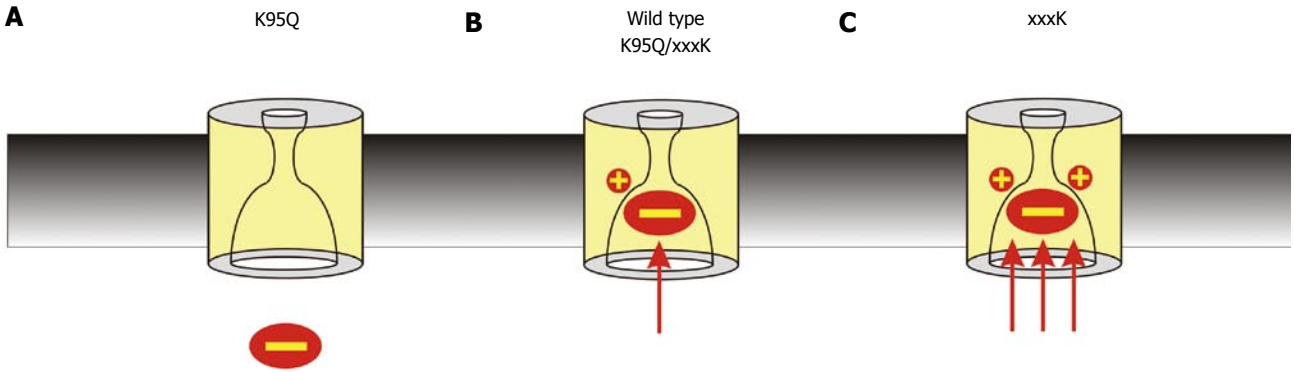

Figure 4 Importance of the number of fixed positive charges in the pore inner vestibule. The importance of electrostatic interactions with the pore inner vestibule is demonstrated by the finding that the strength of block can be decreased or increased by mutations that decrease or increase, respectively, the number of positively charged amino acid side chains in the pore inner vestibule area shown in Figure 3C. A: Block is relatively weak in when the endogenous positive charge is removed, for example as in the K95Q mutation; B: Block is of similar strength to that observed in wild type cystic fibrosis transmembrane conductance regulator when the positive charge is "transplanted" to other, nearby sites, for example as in the double mutants K95Q/I344K, K95Q/V345K, and K95S/S1141K; C: Block is relatively strong when a second positive charge is introduced, for example as in I344K, V345K and S1141K.

interchangeable roles within the pore inner vestibule, in that either could effectively host the positive charge that supports interactions with Cl^- and blocking anions[82] (Figure 4). Substituted cysteine accessibility mutagenesis and disulfide cross-linking experiments indicated that the amino acid side chains at these two positions line the inner vestibule in open channels and that these side chains are in close physical proximity[82]. Subsequent experiments showed that the positive charge from K95 could similarly be transplanted to different pore-accessible positions in TM6 (I344, V345, M348, A349), as well as a site closer to the extracellular end of TM1 (Q98)[29]. Thus it appears that the exact location of the positive charge in the pore inner vestibule is not critical to support channel function. The ability of other sites in TMs 1, 6 and 12 to accommodate the positive charge that normally resides at K95 then allowed investigation of the effects of introducing a second positive charge at these sites (by mutagenesis to lysine) while retaining the positive charge at K95 - in effect, increasing the number of positive charges located deep in the pore inner vestibule from one to two (Figure 4). Interestingly, at no site tested (Q98K, I344K, V345K, M348, A349K, S1141K) did the addition of a second positive charge increase single channel conductance[29,82]. This suggests that, while the presence of one positive charge is essential for normal Cl^- conductance, a second positive charge provides no additional benefit. However, a second fixed positive charge (in S1141K) increased the strength of block by cytoplasmic NPPB, and also induced apparent voltage-dependent channel block by polyvalent anions present in the experimental solutions (ATP, pyrophosphate)[82], suggesting that the number of positive charges was correlated with open channel blocker potency (Figure 4). This suggestion was most strongly supported using the small divalent anion $Pt(NO_2)_4^{2-}$, which also causes voltage-dependent open

channel block in a K95-dependent manner[84,85]. Addition of a second positive charge to nearby pore-lining sites in TM6 (I344K, V345K) or TM12 (S1141K) led to a dramatic (40-100 fold) increase in the apparent affinity of intracellular $Pt(NO_2)_4^{2-}$ block[29,82], suggesting that increasing the number of positive charges in the pore has a greater impact on interactions with multivalent anions (such as $Pt(NO_2)_4^{2-}$ and ATP) than monovalent anions such as Cl^-. Positive charges introduced at other sites within the pore inner vestibule (Q98K, S341K, M348K, A349K) also supported strengthened $Pt(NO_2)_4^{2-}$ block, albeit to a lesser extent. These findings, summarized in Figure 4, led to the hypothesis that one positive charge in the inner vestibule (as in wild type CFTR) was optimum for CFTR channel function[82]. Thus, removal of the one endogenous positive charge (as in K95Q or K95S) decreases channel function due to reduced electrostatic attraction of Cl^- ions and a resulting dramatic decrease in channel conductance. This effect can be "rescued" by introducing a positive charge at other, nearby positions (as in K95S/S1141K and K95Q/V345K). Conversely, addition of a second positive charge (as in I344K, V345K, S1141K and other lysine substitutions) results in no further increase in Cl^- conductance but increases the electrostatic attraction of multivalent anions that block the pore, resulting in an overall decrease in channel function. Thus the greatest importance of a single fixed positive charge in the inner vestibule may be in conferring preference for monovalent anions, including the physiological channel transport substrates Cl^- and HCO_3^-.

If one is the optimum number of positive charges in the inner vestibule to maximize channel function, where is the optimal location for this charge? Since normal channel function can be rescued by moving the positive charge to other, nearby sites in TM1, TM6 or TM12, the exact location of this charge does not appear to be critical[29]. Of all sites tested as hosts of the positive charge (K95, Q98 in TM1; S341, I344, V345, M348, A349 in TM6; S1141 in TM12), K95 appears optimal in terms of maximizing single channel conductance[29]. In terms of both single channel conductance and blocker binding properties, I344, V345 and S1141 appeared to be the best locations for a positive charge to reproduce wild type properties, with Q98, S341, M348 and A349 also being able to host this positive charge to some extent[29]. Similarly, a second positive charge at I344, V345 or S1141 had the greatest impact on divalent $Pt(NO_2)_4^{2-}$ block[29]. These ideas are presented graphically, within the framework of a recent atomic homology model of CFTR, in Figure 3C. Within this model, the side chains of I344, V345 and S1141 appear to be at approximately the same "depth" into the channel pore as K95; with Q98 and S341 being located more deeply into the pore from its cytoplasmic end, and M348 and A349 closer to the cytoplasmic mouth of the pore. This relative location of amino acids is also supported by experimental evidence that disulfide bonds can be formed between cysteine side chains substituted for K95 and S1141[82], as well as between K95C and I344C and between Q98C and I344C[86]. This model suggests that it is location along the axis of the channel pore that is most important in defining the functional effects of a positive charge in the pore inner vestibule: residues close to the endogenous site at K95 are best able to substitute and rescue pore function, while residues either further from, or closer to, the cytoplasmic entrance of the pore are less able to host this important positive charge. This is consistent with molecular modeling studies that show open channel blockers docked within the pore inner vestibule and with their negative charges close to the positive charge of K95[20].

As described above, interaction with the positive charge at K95 and occlusion of the pore inner vestibule appears to be the molecular mechanism of many different kinds of CFTR open channel blockers, including those shown in Figure 2. However, a second blocker binding site was identified using the large, polyvalent organic anion suramin[87]. Suramin causes potent, voltage-independent block of CFTR channels exclusively from the intracellular side of the membrane[87,88]. Furthermore, block by intracellular suramin is independent of extracellular Cl^- concentration, and completely unaffected by removal of the key positive charge in the inner vestibule in the K95Q mutant[87]. This suggests that suramin does not enter deeply enough into the pore inner vestibule to experience electrostatic interaction with K95, perhaps because the suramin molecule is simply too big to pass into this restricted pore region. In contrast, suramin block was weakened in an electrostatic fashion by mutagenesis of another positively charged amino acid, R303 at the cytoplasmic end of TM5[87] (Figure 1D and E). This result was consistent with the previous finding that the positive charge of R303 attracts intracellular Cl^- ions to the cytoplasmic entrance of the pore[83] and suggests that the large suramin molecule may be able to occlude the cytoplasmic mouth of the pore to prevent Cl^- movement into or out of the pore. As shown in Figure 5, this proposed molecular mechanism of suramin action is consistent with observed biophysical differences between suramin block and block by other (smaller) open channel blockers that interact with K95 (see above). Because of its size, suramin does not penetrate deeply into the inner vestibule; as a result, it does not traverse enough of the transmembrane electric field to generate measurable voltage-dependence of block, it does not reside in close proximity to Cl^- ions bound within the channel pore (perhaps in the narrow pore region or close to the outer extent of the inner vestibule) and so does not experience the kind of repulsive electrostatic interactions that are thought to underlie extracellular Cl^- dependence, and it does not approach close enough to K95 to experience attractive electrostatic interactions with this positively charged residue (Figure 5). Electrostatic interaction with R303 near the cytoplasmic mouth of the pore may also contribute to the inhibitory effects of other substances on CFTR, for example arachidonic acid[89].

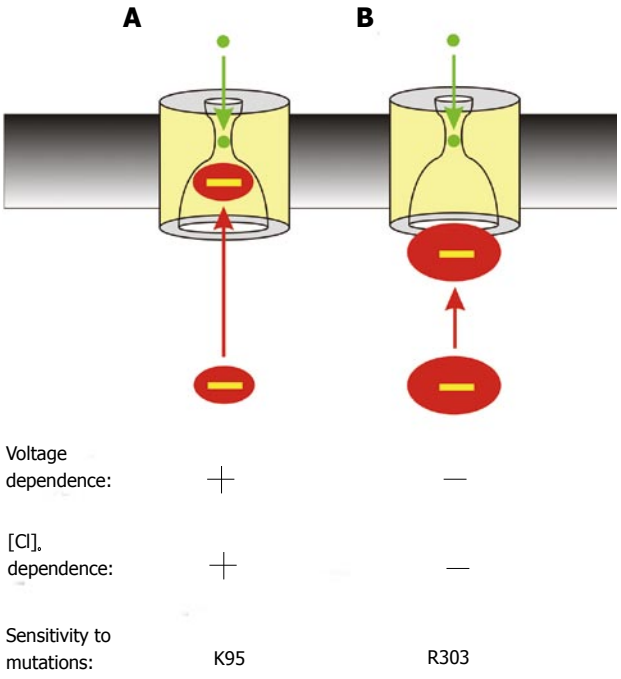

Figure 5 Two distinct proposed mechanisms of block by cytoplasmic anions. A: The effects of most blockers are voltage- and Cl^- dependent (as described in Figure 2) and are sensitive to mutations that remove the positive charge at K95; B: The large multivalent anion suramin blocks the channel in a voltage- and Cl^- independent fashion, and its effects are dependent on a positive charge at R303 but independent of K95. This is interpreted as the large suramin molecule blocking the cytoplasmic entrance to the pore; at a site that does not involve entering significantly into the transmembrane electric field or approaching close enough to Cl^- ions inside the pore to experience repulsive electrostatic interactions.

PHYSIOLOGICAL RELEVANCE

CFTR channel currents are routinely studied in excised, inside-out membrane patches, where the current-voltage relationship is uniformly linear[90,91] (Figure 6). Conversely, CFTR channel currents in intact cells, including native epithelial cells[92-94], cardiac myocytes[95,96], and many different heterologous expression systems[82,97-99], exhibit outward rectification of the current-voltage relationship, such that outward currents (carried by Cl^- influx) show greater conductance than inward currents (carried by Cl^- efflux) (Figure 6). This rectification - and its disappearance in cell-free membrane patches - led to the longstanding suggestion that CFTR channels in intact cells are subject to voltage-dependent block by unknown cytosolic anions[58,97,100]. This appears to reflect predominantly a voltage-dependent flickery block by cytosolic anions that is lost when the membrane patch is excised from the cell[94,97,98,100], although differences in single channel conductance in cell-attached and excised patches have also been reported[92,95,97]. Detailed single channel recording experiments from cell-attached membrane patches suggested that the flickery blocking mechanism was functionally analogous to that generated by exogenous voltage-dependent blocking anions with intermediate blocking and unblocking kinetics[100]. Open channel block as the mechanism of outward rectification was further

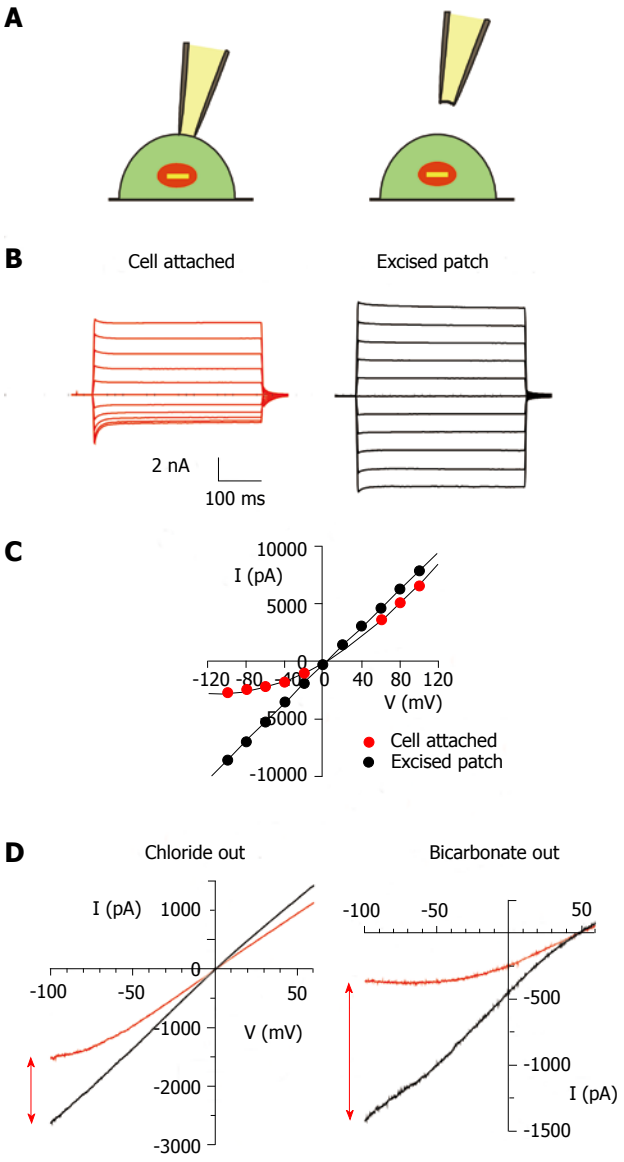

Figure 6 Channel block by cytoplasmic anions in intact cells and its dependence on extracellular anions. A: During patch clamp recording from intact cells, cystic fibrosis transmembrane conductance regulator (CFTR) channels in the cell membrane are subject to block anions present in the cytoplasm of the cell (left). This blocking effect is lost when the patch of membrane is excised into the inside-out patch configuration (right); B: Example of this effect during macroscopic CFTR current recording from a baby hamster kidney cell expressing human CFTR, as described in detail[82]. Currents were recorded before (red) and after (black) excision of the patch from the cell, during voltage steps to between -100 mV and +100 mV in 20 mV increments from a holding potential of 0 mV. Dotted line represents the zero current level; C: Current-voltage relationships for the currents shown in (B). Note the outward rectification of the relationship in cell-attached recording (red) due to voltage-dependent channel block, and loss of this blocking effect following patch excision (black); D: Similar example current-voltage relationships from baby hamster kidney cell membrane patches when the extracellular solution contained 150 mmol/L NaCl (left) or 150 mmol/L $NaHCO_3^-$ (right), as described in detail[101]. Note that the apparent degree of block in cell attached patches (red) is stronger when the extracellular solution contains HCO_3^- compared to Cl^-, an effect quantified in detail in ref. [101].

supported by the more recent finding that inhibition of currents in intact cells was reduced in K95Q-CFTR and (to a lesser extent) R303Q-CFTR[101]. This indicates that the unknown cytosolic blocking anions interact with

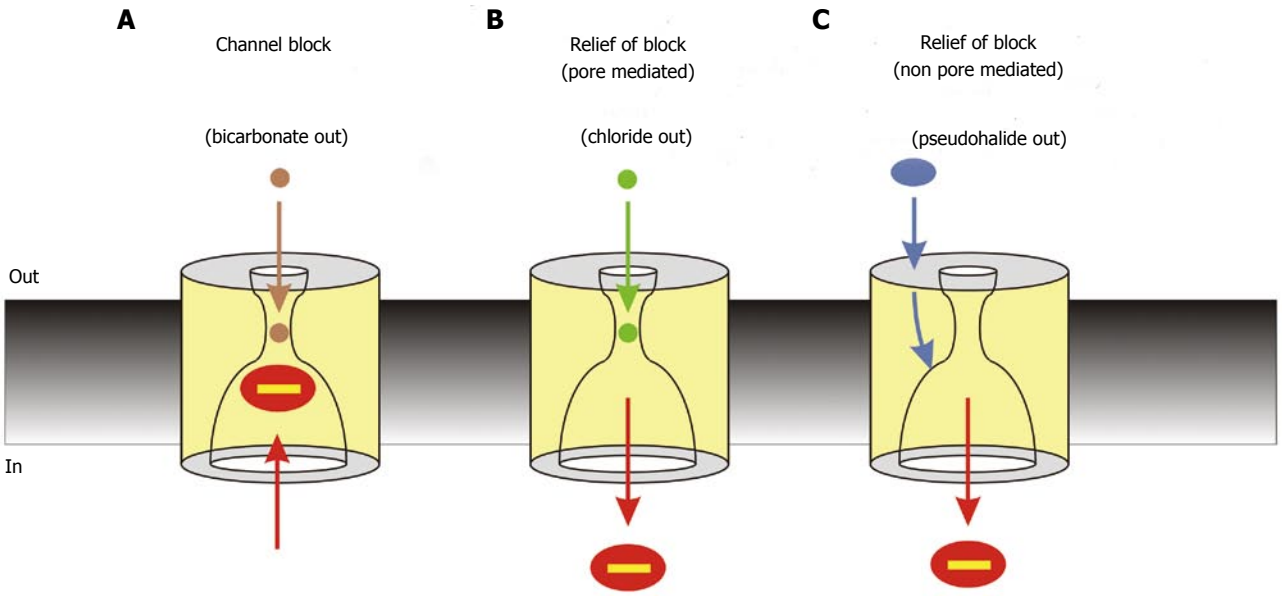

Figure 7 Interactions between cytoplasmic blocking anions and extracellular anions. Cytoplasmic block is modified by extracellular anions by different mechanisms, leading to different degrees of block under different conditions. A: Block is strong in the absence of modulation of block by extracellular anions; physiologically, such a condition may occur during periods of epithelial HCO_3^- secretion, leading to strong block of cystic fibrosis transmembrane conductance regulator (CFTR) channel currents under these conditions[101]; B: Block is weakened by extracellular anions that can enter the channel pore, such as Cl^-, due to an electrostatic "knock-off" mechanism. This may lead to increased CFTR channel currents during periods of epithelial Cl^- secretion[101]; C: Block is weakened by extracellular anions that interact with an extracellular part of the protein involving extracellular loop 4. This is presumed to result in a long-range conformational change in CFTR that decreases the affinity of the cytoplasmic blocker binding site. This mechanism may allow pharmacological manipulation of CFTR activity by compounds that interact with the extracellular anion binding site[114]. Note that Cl^- ions may also interact with intracellular blocking anions by the non-pore mediated effect shown in (C)[114].

these positively charged residues in the CFTR pore in intact cells, much as had previously been shown for exogenous channel blockers.

