EGFR Ligands Drive Multipotential Stromal Cells to Produce Multiple Growth Factors and Cytokines via Early Growth Response-1

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Cell therapy with adult bone marrow multipotential stromal cells/mesenchymal stem cells (MSCs) presents a promising approach to promote wound healing and tissue regeneration. The strong paracrine capability of various growth factors and cytokines is a key mechanism of MSC-mediated wound healing and tissue regeneration, and the goal of this study is to understand the underlying mechanism that supports the strong paracrine machineries in MSCs. Microarray database analyses revealed that early growth response-1 (EGR1) is highly expressed in MSCs. Our previous studies showed that epidermal growth factor (EGF) treatment induces growth factor production in MSCs *in vitro*. Since EGF strongly upregulates EGR1, we hypothesized that EGF receptor (EGFR)–EGR1 signaling plays a pivotal role in MSC paracrine activity. EGF treatment upregulated the gene expression of growth factors and cytokines, including EGFR ligands in a protein kinase C (PKC)- and/or mitogen-activated protein kinase–extracellular-signal-regulated kinase-dependent manner, and it was reversed by shRNA against EGR1. PKC activator phorbol 12-myristate 13-acetate enhanced EGFR tyrosyl phosphorylation and upregulated the gene expression of growth factors and cytokines in a heparin-binding EGF-like growth factor (HBEGF) inhibitor CRM197 sensitive manner, indicating an involvement of autocrined HBEGF in the downstream of PKC signaling. Moreover, stimulation with growth factors and cytokines induced the expression of EGFR ligands, presumably via EGR1 upregulation. These data indicate EGR1 as a convergence point of multiple signaling pathways, which in turn augments the production of multiple growth factors and cytokines by enhancing the autocrine signaling with EGFR ligands.

Introduction

CELL THERAPY with adult multipotential stromal cells or mesenchymal stem cells (MSCs) is a promising approach against various diseases, such as chronic nonhealing wounds or coronary arterial disease, as these cells promote angiogenesis and tissue regeneration upon cell transplantation [1–11]. These cells were originally isolated as colony-forming adherent fibroblast-like cells or colony-forming unit fibroblastic cells from bone marrow suspension [12], but it was subsequently realized that these cells carry multipotency, that is, capable of differentiating into multiple cell lineages, including osteoblasts, chondrocytes, adipocytes, and other types of cells [7–10,13,14].

Initially, MSC differentiation and direct incorporation into local tissues undergoing wound healing and tissue regeneration was regarded as the primary mechanism of MSC action; however, the strong paracrine capability of various growth

factors and cytokines secreted by MSCs is now recognized as a key underlying mechanism of MSC-mediated wound healing and tissue regeneration [15]. The conditioned medium of MSCs, which contains these growth factors and cytokines, was shown to exert tissue regenerative effects as well as further support for the importance of this mechanism [16–19]. This study was aimed to understand the molecular mechanism that supports the strong paracrine machineries in MSCs.

Early growth response-1 (EGR1) encodes a zinc finger transcription factor that exemplifies a group of immediate-early response genes, since a variety of stimulations, including growth factors, cytokines, and even noxious stimuli, rapidly and transiently induce its expression. The induced EGR1 in turn binds to the EGR response element in the promoter regions of growth factors and cytokines and upregulates these genes. Thus, EGR1 may function as a converging point for many signaling pathways [20,21].

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Microarray database analyses revealed that EGR1 is highly expressed in MSCs. We and others showed that the treatment with epidermal growth factor (EGF) or transforming growth factor alpha (TGF α), another EGF receptor (EGFR) ligand, induces growth factor production in MSCs in vitro [22–24]. Moreover, the pretreatment with TGF α was shown to confer additional organ-protective effects on the transplanted MSCs by enhancing paracrine activity of MSCs [25,26]. Since EGF is a strong inducer of EGR1 in MSCs (Fig. 2), we hypothesized that EGFR–EGR1 signaling plays a pivotal role in MSC paracrine activity.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM) culture medium, cell culture supplements, and all of the reagents for polymerase chain reaction (PCR) were from Life Technologies. Fetal bovine serum (FBS) and Lentivirus Mission® shRNA vectors were from Sigma. All growth factors and cytokines were from Life Technologies or Peprotech. P42/44 mitogen-activated protein kinase–extracellular-signal-regulated kinase (MAPK-ERK) kinase (MEK) inhibitor PD98059 was from EMD Biosciences. Pan protein kinase C (PKC) inhibitor bisindolylmaleimide I (BIM) was from Cayman Chemical. All other reagents were from Sigma or Life Technologies, unless described otherwise.

This study was approved by the IBC committee of the University of Pittsburgh for recombinant DNA research and biohazard agents in obedience to National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

Cell culture

Human telomerase reverse transcriptase–immortalized human bone marrow MSCs were the kind gift of Dr. Junya Toguchida (Kyoto University, Kyoto, Japan) [27], and were

cultured in DMEM supplemented with 10% FBS (Atlanta Biological), 2 mM L-glutamine, 1 mM pyruvate, and 100 mM nonessential amino acid. We confirmed the multipotency of the immortalized MSCs into osteogenic, adipogenic, and chondrogenic lineages previously [7,14].

