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Multipotent mesenchymal stem cells from human placenta: critical parameters for isolation and maintenance of 'stemness' after isolation

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Condensation

Sequential enzymatic digest of term human placenta effectively isolates multipotent mesenchymal stem cells of maternal origin that remain multipotent in conditions of sub confluent expansion.

Abstract

OBJECTIVE: This study was undertaken to isolate and characterise multipotent mesenchymal stem cells from term human placenta (PD-MSCs).

STUDY DESIGN: Sequential enzymatic digestion was used to isolate PD-MSCs in which trypsin removes the trophoblast layer, followed by collagenase treatment of remaining placental tissue. Karyotype, phenotype, growth kinetics, and differentiability of PD-MSCs isolates from collagenase digests were analyzed.

RESULTS: PD-MSC isolation was successful in 14 out of 17 cases. Karyotyping of PD-MSC isolates from deliveries with male foetus, revealed that these cells of maternal origin. Flow cytometry and immunocytochemistry confirmed the MSC phenotype. Proliferation rates of PD-MSCs remained constantly high up to passage 20. These cells could be differentiated towards mesodermal lineage *in vitro* up to passage 20. Non-confluent culture was critical to maintain the MSC stemness during long-term culture.

CONCLUSION: Term placenta constitutes a rich, very reliable source of maternal MSCs that remain differentiable even at high passage numbers.

Key words: Human placenta, mesenchymal stem cells, tissue regeneration

Introduction

Mesenchymal stem cells (MSCs) represent an interesting cell type for research and therapy because of their ability to differentiate into mesodermal lineage cells such as osteocytes, chondrocytes, cardiac muscle, or endothelial cells [1]. In addition, they secrete large amounts of pro-angiogenic and anti-apoptotic cytokines [2] and possess remarkable immunosuppressive properties [3]. MSCs have been derived from many different organs and tissues [4] Evidence has emerged that also different parts of human placenta, umbilical cord, amniotic membrane, as well as umbilical cord blood harbor MSCs. [5-9] These tissues are normally discarded after birth, avoiding ethical concerns. [10] Mechanical as well as enzymatic methods for MSC isolation from different regions of human placenta of different gestational ages were reported (Table 1, see references therein). [5, 11-26] Knowledge about vitality, karyotype, phenotype and expandability of such placenta-derived MSC isolates is a prerequisite for therapeutic application, however systematic investigations into reliability of this MSC source and phenotypic stability have not yet been attempted. Further, former reports on placenta-derived MSCs often lack information about the karyotype of the cell isolates. Here we describe enzymatic fractionation of term human placenta that allows recovery of multipotent, fibroblast-like cells, which we tentatively term as placenta-derived mesenchymal stem cells (PD-MSCs) with high fidelity. Unexpectedly, as demonstrated by genotypic analyses of cell isolates from male deliveries, the resulting isolates were of maternal, not fetal origin. Our systematic characterization of cell isolates from multiple cases showed that these cell isolates reproducibly fulfil the general definition of MSCs both by phenotypic and functional criteria. [27] We demonstrate that maternally-derived PD-MSCs can be greatly expanded, maintain they differentiation capacity and stable phenotype up to passage 20.

Materials and Methods

Placenta collection

The Ethical Committee of the District of Zurich approved the protocol (study Stv22/2006). Following written consent, placentas were collected from 17 women donors immediately after elective caesarean section in the absence of labor, preterm rupture of membranes, chorioamnionitis, or chromosomal abnormalities. Mean maternal age was 32 years (between 28 and 39 years) and mean gestational age was 38 ±1 weeks. Mean placental weight was 573 ±113 g.

Cell isolation

Figure 1 depicts the isolation procedure. After removal of decidua and fetal membranes, approximately 30g placental tissue was minced and washed three times in physiological saline. Blood vessels and clots were removed mechanically. The minced placental tissue was subjected to sequential digests with trypsin and collagenase I. First, to remove the trophoblastic epithelial cell layer, tissue was incubated in 50mL 0.25% trypsin solution containing 80U/mL DNase I (Roche AG, Basel, Switzerland) for 1 h at 37°C. The remaining placental fragments were separated in a 250μm metal sieve from the 'trypsin cell suspension'. Approximately 15g of placental fragments were subjected to a second digest with collagenase. For that, tissue fragments were incubated with 50mL of 12.5U/mL collagenase I (Sigma-Aldrich AG, Buchs, Switzerland), 80U/mL DNase I for one hour at 37°C. Cell suspensions from both trypsin and collagen digests were filtered twice through 100μm cell strainers (BD Bioscience, San Jose, CA), then the cells were collected by centrifugation for 5min at 300g. The cell pellets were shortly resuspend in hypotonic red blood cell lysis buffer (physiological saline with 2mm EDTA, 0.5% BSA, without Ca und Mg, diluted 1:10 with distilled water), pelleted again by 5min centrifugation at 300g. Finally, the cells were suspended in 10ml Non Hematopoietic Stem Cell Expansion Medium (NH expansion medium; Miltenyi Biotec

GmBH, Bergisch-Gladbach, Germany) and plated into a single $75cm^2$ tissue culture flask (TPP AG, Trasadingen, Switzerland) and cultured at 37°C.

Colony assay

Freshly isolated PD-MSCs, passage 0, were replated at low density, i.e. 50 cells per well of 6-well plates (TPP), and cultured at 37˚C, 5% CO_2 in Non Hematopoietic Stem Cell Expansion Medium (NH expansion medium). Outgrowing colonies of spread cells were visualized and counted by fluorescence microscopy using rhodamine-labelled phalloidin to stain actin cytoskeleton (Invitrogen, Basel, Switzerland), and 4'6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR) to stain cell nuclei. Images were acquired with a Zeiss Axiovert 200M (Carl Zeiss AG, Feldbach, Switzerland) equipped with a digital camera AxioCam MRc, (Carl Zeiss AG, Feldbach, Switzerland).

Growth kinetics

PD-MSCs of passages 1, 10, and 20 taken from four different cases were plated at 5×10^3 cells per well in 12 well-plates (TPP) and cultured at 37˚C and 5% CO_2 in Non Hematopoietic Stem Cell Expansion Medium (NH expansion medium). All experiments were performed in triplicate. Cell counts were determined after 24, 48, and 72h culture. For that, cells were detached with 0.25% trypsin solution (GIBCO-Invitrogen AG, Basel, Switzerland) and counted with a Coulter Z1 cell counter (Instrumenten Gesellschaft AG, Zurich, Switzerland). Dead cells were identified by staining with 0.4% trypan blue staining solution (Sigma - Aldrich AG).