While outward rectification of CFTR currents in intact cells-and the weak form of voltage-dependence it confers on CFTR channel currents (Figure 6) has long been recognized, only recently has it been suggested that the voltage-dependent channel block that underlies this voltage-dependence might fulfil some kind of channel regulatory role. Just as block by exogenously applied open channel blockers is sensitive to extracellular Cl^- (Figure 2D), so too is block by endogenous cytosolic anions in intact cells[82,100,101] (Figures 6D and Figure 7). This is not surprising if, as described above, these two intracellular voltage-dependent blocking effects share a common molecular mechanism. Recently it was proposed that this Cl^- dependence might be one mechanism that allows CFTR conductance to be regulated by the composition of secreted fluid bathing the extracellular face of epithelial cells[101]. Most CFTR-expressing epithelia secrete substantial amounts of Cl^- and HCO_3^- (up to 140 mmol/L HCO_3^- in the case of the pancreas[102]) in a CFTR-dependent fashion[2,103,104]. Furthermore, in many epithelia the concentrations of Cl^- and HCO_3^- in secreted fluid vary greatly under physiological conditions[102,104-110]. Interestingly, it was shown that voltage-dependent block of CFTR in intact cells was significantly stronger under high extracellular (HCO_3^-) conditions than under high extracellular Cl^- conditions[101]. This suggests that extracellular HCO_3^- is unable to substitute for Cl^- in relieving the blocking effects of endogenous cytoplasmic blocking anions. As a result, overall CFTR activity will be increased under high extracellular Cl^- conditions (*i.e.*, during periods of epithelial Cl^- secretion) and decreased under high HCO_3^- conditions (*i.e.*, during periods of secretion of relatively HCO_3^--rich fluid)[101] (Figure 7). These findings led to the suggestion that endogenous cytoplasmic blocking anions are physiologically relevant regulators of CFTR channel function, in that they confer upon the channel sensitivity to physiologically relevant changes in extracellular fluid composition[101]. In epithelial cells, this may be one mechanism by which CFTR channel function is fine-tuned by the concentration of its transport substrates Cl^- and HCO_3^- at the apical face of these cells[111-113].

While extracellular Cl^- may be an endogenous substance regulating CFTR channel function *via* modulation of the blocking effects of cytoplasmic anions, this mechanism of channel regulation may also be subject to pharmacological manipulation. Thus, millimolar concentrations of extracellular multivalent psuedohalide anions ($Co(CN)_6^{3-}$, $Co(NO_2)_6^{3-}$, $Fe(CN)_6^{3-}$, $IrCl_6^{3-}$, $Fe(CN)_6^{4-}$) were shown to mimic the effects of high extracellular Cl^- on channel block in intact cells, leading to an overall stimulation of CFTR channel function[114] (Figure 7). It was suggested that these anions represent the founder members of a new class of CFTR potentiators, and that their effects identify a novel mechanism by which CFTR function could potentially be increased therapeutically in the treatment of CF. Interestingly, these pseudohalide anions do not enter into the CFTR channel pore[114] and as such presumably do not interact electrostatically with blocking anions inside the channel pore; such an electro-

static "knock off" mechanism is commonly proposed to underlie the effect of extracellular Cl^- ions on intracellular open channel blockers[115,116] (Figure 7), as well as permeant ion effects on blocker binding in many other ion channel types[59]. Instead, pseudohalide anions were shown to exert their effects *via* interation with extracellular parts of CFTR, in particular ECL4[114,117]. The molecular mechanism of action of these substances, acting on extracellular parts of the protein, is therefore distinct from those of known CFTR potentiators, perhaps allowing additive or synergistic effects with other types of potentiators. Furthermore, the suggestion that a novel potentiator "binding site" might exist on ECL4 raises the possibility that this externally-accessible part of the CFTR protein could in future be targetted by drugs that can manipulate CFTR function therapeutically.

CONCLUSION

The architecture and Cl^- permeation mechanism of CFTR likely results in a susceptibility to relatively low affinity, voltage dependent open channel block by a very broad range of structurally diverse organic anions, including unidentified anions that the channel normally encounters in the cytoplasm of the cell. Because the channel is normally involved in the secretion of Cl^- and HCO_3^- ions at hyperpolarized cell membrane potentials, the channel has a relatively large intracellular vestibule that contains fixed positive charges to allow it to capture these anions from the cytoplasm by the process of electrostatic attraction. As the vestibule narrows toward the centre of the pore (Figure 1C and D), a single, functionally unique positive charge (K95 in TM1) ensures efficient attraction of monovalent anions (Figure 3). Beyond this point, permeating anions pass into a narrow, uncharged pore region that may allow some level of discrimination between different anions, and also acts as a size selectivity filter to prevent larger organic anions from escaping the cell. While this architecture appears efficient at maximizing channel Cl^- conductance (Figure 4), it also probably results in some degree of channel inhibition by cytoplasmic anions that are attracted by the positive charge at K95, but which are too large to pass into the narrow pore region (Figures 2, 3, 4 and 5). CFTR experimentalists have long taken advantage of these voltage dependent blocking anions to investigate CFTR-dependent processes, to think about the possible advantages of inhibiting CFTR function in disease states associated with inappropriately elevated CFTR function, and as relatively high-affinity probes to investigate the structure and function of the wide inner vestibule of the channel pore. This has allowed the development of functional (Figures 3, 4 and 5) and structural[20] models of the pore. More recently, it has been suggested that endogenous substances that act in this fashion may in fact play a role in tying CFTR function to the content of epithelial cell secretions (Figures 6 and 7), perhaps allowing CFTR activity to be fine-tuned directly by the amount of its substrate(s) being secreted from epithelial cells. In the future, this mechanism of CFTR regulation could be targetted by new drugs that act at an extracellular site on the CFTR protein to reduce the voltage-dependent blocking effects of endogenous cytoplasmic anions and so increase overall CFTR function in CF patients.

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REVIEW

Role of 3'-untranslated region translational control in cancer development, diagnostics and treatment

Andrii Vislovukh, Thaiz Rivera Vargas, Anna Polesskaya, Irina Groisman

Andrii Vislovukh, State Key Laboratory of Molecular and Cellular Biology, Institute of Molecular Biology and Genetics, NAS of Ukraine 150, Kyiv 03680, Ukraine
Thaiz Rivera Vargas, Anna Polesskaya, Irina Groisman, Université Paris Sud, Laboratoire Epigénétique et Cancer, Formation de Recherche Evolution 3377, F-91191 Gif-Sur-Yvette, France
Thaiz Rivera Vargas, Anna Polesskaya, Irina Groisman, Centre National de la Recherche Scientifique (CNRS), F-91191 Gif-Sur-Yvette, France
Thaiz Rivera Vargas, Anna Polesskaya, Irina Groisman, Commissariat à l'Energie Atomique (CEA), Saclay, F-91191 Gif-sur-Yvette, France
Author contributions: Vislovukh A, Vargas TR, Polesskaya A and Groisman I contributed to this paper.
Correspondence to: Irina Groisman, PhD, Commissariat à l' Energie Atomique (CEA), Saclay, Bat. 144-Point courrier n 22, F-91191 Gif-sur-Yvette, France. irina.groisman@cea.fr
Telephone: +33-1-69085376 Fax: +33-1-69084712
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Abstract

The messenger RNA 3'-untranslated region (3'UTR) plays an important role in regulation of gene expression on the posttranscriptional level. The 3'UTR controls gene expression *via* orchestrated interaction between the structural components of mRNAs (cis-element) and the specific trans-acting factors (RNA binding proteins and non-coding RNAs). The crosstalk of these factors is based on the binding sequences and/or direct protein-protein interaction, or just functional interaction. Much new evidence that has accumulated supports the idea that several RNA binding factors can bind to common mRNA targets: to the non-overlapping binding sites or to common sites in a competitive fashion. Various factors capable of binding to the same RNA can cooperate or be antagonistic in their actions. The outcome of the collective function of all factors bound to the same mRNA 3'UTR depends on many circumstances, such as their expression levels, affinity to the binding sites, and localization in the cell, which can be controlled by various physiological conditions. Moreover, the functional and/or physical interactions of the factors binding to 3'UTR can change the character of their actions. These interactions vary during the cell cycle and in response to changing physiological conditions. Abnormal functioning of the factors can lead to disease. In this review we will discuss how alterations of these factors or their interaction can affect cancer development and promote or enhance the malignant phenotype of cancer cells. Understanding these alterations and their impact on 3'UTR-directed posttranscriptional gene regulation will uncover promising new targets for therapeutic intervention and diagnostics. We will also discuss emerging new tools in cancer diagnostics and therapy based on 3'UTR binding factors and approaches to improve them.

Key words: Translational control; 3'-untranslated region; MicroRNAs; RNA binding proteins; Cancer

Core tip: The messenger RNA 3'-untranslated region (3'UTR) plays an important role in regulation of gene expression on the posttranscriptional level. 3'UTR controls gene expression *via* orchestrated interaction between structural components mRNAs (cis-element) and specific trans-acting factors (RNA binding proteins and non-coding RNAs). Alteration of any of these components can lead to various pathologies. In this review we will discuss how alteration of these factors or a change in the crosstalk between them can affect cancer development and promote or enhance the malignant phenotype of cancer cells. Understanding these regulatory mechanisms and their impact on 3'UTR-directed posttranscriptional gene regulation may uncover promising new targets for therapeutic intervention and diagnostics.

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INTRODUCTION

During tumor growth, characteristic alterations in gene expression result in modification of the quantity of the corresponding proteins. The alterations have been extensively documented at the mRNA transcription and protein degradation levels; both have a strong impact on the accumulation of critical proteins involved in tumorigenesis. While translational control is a key mechanism involved in the regulation of the gene expression[1], the impact of the misregulation of gene expression during carcinogenesis at the translational level has long been widely underestimated.

Translation of mRNA into proteins can be specifically regulated by a combination of RNA-binding factors (proteins and antisense RNA) that act positively or negatively on translation initiation and elongation, mRNA stability and mRNA localization. This regulation is mostly controlled by sequence elements in 3'-untranslated region (3'UTR) of the transcripts, located downstream from the open reading frame. The importance of the 3'UTR was not fully appreciated until the discovery of small non-coding regulatory RNAs (microRNAs or miRNAs). MiRNAs interact with a protein complex called RNA-induced silencing complex (RISC), which controls gene expression by binding to miRNA target sites in mRNA 3'UTRs. MiRNAs have proven to be not only important markers but also key players in the control of gene expression during cancer development. Multiple 3'UTR regulatory elements are usually involved in the regulation of translation. One of the best characterized of them is the cytoplasmic polyadenylation element (CPE) which, upon binding by the CPE-binding protein (CPEB), regulates specific target mRNAs. CPEB1 directly controls the mammalian cell cycle, particularly during senescence, suggesting a role in cancer and aging. According to the literature and to our unpublished data, members of CPEB family are misregulated in many cancers and can play important role in carcinogenesis[2,3].

The insulin-like growth factor (IGF)-2 mRNA-binding proteins 1, 2 and 3 [IGF2BP1-3/Insulin-like growth factor 2 mRNA-binding protein 1 (IMP1-3)] belong to another well-known family of proteins that bind to 3' UTR and control the expression of proteins important in the normal cell cycle and in cancerous transformation[4,5]. IGF2BP1-3 and IMPs are highly over-expressed in a number of cancers[6].

The aim of this review is to show that regulatory factors controlling gene expression *via* binding to 3'UTR do not act separately but in cooperation. Crosstalk of these factors is based on the binding sequences and direct protein-protein interaction. The functional and physical interactions of factors binding to 3'UTR can change the character of their action, according to physiological conditions[7]. Disruption of the coordinated action of these factors can have a big impact on the expression of proteins involved in cancer induction and development. A detailed understanding of these mechanisms can help in development of new tools for cancer diagnostics and treatment.

MIRNA AND CANCER

One of the main breakthroughs in cellular and molecular biology in the last decade was the discovery of gene expression regulation by non-coding RNAs. The number of classes of non-coding RNAs continues to grow rapidly. Major among them are miRNAs, piRNAs, endo-siRNAs, exo-siRNAs, rasiRNAs, scnRNAs, tasiRNAs, natsiRNAs, 21U-RNA, lncRNAs and tRNA-derived RNA fragments[8]. We will focus this review on miRNAs, which is the most widely studied group of non-coding regulatory RNAs. MiRNAs are small (21-23 nt) RNAs. MiRNAs originate from Pol Ⅱ-transcribed precursors (pri-miRNAs). Then the Drosha enzyme recognizes a 70 nt stem-loop structure and produces pre-miRNA, which is transported from the nucleus by Exportin 5. In the cytoplasm, Dicer enzyme forms a double-stranded 22 nt RNA from pre-miRNA. One of the RNA strands is degraded, whereas the other one inserts into the RISC complex, binds to the target sequence in 3'UTR, and carries out its regulatory function[9]. These tiny molecules are involved in the regulation of almost all cellular processes[10-12]. Since single miRNA can potentially have hundreds of targets, alteration of its expression can easily influence cellular homeostasis, which in the most extreme case may result in cell death or in malignant transformation of the cell. Indeed, the first evidence of involvement of miRNAs in tumorigenesis was shown in 2002 by Calin *et al*[13]. These authors found that in 68% of chronic lymphocytic leukemia (CLL) cases, deletions and down-regulation of miRNA genes *miR-15* and *miR-16* at 13q14 locus were observed. Since then, thousands of publications have been devoted to miRNAs involvement in various types of cancer.

Misregulation of miRNA expression in various cancers

Involvement of miRNA in cancer has been proven by genome-wide expression studies using microarray technology and techniques based on quantitative polymerase chain reaction (qPCR), which have helped to establish the miRNA profiles of normal and neoplastic tissues[14,15]. These studies revealed a global decrease in miRNA expression in many tumors. Various tumors also correlate with changes in specific miRNA expression. The above studies were supported by a number of investigations of individual types of neoplasms[16-29] (and many others). About 200 miRNAs have at least once been reported as being up- or down-regulated in tumors.

Overall, these studies prove that each neoplasm could exhibit a distinct miRNA expression profile that differs from one of the other neoplasms and its normal tissue counterpart. However, a group of miRNAs was shown to have a similar expression profile in multiple cancers, suggesting that their involvement in tumorigenesis is common for many cancer types. At the same time, there are many miRNAs that are differentially misregulated in different cancers[30]. The reason for this is not yet clear, but it is likely that the function of a miRNA may vary because of tissue-specific expression of their targets. On the other hand, specific miRNAs can have different cofactors and build different networking in different cancers. Thus, it becomes possible for a given miRNA to act either as an oncogene or as a tumor suppressor, according to the context.

One of the best examples for tissue-specific target regulation is the let-7 family of miRNAs, which according to many reports acts as tumor suppressors[31-34]. It has been shown that let-7 is frequently down-regulated in many cancers, leading to up-regulation of the proto-oncogenes RAS[35], High Mobility Group A2 (HMGA2)[36-38], Myc[39], integrin beta 3[40], the oncofetal gene *IMP-1*[41] and the miRNAs maturation enzyme Dicer[42]. Let-7b was shown to down-regulate the expression of cyclin D1, D3, A and cyclin-dependent kinase (Cdk 4) in melanoma cells[43].

