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Original Article

# EphA2 is a biomarker of hMSCs derived from human placenta and umbilical cord

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ABSTRACT

*Objective:* The heterogeneous nature of mesenchymal stem cells (MSCs) and the absence of known MSC-specific biomarkers make it challenging to define MSC phenotypes and characteristics. In this study, we compared the phenotypic and functional features of human placenta-derived MSCs with those of human dermal fibroblasts *in vitro* in order to identify a biomarker that can be used to increase the purity of MSCs in a primary culture of placenta-derived cells.
*Materials and methods:* Liquid chromatography-tandem mass spectrometry analysis was used to analyze and compare the proteome of human placenta-derived MSCs with that of fibroblasts. Quantitative real-time polymerase chain reaction, immunofluorescence, and flow cytometry were used to determine expression levels of EphA2 in placenta-derived MSCs. EphA2-positive cells were enriched by magnetic-activated cell sorting or with a cell sorter. An shRNA-mediated EphA2 knockdown was used to assess the role of EphA2 in MSC response to Tumor necrosis factor (TNF)-α stimulation.
*Results:* Analysis of proteomics data from MSCs and fibroblasts resulted in the identification of the EphA2 surface protein biomarker, which could reliably distinguish MSCs from fibroblasts. EphA2 was significantly upregulated in placenta-derived MSCs when compared to fibroblasts. EphA2 played an important role in MSC migration in response to inflammatory stimuli, such as TNF-α. EphA2-enriched MSCs were also more responsive to inflammatory stimuli *in vitro* when compared to unsorted MSCs, indicating a role for EphA2 in the immunomodulatory functionality of MSCs.
*Conclusion:* EphA2 can be used to distinguish and isolate MSCs from a primary culture of placenta-derived cells. EphA2-sorted MSCs exhibited superior responsiveness to TNF-α signaling in an inflammatory environment compared with unsorted MSCs or MSC-like cells.


## Introduction

Mesenchymal stem or stromal cells (MSCs), which are multipotent cells of embryonic mesodermal origin, have a fibroblast-like morphology. Depending on the stimuli and culture conditions, these cells can differentiate into adipocytes, osteocytes, chondrocytes, neural lineage cells, or myocytes among other cell types. Although the plasticity of human MSCs (hMSCs) and their role in tissue repair and regeneration have been extensively studied, there has been a recent focus on their immunological trophic properties [1,2]. hMSCs have been isolated from a variety of tissues, with the

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most frequently used source of MSCs being the bone marrow (BM). However, the procedure to isolate MSCs from bone marrow is extremely invasive. Tissues, such as human umbilical cord and placenta, which are normally discarded after labor, have been considered as attractive alternative sources for noninvasive isolation of hMSCs, and previous studies have reported efficient isolation of hMSCs from umbilical cord or placenta [3].

MSCs represent a more complex stromal cell subpopulation contained in mesenchymal tissue. Due to the heterogeneous nature of MSCs and the absence of known MSC-specific biomarkers, it is a challenging task to define MSC phenotypes and characteristics [4–6]. The molecular components responsible for MSC functionalities, particularly those on the plasma membrane, remain largely unknown. Additionally, lack of specific cell-surface markers increases the risk of contamination of MSC cell cultures with other cell types, specifically mature stromal cells, such as fibroblasts, which are abundant in mesenchymal tissue [4–6]. Isolation of MSCs from placenta-derived tissues is complicated by the fact that non-MSCs, such as fibroblasts, placenta-derived epithelial cells, and placenta-derived reticular cells, often coexist with MSCs. Fibroblasts, in particular, are the main source of contamination.

Fibroblasts are considered mature mesenchymal cells that are particularly abundant in the connective tissue, and are consequently the most frequent contaminating-cell phenotype in many cell-culture systems. Not only is it difficult to successfully eliminate fibroblasts from a culture, it is also particularly complex to distinguish MSCs from fibroblasts in the same culture. Fibroblasts and MSCs have a similar morphological appearance; they both proliferate well and have many identical cell-surface markers [7,8]. MSCs are currently defined by the International Society of Cellular Therapy as plastic adherent, multipotent, fibroblast-like cells expressing CD73, CD105, and negative for the hematopoietic markers CD14, CD34, and CD45. However, since these properties and markers are also shared by fibroblasts, the current definition fails to distinguish MSCs from generic fibroblasts. Currently, the best way to distinguish MSCs from fibroblasts is based on functional properties; MSCs retain multipotent stemness and immunomodulation capacity, while fibroblasts seem more limited in both of these functional areas.

Since Friedenstein et al's [9] pioneering work on identification of MSCs, there have been no reports on distinct differences in culture-derivation methodology, morphology, or gene expression signatures that would consistently and unequivocally distinguish *ex vivo* culture-expanded MSCs from fibroblasts [10–13]. There is currently also no accepted criterion or single cell-surface marker that can be used for separating MSCs from fibroblasts. Due to the fact that the fibroblast is the most common contaminant in MSC cultures derived from the placenta, it is crucial to identify a novel surface protein that can be used as a biomarker to distinguish MSCs from fibroblasts in order to ensure the homogeneity of primary cultures of placenta-derived MSCs.

Human erythropoietin-producing hepatocellular (Eph) receptors include transmembrane proteins comprising the largest family of receptor tyrosine kinases. The first identified function of Eph receptors was their role in the complicated and sophisticated mechanism underlying axon guidance. Eph receptors are now known to regulate a wide range of cell-to-cell communication events involved in cell positioning and tissue patterning during embryonic development. They also play a role in the pathology of cancer and vascular complications [14–18]. Additionally, these receptors are important regulators of specialized cell functions involved in synaptic plasticity, insulin secretion, bone remodeling, epithelial homeostasis, as well as inflammatory and immune responses [14,15,19]. They are expressed by a wide variety of cell types, such as neurons, vascular cells, epithelial cells, inflammatory cells, immune cells, and tumor cells, including cancer stem cells [20–23].

The EphA2 gene belongs to the Eph-receptor subfamily of the protein-tyrosine kinase family. EphA2 has previously been shown to play a role in mediating developmental events, particularly in the nervous system. EphA2 functions in specific aspects of pattern formation during development and subsequently in the development of several fetal tissues. It is involved in vasculogenesis, neural tube development, axial mesoderm formation, early hindbrain development, neuron differentiation, regulation of cell migration, bone remodeling through regulation of osteoclastogenesis and osteoblastogenesis, mammary gland epithelial-cell proliferation, and branching morphogenesis during mammary gland development [24]. In particular, the role of EphA2 in nervous system embryonic development is well-defined [25], and it has been implicated in the process by which neurons send out axons to reach the correct targets.

