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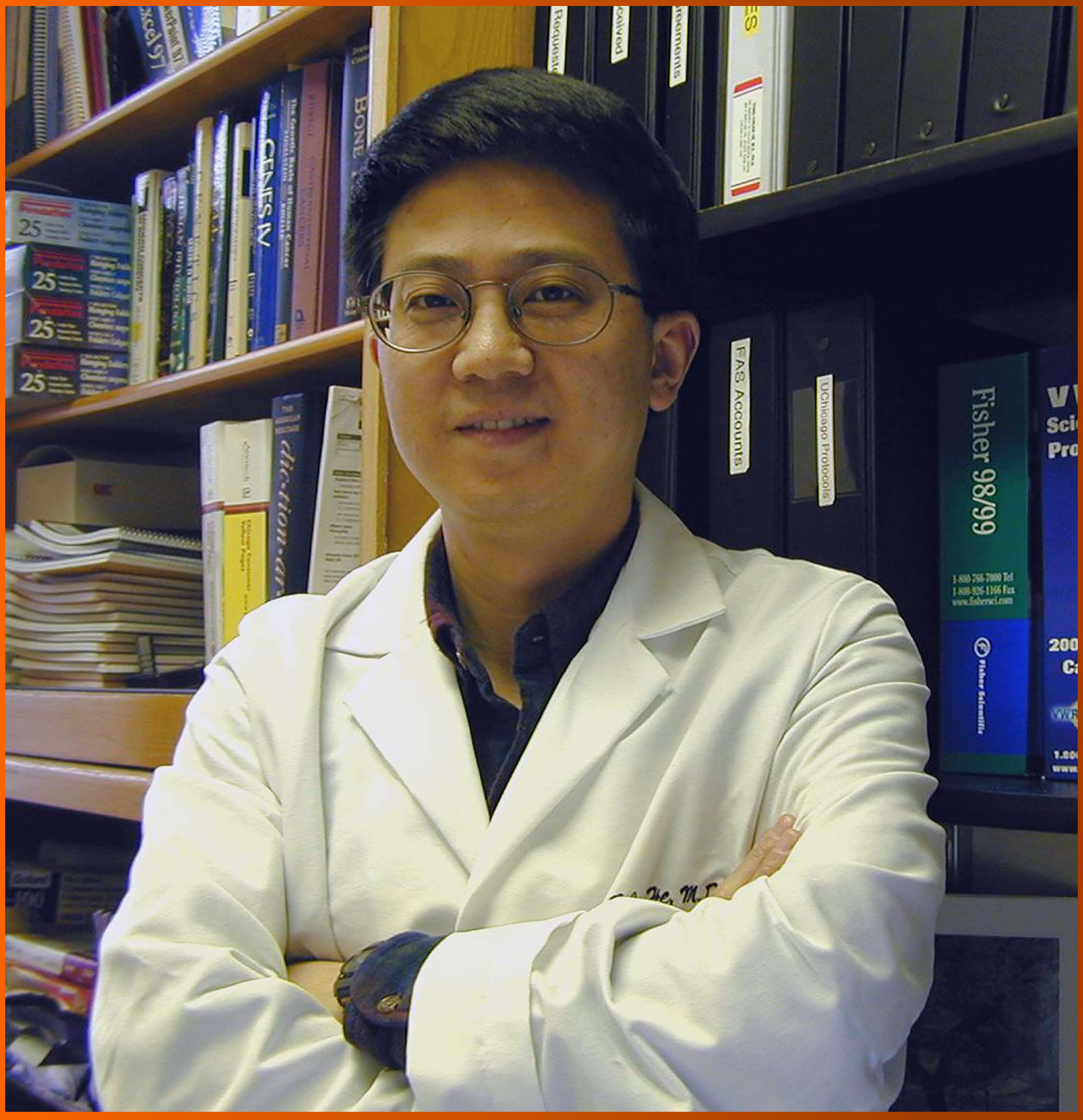

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FRONTIER

Immunomodulation by mesenchymal stem cells: Interplay between mesenchymal stem cells and regulatory lymphocytes

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Abstract

Mesenchymal stem cells (MSCs) possess immunomodulatory properties, which confer enormous potential for clinical application. Considerable evidence revealed their efficacy on various animal models of autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus and uveitis. MSCs elicit their immunomodulatory effects by inhibiting lymphocyte activation and proliferation, forbidding the secretion of proinflammatory cytokines, limiting the function of antigen presenting cells, and inducing regulatory T (T_{reg}) and B (B_{reg}) cells. The induction of T_{reg} and B_{reg} cells is of particular interest since T_{reg} and B_{reg} cells have significant roles in maintaining immune tolerance. Several mechanisms have been proposed regarding to the MSCs-mediated induction of T_{reg} and B_{reg} cells. Accordingly, MSCs induce regulatory lymphocytes through secretion of multiple pleiotropic cytokines, cell-to-cell contact with target cells and modulation of antigen-presenting cells. Here, we summarized how MSCs induce T_{reg} and B_{reg} cells to provoke immunosuppression.

Key words: Mesenchymal stem cells; Regulatory T cells; Regulatory B cells; Immunomodulation; Autoimmunity

Core tip: In this review, we summarized the mechanisms involved in regulatory T (T_{reg}) and B (B_{reg}) cell induction by mesenchymal stem cells (MSCs). In an inflammatory environment, MSCs secrete various anti-inflammatory cytokines, actively interact with immune cells and modulate them to acquire regulatory properties, thus, generate a tolerogenic environment. Particularly, by

inducing T_{reg} and B_{reg} cells, the immunomodulation of MSCs is amplified. Therefore, genetic engineered MSCs to enhance their ability to induce T_{reg} and B_{reg} cells may increase their therapeutic efficacy.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are mesodermal progenitor cells that have a wide range of differentiation capacity. They can differentiate into adipocytes, osteocytes, chondrocytes, myocytes, fibroblasts and stromal cells[1]. In addition, some research studies have shown that MSCs, under certain conditions, can trans-differentiate to cells from ectodermal and endodermal lineage[2,3]. Among them, the ability of MSCs to develop into neurons is of particular interest. Considering that neural stem cells are limited in number and extremely difficult to be isolated while, comparatively, massive numbers of MSCs can be derived from numerous adult tissues, including, liver, kidney, adipose tissue, bone marrow, dental pulp, peripheral blood and umbilical cord blood. MSCs may serve as a reliable source of neural cells for potential cell replacement therapy or regenerative medicine.

Aside from its diverse differentiation capacity, their immunomodulatory properties also prompt researchers to study profoundly. MSCs are capable of regulating both innate and adaptive immunity. They secrete a large variety of soluble factors, including interleukin (IL)-6, IL-8, transforming growth factor-β1 (TGF-β1), indoleamine 2,3-dioxygenase (IDO), human leukocyte antigen-G (HLA-G) and prostaglandin E2 (PGE2)[4]. These factors allow MSCs to interact with components of the innate and adaptive immunity, subsequently modulate inflammation and immune tolerance. Monocytes, for instance, under the influence of MSCs-secreted IL-6, IDO and PGE2, tend to develop into anti-inflammatory M2 macrophages instead of proinflammatory M1 macrophages[5-9]. In addition, recent reports showed that human gingiva derived MSCs have converted M1 macrophages to M2[5]. Natural killer (NK) cells, on the other hands, express CD73 and acquires regulatory phenotype when exposed to MSCs[10,11]. Similarly, regulatory dendritic cells (DC) induced by MSCs were capable of secreting IL-10, a powerful anti-inflammatory cytokine[12-14]. Thus, MSCs are able to suppress innate immunity by skewing their differentiation into regulatory subtype (Figure 1).

MSCs can regulate adaptive immune system by suppressing the proliferation, differentiation and activation of T cell and B cell. A number of studies have demonstrated that MSCs can inhibit the proliferation of Th1 and Th17 cell, decrease the production of interferon (IFN)-γ, IL-2, IL-6 and IL-17, and downregulate the T cell activation markers, CD38 and HLA-DR[15-19]. When MSCs were co-cultured with B cell and in the presence of different B cell trophic stimuli, B cell proliferation was inhibited and they were arrested in G_0/G_1 phase. Moreover, B cell differentiation was prohibited as indicated by limited production of IgG, IgM and IgA[20]. In addition, the regulatory-skewing propensity of MSCs observed in innate immune system also applies to T and B lymphocyte. In fact, the ability of MSCs to expand regulatory T (T_{reg}) cells and regulatory B (B_{reg}) cells have been intensively studied. However, the mechanism of how T_{reg} and B_{reg} cells are induced by MSCs has not been fully understood. Some suggest regulatory lymphocytes induction by MSCs requires mediation of other immune cells, while others propose MSCs-released cytokines are sufficient to expand T_{reg} and B_{reg} cell populations, but more and more researchers have come to the consensus that MSCs can use multiple pathways to generate regulatory lymphocytes and which pathways are more favorable is determined by the microenvironment that MSCs encounter[21]. Altogether, MSCs modulate immune cells to acquire regulatory phenotype, hence, alter the inflammatory milieu into a tolerogenic one (Figure 1).

There is another advantage of using MSCs for cellular therapy. MSCs have low immunogenicity, implying that MSCs can be used for allogeneic transplantation. This property is particularly helpful to the patient whose MSCs are compromised. Thereby, MSCs possess valuable therapeutic potential to treat immune-mediated disorders[22].

Although MSCs have demonstrated as a promising immunoregulator for clinical use, the immunomodulatory and low-immunogenicity properties of MSCs are not constitutive. The function of MSCs is based on the signals from the vicinity. MSCs, in the absence of tumor necrosis factor (TNF)-α and IFN-γ may adopt pro-inflammatory phenotype, which activate T cells to response. On the contrary, when MSCs are exposed to high level of TNF-α and IFN-γ they will behave as an anti-inflammatory regulator by producing TGF-β1, IDO, and PGE2[23]. Likewise, depending on the level of IL-6, MSCs can convert monocyte into M1 or M2 macrophages[22,24-26]. Thus, before any clinical application, the plasticity of MSCs should be carefully considered. In this review, we summarized current understandings on how MSCs interact with regulatory lymphocytes, T_{reg} and B_{reg} cells particularly, to attenuate autoimmunity, and how this knowledge can contribute to therapeutic development.

T_{reg} LYMPHOCYTE

The notion of "suppressive" T cells has long been proposed in 1970s. Due to technical limitation, their identities and phenotypic characteristics cannot be described until 1995, Sakagucho *et al*[27] isolated a unique $CD4^+$ $CD25^+$ T cells that can suppress immune responses and maintain immunologic self-tolerance[28]. Later, this subpopulation

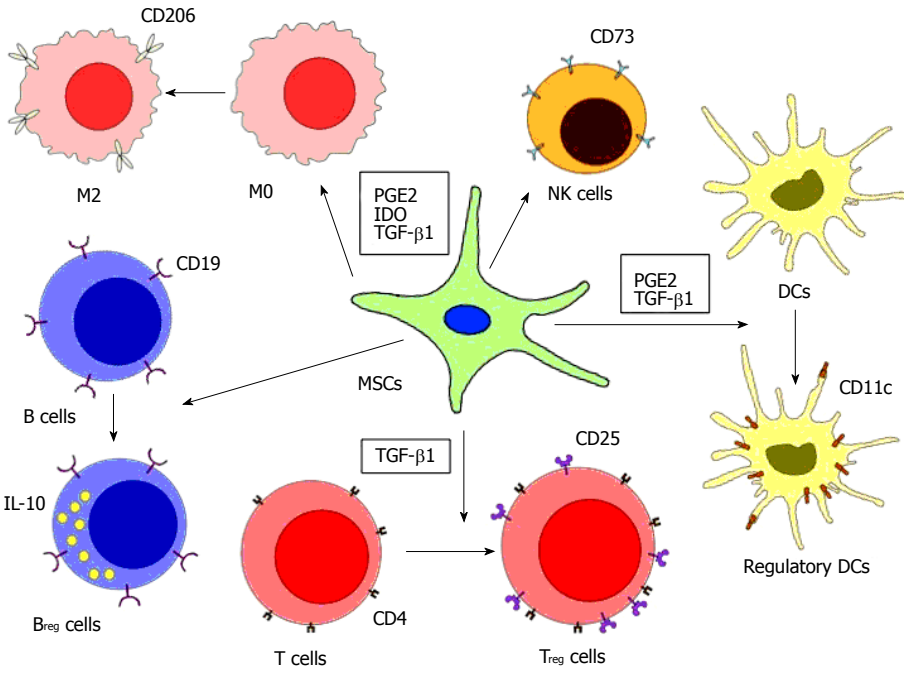

Figure 1 Immunosuppression by mesenchymal stem cells. MSCs suppress innate and adaptive immune responses by enhancing regulatory immune cells with tolerogenic properties. MSCs suppress macrophages by favoring monocyte polarization to anti-inflammatory M2 macrophages, increasing the production of IL-10, and decreasing the production TNF-α and IL-12. MSCs can also regulate DCs by downregulating the expression of MHC, CD40, CD80, CD83 and CD86, thus, diminishing their antigen presenting ability, while upregulating the expression of IL-10. MSCs can reduce the NK cell cytotoxicity and decrease their production of TNF-α and IFN-γ. T_{reg} and B_{reg} cells can be induced by MSCs, further increase the production of anti-inflammatory cytokines (IL-10 and TGF-β1). However, the mechanisms of how B_{reg} cells are induced by MSCs are still not clear. MSCs: Mesenchymal stem cells; TNF: Tumor necrosis factor; IL: Interleukin; NK: Natural killer; DCs: Dendritic cells; IFN-γ: Interferon-γ; T_{reg}: Regulatory T; B_{reg}: Regulatory B; TGF: Transforming growth factor; PGE2: Prostaglandin E2; IDO: Indoleamine 2,3-dioxygenase.

of T cells was named as T_{reg} cells. For those T_{reg} cells that undergo maturation in thymus, are referred to as thymus-dervied T_{reg} (tT_{reg}) cells. Three days post-maturation, tT_{reg} cells will relocate from thymus to periphery[29]. Surprisingly, tT_{reg} cells only comprise 5%-10% of peripheral T cells, but they are the critical regulator of autoimmunity. This is evidenced in mice lacking peripheral T_{reg} cells. They were lethal due to various autoimmunity enhancements[29,30].

