<Original Article>

Intravenous Injection of iPS Cell-Derived Endothelial Progenitor Cells Prevents Miscarriage by Releasing Pro-Angiogenic Factors in a Mouse Model of Recurrent Spontaneous Abortion

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ABSTRACT

Insufficient production of cytokines and fluid factors involved in angiogenesis during early pregnancy causes abortion due to placental dysplasia. Endothelial progenitor cells (EPCs) are immature endothelial cells that play important roles in vascular repair and neovascularization. In this study, we investigated whether human induced pluripotent stem cell-derived EPCs (hiPS-EPCs) could improve abortion rates in a mouse model of recurrent spontaneous miscarriage. After the differentiation induction of hiPSCs, CD31/CD34 double-positive cells increased and showed the presence of tube-like structures, confirming successful differentiation into hiPS-EPCs. Intravenous transplantation of hiPS-EPCs significantly reduced abortion rates in the mouse model. To elucidate the mechanism by which miscarriages were ameliorated, we investigated whether the cells were preserved in the embryos. We differentiated GFP-labeled iPSCs into EPCs (GFP-EPCs), and intravenously injected them into the model mouse. Whole-mount immunostaining analysis showed the presence of injected cells expressing GFP in the uterine vasculature adjacent to the embryos from mice injected with GFP-EPCs. Furthermore, the gene expression of fluid factors such as VEGF and PlGF was increased in the placentas of mice implanted with hiPS-EPCs. These data indicate that hiPS-EPC transplantation therapy may reduce miscarriage *via* transplanted cells that remain in the uterine vasculature and secrete pro-angiogenic factors.

INTRODUCTION

Spontaneous abortion, or miscarriage, is the natural death of an embryo or fetus before 22 weeks of pregnancy. It is one of the most common pregnancy complications; approximately 15 % of pregnant women experience miscarriages. Recur-

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rent spontaneous abortion (RSA) is defined as three or more spontaneous abortions. Recently, spontaneous abortion rates in pregnant women have been found to increase with the woman's age. Several mechanisms have previously been suggested for the pathogenesis of miscarriage, including aging, chromosomal abnormalities, hormonal imbalance, uterine abnormalities, infections, and autoimmune disorders; however, the cause is unknown in 40–50 % of cases [1–3]. There is currently no proven treatment for spontaneous abortions of unknown etiology.

The body undergoes several anatomical and physiological changes during early pregnancy. Particularly in the placenta, increased humoral factors, cytokine production in angiogenesis, and infiltration of extravillous trophoblasts into the spiral arteries, known as uterine spiral artery remodeling, are essential to promote blood flow to the placenta and fetal development. Placental insufficiency is one of the major risk factors for abortion, causing fetal growth restriction and hypertensive disorders in pregnancy [4–7].

Endothelial progenitor cells (EPCs), CD34$^+$, KDR$^+$, CD133$^+$, and CD45$^-$ cells, represent a heterogeneous population of resident mononuclear cells derived from the bone marrow. EPCs regulate neovascularization through physiological and pathological processes throughout the body and have therapeutic potential for various diseases [8,9]. Angiogenesis is the proliferation of existing vascular endothelial cells stimulated by various humoral factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), which are secreted by EPCs, whereas vasculogenesis is the process in which EPCs are taken directly into the area where blood vessels are being formed and then proliferate and differentiate [10,11].

Previously, we established a mouse embryonic stem cell (ESC) line from female CBA/J mice crossbred with male DBA/2 mice and performed ESC-derived EPC transplantation therapy using an RSA mouse model [12]. This treatment reduced the fetal resorption rate in this mouse model and significantly promoted fetomaternal neovascularization through the direct formation of spiral arteries. However, mouse ESCs can cause rejection when transplanted into humans, and there are ethical problems because they are derived from fertilized egg embryos. In contrast, human induced pluripotent stem cells (hiPSCs) are established from ordinary human somatic cells [13] and do not have the ethical problems associated with ESCs [14]. Due to their pluripotency, meaning that they can differentiate into various cell types, and their ethical ease of use, hiPSCs have been used in many clinical trials for the treatment of various diseases, such as age-related macular degeneration, heart failure, and Parkinson's disease [15]. This study focused on the clinical applications to determine whether hiPSC-derived EPCs (hiPS-EPCs) can improve abortion rates in the RSA mouse model.

MATERIALS AND METHODS

Culture of hiPSCs

The hiPSC line used in this study was WTC-11, obtained from Coriell Institute. The hiPSCs were single-cell passaged every 7 days using Accutase (AT104, Innovative Cell Technologies, San Diego, CA, USA). StemFit AK02N medium (RCAK02N, Ajinomoto, Tokyo, Japan) supplemented with 10 µM Y-27632 (10-2301, Focus Biomolecules, Plymouth Meeting, PA, USA) and 1.67 µg/ml iMatrix-511 silk (892021, Nippi, Tokyo, Japan) was used to seed hiPSCs at each passage. After 24 h, the StemFit AK02N medium was replaced with fresh one without Y-27632 and the medium was changed every other day. The plating density was 14,000 cells/1.5 ml per well in a 6-well plate. Cells were counted on a TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA). A GFP-labeled hiPSC line was kindly provided by Dr. K. Tomoda (Gladstone Institutes, CA, USA) and was cultured in the same manner as the hiPSCs described above.

