Effect of 17β-estradiol on mediators involved in mesenchymal stromal cell trafficking in cell therapy of diabetes

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Abstract

Background aims. Mesenchymal stromal cells (MSCs) have shown great promise for cell therapy of a wide range of diseases such as diabetes. However, insufficient viability of transplanted cells reaching to damaged tissues has limited their potential therapeutic effects. Expression of estrogen receptors on stem cells may suggest a role for 17β-estradiol (E2) in regulating some functions in these cells. There is evidence that E2 enhances homing of stem cells. Induction of hypoxia-inducible factor-1α (HIF-1α) by E2 and the profound effect of HIF-1α on migration of cells have previously been demonstrated. We investigated the effect of E2 on major mediators involved in trafficking and subsequent homing of MSCs both in vitro and in vivo in diabetic rats. Methods. E2 has been selected to improve the poor migration capacity of MSCs toward sites of injury. MSCs were incubated with different concentrations of E2 for varying periods of time to investigate whether estradiol treatment could be effective to enhance the efficiency of MSC transplantation. Results. E2 significantly enhanced the viability of the cells that were blocked by ICI 182,780 (estrogen receptor antagonist). E2 also increased HIF-1α, CXC chemokine receptor 4 and C-C chemokine receptor 2 protein and messenger RNA levels measured by Western blot and reverse transcription–polymerase chain reaction. The enzymatic activity of matrix metalloproteinase 2 and metalloproteinase 9 was elevated in E2-treated cells through the use of gelatin zymography. Finally, the improved migration capacity of E2-treated MSCs was evaluated with the use of a Boyden chamber and in vivo migration assays. Conclusions. Our data support that conditioning of MSCs with E2 promotes migration of cells in cultured MSCs in vitro and in a diabetic rat model in vivo through regulation of major mediators of cell trafficking.

Key Words: CCR2, CXCR4, HIF-1α, mesenchymal stromal cell migration, 17β-estradiol

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by insulin deficiency caused by destruction of pancreatic β cells [1]. Insulin therapy is the treatment of choice for T1D; however, in most cases it is unable to provide tight glycemic control, and this leads to long-term progressive complications [2]. Although the use of β-cell islet transplantation is known as ideal alternative option, it is associated with some important problems including adverse effects of immunosuppressive drugs, inadequate organ donors and graft rejection [3]. Over the past decade, cell therapy has been considered as a promising strategy for the treatment of various disorders such as T1D and as an alternative approach for β-cell transplantation [4]. Ease of isolation, high expansion capability, trophic action and ability to move toward sites of injury have made mesenchymal stromal cells (MSCs) a suitable candidate for regenerative medicine and cell-based therapies [5,6]. Moreover, immunomodulatory properties of MSCs also make them an attractive treatment option, particularly for disease with immune-based etiology [7].
One of the key features of MSC therapy is homing, a unique ability of transplanted cells migrating toward injured targets, but with some limitations [8]. Previous studies have demonstrated that only a small percentage of the transplanted cells reach the target tissues [9]. Thus, enhancing the number of MSCs reaching the site of injury may improve the overall therapeutic efficacy of MSC transplantation.

Migration of MSCs toward the target sites has multiple steps in which chemokines and their receptors are the key players [10]. The pivotal importance of stromal cell–derived factor 1 (SDF-1) and its chemokine receptor, CXCR4, in migration of stem cells toward injured tissues has been demonstrated [11]. Another important chemokine in trafficking of stem cells is monocyte chemoattractant protein (MCP)-1 and its chemokine receptor, CCR2 [12]. CCR2 also mediates hematopoietic stem and progenitor cells trafficking to sites of inflammation in mouse [13]. In addition to chemokines, matrix metalloproteases (MMPs), enzymes that degrade extracellular matrix, facilitate transmigration of cells during chemotaxis [14]. There is evidence that MSCs secrete MMPs [15].