Recent studies reported the role of Eph receptors in stem-cell biology, both during embryonic development and in adult stem cells. Eph receptors are expressed in most adult stem-cell niches. Stem cells are located in specialized microenvironments, or niches, defined as the combination of cellular and microenvironmental determinants orchestrating the self-renewal and differentiation of stem-cell pools within specialized tissue locations. The expression of Eph receptors and ephrin ligands during embryogenesis and tissue homeostasis is consistent with their involvement in stem-cell regulation during development and in adult tissue homeostasis [26,27]. It has been suggested that the Eph/ephrin system has a spatiotemporal regulatory function in the balance between stem-cell quiescence, self-renewal, and differentiation [28]. However, the mechanism by which Eph is involved in maintenance of the stem-cell niche and its role in stem-cell regulation are not well understood. EphA2 is highly expressed in embryonic stem cells [29]. Quantitative proteomics studies showing high expression of EphA2 in human bone marrow MSCs and human umbilical cord perivascular cells (HUCPVCs) confirmed that it is upregulated in mesenchymal progenitor populations [30]. Nevertheless, the majority of the EphA2 functional studies in stem cells have been focused on the nervous system. EphA2 is highly expressed in the central nervous system, including precursors in neuronal and glial lineages [25,27]. Recent studies provided evidence that ephrin-A1 promotes the motility of EphA2-positive cardiac stem cells, resulting in enhanced regeneration and cardiac function after myocardial infarction [31]. Besides these findings, the expression profile and functions of EphA2 in stem-cell science are not yet well understood.

In this study, we compared the phenotypic and functional features of human placenta-derived MSCs with those of normal human dermal fibroblasts *in vitro*. Our proteomics data showed that expression of the EphA2 surface-specific marker can be used to distinguish MSCs in a primary culture of cells derived from placenta-related tissue. Our study provides a method of increasing the purity of the MSC population in a primary culture of cells derived from placenta-related tissue, based on the expression level of EphA2. Additionally, *in vitro* functional assays showed that the EphA2-enriched MSC population was responsive to tumor necrosis factor (TNF)-α-dependent signaling in an inflammatory environment.

## Materials and methods

### *Isolation of MSCs from placenta and umbilical cord*

Placental tissues were collected from healthy full-term placenta ($n = 8$). Written informed consent was obtained from individual mothers before the study, which was approved by the Ethics Committee of the Cardinal Tien Hospital. The age range of the

maternal donors was 20–45-years old. The placentas were kept at 4°C until placement in a biological safety cabinet. Placental-derived tissues were cut into small pieces, 1–2 $mm^3$ in size, digested with 10 U/mL collagenase, 2.5 U/mL dispase, and 0.05% Trypsin–EDTA for 90 minutes at 37°C. Samples were thoroughly washed three times with sterile phosphate-buffered saline (PBS). Tissue samples were then collected in 15 mL tubes and centrifuged at 250 g for 5 minutes. The cell pellet fraction was resuspended in α minimal essential medium (αMEM; Invitrogen, Waltham, NA, USA) with 10–15% fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine, 1 ng/mL basic fibroblast growth factor (FGF; Peprotech, Rocky Hill, NJ, USA), and PSF (100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL Fungizone; Invitrogen), then plated in T75 flasks. Cultures were washed from three to five times with PBS after 7 days to remove nonadherent cells from plastic-adherent colonies, which were further cultured up to 2 weeks with medium change every 3 days. The culture was maintained in αMEM supplemented with 10–15% FBS, 2 mM L-glutamine, 1 ng/mL basic FGF, and PSF at 37°C with saturated humidity and 5% $CO_2$. Cells were passaged at approximately 70–90% confluence. The stem cells were subcultured by treating with TrypLE (Life Technologies, Carlsbad, CA, USA) for 1 minute at 37°C. The cells were washed and harvested by centrifugation at 300 g for 5 minutes, then replated at a lower density (5000 cells/$cm^2$). The stem cells were maintained in αMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 ng/mL basic FGF at 37°C in saturating humidity and 5% $CO_2$. MSCs were characterized by analyzing the expression of CD markers (CD44, CD73, CD90, CD105, CD11 b, CD19, CD34, CD45, and HLA-DR; BD Stemflow hMSC Analysis Kit; BD Biosciences, San Jose, CA, USA) using flow cytometry. The capability of trilineage differentiation (osteocyte, chondrocyte, and adipocyte) were also examined and demonstrated positive results (data not shown).

*Sample preparations for liquid chromatography-tandem mass spectrometry*

Briefly, the MSC pellet was suspended in radioimmunopre-cipitation assay buffer (with 0.25 M sucrose and 0.1 M EDTA, final concentration) at pH 7–8, and then homogenized at 4°C using a Tissue-Tearor homogenizer (Micro-Grinder, RPI Corp, IL USA). Cell extracts were centrifuged at 1000*g* for 10 minutes at 4°C, and then the supernatant was centrifuged at 100,000*g* for an additional 60 minutes at 4°C. The supernatant fraction (containing the nuclear and cytoplasmic fractions) was saved for in-gel digestion. The crude membrane fraction pellet was resuspended in 6M urea, 5 mM EDTA, and 2% (w/v) sodium dodecyl sulfate for a separate in-gel digestion. After trypsinization, samples were further processed for liquid chromatography-tandem mass spectrometry (LC-MS/MS).

*Immunophenotypic characterization of placenta-derived MSCs and fibroblasts*

Full-term placentas were collected and MSCs were isolated from amniotic membrane (AM), chorionic disk (CD), chorionic membrane (CM), and umbilical cord (UC). Placenta-derived cells were cultured, expanded, and maintained in αMEM with FBS and basic FGF at 37°C and at saturating humidity and 5% $CO_2$. Cells were subcultured at 80% confluence, and then phenotypically characterized by flow cytometry. Immunostaining was performed by incubating cells with the specified antibodies according to manufacturer instructions (BD Stemflow hMSC Analysis Kit; BD Biosciences). Nonspecific immunoglobulin G of the corresponding class served as the negative control. Cell suspensions were subjected to flow cytometry (BD Biosciences FACSCanto II) and data analysis performed using Flowjo 7.6.1 software (http://www.flojo.com).

*Sorting EphA2-enriched MSCs by magnetic-activated cell sorting or using a cell sorter*

The magnetic-activated cell sorting (MACS) method allows cells to be separated by incubating with magnetic nanoparticles coated with antibodies against EphA2-surface antigen. Primary cultures of placenta-derived MSCs were incubated with the fluorescence-conjugated anti-human antibodies against EphA2 and sorted with R-Phycoerythrin (PE) Magnetic Particles according to manufacturer instructions (MACS Technology, MiltenyiBiotec, Bergisch Gladbach, Germany). For sorting by cell sorter, cells derived from placentas were harvested and sorted by anti-EphA2 antibodies using the JAZZ cell sorter (BD Biosciences) at P0.