Differentiation of hiPSCs into EPCs

To differentiate hiPSCs into EPCs, 10,000 hiPSCs were seeded into 12-well plates 3 days prior to differentiation and cultured in StemFit AK02N medium until the start of differentiation. At the start of differentiation (day 0), 10 mM CHIR99021 (034-23103, FUJIFILM Wako Pure Chemical, Osaka, Japan) was added in a ratio of 1/1000 to StemPro-34 SFM Complete Medium (10639011, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with GlutaMAX Supplement (35050061, Thermo Fisher Scientific) and 60 µg/ml L-ascorbic acid 2-phosphate (A8960, Merck KGaA, Darmstadt, Germany) for 48 h. The medium was replaced with a differentiation induction medium without CHIR99021, which was changed every other day.

Mice

Female CBA/J mice mating with male DBA/2 mice are susceptible to abortion because of numerous immunological disorders and poor placentation and are commonly used as a mouse model of recurrent spontaneous abortion [16]. Female CBA/J (9–15 weeks old) and male DBA/2 (6–14 weeks old) inbred mice were purchased from the Charles River Laboratory. The day of vaginal plug detection was considered embryonic day 0.5 (E0.5) of gestation. Differentiated hiPSCs on day 5 of endothelial differentiation culture (500,000 cells in 200 µl of PBS/mouse) or PBS alone (control group) were injected into the tail vein of pregnant female mice on E6.5–7.5. Dams were euthanized on E13.5–14.5, at which time pregnancies undergoing resorption could be easily assessed visually, and embryo resorption rate could be counted. The total number of implantation and resorption sites were recorded. In addition, the decidua, including the placenta and embryo, were collected on E13.5–14.5. All mice were maintained in a laminar airflow cabinet at a barrier facility. All

experimental procedures involving animals were performed in accordance with the guidelines of the Animal Care and Use Committee of Osaka Medical and Pharmaceutical University (No. 21063-A).

Isolation of total RNA

Total RNA was purified from hiPSCs, hiPS-EPCs, or mouse placental tissue using RNAzol RT (RN190; Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The absorbance of the RNA was measured at 260 and 280 nm using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

Reverse Transcription and quantitative RT-PCR

Total RNA (1 µg/sample) was reverse transcribed to generate a cDNA template using PrimeScript RT Reagent Kit (RR037A, Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The RT mixture was prepared in a 10 µl reaction volume, and RT was performed using a Takara PCR Thermal Cycler Dice (Takara Bio) with the following settings: 37 °C for 15 min, 85 °C for 5 s, and 4 °C until finished. RT-qPCR was used to assess the relative levels of our target genes using the StepOne Plus Real-Time PCR system (Thermo Fisher Scientific) with Probe qPCR Mix (RR391A, Takara Bio) and TaqMan probes (Thermo Fisher Scientific) as follows: denaturation at 95 °C for 20 s; 40 cycles of annealing and extension at 60 °C for 20 s. PCR primers, annealing cycles, and temperatures were summarized, and the fold change was calculated by the delta C_T method using GAPDH amplification as an internal control. TaqMan primers were purchased from Thermo Fisher Scientific and are summarized in **Table 1**.

Flow cytometry analysis

On day 5 of differentiation, the cells were washed with PBS and incubated with Accutase for 5 min at 37 °C and the cells were collected. The cells were neutralized by the addition of DMEM, thoroughly suspended, and centrifuged at 5,000 rpm for 5 min. Cells were resuspended in 600 µl of wash buffer and stained with the following antibodies (1:10) for 30 min on ice: CD31 (Anti-human CD31 PE, 12-0319-41, Thermo Fisher Scientific), CD34 (APC anti-human CD34, 343510, BioLegend, San Diego, CA, USA), CD45 (FITC anti-human CD45, 304006, BioLegend), centrifuged at 2,000 rpm, 4 °C for 2 min and resuspended in wash buffer again. The percentage of stained cells was analyzed using a BD FACSAria Fusion Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Matrigel tube formation assay

Differentiated cells were dissociated using Accutase, replated into the wells of a 48-well plate coated with 150 µl of Matrigel Basement Membrane Matrix (354234, Corning, Corning, NY, USA) and incubated in the medium used before replating for 24 h. Tube formation was observed using an EVOS XL Core Imaging System (AMEX1000; Thermo Fisher Scientific). Representative images of the robust tube-like structures were selected.

