Signal transducer and activator of transcription 3–stimulated hypoxia inducible factor-1 α mediates estrogen receptor- α –induced mesenchymal stem cell vascular endothelial growth factor production

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Objective: Vascular endothelial growth factor, a critical factor in angiogenesis, mediates stem cell paracrine protective effects on ischemic myocardium. Studies on the role of sex in stem cell function have demonstrated that female mesenchymal stem cells produce greater vascular endothelial growth factor and provide better cardiac protection compared with male mesenchymal stem cells. The purpose of this study was to determine the mechanisms by which estrogen affects mesenchymal stem cell function as a potential therapeutic measure during ex vivo expansion, before therapeutic use.

Methods: A single-step purification method using adhesion to cell culture plastic was adopted to isolate mesenchymal stem cells from wild-type, estrogen receptor- α knockout, estrogen receptor- β knockout, and signal transducer and activator of transcription 3 knockout mice. Mesenchymal stem cells were treated with or without 17 β -estradiol, estrogen receptor- α agonist (propyl pyrazoletriol), and estrogen receptor- β agonist (diarylpropionitrile).

Results: 17 β -estradiol significantly increased mesenchymal stem cell vascular endothelial growth factor production in a dose-dependent manner. Both estrogen receptor- α and estrogen receptor- β were expressed in mesenchymal stem cells. Administration of 17 β -estradiol or estrogen receptor- α agonist (not estrogen receptor- β agonist) elevated mesenchymal stem cell vascular endothelial growth factor, hypoxia inducible factor-1 α expression, and signal transducer and activator of transcription 3 activation. However, these effects were neutralized in estrogen receptor- α knockout mesenchymal stem cells, not estrogen receptor- β knockout. Signal transducer and activator of transcription 3 knockout abolished estrogen receptor- α –induced hypoxia inducible factor-1 α and subsequent vascular endothelial growth factor production.

Conclusion: 17 β -estradiol–induced vascular endothelial growth factor production from mesenchymal stem cells appears to be mediated through estrogen receptor- α –activated signal transducer and activator of transcription 3–mediated hypoxia inducible factor-1 α expression.

Supplemental material is available online.

A rapidly growing body of evidence indicates that stem cells may exert their beneficial effects on the injured heart in part by paracrine actions.^{1–3} A number of in vitro and in vivo experiments have suggested that vascular endothelial growth factor (VEGF), a growth factor with potent angiogenic, chemotactic and vasodilatory effects, is one important mediator

of these cardioprotective paracrine actions of stem cells.^{4–7} Additional studies have established that bone marrow cells genetically modified to overexpress VEGF appear to be even more effective for cardiac repair.⁶ Furthermore, our recent study has shown that decreasing VEGF production by mesenchymal stem cells (MSCs) with the use of small interfering RNA abolishes their ability to protect myocardial function after acute ischemia/reperfusion injury.² Taken together, these data highlight the need for understanding mechanisms of MSC VEGF production so that its production may be increased and the therapeutic effectiveness of stem cells may thereby be enhanced.

Sex differences exist in MSC paracrine growth factor production. Female MSCs produce greater levels of VEGF compared with male MSCs in response to toxic stimuli in vitro and may also provide better cardioprotection.⁸ In addition, 17 β -estradiol (E2) pretreatment of MSCs has been reported to increase MSC VEGF production and may also improve MSC-mediated cardiac protection.⁹ These results indicate that estrogen may have a favorable effect on MSC function. In addition, estrogen has been shown to regulate cell function in a variety of stem/progenitor cells, including

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Abbreviations and Acronyms

E2	= 17 β -estradiol
ELISA	= enzyme-linked immunosorbent assay
ER	= estrogen receptor
DPN	= diethylpropionitride
HIF	= hypoxia-inducible factor
KO	= knockout
MAPK	= mitogen-activated protein kinase
MSC	= mesenchymal stem cell
PPT	= propyl pyrazoletriol
STAT3	= signal transducer and activator of transcription-3
VEGF	= vascular endothelial growth factor
WT	= wild type

MSC-derived osteoblasts, human endothelial progenitor cells, and embryonic cortical progenitor cells.¹⁰⁻¹³ Furthermore, estrogen treatment also up-regulates VEGF production by differentiated cells.^{14,15} However, the mechanisms by which E2 mediates VEGF production in MSCs remain unknown.