*Quantitative real-time polymerase chain reaction evaluation of EphA2 transcript in placenta-derived MSCs and fibroblasts*

Total RNA was isolated from 64 populations of placenta-derived cells ($n = 8$, including passage 1 and passage 3, from AM, CD, CM, and UC samples, as well as from Human Foreskin Fibroblasts (neonatal, PC501A-HFF; System Biosciences, Mountain View, CA, USA) using the Direct-zolminiprep Kit (Zymo Research Corporation, Irvine, CA, USA). Complementary DNA (cDNA) was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) from 100 ng total RNA. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the Roche Universal Probe Library System with a LightCycler480 II (Roche) according to manufacturer instructions using the EphA2 5′ primer sequence ACC CCC ACA CAT ATG AGG AC and the 3′ primer sequence TGG ATG GAT CTC GGT AGT GA.

*EphA2 knockdown by lentiviral transduction*

Lentiviral transduction was used for shRNA-mediated knockdown of EphA2 in order to evaluate the role of EphA2 in MSCs. Each construct had an enhanced green fluorescent protein (EGFP) reporter to monitor transduction efficiency. Four different shRNA sequences (Target #1, GCGTCATCTCCAAATACAA; Target #2, GGCTGAGCGTATCTTCATT; Target #3, GCGCCTGTTCACCAAGATT; and Target #4, GCAGCAAGGTGCACGAATT) were tested, each with three different multiplicity of infection values (2, 5, and 10), with each experimental condition was performed in triplicate. Knockdown efficiency was evaluated by qRT-PCR. The best knockdown efficiency achieved was 50%. The clone which conferred the best knockdown efficiency was used in all subsequent experiments.

*Trans-well migration analysis of EphA2-knockdown MSCs*

MSCs transduced with the wild-type, sh-scramble, and sh-EphA2 knockdown constructs were plated in 8 μm trans-wells at a density of 30,000 cells/well. FBS (0.2%) and TNF-α were added in the lower chamber to stimulate migration of MSCs. After 6 hours, the number of viable migrated cells was determined using Cell Titer-Glo Luminescent reagents (Promega, Madison, WI, USA) according to manufacturer instructions.

*Trans-well migration analysis of $EphA2^{high}$ MSCs*

Primary cultures of placenta-derived cells were incubated with magnetic beads conjugated to anti-human antibodies against EphA2, and then sorted by positive selection. Flow cytometry analysis was used to confirm the $EphA2^{+}$ and $EphA2^{-}$ MSC cell populations after MACS sorting. Cells were plated in 8 μm trans-

wells at a density of 30,000 cells/well. FBS (0.2%) and TNF-α were added in the lower chamber to activate migration of MSCs. After 6 hours, the number of viable migrated cells was determined using Cell Titer-Glo Luminescent reagents (Promega) according to the manufacturer's instructions.

### *Statistical analyses*

A Student *t* test or two-way analysis of variance was used to calculate significance. A $p$ value $< 0.05$ was considered statistically significant.

## Results

### *Immunophenotypic characterization of placenta-derived MSCs and fibroblasts*

MSCs were derived from AM, CD, CM, and UC. The phenotypic features of human placenta-derived MSCs were compared with those of normal human dermal fibroblasts using differential proteomics. This approach employed protein extraction coupled with gel separation, followed by LC-MS/MS to analyze the membrane proteome of human placenta-derived MSCs. These data were compared to the results derived from fibroblasts. EphA2 was significantly upregulated in placenta-derived MSCs as compared to fibroblasts. The expression levels of EphA2 in fibroblasts were below the limit of detection sensitivity of the LTQ-Orbitrap XL instrument.

We assessed the expression of CD11b, CD19, CD34, CD45, CD73, CD90, CD105, HLA-DR, and EphA2 using flow cytometry. MSCs isolated from different placenta locations were positive for CD73, CD90, CD105, and EphA2, and negative for CD11b, CD19, CD34, CD45, and HLA-DR. Flow cytometry analysis showed that fibroblasts were positive for CD73, CD90, CD105, negative for CD11b, CD19, CD34, CD45, HLA-DR, and negative or had low expression of EphA2. Placenta-derived MSCs consistently had a high percentage of EphA2-positive cells, whereas fibroblasts did not. The results are shown in Table 1.

**Table 1**
Immunophenotypes of placenta-derived mesenchymal stem cells and fibroblasts (percentage of positive cells following flow cytometry).

| Donor/tissue | Cell surface markers | | | | |
|---|---|---|---|---|---|
| | CD73 | CD90 | CD105 | EphA2 | Negative cocktail[a] |
| D12AM | 99.6 | 99.6 | 75.9 | 80.0 | 0.6 |
| D12CD | 99.3 | 99.5 | 97.0 | 72.9 | 0.5 |
| D12CM | 99.3 | 98.0 | 98.0 | 77.7 | 2.3 |
| D12UC | 99.4 | 99.7 | 90.6 | 80.2 | 0.7 |
| D17AM | 99.7 | 95.7 | 89.4 | 62.7 | 0.6 |
| D17CD | 99.7 | 95.7 | 92.1 | 45.0 | 0.6 |
| D17CM | 99.6 | 77.8 | 88.2 | 80.7 | 1.0 |
| D17UC | 99.6 | 99.8 | 84.9 | 65.9 | 1.4 |
| Fibroblasts | 99.3 | 97.7 | 75.6 | 18.6 | 0.6 |

AM = amniotic membrane; CD = chorionic disk; CM = chorionic membrane; D = donor; MSC = mesenchymal stem cell; P = passage; UC = umbilical cord.
[a] Negative Cocktail included the antibodies against CD11b, CD19, CD34, CD45, and HLA-DR (Human MSC Analysis Kit, BD Stemflow, catalog number 562245; BD Biosciences San Jose, CA, USA). The immunophenotypes of placenta-derived MSCs were derived from samples from donors #12 and #17 at P0. Flow cytometry analysis reveals that the MSC populations are ~99.3–99.7% CD73 positive, ~77.8–99.8% CD90 positive, ~75.9–98.0% CD105 positive, and ~45.0–80.7% EphA2 positive at P0. By contrast, hematopoietic cell lineage-specific markers, such as CD11b, CD19, CD34, CD45, and HLA-DR are not expressed in MSCs. Flow cytometry analysis reveals that fibroblast populations are 99.3% CD73 positive, 97.7% CD90 positive, 75.6% CD105 positive, and 18.6% EphA2 positive. Hematopoietic cell lineage-specific markers, such as CD11b, CD19, CD34, CD45, and HLA-DR, are not expressed in fibroblasts.