A similar effect was observed for the miR-34 family, another potential tumor-suppressor in a variety of cancers. Localized to chromosomes 1 (34a) and 11 (34c and b), this family is frequently deregulated in various cancers, including lung, ovarian, CLL and colorectal[44-47]. In addition, miR-34b/c polymorphism has been linked to risk of developing hepatocellular carcinoma[48]. The miR-34 family appears to be the direct transcriptional target of p53[49,50] and has few validated targets, SNAIL (zing finger protein SNAL1, epithelial-mesenchymal transition), Wnt, SIRT1 (silent mating type information regulation homolog), cyclin-dependent kinase 6 (CDK6) and others[51-54].

The miR-29 family (a, b and c) also has often been found to be decreased in tumors, such as CLL, hepatocellular carcinoma and breast cancer[55-57], and has been validated to target key components of cellular survival as MCL-1 (induced myeloid leukemia cell differentiation protein), cell cycle CDK6 and dedifferentiation Krüppel-like factor 4[26,55,58]. The most interesting observation concerning miR-29 is that it can globally alter methylation status through targeting of DNA methyltransferases 3A and B (DNA methyltransferases 3A and B) and lead to the derepression of critical tumor suppressors[59].

The miR-17-92 cluster acts as a group of oncogenes when over-expressed. This group includes 14 homologous miRNAs that are encoded by three gene clusters on chromosomes 7, 13 and X[25,60]. The cluster on chromosome 13 is amplified in human B cell lymphomas[61], which leads to increased expression of various miRNA members. Forced expression of the miR17-92 cluster along with myc proto-oncogene (MYC) accelerates tumor development in mouse B cell lymphoma[62], thus acts as an oncogene. Up-regulation of members of this large miRNA group protects cells from apoptosis by inhibiting the expression of E2F, p21 and Bim[63,64].

Among oncogenic miRNAs families, the most therapeutic and diagnostic potential is the miR-21 family, located on chromosome 17. It is over-expressed in several cancers, including breast, colorectal and lung[65-67], and has few validated targets: TPMI (tropomyosin), PDCD4 (program cell death protein 4) and PTEN (phosphatase and tensin homolog)[68-70].

These and other observations found in the literature prove that miRNAs play very important roles in cancer, although their mode of action can differ according to the composition of the targets and a combination of other factors. Knowledge of the mechanisms of miRNA action in particular cancers, especially understanding of their collaborators or inhibitors, will help to develop proper tools for miRNA-based therapy and diagnostics.

Cancer processes associated with misbalance of miRNA expression

Epithelial-mesenchymal transition: To date, it is believed that one of the causes of failure in the treatment of cancer is the existence of cancer stem cells[71]. In cancer, epithelial-mesenchymal transition (EMT) is a process by which epithelial cells are reprogrammed to lose their differentiation and become undifferentiated stem cells with mesenchymal properties. Despite the fact that genes responsible for EMT are well known[72], data devoted to the involvement of miRNAs in this process are still accumulating. Thus, Nairismägi *et al*[73] showed that miR-580 and CPEB1/2 down-regulate TWIST1 expression, one of the main inductors of EMT in a cooperative way. Another miRNA that suppresses EMT belongs to the miR-200 family. These miRNAs increase E-cadherin expression by targeting the mRNA of the E-cadherin transcriptional repressors zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2[74,75]. It was later shown that the miR-200 family is downregulated in the initial stages of stromal invasion, but restored at metastatic sites[76]. In cases of hepatocellular carcinoma, miR-612 was found to have an inhibitory effect on EMT targeting of the AKT (also known as protein kinase B) signal cascade[77]. On the other hand, a set of miRNAs is correlated with EMT progression. MiR-21 is thus over-expressed during EMT, whereas blockage of miR-21 inhibits metastasis development[78,79]. During EMT, the Twist transcription factor induces expression of miR-10b. In turn, over-expression of miR-10b in non-metastatic breast tumors initiates intense invasion and metastasis[80]. Furthermore, in hepatocellular carcinoma, miR-106b promotes cell migration and metastasis by activating the EMT process[81].

Angiogenesis and proliferation: The tumor growth rate is one of the most critical characteristics that define the level of cell malignancy. However, while growing, a tumor must supply itself with nutrients, which are provided by active angiogenesis. Deregulation of miRNA

expression is also involved in these processes. For instance, upregulation of the miR-17-92 cluster in adenocarcinoma leads to downregulation of its predicted targets: anti-angiogenic thrombospondin-1 and connective tissue growth factor, resulting in enhanced neovascularization[82]. Lee *et al*[83] showed that miR-378 increases cell survival, tumor growth and angiogenesis. Detailed analyses revealed that the main targets of the miR-378 were SuFu (inhibitor of Hedgehog signal cascade) and tumor suppressor Fus-1. Regulation of proliferation is mainly carried out by forced entry and progression of the cell cycle. Cyclins and their CDKs regulators are key players in the above-mentioned process. Thus, miRNAs can potentially inhibit key components, resulting in the inhibition of proliferation or in decreased expression of cyclin inhibitors. Indeed, Linsley *et al*[84] showed that the miR-16 family regulates cell cycle progression. Furthermore, the miR-497-195 cluster has been shown to target multiple cell cycle regulators, including CDKs, but it is transcriptionally silenced in hepatocellular carcinoma[85]. In the case of breast cancer, the same miRNA was able to decrease the cyclin E1 level[86]. Wee-1, a well-known cell cycle regulator, is also a target of miR-497[87]. There are other cases in which miRNAs inhibit cell cycle inhibitors. Thus, analyses of miRnome from a broad set of different cancer samples demonstrated that a number of miRNAs were over-expressed in all cases. Interestingly, one of the main targets of these miRNAs was RB1, a well-known negative regulator of the cell cycle[15].

Mechanisms of alterations of miRNA-mediated control of gene expression in cancer

Genetic mechanisms: It is well known that genome instability characterizes malignant cells. The discovery of DNA alteration involvement in trespassing of miRNA gene expression came from the observation that 30%-50% of *miRNA* genes are located in fragile sites[88,89]. Fragile DNA sites are regions that possess high levels of instability and are susceptible to such processes as genomic rearrangement, which include multiplication and deletion of loci, translocation, high rates of mutation *etc*[90]. Such a process is deletion of oncosuppressive *miR-15a*/*miR-16a* miRNAs that target the anti-apoptotic B-cell lymphoma 2 (BCL-2) protein[91], which was found in the majority of CLL cases[13]. Another rearrangement, translocation, was shown to alter the 17-92 cluster that contains a set of miRNAs among which is leukemogenic miR-19[92]. Translocation can also alter miRNA targets, which results in the disruption of miRNA-mediated proto-oncogenes repression. For instance, Mayr showed that translocation of High Mobility Group A2 (HMGA2) 3'UTR disrupts its repression by let-7 miRNA[37]. During amplification, the number of pro-oncogenic miRNA genes is often increased. Thus, miR-26a, a direct regulator of PTEN, is frequently amplified at the DNA level in human glioma[93]. Amplification of growth-promoting miR-23a is widely observed in gastric cancer[94]. While there is little data concerning the role of mutations in miRNA-mediated control in cancer, the number of publications dedicated to the role of single-nucleotide polymorphism (SNP) on miRNA action is growing fast. SNPs are single-nucleotide variations that naturally occur in the genome. They can potentially alter miRNA seed sequence, which results in alterations in miRNA target sites and deprivation of proto-oncogene expression control. It may also influence miRNA secondary structure and cause disruption of pri-miRNA recognition by miRNA processing enzymes. So far, numerous genomic studies have shown that SNPs in the miRNA seed sequence or target site may be associated with the risk for different types of cancer and in the prognosis of cancer treatment[95-99].

Transcriptional mechanisms: MiRNAs can be processed from RNA intron (mirtron) or transcribed as independent transcripts. In the latter case, an *miRNA* gene has its own promoter and is transcribed by Pol Ⅱ[9]. Since tissue transcription factors in cancer are often misregulated, it is logical to assume that this also influences miRNA expression. Indeed, regulation of miRNA expression by such well-known cancer-related transcription factors such as E2F, RAS, MYC and P53 has been shown[100,101]. Moreover, miRNAs and their transcription factors often work in feedback loops. Thus, E2F is responsible for up-regulation of the above-mentioned 17-92 cluster of miRNA in gliomas. E2F1 acts as a transcriptional activator of the *miR-17-92* cluster and binds directly to the *miR-17-92* promoter[102]. However, the set of miRNAs produced from this cluster directly inhibits E2F1. This is an example of a negative feedback loop[102,103]. Since E2F1 activates its own transcription by a positive feedback loop, miRNAs in this case act as a fuse for E2F1 over-saturation. MiRNAs miR-449a and miR-449b are other targets of E2F1. In this case, both miRNAs form a negative feedback loop indirectly by targeting the pRb-E2F1 pathway through cell cycle arrest[104]. High expression of miR-375 and estrogen receptor α (ERα) in breast cancer cells is an example of a positive feedback circuit. MiR-375 targets dexamethasone-induced ras-related protein 1 mRNA, an ERα inhibitor, whereas ERα increases *miR-375* expression[105].

Epigenetic mechanisms: Methylation of DNA, especially gene promoter regions of the genes, causes alteration in gene expression[106]. During cancer progression, two cases could potentially be realized: hypermethylation of oncosuppressors and hypomethylation of oncogenes. The fact that most miRNAs are associated with CpG islands[107] allows us to assume that miRNA genes are potential targets of DNA methylation machinery. Indeed, treatment of cells with inhibitors of DNA methylation (5-aza-2'deoxycytidine) led to upregulation of the subset of oncosuppressor miRNAs in human cancer cells[108]. Another example is the oncosuppressor *miR-663* gene, which targets well-known proto-oncogenes such as *EEF1A2, TGFβ, JunB* and *JunD*[109-111]. It was found to be downregulated *via* methylation in samples of human acute myeloid leukemia, hepatocellular carcinoma

and breast cancer, as well as in the K-562 leukemia cell line[112-115]. Similar processes occur with miR-129-2, a tumor-suppressive miRNA that is frequently methylated in lymphoid but not myeloid malignancies[116]. The process of hypomethylation can also be influenced in cancer-related alterations of miRNA expression. Thus, Li *et al*[117] observed hypomethylation of miR-200a/200b promoters with subsequent overexpression of these miRNAs. MiR-200a and miR-200b target *SIP1*, a protein product that suppresses E-cadherin expression and contributes to epithelial mesenchymal transition[74,117]. In renal cell carcinoma, the promoter of the well-known oncogene miR-21 was found to be hypomethylated, which correlates with upregulated miRNA expression level[118].

The stoichiometry of miRNAs and their targets: Each miRNA potentially targets hundreds of transcripts. Depending on the strength of the miRNA binding site, the target can be more or less inhibited. Thus, constant levels of miRNAs and mRNAs expression are in equilibrium, which provides cell homeostasis. However, several mechanisms that might decrease the miRNA level by using "miRNA sponges" have been discovered. The most well-known example is regulation of PTEN oncosuppressor expression by its pseudogene *PTENP1,* which harbors the same conserved miRNA binding site as *PTEN* mRNA[119]. In samples of colon cancer, a decrease in the *PTENP1* pseudogene copy number was found, which potentially increases the miRNA pool that targets PTEN. A pseudogene sequestering the miRNA pool was also shown in the case of *KRAS1P* pseudogene that possess binding sites for miR-143 and let-7 family[120]. Another example of a "miRNA sponge" is circular RNAs (circRNAs). These non-coding RNAs are processed from introns during splicing and carry multiple miRNA binding sites. Hansen *et al*[121] has shown that ciRS-7 (circular RNA sponge for miR-7) contains more than 70 selectively conserved miRNA target sites and strongly inhibits miR-7 oncosuppressor activity[122,123].

3'UTR BINDING PROTEINS AND CANCER

Modulation of the protein expression on the posttranscriptional level during oncogenic transformation often depends on 3'UTR and takes place by changing cis-elements or trans-binding factors that dictate stability and translation efficiency of cancer-related protein mRNAs.

There are few well-characterized cis-elements present in the 3'UTR region. One of them is the CPE, which has a consensus sequence of U4-8A1-2U and is located in relatively close proximity to the ubiquitous nuclear polyadenylation hexanucleotide AAUAAA[124-126].

CPE binds CPEB, one member of a family of four conserved sequence-specific RNA-binding proteins that contain a zinc finger and two RNA recognition motifs[127]. During Xenopus oocyte maturation, CPEB controls meiosis progression from prophase I to metaphase Ⅱ[127]. Translational control by CPEBs was later also shown to be involved in learning and memory[128,129] and in the regulation of the mammalian cell cycle[130]. CPEB is also implicated in senescence in mammals[131,132] and in controlling the translation of proteins involved in cell cycle checkpoints[133]. Xenopus studies have shown that CPEB can both promote and inhibit RNA translation by respectively elongating and shortening the poly(A) tail. The balance between the two CPEB-associated activities is altered during progression of the cell cycle, depending on post-transcriptional modifications as well as on the number and location of CPEs to which CPEB binds and recruits associated adenylating and de-adenylating protein complexes. The CPEB-containing complex in Xenopus include: symplekin, which may be a platform protein upon which multi-component complexes are assembled, poly(A) ribonuclease, a de-adenylating enzyme and germline-development factor 2 (Gld2), an atypical poly(A) polymerase[134,135]. The induction of cytoplasmic polyadenylation is mediated by activation of the serine/threonine kinase Aurora A/Eg2, possibly through repression of glycogen synthase kinase 3[136,137]. When phosphorylated on S174 or T171 (species-dependent), CPEB promotes polyadenylation by stimulating the activity of Gld-2[138]. The newly elongated tail bound by the poly(A)-binding protein promotes translation by augmenting the assembly of the eIF4F initiation complex[139].

CPEB family members were found to be misregulated in various cancers[3]. One of them, CPEB4, was recently shown to be not only over-expressed in pancreatic cancer and glioblastoma in comparison with healthy pancreatic and brain tissues, but also plays a role as a key regulator of cancer transformation and controls translation of hundreds of mRNAs. SiRNA down-regulation of CPEB4 expression in RWP-1 (human pancreatic cancer lines) and Capan pancreatic cancer cells reduce their ability to form tumors after injection into nude mice[2]. This group found that one of the most enriched CPEB4-associated mRNAs, tissue type plasminogen activators (tPA), which is known to be over-expressed in pancreatic tumors, has a short poly(A) tail in normal tissue, whereas in ductal tumors and pancreatic ductal adenocarcinoma cell lines, the tPA poly(A) tail is elongated. This observation supports the idea that misregulation of protein expression during cancer transformation can be controlled by the length of the poly(A) tail, which depends on the presence of CPEB proteins[2].

Insulin-like growth factor-2 mRNA-binding proteins (IGF2BPs or IMPs) are oncofetal proteins that were first discovered in human embryonic Rhabdomyosarcoma and are highly expressed in a number of human cancers[6]. IMPs belong to a conserved family of RNA-binding proteins implicated in the post-transcriptional regulation of multiple mRNAs, IGF2, MYC, CD44, PTEN, G_1/S-specific cyclin-D1 (CCND1), CCND3, G_1/S-specific cyclin-G1 (CCNG1) and others[4,5,140,141]. All these IMP targets are implicated in the proliferation and invasion of human cancer cells. Moreover, several studies have shown that IMPs participate in essential cell functions alienated during cancer transformation, such as cell polarization, migration, morphology, metabolism, proliferation and

differentiation[142].

IMPs are mainly expressed in the embryo and are important during development. However, because of their abnormal re-expression in several types of cancer, IMPs are considered as oncofetal proteins. Typically, IMP1 and IMP3 have been implicated in colon, liver, kidney, pancreas and female reproductive tissue cancers. IMP3 is reported in over 50 publications as being over-expressed in multiple cancer types. IMP3 expression actually correlates with tumor aggressiveness. Concerning IMP2, a few studies have linked its expression to liposarcoma, liver cancer and endometrial adenocarcinomas[142].

IMPs are generally observed in the cytoplasm, where they associate with target mRNAs in cytoplasmic ribonucleoprotein complexes (mRNPs). Actually, in complex with a wide range of other RNA binding proteins (RBPs), IMPs are able to control mRNA turnover, transport, localization and translation.

Other studies provide evidence suggesting an important role for IMPs in cell migration. For instance, IMP2 binds and controls the expression of PINCH-2 (particularly interesting new cysteine-histidine-rich protein) and MURF-3 (muscle specific RING finger protein2) mRNAs to modulate cell motility[143].

Despite controversial observations regarding a potential nuclear role of IMPs, increasing evidence suggests that IMPs can recruit their target mRNAs in the nucleus during their transcription[144-146]. Moreover, a recent study actually shows that in contrast with IMP1 and IMP2, IMP3 has nuclear localization in a large number of human cancer cell lines. For example, IMP3 is almost 100% nuclear in hepatocellular carcinoma, breast and ovarian cancer cells[4].

Among other well known proteins that bind mostly to the AU-rich sequences in 3'UTR and are involved in cancer transformations are Hu/elav proteins, known to bind AU-rich sequences in the 3'UTR and enhance mRNA translation or increase its stability[147,148]. HuR is ubiquitously expressed and HuB, -C and -D are primarily neuronal. HuR is also known as embryonic lethal, abnormal vision, Drosophila-like 1. A link between HuR and malignant transformation has been suggested in cancers such as breast, colon, lung and ovary[149]. Their targets are involved in several processes, such as cell growth and survival, proliferation, stress response, senescence and cancer[150,151].