Antibodies

Information about primary and secondary antibodies used for flow cytometry and immunochemistry is provided in Table 2.

Flow cytometry

Cells were collected by trypsinization. Reaction was performed on live cells, using 10^5 cells per antibody reaction. Cells were incubated for 25min at 4°C. Unstained, non-incubated cells serve as controls. Cells were fixed in 4% buffered formalin and analyzed with a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). A minimum of 10^4 gated events, were acquired for each sample. For staining of the intracellular markers CK-7, α-SMA, and vWF, cells were fixed in 4% buffered formalin, shortly permeabilized by 10 min incubation in 0.9% saponin solution, and then incubated with specific antibodies for 25 min at 4°C.

Immunocytochemistry

Briefly, cells on 15 mm glass cover slips (Menzel GmbH + Co KG, Germany) were fixed for 5 min in 100% acetone, then rehydrated in PBS and blocked with Ultra V Blocking buffer (Bio Vision Corporation, England). For staining for intracellular proteins, cells were permeabilized with 0.1% Triton X-100 for 3min, then incubated with primary antibodies for 1h at room temperature. In case of using unconjugated primary antibodies, samples were subsequently incubated with secondary polyclonal FITC conjugated goat anti-mouse Ig (BD Pharmingen, Switzerland) or FITC-conjugated rabbit anti-rat Ig antibodies (Dako Cytomation, Glostrup, Denmark) for 30min at room temperature. All cell samples were additionally counterstained with DAPI. Images were taken with a Zeiss Axiovert 200M (Carl Zeiss AG) equipped with a digital camera AxioCam MRc, (Carl Zeiss AG) and analysed with AxioVs40 V 4.5.0.0 imaging software (Carl Zeiss AG).

Cytogenetic analysis

Chromosome analysis of PD-MSCs cultures, passage 3, from six donors was performed. Briefly, metaphase chromosome preparations were performed according to standard

procedures at a 400-500 GTG band level. Analysis and identification of the chromosomes was performed using a Zeiss Axioplan microscope (Carl Zeiss AG, Feldbach, Switzerland). Images were recorded with a Photometrics CCD KAF1400 camera (Photometrics, Tucson, AZ) and controlled with smart capture imaging software (Vysis, Inc., Downers Grove, IL).

Fluorescent in situ hybridization analysis for detection of sex determination region

Fluorescence in situ hybridisation (FISH) studies for detection of sex determination region (SRY) were performed using chromosome X (Xp11.1-q11.1, locus DXZ1) and chromosome Y (Yp11.1-q11.1, locus DYZ3, alpha satellite) probes Vysis (Abbott Molecular Inc., IL) according to the manufacturer's instructions (Abbot Molecular Inc., Des Planes, IL 60018). Analysis of at least 200 metaphases and interphases was performed on PD-MSCs isolated from three male deliveries at passage 3 using a Zeiss Axioplan epifluorescence microscope (Carl Zeiss AG). Images were recorded by Photometrics CCD KAF1400 camera (Photometrics) and controlled with smart capture imaging software (Vysis, Inc.)

Multipotent differentiation assays

PD-MSCs, passage 6, from three donors were tested for differentiation towards mesodermal lineage *in vitro*. Differentiation was induced by their culture in commercial differentiation media: AdipoDiff, ChondroDiff, and OsteoDiff Induction Media (Miltenyi Biotec GmbH) following the manufacturer's protocol. Control cultures were grown in NH expansion medium (Miltenyi Biotec GmbH). After differentiation, cells were examined microscopically with a Zeiss Axiovert 200M (Carl Zeiss AG).

Adipogenic differentiation

For adipogenic differentiation, PD-MSCs were cultured at 50×10^3 cells per well of 12 well-plates in AdipoDiff induction medium (Miltenyi Biotec GmBH) for 3 weeks, with fresh

media added every 48h. Oil Red O was utilized to visualize fat droplets. Briefly, cells were washed twice with PBS, fixed in pre-cooled methanol for 5 min, rinsed with deionised water, and then incubated with 0.5% Oil Red O (Sigma-Aldrich AG) in isopropanol for 20 min at room temperature. Finally, stained cells were washed with water and analysed microscopically.

Chondrogenic differentiation

Chondrogenic differentiation was performed under micromass conditions in ChondroDiff induction medium (Miltenyi Biotec GmBH), according to the manufacturer's instructions. Briefly, 5×10^6 MSCs were pelleted by 5 min centrifugation at $300g$ in a cell culture centrifuge. The micromass was kept in ChondroDiff medium for 24 days, with fresh media added every 48 h. The pellets were fixed in 10% formalin, and then finally embedded in paraffin. Staining for proteoglycans was performed on deparaffinised 5 μm sections by 30 min incubation with 3% Alcian Blue (Sigma-Aldrich AG) in 3% acetic acid, pH 2.5. Counterstain was performed with Nuclear Fast Red (DAKO Cytomation).

Osteogenic differentiation

For osteogenic differentiation, 3×10^4 PD-MSCs were seeded in 12 well plates (TPP) and maintained in OsteoDiff induction medium (Miltenyi Biotec GmBH) for 3 weeks, with fresh medium added every 48h. After 21 days, cells were fixed in 70% ethanol. Extracellular calcium deposition was stained by 45 min incubation with 2% Alizarin Red S (Sigma-Aldrich AG) in water. Finally, stained cells were washed with deionised water and analysed microscopically.

Angiogenic differentiation

Angiogenic differentiation was performed as described [28]. Briefly, PD-MSCs were seeded at the density of 2.5 x 10^4 cells per well of 12 well plates and cultured for 7 days in Dulbecco's Modified Eagle Medium (Invitrogen AG) supplemented with 2% fetal bovine serum (Invitrogen AG) and 50 ng/ml vascular endothelial growth factor 121 [29]. Control cultures were performed without VEGF. After 7 days, cells were fixed and stained with mouse anti-human CD34 antibodies (Miltenyi Biotec GmBH).

Statistical analysis

Data are shown as mean ± SD. Mann-Whitney (nonparametric) test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Data are given as mean ± standard deviation. Significance level was set at $p < 0.05$.