Previous studies have indicated that preconditioning of MSCs by various agents such as chemical agents, drugs and also low oxygen status such as hypoxia may improve stem cell homing by enhancing the mechanisms involved [16,17]. Rochefort et al. [18] demonstrated a 15-fold increase in the number of circulating MSCs when rats were placed in a hypoxia chamber for 3 weeks. Hypoxia and agents that mimic hypoxic conditions can produce a protein named hypoxia-inducible factor (HIF), which is the most important regulator of oxygen homeostasis in normoxia and hypoxia [19].

HIF-1 is a heterodimer transcription factor that consists of an oxygen-inducible α-subunit and constitutively expressed β-subunit. HIF-1α is sensitive to the oxygen level, and, in normoxia, is quickly subjected to prolyl hydroxylation. Hydroxylated HIF-1α is targeted for rapid degradation through the ubiquitin-proteasome pathway [20,21]. Activated HIF-1 binds to the hypoxia-response element and initiates transcription of a broad range of genes that involve in cell proliferation, differentiation, cell survival and stem cell homing [22,23].

HIF-1α is activated by a number of cytokines, growth factors and hormones; recent evidence has indicated that 17β-estradiol (E2) can also induce HIF-1α [24,25]. The presence of estrogen receptors (ER) on stem cells could suggest the crucial role of estrogen in the regulation of different functions of stem cells such as cell proliferation and vascular endothelial growth factor production [26–28]. It is well known that E2 exerts its biological activity through ER-α and ER-β, and activated ERs can induce HIF-1α expression [29].

Therefore, in the present study, we examined the effects of 17β-estradiol preconditioning, accompanied by enhancing the mediators involved in MSC trafficking and consequent improvement of poor homing of transplanted MSCs to injured tissues in cell-based treatment of diabetes.

**Methods**

**MSC isolation and cell culture**

Bone marrow–derived MSCs were isolated from the bilateral femurs and tibias of male Wistar rats (8 weeks old; weight, 150–200 g) by removing the epiphyses and flushing the shaft with minimal essential medium–α (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen) and 1% penicillin-streptomycin (Gibco, Invitrogen). Subsequently, the cells were centrifuged and cultured in 25-cm² culture flasks and incubated at 37°C in a humidified chamber of 95% air and 5% CO₂. After 48 h, non-adherent cells were removed and culture medium was changed every 3 days. Cells at passage 2–4 were used to perform the experiments. E2 (Sigma-Aldrich, St Louis, MO, USA) was dissolved in ethanol (99.5%, Merck, Darmstadt, Germany) and stock solution stored at 4°C. Cells were left untreated (control) or treated in a concentration-dependent manner of E2 (1–1000 nmol/L) for various periods of time. ER antagonist (ICI 182,780; 1 μmol/L; Sigma-Aldrich) and HIF-1α–specific blocker (SC205346, 30 μmol/L, Santa Cruz Biotechnology, Dallas, TX, USA) were used to confirm the role of E2 and HIF-1α in the regulation of CXCR4 and CCR2 expression and MMP 2 and MMP 9 activity.

**Characterization of MSCs**

Cell surface markers of MSCs were quantified by means of fluorescence-activated cell sorting (FACS) analysis of a homogenous population of fibroblast-like cells obtained from passages 2–4. For direct labeling a total of 2 × 10⁵ cells were incubated with fluorescein isothiocyanate–conjugated monoclonal rat antimouse antibodies positive to CD44, CD90 (BD Biosciences Pharmingen, San Diego, CA, USA), CD73, CD105 (Santa Cruz Biotechnology) and CD106 (Biolegend, San Diego, CA, USA) and negative to CD31, CD45 (BD Biosciences Pharmingen) and CD34 (Santa Cruz Biotechnology) for 40 min on ice in darkness. Unstained cells were used as negative control [30]. The analysis was then carried out with the use of a FACSCalibur cytometer (Becton Dickinson, NJ, USA) and CellQuest software (Becton Dickinson).
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

This colorimetric assay is an established method of determining viable cell number in cytotoxicity and proliferation studies. Reduction of tetrazolium salts by the mitochondrial succinate dehydrogenase to a blue formazan product is directly proportional to the number of living cells. Briefly, MSCs were seeded in 96-well plates at a density of 5000 cells per well; the cells were then exposed to different concentrations of E2 (1−1000 nmol/L) in the presence or absence of ICI 182,780 for 24 h and 48 h. Non-treated MSCs were used as control (without FBS for negative and with 15% FBS for positive control). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) was added to each well and incubated for 2 h at 37°C in darkness. Medium was then discarded and 100 μL of dimethyl sulfoxide (Sigma-Aldrich) was added. At 570 nm, absorbance was measured by use of a microplate reader (Bio-Tek ELX800, WA, USA).