Accumulating evidence demonstrates that hypoxia-inducible factor (HIF)-1 α plays a critical role in mediating VEGF expression in a wide range of cell types *in vitro*.¹⁶⁻¹⁸ A number of growth factors, cytokines, and hormones have been reported to activate HIF-1 α -induced VEGF production.^{14,16} More important, HIF-1 mediates prostaglandin E2-induced VEGF gene expression in cancer cells, suggesting that estrogen may mediate HIF-1-induced VEGF expression.¹⁷ It is also known that estrogen initiates its biological activity by binding to estrogen receptor (ER)- α or ER β . Activated ER has been reported to induce HIF-1 α activation and thereby stimulates VEGF-mediated angiogenesis in osteoblasts.¹⁹ In addition, estrogen induces VEGF production in epithelial cells through activation of HIF-1 α and its recruitment to the VEGF promoter by ER α .¹⁴ No information exists regarding the effects of estrogen or its associated receptors on HIF-1 α -mediated VEGF production in MSCs. It is known, however, that activation of HIF-1 α by hypoxia may be linked to the signal transducer and activator of transcription-3 (STAT3) pathway.^{20,21} Estrogen-activated STAT3 has also been noted to mediate physiologic and pathologic processes in several cell types.²²⁻²⁴ Therefore, an investigation into whether estrogen induces HIF-1 α -mediated VEGF production through activation of STAT3 in MSCs is worthwhile.

In the present study, we examined the effects and mechanisms of action of E2 on VEGF production by MSCs. We hypothesized that E2 would increase the stem cell release of VEGF by ER α -induced STAT3 activation and subsequent HIF-1 α expression.

MATERIALS AND METHODS**Animals**

C57BL/6J mice with and without targeted deletion or mutation of ER α (ER α knockout [KO]; The Jackson Laboratory, Bar Harbor, ME) or ER β (ER β KO; Taconic Farms, Inc, Hudson, NY) and the STAT3 deficiency mouse line²⁵ (a gift from Dr Fu's group) were maintained in a quiet quarantine room for 2 weeks before the experiments. The animal protocol was reviewed and approved by the Indiana Animal Care and Use Committee of Indiana University. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication No. 85-23, revised 1985).

Preparation of Mouse Bone Marrow Mesenchymal Stromal Cells

A single-step purification method using adhesion to cell culture plastic was used as previously described.²⁶ Briefly, mouse bone marrow mesenchymal stromal cells were harvested from male bilateral femurs and tibias by removing the epiphyses and flushing the shaft with complete media (Isocove's Modified Dulbecco's Medium with 10% fetal bovine serum and 1% pen-strep; GIBCO Invitrogen, Carlsbad, CA). Cells were washed by adding complete media, centrifuging for 5 minutes at 300 rpm at 24°C and removing supernatant. The cell pellet was then resuspended and cultured with complete media at 37°C, 5% CO₂, and 90% humidity. MSCs preferentially attached to the polystyrene surface; after 48 hours, nonadherent cells in suspension were discarded. Fresh complete medium was added and replaced every 3 days thereafter. MSCs were measured for CD markers at passage 3 by flow cytometry, and they were double negative for the hematopoietic markers CD34 and CD45 (>90%), negative for the macrophage marker CD11b (>95%), and positive for the MSC marker CD44 (>95%).² MSCs were used for experiments until passage 5.

Experimental Groups

After 5 passages, MSCs were plated in 12 well plates at a concentration of 1×10^5 cells/well/mL. First, a dose response experiment was performed to determine the concentration of E2 that would cause maximum VEGF production in wild-type (WT) stem cells. MSCs were treated with E2 (Sigma, St Louis, MO) at 0, 1, 10, 100, 1000, and 10,000 nM, respectively, for 24 hours. Supernatants were then collected for VEGF assay (enzyme-linked immunosorbent assay [ELISA]). The experiment was repeated on 3 separate occasions.