AU-binding factor 1 (AUF1), also known as heterogeneous nuclear ribonucleoprotein D, belongs to a big family of hnRNPs that includes hnRNNP A, B, C, D, E, F, H, I, K, L, M, Q and R. AUF1 binds to the AU-rich sequence in the 3'UTR of target mRNAs and promotes degradation of the target transcript, most probably by recruiting them to exosomes for degradation[152,153]. However, AUF1 was found to enhance stability and translation of some mRNAs[154,155]. AUF1 was also shown to be involved in several processes: cell cycle, stress response, apoptosis and carcinogenesis.

T-cell intracellular antigen 1 (TIA-1) TIA-1-related (TIAR) binds to AU/U-rich sequences in the 3'UTR of the target transcript and suppresses mRNA translation[156]. Under stress conditions, these proteins are thought to halt mRNA-to-protein aggregations known as stress granules[157].

Nuclear factors 90 interacts with AU rich sequences and suppresses translation of mRNAs involved in the cell cycle[158].

Tristetraprolin (TTP), zinc finger protein, binds AU-rich sequences in mRNAs to promote their decay. It is involved in the cell cycle, inflammation and carcinogenesis[159,160].

KH-type splices regulatory protein (KSRP). RBP binds to AU-rich sequences of target transcripts, promoting mRNA decay. Its targets encoded cytokines, transcription factors, proto-oncogenes and cell cycle regulators[161].

Nucleolin interacts with mRNAs bearing AU-rich or G-rich sequences and regulates mRNA stability and translation. Its targets are involved in the cell cycle, cell morphology, development, cell proliferation and cancer genesis[148].

Obviously, two or more RBP may functionally interplay among themselves and with microRNAs through binding to the same mRNA 3'UTR.

INTERACTION BETWEEN 3'UTR BINDING FACTORS AND THEIR FUNCTION IN NEOPLASTIC TRANSFORMATION

RNA binding proteins interaction

Significant evidence has accumulated to support the idea that several RNA binding proteins can bind the same mRNA target on either the non-overlapping binding sites or on common sites in a competitive fashion. Different RBPs that are capable of binding to the same RNA can cooperate or compete in their actions (Figure 1). The outcome of the combined action of all factors bound to the same mRNA 3'UTR depends on many circumstances, such as expression of different RBPs, their affinity for the binding sites, and their localization in the cells. This can be controlled by different physiological conditions.

For instance, interleukin (IL)-8 plays an integral role in promoting a malignant phenotype in breast cancer and its production is directly influenced by inflammatory cytokines in the tumor microenvironment. Subsequently, activation of the IL-1 receptor on malignant breast cancer cells strongly induces IL-8 mRNA expression. HuR, KSRP and TIAR were found to bind one or more locations within the IL-8 3'UTR, although affinity of the stabilizing factor HuR was 20-fold stronger than that of the KSRP destabilizing factor[162]. HuR, AUF1 and nucleolin bind to BCL-2 mRNAs. HuR and nucleolin both stabilized the BCL-2 transcript, while AUF1 enhanced degradation[163-166]. Thus HuR and nucleolin can have a cooperative effect that is antagonized by AUF1. Another example is related to regulation of GADD45A mRNA stability and translation efficiency. Nucleolin stabilized

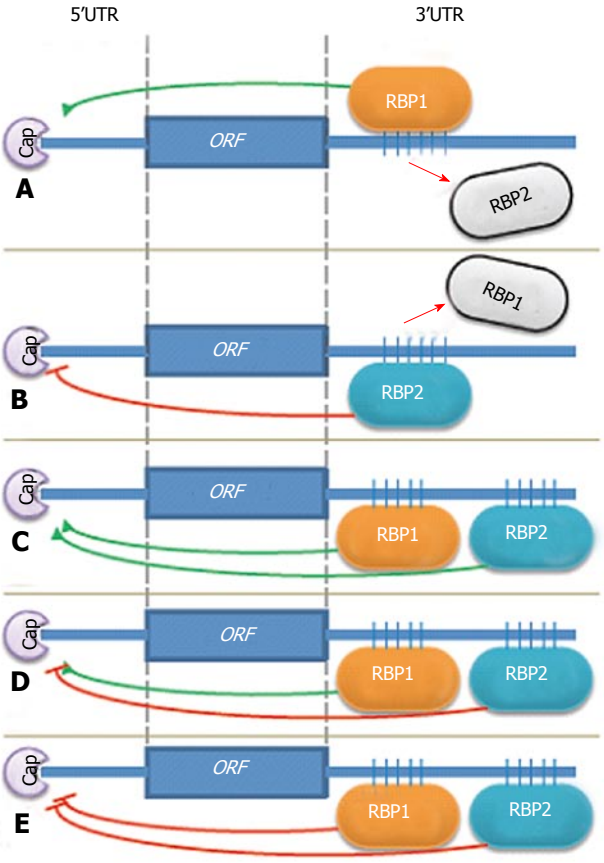

Figure 1 Models of RNA binding proteins interplay in regulation of the same target. A, B: Two RBPs compete for the same binding site in 3'UTR; A: RBP1 binds to the site, repels RBP2, and stimulates translation; B: RBP2 binds to the site, repels RBP1, and inhibits translation; C, D, E: Two RBPs bind to the different sites and cooperate (C, E) or compete (D) in their actions; C: Cooperative action of two RBPs stimulating expression; D: Two RBPs antagonized their effects; E: Cooperative action of two RBPs inhibiting expression. RBPs: RNA binding proteins; 3'UTR: 3'-untranslated region; ORF: Open reading frame.

GADD45A mRNA and was antagonized by AUF1, which promotes decay of this mRNA, and by TIAR, which suppresses translation[167].

In addition, HuR and AUF1 formed a stable ribonucleoprotein complex in the nucleus, whereas in the cytoplasm, HuR and AUF1 bound to target mRNAs individually. HuR colocalizes with the translational apparatus and AUF1 with exosomes[168].

The nuclear localization of IMP3 depends on its protein partner, HNRNPM. Nuclear IMP3 is important for the efficient synthesis of CCND1, D3 and G1 proteins and for the proliferation of human cancer cells. Curiously, IMP3 can be differentially localized in normal versus cancerous adult cells, which in turn will determine the efficiency of protein synthesis of CCND1, D3 and G1 in these cells and have an impact on their proliferation[4]. These studies suggested that IMPs are controlling the transcript destiny of targeted mRNAs in the nucleus and subsequently influence their stability and translation in the cytoplasm. IMP is also found in complex with other RNA-binding proteins, such as HNRNP A2/B1, HNRNP A1, HNRNP A3, Polypyrimidine tract-binding protein 1, interleukin enhancer-binding factor 3, an RNA helicase DHX9 and a mRNA-stabilizing protein HuR[4]. Some of these IMP3 partners, such as HuR, are already known to positively regulate CCND1 mRNA stability and translation[168].

Members of the CPEB and PUF (drosophila pumilio (Pum) protein is a founder member of a novel family of RNA-binding proteins, known as the PUF family.) (Pomelia/Fem-3 mRNA-binding factor 1) families collaborate to regulate mRNA expression throughout eukaryotes. PUF was shown to directly interact with CPEB in C. elegans and humans (CPEB3) and to inhibit translation of common targets[169].

3'UTR binding factors can control translation efficiency *via* interaction with translation, initiation and elongation factors. An example of the interaction with initiation factors has been described for CPEB1 in a previous chapter. Recently, the eukaryotic translation elongation factor 1A1 (eEF1A1) was shown to be involved in EMT regulation. The main function of eEF1A1 is delivery of aminoacyl tRNA to the A-site of the ribosome[170-172]. However, Hussey *et al*[173] discovered a new mechanism of EMT control when eEF1A1 in complex with hnRNP E1 binds to the BAT element in the 3'UTR of the EMT, inducing Dab2 and ILEI transcripts. This results in the inhibition of eEF1A1 release from the ribosomal A site, which causes a stall in translational elongation of the above-mentioned transcripts[173].

Moreover, PUF and Ago can interact with eEF1A proteins to repress translation elongation in both C. elegans and mammalians. This repression occurred after translation initiation and led to ribosome accumulation within the open reading frame, roughly at the site where the nascent polypeptide emerged from the ribosomal exit tunnel. Together, these data suggest that a conserved PUF-Ago-eEF1A complex attenuates translation elongation[174].

RNA binding protein and microRNA interaction

Proteins that bind to the same mRNAs 3'UTR can modulate the function of miRNAs. They either enhance the inhibitory function of miRNA or prevent it. On the other hand, miRNA also can assist the function of RNA binding protein or inhibit it. This can happen simply through binding site competition or collaboration (*via* RNA remodeling), direct protein-protein interaction of 3'UTR-binding complexes, or just functional interplay when a few factors act separately but their actions augment or negate each other (Figure 2).

Interestingly there are few cases described in the literature in which miRNA in collaboration with RNA binding proteins can change their mode of action during the cell cycle or under physiological conditions such as oxidative stress and others.

It has been shown that upon cell cycle arrest, the ARE (AU-rich element) in tumor necrosis factor-α mRNA acts as a translation activation signal, recruiting AGO (argonaute RISC catalytic component) and fragile

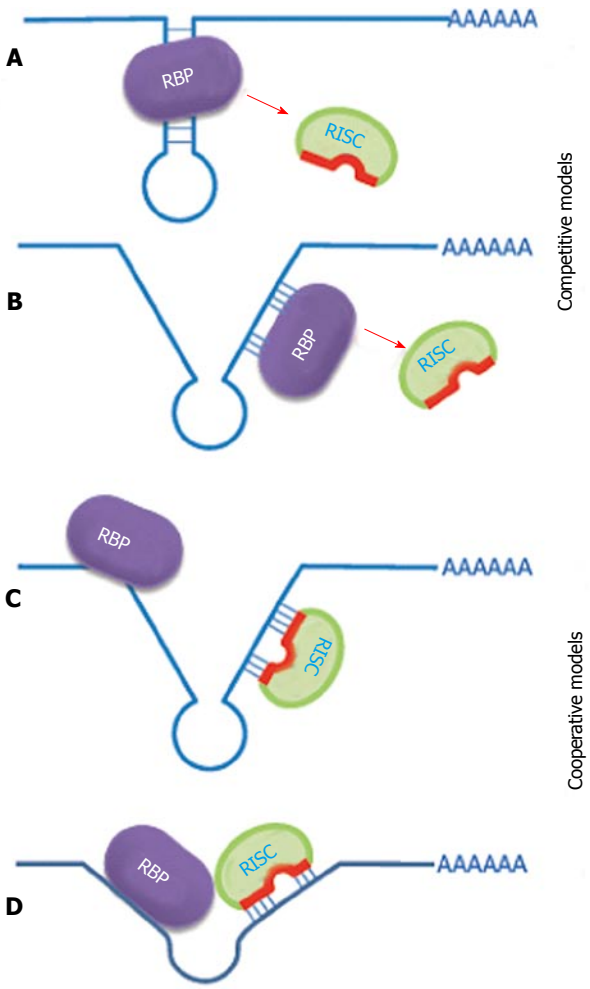

Figure 2 Models of RNA binding protein and miRNA interplay targeting the same mRNA. A, B: Competitive interaction. A: RBP stabilizes the secondary structure in 3'UTR, and prevents miRNA binding; B: RBP competes with miRNA for the same binding site; C, D: Cooperative interaction; C: RBP facilitates miRNA function by opening secondary structure in 3'UTR and liberating its binding site; D: RBP directly interacts with RISC (RNA-induced silencing complex) complex, stabilizing binding of the latter. RBPs: RNA binding proteins; 3'UTR: 3'-untranslated region.

X mental retardation-related protein 1 factors associated with miRNPs. Human miRNA mir-369-3 directs the association of these proteins with the AREs, leading to the activation of translation[7]. Moreover, two well-studied miRNAs, let-7 and the synthetic miRNA miRcxcr4, also induce translational up-regulation of target mRNA upon cell cycle arrest. However, they repress translation in proliferating cells. It has been proposed that translation regulation by miRNPs oscillates between repression and activation during the cell cycle[7].

Another example of inactivation, storage and reactivation is calcium transport protein (CAT)-1 mRNA targeting by mir-122 under stress conditions. The derepression of CAT-1 mRNA is accompanied by its release from cytoplasmic P-bodies and its recruitment to polysomes. Derepression requires binding of HuR, an AU-rich-element-binding protein, to the 3'UTR of CAT-1 mRNA[175]. Thus, interaction with RNA binding proteins can change the miRNA mode of action directed to the same target, according to conditions.

Some difficulty in understanding the affiliation of certain RBPs for oncogenes or tumor suppressors came from the observation that the same RBP interacting with different miRNAs in the regulation of different targets could lead to enhanced or suppressed cancer transformation, according to the nature of the target. The stimulation effect of Pomelia on miRNA function, most probably through mRNA remodeling, is directed towards the targets acting in opposite ways, as oncogene or tumor suppressor. It was shown by Kedde and co-workers that Pomelia RBP pumilio RNA-binding family member 1 (PUM1) and PUM2 promote the regulation of miR-221/222 on the $p27^{kip1}$ check-point protein and tumor suppressor mRNAs by opening of the secondary structure of the p27 3'UTR and exposing the binding sequence to miR-221/222. This causes down-regulation of $p27^{kip1}$ accumulation and stimulates cell proliferation and breast cancer development[176]. On the other hand, Pomelia collaborates with some miRNAs to repress E2F3, transcription factor and strong oncogene. This prevents cell proliferation and down-regulates bladder cancer development[177].

Another example of miRNA and RBP collaboration was shown by Nairismägi *et al*[73] who showed that miR-580 and CPEB1/2 down-regulate TWIST1 expression, one of the main inductors of EMT in a cooperative way. On the other hand, Dnd1 is an example of RBP that prevents binding of miRNA to their target sequences in a few genes, such as $p27^{kip1}$ and LATS2, and suppresses formation of germ cell tumor[178]. It also prevents miR-21 function on its MutS protein homolog 2 target, which suppresses tumorigenesis in skin[179]. Thus by preventing miRNA down-regulation of tumor suppressors, Dnd1 inhibits the development of certain tumors.

The same RNA binding protein can cooperate or antagonize miRNA functions, according to the mRNA-target. One of the most investigated examples is HuR[147], which was found to recruit let-7 to suppress c-MYC mRNA translation[8] but competes with miR-494 and miR-548-3p for the regulation of nucleolin and TOP2A mRNA, respectively[180,181].

Some RBPs not working alone but in complex with other RNA binding proteins can prevent miRNA actions. IMP1 in complex with heterogeneous nuclear ribonucleoprotein U, synaptotagmin binding, cytoplasmic RNA interacting protein, YXB1 (transcriptional regulator) and DHX9 [DEAH (Asp-Glu-Ala-His) box helicase 9] is able to stabilize the mRNA of MYC, possibly by inhibiting its translation-coupled degradation[182]. However, some studies showed that MYC is repressed by members of the let-7 microRNA family, suggesting a possible function of IMP1 in protecting MYC mRNAs from microRNA silencing. This was previously proposed as a mechanism for the stabilization of the BTRC (beta-transducin repeat containing E3 ubiquitin protein ligase) mRNA by IMP1[183,184].

Not only RNA-binding protein can influence miRNA function, but reciprocal action has already also been

documented in the literature. Some miRNAs can affect the function of RNA binding protein. For example, interaction of mir-16 (a member of the mir-15/16 family of miRNPs) and an ARE-binding protein TTP (tristetraprolin) has been shown to occur through association with AGO/eiF2C family members. Mir-16 assists TTP in targeting ARE, which appears to be an essential step in ARE-mediated mRNA degradation[185].

From all of these examples, one can see that interaction among factors binding to 3'UTR brings a new level of complexity to the mechanisms of action of these factors and their influences on cancer transformation. It is becoming clear that to understand the true picture of the post-transcriptional control of certain genes *via* 3'UTR, especially that involved in cancer transformation, one needs to take into account all proteins and miRNAs binding to their 3'UTRs.

APPLICATION OF 3'UTR-BINDING FACTORS TO CANCER DIAGNOSIS AND TREATMENT

From the very early investigations that suggested miRNA involvement in cancer, scientists began to think about using it as a tool for cancer diagnosis and therapy. A number of studies have been initiated utilizing miRNA expression profiling to determine markers for diseases. An early study comparing a limited number of available miRNAs in cancer and normal tissues drove the conclusions that miRNAs expression signatures are able to classify tumors based on the development lineage and the differentiation state, suggesting miRNAs as a potential biomarker[14]. Following works used the miRNA expression profile to define a number of normal and cancerous tissues from thyroid, kidney, bladder, liver *etc*[186-193]. Furthermore, miRNA profiling has also been used to classify tumor subtypes in breast cancer in development[194,195]. Mir-342 is differentially expressed in breast cancer subtypes with high expression in Luminal B-type tumors and decreased expression in therapeutically difficult estrogen receptor/human epidermal growth factor receptor 2-negative tumors[196]. This observation suggested that select miRNAs expression could differentiate tumor subtypes that can be more sensitive or resistant to particular treatments.

Radiation therapy (RT) is one of the most often used procedures in cancer treatment; however, not all patients respond well to it. So, it is very important to develop markers that can predict a patient's response to RT. MiRNA profiling has a big potential for this type of diagnosis.

One of the first reports identifies the let-7 family for its role in modulating sensitivity for RT in lung cancer[197]. It has been demonstrated that over-expression of let-7 promotes radio-sensitivity while knockdown increases resistance both *in vitro* and *in vivo*. Mir-181a has been identified as an important miRNA for radio-sensitivity in glioma cells. Transient over-expression of miR-181a prevented radio-sensitivity that correlated with decreased quantities of Bcl-2, an anti-apoptotic protein[198]. Similarly, over-expression of mir-451 in colorectal cancer cell lines decreases proliferation and increases RT sensitivity of colorectal cancer cells[199].