Reverse transcription—polymerase chain reaction

Total RNA was extracted with the use of Trizol reagent (Invitrogen Merelbeke, Belgium) according to the manufacturer’s protocol and quantified by means of spectrophotometer. For complementary DNA synthesis, 1 μg of extracted RNA was reverse-transcribed with the use of 2 μmol/L oligo dT (Fermentas, Loughborough, UK) and 200 U Moloney murine leukemia virus reverse transcriptase (Fermentas) in a final volume of 20 μL and stored at −20°C. Reverse transcription reaction was carried out at 42°C for 1 h and 72°C for 10 min and stored at 4°C until polymerase chain reaction (PCR) analysis. Afterward, complementary DNA was subjected to PCR. The primers were as follows: CXCR4, forward 5’-GGAAGGAACTGAAAGCTCCAGAA-3’; reverse 5’-GAAACCAACAGCACAACAAACAC-3’; CCR2, forward 5’-TGATCCTGCCCCCTACTTGTCAT-3’ and reverse 5’-ATGGCCTGGCTCAT-3’; β-actin, forward 5’-TGTCACACCTCCAGCATGTG-3’; reverse 5’-AGCTCAGTAGAGCCTCGCTAGA-3’.

Cyclic conditions were carried out in an Eppendorf Mastercycler (Hamburg, Germany). Each cycle consisted of 5 min at 94°C, followed by 40 cycles of 30 seconds at 94°C, 1 min at 52°C, and a final step of 5 min at 72°C for CXCR4, 5 min at 94°C, 30 seconds at 94°C, 1 min at 55°C and 5 min at 72°C, 35 cycles for CCR2 and β-actin; 5 min at 94°C, 1 min at 56°C and 1 min at 72°C, 35 cycles. The PCR products were separated on 1.5% agarose electrophoresis gel and visualized by means of red-safe staining (iNtRON Biotechnology, Seongnam, Korea).

Protein extraction/Western blot analysis

Cells were cultured in the presence or absence of 17β-estradiol (10, 100 nmol/L) for 1−24 h. At the end of each incubation time, cells were washed twice with ice-cold phosphate-buffered saline and lysed by use of radio-immunoprecipitation lysis buffer (Roche, Applied Science, penzberg, Germany), including protease inhibitors and centrifuged for 30 min at 12,000g at 4°C. Total protein concentration was measured by use of the Bradford assay (Bradford 1976). Next, equal amounts of samples (50 μg) were loaded and separated by use of 15% sodium dodecyl sulfate—polyacrylamide gel and transferred onto a Polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA, USA). The membranes were incubated with the following primary antibodies: mouse monoclonal antibody anti—HIF-1α (1:500; Sigma Aldrich), rabbit polyclonal antibody anti-CXCR4 (1:1000, Abcam, Cambridge, UK), rabbit anti-CCR2 (1:1000, Abcam) and rabbit anti—β-actin (1:5000, Cell Signaling, Danvers, MA, USA). The membranes were then incubated with secondary antibody (antimouse immunoglobulin G and anti-rabbit antibody conjugated with horseradish peroxidase; Cell Signaling) for 1 h. The immunocomplexes were visualized with the use of an enhanced chemiluminescence kit (Amersham Biosciences, Buckinghamshire, UK). Band density was quantified by use of Image J software (National Institutes of Health, Bethesda, MD, USA).