WT, ER α KO, ER β KO, and STAT3KO MSCs were divided into 4 experimental groups (triplicate wells per group: 1) control; 2) 100 nM of E2; 3) 100 nM of ER α agonist (propyl pyrazoletriol [PPT]; Tocris Cookson Inc, Ellisville, MO); 4) 100 nM of ER β agonist (diethylpropionitride [DPN]; Tocris Cookson Inc). To distinguish acute or chronic effects of E2 on VEGF production, supernatants were collected for VEGF assay (ELISA) after 6-hour or 24-hour incubation. The experiment was repeated on 3 separate occasions ($n = 6-11$ wells/group). In addition, after different time incubation (15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours), cell lysates were harvested for the assay of STAT3 activation by Western blot. Furthermore, activation of mitogen-activated protein kinases (MAPKs: p38 MAPK, JNK, and ERK1/2) and PI3K/Akt was also analyzed. Expression of nuclear HIF-1 α , ER α , and ER β was examined by using nuclear extracts.

Enzyme-Linked Immunosorbent Assay

VEGF in the MSC supernatant was determined by ELISA using a commercially available ELISA set (R&D Systems Inc, Minneapolis, MN). ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate.

RNA Extraction, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Total RNA was extracted from 4-hour incubated WT, ER α KO, ER β KO, and STAT3KO MSCs with or without E2, ER α agonist, and ER β agonist by

using RNA STAT-60 (TEL-TEST, Friendswood, TX). Total RNA (0.5 μ g) was subjected to cDNA synthesis using cloned avian myeloblastosis virus first-strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA). cDNA from each sample was analyzed for 18S rRNA (assay ID Hs99999901_s1) and VEGFa (assay ID Mm00545913_s1) by using TaqMan gene expression assay (real-time polymerase chain reaction) (Applied Biosystems, Foster City, CA). Each sample was assayed in duplicate. Values were normalized to corresponding 18S rRNA real-time reverse transcriptase-polymerase chain reaction values and expressed as the fold increase relative to corresponding control.

Western Blotting

Western blot analysis was performed to measure MAPKs (p38 MAPK, JNK, and ERK1/2), STAT3 activation, PI3K/Akt signaling, and HIF-1 α . Cells were lysed in cold RIPA buffer (Product No. R 0278: Sigma) and centrifuged at 12,000 rpm for 10 minutes to get whole cell extracts. Nuclear extracts were isolated by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL). The protein extracts (5 μ g/lane) were subjected to electrophoresis on a 4% to 12% precise protein gel (Pierce Biotechnology) and transferred to a nitrocellulose membrane. The membranes were incubated in 5% dry milk for 1 hour and then incubated with the following primary antibodies: STAT3, phosphor-STAT3 (Tyr705), p38 MAPK, phosphor-p38 MAPK, JNK, phosphor-JNK (Thr183/Tyr185), ERK1/2, phosphor-ERK1/2 (Thr180/Tyr182), PI3K, phosphor-PI3K, Akt, phosphor-Akt (1:1000 dilution, Cell Signaling Technology, Beverly, MA), HIF-1 α , ER α (MC-20), ER β (H-150) (1:200 dilution, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), GAPDH (1:5000 dilution, Biodesign International, Saco, ME), or TATA binding protein (1:2000 dilution, Abcam Inc, Cambridge, MA). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin-G secondary antibody (Pierce Biotechnology) and detection using SuperSignal West Pico Stable Peroxide Solution (Pierce Biotechnology). Films were scanned using an Epson Perfection 3200 Scanner (Epson America, Long Beach, CA), and band density was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Presentation of Data and Statistical Analysis

All reported values are mean $\pm$ standard error of the mean. Data were compared using 1-way analysis of variance with post hoc Tukey test or Student *t* test.

RESULTS

E2 increases MSC production of VEGF in a dose-dependent manner. Sex differences were noted in MSC production of VEGF in response to injury, with higher levels of VEGF observed in female MSCs.²⁷ To determine what concentration of E2 would cause maximum VEGF production in WT stem cells, different doses of E2 at 1, 10, 100, and 1000 nM, and 10 μ M were adopted. Our data demonstrated

FIGURE 1. A, E2 increased MSC production of VEGF in a dose-dependent manner (1, 10, 100, and 1000 nM, and 10 μ M) (24-hour incubation). B, Expression of ER α and ER β in MSCs. Nuclear extracts from 6 individual samples were analyzed by Western blot. Results are mean $\pm$ standard error of the mean (SEM) (the experiment was repeated on 3 separate occasions). **P* < .01 versus control (0). #*P* < .01 versus 1 nM of E2. \$*P* < .01 versus 10 nM of E2. VEGF, Vascular endothelial growth factor; ER, estrogen receptor.

that E2 increased MSC VEGF production in a dose-dependent manner in WT (Figure 1, A). Pharmacologic concentrations of E2 at 100 nM yielded maximal VEGF release in WT MSCs.