Chemotherapy is another widely used treatment in cancer therapy. The miRNA profile also has a big potential as a marker for chemo-sensitivity. Inhibition or introduction of some miRNAs to certain cancers can improve their chemo-sensitivity. Inhibition of mir-21 sensitizes U251 glioma cells to etoposide and glioma in mice to tumor necrosis factor-related apoptosis, inducing the ligand S-TRAIL (TNF-related apoptosis-inducing ligand)[200-202]. Mir-451 is downregulated in the glioblastoma stem cell population. Reintroduction of mir-451 in combination with the frequently used glioblastoma treatment imatinib inhibits the growth of glioblastoma stem cells and the formation of neurospheres[203].

Mir-122 was shown to be downregulated in hepatocellular carcinoma (HCC) cells, which promotes RT resistance as well as growth, proliferation and metastasis[204]. Insulin growth factor 1 tyrosine kinase receptor is targeted and suppressed by miR-122 in normal liver cells. However, depletion of mir-122 in HCC increases the IgfIR level. Reintroduction of mir-122 in HCC promotes sensitivity to the tyrosine kinase inhibitor sorafenib[204].

In colorectal cancer, a number of miRNAs have been associated with predicting the response to nucleoside analogs. Mir-143 is downregulated in colon cancer. It targets NF-κB, Bxl-2 and ERK5 and has been shown to increase sensitivity to fluorouracil in HCT-166 colon cancer cell lines[205]. In rectal cancer, mir-125b and mir-137 were associated with poor response to capecitabine, a prodrug that is enzymatically converted to fluorouracil[206]. In colon cancer, mir-519c targets and suppresses ATP-binding cassette sub-family G member 2 (ABCG2) in cell lines that are sensitive to mitoxantrone, whereas mir-519c inhibition increases the ABCG2 level and chemoresistance. In the ABCG2 resistant cell line, mRNA possess a shortened 3'UTR, which results in the loss of a mir-519c target site and a high-level of ABCG2 protein[207].

All these examples clearly show that miRNA profiling of each cancer could provide useful information for choosing the right treatment strategy. Few bio-pharmaceutical companies are working on developing miRNA-profiling platforms for more detailed identification of cancer subtypes that could improve recommendation of treatment. There are more than 100 ongoing trials incorporating miRNA as biomarkers underway in various bio-pharmacological companies.

Direct miRNA therapeutics, the fundamental principle of miRNA therapy, involves either directed silencing or reduction in tumor-promoting miRNAs versus enrichment of tumor suppressive miRNAs. *In vivo*, these approaches include genetically engineered animals and different ways of delivery, such as viral vectors, nanoparticle-based delivery, mimics and antimiRs. Targeting miRNA for suppression through the use of antimiRs is perhaps the most promising model. Through complementary binding to the target miRNA (working strand),

these molecules can repress the action of select miRNAs.

To improve stability and target specificity, investigators have developed various modifications. Three types of modification currently give the most promising results: replacement of 2-OH residues by 2'-O-methyl modified oligonucleotides, 2'-O-methoxyethyl and locked nucleic acid. In addition, conjugation of cholesterol may be used to improve target specificity[208].

Sponge is another tool for RNA-silencing. By having multiple target binding sites, sponges essentially compete with target mRNA for miRNA occupancy, thus decreasing binding miRNA to its real target[209].

To target a few miRNAs involved in the same cancer formation, investigators started using tiny 8-mer locked nucleic acids with a phosphorothioate backbone to enhance the stability level[210]. They were shown to inhibit families of miR-221/222 and let-7 with high specificity.

Viral vector-based delivery systems, including adenoviral, retroviral and lentiviral systems provide some advantages. For example, lentiviral let-7 delivery has been successfully used in murine models of lung cancer[32]. Several nanoparticles with lipid-based formulations were perhaps the most effective in delivery while minimizing toxicity. Lipid emulsions have been used to deliver miRNAs in lung cancer and lymphoma[211-213].

In spite of big efforts, only mir-122 has successfully reached the clinical trial in targeted therapy[214,215]. The systematic delivery of antimiR-122 could reduce the hepatitis C virus (HCV) viral load chimpanzee model of chronic HCV infection with minimal toxicity[216]. Santaris Pharma conducted a human phase Ⅱa trial safety antiviral function using miravirsen (a locked nucleic acid-modified miR-122 antagonist).

RNA binding proteins similarly can be used as markers for proper cancer diagnostics, leading to better treatment selection. For example, IMP3 over-expression has been associated with distinct cancer types. Several studies have suggested IMP3 as an important marker for poor prognosis in cancer[217,218]. Moreover, it was demonstrated that IMP3 promotes cell growth, proliferation and resistance to ionic irradiation in an IGF2-dependent manner[219,220]. Since CPEB4 was found to be a key protein for pancreatic cancer and glioblastoma development, one can try to apply siRNA-dependent direct down-regulation of CPEB4 protein in this type of tumor using delivery methods that are discussed in this chapter.

In conclusion, 3'UTRs of human mRNAs contained many cis-elements that bind trans factors and are important for the development of various diseases, including cancer. Additional work is required to identify the complete set of 3'UTR cis-elements and the trans-regulatory factors that interact with them and to determine functional consequences of these interactions and their role in cancer transformation. Powerful transcriptome-wide computational and experimental methods are now being used to address these questions. Along with lower-throughput reductionist approaches, they should move us closer to a system biology understanding of how 3'UTRs contribute to gene regulation during cancer transformation. This will allow developing new, more powerful drugs in cancer therapy.

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REVIEW

Roles and mechanisms of the CD38/cyclic adenosine diphosphate ribose/Ca^{2+} signaling pathway

Wenjie Wei, Richard Graeff, Jianbo Yue

Wenjie Wei, Richard Graeff, Jianbo Yue, Department of Physiology, the University of Hong Kong, Hong Kong, China
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Correspondence to: Jianbo Yue, PhD, Department of Physiology, the University of Hong Kong, 102 Pok Fu Lam Road, Hong Kong, China. jbyue@me.com
Telephone: +86-852-29199202 Fax: +86-852-28559730
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Abstract

Mobilization of intracellular Ca^{2+} stores is involved in many diverse cell functions, including: cell proliferation; differentiation; fertilization; muscle contraction; secretion of neurotransmitters, hormones and enzymes; and lymphocyte activation and proliferation. Cyclic adenosine diphosphate ribose (cADPR) is an endogenous Ca^{2+} mobilizing nucleotide present in many cell types and species, from plants to animals. cADPR is formed by ADP-ribosyl cyclases from nicotinamide adenine dinucleotide. The main ADP-ribosyl cyclase in mammals is CD38, a multi-functional enzyme and a type Ⅱ membrane protein. It has been shown that many extracellular stimuli can induce cADPR production that leads to calcium release or influx, establishing cADPR as a second messenger. cADPR has been linked to a wide variety of cellular processes, but the molecular mechanisms regarding cADPR signaling remain elusive. The aim of this review is to summarize the CD38/cADPR/Ca^{2+} signaling pathway, focusing on the recent advances involving the mechanism and physiological functions of cADPR-mediated Ca^{2+} mobilization.

Key words: Cyclic adenosine diphosphate ribose; CD38; Ca^{2+}; Ryanodine receptors; Nicotinamide adenine dinucleotide

Core tip: This is a comprehensive review regarding the role and mechanism of the CD38/Cyclic adenosine diphosphate ribose (cADPR)/Ca^{2+} signaling pathway in various cellular processes. We introduce the structure and function of cADPR, together with its production and degradation pathways. We also describe CD38, the main enzyme that is responsible for synthesis of cADPR, through its structure and topology. Finally, we summarize the functions of the CD38/cADPR/Ca^{2+} signaling pathway under both physiological and pathological conditions.

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INTRODUCTION

Discovered more than two decades ago, cyclic adenosine diphosphate ribose (cADPR) has been established as a second messenger, according to criteria first proposed by Sutherland and co-workers[1]. Together with inositol 1,4,5-trisphosphate (IP_3) and nicotinic acid adenine dinucleotide phosphate (NAADP), cADPR has been recognized as a principal second messenger involved in cellular Ca^{2+} mobilization. Extracellular stimuli can induce cADPR production, which leads to Ca^{2+} mobilization from intracellular stores as well as Ca^{2+} entry from the extracellular compartment to initiate diverse cellular responses. cADPR is synthesized by ADP-ribosyl cyclases and the major ADP-ribosyl cyclase in mammals is CD38 (Figure 1). In this review, we will first introduce the structure and

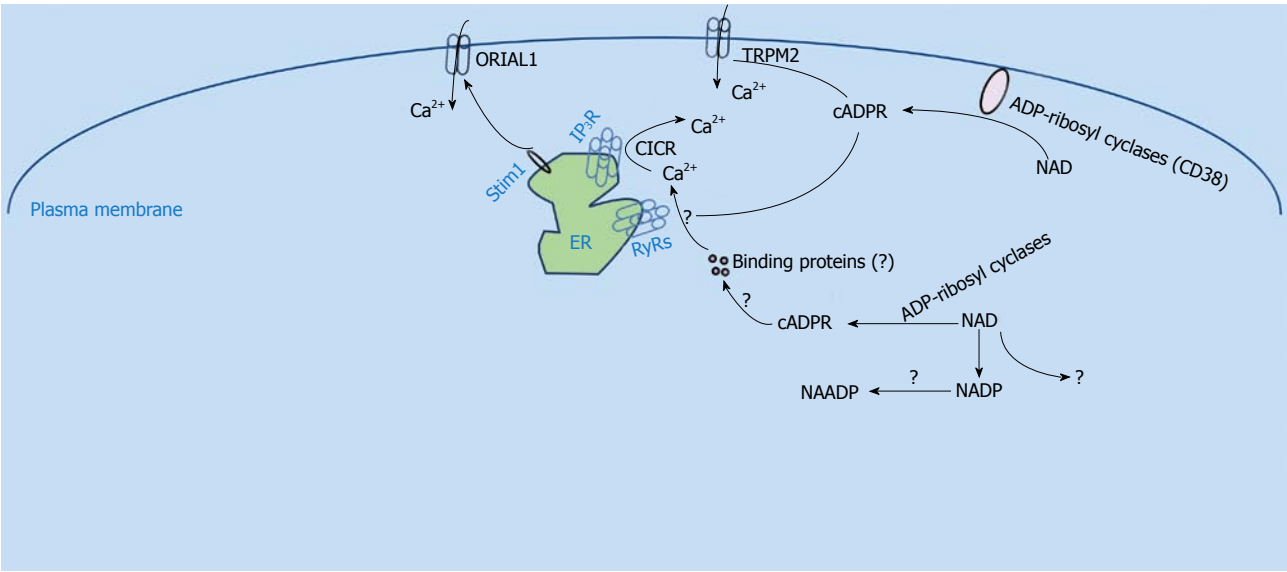

Figure 1 Cyclic adenosine diphosphate ribose mediated Ca^{2+} signaling. TRPM2: Transient receptor potential cation channel M2; cADPR: Cyclic adenosine diphosphate ribose; NAADP: Nicotinic acid adenine dinucleotide phosphate; NAD: Nicotinamide adenine dinucleotide; ER: Endoplasmic reticulum.

function of cADPR. Next, the structure and topology of CD38 will be reviewed. Finally, the physiological functions of CD38/cADPR/Ca^{2+} signaling and their involvement in pathological processes will be summarized.

THE STRUCTURE AND FUNCTION OF CADPR

A suitable model system is the foundation of any novel finding and this concept is also true for the discovery of cADPR. Sea urchin eggs are large and amenable for microinjection studies so that Ca^{2+} mobilizing activities during fertilization can be readily observed, and it is easy to isolate endoplasmic reticulum (ER) from sea urchin eggs, making them the perfect system to investigate mechanisms of intracellular Ca^{2+} mobilization[2]. Taking advantage of the sea urchin homogenate preparation and use of the fluorescent Ca^{2+} indicator Fura 2, Lee *et al*[3] and Clapper *et al*[4] found that the pyridine nucleotide nicotinamide adenine dinucleotide (NAD) can invoke a delayed Ca^{2+} release from ER independent of IP_3. They then determined that this delay was due to enzymatic conversion of NAD to cADPR by the homogenate. Later, Lee *et al*[5] solved the structure of cADPR by x-ray crystallography and showed that it is a novel cyclic nucleotide formed by the covalent linkage of the N1 nitrogen of the adenine ring to the anomeric carbon of the terminal ribose to become a closed cyclic structure (Figure 2). Benefiting from the identified structure, multiple cADPR analogs have been synthesized, which greatly promoted research on the role and mechanism of cADPR-mediated Ca^{2+} signaling[6-9].

From the very beginning of research on cADPR, several pharmacological studies have clearly shown that the mechanism of cADPR-induced Ca^{2+} release is different from that of IP_3. For example, desensitization experiments demonstrated that the sea urchin homogenates which were desensitized to IP_3 would still respond to cADPR[4], and the IP_3 inhibitor heparin had no effect on the cADPR-induced Ca^{2+} release[10]. Using the sea urchin homogenate as the model, Galione *et al*[11] proposed that calcium-induced calcium release (CICR) may be modulated by cADPR, since concentrations of cADPR in the nanomolar range could greatly increase the sensitivity to Ca^{2+} during the CICR process. Thus, ryanodine receptors (RyRs) were proposed to be the cADPR receptors through which the CICR functions, and this idea was supported by several subsequent studies. For example, cADPR was shown to directly activate RyR2 that was incorporated into lipid bilayers[12]. In HEK293 cells transfected with an islet type RyR, which is a splice variant of the *RyR2* gene by alternative splicing of exons 4 and 75, Ca^{2+} release was enhanced in the presence of 100 μmol/L cADPR, and the effect could be reversed by pre-incubating with a cADPR antagonist, 8-bromo-cADPR (8-Br-cADPR)[13]. Similarly, cADPR triggered a marked Ca^{2+} transient in HEK293 cells that stably expressed RyR1 and RyR3, and this Ca^{2+} transient was abolished by dantrolene, an RyR antagonist[14]. In summary, all these results suggested that RyRs might serve as cADPR receptors (Figure 1).

However, further experiments argued that the action of cADPR on ryanodine receptors might require the assistance of additional protein factors (Figure 1). For example, both calmodulin and FK506 binding protein (FKBP) have been shown to be required for cADPR action[15-20]. These data suggested that cADPR does not directly bind to the ryanodine receptors, but acts through some intermediate proteins, whose definitive identities remain to be established. Zheng *et al*[21] demonstrated in mouse bladder smooth muscle that Ca^{2+} release induced by cADPR is actually mediated by FKBP12.6 proteins. Nevertheless, additional research such as genome-wide

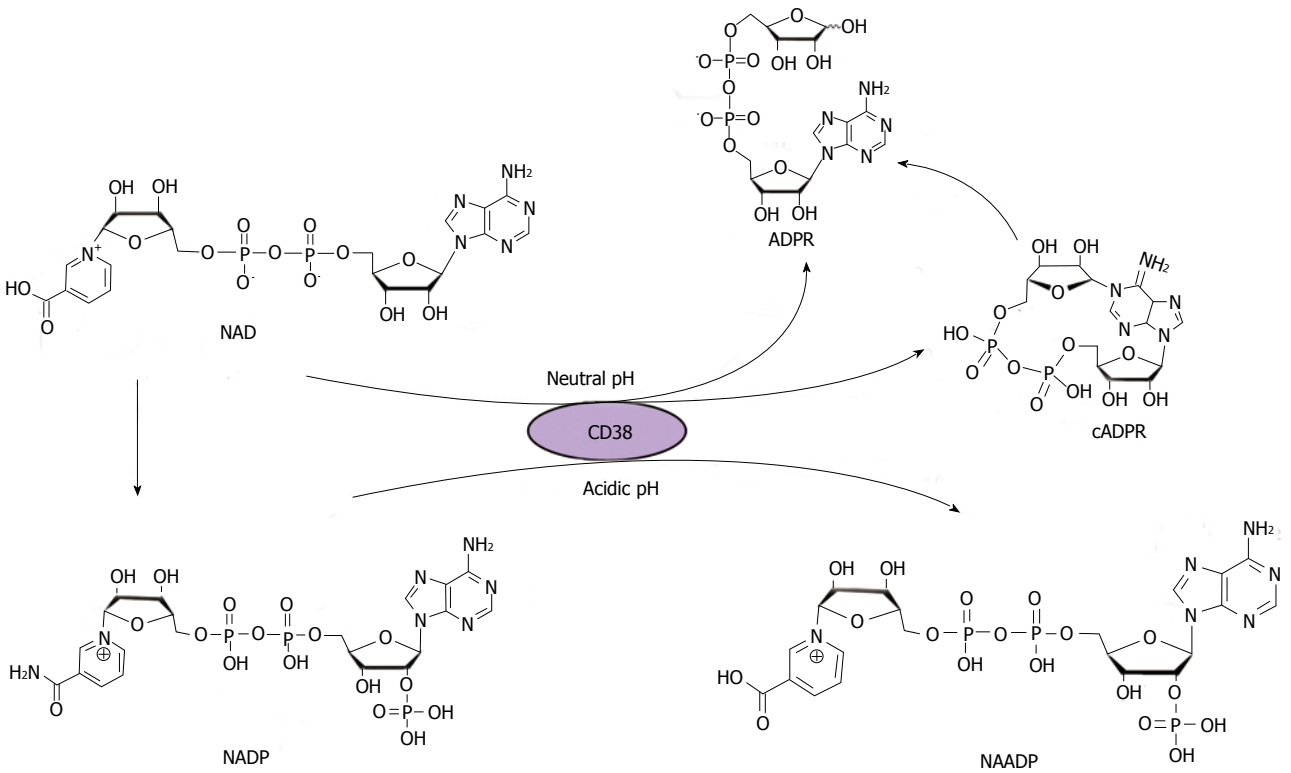

Figure 2 Schematic of the structure and synthesis of cyclic adenosine diphosphate ribose. cADPR: Cyclic adenosine diphosphate ribose; NAADP: Nicotinic acid adenine dinucleotide phosphate; NAD: Nicotinamide adenine dinucleotide.

