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Review Article

Early Growth Response Genes Signaling Supports Strong Paracrine Capability of Mesenchymal Stem Cells

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MSCs provide a promising method for cell therapy through their wound healing and tissue regenerative properties. Originally, MSCs' role in wound healing was thought to be tied to their multipotency, but it is now accepted that MSCs mediate the healing process through their strong paracrine capability. EGF was shown to facilitate *in vitro* expansion of MSCs without altering multipotency. Our previous data suggest that the molecular machinery underlying MSCs' strong paracrine capability lies downstream of EGFR signaling, and we focus on transcription factors EGR1 and EGR2. Evidence suggests that EGR1 regulates angiogenic and fibrogenic factor production in MSCs, and an EGFR-EGR1-EGFR ligands autocrine loop is one of the underlying mechanisms supporting their strong paracrine machinery through EGR1. EGR2 appears to regulate the expression of immunomodulatory molecules. Chronic nonhealing wounds are ischemic, inflammatory, and often fibrotic, and the hypoxic micro-environment of these wounds may compromise MSCs' wound healing properties *in vivo* by upregulating the EGR1's fibrogenic effects and downregulating the EGR2's immuno-modulatory effects. Thus, these transcription factors can be potential targets in the optimization of cell-based therapies. Further study *in vitro* is required to understand MSCs' paracrine machinery and to optimize it as a tool for effective cell-based therapies.

1. Overview of MSCs

Adult bone marrow multipotential stromal cells or mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into multiple cell lineages, such as osteocytes, adipocytes, and chondrocytes [1–7]. Because of their strong tissue regenerative, wound repair, and immunomodulatory effects, cell therapy with MSCs is highly promising against various diseases in the fields of regenerative medicine and immunology [8–15].

MSC-based therapeutics was shown to accelerate the wound repair process in various animal models and pilot clinical studies including limb ischemia and coronary arterial diseases [8–14, 16–19]. However, beneficial results of stem/progenitor cell therapeutics in initial small-scale clinical studies have not been reproduced by subsequent randomized controlled trials, strongly indicating *the urgent needs of further optimization of cell-based therapy* [20].

Initially, these cells were simply viewed as cellular blocks to resupply the regenerating and repairing tissues through their multidifferentiation potential; however, it is now widely accepted that MSCs' strong paracrine capability of various bioactive molecules such as vascular endothelial growth factor (VEGF) or indoleamine dioxygenase-1 (IDO1) plays a key role in MSC-based therapeutics actions [8, 15, 21–23]. In effect, MSCs, which reside within the perivascular space [24], can be viewed as paracrine delivery vehicles. Understanding of the molecular mechanism of the strong paracrine machinery of MSCs could lead to the identification of novel therapeutic targets and maximization of immuno-modulating, wound healing, and tissue regenerating effects of MSC-based therapeutics [25].

2. Roles of Epidermal Growth Factor Receptor Signaling in MSCs

In vitro, MSC expansion with animal component-free artificially-defined culture media is ideal for MSC preparation

for clinical use to maximize the safety of MSC-based therapeutics [26–29]. Identification of key molecular factors for *in vitro* MSC expansion and understanding the molecular mechanism of MSCs' strong paracrine capability should provide key knowledge for *in vitro* MSC expansion without using any animal components while maintaining MSCs' paracrine capability. Moreover, advanced knowledge of molecular regulation of the angiogenic, mitogenic, fibrogenic, and immunomodulatory properties would allow for the MSC preparation of personalized properties to best fit the clinical needs of individual patients.

We previously showed that epidermal growth factor (EGF) could be used for *in vitro* MSC expansion without compromising their multidifferentiation potential [6, 30]. Moreover, EGF stimulation enhances the production of multiple growth factors and cytokines including VEGF, hepatocyte growth factor (HGF), Heparin-binding epidermal growth factor-like growth factor (HBEGF), and interleukin-6 (IL6) [25, 26]. These data strongly suggest that EGF can be used for *in vitro* MSC expansion and enhancement of their paracrine capability.