Apart from tT_{reg} cells, T_{reg} cells can also be generated in periphery[31,32]. Periphery-derived T_{reg} (pT_{reg}) cells are converted from naïve T cells ($CD4^+CD25^-Foxp3^-CD45RB^{hi}$). Upon activation of naive T cells and in the presence of particular cytokines, two main types of T_{reg} cells can be differentiated in the periphery and *in vitro*, namely, T helper 3 (Th3) cells and type 1 regulatory T (Tr1) cells. Th3 cell and Tr1 cell differentiation are promoted by TGF-β and IL-10, respectively[33-35]. Both Th3 and Tr1 cells are suppressive to effector and memory T cells, and they are able to secrete cytokine for self-activation. However, one distinct phenotypical difference is Th3 cells are $Foxp3^+$ whereas Tr1 cells are $Foxp3^-$.

Forkhead box P3 (Foxp3) is a transcription factor that constitutively express in tT_{reg} cells and some types of pT_{reg} cells. It has been recognized as the master regulator of T_{reg} cells. Scurfy, a *Foxp3* gene mutated mouse, is lethal by one month after birth, displays hyperactivation of $CD4^+$ T cells and overproduction of proinflammatory cytokines[36]. In human, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is X-linked recessive disorder caused by mutation in *Foxp3* gene[37]. T_{reg} cells from the patients with IPEX are either dysfunction or completely vanished. As a result, IPEX patients are afflicted with various autoimmune diseases, allergy and/or inflammatory bowel disease[38]. The provoked inflammation on IPEX patients indicates the failure of immune tolerance. Foxp3 promotes its regulatory effect by enhancing the expression of IL-2 receptor (CD25), cytotoxic T cell-associated antigen-4 (CTLA-4), and glucocorticoid-induced TNF receptor family-related protein (GITR), meanwhile suppressing the production IL-2, IL-4 and IFN-γ[39]. T_{reg} cells monitor the inflammatory status by the exogenous level of IL-2. Binding of IL-2 to CD25 would enhance the expression of T_{reg}-cell associated genes and regulate the inflammation by suppressing effector T cell proliferation or by altering the function of antigen presenting cells[40]. Retroviral transfer of *Foxp3* to naïve T cells ($CD4^+CD25^-Foxp3^-$) can upregulate the expression of some T_{reg} cell-associated genes, including CD25, CTLA-4, GITR and CD103, and the *Foxp3*-transduced T cells were shown to be suppressive[41]. Altogether, Foxp3 is critical to the function and the development of T_{reg} cells and to a greater extent, the maintenance of immune homeostasis[42,43].

T_{reg} LYMPHOCTYE INDUCTION BY MSCs

MSCs are able to induce $Foxp3^+$ T_{reg} cell population *in vitro* and *in vivo*. So far, several mechanisms have been proposed, including: (1) secretion of soluble mediators; (2) cell-cell interaction; and (3) modulation of antigen

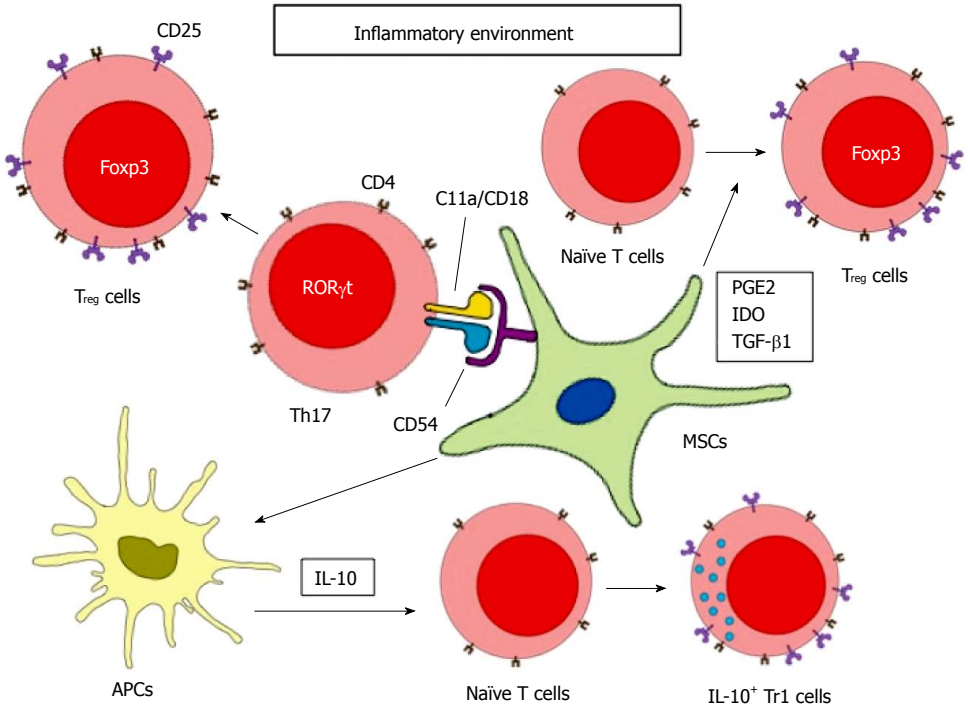

Figure 2 Mesenchymal stem cells-mediated regulatory T cell induction. MSCs induce T_{reg} cells through soluble mediators stimulation, cell-cell interaction, and modulation of antigen-presenting cells. Under inflammatory environment, MSCs secretes TGF-β1, PGE2 and IDO to facilitate the differentiation of naïve T cells to $Foxp3^+T_{reg}$ cells. MSCs can also interact with Th17 cells by direct contact *via* CD54 and C11a/CD18. With the presence of PGE2, differentiated Th17 cells can be converted to functional $Foxp3^+T_{reg}$ cells. MSCs can increase the secretion of IL-10 by antigen presenting cells, which will then induce Tr1 cells differentiation. MSCs: Mesenchymal stem cells; IL: Interleukin; T_{reg}: Regulatory T; TGF: Transforming growth factor; PGE2: Prostaglandin E2; IDO: Indoleamine 2,3-dioxygenase.

presenting cells (Figure 2).

Secretion of soluble mediators

TGF-β1: MSCs can secrete TGF-β1 to promote T_{reg} cell differentiation, especially when MSCs are placed in an inflammatory environment[21]. TGF-β1 is a potent immunosuppressor secreted by every leukocyte lineages, including macrophages, DCs, NK cells, T cells and B cells. Both TGF-β1 knockout mice and T-cell specific TGF-β receptor Ⅱ knockout mice develop severe autoimmunity, leading to multiple organs failure and death, suggesting the importance of TGF-β1 in regulating peripheral tolerance[44,45]. Generally, TGF-β1 can suppress the proliferation of T cells, the activation of B cells, the maturation and antigen presentation of DCs, the cytotoxicity of NK cells, and phagocytic effect of macrophages[46]. Moreover, as mentioned earlier, TGF-β1 is able to convert naïve T cells to $Foxp3^+$ Th3 cells, although such conversion seems to be concentration-dependent. High concentrations of TGF-β1 suppresses the expression of IL-23R and shifts the conversion to $Foxp3^+$ Th3 cells, whereas at lower concentrations and in the presence of IL-6 and IL-21, the expression of IL-23R is enhanced and results in $RORγt^+$ Th17 differentiation[47]. In addition, neutralizing TGF-β1 reduced mRNA and protein level of Foxp3 and CD25, further confirms its essential role in promoting T_{reg} cell differentiation[48]. In conclusion, MSCs-secreted TGF-β1 not only acts as a suppressor of innate and adaptive immune response, it can also induce development of T_{reg} cells from naive T cells, which further enhance the regulatory effects.

PGE2: MSCs can also secrete PGE2 to induce T_{reg} cells. PGE2 plays a major role in suppressing chronic inflammation. PGE2 can reduce IFN-γ production of NK cells, limit the phagocytic ability of macrophages and interfere early activation of B cells[49-52]. Although PEG2 can suppress early development of DCs, it is surprising that PGE2 also stabilize matured DCs and enhance its antigen presenting capacity[53-55]. Moreover, despite PGE2 is able to shift the differentiation of naïve T cells from Th1 to Th2 cells, PGE2 also promote proinflammatory Th17 cell development by elevating IL-23 production[56]. Thereby, PEG2 is not exclusively anti-inflammatory. It also possesses the ability to provoke inflammation. Nevertheless, like TGF-β1, PGE2 can induce $Foxp3^+T_{reg}$ cell differentiation and it is one of many soluble mediators that produce by MSCs. Diminishing PGE2 signaling when co-culture $CD4^+$ T cells with MSCs by antagonist indomethacin fail to upregulate Foxp3 and CD25 expression. In fact, when inhibiting both TGF-β1 and PGE2 signaling, the expression of Foxp3 and CD25 further decreased[48]. Furthermore, after transferring adipose tissue-derived MSCs in asthmatic mice, the number of infiltrated inflammatory cells was significantly reduced and no obvious goblet cell hyperplasia was found in the lung. Meanwhile, the number of T_{reg} cells was elevated. When TGF-β1 neutralizing antibodies or indomethacin was added to MSCs-treated asthmatic mice, the anti-

inflammatory effects promoted by MSCs as well as the T_{reg} cell expansion. These results demonstrated the necessity of TGF-β1 and PGE2 for T_{reg} cell induction as well as the anti-inflammatory effect of MSCs[57].

IDO: IDO is a rate-limiting enzyme that catalyzes the degradation of tryptophan *via* kynurenine pathway. IDO is expressed in various cell types, including macrophages, DC and MSCs. Interestingly, IDO expression can be induced by IFN-γ and other proinflammatory cytokines. Munn *et al*[58] treated pregnant mice carrying allogeneic or syngeneic fetus with 1-methyltryptophan, an IDO inhibitor. As a result, allogeneic, but not syngeneic, fetuses provoked severe immune rejection[58]. Also, some studies suggested the association of tryptophan catabolism with inhibition of T cell proliferation, emphasizing its tolerogenic potential[59,60]. In addition, kynurenines, a tryptophan catabolite, can promote T_{reg} cell induction[61]. Infusion of MSCs to kidney allograft murine model prevented graft rejection, and the T_{reg} cell population was elevated. In contrast, allograft tolerance and T_{reg} cell expansion diminished when the recipients were treated with IDO-deficient MSCs. These results demonstrated the importance of IDO in MSCs-mediated Treg cell induction and graft tolerance[62]. Other soluble factors, like human leukocyte antigen-G5 and haem oxygenase 1, are also shown to be involved in MSCs-mediated T_{reg} cell induction[63,64]. However, the underlying mechanisms are not clear. More studies need to be done in order to further increase the efficacy of MSCs-based therapy and to reveal the potential risk that could cause to the patients.

Cell-cell interaction

Apart from soluble mediators, cell-cell interaction is also important to the modulatory function of MSCs and T_{reg} cell induction. MSCs are known to express adhesion molecules on their surface, although only low level of expression can be detected in normal condition. However, after placing MSCs in inflammatory conditions, adhesion molecules, ICAM-1 and VCAM-1, chemokine ligands of CCR5 and CXCR3 are upregulated. Through these molecules, T cells are attracted and anchored to MSCs. With close proximity, adhesion molecules co-operate with IDO and NO, suppress T cell activity by inducing their apoptosis or cell arrest[65-68]. It is also worth to note that MSCs can inhibit the expression of ICAM-1, CXCR3 and α-integrin on $CD3^+$ T cell, reduced the interaction between T cells and endothelial cells, thus, disrupted T cells from infiltrating into CNS[69]. On the other hand, MSCs can attach to Th17 cells *via* CCR6 and CD11a/CD18 and facilitate Th17 to adopt regulatory phenotype[70]. Moreover, when co-culture MSCs with $CD4^+$ T cells in transwell system; T_{reg} cells cannot be induced, even in the presence of PGE2 and TGF-β[48]. These results further confirmed cell-cell interaction is essential to the overall suppressive effect of MSCs. However, T_{reg} cell induction ability was recovered if MSCs were co-cultured with peripheral blood mononuclear cells instead of isolated $CD4^+$ T cells, suggesting there is an alternative pathway that does not require cell-cell contact, and it is likely, through soluble mediators in peripheral blood mononuclear cells[48].

Modulation of antigen presenting cells

Increasing evidence has indicated MSCs are able to shift macrophages, DCs and NK cells to a regulatory phenotype and alter their cytokines production. For example, MSCs skew monocyte toward M2 macrophage differentiation. Subsequently, M2 macrophages secrete CCL18 and IL-10 to exert suppressive response and induce T_{reg} cell differentiation[26]. As discussed above, IL-10 is able to induce naïve T cell to $Foxp3^-$ Tr1 cell, which secrete high level of IL-10 and TGF-β to modulate the inflammatory microenvironment. Interestingly, although MSCs express neither IL-10 nor its receptor, MSCs are able to induce NK cells, DCs, macrophages, T cells and B cells to produce IL-10[5,10-12,17]. In addition, IL-10 is a powerful anti-inflammatory cytokine that suppresses antigen-specific immune responses, reduces pathological immune responses and promotes allograft tolerance.

In conclusion, the mechanisms underlying MSCs-mediated T_{reg} cell development are complicated, which involve synthesis and secretion of multiple mediators, direct interaction with target cells and modulation of certain antigen-presenting cells. Apparently, there is no single pathway that governs the whole induction process, indicating that MSCs possess certain degree of plasticity. Regardless of how T_{reg} cells are enhanced by MSCs, MSCs-activated T_{reg} cells play a significant role on immunoregulation and affect a wide spectrum of immune responses[43,71,72]. Certainly, T_{reg} cells can massively amplify the immunomodulatory effect of MSCs. However, the mechanism in regard to T_{reg} cell induction is far from elaborate and additional researches are required.