Tissue clearing and whole-mount staining of mouse uterus containing embryos

Three hours after GFP-labeled hiPS-EPCs (GFP-EPCs) were transplanted into pregnant mice on E7.5, the uterus which contained embryos were collected from the mice and immediately placed in 4 % paraformaldehyde in PBS at 4 °C for 60 min. Subsequently, specimens were treated with a quenching solution (0.1 M glycine in PBS) for 60 min, washed well several times with 0.2 % Triton X-100 in PBS (PBT) solution, and incubated in PBT containing 1 % bovine serum albumin (BSA) (01863-35, Nacalai Tesque, Kyoto, Japan) and whole rat IgG (5 mg/ml) (147-09521, FUJIFILM Wako Pure Chemical) in PBT at 4 °C overnight. After washing in 1 % BSA in PBT, the specimens were stained with Alexa Fluor 647-conjugated anti-CD31 (clone MEC13.3, 102516; BioLegend) and Alexa Fluor 488-conjugated anti-GFP antibodies (clone RQ2, D153-A48; MBL, Tokyo, Japan). DAPI was used as a counterstain. A 50 % benzyl alcohol/50 % benzyl benzoate solution, also known as Murray's clear, was used for the tissue clearing of the stained tissues [17–19]. Confocal images of the specimens were obtained using a Leica TCS-SP8 confocal laser-scanning microscope (Leica, Wetzlar, Germany) with a 20× objective lens.

Statistical analysis

Statistical comparisons between two groups were performed using an unpaired two-tailed Student's *t*-test, and multiple comparisons were evaluated using one-way ANOVA with Dunnett's post-hoc test. Data are shown as means ± SD. *P* value of < 0.05 was considered to be statistically significant.

Table 1 List of TaqMan probes used in this study

Gene	Assay reference
GAPDH	Hs02786624_g1
NANOG	Hs02387430_g1
OCT-4	Hs04260367_gh
CD34	Hs02576480_m1
CD31	Hs01065279_m1
KDR	Hs00911700_m1
CD133	Hs01009259_m1
VEGF	Hs00900055_m1
PlGF	Hs00182176_m1
GAPDH	Mm99999915_g1
VEGF	Mm00437306_m1
PlGF	Mm00435613_m1

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RESULTS

Differentiation and characterization of hiPS-EPCs

To confirm the success of EPC differentiation from hiPSCs, the expression levels of pluripotency markers, such as *NANOG* and *OCT-4*, and EPC-related markers (*CD34*, *CD31*, *CD133*, and *KDR*) were analyzed by RT-qPCR on days 2, 5, and 8 of differentiation. On day 2 of differentiation, the expression levels of *NANOG* and *OCT-4* were significantly reduced, indicating that only a few undifferentiated cells remained (**Fig. 1A**). In contrast, the expression levels of *CD34*,

CD31, and *KDR* significantly increased on day 5 of differentiation. In addition, *CD133* expression showed a tendency to increase on day 5 of differentiation (**Fig. 1B**). Similarly, flow cytometric analysis was performed on cells on day 5 of differentiation to determine the percentages of CD34-, CD31-, and CD45-positive cells. On day 5 after differentiation, CD31/CD34 double-positive endothelial lineage cells were induced (CD31+/CD34+, 7.4 %; CD31+, 7.7 %; CD34+, 16.9 %), and the hematopoietic marker CD45 was downregulated (CD45+, 0.7 %) (**Fig. 1C**). Furthermore, to confirm EPC ability, a tube formation assay was performed. After 1 day of culture on
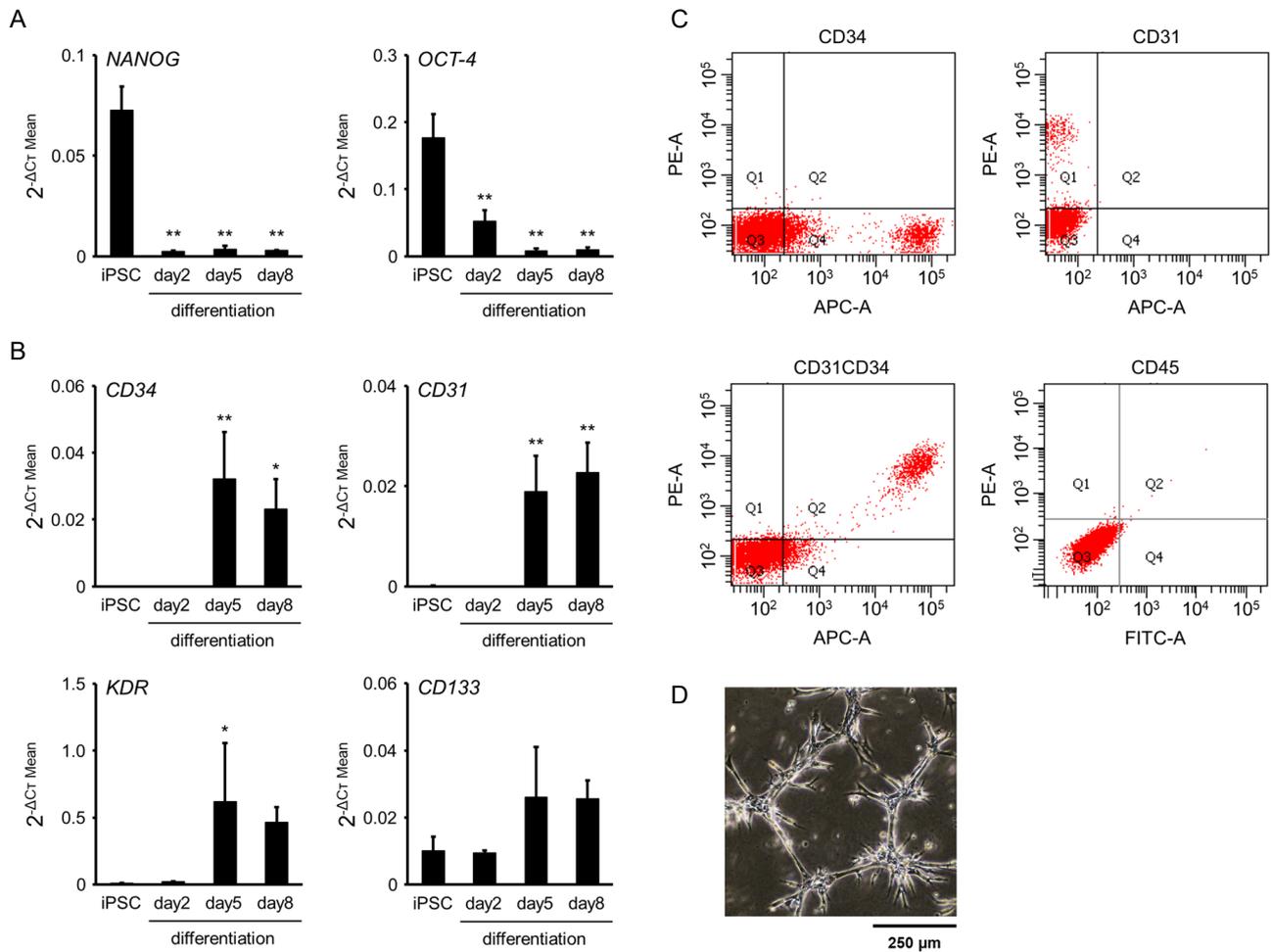
Figure 1 Differentiation of hiPSCs into EPCs and their characterization