**Table 2**
The immunophenotypic characterization of EphA2-sorted placenta-derived MSCs at P0 (percentage of positive cells following flow cytometry).

| Cell population | Cell surface markers[a] | | | |
|---|---|---|---|---|
| | CD73 | CD90 | CD105 | EphA2 |
| D17CDP0_unsorted | 99.7 | 95.7 | 92.1 | 45.0 |
| D17CDP0_EphA2-sorted | 100.0 | 97.2 | 98.0 | 96.6 |

CD = chorionic disk; D = donor; MSC = mesenchymal stem cell; P = passage.
[a] The immunophenotypic characterization of the EphA2-sorted MSCs is demonstrated in MSCs derived from the CD of donor #17 at P0. The results show that EphA2-positive cells are also CD73 positive, CD90 positive, and CD105 positive.

### *Immunophenotypic characterization of EphA2-sorted placenta-derived MSCs*

#### *Flow cytometry analysis of EphA2-enriched MSCs by MACS*

Flow cytometry analysis of MACS-sorted MSCs at P0 revealed a homogenous cell population in 100% of CD73-positive, ~97.2–99.5% of CD90-positive, ~96.0–~99.9% of CD105-positive, and ~96.6–100% of EphA2-positive cells since P0 (Table 2). This suggested that EphA2 sorting using antibody-conjugated magnetic beads could dramatically improve the MSC purity from P0. The enriched EphA2-positive MSC population could be maintained and expanded *in vitro* (Table 3).

#### *Flow cytometry analysis of EphA2-enriched MSCs sorted by flow cytometry cell sorter*

Flow cytometry analysis of EphA2-sorted MSCs obtained using the flow cytometry cell sorter revealed that there were ~99.5–100% CD73-and CD90-double-positive, ~99.6–100% CD105-and CD90-double-positive, ~99.5–100% EphA2-and CD90-double-positive, ~99.8–100% CD73-and EphA2-double-positive, ~99.5–100% CD105-and EphA2-double-positive, and ~99.7–100% CD73-and CD105-double-positive populations in passages ~2–6 (Table 4). Our data showed that the EphA2 protein was continuously expressed and maintained in MSC cultures even at later passages.

### *qRT-PCR evaluation of EphA2 transcripts in placenta-derived MSCs and fibroblasts*

We used qRT-PCR to evaluate EphA2 expression in placenta-derived multipotent MSCs and compared it with that in fibroblasts. Gene expression was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase in the different cell populations. The expression of EphA2 transcripts in MSCs was calculated by fold enrichment compared to the expression of EphA2

**Table 3**
The immunophenotypic characterization of the EphA2-MACS-enriched population during *in vitro* expansion (percentage of positive cells following flow cytometry).

| Cell population | Cell surface markers[a] | | | |
|---|---|---|---|---|
| | CD73 | CD90 | CD105 | EphA2 |
| D17CDP0_EphA2-sorted | 100.0 | 97.2 | 98.0 | 96.6 |
| D17CD_EphA2+_P1 | 100.0 | 99.5 | 99.9 | 100.0 |
| D17CD_EphA2+_P2 | 100.0 | 99.3 | 99.9 | 99.9 |
| D17CD_EphA2+_P3 | 100.0 | 99.5 | 99.9 | 100.0 |

CD = chorionic disk; D = donor; MACS = magnetic-activated cell sorting; MSC = mesenchymal stem cell; P = passage.
[a] The immunophenotypic characterization of the EphA2-sorted MSCs in later passages. Immunophenotype is demonstrated in the MSCs derived from CD from donor #17. MSCs were sorted by EphA2-antibody-conjugated magnetic beads at P0 and maintained in optimized MSC culture conditions during *in vitro* expansion. The results show that expression of cell-surface marker EphA2 was maintained in later passages in optimized MSC culture conditions.

**Table 4**
The immunophenotypic characterization of EphA2-FACS-enriched MSC population during *in vitro* expansion.[a]

| % | CD73+CD90+ | CD105+CD90+ | EphA2+CD90+ | CD73+EphA2+ | CD105+EphA2+ | CD73+CD105+ |
|---|---|---|---|---|---|---|
| P2 | 100 | 100 | 99.5 | 100 | 99.8 | 99.7 |
| P3 | 99.5 | 99.6 | 99.5 | 99.8 | 99.5 | 99.7 |
| P4 | 99.9 | 99.7 | 99.9 | 100 | 99.6 | 99.7 |
| P6 | 100 | 99.9 | 100 | 99.9 | 100 | 100 |

D = donor; FACS = fluorescence-activated cell sorting; MSC = mesenchymal stem cell; P = passage; UC = umbilical cord.
[a] The immunophenotypic characterization of the EphA2-enriched MSCs during later expansion. Immunophenotype is determined in MSCs derived from the UC from donor #7. MSCs at P0 were sorted by anti-EphA2 antibodies using the cell sorter, and then maintained in optimized MSC culture conditions during later passages. The expression of the EphA2 cell-surface marker was preserved in later passages.

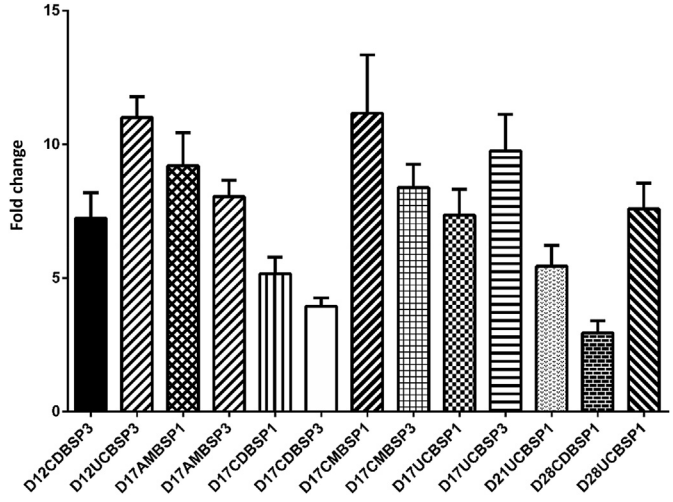



**Figure 1.** Real-time polymerase chain reaction quantitation of EphA2 mRNA. EphA2 mRNA levels in placenta-derived MSCs were evaluated by fold enrichment compared to the expression of EphA2 in fibroblasts, i.e., by comparison of EphA2 mRNA levels in MSCs to EphA2 mRNA levels in fibroblasts (MSCs/fibroblasts). MSC samples from donors #12, #17, #21, and #28 were used to determine EphA2 transcript levels. The results show that EphA2 is highly enriched in MSCs compared to fibroblasts. AM = amniotic membrane; BS = fetal bovine serum; CD = chorionic disk; CM = chorionic membrane; D = donor; MSC = mesenchymal stem cell; P1 = passage1; P3 = passage3; UC = umbilical cord.

