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Full Length Research Paper

Evaluation of the potential of human mesenchymal stem cell engrafted after in utero transplantation in murine model

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Mesenchymal stem cells derived from human placenta (hPMSc) is an important alternative source of adult stem cells. These cells with multilinear capacity represent a biological material important for regenerative medicine. In this study, we established a mouse model for *in utero* transplantation of hPMCs to investigate if these cells would affect long-term, organ-specific engraftment. Murine model results can be extrapolated in human medicine in the treatment of various diseases.

Key words: Placenta, mesenchymal stem cells, engraftment, *in utero* transplantation.

INTRODUCTION

The human placenta is a fetomaternal organ, formed by both fetal and maternal tissue (Linju et al., 2005). Its successful formation is a critical process in embryogenesis, and the normal development and function of the placenta is crucial to the wellbeing of the fetus. This organ is discarded postpartum, after having performed its necessary function of supporting the embryo and fetus (Geordias et al., 2002; Linju et al., 2005). The intrauterine transplantation of stem cells provides in some instances a therapeutic option before definitive organ failure occurs (Shapiro et al., 2000). The early fetus is uniquely tolerant to foreign antigens, accepting allogeneic or xenogeneic cells without the need to match major histocompatibility complex (MHC) antigens or induce immunosuppression (Muench, 2005). Multiple clinical experiences show that certain diseases such as immune deficiencies and inborn errors of metabolism can be successfully treated using adult stem cells.

The major problem is the low level of engraftment. Some experiments in mice show similar early homing of allogeneic and xenogeneic stem cells and reasonable early engraftment of allogeneic murine fetal liver cells (17.1% donor cells in peripheral blood 4 weeks after transplantation) (Troegera et al., 2006). Multiple researches on animal models are designed to optimize engraftment and recipient microenvironment in order to increase levels of grafting. It is known that some diseases such as hemoglobinopathies (Fanconi´s anemia and thalassaemia), immunological defects (SCID), and certain inborn errors of metabolism can be treated by stem cell transplantation (Troegera et al., 2006). In this study, we established a mouse model for *in utero* transplantation of hPMCs stem cells to investigate if these cells would affect long-term, organ-specific engraftment.

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Abbreviations: hPMSc, Human placental mesenchymal stem cells; **MHC,** major histocompatibility complex; **PBS,** phosphate-buffered saline; **EDTA,** ethylenediaminetetraacetic acid; **FITC,** fluorescein isothiocyanate; **FACS,** fluorescence-activated cell sorting; **MSCs,** mesenchymal stem cells; **SD,** standard deviation; **E13.5,** 13.5 days embryos; **E 20,** 20 days embryos.

MATERIALS AND METHODS

Harvest and preparation of placenta-derived cells

Biological material, clinically normal human term placentas (37 to 40 weeks of gestation, n =3) were collected after Cesarean section. Term placentas from healthy donor mothers were obtained with informed consent approved according to the procedures of the institutional review board. The harvested pieces of tissue were washed several times in phosphate-buffered saline (PBS) (Sigma)

and then mechanically minced and enzymatically digested with 0.25% trypsin- ethylenediaminetetraacetic acid (EDTA) (Gibco) for 30 min at 37°C. After centrifugation the cell suspension was filtered to eliminate undigested fragments. For erythrocytes lysis, cells suspensions were treated with fluorescence-activated cell sorting (FACS) Lysing Solution 10x (BD Biosciences) for 15 min. The suspension pelleted by centrifugation (1500 rpm/7 min) and suspended in propagation medium, which consist of Dulbecco's Modified Eagle's medium (DMEM) (Gibco) supplemented by 10% fetal calf serum (FCS), 100 U/ml penicillin-streptomycin (Gibco). Cultures were maintained in DMEM with 10% fetal bovine serum (FBS; Hyclone, USA) at 37°C with 5% CO_2. Approximately 1 week later, some colonies consisting of fibroblast-like cells were observed. These cells were trypsinized and replated for expansion. In order to obtain single cell-derived hPMSc clones, cells were serially diluted in 96-well culture plates (BD Biosciences) at a final density of 60 cells/ plate. Colonies that grew with homogeneous bipolar morphology were expanded.

Flow cytometry

The cell surface phenotype of hPMCs was characterized after the second passage. The cells were trypsinised (0.25% trypsine EDTA), washed twice with PBS and stained according to the recommendation of the manufacturer with the monoclonal antibodies, FITC-CD44, examined with a FACS CantoII Apparatus (Becton–Dickinson).