RNAi screening is needed to elucidate the direct receptor of cADPR.

In addition, growing evidence has shown that cADPR also evokes Ca^{2+} influx (Figure 1)[22]. It has been shown that cADPR can significantly potentiate the transient receptor potential cation channel M2 (TRPM2) channel activity in a temperature dependent manner[23]. Similarly, we recently synthesized a novel fluorescent caged cADPR analogue, coumarin caged isopropylidene-protected cIDPRE (Co-*i*-cIDPRE), and found that it is a potent and controllable cell permeant cADPR agonist. Moreover, we demonstrated that uncaging of Co-*i*-cIDPRE activates RyRs for Ca^{2+} mobilization and triggers Ca^{2+} influx *via* TRPM2[24]. Yet, another experiment showed that TRPM2 is not involved in the effect of another membrane-permeant cADPR agonist, 8-bromo-cyclic IDP-ribose (8-Br-N^1-cIDPR), which induced Ca^{2+} entry in T cells[25]. Thus, the channel that mediates the cADPR induced Ca^{2+} influx still needs to be elucidated.

ENZYMATIC PATHWAY OF CADPR SYNTHESIS AND DEGRADATION

As mentioned above, the effect of NAD to induce Ca^{2+} release in sea urchin eggs was shown to result from its enzymatic conversion to cADPR. Subsequently, a similar enzymatic activity was shown to exist in a wide variety of mammalian tissues[26]. The first purified enzyme shown to produce cADPR from NAD was identified in *Aplysia* and was later named ADP-ribosyl cyclase[27]. Surprisingly, the amino acid sequence of *Aplysia* ADP-ribosyl cyclase, a soluble 30 kDa protein, showed overall about 68% homology with human CD38, a lymphocyte antigen[28,29]. CD38 was indeed able to catalyze the cyclization of NAD to cADPR in pancreatic beta-cells[30]. Moreover, purified murine CD38 was able to convert NAD to cADPR in an *in vitro* assay[28]. Later, CD157, a GPI-anchored antigen that shared 30% homology with CD38, was found to have ADP-ribosyl cyclase activity as well[31].

Overall, these ADP-ribosyl cyclases share about 25%-30% sequence identity[32], and this family is likely to grow since researchers have continued to find ADP-ribosyl cyclase activity that is undefined. In addition, it appears that these unknown cyclases function differently in different tissues. For example, an unidentified cardiac ADPR cyclase can be inhibited by micromolar concentrations of Zn^{2+}, which is different from the effects of this cation on CD38 and CD157[33,34]. A similar ADP-ribosyl cyclase that can be inhibited by the divalent cations Zn^{2+} and Cu^{2+} has also been found in the disks of bovine retinal rod outer segments[35]. Specific inhibitor based analysis confirmed the existence of a distinct ADP-ribosyl cyclase in the kidney since it responded differently to the inhibitor 4,4'-dihydroxy azobenzene (DHAB) treatment than CD38[36].

So far, CD38 is still considered to be the main mammalian ADP-ribosyl cyclase, as shown by the fact that extracts of tissues from CD38 knockout mice have little if any ADP-ribosyl activity compared to those from wild type mice. When incubated with NAD *in vitro*, CD38 only produced a small portion of cADPR, while the major-

ity of the product is ADP-ribose; thus CD38 possesses both cyclase and NADase activities. In addition, CD38 can hydrolyze cADPR to ADP-ribose and, other than CD157, it remains the only ADP-ribosyl cyclase that has been identified in mammals[28]. Moreover, CD38 shows another bifunctional character in that it catalyzes the synthesis and hydrolysis of another secondary messenger, NAADP. In this reaction, CD38 catalyzes the exchange of the nicotinamide group of NADP with nicotinic acid under acidic conditions to generate NAADP; furthermore, NAADP can also be hydrolyzed by CD38 to ADPRP (Figure 2)[37,38]. Understanding the structure and function of CD38 is a crucial part of cADPR/Ca^{2+} signaling research.

STRUCTURE AND ENZYMATIC FUNCTION OF CD38

CD38 is a transmembrane protein, containing a short 21 amino acid residue N-terminal cytoplasmic tail, a 23 amino acid residue hydrophobic transmembrane domain, and a large 256 amino acid residue carboxyl-terminal extracellular domain with four putative glycosylation sites[39]. The extracellular domain of human CD38 with the glycosylation sites removed has been expressed in yeast and purified. Structural analysis of the recombinant CD38 by X-ray crystallography showed that the secondary structure of CD38 is similar to that of the *Aplysia* cyclase. Overall, both CD38 and the cyclase have similar topology although the cyclase forms dimers in the crystals whereas CD38 does not. The middle cleft of both proteins forms a deep pocket as the active site, with a TLEDTL conserved sequence sitting in the bottom of the pocket[40,41]. Site-directed mutagenesis studies identified Glu226 as the catalytic residue of CD38[42]. Two other residues, Glu146 and Thr221, were found to be essential for the cyclization and hydrolysis activity of CD38, respectively[43]. Upon binding of NAD to the active site, the nicotinamide ring interacts with Trp189 by hydrophobic ring stacking, the 2' and 3' hydroxyls of the northern ribose form hydrogen bonds with Glu226, and the ribose diphosphate moiety interacts with amino acids Trp125, Ser126, Arg127, Thr221 and Phe222. Upon cleavage of the nicotinamide ring, the N1 nitrogen of the adenine ring gains access to the anomeric carbon to form a covalent bond and produce cADPR. Alternatively, a water molecule, rather than the adenine ring, attacks the intermediate to form ADP-ribose[44]. In contrast to the formation of cyclic ADP-ribose from NAD, CD38 also catalyzes the formation of NAADP from NADP. Under acidic pH and in the presence of nicotinic acid, the acidic residues in the active site of CD38 are protonated, thereby facilitating the nucleophilic attack of the intermediate of NADP by nicotinic acid to generate NAADP[44].

TOPOLOGY OF CD38

Structurally, CD38 is predicted to be a type-II transmembrane protein with its catalytic C-terminal domain located outside of the cell[39]. This circumstance presents a dilemma because the NAD substrate is located intracellularly whereas the enzyme is positioned extracellularly. If so, cytosolic NAD must be transported out of cells first and then cyclized by CD38 to produce cADPR in the extracellular space. Subsequently, the cADPR product must be transported back into the cytosol to induce Ca^{2+} release from the ER. This scenario obviously presents a "topological paradox" for the cADPR/Ca^{2+} signaling cascade. Two general hypotheses have been proposed to solve this puzzle (Figure 3). The first proposal is based on the presence of transporters, such as connexin 43 hemichannels, which allow intracellular NAD to move to the extracellular space so that it is available for access to the catalytic domain of CD38 to be converted to cADPR[45]. The cADPR product is then transferred back to cells *via* either CD38 or nucleoside transporters[46]. Besides this direct transport model *via* transporters, Zocchi *et al*[47] also suggested that CD38 undergoes an extensive internalization through invaginations of the plasma membrane to form endocytotic vesicles, which makes the active site of CD38 intravesicular and able to convert cytosolic NAD into cADPR. CD38 itself is a unidirectional transmembrane transporter of cADPR that mediates the cADPR efflux into the cytoplasm to reach the Ca^{2+} store, while influx of the cytosolic NAD^+ substrate into the endocytotic CD38-containing vesicles is mediated by other transmembrane transporters, such as connexin 43 hemichannels[48]. The internalization of CD38 has been supported by several studies. For example, the internalization of CD38 can be induced by NADP in Chinese hamster ovary (CHO) cells[49] and hemin treatment can induce internalization of CD38 in K562 cells[50]. Rah *et al*[51] have also demonstrated that association of phospho-nonmuscle myosin heavy chain ⅡA with tyrosine kinase Lck and CD38 is critical for the internalization and activation of CD38. However, mechanisms regarding the transporter mediated CD38 activation process remain elusive. For example, connexin 43 hemichannels are opened for NAD export only when the cellular Ca^{2+} is 100 nmol/L; thus this system is unlikely to operate when Ca^{2+} is elevated above basal levels[45].

The second proposal offered to explain the topological paradox involves a consideration of the orientation of CD38. Bruzzone and coworkers have shown that treatment of granulocytes with 8-Br-cyclic adenosine monophosphate (cAMP), a cell-permeant analog of cAMP, induced serine phosphorylation of CD38, correlating with a cAMP-dependent intracellular cADPR synthesis[52]. Although the exact location of the phosphorylation sites is unknown, it was predicted to be in the catalytic C-terminal domain that contains multiple serine residues. However, if the catalytic domain of CD38 is phosphorylated by protein kinase A (PKA), this domain should be in the cytosol to directly cyclize NAD, thereby synthesizing cADPR intracellularly. This suggests that although CD38 is believed to be a type-Ⅱ protein, at least a portion of the total CD38 is expressed as a type-Ⅲ membrane protein with its C-terminal catalytic domain sitting in the cyto-

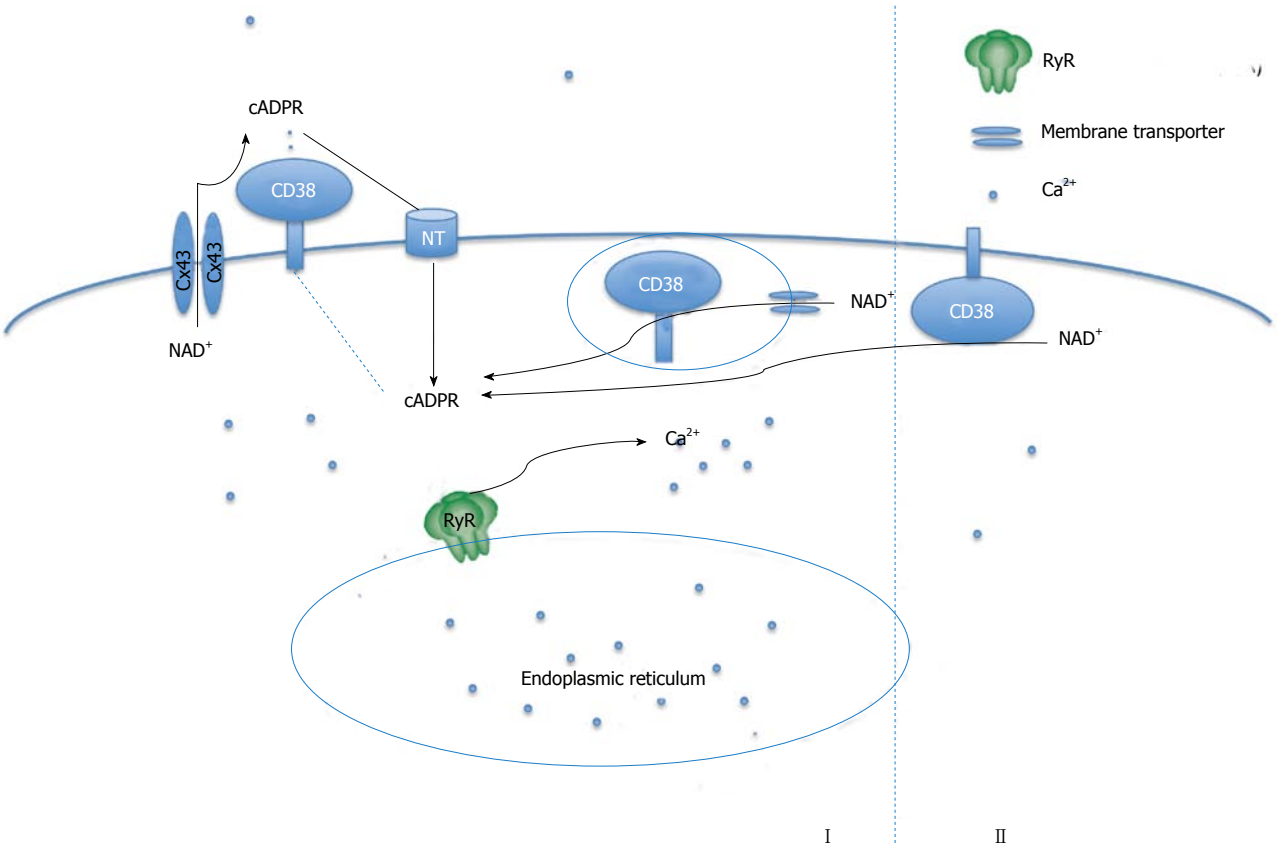

Figure 3 Models of CD38 topology. cADPR: Cyclic adenosine diphosphate ribose; NAD: Nicotinamide adenine dinucleotide; RyR: Ryanodine receptor.

sol[53]. Since the number of positive charges that determine the polarity of membrane protein is equal on each side of the CD38 transmembrane segment, studies from protease digestion[54] and electron microscopy[55] showed that the nuclear CD38 might be a type-Ⅲ membrane protein. Most recently, Zhao *et al*[56] reported that expression of a cytosolic CD38 protein with deletion of both the N-terminal tail and transmembrane domain results in intact disulfides as well as active enzyme in spite of the cytosolic reductive environment; this result appears to solve the fundamental need of the six disulfides for CD38 enzymatic activity. Based on this finding, they consequently proved the co-expression of type Ⅱ and type Ⅲ CD38 on the surface of leukemia HL-60 cells during retinoic acid-induced differentiation and on interferon Y-activated natural human monocytes and U937 cells[57]. They proposed that the type-Ⅲ structure may take part in fast cellular responses, while the type-Ⅱ structure may be more suitable for slower and long term responses (Figure 3)[58].

PHYSIOLOGICAL FUNCTIONS OF THE CD38/CADPR/CA^{2+} PATHWAY

In addition to its role in cADPR production, another function of CD38 is to regulate the NAD level inside cells. It has been well established that NAD plays an essential role in energy metabolism and is involved in diverse signal transduction pathways. A rather surprising finding is that CD38 has a dramatic role in intracellular NAD metabolism. NAD levels in CD38 knockout mice are 10 to 20-fold higher than that in wild-type animals. These results suggest that CD38 is a major regulator of NAD levels in mammalian cells[59].

CD38 was originally identified as a lymphocyte antigen; thus it is not surprising that the CD38/cADPR/Ca^{2+} pathway plays an important role in inflammatory processes. In an ischemic stroke study, $CD38^{-/-}$ mice produced less monocyte chemoattractant protein-1 (MCP-1) after temporary middle cerebral artery occlusion and had fewer infiltrating macrophages and lymphocytes in the ischemic hemisphere than the wild type mice, whereas the amount of resident microglia was unaltered. The same study also demonstrated that CD38 affected immune cell migration as well as activation, two crucial postischemic inflammatory responses in secondary brain damage, suggesting that CD38 might be a therapeutic target to modulate the inflammatory mechanisms after cerebral ischemia[60]. Recently, Ng *et al*[61] used intravital multi-photon microscopy to observe the neutrophil granulocyte traffic into the injury site in the dermis of mice and found that the amplification phase, which is the attraction of more neutrophils toward the damage focus after the initial phase of migration by scouting neutrophils, was mediated by cADPR. cADPR and CD38 were also involved in the regulation of leukocyte adhesion and chemotaxis and were required for the deletion of T regulatory cells during inflammation as well[62]. In addition, 8-Br-cADPR, a

cADPR antagonist, inhibited the MCP-1 induced Ca^{2+} increase, reactive oxygen species (ROS) production and apoptosis in human retinal pigment epithelium, suggesting that cADPR is also involved in the inflammatory responses of age-related macular degeneration (AMD)[63].

Recently, we demonstrated that cADPR is important for regulating cell proliferation and neuronal differentiation in PC12 cells. We found that acetylcholine (Ach) activates the CD38/cADPR pathway to induce Ca^{2+} release and the CD38/cADPR/Ca^{2+} signaling pathway is required for Ach-stimulated cell proliferation in PC12 cells. Interestingly, inhibition of the cADPR pathway accelerated nerve growth factor (NGF)-induced neuronal differentiation in PC12 cells. On the other hand, CD38 overexpression increased cell proliferation but delayed NGF-induced differentiation. Taken together, we demonstrated that cADPR plays a dichotomic role in regulating proliferation and neuronal differentiation of PC12 cells[64].

Abscisic acid (ABA) is an endogenous stimulator of insulin secretion in human and murine pancreatic beta cells. ABA triggered activation of CD38 and production of cADPR before insulin release, suggesting that CD38 is a regulator of insulin release[65]. Also, CD38 expression and cADPR production induced by ABA were required for ABA-induced upregulation of COX-2 and prostaglandin E2 in human mesenchymal stem cells (MSC) and for chemokinesis of MSC[66].