B_{reg} LYMPHOCYTE

In recent decade, B_{reg} cells were being intensively investigated due to its immunosuppressive effect on excessive inflammation. Like T_{reg} cells, B_{reg} cells can produce anti-inflammatory cytokines, like TGF-β and IL-10. Among these, IL-10 is strongly associated with B_{reg} cells since depleting IL-10-producing B cells result in chronic inflammation, outgrowth of proinflammatory T cell after autoimmune induction[73-75]. But unlike T_{reg} cells, there is no "master regulator" being identified in B_{reg} cells, which complicated the process of B_{reg} cell classification. So far, there are several B cell subsets have been identified as B_{reg} cells in mice. They are $CD5^+CD1d^{hi}$ B (B10) cells and $Tim1^+$ B cells[76-78]. In human, there is $CD19^+CD24^{hi}CD38^+CD1d^{hi}$ B cells and $CD19^+CD24^{hi}CD27^+$ B cells[79,80]. B_{reg} cells control inflammation by suppressing IL-12 secretion from DCs, thus inhibiting Th1 and Th17 differentiation[81]. Through

the secretion of TGF-β, B_{reg} cells can induce $CD4^+$ T cell apoptosis and anergy in $CD8^+$ cytotoxic T cells[82,83]. Recent studies indicated that B_{reg} cells play a role in T_{reg} cell development and function. As B_{reg} cells are one of the major sources of IL-10, which drive Tr1 differentiation, it is not surprising that B_{reg} cells can expand T_{reg} cell population during inflammation. Additionally, when B cell specific IL-10 defective mice (DBA/1IL-10 $KO^{-/-}$ mice) were induced with arthritis, the percentage of Tr1 was significantly decreased, indicating effects of IL-10^+B_{reg} cells on T_{reg} cell formation[75]. Besides TGF-β and IL-10, recent studies reported that IL-35 is another pleiotropic cytokine that regulate overwhelming inflammation and autoimmunity[84,85]. Antigen-driven proliferation assay revealed that IL-35 was able to suppress $CD4^+$ T cell proliferation[86]. Treatment with IL-35 ameliorated disease severity and reduced Th1 and Th17 cells in mice with experimental autoimmune uveoretinitis (EAU)[85]. More importantly, IL-35 can increase T_{reg} and B_{reg} cell populations. Similar to IL-10, IL-35-induced T_{reg} (iTr35) cells are $Foxp3^-$. However, adoptive transfer of iTr35 cells to various autoimmune disease animal models has sufficiently alleviated their clinical severity, and the effect was comparable to tT_{reg} cells-treated mice[35]. On the other hand, when recombinant IL-35 was injected into the EAU mice, the frequency of $B220^+$ IL-10^+B_{reg} cells, IL-35^+B_{reg} cells and B10 cells were upregulated in the spleen and draining lymph nodes[85]. Collectively, B_{reg} cells exhibit anti-inflammatory and immunoregulatory effects, at least in part, by secreting multiple anti-inflammatory cytokines (TGF-β, IL-10 and IL-35), promoting differentiation of other regulatory cells, and inhibiting the proliferation and function of effector T cells.

B_{reg} LYMPHOCYTE INDUCTION BY MSCs

Although MSCs do not constitutively express IL-10, and currently there is no evidence to indicate that MSCs produce IL-35, several studies have reported that MSCs induce IL-10^+B_{reg} cell differentiation in mouse model[87-89]. Our group studied the effects of human bone marrow-derived MSCs in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, and observed attenuation of clinical severity and neuroinflammation; and excitingly, these were associated with expansion of $CD1d^{hi}$ $CD5^+$ B_{reg} cells after MSCs administration[87]. Subsequently, another study demonstrated intravenous infusion of adipose tissue-derived MSCs to $Roquin^{san/san}$ mice, an animal model of systemic lupus erythmatosus (SLE), lead to increased numbers of B10, B10pro and naïve T_{reg} cells[89]. Moreover, the MSCs-mediated B_{reg} cell induction is not restricted to murine models. Administrating MSCs into refractory chronic graft *vs* host disease (cGvHD) patients have improved patients' overall clinical conditions. Consistent with murine models, MSCs increased the frequency and the function of $CD5^+$ IL-10^+B_{reg} cells by enhancing their proliferation and survival[88]. Momentarily, we are still not clear about the mechanism regarding to MSCs-mediated B_{reg} cell induction. It is worthwhile to ask whether the induction is IL-35 or IL-10-dependent since MSCs can induce IL-10 production by T_{reg} cells, DCs, and M2 macrophages, implying the possibility of creating a positive feedback loop for B_{reg} cell generation. Further understanding the mechanisms of how MSCs induce T_{reg} and B_{reg} cells can definitely contribute to the therapeutic development of MSCs and further improve their potential therapeutic efficacy.

THERAPEUTIC POTENTIAL OF GENETIC ENGINEERED MSCs

MSCs contain multiple properties that are suitable for therapeutical use. Wide-spectrum of differentiation capacity made it a perfect candidate for regenerative medicine. MSCs have been used to generate cartilage, bone, liver, intervertebral disc, and cardiac tissue[90]. Recent reports have suggested using MSCs for neural cell replacement. However, rather than direct neural differentiation, MSCs tend to recruit neural progenitor cells (NPCs) to the injury sites and support NPCs proliferation and differentiation[91]; Immunomodulatory properties of MSCs are potentially useful for the treatment of autoimmune diseases and GvHD. Transplanted MSCs suppressed the proliferation and activation of T cells and NK cells in type 1 diabetes animal model. Also, the level of IFN-γ and TNF-α were reduced. When MSCs were co-transplanted with pancreatic islets, MSCs protected grafted islets from immunorejection and secreted various trophic factors to promote graft vascular network[92,93]. Another intriguing advantage of using MSCs to treat immune diseases is that, unlike traditional immunotherapy in which a certain modulator act on a particular pathway, MSCs elicit their suppression on multiple immune cell types *via* various mechanisms. Although the immunosuppressive effects of MSCs appear very promising, further investigations are required to elucidate the underlying mechanisms, so as to prevent complications and maximize the therapeutic efficacy.

One current issue on immunotherapy is that a particular modulator or antibody may be seemingly effective, however, the therapeutic efficacy is limited since such modulator may also compromise certain cells or mediators beneficial to the disease recovery. Rituximab, for example, is a CD20 neutralizing antibody and it is believed to be an effective treatment for B and T-cell-mediated diseases, such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus[94-96]. Rituximab-induced B-cell depletion depends on the expression of CD20 on the cell surface, but the expression of CD20 gradually disappeared upon plasma cell differentiation[97,98]. Moreover, B_{reg} cells were also depleted, thus, exacerbates the disease symptoms[73]. In EAE, B10 cells play an important regulatory role during the initiative phase whereas they are less involved at the late phase of the disease[99,100]. Therefore, depleting B cells by rituximab at the early phase have a potential risk of worsening the

clinical conditions. As a consequence, it is necessary to develop an alternative strategy.

The immunosuppressive properties of MSCs on different murine autoimmune disease animal models support its potential clinical application. However, the immunomodulatory secretome of MSCs vary and greatly rely on the host inflammatory environment[21]. To minimize this uncertainty, a novel therapeutic strategy, in which MSCs are genetically engineered with defined immunoregulatory cytokines, has been developed. Transplantation of IL-10-engineered adipose-derived MSCs attenuated EAE by reducing the number of immune cell infiltration to the CNS, decreasing the secretion level of IL-17A, TNF-α and IL-2, and inhibiting antigen-presenting function of DC[101]. Since the immunosuppressive effect of MSCs is enhanced if they are placed proximal to the inflammatory area, Liao *et al*[102] engineered MSCs with CNS homing ligand genes, P-selectin glycoprotein (PSGL-1) and Sialyl-Lewis^x (SLeX), along with IL-10 to EAE model. Consequently, EAE was attenuated, CNS homing ability was enhanced and their therapeutic efficacy was increased[102]. Genetic engineering of MSCs has been well studied in regenerative medicine. Different combination of treatments is documented and aims to redirect the MSCs differentiation propensity. Comparatively, genetic modification of MSCs for the treatment of autoimmune diseases is currently under development. Considering that the effect of MSCs may vary between patients with different severity of neuroinflammation, information on the clinical condition and pathology of the individual patient will probably help to predict treatment efficacy. Moreover, questions like in what phase of a particular disease introducing MSCs can improve the clinical outcome, or to what extent MSCs can elicit their suppressive effect and meanwhile, does not compromise the immunity in response to pathogens or infectious agents, are worthwhile to explore in order to safely use in human patients.

SAFETY AND CONCERNS OF MSCs AS CELLULAR THERAPIES IN PATIENTS

To date, there are nearly 500 ongoing MSC-based clinical trials. They aim to investigate the effectiveness of MSCs on treating different diseases, including GvHD, diabetes, cardiovascular diseases, hematological diseases and neurological diseases[103]. Although most of these clinical trials reported the patients were well tolerated to the MSC infusion and administration, there are some safety concerns requiring caution[104]. During *in vitro* expansion, MSCs can give rise to replicative senescence, which may affect the activity of surrounding healthy cells and therefore, reduce the clinical efficacy[105]. Moreover, although MSCs have low immunogenicity due to the reduced expression of co-stimulatory receptors and major histocompatibility complex (MHC) class Ⅱ antigens, *in vitro* stimulation of pro-inflammatory cytokines on MSCs can upregulate MHC class Ⅰ and MHC class Ⅱ expression, compromising the hypo-immunogenicity property of MSCs.

CONCLUSION

The immunomodulatory properties of MSCs have been massively studied due to its intriguing suppressive effects on various immunological diseases. Broad-range of immune cells can be regulated by MSCs through a series of soluble mediators stimulation, chemokine attraction, and cell-to-cell interaction. MSCs-induced T_{reg} and B_{reg} cells enhance the immunosuppressive capacity and generate a tolerogenic microenvironment against overwhelmed inflammation. This hypothesis supports the observation that infused MSCs can only survive in the recipient for a short period of time, however, the regulatory effects of MSCs are long lasting, suggesting MSCs may act as an activator or a switcher that initiate certain cells, possibly T_{reg} and B_{reg} cells, to react to the inflammation and at the same time, alter the microenvironment for those cells to sustain their immunosuppressive effects. Although MSCs appear very promising as treatment in experimental models of autoimmune diseases, there are still many challenges need to overcome before MSCs can be widely use in clinical medicine.

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MINIREVIEWS

Racial disparity in colorectal cancer: Gut microbiome and cancer stem cells

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Abstract

Over the past two decades there has been remarkable progress in cancer diagnosis, treatment and screening. The basic mechanisms leading to pathogenesis of various types of cancers are also understood better and some patients, if diagnosed at a particular stage go on to lead a normal pre-diagnosis life. Despite these achievements, racial disparity in some cancers remains a mystery. The higher incidence, aggressiveness and mortality of breast, prostate and colorectal cancers (CRCs) in African-Americans as compared to Caucasian-Americans are now well documented. The polyp-carcinoma sequence in CRC and easy access to colonic epithelia or colonic epithelial cells through colonoscopy/colonic effluent provides the opportunity to study colonic stem cells early in course of natural history of the disease. With the advent of metagenomic sequencing, uncultivable organisms can now be identified in stool and their numbers correlated with the effects on colonic epithelia. It would be expected that these techniques would revolutionize our understanding of the racial disparity in CRC and pave a way for the same in other cancers as well. Unfortunately, this has not happened. Our understanding of the underlying factors responsible in African-Americans for higher incidence and mortality from colorectal carcinoma remains minimal. In this review, we aim to summarize the available data on role of microbiome and cancer stem cells in racial disparity in CRC. This will provide a platform for further research on this topic.

Key words: Colorectal cancer; Cancer stem cells; Racial disparity; Microbiome; MiRNA

Core tip: The role of microbial dysbiosis and cancer stem cells (CSCs) in colorectal cancer (CRC) has been studied extensively, however, their implication in racial disparity is not well known. A number of recent studies have shown that different dietary patterns affect gut microbiome. Likewise, dietary patterns also affect intracellular regulatory events which may affect the function of CSCs. Our objective is to consolidate the available data, on the role of gut microbiome and CSCs in racial disparity in CRC, explore a link between them and lay a foundation for further advances.

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INTRODUCTION

Colorectal cancer (CRC) remains the second leading cause of cancer related mortality in the United States. However, the incidence and mortality of colon cancer is different among various racial and ethnic groups. African Americans (AAs) share the largest burden of CRC in the United States. Data from Surveillance, Epidemiology and End Results (SEER) revealed that the age-adjusted incidence of CRC in AAs, based on cases diagnosed between 2008 and 2012 was 52.3 per 100000 for men and women combined per year, compared to 41.5 per 100000 for men and women combined per year among Caucasian Americans (CAs). Similarly, the age-adjusted mortality from CRC in AAs, based on cases diagnosed between 2008 and 2012 was 21.4 per 100000 for men and women per year, compared to 15 per 100000 for men and women per year in Whites/CAs[1]. AAs not only tend to be diagnosed at a younger age but also have a worse prognosis than CAs[2,3]. Many genetic, epigenetic and environmental factors have been reported that are responsible for this racial disparity.

In recent years, there has been an increased focus on differences between microbiota of colon of healthy individuals and of patients with CRC. A relationship between microbial dysbiosis and CRC is now well established[4-7].

The concept that pluripotent and self-renewing cancer stem cells (CSCs) have a pivotal role in the development and progression of many malignancies, including CRC is now well accepted. We have reported a higher proportion of CSCs (specifically $CD44^+$ $CD166^-$ phenotype) in AAs, who also had a significantly higher number of adenomas, compared to CAs[8]. However, underlying regulatory mechanisms remain unknown.

Recent studies have shown that host can alter gut microbiota through external (diet, obesity, *etc.*) and internal factors (microRNAs in intestinal epithelial cells)[9]. MicroRNAs (miRNAs) have also been reported to regulate CSCs[10]. Thus it is possible that the gut microbiota and CSCs are not entirely isolated domains and in AAs, the higher frequency of unfavorable mutations, through miRNAs, facilitates pathogenic microbiota over commensal bacteria (Figure 1).

DIETARY REGULATION OF MICROBIOTA AND RACIAL DISPARITY IN CRC

Human gut is a major harbinger of a wide variety of microbial cells containing approximately 10^{14} cells estimating 1000 species. The dominant composition is bacteria with 90% of species belonging to Firmicutes and Bacteroides[11]. These bacteria are in a symbiotic relationship with the intestine, utilizing undigested nutrients as substrates and in return produce various vitamins, amino acids, transform bile salts and assist in maintenance of intestinal barrier, appropriate immune response against pathogens[12]. This homeostasis is altered in a state of dysbiosis, which is overgrowth of pathogenic bacteria that are normally inhibited by commensal bacteria.

Numerous studies have been performed to examine whether and to what extent the dietary changes may affect gut microbiota. In general, these studies suggest that changes in diet and their interaction with gut microbiota exert profound effects on intestinal homeostasis through various metabolites[13]. Emergence of metagenomic sequencing has enabled identification of microorganisms not possible with 16S ribosomal RNA gene (*rRNA*) sequence-based methods and traditional culture methods[13-15]. Collective genomes of the members of a microbial community are analyzed against widely available microbial databases, thus allowing identifying microbial communities, which are virtually uncultivable. This has led to discovery of hundreds of microbial genus not previously known to exist in the human gut.

Qin *et al*[11] published a comprehensive catalogue of human gut microbial genes in 2010. Among the various conclusions, one was that *Fusobacterium* genus is not an abundant constituent of the normal gut microbiota. It is a genus of anaerobic gram-negative bacilli and has been known to cause periodontal disease. *Fusobacterium* species esp. *F. nucleatum* has been isolated from colon and fecal samples of patients with CRC in multiple studies[16-18]. Castellarin *et al*[19] even found a positive association between *Fusobacterium* and lymph node metastases.