Quantitative reverse transcription-PCR and PCR array

The level of mRNA transcripts was assessed by 2-step quantitative reverse transcription–polymerase chain reaction (qRT-PCR) for both Taqman® PCR (Life Technologies) and Sybr-green-based PCR array (Qiagen). Mx3005P (Stratagene) was used as a quantitative real-time PCR thermal cycler.

For Taqman PCR, total RNA was prepared with TRIzol plus according to the manufacturer's instruction. In brief, after DNase treatment for the removal of possible contaminated genomic DNA, cDNA was synthesized from total RNA with High Capacity RNA-to-DNA kit (Life Technologies) for Taqman PCR. Then, cDNA was mixed with Brilliant III Ultra Fast QPCR Master Mix (Agilent Technologies) and each Taqman gene-specific probe/primers and subjected to the following PCR: 95°C for 3 min, and then 40 cycles of 2-temperature PCR at 95°C for 15 s for denaturing and 60°C for 30 s for annealing and extension.

The Taqman probe/primers (Life Technologies) used in this study are listed in Table 1. Taqman probe/primers for human ribosomal protein, large, P0 (RPLP0) (4333761F) were used as an endogenous invariant control (housekeeping gene) (Life Technologies). All PCR primers were designed to span intron(s) to discriminate cDNA amplicons from genomic amplicons according to the manufacturer. Amplification efficacy of each PCR was assessed initially with serial dilution of control samples; those fell into 95%–105%. Comparative Ct method was utilized to assess the levels of each mRNA transcript relative to that level of RPLP0 mRNA transcript.

The Human Growth Factors RT² Profiler™ PCR Array (PAHS-041) (Qiagen) was also utilized to profile the

TABLE 1. TAQMAN PRIMERS USED IN THIS STUDY

EGR1	Early growth response-1	Hs00152928_m1
EGF	Epidermal growth factor	Hs01099999_m1
HBEGF	Heparin-binding EGF-like growth factor	Hs00961131_m1
AREG	Amphiregulin	Hs00950669_m1
VEGFA	Vascular endothelial growth factor A	Hs00900055_m1
PDGFA	Platelet-derived growth factor alpha	Hs00234994_m1
PDGFB	Platelet-derived growth factor beta	Hs00966522_m1
HGF	Hepatocyte growth factor	Hs00300159_m1
FGF2	Fibroblast growth factor 2 (basic)	Hs00266645_m1
LIF	Leukemia inhibitory factor	Hs00171455_m1
IL6	Interleukin 6	Hs00985639_m1
IL11	Interleukin 11	Hs00174148_m1
EGFR	Epidermal growth factor receptor	Hs01076078_m1
ERRB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	Hs01001580_m1
PDGFRA	Platelet-derived growth factor receptor, alpha	Hs00998018_m1
PDGFRB	Platelet-derived growth factor receptor, beta	Hs01019589_m1
MET	Met proto-oncogene (hepatocyte growth factor receptor)	Hs01565584_m1
KDR	Kinase insert domain receptor	Hs00911700_m1
FLT1	Fms-related tyrosine kinase 1	Hs01052961_m1
LIFR	Leukemia inhibitory factor receptor alpha	Hs01123581_m1
IL6R	Interleukin 6 receptor	Hs01075667_m1
IL11RA	Interleukin 11 receptor, alpha	Hs00234415_m1

expression of 84 genes related to growth factors (Supplementary Table S1; Supplementary information is available online at www.liebertonline.com/scd), according to the manufacturer's instruction. In brief, after DNase treatment on the total RNA, cDNA was synthesized from total RNA with RT² First Strand Kit (Qiagen). Then, cDNA was mixed with SA-Biosciences RT2 qPCR Master Mix (Qiagen), loaded to the PCR array well preloaded with specific probes, and subjected to the following thermocycle: 95°C for 10 min, and then 40 cycles of 2-temperature PCR at 95°C for 15 s for denaturing and 60°C for 1 min for annealing and extension. At the completion of the thermocycling program, melting curve analysis was conducted to ensure the specificity of the amplicons. The resulting Ct values for all wells were uploaded at [<http://sabiosciences.com/pcrarraydataanalysis.php>] for Web-based PCR array data software that analyzes the gene expression with delta-delta Ct method with endogenous controls listed in Supplementary Table S1. The relative expression levels for each gene in the 2 samples are plotted against each other in the scatter plot.

Gene knockdown with shRNA

The anti-EGR1 shRNA (TRCN0000273850: CCGGCATC TCTCTGAACAACGAGAACTCGAGTTCTCGTTGTTTCTGAGATGTTTTT) and nontarget shRNA control vector (CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGT GCTCTTCATCTTGTGTTTTT) were purchased from Sigma-Aldrich. Immortalized MSCs plated on 24-well plate at 4.4×10^3 cells were infected with shRNA lentivirus at MOI of 100 in the presence of 8 µg/mL polybrene.

Puromycin-containing selection medium was used after day 3 to isolate the cells stably expressing transfected shRNAs. Lentivirus-mediated nonspecific interferon response was evaluated by IFN γ qRT-Primers (Invivogen) and was shown to be minimal (data not shown).