Gelatin zymography

MMP 2 and MMP 9 activities were determined through the use of a zymography assay. Briefly, equal amounts of cell medium (20 μL) were mixed with ×3 loading buffer (125 mmol/L Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate and 0.002% bromophenol blue, pH 6.8) and electrophoretically separated on 10% sodium dodecyl sulfate—polyacrylamide gel containing 0.1% gelatin A (MMP 9) or gelatin B (MMP 2) (Sigma-Aldrich). After electrophoresis, gel was placed into Triton X-100 and was then incubated in developing buffer (50 mmol/L Tris-HCl, 200 mmol/L NaCl, 5 mmol/L CaCl2 and 0.01% NaN3, pH 7.5) overnight at 37°C. Gel was then stained with Coomassie brilliant blue (40% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue G-250 and de-ionized water) for 1 h and washed with destaining solution (40% methanol, 10% acetic acid and de-ionized water). When the white bands of gelatinolysis appeared on the dark blue background, a photo of the gel was taken for densitometry analysis.
Transmigration assay

MSC migration was analyzed by use of the Chemo- 
icon QCM 96-well cell migration assay kit (8-μm 
pore size, polycarbonate membrane; Millipore, Bill-
erica, MA, USA); 4 × 10^5 cells were seeded in 
the upper chamber, and the lower chamber was filled 
with different concentrations of potent chemo-
attractants, SDF-1α (Biomol, Enzo Life Sciences, 
Farmingdale, NY, USA) or MCP-1 (Biomol, Enzo 
Life Sciences). After incubation time (1–24 h) at 
37°C with 5% CO_2, non-migrated cells were 
removed and migrated cells were collected according 
to the manufacturer's instructions and stained with 
CyQuant GR dye. Total cell migration was deter-
mined by measuring the optical density of the 
migrated cells with the use of a 480/520-nm filter set.

Animal model of diabetes

Diabetes was induced by means of a single intra-
peritoneal injection of streptozocin (STZ; Sigma-
Aldrich, 60 mg/kg) diluted in citrate buffer, and the 
control group received normal saline. Three days 
after diabetes induction, blood glucose levels were 
measured by use of a glucometer (Roche Diagnostic, 
NSW, Australia). Rats with fasting blood glucose of 
>200 mg/dL or above were considered diabetic. This 
study was approved by Ethics Committee of Iran 
University of Medical Sciences.

MSC labeling

MSCs at passage 3 were labeled with chlor-
omethylbenzamido (CM-DiI, red fluorescence; 
Invitrogen). Briefly, the cells were incubated with 
CM-DiI for 5 min at 37°C and 15 min at 4°C. The 
cells then were preconditioned with E2 (100 nmol/L) or 
without E2 (control) at 37°C and 5% CO_2. After 
24 h, 1 × 10^6 cells were harvested and resuspended 
in 1 mL of normal saline. Trypan blue staining 
showed that CM-DiI labeling had no adverse effect 
on the viability of cells compared with non-labeled 
MSCs.

Cell transplantation

Experimental groups were divided into 3 groups: (i) 
the sham group received normal saline (perfusate 
vehicle, n = 4 in each group), (ii) the diabetic control 
group received CM-DiI–labeled untreated MSCs and 
(iii) the diabetic group received 1 × 10^6 CM-
DiI–labeled E2-treated MSCs through tail vein injec-
tion. After 24 h of cell transplantation, animals 
were perfused intracardially with normal saline and 
paraformaldehyde (4%). The pancreases were 
excised and placed in paraformaldehyde for at least 
48 h at 4°C; fixed tissues were subjected to paraffin 
embedding and tissue processing. After processing, 
sections were obtained and stained with Hoechst 
(Sigma-Aldrich). To measure the migrated cell number, 
sections were visualized with the use of a 
fluorescence microscope.

Statistical analysis

The results are presented as mean ± standard error 
of the mean. Statistical significance was analyzed by 
means of 1-way analysis of variance for multiple 
comparisons and an unpaired Student's t-test for 
comparisons between 2 groups. Statistically, differ-
ces were assumed to be significant at P < 0.05.

Results

MSC characterization

MSCs were isolated from tibia and femur of the rats 
and cultured under specified conditions. The puri-
ified MSCs displayed a uniform fibroblast-like 
appearance during the culturing process (Figure 1a,b). 
At passage 3, CD markers of MSCs were measured 
by flow cytometry, and the evaluations showed that 
the majority of the adherent cells (>95%) were 
positive for CD44, CD73, CD90, CD105 and 
CD45 (a leukocyte marker), which suggests their mesen-
chymal origin (Figure 1c,d).