Results

Figure 1 depicts the flow chart of the enzyme-mediated fractionation of human term placenta for derivation of fibroblast-like cells, which we tentatively term placenta-derived multipotent mesenchymal stem cells (PD-MSCs). Selection for MSCs rested on the classic adhesion method on tissue culture plastic. The protocol proved successful in 14 out of 17 test cases. Cell suspensions produced from by final collagenase I digest of approximately 15 g placental tissue fragments typically produced 300-500 colonies of variable sizes that contained outgrowing fibroblast-like cells. Typically, approximately 4 to 6 x 10^4 cells were obtained within two weeks after plating. Plating of cell suspensions from the first digest with trypsin did not produce any colonies. Outgrowing cells when harvested and replated in high dilution rapidly formed secondary colonies from single cells (Figure 2A). PD-MSCs were expandable up to passage 20 (as far as we cultured) without change of fibroblast-like morphology (Figure 2B) or proliferation rate (Figure 2C). Chromosomal analysis of cell isolates from six different placental cases (3 of female and 3 of male deliveries) did not show any chromosomal abnormalities. Figure 3 gives one example. Karyotypes were normal 46, XX in all test samples, including the three PD-MSC isolates obtained from male deliveries (Table 3). FISH analysis for the SRY gene confirmed the absence of male cells in our PD-MSC isolates. Thus, our procedure isolated only PD-MSCs of maternal origin, which was unexpected. The phenotype of the cell isolates, passage 1, was examined by flow cytometry for five cases (Figure 4) and further examined by immunocytochemistry with a panel of antibodies against 30 antigens for a single case (Table 2; Figure 5). Flow cytometry revealed very little scatter in the phenotypic marker profile of placenta derived isolates between cases. The expression profile conformed to the criteria recently defined for multipotent mesenchymal stem cells. [27] (Figure 4-5) Cell isolates were uniformly positive for mesenchymal stem cell markers CD44, CD73, CD90, CD105, and CD166. Hematopoietic markers CD34, CD45 as well as CD11b, CD14, CD19, and CD79alpha were not expressed. Cells stained also negative for macrophage

restricted antigen CD163 which marks Hofbauer cells, [30] the cytotrophoblast-specific marker cytokeratin 7 [31] and syncytiotrophoblast-specific marker placental alkaline phosphatase (PLAP). [31] Curiously, the entire cell population was positive for two markers of end-differentiated cells such as the endothelial protein vWF, and the smooth muscle cell protein α-smooth muscle actin. PD-MSCs were negative for KDR, cytokeratin 18, CD117 and CD271. We found high levels of HLA-ABC but no HLA-DR. Both flow cytometry and immunocytochemistry revealed that part of the passage 1 cells expressed the antigen CD133 which is considered as a marker of non-committed early progenitors of blood cells, endothelial cells and other stem cell types. [32,33] Immunocytochemistry did no detect expression of human stem cell markers of pluripotency [34] such as the transcription binding domains Oct3/4, Stro-1, Tra 1-60, or Tra-1-81. Flow cytometry and immunocytochemistry revealed that PD-MSCs were positive for stage-specific embryonic antigen SSeA-3 but negative for SSeA-4 (Table 4; Figures 4,5).

Specific induction of differentiation was investigated with PD-MSCs, passage 6, from three cases. This confirmed that the 'mesenchymal stemness profile' by PD-MSCs populations indeed associated with the ability to generate different mesodermal lineage cell types upon their exposure to soluble factors *in vitro*. Figure 6 displays representative results of adipogenic (A,B) , chondrogenic (C,D) , and osteogenic (E,F) differentiation assays, visualizing lipid vacuoles, chondrogenic matrix and calcium deposits, respectively. Moreover, culture in presence of the angiogenic growth factor VEGF induced expression of CD34, which is marker of hematopoietic as well as endothelial precursors. [35] (Figure 6G,H) The latter finding points to a broader mesodermal differentiation capacity of PD-MSCs.

Sub-confluent culture was found critically important to maintain the 'stemness' phenotype of PD-MSCs during expansion. Figure 7 display the results of a side-by-side screen of marker profiles of PD-MSCs that were grown in confluent *versus* sub-confluent culture till passage 10. The screen was performed with four cases of each condition. The phenotypic profile of

PD-MSCs when sub-cultured at 50-70% cell density remained unaffected. In sharp contrast, PD-MSCs grown in confluent or near-to confluent culture lost mesenchymal stem cell markers almost completely. Expression of CD133 antigen disappeared during culture in both conditions.

PD-MSCs passaged in sub-confluent culture maintained their initial marker profile and their ability to differentiate as well. Figures 4B,C depict a direct comparison of the marker profiles of passage 1 PD-MSCs *versus* passage 20 PD-MSCs. Passage 20 PD-MSCs could be induced towards chondrogenic and osteogenic lineage *in vitro*, as demonstrated by staining of chondrogenic matrix and calcium deposits, respectively (Figure 8), while adipogenic differentiation failed (data not shown).

Comment

In demonstrating that term human placenta, which is a readily available and ethically relatively unproblematic tissue, constitutes a robust source of MSCs, we investigated several parameters, namely (1) chromosome number, (2) origin, (3) methods of isolation and (4) propagation that are important for their principal utility for cell-based therapy, and could influence their proliferative as well as differentiation capacities. Chromosome number was found normal in all analyzed PD-MSC isolates (N=6). Looking at maternal or fetal origin, we found that PD-MSC isolates obtained with our isolation procedure were always of maternal origin, which was contrary to our expectations. This finding corroborates the very recent observation by Barlow et al., that cells isolated from placentas of male delivered babies were not of fetal origin. In contrast, the study by Fukuchi et al., which is the only investigation, reported the detection of fetal origin cells isolated from human term placenta. However, looking at the image provided in the same study, a fraction of the isolated cells was of fetal origin, while the remaining cells were of maternal origin. The precise placental anatomical origin of the maternal cells obtained with our procedure is still unclear. One source could be maternal decidua, despite our careful attempts to remove this layer completely before digestion of placenta. Placental septa constitute another potential source. One explanation for the obvious difficulty to capture fetal mesenchymal stem cells from term human placenta is offered by the fact that most of stem cells observed in the first trimester of pregnancy are differentiated to cytotrophoblast and endothelium cells in terminal and intermediate villi, while the few remaining stem cells were only detectable around the stem villi. [36,37] Furthermore, the thinning of the stromal layer and decreased cell density towards the end of gestation is known as a positive adaptation to ensure the increasing nutritional supply of the growing fetus. [38] We think that the most likely origin of the maternal cells obtained in our study is decidual tissue that remained on placental septa, which is difficult to remove.