Cell signaling immunoassay

Luminex[®] xMAP[®] technology-based MILLIPLEX MAP Cell Signaling MAPmates[™] multiplex assay kit (Millipore) was used for intracellular signaling immunoassays. Briefly, cells were lysed on ice in a MILLIPLEX MAP Lysis Buffer containing proteinase and phosphatase inhibitors (Millipore). After removing particulate matter by filtration, lysates were mixed with MILLIPLEX MAP Assay Buffer (Millipore) containing MILLIPLEX MAP capture beads (Millipore) for the target analytes and incubated overnight at 4°C. After removing the supernatant and washing, the beads were incubated with MILLIPLEX MAP Detection Antibodies (Millipore) for 1 h at room temperature in a light-protected condition. After removing supernatant and washing, the beads were re-suspended in MILLIPLEX MAP Assay Buffer (Millipore) and analyzed by Luminex 200[™] (Luminex Corp.). Nonstimulated HeLa cell lysate (Millipore) was used as a negative cell lysate control, whereas EGF-stimulated A431 cell lysate (Millipore) was used as a positive cell lysate control.

Quantitation of secreted heparin-binding EGF-like growth factor and IL6

After rinsing with PBS, MSCs were cultured in 0.1% FBS-containing serum-free medium for 24 h during treatment with 10 nM EGF. The conditioned media were collected and the secreted

heparin-binding EGF-like growth factor (HBEGF) and interleukin 6 (IL6) were measured by quantitative ELISA kits according to each manufacturer's instructions (Siloam Biosciences, Inc.) (RayBiotech, Inc.). The readouts were adjusted per 1×10^6 cells.

Microarray database analysis

DNA microarray dataset already deposited to the Gene Expression Omnibus (GEO) by Kubo et al. [28] (GSE9451) was used in this study. This dataset was generated from total RNA isolated from human iliac MSCs and human skin and gingival fibroblasts, which were obtained from commercial sources (BioWhittaker, Inc., now Lonza and KURABO) or from patients at Hiroshima University Hospital (Hiroshima, Japan) under the protocol approved by ethical authorities at Hiroshima University [28,29]. The DNA microarray dataset was further analyzed with the online ingenuity pathway analysis software [www.ingenuity.com] and each identifier was mapped to its corresponding gene through core analysis. The average gene expression was calculated through comparison analysis for fibroblasts as well as MSCs in biological triplicate.

According to the GEO database, the microarray dataset was produced by using KURABO GeneChip Custom Analysis Service with Human Genome U133 Plus 2.0 chips (Affymetrix, Inc.) for fibroblasts and MSCs in biological triplicate and deposited to the GEO after normalization by using GeneSpring (Silicon Genetics) [28].

Statistical analysis

All experiments were performed in duplicate or triplicate. Data were analyzed using Student's *t*-tests. Significance was set at $P < 0.05$ or more stringent as noted in the text and figure legends.

Results

MSCs express EGR1 higher than fibroblasts in microarray database

By using GEO repository, we found that EGR1 expresses higher in MSCs than in fibroblasts in baseline condition in GSE9451 (Fibroblast, 325.5; MSC, 1223.8; $P = 0.002$) [28]. This strong expression of EGR1 also indicates that cultured MSCs are in an activated state, as suggested by Caplan [15].

The encyclopedia of DNA elements (ENCODE) project consortium was launched with the aim to build a comprehensive parts list of functional elements in the human genome [30,31]. In the ENCODE database, EGR1 was shown to bind to the putative promoter regions of various growth factors and cytokines and their cognate receptors, including HBEGF; platelet-derived growth factor alpha (PDGFA); platelet-derived growth factor beta (PDGFB); vascular endothelial growth factor A (VEGFA); IL6; IL11; PDGF receptor, alpha (PDGFRA); PDGF receptor, beta (PDGFRB); met proto-oncogene (MET); IL6 receptor (IL6R); and IL11 receptor alpha (IL11RA) (data not shown).

PKC activator phorbol 12-myristate 13-acetate strongly induces EGR1 and multiple growth factor/cytokine production

One of the upstream signaling pathways strongly inducing EGR1 expression is the PKC pathway [32],

FIG. 1. Effects of phorbol 12-myristate 13-acetate (PMA) stimulation on the gene expression in mesenchymal stem cells (MSCs). MSCs were treated with 100 ng/mL PMA for 1 h (**B**) or 4 h (**A**, **C**, **D**, and **E**) and total RNA was collected for evaluation of the gene expression. (**A**) Human growth factor polymerase chain reaction (PCR) array data. The relative expression levels for each gene in the control and PMA-stimulated samples are plotted against each other in the scatter plot. The genes upregulated or downregulated by at least 2-fold in the PMA-treated MSCs relative to nontreated MSCs are indicated in the figure. The upregulated genes are shown with *filled dots*, whereas the downregulated genes are shown with *faint dots*. (**B–E**) The gene expression of early growth response-1 (EGR1) (**B**), growth factors and cytokines (**C**), and cognate receptors (**D** and **E**) evaluated by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) with Taqman[®] primers (**P* < 0.05, ***P* < 0.005 to vehicle control in each analyte).

which is activated by various growth factors and cytokines [33]. PKC pathway activator phorbol 12-myristate 13-acetate (PMA) upregulated the gene expression of various growth factors/cytokines, including HBEGF and amphiregulin (AREG), which are EGFR ligands (Fig. 1A,

C), in addition to EGR1 (Fig. 1B). Besides growth factors and cytokines, PMA treatment also upregulated cognate receptors of these growth factors and cytokines (Fig. 1D, E), indicating the possible presence of autocrine loop(s), possibly via EGR1.