In utero transplantation

For *in utero* transplantation of hPMCs, were prepared single cell suspensions. On day 13.5 after mating, pregnant mice were anesthetized with 4 mg/kg Kethamine and 40 mg/kg Xylazine cocktail administered by intraperitoneal injection. Under aseptic conditions, the uterine horns were exposed and donor cells were injected through a glass micropipette (inserted through the uterine wall and into the peritoneal cavity of each fetus under direct visualization. The injection consisted of 1 x 10^6 hPMCs in 5 µl of PBS. The abdominal incision was closed in two layers using 4-0 silk, and the mice were allowed to complete pregnancy to term.

Engraftment analysis

On E20, a low abdominal midline incision was made and the number of live fetuses in each uterine horn was recorded. Then, placenta, fetal blood and fetal organs including brain, heart, lung, liver, spleen and bone marrow were collected. To obtain single cell suspension as chopped tissues were processed by the Medimachine device. The percentage of cells of donor origin was evaluated by FACS using a flow cytometer (FACS Canto II). Red blood cells were lysed with whole-blood lysing solution (BD Biosciences). Cells were washed twice with cold PBS (Sigma) containing sodium azide (0.1%) and 0.5% bovine serum albumin (BSA) and incubated in the dark at room temperature for 30 min with 20 µl fluorescent antibody (anti - human CD45 PE-Cy5 antibody (PE-Cy5: phycoerythrin-Cy5), (FITC: fluorescein isothiocyanate), anti – human CD34-FITC antibody (FITC: fluorescein isothiocyanate) and anti- human CD44 antibody). Have prepared two samples for each antibody in the study: a sample and a sample labeled with antibody as blank unmarked. For positive control were used MSCs isolated from placenta ($CD44^+$). Cells were then washed two more times with PBS/azide and analyzed. In order to perform serial sections of murine fetuses were embedded in paraffin. Sections (5 mm) were air-dried and fixed in ice-cold acetone or 4% paraformaldehyde for 10 to 20 min. To highlight hPMCs were used antibody against anti-human CD44.

Statistical analysis

The data are described as mean±SD. Differences were assessed by using the independent-samples t-test, paired-samples t-test. A p-value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

To isolate and determine the multipotent potential of hPMC in term placentas, we extracted 4.5± 10^7 nucleated cells from 3 (n=3) placentas delivered at a mean gestational age of 38.66 ± 1.52 weeks. After 7 to10 days, adherent cells with fibroblastic morphology were detected. The hPMCs were cultured for more than 7 passages without any spontaneous differentiation. In utero stem cell transplantation was performed in 10 (n=10) female carrying a total of 65 fetuses. To show that hPMCs injected in utero on E13.5 engrafted in fetal organs, we collected fetal organ samples at E20. Most fetal tissues had demonstrable hPMCs engraftment at E20. Although the distribution pattern and numbers of cells in individual fetuses varied, hPMCs were detectable in more than 60% of the fetus. Engraftment analysis was done using FACS Diva software and results are presented as histograms. We assessed the presence of hPMCs in various fetal mouse tissues (Figure 1). Grafting percentages shown ranged between 1.2 and 7.2% at a mean 4.04%±2.26 (93.37±4, 67 positive control), a low but consistent with published data in literature. Postmortem analysis of the organs from E 20 fetal mice confirmed that hPMCs engrafted in more than 60% of fetal organs after *in utero* transplantation (Figure 2).

Placenta derived mesenchymal stem cells are generally negative for CD34, CD45 and HLA-DR expression and positive for CD29, CD44, CD73, CD90, CD105 and CD166 (Barry et al., 1999, 2001; Parolini et al., 2008). HPMCs can proliferate *in vitro*, maintaining a homogenous morphology, consistent phenotype, and the capacity to differentiate into bone, cartilage, adipose tissue, hepatocytes or insulin secretion cells (Fukuchi et al., 2004; Yen et al., 2005; Chien et al., 2006; Zhang et al., 2006). Besides these capabilities they have a direct immunosuppressive effect on the proliferation of $CD4^+$ and $CD8^+$lymphocytes from human peripheral blood and umbilical cord blood *in vitro*, and are expected to have a potential application in allograft transplantation (Li et al., 2007). Cells of different origins have been used for *in utero* transplantation in a number of models. Tran's species animal models have been widely used in the study of stem cell migration and engraftment (Liechty et al., 2000; Saito et al., 2002). Human bone marrow-derived mesenchymal stem cells have been transplanted into fetal sheep and shown to persist for as long as 13 months with multilineage differentiation potential (Liechty et al., 2000*).*