Gao *et al*[4] examined microbiota from cancerous tissues of CRC patients and found a significant abundance of *Firmicutes* and *Fusobacteria* compared to

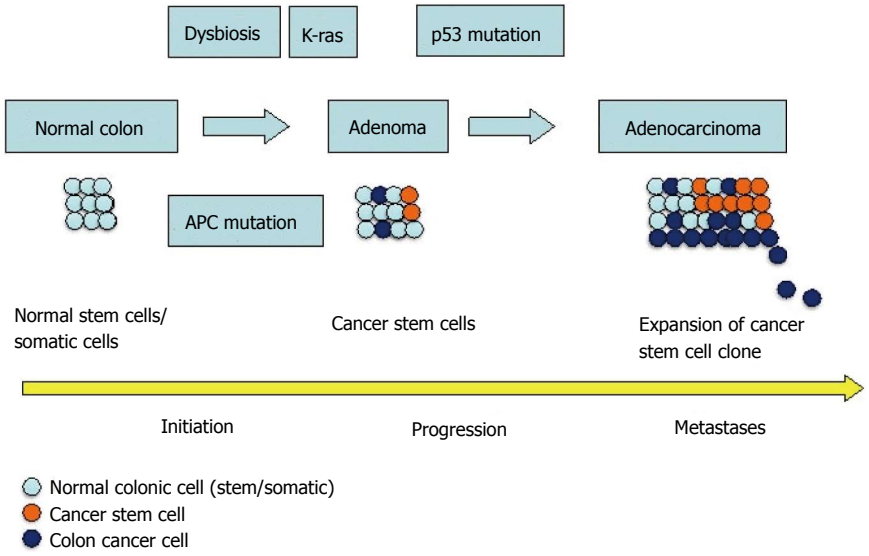

Figure 1 Cancer stem cells during development and progression of colorectal cancer. Schematic representation of the role of dysbiosis caused by microbiome alterations and accumulation of mutations in colonic stem cells leading to development and progression of colorectal cancer. APC: Adenomatous polyposis coli.

healthy individuals. Interestingly they also found that *Proteobacteria* was less abundant in patients with CRC. In the first large series sequencing of stool samples, Sobhani *et al*[5] reported that Bacteroides/Prevotella were markedly increased in patients with CRC.

Dietary components like vegetables, fiber, vitamin D are shown to be associated with a lower risk of colon cancer whereas red meat and a diet rich in saturated animal fat has been shown to be responsible for an increased risk of colon cancer[20,21]. Two major biotransformation pathways for dietary components mediated by microbiome have been reported.

A diet rich in fiber stimulates saccharolytic fermentation and production of short chain fatty acids (SCFAs) namely butyrate, acetate and propionate. These metabolites, particularly butyrate have anti-inflammatory, anti-proliferative and antineoplastic properties, while a fat rich diet stimulates the synthesis and release of bile acids in the gut[22,23].

In their study involving four racial groups, Hester *et al*[24] found that SCFA level was lower in stool from African-Americans than other racial groups. Interestingly, they also found a decreased prevalence of bacteria of *Lachnospiraceae* family in stool from African-American patients. Bacteria of *Lachnospiraceae* family have been previously shown to be associated with butyrate production in colon tissue[25]. A summary depicting bacteria, whose presence has been shown to have or probably has a positive or negative association with colon cancer in AAs has been shown in Table 1.

It has been widely reported that the higher amount of butyrate is seen in stool of healthy controls than CRC patients[26]. On the other hand secondary bile acids have been postulated to have a carcinogenic role[27].

Ou *et al*[28] examined stool from healthy AAs and from age and sex-matched native Africans and found a higher concentration of fecal secondary bile acid in AAs and a higher concentration of short-chain fatty acids in native Africans. Although the reason(s) for these increases are not known, it is possible that changes in dietary habits are responsible for these differences.

Majority of the primary bile acids are returned to the liver by the enterohepatic circulation. A fraction of the primary bile acids escapes the enterohepatic circulation and reaches the colon. In the colon, 7-DHC (Dehydrocholesterol), converts primary bile acids into secondary bile acids, like deoxycholic acid and lithocholic acid. There is plenty of evidence to suggest that when exposed to high levels of bile acids, gastrointestinal cells undergo oxidative and nitrosative stress leading to anti-apoptotic and mutagenic properties[29]. De Boever *et al*[30] demonstrated the protective effect of *Lactobacillus* against bile salt cytotoxicity. Many studies have found that African-Americans have a lower prevalence of *Lactobacillus*, compared to other racial groups[31,32].

Moore and Moore[31] studied the stool microbial composition in populations with higher (CAs, patients with polyp) and lower CRC risk (South African blacks, native Japanese). They found a positive association of *Bacteroides* and *Bifidobacterium*, and a negative association of *Lactobacillus* and *Eubacterium aerofaciens*, with colon cancer risk.

These studies provide ample evidence that a variation in microbial composition between ethnic groups may partly be responsible in colorectal carcinogenesis and that diet plays a role in this microbial diversity.

CSCS AND RACIAL DISPARITY IN CRC

CSCs

According to the stem cell model of carcinogenesis, only some cells in a tissue possess the ability to initiate and sustain tumor growth. These cells, characterized as CSCs have two important properties: Indefinite proliferation and pleuripotency (ability to differentiate itself into more than one cell lineage)[33]. The critical role of CSCs in initiation, development and progression of CRC is now well established[34]. Mutations in normal stem, progenitor or terminally differentiated cells, are believed to be responsible for origin of CSCs, but the

Table 1 Depicting bacterial genus/families, whose presence has been shown to have or probably has a positive or negative association with colon cancer in African-Americans

Positive association	Negative association
Fusobacterium	Lactobacillus
Firmicutes	Lachnospiracea
Bacteroides	Eubacterium
Bifidobacterium	

processes responsible are not completely known.

Colon stem cells are believed to exist as undifferentiated cells at the base of the crypt of lieberkuhn in the proliferative zone. The undifferentiated cells differentiate in to specialized cells as they move up the crypt-villic axis towards the luminal surface. It is estimated that, in human adults in every square centimeter of colon, there are about 14000 crypts and at a given rate of 5 d for colonic epithelium renewal; over 6×10^{14} colonocytes are produced during the individual lifetime[35,36]. The lifelong proliferation of the stem cells makes them more prone to accumulation of mutations than other short-lived cells.

Various pathways tightly regulate the processes involved in maintenance of a normal intestinal epithelium. The central among those is the canonical Wnt pathway. Canonical Wnt signals are transduced through an interaction with Frizzled family receptors (Fz) and LRP5/LRP6 (low-density lipid receptor) co-receptor to the β-catenin signaling pathway. In the absence of Wnt signaling, β-catenin becomes a part of a multiprotein degradation complex, containing tumor suppressor gene product adenomatous polyposis coli (APC), scaffold protein Axin and is phosphorylated by casein kinase Iα and glycogen synthase kinase 3β, and then ubiquinated for subsequent proteosome degradation.

In the presence of Wnt signaling, after signal transduction, Axin is recruited to cell membrane by a Fz-Disheveled (DVL) or LRP5/6 interaction. This leads to degradation of the degradation complex described above and β-catenin buildup in the nucleus. This stable nonphosphorylated β-catenin complexes with several factors and leads to activation of the transcription of several genes like *c-Myc*, *CD44*, *CCND1*, essential for DNA replication, cell cycle control and altered mitosis[37,38].

Characterization of CSCs: Identification of CSCs is a challenging task given the complexity of the cell surface markers, and their difference between various tissues, apart from the technical issues involved. One of the methods used to identify CSC is by the cell surface markers, also known as epitopes. Colonosphere formation, a functional assay is also used to characterize CSCs.

Colon CSCs have been identified by expression of numerous surface epitopes CD133, CD24, CD44, CD166, EPCAM (epithelial specific antigen/ESA), *etc*[39]. CD166 expression has been linked with shortened survival[40]. Similarly, CD44's role in tumor invasiveness and progression has prompted it to be described as a potential CSC marker for CRC[41]. It has been shown that CSCs form tumors in SCID mice at much-diluted concentrations, which histologically resemble the primary tumor[42].

We studied the role of CD44, CD166 and ESA in CRC and reported their expression in premalignant adenomatous polyp[43] and also showed an age dependent increase in their expression, suggesting their role in tumor development and progression[43].

We have also recently observed that $CD44^+CD166^-$ cell proportion in the colonic effluent as well as in the colonic mucosal cells is significantly increased in AAs with adenomas than CAs. We also observed that the colonic effluent from high risk AAs (more than 3 adenomas) contained markedly higher proportion of $CD44^+$ $CD166^-$ cells than low risk AAs (subjects with no adenomas). We were not able to duplicate these results in colonic effluent from white population[8]. Taken together, the above observations point towards the substantial role of CSCs, not only in higher incidence, but also progression of CRC in AAs.

Racial disparity in miRNAs and signaling pathways regulating CSCs: According to the well-accepted Fearon and Vogelstein model of CRC progression, development of CRC is an outcome of accumulation of mutations in tumor suppressor genes, oncogenes; and accumulation of changes is more important than the sequence of changes[44]. This is also the basis of "adenoma-carcinoma model" in which *APC* gene mutation initiates the sporadic CRC, which accounts for 80%-85% CRC, followed by mutations in other genes-notably *K-ras*, deleted in colorectal carcinoma and *p53*[45]. Mutated *APC*, in association with β-catenin up regulates many oncogenes, notably CCND1 and c-myc[46,47]. We have recently reported that AAs had higher (48%) number of adenomas, recorded during colonoscopy, than CAs[8]. These findings confirm the data in separate studies by Corley *et al*[48] and Lebwohl *et al*[49]. In line with the Fearon and Vogelstein model, one of the reasons, in AAs, for a higher incidence of CRC could be the higher number of adenomas in them.

We also examined the rates of mutation of APC and β-catenin, in a small cohort of AAs (n = 10) and CAs (n = 10), The agarose gel electrophoresis of the PCR products of wild type and mutant *APC* gene (hAPC) in colonic biopsies from 5AAs and 6 CAs without adenomas is shown in Figure 2. Out of 10 AAs, 2 showed mutation in *APC* gene, whereas none of the CAs showed mutation in the gene. Similarly, 3 AAs showed mutation in β-catenin, as compared to none of the CAs. This preliminary data clearly supports the role of APC and β-catenin mutations in higher incidence of CRC in AAs.

MicroRNAs (miRNAs) are an expansive class of small non-coding RNAs, 18-23 nucleotides long, and regulate gene expression, either by translational repression or by mRNA degradation through cleavage. MiRNAs are atypically expressed in numerous pathological states,

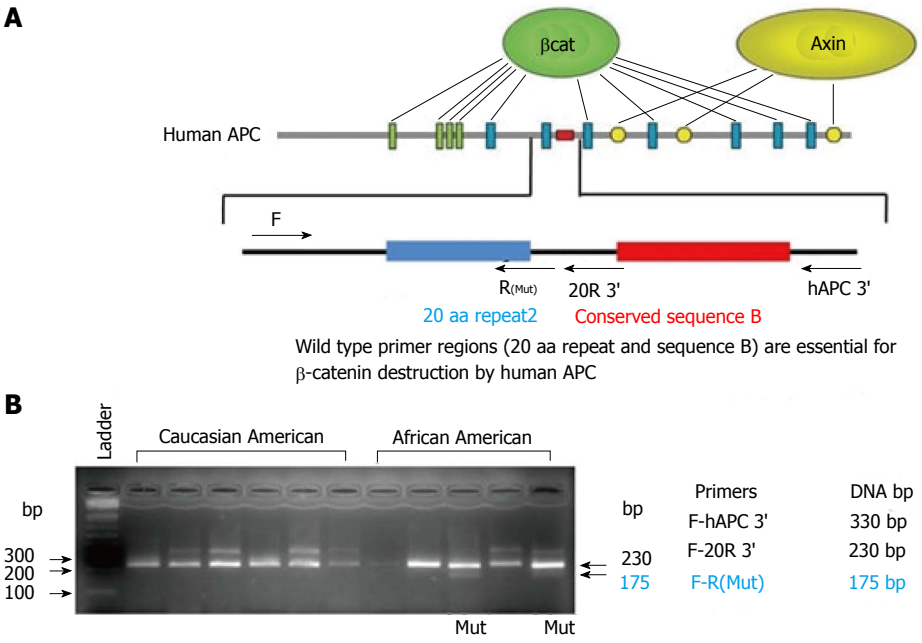

Figure 2 Schematic representation of human *APC* gene and design of appropriate primers for the wild type and mutant gene. A: Human *APC* gene with β-catenin (green and blue bars) and Axin (yellow circles) binding sites. Red bar represents conserved sequence of *APC* gene. Forward (F) and reverse (R) primers were designed to demonstrate mutation in *APC* gene; B: Agarose gel electrophoresis of PCR products showing higher rate of *APC* gene mutation (Mut: 175 bp) in the colonic mucosa of AAs without adenomas than their CA counterparts. AAs: African Americans; CA: Caucasian American; APC: Adenomatous polyposis coli.

and depending on the target, may work as oncogenes or tumor suppressors. MiRNAs' role in CRC regulation through CSCs is well researched[50-52]. We have examined the role of miRNAs 21 and 145 in regulating colon CSCs and reported that the expression of miR-21 is significantly increased and that of miR-145 markedly decreased in chemotherapy-resistant colon cancer cells, highly enriched in CSCs[53]. In colon cancer cells, forced expression of miR-145, significantly inhibits CSCs and tumor growth, whereas up-regulation of miR-21 augments the same[8]. We have also shown the role of miR-21 in age related rise of colon cancer. Upregulated miR-145 was associated with reduced levels of CD44, and β-catenin[53], both of which, we have been shown to be independently associated with racial disparity of CRC.

These observations led us to explore the role of miR-21 in ethnic differences in CRC and/or its precursor, adenoma. Ongoing studies (unpublished data) from our lab revealed that miR-21 levels in normal looking colon mucosa of AAs with adenomas were significantly higher than their CAs counterparts.

Mutation in *K-ras* gene, second most common in CRC progression, is not required for initiation of CRC. Reduced expression of miR-145 has been shown to contribute to CRC development through K-ras expression[54]. We have reported that K-RAS' lack in chemo-resistant colon cancer cells upregulates miR-145, downregulates miR-21, as well as disrupts the negative cooperation among miR-21 and miR-145[53]. Epidermal growth factor receptor (EGFR) is another transmembrane protein, whose role is well established in CRC pathogenesis. We have reported that EGFR inhibitor Cetuximab decreases miR-21 expression, suggesting another link between stem cells and definitive mutations in CRC[55].