FIG. 2. (**A**) Effects of MSC treatments with various growth factors and cytokines on the induction of EGR1. MSCs were stimulated with epidermal growth factor (EGF) (10 nM), PDGF-AA (30 ng/mL), PDGF-BB (30 ng/mL), vascular endothelial growth factor A (VEGFA) (100 ng/mL), hepatocyte growth factor (HGF) (50 ng/mL), FGF2 (30 ng/mL), leukemia inhibitory factor (LIF) (10 ng/mL), interleukin 6 (IL6) (10 ng/mL), and IL11 (10 ng/mL) for 1 h and the total RNA was harvested for gene expression analysis (**P* < 0.05, ***P* < 0.005 to vehicle control in each analyte). (**B–E**) Effects of EGF stimulation on the gene expression in MSCs. MSCs were treated with 10 nM EGF for 4 h (**B**); 1, 4, and 16 h (**C**); or 24 h (**D** and **E**) and gene expression was evaluated in mRNA (**B** and **C**) or in protein (**D** and **E**). (**B**) Human growth factor PCR array data. The relative expression levels for each gene in the control and EGF-stimulated samples are plotted against each other in the scatter plot. The genes upregulated or downregulated by at least 2-fold in the EGF-treated MSCs relative to nontreated MSCs are indicated in the figure. The upregulated genes are shown with *filled dots*, whereas the downregulated genes are shown with *faint dots*. (**C**) The gene expression of various growth factors and cytokines evaluated by qRT-PCR with Taqman primers (**P* < 0.05, ***P* < 0.005 to 0 h control in each analyte). (**D** and **E**) Production of heparin-binding EGF-like growth factor (HBEGF) (**D**) and IL6 (**E**) in the conditioned media by ELISA (**P* < 0.05, ***P* < 0.005 to vehicle control in each analyte). (**F**) The scatter plot of growth factor profiles between EGF-stimulated MSCs and HBEGF-stimulated MSCs by human growth factor PCR array.

EGFR ligand strongly induces EGR1 and upregulates the production of growth factors and cytokines including EGFR ligands

Among the growth factors and cytokines studied in Fig. 1, EGF, PDGF-AA, PDGF-BB, hepatocyte growth factor (HGF), and fibroblast growth factor-2 (FGF2) strongly induced EGR1 expression (Fig. 2A). These data strongly suggest that the PKC-EGR1 pathway is a common signaling pathway acti-

vated by multiple growth factors, and the pathway activation in turn upregulates the gene expression of various growth factors and cytokines. Among these growth factors and cytokines, we singled out EGFR ligands, because EGFR ligands are the only ligands that are strongly upregulated along with their cognate receptor EGFR by PMA treatment (Fig. 1) and in turn strongly induced EGR1 in MSCs (Fig. 2A). We initially chose EGF as a representative EGFR ligand, because it is the most-studied one [7,34]. EGF treatment also upregulated

FIG. 3. Effects of pan protein kinase C (PKC) inhibitor bisindolylmaleimide I (BIM) and mitogen-activated protein kinase-extracellular-signal-regulated kinase (MAPK-ERK) kinase (MEK) inhibitor PD98059 on the gene expression of EGR1 (A) and various growth factors and cytokines (B–I) by qRT-PCR with Taqman primers and the phosphorylation of EGFR (J), MAPK-ERK (K), and c-Jun N-terminal kinase (JNK) (L) by Luminex® xMAP® technology-based immunoassay. MSCs were stimulated with 10 nM EGF in the presence of DMSO (vehicle), BIM (5 μ M), and PD98059 (10 μ M) for 1 h (A–I) or an indicated time (J–L), and total RNA (A–I) or protein (J–L) was harvested for further analysis [$*P < 0.05$, $**P < 0.005$ to DMSO (vehicle)-treated group in each condition; $\#P < 0.05$, $\#\#P < 0.005$ to DMSO (vehicle)-treated control in baseline or 0 min].

multiple growth factors and cytokines in mRNA transcripts (Fig. 2B, C). The EGF-initiated upregulation of growth factors and cytokines was further confirmed in protein levels for HBEGF (5.67 ± 0.24 pg/ 10^6 cells under EGF stimulation; 2.03 ± 0.10 pg/ 10^6 cells in vehicle control, $P < 0.005$) and IL6 (1.07 ± 0.06 ng/ 10^6 cells under EGF stimulation; 0.71 ± 0.02 ng/ 10^6 cells in vehicle control, $P < 0.05$) (Fig. 2D, E), as HBEGF is a representative EGFR ligand as well as the key molecule of the EGFR signaling in MSCs in this study, whereas previous publications suggested the critical roles of IL6 in MSC biology [35,36]. We have already published the protein concentrations of VEGF, HGF, and FGF2 in EGF-stimulated MSC supernatants [23]. Interestingly, EGF treatment upregulated other EGFR ligands, HBEGF and AREG (Fig. 2B–D).