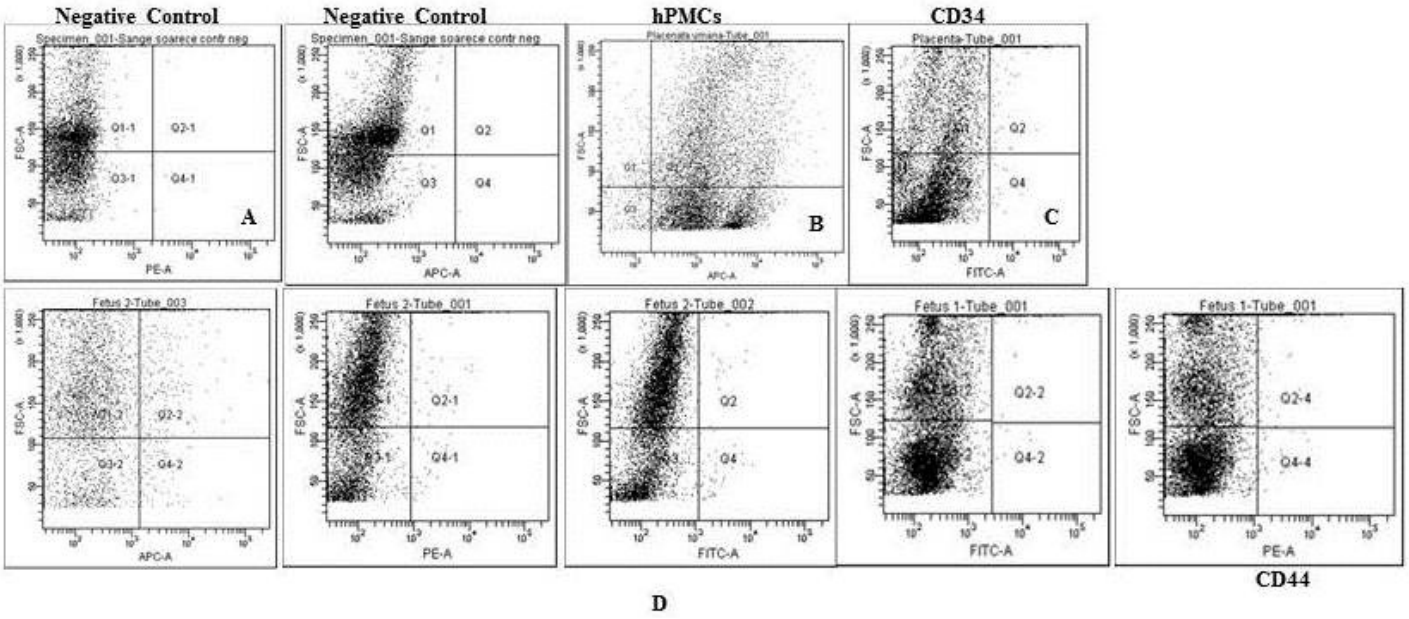

Figure 1. Flow cytometric analysis of CD34 positive hPMCs in the fetus and placenta after *in utero* transplantation of hPMCs. Representative data showing percentages of human CD44-positive hPMCs in placenta; **C** or fetus (organ mixture); **D** of recipient mice at E20; Control negative: mouse peripheral blood without hPMCs transplantation; **A** positive control hPMCs; **B.**