Mutation in *p53* gene has been shown to facilitate not only growth, but also invasiveness in colorectal adenomas. Therefore, *p53* is implicated in the adenoma-carcinoma sequence[56]. *P53* mutations have also been associated with altered miRNA processing[57].

We have recently reported a significant increase in miR-1207-5p in colonic mucosal cells cultured in stem cell media (enriched for CSCs) and $CD44^{+}CD166^{-}$cells isolated *via* flow cytometry, from AAs with adenomas. Additionally, colon cancer cell lines HCT-116 and HT-29 showed a significant increase in miR-1207-5p, compared to normal colonic epithelial cells, HCoEpiC and CCD841[8]. This lays further weight to the role of miRNAs in promoting stem cell-like properties in colon epithelial cells.

A recent whole exome sequencing study on tissues from AAs with CRC identified somatic mutations in *APC*. This also supports the role of mutations in the key protein in Wnt/β-catenin signaling pathway-APC in pathogenesis of CRC[58].

Stemness and epithelial to mesenchymal transition: A tremendous problem in management of cancer is cancer recurrence. Inspite of modern breakthroughs, in CRC, the degree of recurrence is as high as 40%-60%[59].

In any cancer, the capacity of few cells to isolate themselves from an initial site and generate a secondary tumor after implantation at a second site, defines the property of recurrence and metastases. A variety of genetic changes take place *via* a process called EMT (epithelial-mesenchymal transition) that equips CSCs to invade other tissues and survive under attachment free

conditions. In addition to mutations in *APC*, *K-RAS*, *p53* described above, activation of signaling pathways like Wnt/β-catenin, TGF-β, notch, and hedgehog is a very critical step in EMT[60,61].

The Wnt/β-catenin signaling described above regulates EMT by downstream controlling of SNAIL, TWIST, SLUG, which in turn control the expression of effectors of EMT like Vimentin, E-cadherin, and N-cadherin[62-64].

TGF-β signaling is another key pathway regulating EMT progression and is affected by activation of certain transcription factors like TWIST, SNAIL, SLUG and ZEB. In addition to activation of canonical TGF-β signaling, it is also involved in downstream activation of other canonical pathways, including Hedgehog, Notch, and Wnt and for this reason, is considered to be the master switch of the EMT process[65-67].

Notch signaling is another central mechanism for EMT development. Bao *et al*[68] demonstrated that Notch pathway increases ZEB1 expression, which leads to EMT induction by inhibiting miRNA-200. Notch expression has also been correlated with the EMT markers such as, E-cadherin and Vimentin in prostate cancer[69].

There is ample evidence to suggest that cells that undergo EMT have CSC like properties. After invading the new site, these cells initiate secondary tumor, much like CSCs[70]. The regulatory role of miRNA-200 in Notch signaling further supports the CSC theory.

Although the specific differential expression of miRNA-200 in AAs and CAs is not yet elucidated, the direct association of notch signaling with miRNA-200 inhibition, opens avenues for further investigation in the area of racial disparity (see miRNA section). We have also shown that the induced overexpression of miR-1207-5p in normal human colonic epithelial cells (HCoEpiC and CCD841) induces stemness, as well as expression of EMT markers TGF-β, CTNNB1, MMP2, Slug, Snail, and Vimentin associated with an elongated cell morphology[8], indicating its role in regulating stem cell-like properties in colon mucosal cells.

TGF-β stimulation has been shown to cause increased motility in $CD133^+$ cells as compared to $CD133^-$ cells in non-small-cell lung carcinoma. We have discussed the differential proportion of $CD44^+CD166^-$ cells in the colonic effluent as well as in colonic mucosal cells of AAs and CAs[8]. CD44 has been shown to be associated with tumor progression and metastases in CRC in various studies[71].

CONCLUSION

The conventional therapies for colon cancer do not account for CSCs. This has been postulated as one of the reasons for recurrence. It is well known that the recurrence rates are higher in AAs than CAs. In various studies, racial disparity in survival/recurrence is not well explained by differences in socioeconomic conditions, and general patterns of treatment[72-74].

It is possible that the higher rate of recurrence in AAs is in part due to prevalence of those CSCs with a less favorable mutation.

The current cytotoxic therapies act by interfering with the cell cycle of rapidly growing cells. This provides selective advantage to the slow replicating stem cells, which in fact may be enriched after chemotherapy. Data from several studies suggest a pivotal role for CSCs in regulating many malignancies, including CRC. Numerous studies have reported that CSCs or CSC like cells are highly enriched in chemotherapy resistant cancer cells. These include glioma, breast cancer, and colon cancer[75]. Results from our own studies have demonstrated that although the combined therapy of 5-FU and Oxaliplatin inhibited the growth/proliferation of human colon cancer cells (HCT-116 or HT-29), the remaining cells showed enrichment of CSCs[76].

It is well known that butyrate induces differentiation of colon cancer cells[77,78]. Forced cell differentiation has not only been shown to deplete CSCs in colon cancer but also to sensitize colon cancer cells to chemotherapy[79,80]. When these findings are viewed in light of the observations of lower amount of butyrate in stool from AAs with colon cancer than other racial groups (see section on dietary regulation of microbiota and racial disparity), it provides an interesting link between racial disparity, CSCs and CRC.

In order to successfully tackle the disparity and recurrence issues in colon cancer, a better understanding of the biological pathways is needed. Further, the focus needs to be shifted from a uniform treatment approach to a more personalized medicine. An understanding of specific CSC markers responsible for differential initiation, progression and recurrence in AAs, will help develop therapies, which target the same.

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Roles and regulation of bone morphogenetic protein-7 in kidney development and diseases

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Abstract

The gene encoding bone morphogenetic protein-7 (*BMP7*) is expressed in the developing kidney in embryos and also in the mature organ in adults. During kidney development, expression of *BMP7* is essential to determine the final number of nephrons in and proper size of the organ. The secreted BMP7 acts on the nephron progenitor cells to exert its dual functions: To maintain and expand the progenitor population and to provide them with competence to respond to differentiation cues, each relying on distinct signaling pathways. Intriguingly, in the adult organ, BMP7 has been implicated in protection against and regeneration from injury. Exogenous administration of recombinant BMP7 to animal models of kidney diseases has shown promising effects in counteracting inflammation, apoptosis and fibrosis evoked upon injury. Although the expression pattern of *BMP7* has been well described, the mechanisms by which it is regulated have remained elusive and the processes by which the secretion sites of BMP7 impinge upon its functions in kidney development and diseases have not yet been assessed. Understanding the regulatory mechanisms will pave the way towards gaining better insight into the roles of BMP7, and to achieving desired control of the gene expression as a therapeutic strategy for kidney diseases.

Key words: Bone morphogenetic protein-7; Therapeutics; Kidney; Development; Nephron progenitor cells; Disease; Regeneration; Chromatin conformation; Gene expression; Gene regulation

Core tip: Bone morphogenetic protein-7 (BMP7) plays crucial roles in both the development and regeneration of the kidney. The functions and mechanisms of this protein have been clarified extensively for these processes in the fetus and adult kidney. However, the functional differences of BMP7 secreted from different sites in the kidney remain

undefined. We propose that uncovering the regulatory mechanism underlying *BMP7* expression will help to solve that issue. Moreover, those data should pave the way towards development of a novel therapeutic strategy for kidney diseases *via* hyperactivation of the endogenous action of BMP7.

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INTRODUCTION

Bone morphogenetic protein-7 (BMP7) belongs to the transforming growth factor-β (TGFβ) superfamily. It was first identified and cloned as a human homolog of the bovine osteogenic proteins, and designated as the osteogenic protein-1 (OP-1)[1,2]. Knockout mouse models of the *BMP7* gene were reported subsequently. Most strikingly, these models exhibited severe retardation of kidney development and died soon after birth due to renal dysplasia. Additionally, these mice exhibited anophthalmia and polydactyly in the hind limbs. Other phenotypic changes were also observed in ribs and craniofacial bones, but the effects were not fully penetrant[3-5]. Since then, the roles of BMP7 in the various stages of kidney development have been extensively studied. Interestingly, *BMP7* was found to be expressed not only during embryogenesis but also in the adult organ[6-8]. Series of studies have now shed light on the protective and regenerative functions of its expression in the mature kidney[9]. Furthermore, exogenous administration of BMP7 and its mimetic has been considered as a promising therapeutic strategy for treatment of severe kidney diseases[10].

Despite the original implication of an osteogenic property for BMP7, the *BMP7* knockout mice showed a severe phenotype in the kidney. This finding clearly illustrated that the function of the gene is critically determined by its expression pattern. In support of this, *Bmp4* under the control of the *BMP7* locus rescued the loss of the developmental function of *BMP7* in the kidney in mice[11]. Thus, uncovering the regulatory mechanism for *BMP7* will be pivotal for gaining a better understanding of its functional roles and to developing therapeutic applications based upon it. In accordance with this perspective, in this review we first summarize the current knowledge regarding the function of BMP7 in kidney development and diseases, after which we provide an overview of the recent findings in the regulation of its expression, finally discussing the future directions that will most likely advance the knowledge and clinical applications of this field.

BMP7 IN EMBRYONIC KIDNEY DEVELOPMENT

During embryonic development, *BMP7* is expressed in multiple tissues including the kidney, eyes, heart, limbs, forebrain, branchial arches, bones and cartilage[6,12,13]. In the mouse, expression in the developing kidney appears first in the Wolffian duct, at embryonic day (E)9.5, and persists in the ureteric bud evaginated from the duct[6]. At E11.5, *BMP7* expression appears in the condensing mesenchyme that is induced by the ureteric bud. Slight expression is found in the uninduced metanephric mesenchyme as well[12]. At E13.5, the expression area extends to the pretubular aggregates and others derived from the condensed mesenchyme, including the comma-shaped and S-shaped bodies, the distal tubules and the podocytes of the developing glomeruli[6,12,14], although expression in the comma-shaped and S-shaped bodies and the distal tubules was found by some of the studies to be very weak or absent[4,14]. By E16.5, when the ureter has developed substantial branching, the expression in the ureteric epithelium becomes weaker in the medullar region, while its expression in the condensed mesenchyme in the nephrogenic zone of the developing kidney remains robust. Podocytes continue to strongly express *BMP7* after their folding in glomeruli[3,4,12,13,15].

In mice, development of the kidney is severely retarded in the absence of *Bmp7*. In addition to the mutant kidney being smaller in size at birth, the number of nephrons is greatly reduced. These effects are accompanied by abnormal expansion of collecting ducts, which are interspersed by stromal cells and extracellular matrix. Mesenchymal stem cells and glomerulogenesis are also absent in the mutant kidney[3,4].

These developmental defects appear as early as E12[4]. As stated, the size of the mutant kidney is smaller than that of the control kidney, with the condensation of the mesenchyme appearing reduced at E12.5, although formation of pretubular aggregates was also observed[4]. At E14.5, cessation of nephrogenesis becomes apparent with loss of mesenchymal populations in the cortical region[3,4]. However, the comma-shaped and S-shaped bodies are present at this stage[3,4] and the ureteric buds are branched[3]. Moreover, the expression of marker genes, such as *Pax2*, *Pax8* and *Wnt4*, seems more or less normal in the two lineages, as long as the corresponding structures are present[3,4].

These results suggested that the initial reciprocal inductive interaction between the ureteric epithelium and the metanephric mesenchymal cells takes place in the absence of BMP7[3]. Studies using terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (commonly known as the TUNEL assay) showed massive apoptosis in the uninduced metanephric mesenchymal cells occurring from E13.5 to E14.5, explaining the

loss of the cell population that was observed in the mutant kidneys[4,12]. Thus, BMP7 is a survival and/or proliferative factor that acts to maintain and expand nephron progenitor cells, the loss of which leads to severe retardation of kidney development[3,4,12].

The molecular mechanisms that underlie the roles of BMP7 in kidney development have been uncovered by recent studies[16]. The collective BMPs are known to activate SMAD1, SMAD5 and SMAD8 transcription factors, which are associated with receptors of BMPs and are phosphorylated upon their binding. Following phosphorylation, these SMADs form a complex with SMAD4, which then binds to *cis*-regulatory regions to activate target genes. On the other hand, BMPs can also activate the mitogen-activated protein kinase (MAPK) pathway, mediating their downstream effects[17].

An *in vitro* study using a primary culture system of nephrogenic progenitor cells revealed that the proliferative role, but not the survival role, of BMP7 in this cell population is dependent on activation of the jun N-terminal kinase (JNK)-MAPK pathway[18]. Mice with knockout of the *Trps1* gene, which encodes the tricho-rhino-phalangeal syndrome-1 zinc-finger transcription factor, show reduced epithelialization of mesenchymal progenitor cells[19]. Interestingly, expression of *Trps1* in the kidney is dependent on BMP7, *via* p38 MAPK activation[19]. Therefore, a part of the kidney-related phenotype of the *Bmp7*-null mice should be due to the deficiency in *Trps1* activation[19].

An additional role of BMP7 involving the SMAD pathway was recently reported[20]. In the nephrogenic zone, the progenitor populations are partitioned into two distinct compartments: One expressing detectable levels of both CITED1 and Six2 expression, and the other of Six2 only. Importantly, the Six2-only compartment is responsive to the canonical Wnt9/β-catenin signaling, showing appropriate differentiation and epithelialization; meanwhile, the CITED1$^+$/Six2$^+$ compartment is refractory to it[20]. It has been shown that BMP7 promotes the transition of nephron progenitor cells from the CITED1$^+$/Six2$^+$ compartment to the Six2-only compartment *via* the activation of the SMAD pathway. Thus, BMP7 has multiple roles both in the proliferation and differentiation processes of the metanephric mesenchymal progenitor cells, most likely involving distinct signaling pathways.

Use of the two distinct pathways, MAPK and SMAD, in kidney development has also been suggested by the findings from *in vitro* studies. In an *ex vivo* experiment, BMP7 was shown to control branching morphogenesis of the ureteric buds in a dose-dependent manner; specifically, low dosage of BMP7 was shown to induce morphogenesis, while high dosage was shown to exert an inhibitory effect[21]. A subsequent study showed that while the low-dose BMP7 induced p38 MAPK signaling, the high-dose triggered the SMAD pathway, which in turn caused negative regulation of the MAPK activity[22]. Such bimodal regulation might also take place in the developmental context. In this sense, it would be intriguing to understand how secretion of BMP7 from different sites, particularly the ureteric bud or the metanephric mesenchyme, impinges on the differential functional roles in the developmental process. To date, however, this process has remained largely unstudied.