Estrogen Receptor- α Mediates the 17 β -Estradiol-Induced Mesenchymal Stem Cell Release of Vascular Endothelial Growth Factor

To examine which ER mediated the effects of E2 on MSC VEGF production, the expression of ER α and ER β in MSCs was first assessed. Our results indicated that both ER α and ER β were present in nuclear extracts of MSCs as shown by Western blot analysis (Figure 1, B). In addition, PPT, a selective ER α agonist, and DPN, a selective ER β agonist, were

TABLE 1. Protein and mRNA levels of vascular endothelial growth factor in mesenchymal stem cells from wild type, estrogen receptor- α knockouts, or estrogen receptor- β knockouts treated with 17 β -estradiol, the estrogen receptor- α agonist-propyl pyrazoletriol, or the estrogen receptor- β agonist- diarylpropionitrile

	VEGF protein (pg/mL)				VEGF mRNA (folds of control)			
	C	E2	PPT	DPN	C	E2	PPT	DPN
Wild type	701 $\pm$ 32.6	909 $\pm$ 55.4*	912 $\pm$ 74.1*	736 $\pm$ 51.2	1.15 $\pm$ 0.07	1.66 $\pm$ 0.22*	2.04 $\pm$ 0.37*	1.25 $\pm$ 0.40
ER α KO	552 $\pm$ 34.5	543 $\pm$ 59.3	550 $\pm$ 59.8	540 $\pm$ 49.1	1.65 $\pm$ 0.65	1.77 $\pm$ 0.15	1.56 $\pm$ 0.57	1.43 $\pm$ 0.26
ER β KO	603 $\pm$ 23.9	693 $\pm$ 33.7*	718 $\pm$ 25.7*	586 $\pm$ 29.8	1.08 $\pm$ 0.08	1.36 $\pm$ 0.04*	1.47 $\pm$ 0.12 <i>P</i> = .09 vs C	1.05 $\pm$ 0.05

Mean $\pm$ SEM. **P* < .05 vs control.

used to investigate this. An amount of 100 nM of E2 and ER α agonist (PPT) significantly increased VEGF production in WT MSCs by more than 200 pg/mL/10⁵ cells (Table 1). In addition, elevated VEGF mRNA levels, ranging from 1.5- to 2-fold, were also noted in WT MSCs treated with E2 or the ER α agonist (Table 1). However, the ER β agonist (DPN) did not affect stem cell VEGF production (mRNA and protein). To further confirm this observation, ER α KO and ER β KO MSCs were adopted. In line with these results, we found that ablation of ER α abolished E2- or ER α -induced MSC VEGF expression (Table 1). However, application of E2 or the ER α agonist maintained increased levels of VEGF mRNA and protein expression in ER β KO MSCs (Table 1).

17 β -Estradiol-Increased Hypoxia-Inducible Factor-1 α Expression Is Mediated through Estrogen Receptor- α

HIF-1 α has been shown to mediate VEGF expression in a variety of cell types by estrogen and other hormones. To understand the molecular mechanisms of E2-increased VEGF expression, we analyzed the expression of VEGF regulator-HIF-1 α by Western blot. As seen in Figure 2, HIF-1 α expression was increased by more than 2 times in WT MSCs 30 minutes and 1 hour after administration of E2. In addition, the ER α agonist also elevated HIF-1 α expression by 3 times at 30 minutes and 1 hour. However, the selective ER β agonist did not affect HIF-1 α expression. These data suggested that E2 induced HIF-1 α expression via the ER α .

Estrogen Receptor- α Mediates 17 β -Estradiol-Induced Signal Transducer and Activator of Transcription-3 Activation

As seen in Figure 3, A, Western blot analysis showed that there was a 2-fold increase in STAT3 activation (exhibited as phosphorylated STAT3) 15 minutes after E2 or ER α agonist treatment. This elevated activation of STAT3 was also noted within 30 minutes of treatments, whereas total STAT3 levels were not changed. However, DPN, the selective ER β agonist, did not increase STAT3 activation in MSCs. In addition, the trend of increased p-STAT3 was noted to be maintained until 2 hours after administration of E2 or ER α agonist in WT MSCs. After 4 hours of treatment, the ER α agonist-induced STAT3 activation normalized.