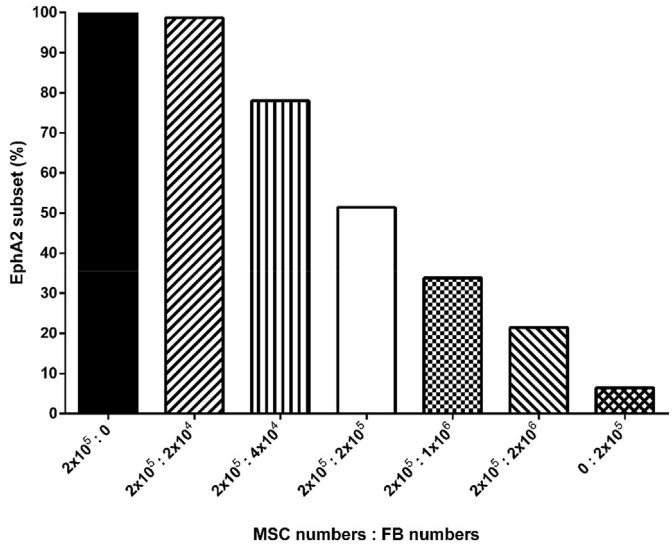



**Figure 2.** Flow cytometry analysis of mixed populations of MSCs and fibroblasts. MSCs derived from the UC from donor #23 were mixed with fibroblasts at different ratios. The percentage of EphA2+ detected by flow cytometry decreased proportionally in response to the increased fibroblast population. FB = fibroblasts; MSC = mesenchymal stem cell; UC = umbilical cord.

in fibroblasts. The results showed that EphA2 was highly expressed in MSCs as compared to fibroblasts (Figure 1).

### *Flow cytometry analysis of mixed populations of MSCs and fibroblasts*

In order to evaluate the feasibility of using EphA2 as a biomarker to separate placenta-derived MSCs from fibroblasts, MSCs derived from the UC of donor #23 were mixed with fibroblasts in the following ratios (MSC:fibroblasts in cell numbers): $2 \times 10^5$:0, $2 \times 10^5$:$2 \times 10^4$, $2 \times 10^5$:$4 \times 10^4$, $2 \times 10^5$:$2 \times 10^5$, $2 \times 10^5$:$1 \times 10^6$, $2 \times 10^5$:$2 \times 10^6$, and 0:$2 \times 10^5$ (Figure 2). The EphA2$^+$ population in each sample was determined using flow cytometry. We showed that the EphA2$^+$ population decreased in proportion to the increase in the fibroblast population.

### *The response of MSCs to TNF-α signaling was compromised by EphA2 knockdown*

shRNA-mediated EphA2 knockdown vectors (shEphA2) were used to evaluate the functions of EphA2 in MSCs. Each lentiviral construct had an EGFP reporter to monitor transduction efficiency

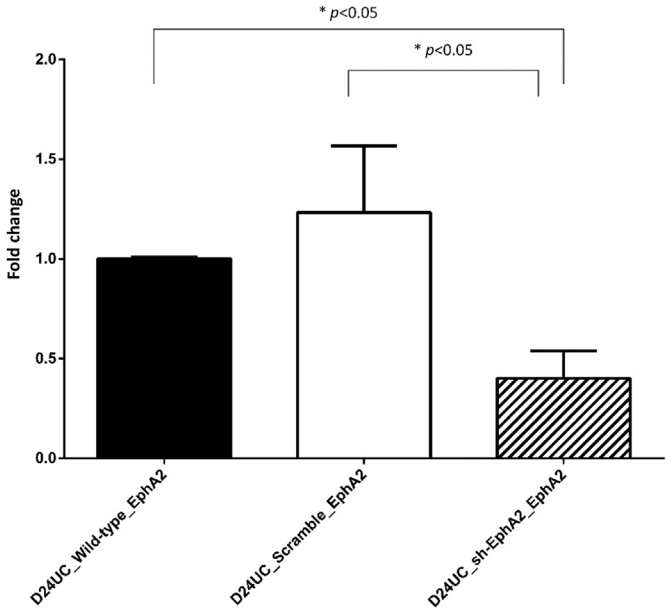
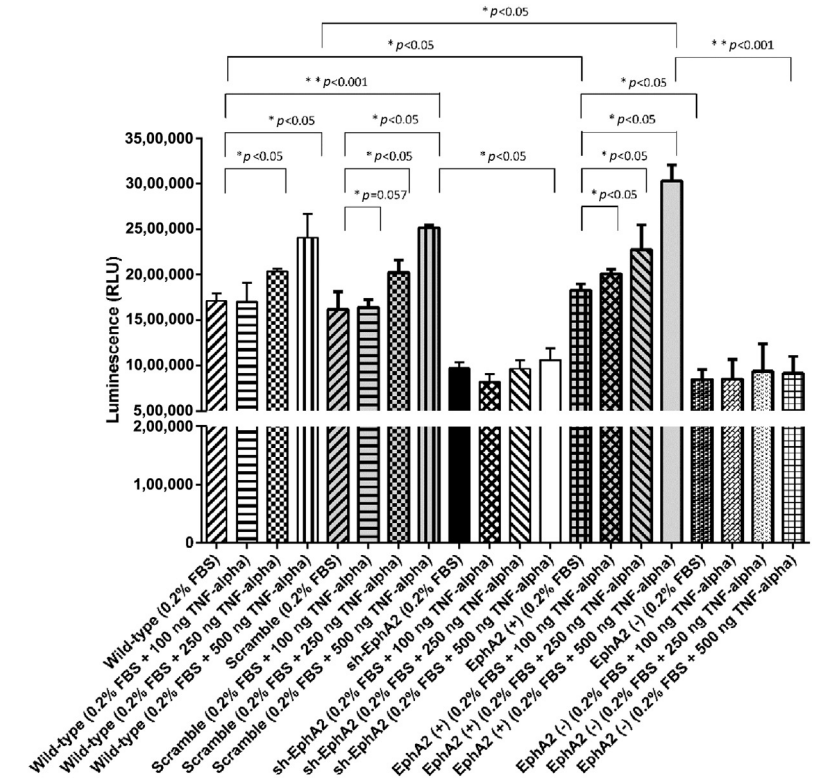
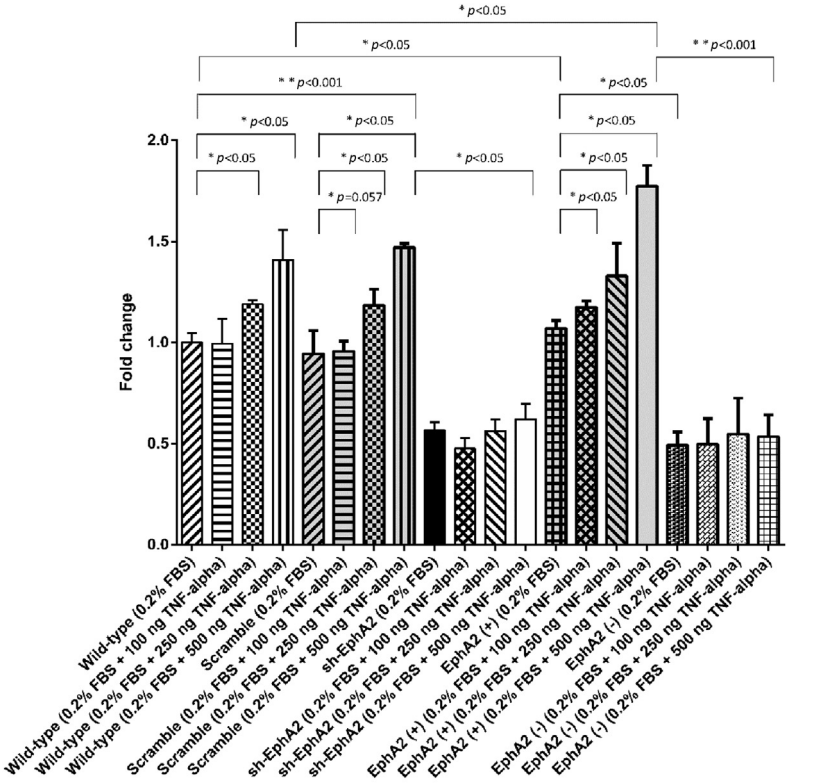