E2 promoted proliferation of MSCs through ERs

MTT assay was performed to elucidate the effects of 
E2 on MSC proliferation. The results showed that E2 
(1000, 100 nmol/L) significantly increased prolifera-
tion of MSCs in a dose-dependent manner by 24 h 
(control: 99.5% ± 7.3%, 100 nmol/L: 125.5% ± 
7.7%, 1000 nmol/L: 132.5% ± 8.1%, Figure 2, n = 8, 
P < 0.05). The effect of E2 was time-dependent from 
24 h to 48 h, with a peak of 140% ± 7.89% of control 
at 48-h application. This effect was blocked by the ER 
antagonist ICI 182,780 (1 μmol/L, 98.5% ± 6.2%, 
n = 8), which indicates that E2 performs the prolif-
eration effect through ERs.

E2 preconditioning increases HIF-1α protein expression 
in MSCs

To investigate the effect of E2 on HIF-1α expres-
sion, Western blot analysis was performed. MSC 
conditioning with E2 enhanced HIF-1α protein 
expression (control: 102.45 ± 7.9, 10 nmol/L: 
125 ± 2.8, 100 nmol/L: 172.05 ± 3.1, Figure 3a,
HIF-1α induction was also quantified 1 h, 12 h and 24 h after being pretreated with 100 nmol/L estradiol. The level of induction was concentration-dependent and decayed with time (control: 102.3 ± 1.8, 1 h: 130.6 ± 0.6, 12 h: 146.3 ± 23.2, 24 h: 113 ± 0.5, Figure 3b, n = 3). For clarifying the role of ERs in the regulation of HIF-1α expression, cells were pretreated with ICI 182,780 (1 μmol/L) 30 min before exposure to E2 (100 nmol/L). Subsequently, it was observed that in the presence of ER antagonist, HIF-1α expression was prevented, and these results suggest that E2 induced HIF-1α through ERs (ICI: 101.3 ± 3.8, Figure 3b, n = 3). E2 induction of HIF-1α was reversed by pretreatment of MSCs with HIF-1α inhibitor (SC205346: 97 ± 1.15, Figure 3b, n = 3).

E2 enhances CXCR4 and CCR2 messenger RNA and protein expression level

To examine the effect of E2 on CXCR4, CCR2 messenger RNA (mRNA) as well as protein expression levels, reverse transcription–PCR and Western blotting analysis were performed. E2 stimulated a significant increase in CXCR4 (control: 69.3 ± 5.2, 10 mol/LM: 108 ± 6.1, 100 nmol/L: 146.6 ± 2.9, Figure 4a, n = 3) and CCR2 (control: 57.6 ± 2.8, 10 nmol/L: 75.3 ± 2.4, 100 nmol/L: 98 ± 1.1,
Figure 4b, *n* = 3) transcript levels, which can be reversed by ICI 182,780 and HIF-1α inhibitor (Figure 4a,b). These data confirmed that ERs and HIF-1α were involved in upregulation of CXCR4 and CCR2 mRNA expression. Moreover, to verify whether the elevation of mRNA for CXCR4 and CCR2 was translated to an increase in protein level, Western blot analysis was performed. E2-treated (100 nmol/L) MSCs expressed higher levels of CXCR4 (control: 243/63.1, 12 h: 323.3/64.1, 24 h: 402.8/65.1, Figure 5a, *n* = 3) and CCR2 (control: 131.6/61.4, 12 h: 203.8/63.3, 24 h: 286.6/64.3, Figure 5b, *n* = 3) compared with control.