Since cADPR can activate RyRs for Ca^{2+} release from ER and can modulate the CICR process, the CD38/cADPR/Ca^{2+} pathway is predicted to participate in the regulation of cardiac activities, including cardiogenesis and the function of adult cardiac tissue. In fact, ever since the discovery of cADPR, researchers have vigorously explored its role in cardiac tissues. Galione *et al*[67] showed that application of cADPR through a patch electrode resulted in an increase in Ca^{2+} transients with a concomitant increase of the magnitude of contraction in guinea-pig cardiac ventricular myocytes, whereas application of the inhibitor 8-amino-cADPR resulted in a significant reduction in contractions and Ca^{2+} release from the SR. Similarly, in rat cardiac ventricular myocytes, cADPR increased the frequency of Ca^{2+} "sparks", which may contribute to the increase in subsequent whole-cell Ca^{2+} transients[68]. In addition, Prakash *et al*[69] found that microinjection of cADPR into adult rat ventricular myocytes not only induced sustained Ca^{2+} responses in a concentration dependent manner but also increased the frequency and amplitude of spontaneous Ca^{2+} waves, which were completely blocked by 8-amino-cADPR, a cADPR antagonist.

Interestingly, cardiac hypertrophy developed only in CD38 knockout male mice. The expression of RyR protein was increased only in female CD38 knockout mice compared with wild type, suggesting that the CD38/cADPR signaling plays an important role in intracellular Ca^{2+} homeostasis in cardiac myocytes *in vivo*, although its deficiency was compensated differentially according to gender[70].

cADPR was also shown to be involved in angiotensin Ⅱ-induced cardiac hypertrophy[71]. In rat cardiomyocytes, angiotensin Ⅱ evoked a Ca^{2+} increase *via* IP_3R to activate PKC, which then activated the NAD(P)H oxidase to initiate ROS generation. The ROS together with Ca^{2+} then activated the ADP-ribose cyclase to synthesize cADPR, which induced a sustained increase of both Ca^{2+} and ROS and finally led to cardiac hypertrophy[72]. Most recently, Xu *et al*[73] demonstrated that CD38/cADPR was involved in the regulation of superoxide ($O_2^{\bullet-}$) production in mouse coronary arterial myocytes (CAMs). NAD(P)H oxidase is responsible for $O_2^{\bullet-}$ production. Since CD38 can use NAD, an NAD(P)H oxidase product, to produce cADPR and cADPR production can result in an increase in NAD(P)H oxidase activity, the system contains a positive feedback loop. Xu *et al*[73] found that oxotremorine, a muscarinic type 1 receptor agonist, stimulated intracellular $O_2^{\bullet-}$ production in CAMs that was inhibited in CD38 knockout, CD38 knockdown, or nicotinamide-treated (a CD38 inhibitor) cells. On the other hand, direct application of cADPR into CAMs increased intracellular Ca^{2+} and $O_2^{\bullet-}$ production in $CD38^{-/-}$ CAMs. Moreover, CD38 knockout, Nox1 knockdown or Nox4 knockdown blocked oxotremorine-induced contraction in the isolated perfused coronary arteries in mice. Taken together, these data indicate that the CD38/cADPR pathway is an important regulator of Nox-mediated intracellular $O_2^{\bullet-}$ production.

The CD38/cADPR/Ca^{2+} pathway has also been shown to regulate the cardiogenesis process. We recently studied the role of CD38/cADPR/Ca^{2+} in the cardiomyogenesis of mouse embryonic stem (ES) cells. We found that beating cells appeared earlier and were more abundant in CD38 knockdown embryoid bodies (EBs) than control EBs, and the expression of several cardiac markers was increased significantly in CD38 knockdown EBs than control EBs. Similarly, more cardiomyocytes (CMs) existed in CD38 knockdown or cADPR antagonist-treated EBs compared to control EBs. Conversely, CD38 overexpression in mouse ES cells markedly inhibited CM differentiation. Surprisingly, CD38 knockdown ES cell derived CMs possess the functional properties characteristic of normal ES cell derived CMs. In addition, we found that the CD38/cADPR pathway inhibited the Erk1/2 cascade during CM differentiation of ES cells, and transient inhibition of Erk1/2 blocked the enhancive effects of CD38 knockdown on the differentiation of CM from ES cells. Taken together, we demonstrated that the CD38/cADPR/Ca^{2+} signaling pathway inhibits the CM differentiation of mouse ES cells[74].

The mechanism underlying cADPR regulation of Ca^{2+} sparks in cardiomyocyte remains elusive. Zhang *et al*[19]. showed that cADPR markedly increased the Ca^{2+} spark frequency in cardiomyocytes isolated from wild type mice, whereas cADPR failed to initiate Ca^{2+} sparks in cardiomyocytes isolated from FK506 binding protein 12.6 (FKBP12.6) knockout mice. They further demonstrated that cADPR induced FKBP12.6 dissociation from RyRs in a phosphorylation-dependent manner. Yet, another study showed that cAMP signaling is required for the

role of cADPR in the beta-adrenergic receptor induced Ca^{2+}increase in rat cardiomyocytes. They found that the isoproterenol-mediated increase of Ca^{2+} was blocked by pretreatment with 8-Br-cADPR, PKA inhibitor H89 or a high concentration of ryanodine. Moreover, incubation of ventricular lysates with isoproterenol, forskolin or cAMP resulted in activation of ADP-ribosyl cyclase of the ventricular lysates[34]. Interestingly, for comparison, estrogen increased CD38 expression and its cyclase activity, but did not affect its hydrolase activity, while progesterone eliminated the effects of estrogen on CD38 in the rat myometrium[75]. Nevertheless, the mechanism of how the CD38/cADPR is involved in the regulation of cardiac function is still unclear.

CD38/CADPR/CA^{2+} PATHWAY IN PATHOLOGICAL PROCESSES

The CD38/cADPR/Ca^{2+} pathway has been suggested to be involved in various pathological processes. For example, CD38 deficiency accelerated diabetes in a non-obese diabetic (NOD) mice model[76]. It has also been shown that both the specific kidney ADP-ribosyl cyclase activity and cADPR production were increased in the kidneys of diabetic mice, suggesting that cADPR plays a role in the renal pathogenesis of diabetes[77]. Down-regulation of CD38 has also been shown to mediate the intermittent hypoxia induced impairment of glucose-induced insulin secretion, suggesting that CD38 plays a role in type 2 diabetes progression[78]. Numerous studies have been attempted to dissect the molecular mechanism of the role of CD38/cADPR/Ca^{2+} pathway in mediating diabetes in order to identify an alternative therapeutic tool. Tian *et al*[79] found that the content of cADPR was elevated with concomitant enhanced activity of RyR2 in ventricular myocytes isolated from a type 1 diabetic rat model, suggesting that cADPR mediates type 1 diabetes through regulating the function of RyR2. Chen *et al*[80] demonstrated that the ATP-gated ion channel P2X7 was required for the acceleration of type 1 diabetes induced by CD38 deficiency. Taken together, knowledge about the role of the CD38/cADPR/Ca^{2+} pathway in diabetes is accumulating rapidly and there is hope that understanding this pathway will facilitate the development of novel therapeutics for the disease.

The CD38/cADPR/Ca^{2+} pathway has been associated with inflammatory airway disorders. In human airway smooth muscle (ASM) cells, increased ASM contractility in inflammatory diseases such as asthma was due to enhanced Ca^{2+} sensitivity to cytokines, which was correlated with the increase of CD38 expression and cADPR level[81]. This increase of CD38 was induced by TNFα *via* NFκB and could be inhibited by glucocorticoids[82]. In addition, the CD38/cADPR/Ca^{2+} pathway also mediated the 2-arachidonoylglycerol induced rapid actin rearrangement during differentiation of HL-60 cells into macrophage-like cells[83], and extracellular NAD^{+} induced stimulation and recruitment of human granulocytes during the inflammation process[84]. In addition, CD38 was involved in a neuroinflammatory disorder where CD38 expression level was markedly increased in IL-1beta- or HIV-1-activated human astrocytes, whereas CD38 knockdown significantly reduced proinflammatory cytokine and chemokine production in astrocytes[85]. Considering these results, the CD38/cADPR/Ca^{2+} pathway plays important roles in multiple inflammatory processes.

CONCLUSION

The CD38/cADPR/Ca^{2+} pathway modulates various processes of cells, including inflammation, insulin secretion, cardiogenesis, cardiac regulation *etc*. With further investigation, it is likely that other physiological roles of the CD38/cADPR/Ca^{2+} pathway will be revealed. For example, Yue *et al*[64] have shown that the CD38/cADPR/Ca^{2+} pathway delayed the nerve growth factor induced differentiation of PC12 cells; thus it is reasonable to predict that this pathway might also be involved in the regulation of neurogenesis. Using the mouse embryonic stem cell *in vitro* differentiation model, our preliminary results showed that the CD38/cADPR/Ca^{2+} pathway does play a role in neural differentiation of mES (unpublished data); however, further research is needed to decipher the underlying mechanism. A comprehensive understanding of the physiological and pathological roles of the CD38/cADPR/Ca^{2+} pathway in various cellular processes will undoubtedly be helpful for exploiting new molecular therapy targets. In addition, it still remains to be determined whether cADPR binds directly to RyRs or through some unknown proteins. Recently, the long-sought-after store-operated Ca^{2+} entry proteins were identified using a genome-wide RNAi screen by several groups[86-88]. A similar strategy could be applied to identify novel cADPR-interacting proteins or regulators.

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MINIREVIEWS

Thioredoxin and glutaredoxin-mediated redox regulation of ribonucleotide reductase

Rajib Sengupta, Arne Holmgren

Rajib Sengupta, Arne Holmgren, Division of Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, SE-17177 Stockholm, Sweden
Author contributions: Sengupta R and Holmgren A contributed equally to this work, generated the figures and wrote the manuscript.
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Correspondence to: Rajib Sengupta, PhD, Division of Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Scheeles Vag 2, SE-17177 Stockholm, Sweden. rajib.sengupta@ki.se
Telephone: +46-8-52487727 Fax: +46-8-7284716
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Abstract

Ribonucleotide reductase (RNR), the rate-limiting enzyme in DNA synthesis, catalyzes reduction of the different ribonucleotides to their corresponding deoxyribonucleotides. The crucial role of RNR in DNA synthesis has made it an important target for the development of antiviral and anticancer drugs. Taking account of the recent developments in this field of research, this review focuses on the role of thioredoxin and glutaredoxin systems in the redox reactions of the RNR catalysis.

Key words: Ribonucleotide reductase; Thioredoxin; Glutaredoxin; DNA synthesis; Thiol disulfides; Replication

Core tip: Thioredoxin and glutaredoxin-mediated redox regulations of ribonucleotide reductase (RNR) catalysis play a vital role as the RNR catalysis involves different redox active thiol functions, thiyl radicals and thiol proteins. The in depth knowledge of the whole redox catalysis will contribute significantly to designing and developing new RNR inhibitors for improved cancer chemotherapy, antibiotic development and antiviral treatments.

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INTRODUCTION

Ribonucleotide reductase (RNR) catalyzes the rate limiting step of the DNA synthesis where the reduction of ribonucleotides (NTPs) results in the formation of corresponding deoxyribonucleotides (dNTPs)[1-4]. The RNR catalysis involves protein free radicals, redox-active thiols and proteins of the thioredoxin (Trx) superfamily. In the RNR complex, the R1 subunit contains the active site, allosteric sites and redox active thiols/disulfides required for the RNR catalysis; while the R2 subunit provides a dinuclear metal cluster and a tyrosyl free radical essential for the catalytic cycle. The RNR activity can be regulated by expression of different subunits, subcellular localization, post-translational modifications and allosteric regulation involving both activity and substrate specificity[3-8]. The DNA replication is coordinated with the cell growth by different regulatory mechanisms. Development of malignancy and cancer are found to be associated with an increased expression and activity of RNR. In cells, an imbalance in the levels of dNTPs will cause mutagenesis and carcinogenesis. On the other hand, blockage of RNR activity can inhibit DNA synthesis and repair which results in apoptosis. In recent years, several RNR inhibitors have entered clinical trials. Recent developments in the field will provide a new basis for the discovery of more

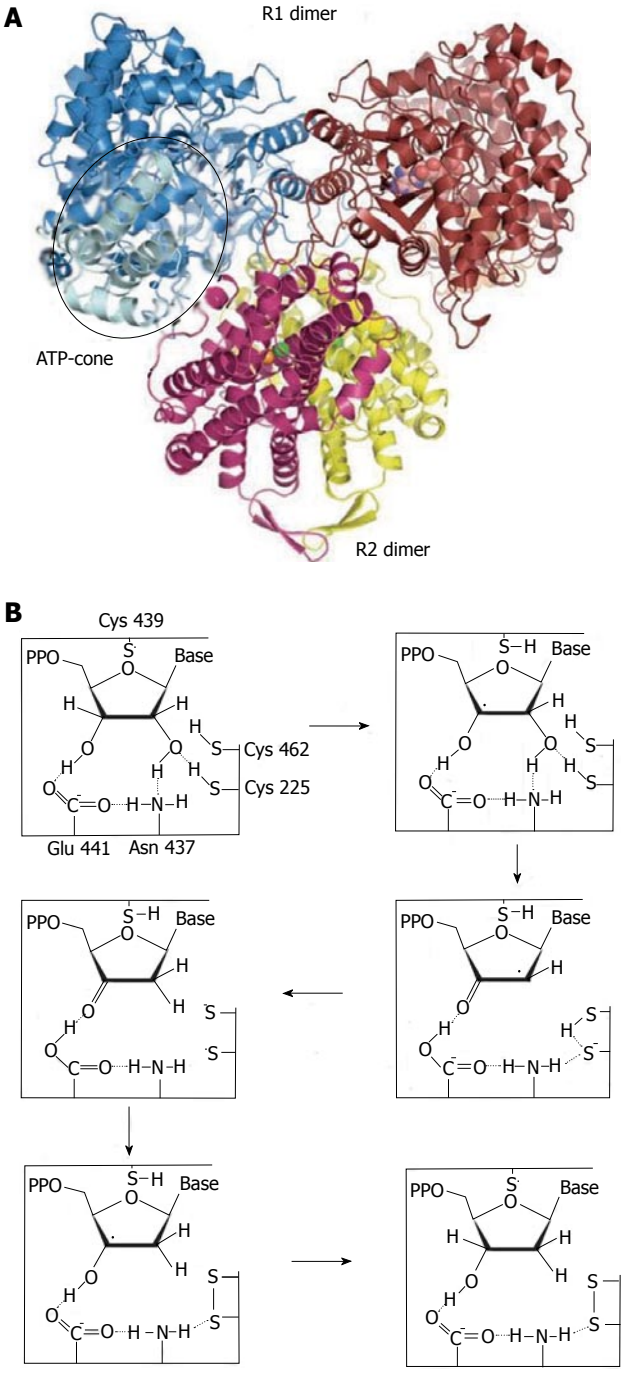

Figure 1 Crystal structure of Class I aerobic ribonucleotide reductase complex (A), and proposed reaction mechanism of ribonucleotide reductase catalysis (B). A: This is based on the crystal structures of the R1 and R2 proteins (Protein Data Bank ID: 1RLR and 1RIB). The figure shows the presence of substrate in R1 subunit and dinuclear iron center in R2 subunit. The ribonucleotide reductase complex (RNR) complex is a tetramer with the dimer of R1 subunit and the dimer of R2 subunit. The allosteric regulatory domain of R1 subunit (ATP-cone) binds either ATP or dATP to regulate the enzymatic activity (adapted from Logan *et al*[9]); B: The figure describes the reduction of nucleoside diphosphate (NDP) to deoxyribonucleoside diphosphates (dNDP) by class I RNR (*E. coli*). The reduction is initiated by a thiyl radical (Cys 439) by abstracting the 3'-hydrogen from the NDP. A water molecule is lost and the two cysteines (Cys 225 and Cys 462) then deliver the required reducing equivalents, generating a 3'-ketodeoxynucleotide which is subsequently reduced to give dNDP (adapted from Holmgren *et al*[4]).

effective RNR inhibitors for cancer therapy. Taking account of the recent progress in this field of research, this review focuses on the role of Trx and glutaredoxin (Grx) systems in the redox regulations of the RNR catalysis.

RNR: CLASSIFICATION AND CATALYSIS

Based on the pathways of radical initiation and requirements of metal cofactors, the RNRs have been divided in to three classes[2-5]. The active site of all three classes of RNR has a very similar structure (Figure 1)[2-5,9]. A conserved cysteine residue plays the vital role for the generation of a thiyl radical in all the classes of RNR (Figures 1 and 2). The reaction mechanisms of different classes of RNR are similar due to the structural similarities of the catalytic domains. The RNR catalysis starts with the generation of a thiyl radical close to the bound substrate[2-5]. Then the abstraction of hydrogen from the C3' of the ribose ring and generation of a substrate radical occur. During the RNR catalysis, a cysteinyl radical, required for the abstraction of hydrogen at the C3' ribose substrate, is derived from a tyrosyl radical for class I or cobalamin cofactor for class II or a glycyl radical for class III. For class I and II enzymes, electrons required for the reduction of the ribonucleotides aare provided by reduced nicotinamide adenine dinucleotide phosphate (NADPH) through Trx or Grx systems. However, for class III enzymes, the electrons are supplied by formate.