EGF receptor (EGFR) is a prototypal receptor tyrosine kinase widely expressed in many types of cells including MSCs [25, 26]. Upon binding of EGFR ligands such as EGF, HBEGF, or amphiregulin (AREG), EGFR undergoes dimerization and autophosphorylation through its intrinsic tyrosine kinase activity and activates numerous signaling pathways including the protein kinase C (PKC) pathway and the p42/44 mitogen-activated protein kinase (MAPK) pathway [6, 25, 26, 31].

Based on our previous studies [6, 26], we speculated that the molecular machinery supporting MSCs' strong paracrine capability should be located downstream of EGFR signaling, and we analyzed the publicly available microarray database (GSE9451) to see whether transcription factors regulating the expression of growth factors and cytokines downstream of EGFR signaling are differentially expressed in human iliac bone marrow MSCs and human skin fibroblasts, another type of mesenchymal cells akin to MSCs but with reduced differentiation and paracrine capability. Our analysis showed that *early growth response genes-1, -2, and -3 (EGR1-3)* are expressed in MSCs at much higher levels than in fibroblasts (Table 1) [25]. High baseline expression of EGR1-3 in MSCs might reflect the activated state of MSCs in culture, as suggested by Caplan [32]. *EGRs* encode a zinc finger transcription factor (TF) whose activity is mainly regulated at the gene transcription level, and gene expression is upregulated in response to various growth factors and cytokines such as epidermal growth factor (EGF). Once induced, EGRs regulate the gene expression of various growth factors, cytokines, their cognate receptors, and other bioactive molecules [25, 33, 34].

Table 1: *EGR1-3* gene expression in human primary fibroblasts (FBs) and human primary mesenchymal stem cells (MSCs) from GEO database (GSE9451). *EGR1* data was published previously [25]. Gene expression was given in arbitrary units.

	FBs	MSCs	*P* value
EGR1	325.5	1223.8	0.002
EGR2	4.1	50.1	0.024
EGR3	5.7	64.7	<0.001

3. Possible Roles of EGR1-3 in MSCs

Although EGR1-3 has a highly conserved DNA-binding domain and share conserved zinc finger DNA-binding sequences [35], each EGR is regulated by distinct signaling pathways; for instance, PKC inhibitor bisindolylmaleimide I (BIM) weakly inhibits EGR1 induction [25], but it moderately inhibits EGR2 induction and totally abolishes EGR3 induction in MSCs (unpublished data). Moreover, each EGR confers functions that are largely distinctive from each other [36–39]. Among EGR1-3, EGR1 is the most studied, and its multiple roles have been proposed. For example, EGR1 is identified as one of the key molecules contributing to the development of atherosclerosis, intimal thickening after acute vascular injury, ischemic pathology, angiogenesis, allograft rejection, and cardiac hypertrophy [33, 40]. EGR1 could promote tumor progression, but at the same time, it could serve as a tumor suppressive or proapoptotic regulator [34, 41]. EGR2 is a key regulator of myelination in the nervous system and of hindbrain development [37, 42, 43]. It was also identified as a positive regulator of fibrosis development [36] and a negative regulator of T cell activation [44]. EGR3 was also identified to be critical in muscle spindle formation [45] and is a key regulator of endothelial cell activation by VEGF [39].

Biological roles of EGRs in MSCs have been addressed in only few studies including ours [25]. In this study, we showed that EGF receptor (EGFR) ligand is one of the strongest inducers of *EGR1* expression among the various growth factors and cytokines we evaluated. Upon EGF stimulation of MSCs, *EGR1* is strongly and transiently induced in a MAPK extracellular signal-regulated kinase (MEK) inhibitor sensitive manner. EGR1 signaling in turn upregulates growth factors and cytokines including EGFR ligands HBEGF and AREG in MSCs. Those data suggest the presence of an autocrine loop with an EGFR-EGR1-HBEGF/AREG axis. Moreover, HBEGF inhibitor CRM197 decreases the expression of *AREG, VEGF, leukemia inhibitory factor (LIF)*, and *interleukin 11 (IL11)* induced by a PKC activator phorbol ester, presumably by inhibiting HBEGF in the autocrine loop. Thus, even though binding of EGFR ligands to EGFR will eventually cause downregulation of EGFR and shutoff of the EGFR signaling [31], EGR1 still functions as a convergence point for multiple signaling pathways, and the EGFR-EGR1 axis could serve as a molecular machinery supporting the strong paracrine capability of MSCs, at least for select growth factors and cytokines described above (Figure 1).