**E2 promotes MSC migration by enhancing MMP 2 and MMP 9 activity**

Effects of E2 on MMP 2 and MMP 9 enzymatic activities were determined by means of zymography assay. Densitometry analysis showed that E2 increased MMP 2 (control: 90 ± 3.1, 10 nmol/L: 105 ± 3.3, 100 nmol/L: 120 ± 4.1, Figure 6a, *n* = 3, *P* < 0.05) and MMP 9 (control: 92.6 ± 2.8, 10 nmol/L: 123.3 ± 2.7, 100 nmol/L: 132.1 ± 3.9, Figure 6b, *n* = 3, **P** < 0.01) activities noticeably compared with control and that this effect can be abolished by SC205346 pretreatment.

**Effect of E2 on in vitro MSC migration**

Effects of E2 on MSC migration in response to chemotactic agents were studied by use of the QCM chemotaxis 96-well cell fluorometric migration kit. E2 promoted MSC migration, probably through upregulation of CCR2 and CXCR4, respectively. MSCs migrated from the upper chamber toward different concentrations of SDF-1α and MCP-1 located in the lower chamber during the experiment period. MSCs exposed to E2 displayed a more significant migratory capacity compared with untreated cells (Figure 7a,b). E2 induced MSC migration in a dose-dependent manner by 24 h (control: 80 ± 1.15, 1 nmol/L: 90.3 ± 6.3, 10 nmol/L: 116.3 ± 8.5, 100 nmol/L: 159.6 ± 9.8, Figure 7c, *n* = 3). MSC migration was also quantified 1, 6, 12 and 24 h after being pretreated with 100 nmol/L estradiol. The number of migrated MSCs increased time-dependently (control: 80 ± 1.15, 1 h: 86.6 ± 4.3, 6 h: 116.6 ± 14.5, 12 h: 134 ± 12.4, 24 h: 159.6 ± 9.8, Figure 7d, *n* = 3).
E2 preconditioning elevates incorporation of MSCs into injured pancreas

To investigate the effects of E2 preconditioning on MSC migration toward the pancreas, CM-DiI–labeled MSCs were used. Trypan blue staining showed that CM-DiI labeling had no adverse effect on viability of cells compared with non-labeled MSCs. The presence of migrated MSCs was assessed by means of fluorescence microscopy in the pancreas. A significant increase in the recruitment of E2-treated CM-DiI–labeled MSCs was observed in injured pancreas of diabetic animals (pretreated with 100 nmol/L E2 for 24 h), compared with controls, which confirms the pivotal importance of E2 in MSC homing \((P < 0.05, \text{Figure 8})\).

Discussion

There is compelling evidence to indicate that cell therapy has gathered interest as a future restorative treatment for diabetes \([4]\). The unique characteristics of MSCs make them a favorite candidate for cell transplantation purposes \([5]\). Recently, there are abundant data indicating the therapeutic effects of MSC transplantation for treatment of diabetes in animals and humans \([31]\). Despite all the promises, the major obstacle in cell therapy is the poor and insufficient viability as well as the low number of transplanted cells reaching to the target tissues that limit their potential therapeutic effects. Recent evidence has proposed that pharmacological conditioning may improve this process and possibly overcome the barriers \([16,32]\). Therefore, the present study investigated the effect of E2 conditioning on major mediators of MSC trafficking, particularly with the purpose of improving cell therapy in T1D. We have also provided evidence indicating that preconditioning of MSCs with E2 enhances cell migration toward potent chemoattractants such as SDF-1 and MCP-1 \textit{in vitro} and injured pancreas \textit{in vivo}. The effects of E2 have shown through enhancement of homing mechanisms including upregulation of CXCR4 and CCR2 cytokine receptors as well as elevation in activity of MMP 2 and MMP 9, which is mediated by HIF-1\(\alpha\).