Our method of cell isolation by way of sequential digestion of the trophoblast cell layer with trypsin and following digestion of remaining placental tissue with collagenase I proved very effective for obtaining PD-MSCs. Outgrowth of PD-MSCs from collagenase digests was successful in 14 out of 17 test cases, and resulted in populations with remarkably little scatter in their MSC profiles between cases. As to propagation, we found out that PD-MSCs must be propagated in sub-confluent culture to maintain their MSC profile, because confluent culture led to gradual loss of MSC identity. With proper sub-confluent passage, PD-MSCs maintained their phenotypic MSC profile up to the highest passage numbers we tested in this study, passage 20, as well as their differentiation capacity.

As detailed in the Results section, cultures of PD-MSCs, after one passage, were constituted by a homogenous population of fibroblast-like cells which expressed the typical mesenchymal stem cell markers, but showed no contamination with macrophages, hematopoietic cells or trophoblast cells (Figure 4A,B). We observed by flow cytometry and immunocytochemistry that early passage cultures expressed CD133, a protein of unknown functionality that is considered a marker of immature cells. CD133 expression has been found restricted to stem cells in normal adult tissue [39], and expressed by non-committed early progenitors of blood cells and endothelial cells [32], non-malignant neural progenitors but also as tumor-initiating stem cells in brain. [40] Recent studies by Tondreau et al. showed that CD133-positive cell fractions of mononuclear cells human peripheral blood and cord blood, which were isolated by magnetic sorting with microbeads coated with anti-CD133 antibody, give rise to MSCs. [33] Magnetic sorting of CD133-positive cells from placental extracts therefore could provide an alternative to MSCs selection by adhesion to tissue culture plastic.

The growth potential of PD-MSC as assessed by their growth kinetics at passages 1, 10, and 20 was unaffected by subculture. Further, our studies of 'stemness' in functional differentiation assays showed that PD-MSCs retain their ability to generate chondrocytes and osteocytes at passage 20, although adipogenic potential was obviously lost at late passage.

Induction with VEGF of endothelial differentiation in early passage PD-MSCs showed that the induced cells expressed CD34, a marker for endothelial precursors. We strongly caution about an interpretation that PD-MSCs could differentiate into endothelial cells, but rather think that this finding could point to a broader plasticity of PD-MSCs.

In conclusion, we have described a highly reproducible, straightforward methodology that leads to isolation of multipotent mesenchymal stem cells of maternal origin from human term placenta. Further studies are now indicated to evaluate the properties of such maternally-derived PD-MSCs regarding their full differentiation capacity *in vitro*, and ultimately *in vivo*. Aside from evaluating their utility for future cell replacement therapy and tissue engineering, we will seek to resolve whether such PD-MSC isolates could become exploited in transplantation medicine for an ability to exert immunosuppressive effects [41], and/or to secret pro-angiogenic and anti-apoptotic cytokines to aid tissue healing. [2]

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Figure legends

Figure 1. Fractionation scheme for isolation of MSCs from term human placental tissue. The procedure till cell plating takes approximately 5 h.

Figure 2. Morphology and growth kinetics of PD-MSCs. (A) Secondary colony formation from single cells. Cell shape and organization was analysed by fluorescence microscopy to visualize phalloidin-stained F-actin cytoskeleton (red) and DAPI-stained cell nuclei (blue). (B) Phase microscopic images of PD-MSCs at indicated passages. (C) Growth kinetics of PD-MSCs at indicated passages. * indicates significant enhancement (p<0.05) of cell numbers between time points.

Figure 3. Representative example of chromosome analysis of PD-MSC cultures at passage 3 (N=6).

Figure 4. PD-MSC phenotype at passages 1 and 20 as determined by flow cytometry. (A) Representative sample of passage 1 PD-MSCs. Green lines denote unstained control cells. (B) Screen of PD-MSC isolates, passage 1 (N=5). (C) Screen of PD-MSC isolates, passage 20 (N=5).

Figure 5. PD-MSC phenotype at passage 6 (N=1) as determined by immunocytochemistry.

Figure 6. Mesodermal lineage differentiation of PD-MSCs *in vitro*. A representative differentiation of PD-MSCs, passage 6 is shown. Cells were kept in induction medium (Differentiation) or for control, maintained without induction in standard medium (Control) (A,B) Adipogenic differentiation and control as determined by Oil-red-O staining of lipid

droplets. (C,D) Chondrogenic differentiation and control as determined by Alcian Blue staining of proteoglycans (E,F) Osteogenic differentiation and control as determined by Alizarin Red S staining of calcium deposition (G,H) Endothelial differentiation and control as determined by CD34 expression in green.

Figure 7. PD-MSC phenotype in sub-confluent *versus* confluent subculture. (A) Representative example of passage 10 PD-MSCs. (B) Screen of PD-MSC isolates, passage 10 (N=5). Sub-confluent culture was found critically important to maintain MSC marker expression.