Bmp7 expression continues in the developing kidney, even after the stage when the knockout mice present the severe abnormality. Several studies have aimed to uncover its roles in these later stages. A mouse strain that expresses the Cre recombinase under the promoter of *Nphs2* was used to create a conditional knockout mouse in which *BMP7* has near-complete specific deletion in the podocytes of mature glomeruli[23]. These mice presented with hypoplastic kidneys, and proximal tubules of markedly reduced size. Concomitantly, phosphorylation of p38 MAPK was significantly reduced in the proximal tubules[23].

Interesting phenotypes were observed upon deletion of the *BMP7* alleles at E12.5, which was accomplished using a mouse line expressing an inducible Cre[24]. Deletion after the early stage of nephrogenesis resulted in precocious maturation of glomeruli, as well as increased apoptosis of the progenitor cells. *In vitro* assays further showed that BMP7 inhibits epithelialization of the mesenchymal progenitor cells, which might explain the precocious maturation that was observed[24]. These findings might appear to be contradictory to the above-mentioned model in which BMP7 is required for shifting the competence of the mesenchymal progenitor cells for the differentiation cue[20]; however, at an early stage, the metanephric mesenchymal cells do not need the BMP7/SMAD pathway for differentiation[20]. In fact, the *BMP7* knockout mice can develop nephron structures adequately up to E13.5. Therefore, the reduction of BMP7 at E12.5 might guarantee or even accelerate the early phase of nephron formation. The late stage formation observed in the deletion mice might be attributed to the remaining expression of *BMP7* (around 10% as compared to the controls) after the induction by tamoxifen, particularly as BMP7 exhibits dose-dependency in induction of the downstream cascades[22]. However, further studies are necessary to clarify this issue.

Overall, BMP7 mainly acts to determine and balance the fates of the mesenchymal progenitor cells, between proliferation and differentiation, to determine the final size of the mature kidney. Distinct pathways are utilized for these different roles. Although the mechanism to switch between the different pathways is largely unknown, dose-dependent regulation might contribute, at least partially. Therefore, regulation of the expression of *BMP7* is expected to play a critical role in the developmental process, and this topic will be discussed later in this review.

BMP7 IN THE ADULT KIDNEY

Kidney-specific expression of *BMP7* persists in the mature organ of the adult[7]. Its functional significance has been suggested by a series of studies. All the more,

exogenous administration of BMP7 to injured kidneys was also shown to have therapeutic effects, including prevention of fibrosis, inflammation and apoptosis. We first summarize, here, the findings regarding the latter, and then we discuss the endogenous role of the protein at the end of this section.

Systemic administration of recombinant BMP7 to a rat model of ischemia/reperfusion injury was first shown to enhance recovery after acute injury by suppressing inflammatory responses, apoptosis and fibrosis[25]. In subsequent studies, the administration of BMP7 to a rat model of unilateral ureteral obstruction (UUO) using prevention protocols resulted in blunting of the development of injury[26,27].

Renal fibrosis is considered as a hallmark of chronic kidney diseases, although functional benefits of fibrosis have been recognized recently[28]. TGFβ1 is a key mediator of fibrosis in many tissues, including in the kidney in response to injury (reviewed in[29,30]). Binding of TGFβ1 to its type II receptor, TBRII, triggers the receptor to activate the TGFβ receptor type I (TBRI)-kinase, which in turn induces downstream cascades *via* phosphorylation of SMAD2 and SMAD3[31]. On the other hand, ligand binding to the type I activin-like kinase (Alk) receptors and type II serine/threonine kinase receptors (BMPRII) for BMP7 activates SMAD1/5/8 for SMAD signaling[31].

Roles of these signaling pathways in renal fibrosis were investigated in an *in vitro* model[32]. Incubation of mouse distal tubular epithelial cells (NP1) with TGFβ1 led to induction of epithelial-to-mesenchymal transition (EMT) that was associated with nuclear localization of phosphorylated SMAD2/3[32]. However, addition of BMP7 to this culture system reversed the EMT through phosphorylation and activation of SMAD1, which in turn transcriptionally up-regulated the expression of E-cadherin, an important adhesion molecule in epithelial cells[32].

Based on these findings, the counteraction of BMP7 against the TGFβ1-induced EMT was further tested *in vivo*. Intraperitoneal administration of BMP7 to a mouse model of progressive chronic kidney injury with nephrotoxic serum nephritis (NTN) led to reversal of the renal pathology and to a decline in the mortality rate[32]. The same group also showed that the BMP7 treatment could attenuate progression of chronic kidney fibrosis in two genetic mouse models, namely those of Alport's syndrome and lupus nephritis[33].

Recent studies have revealed involvement of epigenetic regulation in the renoprotective effect of BMP7. *Rasal1*, the gene encoding rasGAP-activating-like protein 1, was shown to be aberrantly hypermethylated in the fibrotic condition that is induced by TGFβ1[34]. Reversal of fibrosis by the administered BMP7 was also found to be associated with active removal of methylation at *Rasal1 via* the 10-11 translocation enzyme-3 (Tet3)[35].

In contrast to the above findings showing the therapeutic effects of BMP7, the function of the endogenously expressed molecule in the adult kidney has not been thoroughly assessed to date. This might be partly due to the technical difficulty of eliminating BMP7 specifically in the adult kidney and not in the developing kidney, so as to avoid the developmental arrest that otherwise leads to death. However, several studies have demonstrated the pivotal role of endogenously expressed BMP7 in protection of the kidney from injuries.

Uterine sensitization-associated gene-1 (USAG1) is a BMP antagonist[36-38], and is abundantly expressed in the adult kidney[38]. The *Usag1* knockout mice show resistance to apoptosis and fibrosis, and a down-regulation in the expression of inflammatory genes, all of which were reinduced upon administration of neutralizing antibodies against BMP7[39]. Thus, BMP7 appears to play a renoprotective role endogenously in the kidney, which is negatively regulated by USAG1. As mentioned above, fibrosis and inflammatory responses upon kidney injury seem to have beneficial effects as well for the renal function, serving to sustain the overall structure of the kidney[28]. In this sense, USAG1 and BMP7 might cooperatively serve to balance the progression of fibrosis, and titration of these two proteins in the progression of kidney diseases might be an exciting approach for therapeutics.

Kielin/chordin-like protein (KCP) is, on the other hand, an enhancer of BMP signaling. Interestingly, the knockout mouse of the encoding gene develops susceptibility to kidney injury, further demonstrating the protective role of BMP7 in the adult kidney[40].

Activin-like kinase 3 (Alk3) is one of the three type I receptors for BMP7[31,41]. During the progression of kidney injury, *Alk3* is up-regulated, while the other receptor genes, *Alk2* and *Alk6*, are not. Loss of Alk3 leads to more severe fibrosis and inflammatory response upon NTN-induced chronic kidney fibrosis, further supporting the theory that BMP7 exerts renoprotective functions through binding to Alk3 endogenously[41]. Furthermore, the small peptide agonist THR-123 exhibits therapeutic effects when applied to different models of kidney injuries, through its interaction with Alk3[41].

REGULATION OF *BMP7* EXPRESSION IN THE EMBRYONIC AND ADULT KIDNEY

In the mouse embryo, *BMP7* is expressed not only in the kidney but also in various other tissues. A recent study showed that expression in extra-nephrotic domains is mostly regulated by long-range enhancers that activate gene expression in a tissue-specific manner around the locus[42]. The previous *in vivo* studies had identified some of the enhancers capable of inducing reporter gene expression in the developing kidney. One such element is located in intron 1 of *Bmp7*, which is strongly conserved in tetrapods[43]. When heterologously linked to the lacZ reporter under control of a minimal promoter sequence (Hsp68lacZ)[44], the element induced lacZ expression in the Wolffian

duct, mesonephric tubules, ureteric bud and collecting duct, from E9.5 until E12.5, but not in the metanephric mesenchymal lineage. Of note, this expression could not be recapitulated by the orthologous sequence in *Xenopus tropicalis*. A lacZ reporter construct under control of the endogenous promoter of *BMP7* was also injected into mouse embryos together with the 4-kb upstream and the 3-kb downstream regions of the transcription start site (TSS) at each side, retaining the endogenous context[43]. This entire construct was able to induce reporter expression in the nephrogenic mesenchymal regions. Interestingly, however, none of the separated individual elements covering the upstream and downstream regions was able to drive the expression when linked to Hsp68lacZ[43].

Chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) was performed in $Six2^+$ nephron progenitor cells[45]. A region located at 98-kb downstream of the TSS of *BMP7* was found to be co-bound by Six2 and β-catenin. When tested *in vivo*, this element induced gene expression in a compartment of $Six2^-$ renal vesicles, a part of the *BMP7* expression domain[45]. The ChIP-seq also identified a Six2 binding site in intron 1, an evolutionarily conserved region adjacent to the intron 1 enhancer, though a reporter construct including this region did not show enhancer activity in the developing kidney[43]. These results suggest that *BMP7* expression in the different compartments of the developing kidney, notably the ureteric bud and the metanephric mesenchyme, might be regulated by a distinct set of enhancers that are active in the respective domains, possibly interacting in a cooperative manner with each other. However, to determine whether or not these elements actually contribute to the *BMP7* expression in the kidney, testing by deletion of the respective regions should be performed in future studies.

Bmp7 is highly and specifically expressed in the adult kidney under normal physiologic conditions[7]. The main expression domains are the ureter, collecting duct, thick ascending limb, distal convoluted tubules and podocytes in the glomerulus[46,47]. *BMP7* expression in these cell types might also be regulated by *cis*-regulatory enhancers that are embedded around the locus. However, to date, no such enhancer elements have been described for the expression in adult kidney. Acetylation of K27 of histone H3 (H3K27ac) is associated with enhancer activity of the marked regions. We compared released data from the ENCODE project of ChIP-seq for H3K27ac in kidney tissues at different time points, ranging from E14.5 to the adult stage[48]. In Figure 1, the regions with peaks are more or less common between different stages, but there are some striking differences. Notably, the region of the intron 1 enhancer is highly acetylated at the embryonic stages, but the mark is almost diminished for the adult kidney. These data might suggest that different sets of *cis*-regulatory regions contribute to the expression at different stages.

Once the kidney is damaged, the levels and sites of *BMP7* expression are dynamically altered[15,47]. The injuries cause dramatic response to the cellular states in the kidney *via* the inflammatory response and other signaling cascades, such as that involving TGFβ1. Such responses are expected to lead to alteration of epigenetic states around the *BMP7* locus; as a result, *BMP7* expression would be dynamically regulated. In the following passages of this review, we review the expression dynamics of *BMP7* in several kidney disease models, as reported in the literature to date, and discuss how they are regulated.

In the kidneys of the ischemia/reperfusion rat model, *BMP7* expression dramatically decreases soon after reperfusion, particularly in the outer medulla and glomeruli[47,49]. This reduction might be related to the up-regulation of TGFβ1. The immunostain signal of BMP7 increases in proximal tubular epithelial cells, which are devoid of its expression in the normal situation[50]. Similar up-regulation of *BMP7* in the proximal tubular epithelial cells following ischemia was confirmed in a mouse model[51]. Proximal tubular epithelial cells from human patients with proteinuric nephropathies also showed up-regulation of *BMP7* as compared to that in healthy controls[52]. In an experimental model of diabetic nephropathy, *BMP7* expression was decreased[53], which might be due at least partly to the concomitant increase in *Tgfb1* expression. A tubular injury induced by folic acid resulted in reduced *BMP7* expression at first, but was followed by a gradual recovery of expression in the regenerative phase[15]. In the cisplatin nephrotoxicity model, however, no or only a subtle increase in *BMP7* expression was scored[15,54].

It has long been postulated that TGFβ1, which is an inducer of the fibrotic response upon injury, down-regulates *BMP7* expression (as discussed above). On the other hand, MyoR has been implicated in the activation of *Bmp7*[54,55]. However it has not been assessed adequately to conclude whether these effects are direct or not.

Epigenetic regulation has been studied to understand the direct linkages between various cellular states and *BMP7* expression. In TGFβ1-induced EMT in human renal proximal tubular epithelial cells, *BMP7* is slightly down-regulated[56]. Treatment with trichostatin A, a histone deacetylase (HDAC) inhibitor, however, led to deposition of acetylated histones around the promoter of *BMP7* and to induction of its expression, thereby counteracting the fibrosis[56]. Consistently, in an ischemia/reperfusion mouse model, down-regulation of HDAC5 was found to be involved in the activation of *BMP7* during the regenerative phase following injury, probably *via* acetylation of histones[51].