Basal levels of p-STAT3 were significantly higher in WT MSCs than in ER α KO (7-fold) or ER β KO MSCs (2-fold) (Figure 3, B). However, ablation of ER α or ER β did not change total STAT3 expression. Notably, deficiency of ER α neutralized E2- or ER α -induced STAT3 activation in MSCs (Figure 3, C).

FIGURE 2. Nuclear levels of HIF-1 α are increased by addition of E2 or the ER α agonist-PPT (ER α), but not the ER β agonist-DPN (ER β) in WT MSCs. A, Nuclear HIF-1 α expression 30 minutes after E2, ER α agonist, and ER β agonist treatment. B, Nuclear levels of HIF-1 α at 1 hour after addition of E2, ER α agonist, and ER β agonist. Shown are representative immunoblots. Bar graph indicates relative density (%) of HIF-1 α compared with nuclear loading control TATA binding protein. Mean $\pm$ SEM. * P < .05 versus control. TBP, TATA binding protein; ER, estrogen receptor; HIF, hypoxia-inducible factor.

FIGURE 3. A, Effects of E2, ER α agonist, or ER β agonist on STAT3 activation. Increased STAT3 activation is noted within 15 and 30 minutes after E2 or ER α agonist treatment, and this high level of p-STAT3 is maintained until 2 hours. Shown are representative immunoblots. B, Basal levels of STAT3 activation in MSCs from WT, ER α KO, and ER β KO mice (2 lanes/group). C, E2 or the ER α agonist does not induce STAT3 activation in ER α KO MSCs (2 lanes/group). Bar graph indicates relative density of p-STAT3 compared with total STAT3 (%). Mean $\pm$ SEM. * P < .05. ** P < .01. *** P < .001 versus control or WT. # P < .01 versus ER β KO. STAT3, Signal transducer and activator of transcription-3; ER, estrogen receptor; WT, wild type; KO, knockout.

Estrogen Receptor- α -Activated Signal Transducer and Activator of Transcription-3 Induces Hypoxia-Inducible Factor-1 α Expression and Vascular Endothelial Growth Factor Production

STAT3 activation has been linked to up-regulation of HIF-1 α expression and VEGF production. We already found that E2 induced STAT3 activation and that HIF-1 α expression was mediated through ER α . However, it was

unclear whether ER α -induced STAT3 activation would stimulate HIF-1 α expression in MSCs. Therefore, STAT3KO MSCs were used to examine this mechanism. As shown in Figure 4, deficiency of STAT3 abolished E2- or ER α agonist-induced HIF-1 α expression.

STAT3KO MSCs were also adopted to further determine the effects of STAT3 on ER α -mediated VEGF production. Significantly increased VEGF production was observed in

FIGURE 4. Increased nuclear levels of HIF-1 α by E2 or ER α agonist are absent in STAT3KO MSCs. A, Nuclear HIF-1 α expression at 30 minutes after E2, ER α , and ER β treatment in STAT3KO MSCs. B, Nuclear levels of HIF-1 α 1 hour after administration of E2, ER α , and ER β agonists in STAT3KO MSCs. Shown are representative immunoblots. Bar graph indicates relative density (%) of HIF-1 α compared with nuclear loading control TATA binding protein. Mean $\pm$ SEM. TBP, TATA binding protein; ER, estrogen receptor; HIF, hypoxia-inducible factor.

WT MSCs after 6-hour and 24-hour incubation periods after treatment with E2 or the ER α agonist (Figure 5, A, C). However, these elevated VEGF protein levels were neutralized in STAT3KO MSCs (Figure 5, B, D). In addition, deficiency of STAT3 also abolished E2- or ER α -increased VEGF mRNA levels (Figure 5, E, F).