Figure 8. Induced differentiation of PD-MSCs, passage 20. Cells were kept in induction medium (Differentiation) or for control, maintained without induction in standard medium (Control). (A,B) Chondrogenic differentiation and control as determined by Alcian Blue staining of proteoglycans. (C,D) Osteogenic differentiation and control as determined by Alizarin Red S staining of calcium deposition.
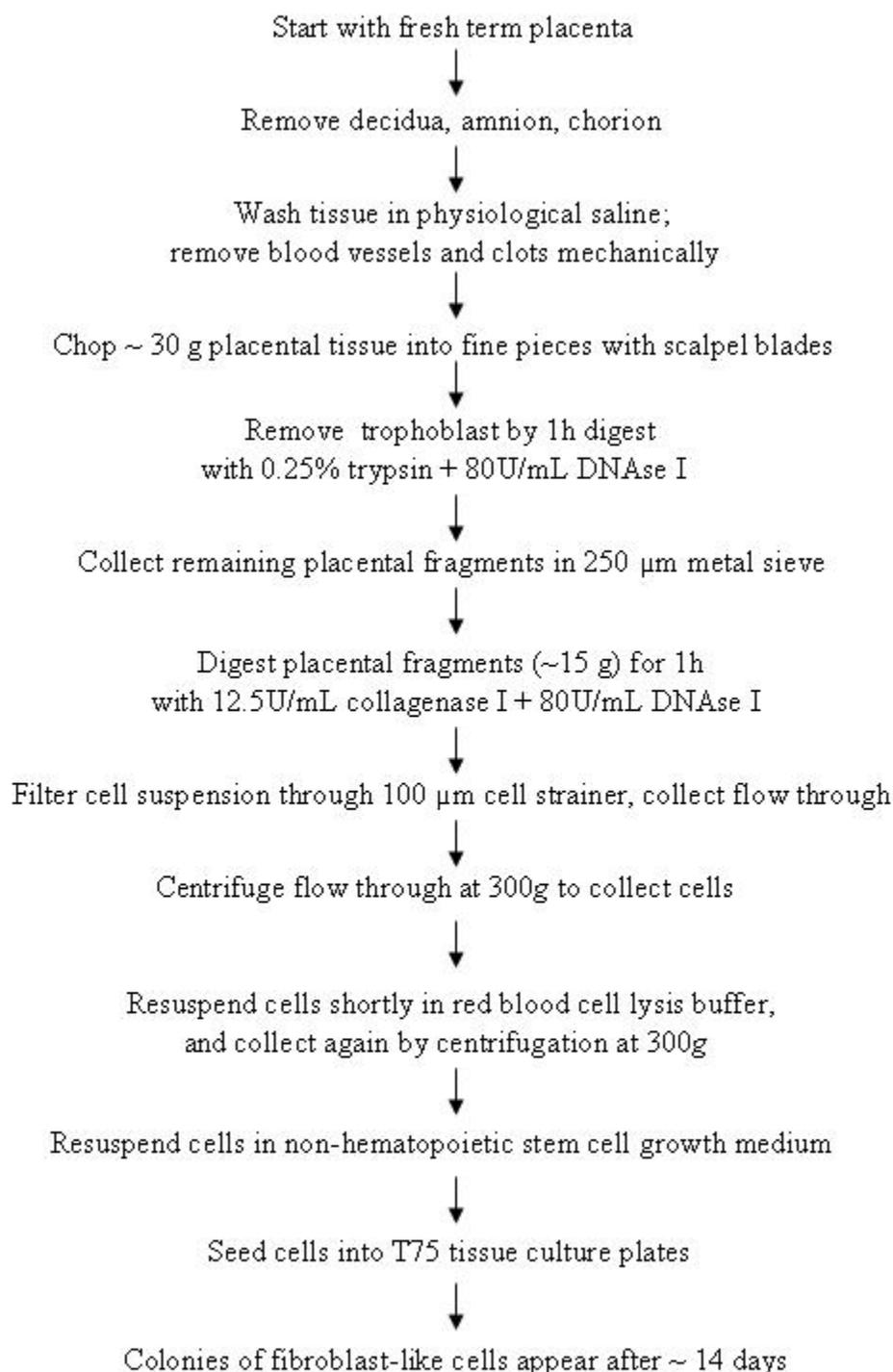
Figure 1.

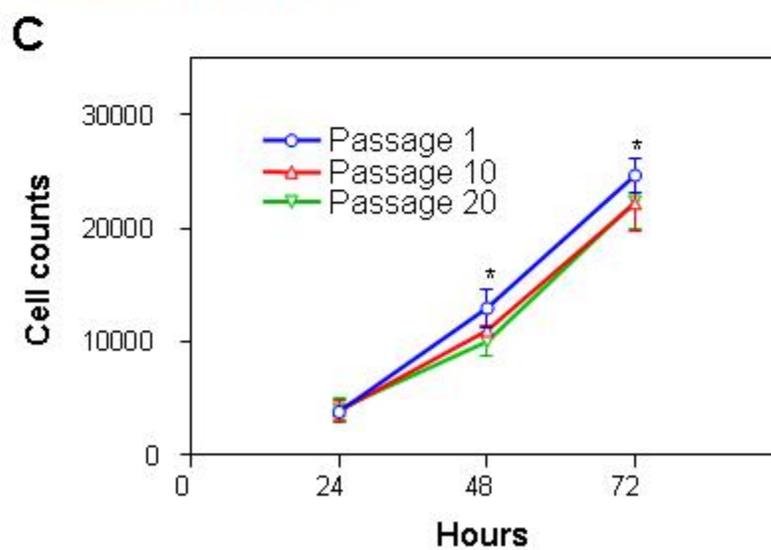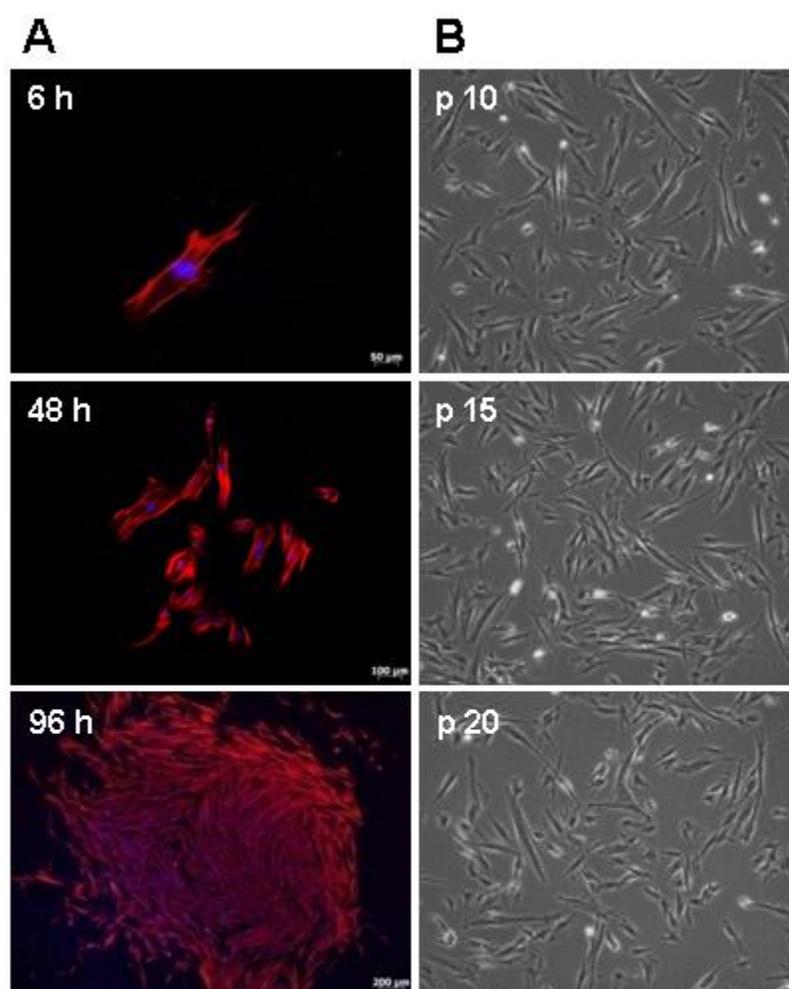Start with fresh term placenta

↓

Remove decidua, amnion, chorion

↓

Wash tissue in physiological saline;
remove blood vessels and clots mechanically

↓

Chop ~ 30 g placental tissue into fine pieces with scalpel blades

↓

Remove trophoblast by 1h digest
with 0.25% trypsin + 80U/mL DNAse I

↓

Collect remaining placental fragments in 250 μm metal sieve

↓

Digest placental fragments (~15 g) for 1h
with 12.5U/mL collagenase I + 80U/mL DNAse I

↓

Filter cell suspension through 100 μm cell strainer, collect flow through

↓

Centrifuge flow through at 300g to collect cells

↓

Resuspend cells shortly in red blood cell lysis buffer,
and collect again by centrifugation at 300g

↓

Resuspend cells in non-hematopoietic stem cell growth medium

↓

Seed cells into T75 tissue culture plates

↓

Colonies of fibroblast-like cells appear after ~ 14 days

Figure 2.