Though HBEGF is known to bind to v-erb-b2 erythroblastic leukemia viral oncogene homolog 4 (ErbB4) in addition to EGFR [37], MSCs express ErbB4 in only trace amounts in the microarray database GSE9451 [28] and ErbB4 protein expression was not observed in human MSCs [38]. Thus, EGFR should be the only functional receptor expressed on MSCs. Indeed, the intracellular signaling elicited by HBEGF stimu-

lation should be similar to that by EGF stimulation, as we showed previously [7]. The gene expression profiles of growth factors and cytokines were also very similar between EGF-stimulated MSCs and HBEGF-stimulated MSCs (Fig. 2F).

Those data are indicative of possible existence of autocrine loops with EGFR ligands via EGR1 in MSCs.

EGF treatment upregulates the gene expression of EGR1, growth factors, and cytokines in a PKC- and/or MAPK-ERK-dependent manner

Next, we investigated the signaling pathways in the downstream of EGFR by using pharmacological inhibitors. Pan PKC inhibitor BIM, as well as MEK inhibitor PD98059, reduced the EGR1 induction by EGF (Fig. 3A). Similarly, the induction of PDGFA, VEGFA, IL6, and IL11 was reduced by BIM as well as by PD98059 (Fig. 3D, F, H, I). The HBEGF induction was reversed by BIM, but not by PD98059 (Fig. 3B), whereas the AREG induction was reversed by PD98059, but not by BIM (Fig. 3C). EGF stimulation activated MAPK-ERK and c-Jun N-terminal kinase (JNK) pathways in the

FIG. 4. Effects of shRNA against Egr1 (shEGR1) on the gene expression of EGR1 (A) and various growth factors and cytokines (B–I) by qRT-PCR with Taqman primers. Nontarget shRNA (shNT) was used as a shRNA negative control. MSCs with shEGR1 or shNT were stimulated with 10 nM EGF or 100 ng/mL PMA for 1 h and total RNA was harvested for gene expression analysis (* $P < 0.05$, ** $P < 0.005$ to control in shNT; # $P < 0.05$, ## $P < 0.005$ to control in shEGR1; P values between shNT and shEGR1 were given in the figure).

downstream of EGFR tyrosyl phosphorylation in MSCs (Fig. 3J–L). Interestingly, BIM treatment appeared to increase the PDGFB and leukemia inhibitory factor (LIF) expression (Fig. 3E, G), even though the expression of those genes was induced by PKC activator PMA (Fig. 1C). The underlying mechanism remains unclear, but disinhibition of PKC-mediated EGFR signal inhibition by BIM [39,40] might induce the enhancement of EGFR downstream signaling (Fig. 3J–L) and cause this paradoxical increase of PDGFB and LIF expression (Fig. 3E, G).

EGR1 knockdown decreases the gene expression of growth factors and cytokines in baseline as well as under stimulation with EGF and PMA

Next, we evaluated the role of EGR1 on the production of growth factors and cytokines by knocking down EGR1 with shRNA against EGR1 (shEGR1). EGR1 knockdown reduced

the gene expression of HBEGF, AREG, PDGFA, PDGFB, LIF, and IL11 upregulated by EGF as well as PMA (Fig. 4), confirming the crucial role of EGR1 in the production of these growth factors and cytokines.

HBEGF inhibitor CRM197 decreases the gene expression of growth factors and cytokines under PMA stimulation

There are several EGFR ligands expressed in MSCs; among them, HBEGF is the most abundantly expressed. Here, we evaluated the roles of HBEGF on the production of growth factors and cytokines.

If HBEGF expressed in MSCs plays any role in the production of growth factors and cytokines, then HBEGF inhibitor CRM197 [41] should alter the production of growth factors and cytokines even without exogenous HBEGF administration to MSCs. Indeed, CRM197 treatment decreased

FIG. 5. Effect of HBEGF inhibitor CRM197 on the PMA-induced EGFR phosphorylation (A) and gene expression (B–I). After pretreatment with 10 μ g/mL CRM197 for 2 h, MSCs were stimulated with 100 ng/mL PMA for 1 and 3 h (A) or 4 h (B–I), and total protein cell lysates or RNA was collected for evaluation of EGFR phosphorylation by Luminex xMAP technology-based immunoassay (A) or the gene expression by qRT-PCR with Taqman primers (B–I). The ratio of phospho-EGFR to total EGFR in EGF-stimulated A431 cell lysate was arbitrarily defined as 1.0 in (A) (* $P < 0.05$, ** $P < 0.005$ to vehicle-treated group in each condition; # $P < 0.05$, ### $P < 0.005$ to vehicle-treated group in each control in baseline or 0 h; † $P < 0.05$, †† $P < 0.005$ to CRM197-treated group in baseline or 0 h).

the production of HBEGF, AREG, VEGFA, LIF, and IL11 up-regulated by PMA (Fig. 5B–I). PMA treatment caused EGFR tyrosyl phosphorylation, which was inhibited by CRM197 treatment at the 3 h time point (Fig. 5A). These data suggest that HBEGF produced and secreted by PMA-stimulated MSCs binds to EGFR and activates the EGFR signaling in an autocrine manner, and this EGFR signaling in turn upregulates the production of other growth factors and cytokines.