Another layer of epigenetic regulation might also impinge on the expression of *BMP7* in kidney. The topological conformation of chromatin has recently been recognized as an important determinant of transcriptional regulation of genes. Particularly, a topologically-associating domain (TAD; a compartmentalized block of the genome,

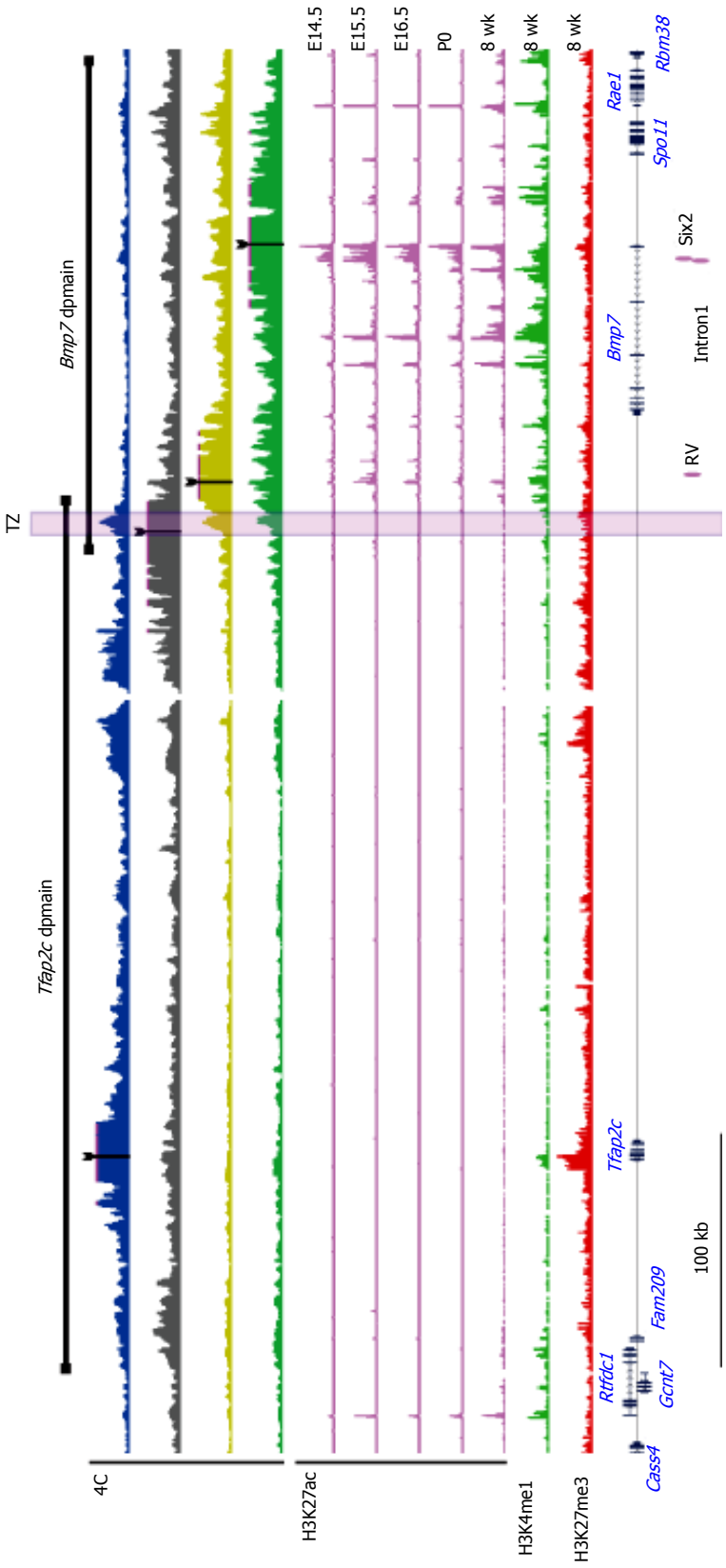

Figure 1 Landscape of the enhances in the kidney and topological chromatin domains around the *BMP7* locus. Exons and introns of the genes within the locus (chr2: 172,250,000-172,850,000 mm9) are represented by blue boxes and arrowed dashes, respectively. Names of the genes are indicated above or below the respective boxes. The ChIP-seq signals of H3K27ac in the kidney tissue at different stages [E14.5, E15.5, E16.5, postnatal day (P)0 and 8-wk-old; indicated to the right] are shown with pink plots. H3K4me1 and H3K27me3 signals in the adult kidney are shown with green and red plots, respectively. The ChIP-seq data were obtained from the ENCODE project[48]; the Data Coordination Consortium accession numbers are ENCSR703ZPF, ENCSR000CAF and ENCSR000CFP for H3K27ac, H3K4me1 and H3K27me3, respectively. Enhancer candidates are represented by pink ovals at the bottom: The RV enhancer is bound by Six2 and β-catenin, and induces reporter expression in the Six2-compartment of the renal vesicle[45]; the intron 1 enhancer drives reporter expression in the developing ureteric buds[43]; the Six2 binding site next to the intron 1 enhancer was identified by ChIP-seq[45] but is not sufficient to induce gene expression in the developing kidney[43]. Note that the H3K27ac mark around the intron 1 enhancer during embryogenesis diminishes in the adult stage. The chromatin domains identified by 4C-seq are shown at the top (indicated by whiskered lines), together with the actual results of the 4C-seq that are shown below[42]. The viewpoints of the 4C-seq are indicated by arrows on the plots: Tfap2c promoter (blue), transition zone (TZ; gray), next to TZ in the BMP7 domain (yellow), and BMP7 promoter (green). The European Nucleotide Archive accession number of the 4C-seq data is ERP005557[42]. The TZ between the two domains is indicated by the purple rectangle that spans the entire diagram.

in which the genomic regions preferentially associate with each other) was characterized as a ground-state structure that facilitates and constrains the interaction between enhancers and promoters of genes within it[57-59].

Bmp7 is flanked by a large intergenic region, at the other side of which a developmental gene *Tfap2c* is located (Figure 1). A recent study showed that the locus is conformationally partitioned into two adjacent domains, one for *BMP7* and the other for *Tfap2c*, by function of a region at the boundary, termed TZ[42]. At

this locus, the action of enhancers is limited to genes located within the same domain that they belong to. In Figure 1, the TZ and 4C (circular chromatin conformation capture) plots that describe the physical domain structure were overlaid on the ChIP-seq map. It is apparent that the acetylation only extends within the *BMP7* domain and not to the neighboring one, underlining the importance of the topological structure for the regulation of *BMP7* in the kidney as well (Figure 1).

TADs seem to be more or less stable in different cell types. This might be due to the fact that CTCF, a ubiquitous DNA binding protein, greatly contributes to the formation of the domain structures[57,60]. However, the topological structure is also a function of other epigenetic modifications, such as transcription of constitutive genes and polycomb group proteins[57,61]. Indeed, the extent that enhancers can activate genes is sometimes different among different enhancers at the same locus[42,59]. Therefore, it might be possible that the topological structure is subject to regulation for the dynamic expression of *BMP7* in response to kidney injuries.

CONCLUSION

BMP7 plays an important role in development and diseases of the kidney. In development, BMP7 is critical both in proliferation and maintenance of the kidney's mesenchymal stem cells and in shifting their competence to respond to differentiation cues. Consequently, BMP7 is a critical determinant of nephron numbers and the size of the organ. At the adult stage, BMP7 is implicated in protection and regeneration of the kidney upon injury. Moreover, administration of BMP7 and its mimetic exerts therapeutic effects in conditions of both acute kidney injuries and chronic kidney diseases.

Precise regulation of the *BMP7* gene is critical to its function in the kidney. At the embryonic stage, the major expression sites are metanephric mesenchyme and ureteric buds, two different lineages that interact with each other. Studies to date have indicated that the active locale of BMP7 is the mesenchymal cells, rather than the ureteric epithelia, but the functional difference of the expression sites remains elusive. In this sense, it will be insightful to identify *cis*-regulatory elements that induce *BMP7* expression in the different cell populations and to test impacts of mutations in the elements on kidney development. The regulatory mechanisms of the adult kidney also remain elusive. Identification of the enhancers will provide insight into the still opaque role of BMP7 in the adult kidney.

Understanding the epigenetic mechanism may not only clarify the dynamic regulation of the gene, but also open up a new avenue for therapeutics for kidney diseases through control of the expression of *Bmp7*. Different layers of epigenetic regulation, such as DNA methylation, histone modifications and higher-order chromatin conformation, almost certainly will bear a role in achieving delicate control of the gene's expression. Each of the layers represents a possible target for therapeutics. Indeed, inhibition of HDAC has already shown a promising effect in augmenting *BMP7* expression in a TGFβ1-induced fibrosis model[56]. Furthermore, recent advances in genome editing tools, such as the CRISPR/Cas9 system, might allow us to control epigenetic modifications, including higher-order chromatin conformation, in a locus-specific manner to optimize the gene expression[62]. To this end, it will be beneficial to further deepen our understanding both of the role and regulation of BMP7 in kidney development and diseases.

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MINIREVIEWS

Stem cell-derived exosomes as a therapeutic tool for cardiovascular disease

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Abstract

Mesenchymal stem cells (MSCs) have been used to treat patients suffering from acute myocardial infarction (AMI) and subsequent heart failure. Although it was originally assumed that MSCs differentiated into heart cells such as cardiomyocytes, recent evidence suggests that the differentiation capacity of MSCs is minimal and that injected MSCs restore cardiac function *via* the secretion of paracrine factors. MSCs secrete paracrine factors in not only naked forms but also membrane vesicles including exosomes containing bioactive substances such as proteins, messenger RNAs, and microRNAs. Although the details remain unclear, these bioactive molecules are selectively sorted in exosomes that are then released from donor cells in a regulated manner. Furthermore, exosomes are specifically internalized by recipient cells *via* ligand-receptor interactions. Thus, exosomes are promising natural vehicles that stably and specifically transport bioactive molecules to recipient cells. Indeed, stem cell-derived exosomes have been successfully used to treat cardiovascular disease (CVD), such as AMI, stroke, and pulmonary hypertension, in animal models, and their efficacy has been demonstrated. Therefore, exosome administration may be a promising strategy for the treatment of CVD. Furthermore, modifications of exosomal contents may enhance their therapeutic effects. Future clinical studies are required to confirm the efficacy of exosome treatment for CVD.

Key words: Exosomes; Messenger RNA; Cardiovascular disease; Mesenchymal stem cells; Stem cells; MicroRNA

Core tip: Exosomes are membrane vesicles that contain and transport specific bioactive molecules, such as proteins, messenger RNAs, and microRNAs, to recipient cells. In this review, we describe the mechanisms of

exosome biogenesis, selective sorting of bioactive molecules into exosomes, and exosome secretion. We also discuss preclinical studies in which stem cell-derived exosomes were successfully used to treat cardiovascular disease (CVD). Finally, we discuss the future possibility of exosome-based clinical treatment of CVD.

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. Owing to recent advances in the treatment of acute myocardial infarction (AMI) using percutaneous coronary intervention or bypass surgery, the survival of patients with AMI has substantially improved. However, many of these survivors develop heart failure (HF) as a result of the death of cardiomyocytes and subsequent tissue remodeling. As the induction of the proliferation and differentiation of the remaining cardiac tissue to regenerate heart structure remains challenging, heart transplantation is still the only treatment option for fatal HF. The development of new therapies for AMI and HF is thus required to improve the outcome in these patients.

Recently, many attempts have been made to improve the outcome of AMI and ischemic HF (IHF) using stem cells in preclinical[1-4] and clinical[5-10] studies. Among of the various stem cells, mesenchymal stem cells (MSCs), particularly bone marrow-derived MSCs, have been used to treat patients with AMI and IHF in clinical trials, with their safety and efficacy demonstrated in some studies[5-10]. The earliest preclinical studies suggested that MSCs have the potential to differentiate into multiple cardiac cell types including cardiomyocytes, vascular endothelial cells, and vascular smooth muscle cells[1-3]. However, subsequent studies did not demonstrate this remarkable differentiation capacity of MSCs. Rather, it was reported that most intravenously injected cells are trapped in the lung rather than engrafted in the heart[11,12]. Even when MSCs are administered to the swine heart *via* the coronary artery following AMI induction, only 6% of the injected cells remained in the infarct zones 14 d after AMI induction[11]. Furthermore, the supernatant of MSC cultures reportedly improves cardiac function[13-15]. These results suggest that MSCs improve cardiac function *via* the secretion of paracrine factors rather than *via* the direct differentiation of MSCs into cardiac cell types. Furthermore, MSC transplantation has several problems such as low survival rate and stem cell tumorigenesis[16]. However, if MSC-secreted paracrine factors can efficiently repair and regenerate cardiac tissues, cell-free therapy is possibly a safer alternative in the future.

Recently, a variety of cell types, including stem cells, have been shown to secrete paracrine factors in not only naked forms but also membrane vesicles, such as exosomes, microvesicles, ectosomes, membrane particles, exosome-like vesicles, and apoptotic bodies[17]. Exosomes are one of the secreted vesicles (also referred to as extracellular vesicles or EVs) that are 30-100 nm in diameter and contain a variety of biologically active molecules, such as proteins, messenger RNAs (mRNAs), and microRNAs (miRs)[18]. In this manuscript, we review the characteristics of exosomes and their possible applications in CVD treatment.

EXOSOME ISOLATION AND IDENTIFICATION

Several strategies have been used to isolate exosomes from tissues. These strategies utilize ultracentrifugation, size-based purification, precipitation using polymers, and immunoaffinity purification as reviewed in some reports[19-21]. Ultracentrifugation is the most established method of exosome isolation which employs sequential centrifugation combined with sucrose density gradient ultracentrifugation. Size-based purification includes ultrafiltration and gel filtration methods. Alternatively, polymers such as polyethylene glycol, widely used to precipitate proteins and viruses, can also be used to precipitate exosomes. As exosomes express specific proteins and lipids on their surface, antibodies recognizing these molecules (frequently conjugated with magnetic beads) are also used in their isolation.

Identification of exosomes is usually achieved by evaluating their morphology and size, their motion in a solution, and the specific molecules they express, as previously reviewed[22,23]. Electron microscopy is commonly employed to measure the size and assess the morphology of exosomes. The number of particles corresponding to exosome size can be counted by nanoparticle tracking analysis. This method utilizes the phenomenon of Brownian motion in a liquid suspension to measure particle size. Because exosomes are derived from endosomes and are finally released from cells as described in the following section, molecules involved in exosome formation, such as tetraspanins (CD81, CD9, and CD63), are expressed in exosomes. These markers can be used to identify exosomes.

EXOSOME BIOGENESIS, SECRETION, AND UPTAKE BY RECIPIENT CELLS

Exosomes are derived from endosomes that are formed by the inward budding of the plasma membrane (Figure 1)[18]. The subsequent inward budding of the endosomal membrane results in the formation of intraluminal vesicles (ILVs) into which cytoplasmic molecules, such as proteins, mRNAs, and miRs are sorted[24,25]. These endosomes containing ILVs, or multivesicular bodies

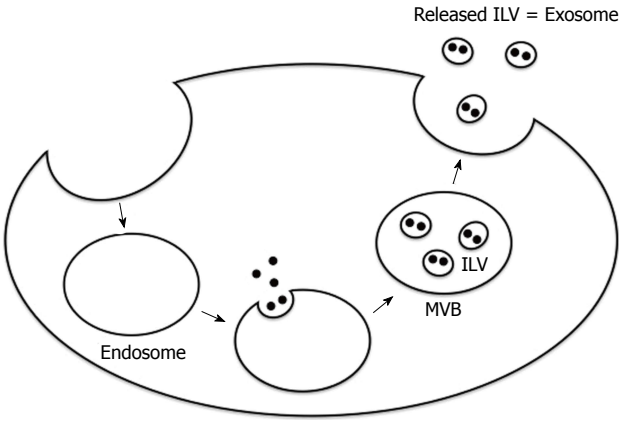

Figure 1 Schematic diagram showing exosome biogenesis and release. ILV: Intraluminal vesicle; MVB: Multivesicular body.

(MVBs)[18], fuse with the plasma membrane and release ILVs into the extracellular environment by exocytosis. These secreted ILVs containing biologically active molecules are referred to as exosomes.