Another possible autocrine loop exists involving platelet derived growth factor (PDGF), as both PDGF-AA and PDGF-BB upregulate *EGR1*, and gene expression of *PDGFA* and *PDGFB* is dependent on EGR1 (Figure 1) [25]. But both PDGF-AA and PDGF-BB do not induce EGR1 as strongly as EGF, and PDGFA and PDGFB expressions are not as high as

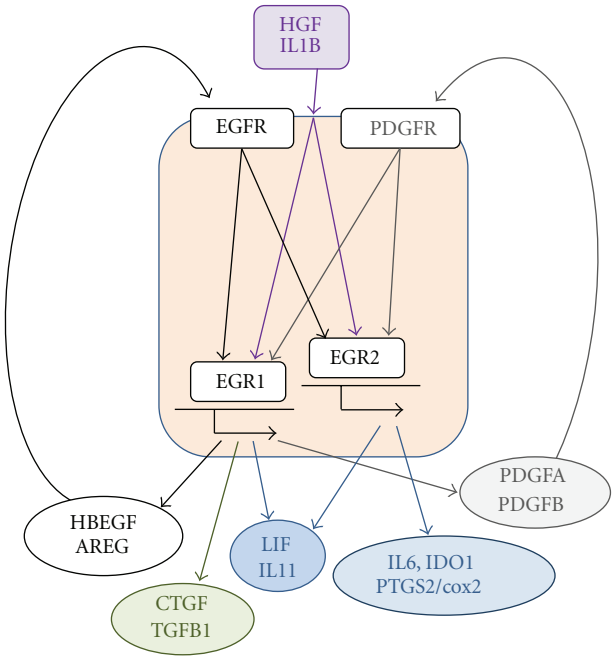

Figure 1: The roles of EGR1 and EGR2 signaling and autocrine loops with EGFR and PDGFR ligands in the production of select bioactive molecules in MSCs. (Abbreviations: CTGF, connective tissue growth factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EGR, early growth response gene; HBEGF, heparin-binding epidermal growth factor-like growth factor; IDO1, indoleamine dioxygenase-1; IL1B, interleukin-1beta; IL6, interleukin-6; LIF, leukemia inhibitory factor; PDGFA, platelet-derived growth factor-A; PDGFB, platelet-derived growth factor-B; PTGS2/cox2, prostaglandin-endoperoxide synthase 2/cyclooxygenase-2; TGFB1, transforming growth factor-beta1; VEGFA, vascular endothelial growth factor-A).

HBEGF. Thus, we speculate that the PDGF-EGR1 autocrine loop is not as strong a contributor to MSCs' paracrine machinery as the EGFR-EGR1 autocrine loop.

Besides *HBEGF, AREG, PDGFA*, and *PDGFB*, our published and unpublished data also showed that EGR1 regulates gene expression of *connective tissue growth factor (CTGF)* and *transforming growth factor-beta 1 (TGFB1)* in MSCs [25]. Although HBEGF, AREG, PDGFA, PDGFB, and CTGF could promote angiogenesis and mitogenesis, these factors could enhance fibrogenesis in the presence of TGFB1 [46, 47], and thus, this group of molecules can be categorized as fibrogenic as well [48–51]. This is in agreement with a recent study showing that EGR1 was also identified as a key factor of fibrogenesis in dermal fibroblasts of patients with scleroderma and systemic sclerosis [52]. In other cell types, EGR1 has been reported to regulate various growth factors and cytokines other than the ones covered in this study [25]; and therefore, we speculate that the EGFR-EGR1 axis probably regulates expression of these factors in MSCs as well.