Stimulation with growth factors and cytokines upregulates HBEGF and AREG

Lastly, we evaluated the gene induction of HBEGF and AREG by other growth factors and cytokines, because multiple growth factors and cytokines upregulated EGR1 expression (Fig. 2A), and the gene expression of HBEGF and AREG is EGR1-dependent (Fig. 4B, C). As expected, the gene expression of HBEGF and AREG was also upregulated by EGF, PDGF-BB, HGF, and FGF2 (Fig. 6), in a similar way that these molecules upregulated EGR1 expression. These results further suggest that the downstream signaling from various growth factors and cytokines converges on EGR1, which in turn enhances the possible autocrine loop of EGFR through upregulation of HBEGF and AREG (Fig. 7).

Discussion

Because paracrine signaling is accepted as one of the primary mechanisms for MSC-based wound healing and tissue regeneration [42], understanding the molecular mechanism

FIG. 6. Effect of MSC treatment with various growth factors and cytokines on the gene expression of HBEGF and amphiregulin (AREG). MSCs were stimulated with EGF (10 nM), platelet-derived growth factor-AA (PDGF-AA) (30 ng/mL), PDGF-BB (30 ng/mL), VEGFA (100 ng/mL), HGF (50 ng/mL), FGF2 (30 ng/mL), LIF (10 ng/mL), IL6 (10 ng/mL), and IL11 (10 ng/mL) for 1 h, and the total RNA was harvested for gene expression analysis by qRT-PCR with Taqman primers (* $P < 0.05$, ** $P < 0.005$ to HBEGF in control; # $P < 0.05$, ## $P < 0.005$ to AREG in control).

FIG. 7. Simplified diagram of autocrine loop with EGFR ligands and select growth factors and cytokines in MSCs.

behind the signaling is important in order to maximize the wound healing and tissue regenerating potential of MSCs. Such knowledge should also help to develop serum-free culture media for in vitro MSC expansion without compromising or even with enhancing paracrine capability [43]. In this study, we indicate the possible existence of an autocrine loop with EGFR in MSCs, and this autocrine loop is most likely mediated by EGFR ligands HBEGF and AREG, which activate the MAPK-ERK, JNK, and/or PKC pathways followed by induction of the EGR1 transcription factor. The activation of this autocrine loop also upregulates the production of other growth factors and cytokines, which further augments the EGFR autocrine loop through induction of EGR1 (Fig. 7). These signaling mechanisms possibly contribute to the strong paracrine machinery of MSCs, and EGR1 would be a converging point of these signaling pathways (Fig. 7).

MAPKs, phospholipase γ and PKCs, phosphatidylinositol 3-kinases, and signal transducers and activators of transcription are the major signaling molecules known to mediate the intracellular signaling from EGFR [34,44]. Among them, the MAPK-ERK pathway is well known to strongly upregulate EGR1 expression via Elk1 [21] and p90 ribosomal S6 kinase-CAMP responsive element binding protein 1 [45]. Other MAPK pathways, such as the JNK or p38 MAPK pathway, are also known to induce EGR1 expression as well [21]. The PKC pathway is also known to upregulate EGR1 expression through activation of the MAPK-ERK, JNK, and p38 MAPK pathways via Raf [21,32,33].

The MAPK-ERK-EGR1 pathway seems to be responsible for the EGF-initiated upregulation of AREG, LIF, and IL11 (Figs. 3 and 4). Although the induction of VEGFA and IL6 was reversed by MAPK-ERK pathway inhibitor PD98059 (Fig. 3), it was not reversed by shEGR1 (Fig. 4), suggesting that the upregulation of VEGFA and IL6 is EGR1 independent. There are other immediate-early response genes such

as c-fos activated in the downstream of MAPK-ERK. EGF stimulation also upregulates EGR2 and EGR3, in addition to EGR1 in MSCs (data not shown). These molecules might be responsible for the induction of VEGFA and IL6 in the downstream of EGFR; however, it is beyond the scope of the current study.

The EGF-initiated upregulation of HBEGF and PDGFB was reversed by shRNA against EGR1 (Fig. 4), but not by PD98059 (Fig. 3), suggesting that the MAPK-ERK pathway might not play a significant role in the upregulation of HBEGF and PDGFB. Similarly, the EGF-initiated upregulation of PDGFA was reversed by shRNA against EGR1 (Fig. 4), but the contribution of MAPK-ERK appears minimal (Fig. 3). JNK might possibly link EGFR to the upregulation of HBEGF, PDGFA, and PDGFB, because the JNK pathway is also activated in the downstream of EGFR (Fig. 3), and it could induce EGR1 via Elk1 as well [21].

Although PKC activator PMA clearly upregulated HBEGF, AREG, PDGFA, PDGFB, VEGFA, LIF, IL6, and IL11 (Fig. 1), pan PKC inhibitor BIM did not reverse the EGF-initiated upregulation of AREG, PDGFB, and LIF (Fig. 3), despite PKC being one of the main signaling pathways in the downstream of EGFR [34,44]. Moreover, pan PKC inhibitor BIM tended to augment the phosphorylation of MAPK-ERK and JNK (Fig. 3K–M), even though the PKC pathway is located in the upstream of these MAPKs [32,33]. The apparent reason for this paradoxical enhancement is unclear, but one explanation is the possible enhancement of EGFR signaling by BIM, which leads to the overall augmentation of EGFR downstream signaling including MAPK-ERK and JNK pathways. In agreement with our data (Fig. 3) and speculation, PKC-mediated downregulation of EGFR signaling has been reported [39,40].