Effects of Estrogen Receptors on the Activation of Mitogen-Activated Protein Kinases and PI3K/Akt Signaling in 17 β -Estradiol-Treated Mesenchymal Stem Cells

Given that estrogen stimulates the MAPK pathway and activates PI3K/Akt signaling, and that both the MAPK pathway and PI3K/Akt have been linked to HIF-1 α expression,^{14,17,28} we determined the effects of E2, the ER α agonist, and the ER β agonist on activation of MAPKs and PI3K/Akt in MSCs. The results from Western blot indicated that administration of E2, PPT, or DPN did not change activation of p38 MAPK or ERK1/2 in MSCs within 30 minutes, 1 hour, or 2 hours after administration (Figure E1, A, C). However, a transient decrease in JNK activation was observed 30 minutes after treatment with E2, ER α agonist, or ER β agonist (Figure E1, B). This decreased pattern in JNK activation was also noted in STAT3KO MSCs (data not shown). This suggests that changes in JNK activation are not involved in STAT3 pathway. In addition, E2, the ER α agonist, and the ER β agonist did not affect activation of PI3K and Akt (Figure E2).

DISCUSSION

VEGF is a critical factor in angiogenesis and has been shown to facilitate stem cell paracrine protection in the ischemic myocardium.^{3,6,7} Accumulating evidence has demonstrated that estrogen plays a role in modulating stem cell function and mediating VEGF production.⁹⁻¹³ Our previous study further suggested that sex differences exist in MSC VEGF production, with relatively higher levels of VEGF being produced by female MSCs.²⁷ These sex disparities in stem cell VEGF production correlate to biologically relevant sex differences in stem cell-mediated cardioprotection, because MSCs from females confer greater cardioprotection compared with male MSCs.⁸ In addition, E2-pretreated MSCs have exhibited increased VEGF production and better cardiac protection.⁹ These studies have led to the important appreciation that estrogen may facilitate the induction of stem cell VEGF production, which may enhance the therapeutic effectiveness of MSCs.

Estrogen initiates its biological effects by interacting with multiple ERs. Two ER subtypes have been identified: ER α and ER β , both of which belong to the nuclear receptor gene family of transcription factors. In this study, we provide the evidence that E2 can increase MSC production of VEGF in a dose-dependent manner. Our results also indicate that both ER α and ER β are expressed in MSCs and that E2-induced VEGF expression (both mRNA and protein levels) is mediated through ER α , but not ER β .

However, the downstream signaling cascades associated with ER α -induced VEGF production in MSCs have not been fully elucidated. HIF-1 is the primary mediator of the physiologic responses associated with reduction in tissue oxygen tension (hypoxia) and plays an important role in VEGF

FIGURE 5. Protein and mRNA levels of VEGF in MSCs from WT and STAT3KO mice after administration of E2, ER α agonist-PPT (ER α), and ER β agonist-DPN (ER β). E2 or ER α agonist increases VEGF secretion at 6-hour (A) and 24-hour (C) treatments, as well as VEGF mRNA (E) in WT MSCs. However, STAT3KO abolishes E2- or ER α -induced VEGF production at 6-hour (B) and 24-hour (D) treatments, as well as VEGF gene expression (F). Mean $\pm$ SEM. * $P < .05$ versus control. STAT3, Signal transducer and activator of transcription-3; ER, estrogen receptor; VEGF, vascular endothelial growth factor; KO, knockout.

transcriptional activation.^{29,30} Transcription factor HIF-1 consists of 2 subunits: HIF-1 α , the hypoxia-induced component, and HIF-1 β , which is expressed constitutively. HIF-1 α is rapidly degraded in normoxic cells. However, under conditions of low oxygen tension, HIF-1 α stabilizes as the result of hypoxia-prevented degradation. HIF-1 α has been reported to regulate the induction of VEGF expression in several cell types under normoxic conditions.^{9,14,15} In vascular smooth muscle cells without hypoxia, angiotensin II up-regulates HIF-1 α -induced VEGF expression.¹⁸ Treatment with insulin-like growth factor 1 has also been shown to elevate HIF-1 α -induced VEGF mRNA and protein levels in cancer cells.¹⁴ Therefore, the activation of HIF-1 α may be required for estrogen-induced VEGF expression via 1 or more

ERs.^{17,19} Indeed, ER α -activated HIF-1 α has been shown to mediate estrogen-induced VEGF production in epithelial cells.¹⁴ In the current study, our data regarding E2- or ER α -increased HIF-1 α expression in MSCs are in line with results from previous studies and further confirm that non-hypoxic stimuli can induce HIF-1 α . In addition, we observed that E2-elevated HIF-1 α is likely mediated by ER α and not ER β . These results suggest that E2-increased VEGF mRNA and protein levels are likely mediated through ER α -induced HIF-1 α in MSCs.