A

6 h

48 h

96 h

B

p 10

p 15

p 20

C

Cell counts

30000

20000

10000

0

Passage 1
Passage 10
Passage 20

0   24   48   72

Hours

Figure 3.

Figure 4.

A

Figure 4 continued.

B

Figure 5.

CD34

CD117

KDR

CD44

CD133/1
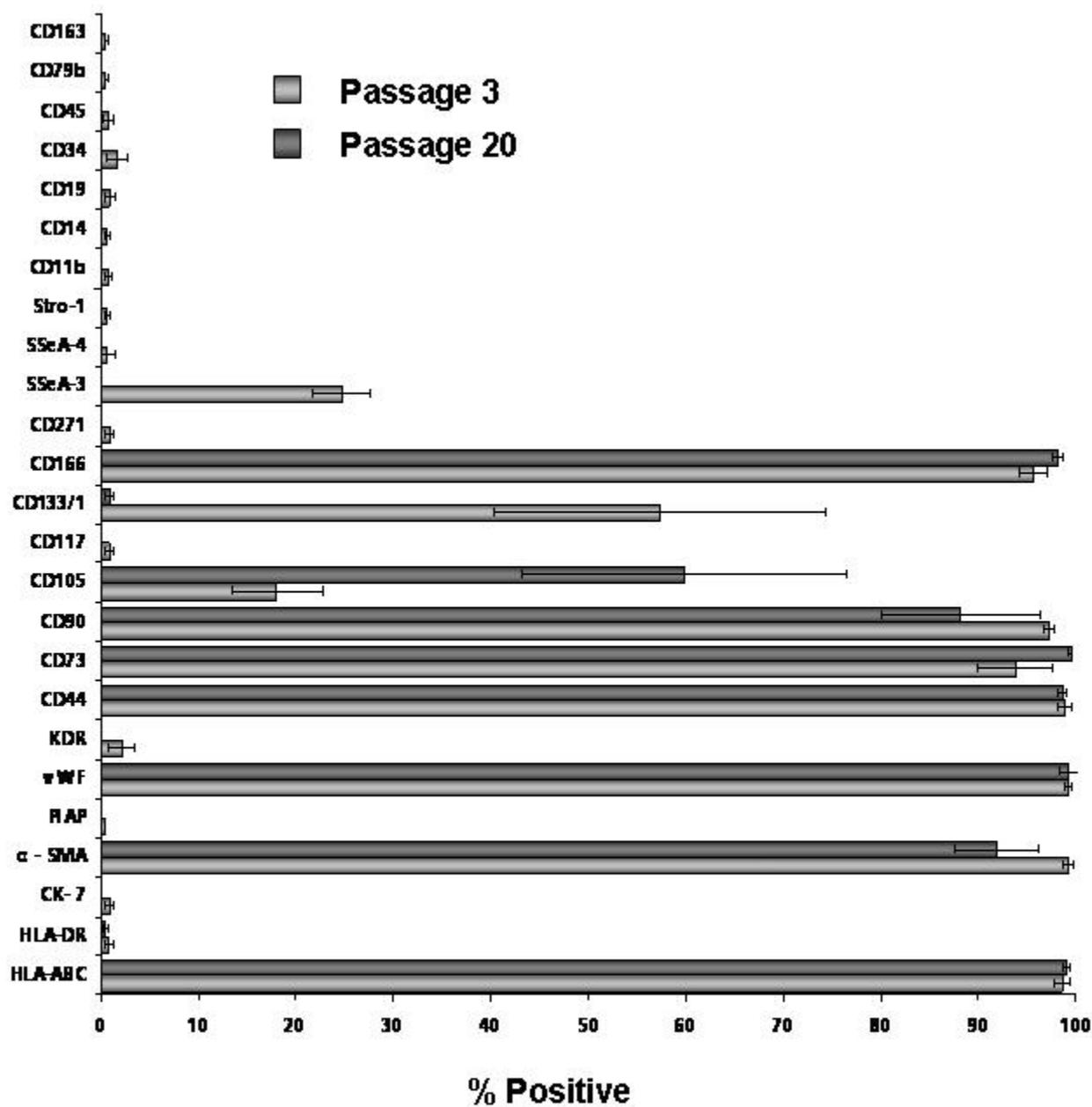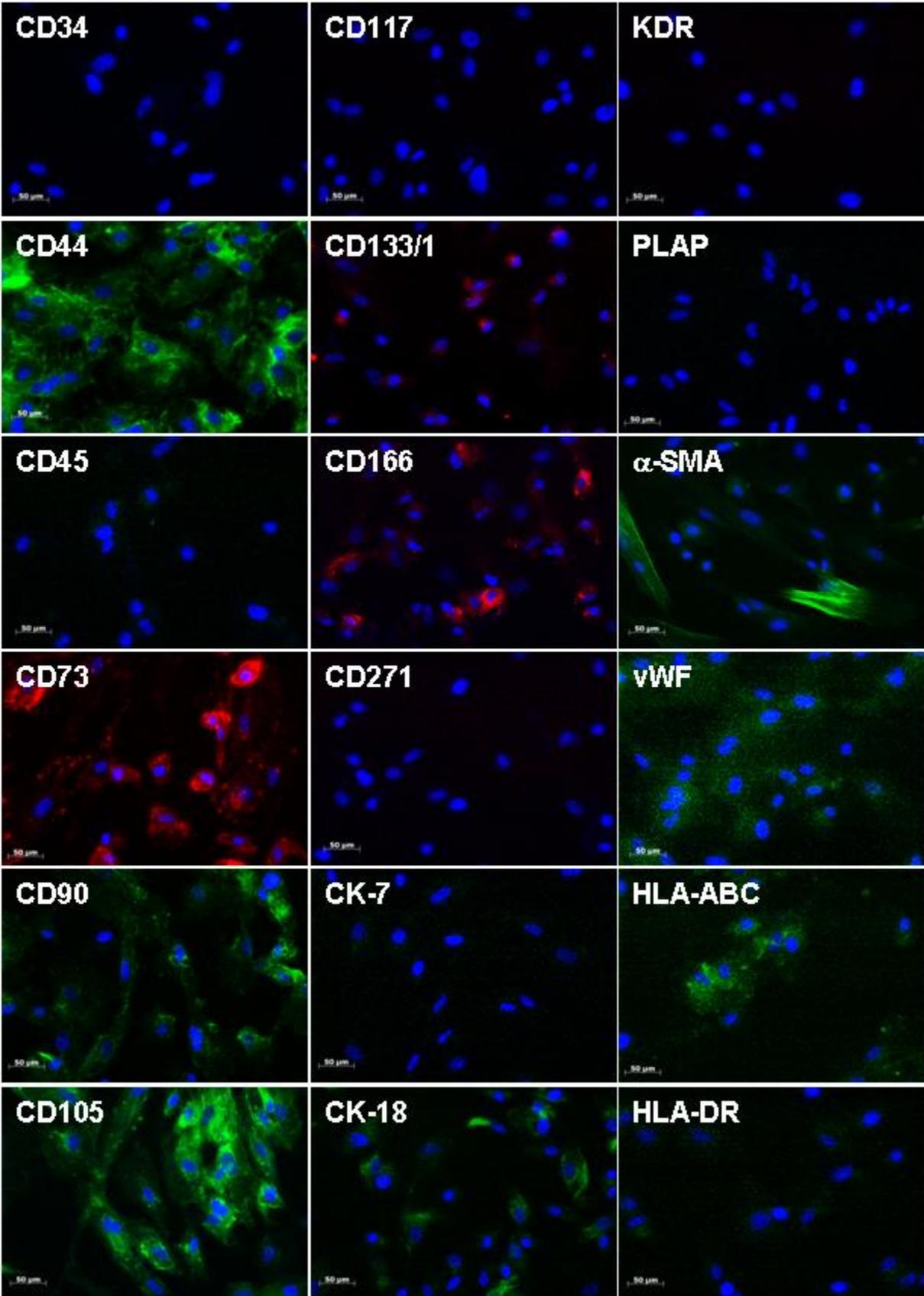
PLAP