We are accumulating data about the roles of EGR2 in MSCs. Our unpublished data showed that EGR2 signaling appears to regulate expression of the molecules including *interleukin-6 (IL6), IDO1, LIF,* and *prostaglandin endoperoxide synthase 2/cyclooxygenase-2 (PTGS2/cox2),* all of which mediate immunomodulatory properties of MSCs (Figure 1) [15]. The role of EGR3 signaling in MSCs is unclear at that moment.

In our TRANSFAC database study, EGR1-3 have multiple consensus EGR-binding elements in their promoters (data not shown) and regulate expression of each other positively as well as negatively in a cell-type specific manner [53]. Indeed, direct EGR1 binding was observed in the putative promoters of *EGR1-3* in the ENCODE database [54–56] available in the UCSC Genome Browser [57], and our preliminary results show direct EGR1 binding to the putative promoters of *EGR1-3* by chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) assays (data not shown). Moreover, EGR3 inhibition by siRNA increases the *EGR1* induction (data not shown). These data indicate the possible presence of the interaction among EGR1-3 in human MSCs.

These findings are, overall, distinct from the results obtained in other types of cells or organs outlined above. Based on our recent data, we speculate that EGRs are key molecular switches regulating the fibrogenic, angiogenic, and immunomodulatory properties of MSCs, and we could target EGR1 and EGR2 to maximize the beneficial effects of MSC-based therapeutics for therapies against various diseases including, but not limited to, chronic nonhealing wounds, ischemic diseases, and immune-mediated diseases [32].

Various other stimuli could induce *EGR1-3* and alter EGR signaling. For example, hepatocyte growth factor (HGF) and interleukin-1beta (IL1B) are strong inducers of EGR1-3 in MSCs [25]. Since these signaling molecules are also involved in wound repair and tissue regeneration [58, 59], they might function to augment EGR signaling in MSCs in wound microenvironments (Figure 1).

4. Hypoxic Microenvironments and EGR Signaling

Wound repair and tissue regeneration play an indispensable role for humans to maintain life. It is also regarded as one of the most complicated biological processes involving various types of cells and bioactive molecules acting in a sophisticated fashion. The normal wound healing process occurs in three distinct, but overlapping stages: inflammation, new tissue formation, and remodeling [58], and any arrests in these processes lead to the formation of chronic nonhealing wounds.

Vascular complications can be the cause of wounds such as ischemic coronary diseases, as well as the direct result of injury or tissue destruction itself. The resultant ischemia is one of the main contributing factors to the arrest of the wound repair and tissue regeneration processes, since the limited supply of oxygen and other nutrients compromises cellular functions in the injured sites and impairs these processes [60]. Therapeutic angiogenesis restores the blood supply to these ischemic lesions and promotes wound repair

and tissue regeneration. Local administrations of single angiogenic factors such as VEGF showed only limited benefit [61], suggesting that an administration of multiple growth factors and cytokines, rather than a single-specific growth factor, is required to attain functional vasculatures through neoangiogenesis [62]. MSCs produce multiple growth factors and cytokines in a coordinated manner in response to environmental cues; thus, MSC-based therapeutics could be one promising solution.

The microenvironments in nonhealing wounds, which require therapeutic interventions such as MSC-based therapeutics for healing, are largely hypoxic due to the compromised blood supply and inadequate angiogenesis [60]. Hypoxia itself activates various intracellular signaling in hypoxia inducible factor (HIF)-dependent and HIF-independent manners [63]. HIF is a master transcription factor regulating the expression of hundreds of genes through binding to HIF response element (HRE) in response to hypoxia. HIF consists of the constitutively expressed β-subunit (HIF-1β) and the regulatory α-subunit (HIF-1α and HIF-2α), which is stabilized in response to hypoxia. In contrast to ubiquitously expressed HIF-1α, the expression of HIF-2α is restricted to certain cell types such as vascular endothelial cells and is less characterized than HIF-1α [64, 65]. MSCs express HIF-2α in addition to HIF-1α [66, 67].