STAT3 has been implicated in a variety of cellular functions, including cell survival/apoptosis, proliferation, inflammation, and angiogenesis.^{26,31,32} Activation of STAT3 has been found in a wide range of tissues and organs, and appears

FIGURE 6. Model for the mechanism of VEGF production mediated by ERα. STAT3, Signal transducer and activator of transcription-3; ER, estrogen receptor; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase.

to be elevated by stress, growth factors, and cytokines. Of interest, STAT3 has been reported to be a direct target gene for estradiol. Not only has chronic estradiol treatment been shown to induce STAT3 activation, but acute treatment with the ERα agonist PPT has also been shown to up-regulate STAT3 expression in the mouse liver.^{22,33} In addition, E2-activated STAT3 plays a role in conveying non-genomic effects of estrogen in endothelial cells.²⁴ Moreover, active STAT3 signaling by raloxifene plus E2 has been shown to modulate proliferation of vascular smooth muscle cells and human thoracic endothelial cells.²³ Our results indicate that administration of E2 or the ERα agonist PPT increases activation of STAT3 in MSCs. In addition, significantly decreased basal levels of active STAT3 have been observed in ER KO MSCs with the lowest levels being noted in ERαKOs. Furthermore, deficiency of ERα abolished E2- or ERα agonist-induced STAT3 activation. Taken together, these data suggest that cross-talk exists between STAT3 and estrogen signaling, and that ERα is required for E2-mediated STAT3 activation in MSCs. However, we recognized that STAT3KO was used in this study and ablation of a complete signaling moiety might cause some nonspecific effects. Therefore, further investigation by using small interfering RNA of STAT3 or transfection of dominant negative STAT3 is required to determine the detailed mechanisms by which STAT3 mediates MSC function.

Recent evidence has demonstrated that STAT3 is a direct transcriptional activator of VEGF expression in a variety of cell types.^{21,26,31,32} Our previous study has indicated that STAT3 is required for both basal and stress-induced VEGF production in MSCs.²⁶ Overexpression of active STAT3 in cardiomyocytes increased myocardial VEGF production,³⁴ and active STAT3-mediated HIF-1α has been reported to up-regulate transcription of the VEGF promoter in human renal carcinoma cells.²⁰ Blockade of STAT3 down-regulates HIF-1α and thereby decreases VEGF expression.³⁵ Moreover, active STAT3 has been shown to stabilize and increase HIF-1α protein levels by inhibition of HIF-1α degradation and acceleration of its synthesis.²⁰ Therefore, it can be proposed that STAT3 signaling may mediate MSC production of VEGF through regulation of HIF-1α. In the current study, we found that ablation of STAT3 neutralized E2- or ERα-induced HIF-1α expression, and thus abolished E2- or ERα-induced VEGF mRNA and protein expression in MSCs. Although Gray and colleagues³⁶ have reported that maximum transcription of VEGF expression requires the binding of both STAT3 and HIF-1α to the VEGF promoter in carcinomas, the detailed mechanistic interactions of STAT3 and HIF-1α in the regulation of VEGF promoter requires further investigation.

CONCLUSIONS

On the basis of these findings, ERα-activated STAT3 increases stem cell HIF-1α-mediated VEGF expression induced by the addition of estrogen (Figure 6). A better understanding of the effects of gender, estrogen, and ER may allow for the modification of stem cells in vitro to maximize stem cell paracrine protective properties, such as the expression of VEGF.

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FIGURE E1. Activation of p38 MAPK, JNK, and ERK1/2 in WT MSCs 30 minutes, 1 hour, and 2 hours after administration of E2, ER α agonist, and ER β agonist. A, p-p38 MAPK and total-p38 MAPK. B, Phosphor- and total-JNK. C, p-ERK1/2 and total-ERK1/2. Two lanes/group are shown. ER, Estrogen receptor.

FIGURE E2. Activation of PI3K and Akt in WT MSCs 30 minutes, 1 hour, and 2 hours after treatment with E2, ER α agonist, and ER β agonist. A, p-PI3K and total-PI3K. B, Phosphor- and total-Akt. Shown are 2 lanes/group. ER, Estrogen receptor.