(A) Expression of pluripotency markers such as NANOG and OCT-4 during differentiation confirmed by RT-qPCR. Values are shown as means ± SD of three independent experiments. **$P < 0.01$ vs. each iPSC, by one-way ANOVA followed by Dunnett's test.

(B) Expression of EPC-related markers, CD34, CD31, CD133, and KDR during differentiation confirmed by RT-qPCR. Values are shown as means ± SD of three independent experiments. **$P < 0.01$, *$P < 0.05$ vs. each iPSC, by one-way ANOVA followed by Dunnett's test.

(C) Flow cytometry was performed to determine the percentages of CD34, CD31, and CD45 positive cells on day 5 of differentiation induction. PE: phycoerythrin, APC: allophycocyanin.

(D) The presence of tube-like structures was confirmed by the results of the tube formation assay. Bar, 250 μm.

Matrigel, the differentiated cells formed tube-like structures (**Fig. 1D**). These data showed that the cells differentiated from hiPSCs expressed EPC-related markers and had vascular endothelial function; therefore, these cells were used as hiPS-EPCs in subsequent experiments.

Intravenous transplantation of hiPS-EPCs reduces fetal anomalies
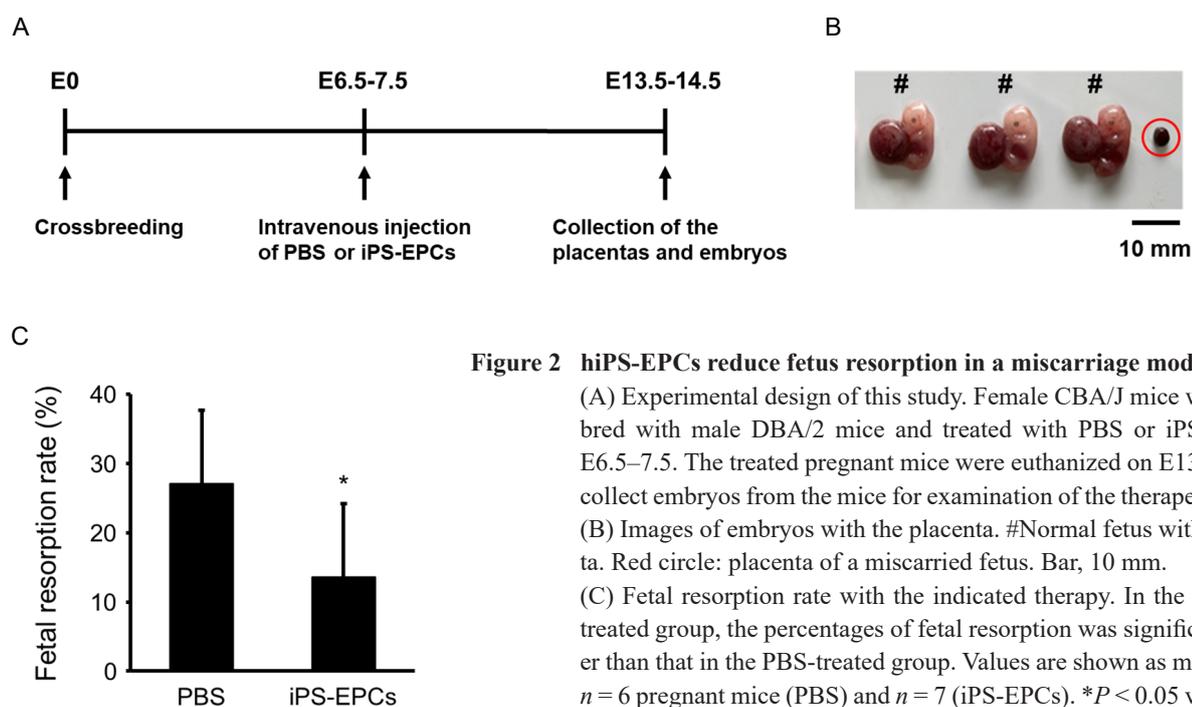
To investigate the therapeutic effect of hiPS-EPC transplantation, we used an RSA mouse model, in which a high frequency of abortion was introduced by crossbreeding female CBA/J mice with male DBA/2 mice [16]. Pregnant CBA/J mice (E6.5–7.5) were intravenously treated with PBS (control group: $n = 6$) or hiPS-EPCs (treatment group: $n = 7$) *via* the tail, and resorption rates were analyzed at E13.5–14.5 (**Fig. 2A**). Resorption rates were calculated by counting the fetuses and placentas. Resorbed infants were defined as those with fetal resorption or placenta only, whereas live infants were defined as those with a confirmed fetal heartbeat at dissection (**Fig. 2B**). The resorption rate in the PBS group was 27.1 %, which decreased to 13.6 % in the hiPS-EPCs group ($P = 0.042$) (**Fig. 2C**). These results show that treatment with hiPS-EPCs improved fetal anomalies in pregnant CBA/J mice.