Figure 4. Effect of E2 treatment on CXCR4 and CCR2 mRNA expression in MSCs. mRNA expression of (A) CXCR4 and (B) CCR2 increased in E2-treated groups. In the presence of ICI and SC205346, mRNA expression levels of CXCR4 and CCR2 reversed to normal level. Values are mean \pm standard error of the mean. \(*P < 0.05; **P < 0.01.\)

Figure 5. Effects of E2 treatment on protein level of CXCR4 and CCR2 in MSCs. MSCs were incubated without E2 (control) and with E2 (100 nmol/L) for 12 and 24 h. E2 conditioning increased CXCR4 and CCR2 protein levels. Values are mean \pm standard error of the mean. \(*P < 0.05; **P < 0.01.\)
In line with current results, evidence indicates that sex differences influence MSC functions so that female MSCs have the ability to limit inflammation more than male MSCs [33]. Another study has also elucidated that rat hearts treated with female MSCs indicated better compliance and contractility compared with hearts treated with male MSCs [34]. In addition, STZ caused more severe hyperglycemia in ovariectomized compared with intact female mice [35]. Also, the lower prevalence of diabetes in pre-menopausal females indicates a possible protective effect of estrogen in diabetes [36].

\[17\beta\text{-Estradiol is an endogenous steroid hormone that plays an important role in expression of many genes and regulating different pathological and physiological processes in men and women [37,38]. On the basis of this background, we used E2 for preconditioning of MSCs to achieve the goal of increasing the survival and higher engraftment of transplanted cells in injured tissues. The current results indicate that E2 significantly promoted viability of cells, which is in agreement with a previous study demonstrating that E2 increased viability and also prevented loss of differentiation capacity of human bone marrow MSCs [39]. Moreover, E2 has also}

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**Figure 6.** Effects of E2 on MMP 2 and MMP 9 enzymatic activity. MSCs were treated with E2 in the absence or presence of SC205346 for 24 h. FBS supernatant free of cultures was collected to perform gelatin zymography assay. E2 enhanced MMP 2 and MMP 9 activity. SC205346 abolished the effect of E2 on MMP 2 and MMP 9 activities. Values are mean ± standard error of the mean. *P < 0.05; **P < 0.01.

**Figure 7.** SDF-1α (100, 500 ng/mL) and MCP-1 (100, 500 ng/mL) were used as potent chemotactic agents in a modified Boyden chamber migration system. The number of migrated MSCs from the upper chamber, across the membrane toward the lower chamber, was measured over 6 h by means of CyQuant Dye staining. E2 treatment increased migratory capacity of MSCs by upregulating CXCR4 and CCR2. Cells without chemoattractant were used as control. E2 treatment enhanced the migration of MSCs toward (A) SDF-1α and (B) MCP-1. Results are reported as the difference in relative fluorescence units (RFU) in the presence or absence of chemoattractants. (C) E2 pretreatment of MSCs for 24 h enhanced migratory capacity of MSCs dose-dependently. (D) Time course of MSC migratory activity pretreated with 100 nmol/L E2. Each experiment was repeated 3 times. Values are mean ± standard error of the mean. *P < 0.05; **P < 0.01.
been shown to improve pancreatic islet transplantation by enhancing human islet-graft survival in vivo [40] and protecting islet cells against cell death induced by pro-inflammatory cytokines in vitro [41]. In contrast, these results were opposite to those of the study performed by Holzer et al. [42] reporting that E2 has growth-inhibitory effects on bone marrow–derived human MSCs [42]. No measurable improvements were reported in neurological recovery and migration of E2-pretreated neuronal stem cells in an animal model of cerebral ischemia [43]. Strong evidence confirmed that E2 mediates its effects such as growth and differentiation through ERs (ERα, β), which have been expressed in various types of stem cells [26,44,45]. In a recent study, it was demonstrated that E2-induced proliferation was reversed in the presence of an ER pure antagonist (ICI 182,780), which indicates that it was a receptor-mediated effect.

It has been speculated that some effects of E2 are mediated by enhancement of HIF-1α. In the present study, E2 (100 nmol/L) enhanced HIF-1α in cultured MSCs (Figure 3a,b). E2 administration in post-stroke has been shown to improve neurological outcomes in rats by increasing HIF-1α and vascular endothelial growth factor protein expression [46]. E2-induced HIF-1α elevation was mediated by ERs and through activation of the phosphatidylinositol 3-kinase/Akt pathway [25].

Currently, the exact mechanism of HIF-1α activation by E2 is not fully understood. In hypoxia, the active complex of HIF-1, along with the transcription coactivator p300, binds to the hypoxia response elements in the promoters of target genes and enhances transcription of genes. It was shown that in normoxia, HIF-1 association with p300 was blocked by hydroxylation of asparagine residue of HIF-1α by asparaginyl hydroxylase, and it was suggested that E2 could regulate expression or activity of this enzyme [20,47,48].