Still, PMA stimulation caused EGFR tyrosyl phosphorylation at the 3 h time point, most likely via autocrine HBEGF produced in the downstream of the PKC pathway (Fig. 5A). We speculate that the PKC-mediated EGFR inhibition would decrease within 3 h, allowing the secreted HBEGF to activate EGFR signaling.

MSCs are known to support hematopoiesis in the bone marrow through production of hematopoietic cytokines, such as granulocyte colony-stimulating factor (G-CSF) or IL6 [46]. Since EGF treatment induces the expression of G-CSF (data not shown) and IL6 in MSCs, EGFR signaling might also play a positive role in MSC-mediated support of hematopoiesis in the bone marrow.

In summary, EGFR signaling upregulates multiple growth factors/cytokines, including HBEGF and AREG, through induction of EGR1, and PKC and/or MAPK-ERK pathways relay most of the signaling. Stimulation with growth factors and cytokines in turn upregulates EGR1, which in turn upregulates HBEGF and AREG. These data indicate the possible presence of autocrine signaling with EGFR and EGFR ligands, and this autocrine loop is further augmented by stimulation with other growth factors and cytokines through EGR1.

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Author Disclosure Statement

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References

1. Tamama K, D Kim and SS Kerpedjieva. Molecular mechanisms of hypoxia-mediated enhanced *in vitro* expansion, augmented self-renewal, and increased therapeutic potential of mesenchymal stem cells. In: *Hypoxia: Causes, Types and Management*. Vordermark D, ed. Nova Science Publishers, Inc., Hauppauge, NY (In press).
2. Tamama K, H Kawasaki, SS Kerpedjieva, J Guan, RK Ganju and CK Sen. (2011). Differential roles of hypoxia inducible factor subunits in multipotential stromal cells under hypoxic condition. *J Cell Biochem* 112:804–817.
3. Huss R. (2010). Stem cell therapeutics: hope for patients and biopharma! *Stem Cells Dev* 19:593–594.
4. Spencer ND, JM Gimble and MJ Lopez. (2011). Mesenchymal stromal cells: past, present, and future. *Vet Surg* 40:129–139.
5. Barry FP and JM Murphy. (2004). Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 36:568–584.
6. Phinney DG and DJ Prockop. (2007). Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views. *Stem Cells* 25:2896–2902.
7. Tamama K, VH Fan, LG Griffith, HC Blair and A Wells. (2006). Epidermal growth factor as a candidate for *ex vivo* expansion of bone marrow-derived mesenchymal stem cells. *Stem Cells* 24:686–695.
8. Prockop DJ. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 276:71–74.
9. Pittenger MF, AM Mackay, SC Beck, RK Jaiswal, R Douglas, JD Mosca, MA Moorman, DW Simonetti, S Craig and DR Marshak. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143–147.
10. Owen M and AJ Friedenstein. (1988). Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* 136:42–60.
11. Kinnaird T, E Stabile, MS Burnett and SE Epstein. (2004). Bone marrow-derived cells for enhancing collateral development: mechanisms, animal data, and initial clinical experiences. *Circ Res* 95:354–363.
12. Friedenstein AJ, JF Gorskaja and NN Kulagina. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 4:267–274.
13. Oswald J, S Boxberger, B Jorgensen, S Feldmann, G Ehninger, M Bornhauser and C Werner. (2004). Mesenchymal stem cells can be differentiated into endothelial cells *in vitro*. *Stem Cells* 22:377–384.
14. Tamama K, CK Sen and A Wells. (2008). Differentiation of bone marrow mesenchymal stem cells into the smooth muscle lineage by blocking ERK/MAPK signaling pathway. *Stem Cells Dev* 17:897–908.
15. Caplan AI. (2009). Why are MSCs therapeutic? New data: new insight. *J Pathol* 217:318–324.
16. Salgado AJ, RL Reis, NJ Sousa and JM Gimble. (2010). Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine. *Curr Stem Cell Res Ther* 5:103–110.