hiPS-EPCs are localized in the uterine vasculature adjacent to the embryos after intravenous injection

To investigate the mechanism by which hiPS-EPCs improve resorption rates, we first examined the size and number of embryos at E7.5 and E10.5 in the pregnant mice intravenously treated with hiPS-EPCs. Three or 72 h after intravenous injection of GFP-EPCs or PBS (as a control) into the tail vein of the pregnant mice on E7.5, the uteruses which contained embryos were collected (**Fig. 3A**). At 3 h post-transplantation (E7.5 + 3 h), almost all the embryos were of the same size and appeared to show normal development (**Fig. 3B**). Additionally, there was no apparent difference in the number of embryos between control and GFP-EPCs-treated mice (**Fig. 3C**). In contrast, at 72 h post-transplantation (E10.5), the GFP-EPCs-treated embryos appeared larger than the control embryos (**Fig. 3B**). Notably, some control embryos were small and exhibited hemorrhage. The number of abnormal embryos was significantly lower in the treated mice than in the control mice ($P = 0.026$) (**Fig. 3D**). These data indicate that, in this mouse model of RSA, major abnormalities appear after E7.5 stage, and EPC transplantation therapy may be effective in the E7.5–10.5 stage.

Next, we examined the *in vivo* localization of transplanted GFP-EPCs by whole-mount immunostaining analysis to determine whether transplanted EPCs differentiate into vascular endothelial cells and are directly involved in placental neovascularization or whether transplanted EPCs secrete angiogenic factors to promote placental neovascularization. At E7.5, the uteruses that contained embryos were isolated 3 h after transplantation and stained with antibodies against GFP and CD31, followed by tissue clearing for confocal microscopic analysis (**Fig. 4A**). The transplanted cells were detected as GFP-stained cells in the uterine vasculature adjacent to

A

E0 — E6.5-7.5 — E13.5-14.5

Crossbreeding | Intravenous injection of PBS or iPS-EPCs | Collection of the placentas and embryos

B

# # #

10 mm

C

Figure 2 hiPS-EPCs reduce fetus resorption in a miscarriage model
(A) Experimental design of this study. Female CBA/J mice were crossbred with male DBA/2 mice and treated with PBS or iPS-EPCs on E6.5–7.5. The treated pregnant mice were euthanized on E13.5–14.5 to collect embryos from the mice for examination of the therapeutic effect.
(B) Images of embryos with the placenta. #Normal fetus with a placenta. Red circle: placenta of a miscarried fetus. Bar, 10 mm.
(C) Fetal resorption rate with the indicated therapy. In the iPS-EPCs-treated group, the percentages of fetal resorption was significantly lower than that in the PBS-treated group. Values are shown as means ± SD. $n = 6$ pregnant mice (PBS) and $n = 7$ (iPS-EPCs). *$P < 0.05$ vs. PBS, by Student's *t*-test.

the embryos that were visualized by CD31 staining in the treated embryos, whereas no specific GFP staining signals were observed in the control embryos (**Fig. 4B**). In addition, transplanted GFP-positive cells were not detectable in uteruses, embryos, and placentas at E10.5 and E14.5 (data not shown).

These data suggest that the mechanism by which EPC therapy reduces miscarriages in a CBA/J × DBA/2 mouse model of RSA is due to the transient localization of transplanted EPCs in the preplacental periphery, rather than the transplanted EPCs themselves forming vascular vessels in the placenta.