We and others previously published the effects of hypoxia or hypoxic priming in MSC survival, the angiogenic factor production by MSCs, and *in vitro* MSC expansion [66, 68–75], but the molecular mechanisms of hypoxia-mediated altered EGR signaling have not been studied except for EGR1, which was reported to be upregulated by hypoxia in glioblastoma cells, monocytes, and hepatoma cells in a HIF-1-independent manner [76, 77]. Consistently, *EGR1* is upregulated by hypoxia in MSCs (unpublished data), and thus, EGR1 signaling in MSCs might be further enhanced in hypoxic microenvironments. Persistent and excessive inflammation is another pathophysiological feature of chronic nonhealing wounds [78, 79], and excessive inflammation also causes fibrosis and scar [80]. EGR1-mediated ECM formation should be a pivotal step in wound healing [81], but hypoxia could cause excessive activation of EGR1 signaling, which might further promote fibrosis formation in chronic wounds.

Our data also showed that hypoxic exposure decreases *EGR2* and *EGR3* induction and expression of their target molecules in MSCs. Interestingly, HIF-2α appears to mediate the decrease of *EGR2* induction, at least partly; however, the role of HIF in the decrease of *EGR3* induction appears minimal if any. Based on these data, we speculate that hypoxia alters EGR2 and EGR3 signaling in MSCs and possibly reduces immunomodulatory properties of MSCs in the hypoxic microenvironments such as nonhealing wounds. MSCs' immunomodulatory properties might be possibly suboptimal through compromised *EGR2* induction in those microenvironments, which could lessen the overall wound repair and tissue regeneration properties of MSCs in the hypoxic microenvironments such as chronic nonhealing wounds because the reversal of persistent inflammation could promote their repair process [80].

5. Exosomes

Recently, exosomes or microvesicles have been recognized as an alternative mechanism of intercellular communication [82]. Exosomes are membranous microvesicles (40–100 nm diameter) released into the extracellular space through exocytic fusion of multivesicular endosomes with the cell membrane [82]. In addition to protein and lipid components, RNAs are responsible for the exosome-mediated intercellular communication [83].

Do exosomes mediate some of the MSCs' paracrine effects? MSCs were shown to be strong producers of exosomes [84]; and indeed, 10% of the total protein present in MSC-conditioned media was estimated to be derived from exosomes [85]. MSCs were shown to exert organ-protective effects via exosome [84, 86–89]. Moreover, MSC-derived exosomes seem to mediate some of MSCs' immunomodulatory effects [84]. However, some reports showed that it is the RNA components, not protein components, that mediate the action of MSC-derived exosomes [86, 89]. Thus, exosomes would mediate some of the MSCs' paracrine effects, but the precise roles of exosomes remain largely unknown at this point.

6. Conclusion

Understanding of MSCs' strong paracrine mechanism should provide molecular targets to optimally personalize the MSC preparations for individual patients. Our previous data suggested that EGR1 and EGR2 play key roles in the production of mitogenic, angiogenic, and immunomodulatory factors in MSCs.

EGR1 functions as a molecular switch of angiogenic, mitogenic, and fibrogenic factor production in MSCs. The EGFR-EGR1-HBEGF/AREG autocrine loop is one of the underlying mechanisms supporting their strong paracrine machinery through EGR1 signaling [25]. EGR2 appears to function as a molecular switch of immunomodulatory molecules in MSCs. Although stimulation with various growth factors and cytokines induces EGR1-3 *in vitro*, it might not necessarily reflect the MSCs' EGR response in harsh microenvironments *in vivo* such as ischemic lesions. Our data showed that hypoxic exposure lessens the induction of *EGR2* in cultured MSCs, suggesting that hypoxic microenvironments *in vivo* might compromise MSCs' immunomodulatory actions by reducing EGR2 signaling.

Through an understanding of their distinct roles in the regulation of various growth factors and cytokines, EGRs may provide a mechanism for altering the wound healing and tissue regenerative capabilities of MSCs through *in vitro* priming prior to patient treatment and/or molecular targeting *in vivo*, and thus, we propose that EGR1 and EGR2 can be potential molecular targets to maximize the paracrine capability of MSCs. Further *in vitro* studies to elucidate the molecular machinery underlying EGRs' paracrine capability are still needed to maximize the benefits of MSC-based cell therapies.

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