The class I RNR occurs in eukaryotes, eubacteria, bacteriophages and viruses. The complex, in its simple form, is a tetramer with the dimer of larger subunit (R1) and the dimer of R2 subunit (Figures 1 and 2)[2-5,9]. Oxygen is required for the generation of a tyrosyl radical (Tyr 122) in the R2 subunit. As described above, during catalysis, the radical is continuously transferred to a cysteine (Cys 439) residue of the R1 subunit and generates a thiyl radical to activate the substrate. The R1 subunit carries the catalytic site, allosteric effector binding sites and redox-active thiol groups required for the catalysis. p53R2 is an additional mammalian RNR protein which functions as a catalytic partner of the R1 subunit[10-12]. The expression of the p53R2 subunit is induced by DNA damage which is mediated by the tumor suppressor protein p53. Both R2 and p53R2 subunits use a diferric iron center generating a tyrosyl free radical required for the RNR catalysis. The R1-p53R2 complex is suggested to be required for basal DNA repair and the R1–R2 complex is suggested to be associated with DNA replication[11]. Moreover, the R1-p53R2 complex has been found to play a significant role in dNTP supply for mitochondrial DNA synthesis.

For class II RNR (archaebacteria, eubacteria), a cofactor (5'-deoxyadenosylcobalamin) replaces the presence of a separate subunit for storage of radicals[2-5]. The cleavage of 5'-deoxyadenosylcobalamin generates a deoxyadenosyl radical which abstracts hydrogen from the active site cysteine residue. Trx system can reduce the C-terminal pair of redox-active cysteines which, in turn, can reduce the active site to continue the RNR catalysis. For anaerobic class III RNR (archaebacteria, eubacteria, bacteriophages), a glycyl radical is generated by the action of activase, S-adenosylmethionine and a reducing

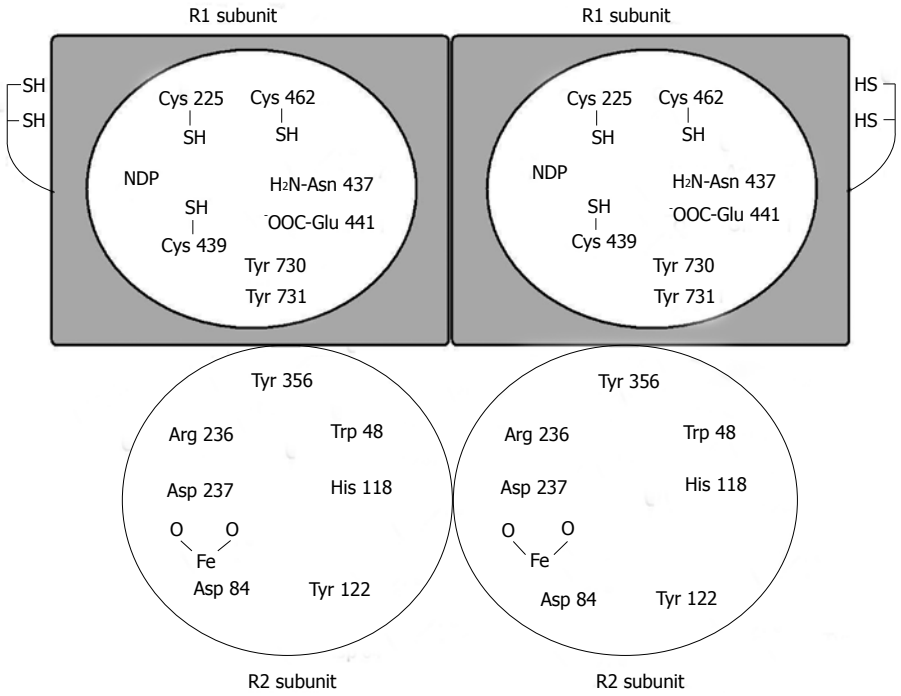

Figure 2 Subunit organization of ribonucleotide reductase complex. Amino acids are shown with *E. coli* numbering which are crucial for the radical transfer and ribonucleotide reductase (RNR) catalysis. The R2 subunit contains the iron-oxygen cluster (Fe-O-Fe) which reacts with dioxygen to generate a stable tyrosyl radical in Tyr 122 required for the RNR catalysis. The radical transfer pathway from Tyr 122 to the active-site Cys 439 in R1 subunit involves the network of Asp 84, His 118, Asp 237, Trp 48, Tyr 356 in R2 subunit and Tyr 730, Tyr 731 in R1 subunit[2-5]. The Cys 225, Cys 462, Asn 437 and Glu 441 residues are involved in binding the substrate nucleoside diphosphate (NDP) in R1 subunit. During the catalysis, the disulfide bond between Cys 225 and Cys 462 is reduced by the C-terminal shuttle dithiols[2-5]. The figure is adapted and modified from Holmgren *et al*[4].

system containing flavoredoxin, NADPH and NADPH-flavoredoxin reductase. Then the glycyl radical generates a thiyl radical required for the catalysis.

TRX AND GRX SYSTEM

Trx is a class of 12 kDa ubiquitous redox proteins found primarily in the cytosol[13]. Trxs possess a catalytically active dithiol function in a Cys-Gly-Pro-Cys motif and are present in all organisms. The complete mammalian Trx system comprising Trx, the selenoenzyme thioredoxin reductase (TrxR) and NADPH also plays a crucial role in redox signaling and thiol homeostasis of cells. Cytosolic Trx1 and mitochondrial Trx2 regulate several metabolic pathways, oxidative/nitrosative stress defence, apoptosis and DNA synthesis[4,13-15]. On the other hand, Grxs are small redox enzymes of approximately 10 kDa and they participate in thiol-disulfide exchange reactions in the presence of glutathione (GSH), glutathione reductase (GR) and NADPH. In the Grx system, Grx is reduced *via* GSH[16]. The glutathione disulfide formed is then reduced by GR and NADPH. Grxs are involved in redox signaling and maintenance of cellular redox environment. Moreover, the maintenance of cytosolic and mitochondrial iron homeostasis have been found to be linked to Grxs[16,17].

Several findings support the redundancy between cytosolic Trx and Grx systems to provide a backup for each other. Yeast and bacterial strains can survive in the absence of either of the disulphide reductase pathways[18]. Moreover, GSH synthesis was found to be essential for mouse development[19], whereas the deletion of Trx gene was reported to be lethal for mouse embryo[20]. Loss of TrxR1 showed no effect on the normal replicative potential[21]. However, the survival of TrxR1-deficient tumor cells was found to be very much dependent on GSH[22]. In a recent study, it has also been shown that the GSH/Grx system can reduce Trx1 in TrxR1-deficient HeLa cells[23].

ROLE OF TRX AND GRX AS EXTERNAL ELECTRON DONORS FOR RNR

Trxs and Grxs belong to related families of low molecular weight redox enzymes catalyzing thiol-disulfide exchange reactions with catalytically active cysteine thiols in a CXXC active site[13,16]. For class I and II RNR enzymes, the electrons are supplied by NADPH through Trx or Grx systems[2-5]. Reduction of ribonucleotide in the RNR catalysis involves the formation of a disulfide in the active site of R1 subunit. Structural studies with *E. coli* RNR show that the active site cleft of the R1 subunit is not very wide to permit the direct reduction by the external redoxin system(s)[3,24]. However, the reduction of active site disulfide is performed by a pair of shuttle cysteine residues in the C-terminal mobile tail of R1 subunit (Figure 3)[4,25,26]. The C-terminal shuttle dithiols of *E. coli* R1 subunit has the CXXXXC sequence; whereas yeast and mammalian R1 has a CXXC sequence. *In vitro*

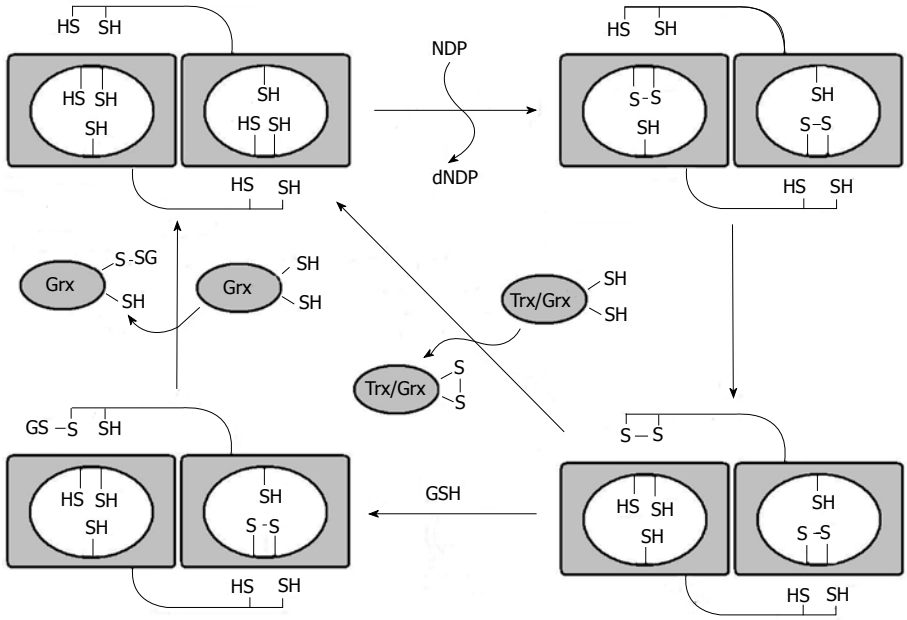

Figure 3 The mechanistic model for the role of thioredoxins and glutaredoxins for the ribonucleotide reductase catalysis. After the completion of one turnover cycle of ribonucleotide reductase (RNR) catalysis, a disulfide bond is formed between the conserved cysteine pair at the active site (shown in the circle). Shuttle dithiol function present at the C-terminal CXXC motif of the neighboring subunit reduces the disulfide bond through disulfide-exchange. Then, the resulting disulfide bond at the C-terminal tail is reduced by the thioredoxin/glutaredoxin (Trx/Grx) systems resulting in an active R1 to continue the next cycle of RNR catalysis. The Grx system can also reduce the C-terminal thiols by the glutathionylation mechanism[4,25,26]. For simplicity, only the reduction of active site of one subunit by the C-terminal shuttle dithiols of the neighboring subunit is shown in the diagram. The figure is adapted and modified from Holmgren *et al*[4].

mutagenesis and kinetic studies support a critical role for the C-terminal cysteine pair of R1 in regeneration of the active site[27,28]. The disulfide exchange reaction results in the formation of a disulfide in the C-terminal tail of R1. Then, the external redoxin systems reduce the disulfide bond to continue the next cycle of RNR catalysis.

Trx and Grx systems were found to act as dithiol electron donors of *E. coli* RNR[29,30]. Recently, Gustafsson *et al*[31] characterized the Trx1 system as the physiologically relevant electron donor for RNR in *Bacillus anthracis*. In *E. coli*, the class I a enzyme requires the dithiol form of at least one of Grx1, Trx1 or Trx2 to be viable[32-34]. The Grx1 system showed 10-fold lower k_m value compared to that of Trx1 system, while both of the redoxins had similar v_{max} (Table 1). This makes Grx1 the most efficient electron donor for the *E. coli* enzyme. However, there is a mechanistic difference between the *E. coli* and the mammalian RNR catalysis involving Trx1 and Grx1 systems as electron donors[26]. Trx1 and Grx1 system showed similar catalytic efficiencies (k_{cat}/k_m) with recombinant mouse RNR complex (Figure 4 and Table 1). In the presence of 4 mmol/L GSH, the Grx1 system showed a higher affinity compared to Trx1 and displayed a higher apparent k_{cat}. The RNR activity with the Grx system was found to be very much dependent on the concentrations of GSH. Here, it is noteworthy to mention that mammalian cells have significantly high concentrations of GSH (5-20 mmol/L)[35]. Moreover, the ability of the monothiol mutant of Grx2 to maintain RNR catalysis demonstrates a glutathionylation mechanism for Grx catalysis in contrast to the dithiol mechanism for the Trx system[26]. However, the *E. coli* RNR complex showed no activity with the monothiol mutant of bacterial Grx1 suggesting the involvement of a dithiol-disulfide mechanism for the catalysis[36].

The advantage of a glutathionylation mechanism may be with the very low levels of R1 involved in the repair and production of dNTPs for mitochondrial DNA synthesis. Trx is present at low levels in many resting postmitotic cells. The sigmoidal curve of Trx activity showed that reduced Trx could not be efficient with a low concentration of R1 in postmitotic cells[26]. The high concentration of GSH[35] would ensure that there is glutathionylated R1 and then monothiol/dithiol Grx should be able to catalyze reduction of the C-terminal disulfide. Several studies reported that the rapidly proliferating cells have increased GSH concentration, while a decrease in GSH concentration limits cell proliferation. GSH acts as a key regulator of cell proliferation and thus the colocalization of GSH with nuclear DNA was observed in proliferating cells[37]. In mammary carcinoma cells, the depletion of glutathione was found to inhibit DNA synthesis[38]. A similar study with 3T3 fibroblast cells showed a significant correlation between progression of cell cycle and the distribution of nuclear GSH[39]. Moreover, accumulation of DNA damage was found in liver, kidney and lung of mice deficient in γ-glutamyl transpeptidase, the enzyme responsible for initiating the catabolism of GSH[40]. In another study, down-regulation of TrxR showed no effects on the dNTP pools in malignant mouse cells[41]. This suggests the role of the GSH/Grx system as an alternative pathway used by the RNR in tumor cells. Moreover, a study in mouse hepatocytes, suggested the importance of a TrxR-independent pathway for the supply of electrons

Table 1 Kinetic parameters of thioredoxin 1, glutaredoxins (1, 2, 2C40S) for reduction of mouse ribonucleotide reductase complex

Electron donor system	V_{max} (nmol/L per second per/ microgram of R1)	k_m (μmol/L)
Trx1	22.2	1.90
Grx1	2.3	0.18
Grx2	1.5	0.30
Grx2C40S	1.5	0.36

Data adapted from Zahedi Avval *et al*[26]. Trx: Thioredoxin; Grx: Glutaredoxins.

to RNR[21]. These studies clearly show the crucial role of the GSH/Grx system in DNA repair *via* RNR catalysis.

ROLE OF TRX FOR CLASS Ⅲ RNR

The class Ⅲ RNR (present in strict and facultative anaerobes) forms an inactive $\alpha_2\beta_2$ complex in resting state[2-5]. The cysteine residues present in the C-terminus of protein α were found to be responsible for the formation of glycyl radical and to participate in radical transfer reactions during enzyme activation. Under the reducing condition, the small β subunit can activate several α proteins. The Trx system was found to activate the enzyme with the same efficiency as dithiothreitol (DTT)[42]. The data suggests that the Trx system keeps the conserved cysteines of the C-terminus of the α-polypeptide in a reduced form which is required for radical generation. Therefore, Trx acts only for the activation of the class Ⅲ RNR. Later, a structural study of the homologous enzyme from bacteriophage T4 revealed the presence of zinc bound to four conserved cysteine residues[43]. It was also shown that the Trx system or DTT is dispensable for the formation of the glycyl radical with the fully Zn-loaded RNR. The radical transfer from glycine to the active-site cysteine to the substrate is controlled by a crucial hydrogen-bond network. Thus, the suggested role of the Trx system (or DTT) was to facilitate the recognition of the network and allow efficient radical transfer.

CONCLUSION

For several years, the RNR inhibitors have been used to treat cancers and viral infections[6,7,44,45]. Most of the RNR inhibitors are either radical scavengers (hydroxyurea) or metal chelators (triapine) which specifically inactivate the R2 subunit[46,47]. On the other hand, several nucleoside analogs and sulfhydryl group inhibitors (such as cisplatin, caracemide, chlorambucil, *etc*.) are used as R1-specific inhibitors[48-50]. Gene expression silencers and R1-R2 polymerization inhibitors (oligopeptides) have also been used to block RNR activity[51-55]. In recent years, many new strategies have emerged in the designing of subunit-specific and more effective RNR inhibitors. Redox regulation of RNR catalysis plays a vital role as the RNR catalysis involves different redox active thiol functions, thiyl radicals and thiol proteins of the Trx superfamily.

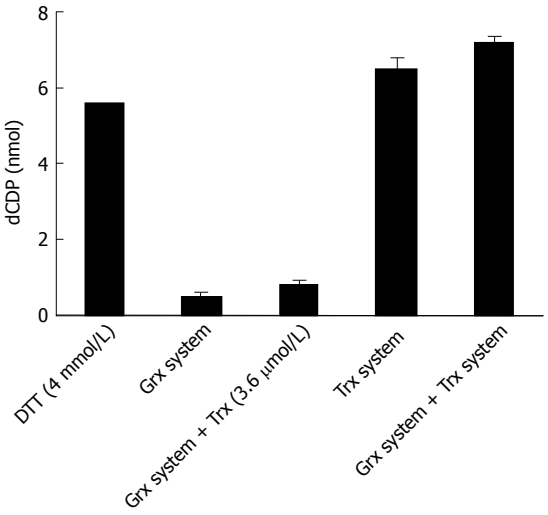

Figure 4 Activity profile of mouse ribonucleotide reductase in the presence of the thioredoxin and glutaredoxins system. Mouse R1 (120 μg/mL) and R2 (40 μg/mL) were assayed with dithiothreitol, thioredoxin (Trx) and glutaredoxin (Grx) systems. The Trx system contained 3.6 μmol/L Trx1, NADPH and TrxR. The Grx system contained 1 μmol/L Grx1, 4 mmol/L glutathione , NADPH and glutathione reductase. Combinations of 3.6 μmol/L Trx1 or the whole Trx system with the Grx system were also monitored (data adapted from Zahedi Avval *et al*[26]).

Therefore, further investigations on the Trx/Grx-mediated redox regulation of RNR catalysis will contribute significantly to design and develop new RNR inhibitors for improved cancer chemotherapy and antiviral treatments.

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Editorial office

World Journal of Biological Chemistry
Room 903, Building D, Ocean International Center,

No. 62 Dongsihuan Zhonglu, Chaoyang District,
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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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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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