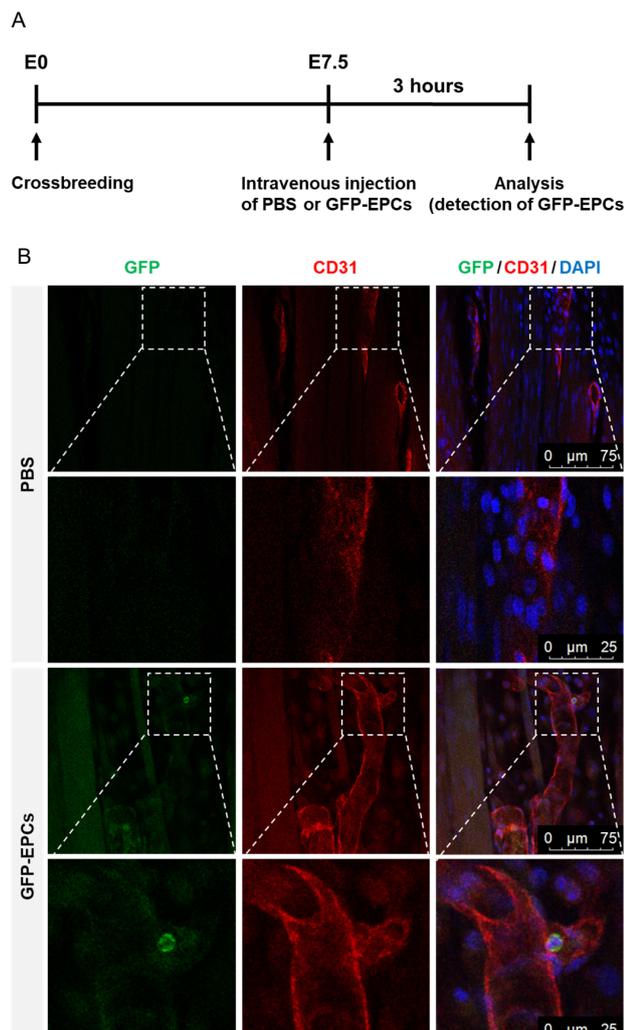### *Increased pro-angiogenic cytokines in mice treated with hiPS-EPCs*

As described above, transplanted hiPS-EPCs were transiently localized in the uterine vasculature of the treated mice. Because EPCs secrete numerous pro-angiogenic factors that contribute to neovascularization during embryogenesis [20], we hypothesized that transplanted hiPS-EPCs would secrete pro-angiogenic factors and support placental and embryonic development. To examine this hypothesis, we first analyzed the expression levels of cytokines that promote placental development in hiPS-EPCs by RT-qPCR analysis. As shown in **Figure 5A**, the expression levels of *VEGF* and *PlGF* were significantly higher in hiPS-EPCs than in undifferentiated hiPSCs *in vitro* culture. Next, to examine whether EPC transplantation therapy improved the environment of placental and embryonic development, we examined the expression levels of pro-angiogenic factors in the placentas which were isolated from the treated mice. RT-qPCR analysis showed that the *VEGF* and *PlGF* expression levels were significantly increased in the E10.5 placentas isolated from the hiPS-EPC-treated mice (**Fig. 5B**). These results indicate that transplanted hiPS-EPCs may increase the expression of pro-angiogenic factors in the placenta. Collectively, these data imply that EPC transplantation reduces miscarriage by secreting pro-angiogenic factors and improving the environment of placental development.
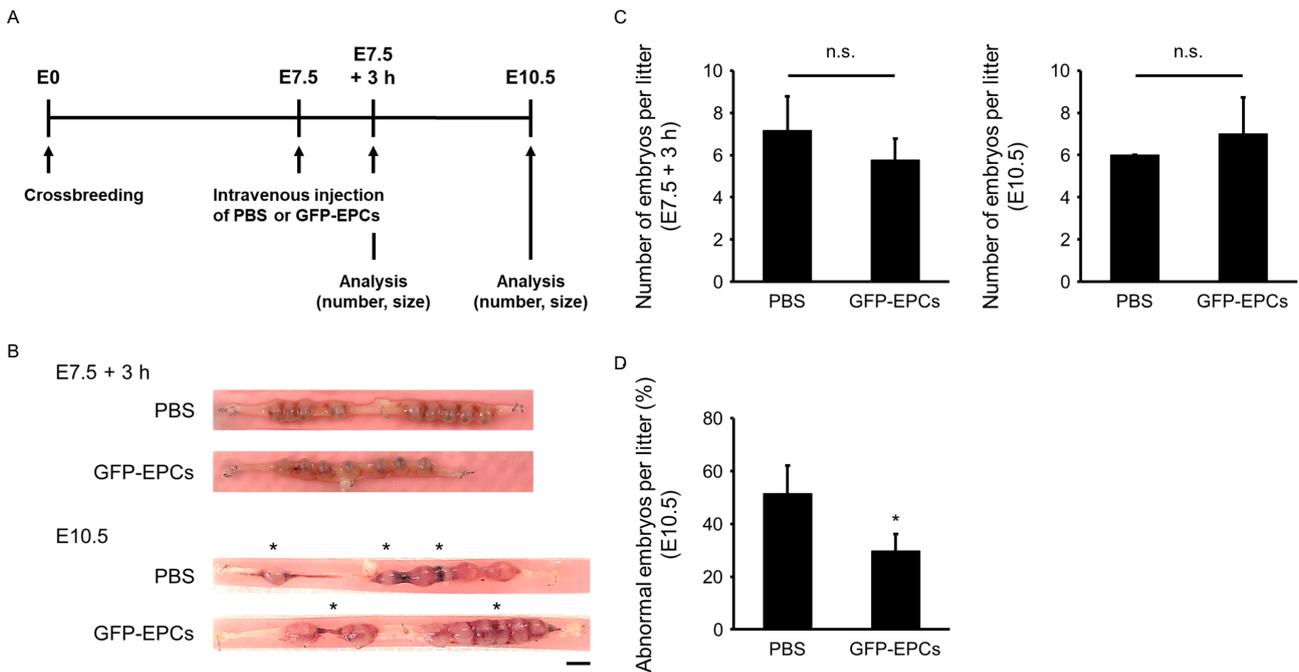
Figure 3 hiPS-EPCs transplantation therapy improves the size of embryos and reduces fetal abnormalities
(A) Experimental design of EPC transplantation therapy and sample collection. At E7.5 stage, pregnant mice were intravenously injected with PBS or GFP-EPCs. After 3 or 72 h, the treated uteruses containing embryos were collected and analyzed. (B) Representative images of PBS- or GFP-EPCs treated embryos 3 or 72 h post-transplantation. Asterisks indicate abnormal embryos. Bar, 5 mm. (C) Histogram showing the number of embryos in pregnant mice at 3 h (E7.5 + 3 h) and 72 h (E10.5) post-transplantation with hiPS-EPCs (PBS-treated mice, $n = 6$ and 4; GFP-EPC-treated mice, $n = 8$ and 3, respectively). Values are shown as means ± SD. n.s. = not significant, Student's *t*-test. (D) Histogram showing the percentage of fetuses with abnormalities per pregnant mouse at E10.5 stage. Values are shown as means ± SD. $n = 4$ pregnant mice (PBS) and $n = 3$ pregnant mice (GFP-EPCs). *$P < 0.05$, vs. PBS, Student's *t*-test.