CD45

CD166

α-SMA

CD73

CD271

vWF

CD90

CK-7

HLA-ABC

CD105

CK-18

HLA-DR

Figure 5 continued.

Figure 6.

Differentiation   Control
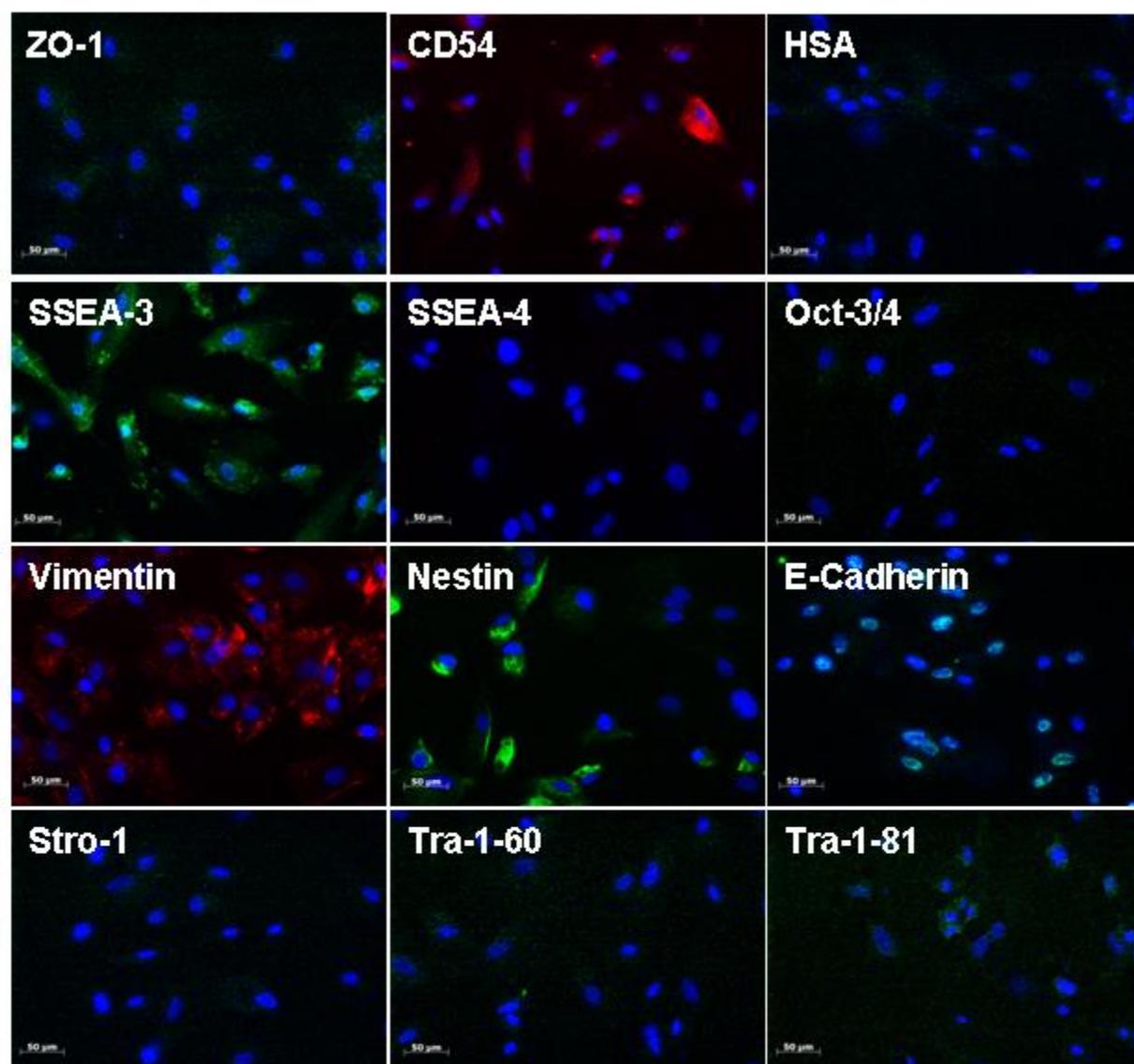