**Figure 3.** EphA2 RNA levels were evaluated by qRT-PCR. Total RNA expression levels in different individual cell populations were normalized to endogenous GAPDH expression levels. "Scramble" represents a scrambled control in the shRNA knockdown experiment. EphA2 mRNA levels in knockdown samples are compared to levels in normal wild-type UC-derived MSCs. qRT-PCR results confirm the sh-EphA2 knockdown efficiency. D = donor; MSC = mesenchymal stem cell; qRT-PCR = quantitative real-time polymerase chain reaction; UC = umbilical cord.


* p<0.05
* p<0.05
* * p<0.001
* * p<0.001
* p<0.05
* p<0.05
* p<0.05
* p<0.05
* p<0.05
* p<0.05
* p<0.05
* p<0.05
* p=0.057
* p<0.05
Luminescence (RLU)
35,00,000
30,00,000
25,00,000
20,00,000
15,00,000
10,00,000
5,00,000
2,00,000
1,00,000
0
Wild-type (0.2% FBS)
Wild-type (0.2% FBS + 100 ng TNF-alpha)
Wild-type (0.2% FBS + 250 ng TNF-alpha)
Wild-type (0.2% FBS + 500 ng TNF-alpha)
Scramble (0.2% FBS)
Scramble (0.2% FBS + 100 ng TNF-alpha)
Scramble (0.2% FBS + 250 ng TNF-alpha)
Scramble (0.2% FBS + 500 ng TNF-alpha)
sh-EphA2 (0.2% FBS)
sh-EphA2 (0.2% FBS + 100 ng TNF-alpha)
sh-EphA2 (0.2% FBS + 250 ng TNF-alpha)
sh-EphA2 (0.2% FBS + 500 ng TNF-alpha)
EphA2 (+) (0.2% FBS)
EphA2 (+) (0.2% FBS + 100 ng TNF-alpha)
EphA2 (+) (0.2% FBS + 250 ng TNF-alpha)
EphA2 (+) (0.2% FBS + 500 ng TNF-alpha)
EphA2 (-) (0.2% FBS)
EphA2 (-) (0.2% FBS + 100 ng TNF-alpha)
EphA2 (-) (0.2% FBS + 250 ng TNF-alpha)
EphA2 (-) (0.2% FBS + 500 ng TNF-alpha)

A


* p<0.05
* p<0.05
* * p<0.001
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* p<0.05
* p<0.05
* p<0.05
* p<0.05
* p=0.057
* p<0.05
Fold change
2.0
1.5
1.0
0.5
0.0
Wild-type (0.2% FBS)
Wild-type (0.2% FBS + 100 ng TNF-alpha)
Wild-type (0.2% FBS + 250 ng TNF-alpha)
Wild-type (0.2% FBS + 500 ng TNF-alpha)
Scramble (0.2% FBS)
Scramble (0.2% FBS + 100 ng TNF-alpha)
Scramble (0.2% FBS + 250 ng TNF-alpha)
Scramble (0.2% FBS + 500 ng TNF-alpha)
sh-EphA2 (0.2% FBS)
sh-EphA2 (0.2% FBS + 100 ng TNF-alpha)
sh-EphA2 (0.2% FBS + 250 ng TNF-alpha)
sh-EphA2 (0.2% FBS + 500 ng TNF-alpha)
EphA2 (+) (0.2% FBS)
EphA2 (+) (0.2% FBS + 100 ng TNF-alpha)
EphA2 (+) (0.2% FBS + 250 ng TNF-alpha)
EphA2 (+) (0.2% FBS + 500 ng TNF-alpha)
EphA2 (-) (0.2% FBS)
EphA2 (-) (0.2% FBS + 100 ng TNF-alpha)
EphA2 (-) (0.2% FBS + 250 ng TNF-alpha)
EphA2 (-) (0.2% FBS + 500 ng TNF-alpha)

B

and knockdown efficiency was evaluated by qRT-PCR. A 50% knockdown efficiency was achieved (Figure 3).

At injury sites, MSCs can be induced and mobilized to exert immunoregulatory effects in response to TNF-α and IFN-γ [32]. MSCs exert their immunosuppressive functionality by suppressing T- and B-cell responses following activation by various cytokines [32]. In inflammatory joint diseases, such as rheumatoid arthritis, MSCs in the bone marrow migrate to the joints via a TNF-α-dependent mechanism and may be partially responsible for the disease process [33]. Increased numbers of MSCs have also been demonstrated in periarticular tissues in osteoarthritis, which may reflect an attempt at joint repair or regeneration following injury [19]. It was proposed that TNF-α release in an inflammatory environment confers immunosuppressive properties upon MSCs when TNF-α binds to TNF-R1 of MSCs and activates the NF-κB pathway [34]. Here, we investigated the role of EphA2 in MSCs in response to inflammatory stimuli, such as TNF-α signaling, and determined if EphA2 was involved in MSC migration during inflammation [2,35]. The mobility of EphA2$^{high}$ and EphA2$^{low}$ MSCs under basal culture conditions or in the presence of TNF-α inflammatory stimuli was examined.

*Trans-well migration analysis of EphA2-knockdown MSCs*

The wild-type, sh-scramble, and sh-EphA2-targeted MSCs were plated in 8 μm trans-wells at a density of 30,000 cells/well. FBS (0.2%) and TNF-α were added into the lower chamber to activate migration of MSCs. The data revealed that EphA2 knockdown compromised the ability of MSCs to migrate, as well as to respond to TNF-α signaling (Figure 4).