A

E0   E7.5

3 hours

Crossbreeding   Intravenous injection   Analysis
of PBS or GFP-EPCs   (detection of GFP-EPCs)

Figure 4 **hiPS-EPCs localize in the uterine vasculature adjacent to the embryos after intravenous injection**

(A) Experimental design of EPC transplantation therapy and sample collection. At E7.5 stage, pregnant mice were intravenously injected with PBS or GFP-EPCs. After 3 h, the whole uteruses containing embryos were collected from the treated mice, and the presence of the transplanted cells was analyzed.

(B) Representative images of immunofluorescence staining for GFP (green) and CD31 (red) in the PBS- or GFP-EPCs-treated embryos. DAPI (blue) was used for nuclear staining.
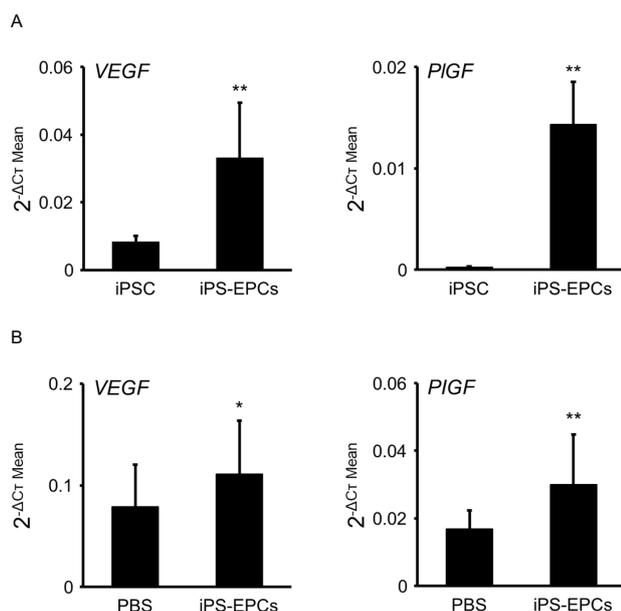
Figure 5 **Increased number of cytokines associated with angiogenesis in the placenta of mice treated with hiPS-EPCs**

(A) The expression of angiogenic factors such as VEGF and PlGF in hiPS-EPCs was confirmed by RT-qPCR. Values are shown as means ± SD of five independent experiments. **P < 0.01 vs. iPSC, by Student's t-test.

(B) The expressions of VEGF and PlGF in the placentas were analyzed by RT-qPCR in E10.5 CBA/J mice treated with hiPS-EPCs. Values are shown as means ± SD. n = 20 placentas from 3 pregnant mice (PBS) and n = 16 placentas from 3 pregnant mice (iPS-EPCs). *P < 0.05, **P < 0.01 vs. PBS, by Student's t-test.