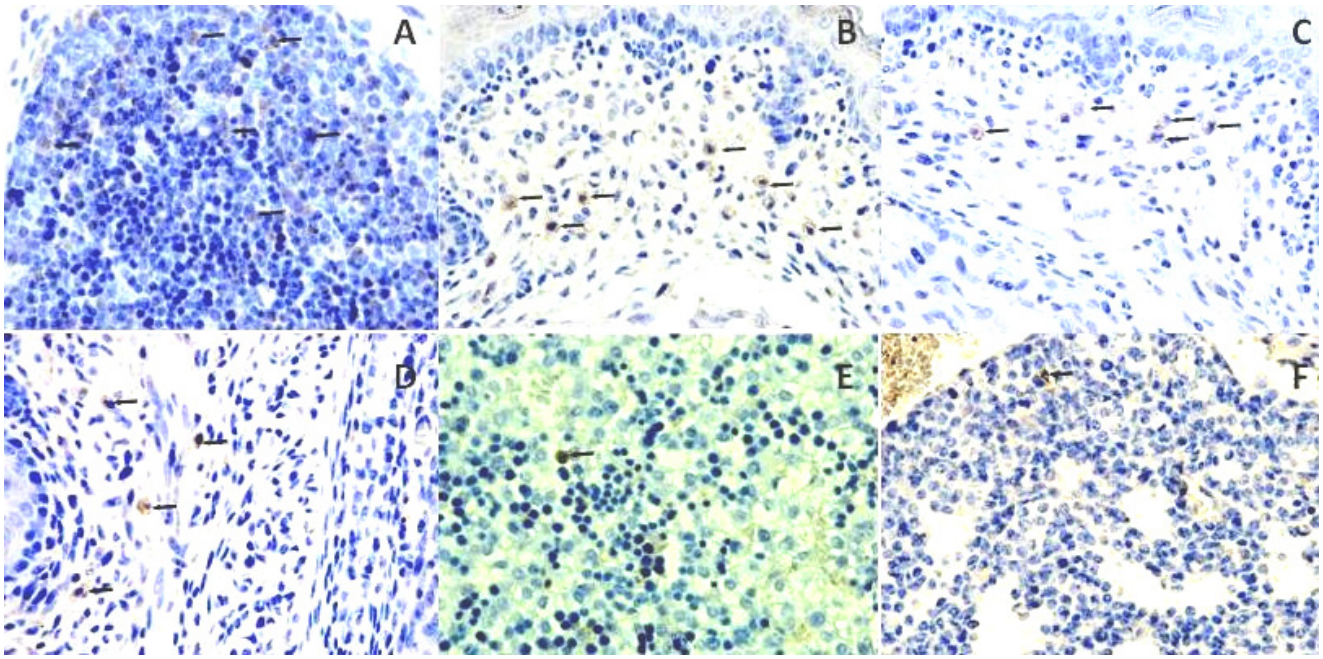

Figure 2. Hematoxylin-eosin (HE) stain and immunofluorescence of fetal mouse organs at embryonic day; **E** 20, were present in various fetal mouse tissues. Mouse tissues were immunostained using fluorescein isothiocyanate (FITC) - conjugated CD44 antibody (A,B,C,D,E,F); A- lymphatic node section with $CD44^+$ cells; **B**, **C** and **D**- murine fetal subcutaneous section with $CD44^+$ human cells, E,F -murine fetal liver section with $CD44^+$ (40x).

In utero transplantation of 1 $x10^8$/kg $CD34^+$ paternal canine bone marrow-derived cells in a canine model achieved a low level of microchimerism (<1%) in various tissues (Blakemore et al., 2004). It has been shown that human cord blood-derived cells can differentiate into hepatocytes in the mouse liver without evidence of cellular fusion (Newsome et al., 2003). Human microchimerism was observed in various organs and tissues at 4

months after transplantation of human amnion and chorion mesenchymal progenitors in neo-natal swine and rats (Bailo et al., 2004). Differences observed in cell numbers may be due to colonization efficiency in different tissue environments or the rate of cell turnover in each organ (Krause et al., 2001). Our study adds to this body of work by establishing an *in utero* (E13.5) model of xenogeneic hPMC transplantation in mice.

Conclusions

In order to identify some sources of stem cell indispensable for regenerative therapy is imperative to identify new sources of mesenchymal stem cells, ethically acceptable, technically accessible and allows isolation of multipotent cells such proliferative potential and multilinear capacity at least similar to mesenchymal stem cells isolated from embryonic or other adult sources. MSCs are widely distributed in a variety of tissues in the adult human body (for example, bone marrow, kidney, lung and liver). These cells are also present in fetal environment (for example, blood, liver, bone marrow and kidney) but MSCs are a rare population in these tissues. The most well studied and accessible source of MSCs is bone marrow, although even in this tissue the cells are present in a low frequency. The human placenta is an attractive new source of MSCs, but the biological characteristics of placenta-derived MSCs have not yet been characterized. Our results show that mesenchymal stem cells are present in the human term placenta and may be a potential source of cells for transplantation therapy. Using routine cell culture techniques, placental derived mesenchymal stem cells can be successfully isolated and expanded *in vitro*. It appears that hPMCs from an allogeneic donor might constitute such a source. A further potential benefit is the exposure of the fetus to allogeneic cells, inducing tolerance such as future treatment.

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