*Trans-well migration analysis of EphA2$^{high}$ MSCs*

Primary cultures of placenta-derived cells were incubated with magnetic beads conjugated to anti-human antibodies against EphA2. Cells were then sorted by positive selection. Flow cytometry analysis confirmed the presence of EphA2$^{+}$ (or EphA2$^{high}$) MSCs and EphA2$^{-}$ (or EphA2$^{low}$) cell populations after MACS. Cells were plated in 8 μm trans-wells at a density of 30,000 cells/well. FBS (0.2%) and TNF-α were added in the lower chamber to activate migration of MSCs. The data revealed that the EphA2$^{high}$ MSC population had an enhanced capability to respond to TNF-α signal and to migrate (Figure 4).

The migration of MSCs was significantly affected by the addition of TNF-α to the basal medium (Figures 4A and 4B). Addition of TNF-α to the basal medium increased MSC migration in a TNF-α-dose-dependent manner (see Figure 4A). By contrast, the mobility of EphA2-knockdown MSCs, as well as EphA2$^{-}$ cells, was abolished. The effect of EphA2 on the migration of MSCs in response to TNF-α was more obvious when the luminescent signal was converted to migrated cell population relative to migrated wild-type MSCs in the 0.2% FBS control in fold change (Figure 4B).

## Discussion

MSCs derived from different sources display similar phenotypic and functional characteristics *in vitro* [30]. However, tissue-origin-specific differences are also apparent, making the definition of a progenitor cell challenging [30]. Previously, Ann E. Canfield's group [30] combined enrichment of cell-matrix interface proteins with quantitative MS and identified a panel of signature proteins that were significantly enriched in bone-marrow-derived MSC and HUCPVC cultures as compared with differentiated mesenchymal cells [adult human dermal fibroblasts (HDFs)]. Comparing bone marrow MSCs or HUCPVCs against HDF preparations identified proteins in membrane-enriched and matrix-enriched fractions that were significantly increased in all adult MSCs and HUCPVCs versus HDFs. Proteins identified as enriched in bone marrow MSCs and HUCPVCs comprised known MSC markers CD106, CD49c, and CD58, and novel markers, including EphA2 [30]. In this approach, multiple sources of MSCs, except HUCPVCs, were derived from adult tissues. The differentiated mesenchymal cells used as internal controls were derived from adult HDFs.

In our study, in contrast to the materials derived from adult tissues, we isolated MSCs from fetal-originated tissues from placenta, including AM, CM, CD, and Wharton's jelly within the UC. Characterization of differential proteomics data from fetal-originated MSCs (from AM, CM, CD, and UC) compared to human fetal fibroblasts led to the identification of EphA2 as a surface-protein marker that could discriminate placental and UC MSCs from fibroblasts during the isolation process.

Human MSCs have been isolated from a wide range of tissues using different techniques. The heterogeneity of the cell population with the different techniques used to isolate, culture, and define MSCs often led to experimental variability and contradictory data. The molecular expressions seemed to vary depending on the tissue source and culture conditions. The debate over the use of a certain surface antigen as a marker to define MSCs implied the difficulty to identify markers independent from the MSC tissue source and culture conditions. According to the data published by Canfield et al [30] and the results from our study, EphA2 was highly expressed in different populations of MSCs derived from fetal, as well as from adult tissues, regardless of the culture conditions. Hence, we would like to propose that the EphA2 protein could serve as an additional universal biomarker to increase the confidence in isolated MSC identification and verification.

One of the therapeutic attributes of MSCs is their ability to mobilize to sites of injury where they participate in repair [32]. Canfield et al [30] used a wound assay to investigate whether cellular migration was affected after knockdown of EphA2 expression. A small, but statistically significant decrease in migration was detected following EphA2 siRNA treatment [30]. We observed similar patterns in our trans-well migration assay (Figure 4).

Recent studies demonstrated that inflammatory factors, such as TNF-α and interleukin-1β, may provide cues to mobilize MSCs to tissue damage sites [32]. Tissue injury is associated with the activation of inflammatory cells. Inflammatory mediators, such as TNF-α, interleukin-1β, free radicals, chemokines, and leukotrienes, are often produced by phagocytes in response to damaged cells and leaked cell contents [32]. These inflammatory molecules and immune cells, together with endothelial cells and fibroblasts, orchestrate changes in the microenvironment that result in the mobilization of MSCs to damaged tissue [32]. Once MSCs have entered the microenvironment of injured tissues, signals from the microenvironment could stimulate the release of an array of cytokines or growth factors by MSCs. These secreted factors modulate the immune response at the damaged site, and promote the development of fibroblasts, endothelial cells, and tissue progenitor cells, carrying out tissue regeneration and repair [32].

**Figure 4.** Trans-well migration assay and detection of cell viability. Cells were plated in 8 μm trans-wells at a density of 30,000 cells/well. FBS (0.2%) and TNF-α were added into the lower chamber to stimulate the migration of MSCs. After 6 hours, the number of viable migrated cells was determined. The data revealed that EphA2 knockdown compromises the capability of sh-EphA2 MSCs to respond to TNF-α signaling. The capability to respond to TNF-α signaling and migration of MSCs is enhanced in EphA2-enriched populations of cells. (A) Viable migrated cells are presented as signal intensity. (B) Viable migrated cells are presented as a relative proportion compared to wild-type MSCs in 0.2% FBS control. FBS = fetal bovine serum; MSC = mesenchymal stem cell; RLU = relative light unit.

In our study, we established a simple trans-well migration assay to mimic the migratory response of MSCs to inflammatory factor TNF-α. MSCs were plated and cultured in the trans-well inserts with basal media, and FBS (0.2%) and TNF-α were added in the lower chamber to create an attractant gradient to stimulate migration of MSCs. The capacity of MSC motility toward inflammatory factor TNF-α could be measured and quantified by the number of migrated cells using a homogeneous method of determining the number of viable cells based on quantitation of the adenosine triphosphate present (an indicator of metabolically active cells). We applied a lentiviral transduction system to knockdown EphA2 expression in MSCs to investigate whether cellular migration toward TNF-α was affected after knockdown of EphA2 expression.

Our results showed that addition of TNF-α to the basal medium in the lower chamber increased MSC migration in a TNF-α-dose-dependent manner (Figure 4). The data revealed that EphA2 knockdown significantly affected the migratory ability of MSCs (~40% decrease), as well as response to TNF-α signaling (Figure 4). By contrast, the EphA2$^{high}$ MSC population after sorting exhibited an enhanced migratory capability and response to TNF-α signaling (Figure 4). We concluded that the EphA2 protein plays a crucial role not only in MSC migration, but also migration in response to inflammatory factor TNF-α.