17. Chen L, EE Tredget, PY Wu and Y Wu. (2008). Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* 3:e1886.
18. Kinnaird T, E Stabile, MS Burnett, CW Lee, S Barr, S Fuchs and SE Epstein. (2004). Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. *Circ Res* 94:678–685.
19. Kinnaird T, E Stabile, MS Burnett, M Shou, CW Lee, S Barr, S Fuchs and SE Epstein. (2004). Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* 109:1543–1549.
20. Silverman ES and T Collins. (1999). Pathways of Egr-1-mediated gene transcription in vascular biology. *Am J Pathol* 154:665–670.
21. Thiel G and G Cibelli. (2002). Regulation of life and death by the zinc finger transcription factor Egr-1. *J Cell Physiol* 193:287–292.
22. De Luca A, M Gallo, D Aldinucci, D Ribatti, L Lamura, A D'Alessio, R De Filippi, A Pinto and N Normanno. (2011). Role of the EGFR ligand/receptor system in the secretion of angiogenic factors in mesenchymal stem cells. *J Cell Physiol* 226:2131–2138.
23. Tamama K, H Kawasaki and A Wells. (2010). Epidermal growth factor (EGF) treatment on multipotential stromal cells (MSCs). Possible enhancement of therapeutic potential of MSC. *J Biomed Biotechnol* 2010:795385.
24. Wang Y, PR Crisostomo, M Wang, TA Markel, NM Novotny and DR Meldrum. (2008). TGF- α increases human mesenchymal stem cell-secreted VEGF by MEK- and PI3-K- but not JNK- or ERK-dependent mechanisms. *Am J Physiol Regul Integr Comp Physiol* 295:R1115–R1123.
25. Herrmann JL, AM Abarbanell, BR Weil, Y Wang, JA Poynter, MC Manukyan and DR Meldrum. (2010). Postinfarct intramyocardial injection of mesenchymal stem cells pre-treated with TGF- α improves acute myocardial function. *Am J Physiol Regul Integr Comp Physiol* 299:R371–R378.
26. Herrmann JL, Y Wang, AM Abarbanell, BR Weil, J Tan and DR Meldrum. (2010). Preconditioning mesenchymal stem cells with transforming growth factor- α improves mesenchymal stem cell-mediated cardioprotection. *Shock* 33:24–30.
27. Okamoto T, T Aoyama, T Nakayama, T Nakamata, T Hosaka, K Nishijo, T Nakamura, T Kiyono and J Toguchida. (2002). Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem Biophys Res Commun* 295:354–361.
28. Kubo H, M Shimizu, Y Taya, T Kawamoto, M Michida, E Kaneko, A Igarashi, M Nishimura, K Segoshi, et al. (2009). Identification of mesenchymal stem cell (MSC)-transcription factors by microarray and knockdown analyses, and signature molecule-marked MSC in bone marrow by immunohistochemistry. *Genes Cells* 14:407–424.
29. Kawahara K and A Shimazu. (2003). Expression and intracellular localization of progesterone receptors in cultured human gingival fibroblasts. *J Periodontol Res* 38:242–246.
30. ENCODE Project Consortium. (2004). The ENCODE (Encyclopedia Of DNA Elements) Project. *Science* 306:636–640.
31. ENCODE Project Consortium. (2011). A user's guide to the encyclopedia of DNA elements (ENCODE). *PLoS Biol* 9:e1001046.
32. Yan SF, E Harja, M Andrassy, T Fujita and AM Schmidt. (2006). Protein kinase C β /early growth response-1 pathway: a key player in ischemia, atherosclerosis, and restenosis. *J Am Coll Cardiol* 48:A47–A55.
33. Nishizuka Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 9:484–496.
34. Wells A. (1999). EGF receptor. *Int J Biochem Cell Biol* 31:637–643.
35. Pricola KL, NZ Kuhn, H Haleem-Smith, Y Song and RS Tuan. (2009). Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism. *J Cell Biochem* 108:577–588.
36. Uccelli A, L Moretta and V Pistoia. (2008). Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 8:726–736.
37. Elenius K, S Paul, G Allison, J Sun and M Klagsbrun. (1997). Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. *EMBO J* 16:1268–1278.
38. Krampera M, A Pasini, A Rigo, MT Scupoli, C Tecchio, G Malpeli, A Scarpa, F Dazzi, G Pizzolo and F Vinante. (2005). HB-EGF/HER-1 signaling in bone marrow mesenchymal stem cells: inducing cell expansion and reversibly preventing multilineage differentiation. *Blood* 106:59–66.
39. Cochet C, GN Gill, J Meisenhelder, JA Cooper and T Hunter. (1984). C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. *J Biol Chem* 259:2553–2558.
40. Friedman B, AR Frackelton, Jr., AH Ross, JM Connors, H Fujiki, T Sugimura and MR Rosner. (1984). Tumor promoters block tyrosine-specific phosphorylation of the epidermal growth factor receptor. *Proc Natl Acad Sci U S A* 81:3034–3038.
41. Mitamura T, S Higashiyama, N Taniguchi, M Klagsbrun and E Mekada. (1995). Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J Biol Chem* 270:1015–1019.
42. Tamama K and SS Kerpedjieva. Acceleration of wound healing by multiple growth factors and cytokines secreted from multipotential stromal cells/mesenchymal stem cells (MSCs). In: *Wound Healing Society Year Book (WHSYB)—Advances in Wound Care*. Sen CK, ed. Mary Ann Liebert, Inc., New Rochelle, NY (In press).
43. Chase LG, U Lakshmipathy, LA Solchaga, MS Rao and MC Vemuri. (2010). A novel serum-free medium for the expansion of human mesenchymal stem cells. *Stem Cell Res Ther* 1:8.
44. Wells A and U Marti. (2002). Signalling shortcuts: cell-surface receptors in the nucleus? *Nat Rev Mol Cell Biol* 3:697–702.
45. Sakamoto KM and DA Frank. (2009). CREB in the pathophysiology of cancer: implications for targeting transcription factors for cancer therapy. *Clin Cancer Res* 15:2583–2587.
46. Caplan AI and JE Dennis. (2006). Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 98:1076–1084.

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