The beneficial role of HIF in cell therapy and migration of stem cells has been confirmed. It has been shown that preconditioning of bone marrow–derived MSCs with hypoxia enhances cell migration and improves treatment of ischemic acute kidney injury caused by HIF-1α enhancement [16]. Moreover, MSC transduction with HIF-1α lentiviral vectors resulted in significant improvement in cell adhesion and migration leading to cardiac healing after myocardial infarction [49]. It is important to mention that HIF-1α impairment plays an important role in diabetes and related complications [50].

As previously shown, HIF-1α activates transcription of a number of genes, and in particular we were interested in those involved in the migration of MSCs. In the present study, we observed that in preconditioned MSCs, E2 could overexpress CXCR4 and CCR2 mRNA and protein levels, which have pivotal importance in migration of these cells. This effect was ER and HIF-1α–mediated, so that in the presence of ICI 182,780, an ER antagonist, and SC205346, an HIF-1α inhibitor, the expression levels of these cytokines reversed to normal levels (Figure 3a,b).
In addition to chemokines, proteolytic enzymes also contribute to cell migration toward target tissues. MSCs secrete MMPs that degrade the extracellular matrix, by which migration of stem cells can be regulated during chemotaxis [14,15]. In this study, we found that E2-induced elevation of MMP activity was through HIF-1α (Figure 6). These results are in agreement with studies indicating that E2 increases MMP 2 and MMP 9 expression in a variety of stem and cancer cells [51,52] that are mediated by HIF-1α, resulting in enhanced migration of stem cells [53]. This result was opposite to that of the study by Miyoshi et al. [54] reporting that MMP expression is HIF-1α-independent in hepatoma cells; also, E2 has been shown to downregulate cell migration and expression of MMP 2 and MMP 9 in colon cancer cells [55]. These differences might be due to variation in source and nature of cells.

Together with in vitro findings, the transmigration assay was performed to confirm the results followed by an in vivo transplantation study in diabetic rats. We found that the migratory potential of E2-preconditioned MSCs toward chemotactic chemokines, SDF-1 and MCP-1 was significantly elevated compared with non-treated cells.

Research indicates that stem cells can be recruited by a variety of stimuli such as tissue injury, ischemia and cytokines [56]. In injured tissue, expression of specific receptors or ligands elevated to facilitate adhesion, trafficking and infiltration of MSCs [57]. SDF-1 is one of the strongest chemokines, upregulated in damaged tissues as a part of response to injury and attracts circulating MSCs that express CXCR4 to enhance repair of injured organs [58]. This is possibly a pattern similar to what we have shown in an in vitro migration assay, with the use of SDF-1. The results of the present study, which are also supported by the results from the study by Kayali et al. [59], confirmed that the SDF-1/CXCR4 axis is important for survival and migration of progenitor cells in the pancreas.

We also reported that E2 treatment increased the number of migrated MSCs toward MCP-1. The MCP-1/CCR2 axis is well known for its role in trafficking of hematopoietic stem and progenitor cells to sites of injury [13]. MCP-1 is expressed at sites of inflammation and represents a chemokine model of homing [12].

To investigate whether the enhanced in vitro migratory capacity of E2-pretreated MSCs contributes to improved MSC recruitment to injured tissue, we established an STZ-induced diabetic model in rats. To track the cells reaching to the target organ, CM-DiI was used for labeling MSCs, and it was found that the number of MSCs homing to the injured pancreas was more significantly increased in the E2-treated DiI-labeled group than that in nontreated 24 h after intravenous infusion.

Finally, it could be concluded that HIF-1α plays a key role in E2-induced MSCs homing through involvement of downstream chemokine receptor genes, CXCR4 and CCR2, from one side and proteolytic enzymes MMP 2 and MMP 9 from the other side to facilitate homing so that conditioning of MSCs with E2 before transplantation could promote migration ability of cells to the injured pancreas.

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