The mechanisms of exosome formation and processing are just starting to be revealed. The formation of MVBs is reportedly mediated by the endosomal sorting complexes required for transport (ESCRT) system or by systems independent of the ESCRT machinery as summarized in some reviews[26-28]. The ESCRT machinery comprises four protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, together with accessory proteins. ESCRT-0 recognizes ubiquitinated proteins and is recruited to the endosomal membrane, where it initiates processes leading to the uptake of ubiquitinated proteins into ILVs. ESCRT-0 subsequently recruits ESCRT-I to the endosomal membrane, which in turn recruits ESCRT-II and ESCRT-III. ESCRT-III induces the inward budding of the endosomal membrane and formation of ILVs, while accessory proteins (particularly the vacuole protein sorting gene 4 ATPase or VPS4) are implicated in the dissociation and recycling of the ESCRT machinery. In addition, other molecular pathways mediate ESCRT-independent MVB formation including tetraspanins[29] such as CD81, CD9, and CD63, and proteolipid proteins such as ceramide[30].

The docking and fusion of MVBs to the plasma membrane appear to be mediated by soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins such as vesicle-associated membrane protein 7 (VAMP7)[31]. The release of ILVs (exosomes) from cells following the fusion of MVBs to the plasma membrane is mediated by several mechanisms. The small GTPases of the Rab family (Rab27a/b, Rab11, and Rab35) are the most studied molecules involved in exosome release[32-34]. Other pathways include WNT5A, glycosphingolipids, flotillins, and stress-induced stimuli such as the increase in intracellular calcium concentration, DNA damage, heat shock, and hypoxia[35-39]. In addition, an acidic environment has been shown to trigger the secretion of exosomes from cells[40].

Once released from cells, exosomes bind to target cells *via* ligand-receptor interactions. Molecules, such as integrins, intercellular adhesion molecules, and tetraspanins seem to be implicated in the binding of exosomes to recipient cells[41-43]. After binding, exosomal contents are reportedly internalized by recipient cells *via* two major mechanisms as summarized in some reviews[23,44]: (1) exosome fusion with the plasma membrane of recipient cells and direct release of contents into the cytoplasm; or (2) internalization by endocytosis into recipient cells. It has been demonstrated that bioactive molecules in exosomes are not only transferred to recipient cells but also exert functional effects[45-47].

Although the precise mechanism remains unknown, a specific set of proteins, mRNAs, and miRs are selectively accumulated within exosomes[48]. It has also been demonstrated that exosomes contain a distinct set of mRNAs compared to the donor cells[49]. Ubiquitination appears to be required for the uptake of some proteins into exosomes[50], although ubiquitination-independent accumulation of proteins has also been reported[51]. The accumulation of miRs into the exosomes of T cells appears to require the recognition of a GGAG sequence located in miRs by the heterogeneous nuclear ribonucleoprotein hnRNPA2B1[49].

Taken together, accumulating evidence indicates that exosomes are a natural vehicle for the efficient and specific transport of biologically active cargo into recipient cells. These properties may be exploited for the delivery of bioactive molecule such as miRs and chemical compounds such as drugs. For instance, stem cell-derived exosomes may be useful for CVD treatment. We review the potential utility of stem cell-derived exosomes for CVD treatment in the following section.

THERAPEUTIC EFFECTS OF STEM CELL-DERIVED EXOSOMES ON CVD

MSC-derived exosomes

Several preclinical studies have demonstrated the efficacy of MSC-derived exosomes for CVD treatment (Table 1). Lai *et al*[52] found that the supernatant of human embryonic stem cell (ESC)-derived MSCs contained small particles (50-100 nm in diameter) corresponding to exosomes. When administered to a mouse model of myocardial ischemia/reperfusion injury, these exosomes remarkably reduced infarct size. The same group also administered exosomes secreted from human ESC-derived MSCs to a mouse model of AMI and demonstrated improved cardiac function[53]. In addition, they found that the tissue levels of ATP and nicotinamide adenine dinucleotide were significantly increased, while those of reactive oxygen species were significantly decreased after exosome administration. Furthermore, they demonstrated that the phosphorylation of Akt and glycogen synthase kinase 3 (that has anti-apoptotic effects) significantly increased and that of c-jun N-terminal kinase (that has proapoptotic

Table 1 Effects of exosome administration on cardiovascular disease models

Origin of exosomes	Experimental model	Findings	Ref.
Human ESC-derived MSCs	AMI	Reduction in infarct size Recovery of cardiac function Decreased oxidative stress Activation of Akt and GSK3 Inhibition of c-JNK	Lai *et al*[52,53]
Human MSCs	AMI	Reduction in infarct size Recovery of cardiac function Increased angiogenesis	Bian *et al*[54]
Mouse MSCs	AMI	Exosomes were enriched in miR-22 miR22 was implicated in the anti-apoptotic effect of exosomes	Feng *et al*[55]
Rat MSCs overexpressing GATA-4	AMI	Reduction in infarct size Recovery of cardiac function Exosomes were enriched in miR-19a	Yu *et al*[56]
Rat MSCs	Stroke	Recovery of neurological function Stimulation of neurogenesis and angiogenesis	Xin *et al*[57]
Rat MSCs overexpressing miR-133b and those whose expression of miR-133b was knocked down	Stroke	Recovery of neurological function was mediated by miR-133b expressed in exosomes	Xin *et al*[58]
Mouse MSCs	Pulmonary hypertension	Reduction in the progression of pulmonary hypertension and right ventricular hypertrophy	Lee *et al*[59]
Mouse CPCs	AMI	Suppression of apoptosis	Chen *et al*[60]
Human CPCs	AMI	Recovery of cardiac function Suppression of apoptosis Stimulation of angiogenesis	Barile *et al*[61]
Human CPCs	AMI	Recovery of cardiac function Suppression of apoptosis Stimulation of angiogenesis miR-146a was enriched in exosomes and partially mediated their function	Ibrahim *et al*[62]
Mouse ESCs	AMI	Recovery of cardiac function Stimulation of angiogenesis and cardiomyocyte survival Stimulation of the survival and proliferation of CPCs miR-294 was enriched in exosomes and miR-294 promoted the survival and proliferation of CPCs	Khan *et al*[63]
Human CD34+ cells	Matrigel plug assay Corneal angiogenesis assay	Promotion of angiogenesis	Sahoo *et al*[64]
Human CD34+ cells expressing SHH	AMI	Recovery of cardiac function SHH was enriched in exosomes and transferred to recipient cells	Mackie *et al*[66]

ESC: Embryonic stem cell; MSCs: Mesenchymal stem cells; CPCs: Cardiac progenitor cells; SHH: Sonic hedgehog; AMI: Acute myocardial infarction; GSK3: Glycogen synthase kinase 3; c-JNK: c-jun N-terminal kinase.

effects) significantly decreased in cardiac tissue following exosome administration. Bian *et al*[54] demonstrated the proliferation and migration of human umbilical vein endothelial cells in response to EVs (100 nm in diameter) collected from human MSCs. They also administered MSC-derived EVs to a rat model of AMI and showed that MSC-derived EV administration significantly reduced infarct size, restored cardiac function, and stimulated angiogenesis in the ischemic zone. Feng *et al*[55] demonstrated that exosomes secreted from mouse MSCs following ischemic preconditioning contained a large amount of miR-22. When administered to mice with AMI, these miR-22-enriched exosomes exerted an anti-apoptotic effect on cardiomyocytes *via* the downregulation of methyl-CpG-binding protein 2. Yu *et al*[56] used MSCs overexpressing the transcription factor GATA-4 (MSC_GATA-4) and demonstrated that the administration of MSC_GATA-4-derived exosomes restored cardiac function and reduced infarct size in a rat model of AMI. The authors also showed that MSC_GATA-4-derived exosomes expressed a greater amount of miRs, particularly miR-19a, than control MSCs and that miR-19a appeared to be involved in the cardioprotective effect of MSC_GATA-4-derived exosomes *via* the downregulation of phosphatase and tensin homolog (PTEN) and subsequent activation of anti-apoptotic Akt and extracellular signal-regulated kinase.

Preclinical studies have also reported favorable effects of exosome administration on neurological recovery following stroke induction. Xin *et al*[57] found that the systemic administration of rat MSC-derived exosomes following the induction of stroke by the ligation of the middle cerebral artery significantly accelerated neurological recovery and stimulated neurogenesis and angiogenesis at the border zone between normal and ischemic tissues. The same group also demonstrated that the administration of MSCs overexpressing miR-133b (MSCs_miR-133b+) enhanced the recovery of neurological function in a rat stroke model whereas MSCs

with miR-133b knockdown (MSCs_miR-133b-) did not[58]. Furthermore, they showed that the level of miR-133b in exosomes isolated from cerebrospinal fluid was higher in the group that received MSCs_miR-133b+. They also demonstrated that MSC-derived exosomes could be transferred to neighboring cells. Finally, they showed that the expression of connective tissue growth factor (CTGF), a target for miR-133b, was significantly reduced in the ischemic boundary zone following MSCs_miR-133b+ administration, while CTGF expression remained unchanged after MSCs_miR-133b- administration. They concluded that miR-133b derived from exosomes was implicated in MSC-mediated recovery of neurological function in this model.

The beneficial effects of MSC-derived exosome administration have also been reported in a mouse model of hypoxic pulmonary hypertension. Lee *et al*[59] demonstrated that the administration of MSC-derived exosomes significantly ameliorated the progression of pulmonary hypertension and right ventricular hypertrophy, possibly *via* the suppression of signal transducer and activator of transcription 3 (STAT3).

Cardiac progenitor cell-derived exosomes

Chen *et al*[60] demonstrated that the injection of exosomes isolated from murine cardiac progenitor cells (CPCs) into the murine heart following ischemia/reperfusion injury significantly suppressed apoptosis. Barile *et al*[61] demonstrated that the administration of EVs (most of which were exosomes) isolated from human CPCs significantly suppressed apoptosis, stimulated angiogenesis, and improved cardiac function in a rat model of AMI. They also showed that specific miRs, such as miR-210, miR-132, and miR-146a-3p, were enriched in CPC-derived exosomes. Ibrahim *et al*[62] reported that the administration of human CPC-derived exosomes in a mouse model of AMI significantly suppressed apoptosis, stimulated angiogenesis, and restored cardiac function. They also demonstrated that miR-146a was enriched in CPC-derived exosomes and that miR-146a administration partially mimicked the beneficial effects of CPC-derived exosomes on cardiac function.

ESC-derived exosomes

Khan *et al*[63] reported that ESC-derived exosomes from mouse stimulated neovascularization, enhanced cardiomyocyte survival, and restored cardiac function in a mouse model of AMI. Furthermore, ESC-derived exosomes augmented the survival and proliferation of CPCs. miR-294 was enriched in ESC-derived exosomes and the treatment of CPCs with miR-294 promoted the progression of the cell cycle to the S phase, suggesting that ESC-derived exosomes transferred miRs, such as miR-294, to CPCs, which promoted the proliferation and survival of CPCs.

CD34+ stem cell-derived exosomes

Sahoo *et al*[64] isolated exosomes from human CD34+ stem cells (which include endothelial progenitor cells[65]) and examined their proangiogenic activity. CD34+ stem cell-derived exosomes stimulated tube formation from cultured endothelial cells in Matrigel (*in vitro* assay), and promoted angiogenesis *in vivo*, as assessed by the Matrigel plug assay and the corneal angiogenesis assay. Mackie *et al*[66] demonstrated that CD34+ stem cells expressing the pro-angiogenic factor sonic hedgehog (SHH) restored cardiac function in a mouse model of AMI. They also showed that SHH was enriched in exosomes secreted from stem cells and that it was transferred to and expressed functionally in recipient cells, suggesting that exosome-mediated transfer of SHH to recipient cells accounts for the beneficial effects of stem cell administration in this model of AMI.

Collectively, these studies provide compelling evidence that exosomes derived from a variety of stem cells exert beneficial effects on animal models of CVD.

FUTURE DIRECTIONS

Clinical trials

Although clinical trials using exosomes for CVD treatment have not yet started, exosome administration in humans has been tested, particularly for cancer immunotherapy[67-69]. Phase I and phase II studies have been performed and the safety of the treatment has been confirmed. Future clinical studies will be required to test the safety and efficacy of exosome treatment for CVD.

Modification of exosomes

Given the low toxicity, high stability in the circulation, and high efficiency of transport to donor cells demonstrated by exosomes, several studies have attempted to augment the therapeutic efficacy by modifying exosomal content. For instance, small RNAs such as small interfering RNAs and miRs have been loaded into exosomes during exosome formation using lipofection or following exosome formation using electroporation[70-74]. These modified exosomes reportedly exerted biological effects in recipient cells[70-74]. Exosomes have also been used as vehicles to transport exogenous chemical compounds to recipient cells stably and efficiently, because some drugs are condensed in the exosomes of donor cells and transferred to recipient cells. Exosomes enriched in curcumin, an anti-inflammatory agent, or chemotherapeutic agents, such as paclitaxel and doxorubicin, have been used to transport these compounds to recipient cells, with their beneficial biological effects confirmed[75-78]. Another strategy that has been examined is the modification of exosomal membrane proteins to improve the efficiency of uptake by recipient cells. Alvarez-Erviti *et al*[70] prepared dendritic cells that expressed Lamp2b, an exosomal membrane protein, fused to a peptide fragment of neuron-specific rabies viral glycoprotein so that exosomes would be accumulated specifically in the brain. The authors demonstrated that these modified exosomes were specifically taken up by

brain tissues when intravenously administered. Therefore, the modification of exosome structure will enhance the specificity and efficiency of transport and the modification of exosome content (for example, by inclusion of specific miRs) will enhance the therapeutic effect in the future.

Exosome-induced tumorigenesis

It has been reported that MSC-derived exosomes promote tumor growth *in vivo via* the stimulation of vascular endothelial growth factor expression in tumor cells[79]. In most cases, the stimulation of angiogenesis appears to be favorable for the regeneration of cardiomyocytes after AMI. However, angiogenesis may stimulate tumor growth in other tissues. Therefore, it is desirable to explore a strategy to specifically deliver exosomes to target tissues.

CONCLUSION

Exosomes are one of the secreted vesicles that contain bioactive molecules, such as proteins, mRNAs, and miRs. Exosomes transfer these bioactive molecules to recipient cells, thus exerting biological effects. Preclinical studies have suggested that exosomes can be used for the treatment of CVD such as AMI and stroke. Future clinical studies are warranted to confirm the efficacy of exosome administration for CVD treatment. Furthermore, modifications of exosomal structure and content will enhance the efficacy of exosome administration for such treatments in the future.

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