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DISCUSSION

Our previous study showed that mouse ESC-derived EPCs reduced miscarriages in a mouse model of RSA [12]. However, this treatment had ethical issues because it used ESCs derived from fertilized eggs. Therefore, this study was performed using hiPSCs, which do not have the same ethical issues as ESCs. To the best of our knowledge, this is the first study to show that the intravenous transplantation of hiPS-EPCs can reduce miscarriages. Since this treatment uses hiPSCs, there are no ethical issues with ESCs, and it is a step closer to clinical application.

It is suggested that abnormal placentation is a major cause of miscarriage. After implantation of the fertilized ovum, trophoblasts provide nutrients to the embryo and develop into a large portion of the placenta. During placentation, trophoblasts differentiate into extravillous trophoblasts and cooperate with various maternal cells to contribute to placental neovasculogenesis and remodeling [21]. In particular, the adequate formation of spiral arteries enables the supply of nutrients and oxygen to the intervillous space, which contributes to the subsequent growth and development of the fetus. Impaired placentation can lead to various perinatal complications, such as miscarriage, intrauterine fetal dysplasia, gestational hypertension, and premature placental abruption. Daimon *et al*. showed that intravenous transplantation of mouse ESC-derived EPCs supported placental development and embryo growth by directly differentiating the cells into vascular endothelial cells and forming spiral arteries in a mouse model of RSA [12]. In this study, we observed an improvement in placental development and embryo growth using the same mouse model by transplantation therapy with hiPS-EPCs. However, we did not find a direct contribution of hiPS-EPCs to the formation of spiral artery vessels. This discrepancy may be due to the differences in cellular origin between mouse ESCs and hiPSCs.

Neovascularization can occur through two mechanisms: vasculogenesis and angiogenesis. In the early stages of placental development, cytotrophoblasts trigger vasculogenesis and angiogenesis, which are spatiotemporally regulated by various pro-angiogenic factors secreted by fetal and maternal lineage cells [22–24]. In this study, hiPS-EPC transplantation therapy indirectly promoted angiogenesis or vasculogenesis because the expression of the pro-angiogenic factors VEGF and PlGF was increased in the cultured hiPS-EPCs and in the embryos treated with hiPS-EPCs (**Fig. 5**). Angiogenic factors are secreted by various cells, including immune cells, placental trophoblasts, and endothelial cells. Sano *et al*. showed that the expression levels of VEGF and PlGF decreased in the placentas of embryos isolated from CBA/J × DBA/2 mice [25], the same mouse model of RSA used in our study. This finding indicates that regulation of angiogenic factors during placentation may be an effective therapy for miscarriages. However, it is difficult to control the actions of angiogenic

factors and related cells spatiotemporally *in vivo*. We believe that transplantation therapy with hiPS-EPCs could make this possible because the transplanted cells can accumulate in the pre-placental zone and continuously secrete various fluid factors promoting placental development. In this study, we could not determine the localization of transplanted cells in embryos at E10.5. In future studies, we will clarify how long transplanted iPS cell-derived cells remain and function in pregnant mice.

In this study, we used cells of human origin for cell transplantation therapy in the RSA mouse model. Although xenotransplanted cells are generally eliminated by the host immune system, there are some reports of cell transplantation therapy in mouse or rat models using hiPSC-derived cells. Bin et al. reported the therapeutic effects of transplanting hiPSC-derived vascular endothelial cells into the region of cerebral ischemia in mice without immunosuppressive drugs [26]. It has also been reported that transplantation therapies using iPSCs or mesenchymal stem cell-derived cells of human origin are less likely to occur in mouse and other animal models [27–29]. Indeed, in this experiment, xenotransplanted hiPSC-EPCs were detected *in vivo* in the treated mice (**Fig. 4B**), and as far as we could observe, the treated mice did not show any obvious rejection-related features such as death, weight loss, or anorexia (data not shown). However, we cannot exclude the possibility that some of the rejection responses associated with xenotransplantation may occur in our model, and further analysis is needed on this point. Immunosuppressive agents are often used in xenotransplantation to reduce rejection. However, the use of immunosuppressive agents seems to be disadvantageous in EPC transplantation therapy in this study. As mentioned above, various cells, including immune cells, are involved in placental development by secreting various fluid factors. In addition, stem cells require inflammatory cytokines released from disease sites to migrate to and function in these sites [30]. Therefore, in this experiment, the use of immunosuppressive agents would have a negative effect on the hiPSC-EPC transplantation therapy for miscarriage.

In summary, we reported that intravenous transplantation of hiPS-EPCs can reduce miscarriages by indirectly promoting placental neovascularization by secreting pro-angiogenic factors in a mouse model of RSA. Furthermore, this study supports the therapeutic potential of hiPSC-derived cell transplantation therapy for pregnant women with recurrent miscarriages.

ACKNOWLEDGMENTS

Declaration of conflicting interests

The authors declare no potential conflicts of interest with respect to the research, authorship, or publication of this article.

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