In summary, our present study reported a method of isolation of placenta-derived MSCs with high homogeneity and at early passage in primary cultures. Using a proteomics profiling approach, we identified a surface biomarker, EphA2, enriched in placenta-derived MSCs. This protein could be used to separate fetal-originated placental MSCs from fibroblasts during the process of isolation. The present study demonstrated that cell sorting using the EphA2 surface marker could distinguish and purify MSCs in a primary culture of cells derived from placenta-related tissues. Expression of EphA2 was stable during the *in vitro* expansion process. The EphA2 protein plays a crucial role in MSC migration. EphA2-sorted MSCs exhibited superior responsiveness to TNF-α signaling in an inflammatory environment, and showed enhanced mobility, as compared with unsorted MSCs. By contrast, the mobility of MSCs and mobility in response to TNF-α were severely compromised in EphA2-knockdown MSCs, as well as EphA2$^{-}$ cells. However, the exploration of *in vitro* functional assays of MSCs was still limited in this study. A broader range of functional analyses is essential to further define the function of EphA2 in MSC biology.

## Conflicts of interest

The authors have no conflicts of interest relevant to this article.

## References


[1] Stagg J, Galipeau J. Mechanisms of immune modulation by mesenchymal stromal cells and clinical translation. Curr Mol Med 2013;13:856–67.
[2] Casado JG, Tarazona R, Sanchez-Margallo FM. NK and MSCs crosstalk: the sense of immunomodulation and their sensitivity. Stem Cell Rev 2013;9:184–9.
[3] Fukuchi Y, Nakajima H, Sugiyama D, Hirose I, Kitamura T, Tsuji K. Human placenta-derived cells have mesenchymal stem/progenitor cell potential. Stem Cells 2004;22:649–58.
[4] Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 2004;32:414–25.
[5] Nombela-Arrieta C, Ritz J, Silberstein LE. The elusive nature and function of mesenchymal stem cells. Nat Rev Mol Cell Biol 2011;12:126–31.
[6] Keating A. Mesenchymal stromal cells: new directions. Cell Stem Cell 2012;10:709–16.
[7] Linge C, Green MR, Brooks RF. A method for removal of fibroblasts from human tissue culture system. Exp Cell Res 1989;185:519–28.
[8] Lorenz K, Sicker M, Schmelzer E, Rupf T, Salvetter J, Schulz-Siegmund M, et al. Multilineage differentiation potential of human dermal skin-derived fibroblasts. Exp Dermatol 2008;17:925–32.
[9] Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation 1968;6:230–47.
[10] Blasi A, Martino C, Balducci L, Saldarelli M, Soleti A, Navone SE, et al. Dermal fibroblasts display similar phenotypic and differentiation capacity to fat-derived mesenchymal stem cells, but differ in anti-inflammatory and angiogenic potential. Vasc Cell 2011;3:5.
[11] Covas DT, Panepucci RA, Fontes AM, Silva Jr WA, Orellana MD, Freitas MC, et al. Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp Hematol 2008;36:642–54.
[12] Haniffa MA, Collin MP, Buckley CD, Dazzi F. Mesenchymal stem cells: the fibroblasts' new clothes? Haematologica 2009;94:258–63.
[13] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.
[14] Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell 2008;133:38–52.
[15] Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signaling and beyond. Nat Rev Cancer 2010;10:165–80.
[16] Nievergall E, Lackmann M, Janes PW. Eph-dependent cell–cell adhesion and segregation in development and cancer. Cell Mol Life Sci 2012;69:1813–42.
[17] Wilkinson DG. Multiple roles of EPH receptors and ephrins in neural development. Nat Rev Neurosci 2001;2:155–64.
[18] Pitulescu ME, Adams RH. Eph/ephrin molecules—a hub for signaling and endocytosis. Genes Dev 2010;24:2480–92.
[19] Miao H, Wang B. Eph/ephrin signaling in epithelial development and homeostasis. Int J Biochem Cell Biol 2009;41:762–70.
[20] Palmer A, Klein R. Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. Genes Dev 2003;17:1429–50.
[21] Hafner C, Schmitz G, Meyer S, Bataille F, Hau P, Langmann T, et al. Differential gene expression of Eph receptors and ephrins in benign human tissues and cancers. Clin Chem 2004;50:490–9.
[22] Binda E, Visioli A, Giani F, Lamorte G, Copetti M, Pitter KL, et al. The EphA2 receptor drives self-renewal and tumorigenicity in stem-like tumor-propagating cells from human glioblastomas. Cancer Cell 2012;22:765–80.
[23] Day BW, Stringer BW, Al-Ejeh F, Ting MJ, Wilson J, Ensbey KS, et al. EphA3 maintains tumorigenicity and is a therapeutic target in glioblastoma multiforme. Cancer Cell 2013;23:238–48.
[24] Huntley RP, Sawford T, Mutowo-Muellenet P, Shypitsyna A, Bonilla C, Martin MJ, et al. The GOA database: gene ontology annotation updates for 2015. Nuc Acids Res 2015;43:D1057–63.
[25] Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia AS, McNamara JO, et al., editors. Neuroscience. Sunderland (MA): Sinauer Associates Inc.; 2001.
[26] Conover JC, Doetsch F, Garcia-Verdugo JM, Gale NW, Yancopoulos GD, Alvarez-Buylla A. Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone. Nat Neurosci 2000;3:1091–7.
[27] Aoki M, Yamashita T, Tohyama M. EphA receptors direct the differentiation of mammalian neural precursor cells through a mitogen-activated protein kinase-dependent pathway. J Biol Chem 2004;279:32643–50.
[28] Gucciardo E, Sugiyama N, Lehti K. Eph- and ephrin-dependent mechanisms in tumor and stem cell dynamics. Cell Mol Life Sci 2014;71:3685–710.
[29] Lickliter JD, Smith FM, Olsson JE, Mackwell KL, Boyd AW. Embryonic stem cells express multiple Eph-subfamily receptor tyrosine kinases. Proc Natl Acad Sci USA 1996;93:145–50.
[30] Holley RJ, Tai G, Williamson AJ, Taylor S, Cain SA, Richardson SM, et al. Comparative quantification of the surfaceome of human multipotent mesenchymal progenitor cells. Stem Cell Reports 2015;4:473–88.
[31] Goichberg P, Bai Y, D'Amario D, Ferreira-Martins J, Fiorini C, Zheng H, et al. The ephrin A1-EphA2 system promotes cardiac stem cell migration after infarction. Circ Res 2011;108:1071–83.
[32] Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. Cell Death Differ 2014;21:216–25.
[33] MacFarlane RJ, Graham SM, Davies PS, Korres N, Tsouchnica H, Heliotis M, et al. Anti-inflammatory role and immunomodulation of mesenchymal stem cells in systemic joint diseases: potential for treatment. Expert Opin Ther Targets 2013;17:243–54.
[34] Pistoia V, Raffaghello L. Unveiling the role of TNF-α in mesenchymal stromal cell-mediated immunosuppression. Eur J Immunol 2014;44:352–6.
[35] Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2008;2:313–9.