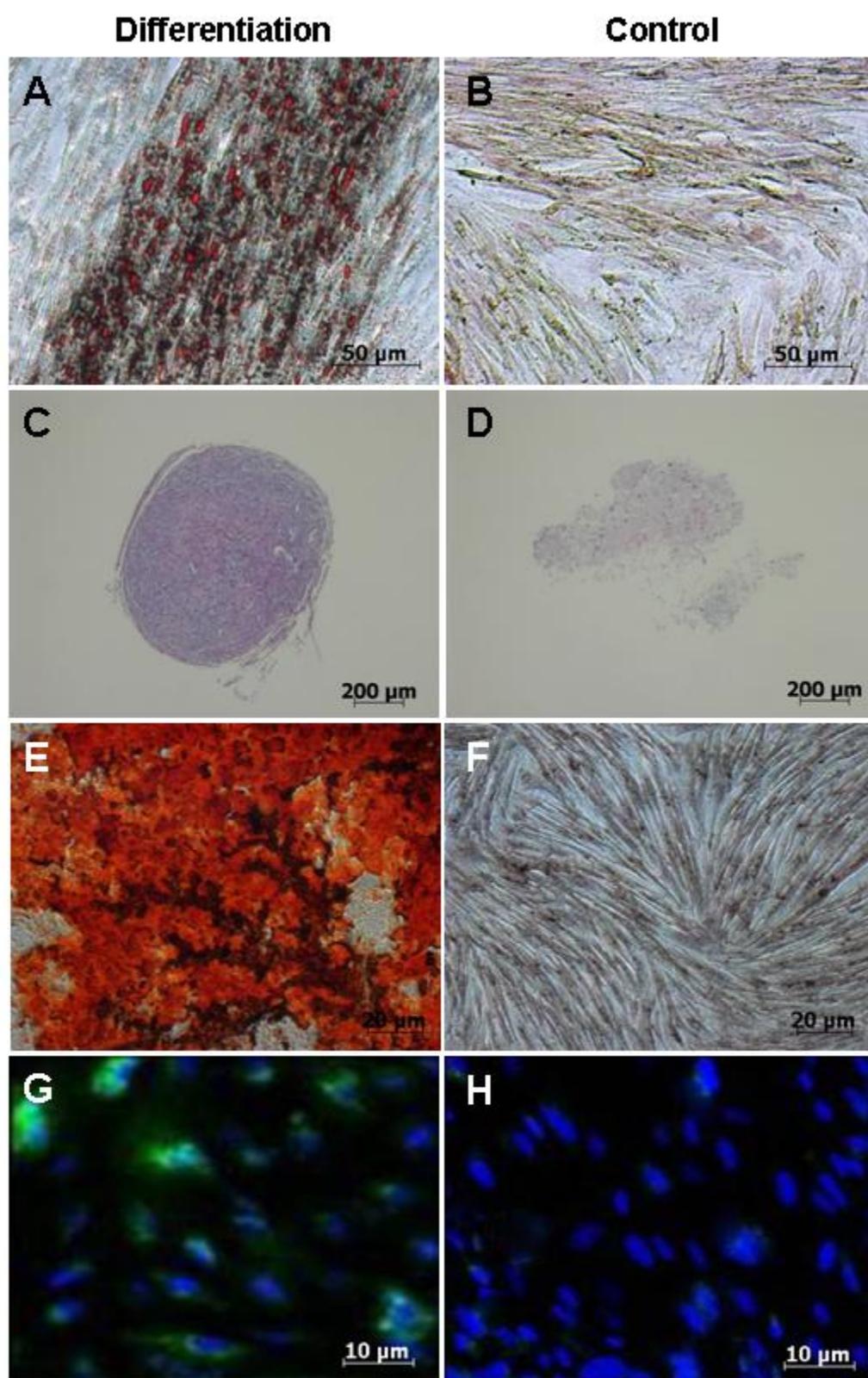
Figure 7.

A

Figure 7 continued.

B

Figure 8.

Differentiation   **Control**
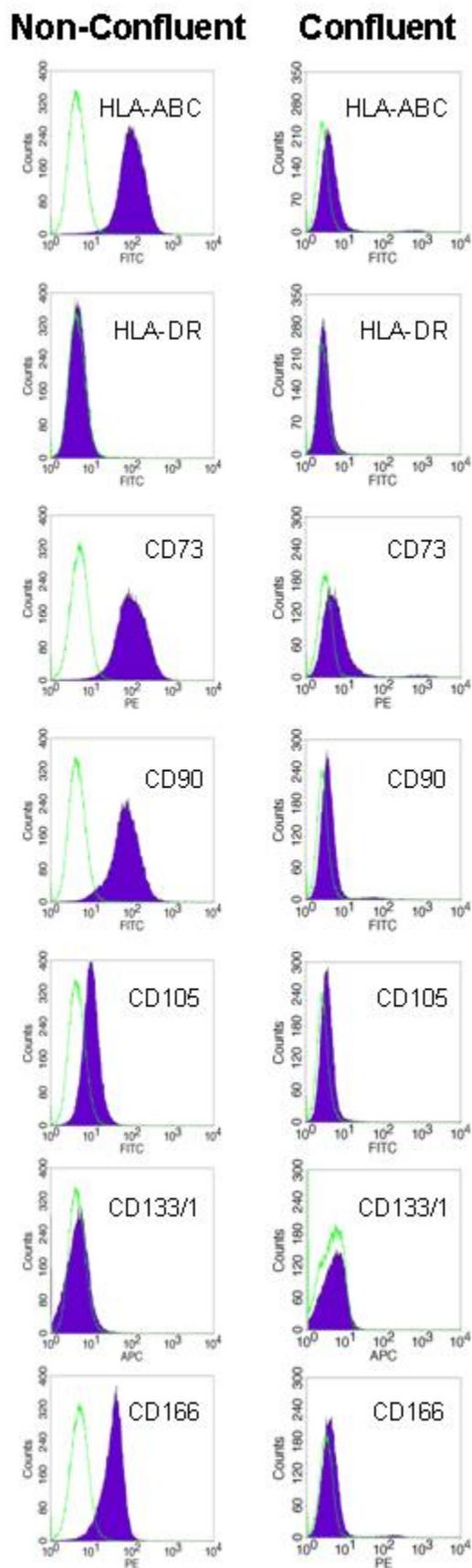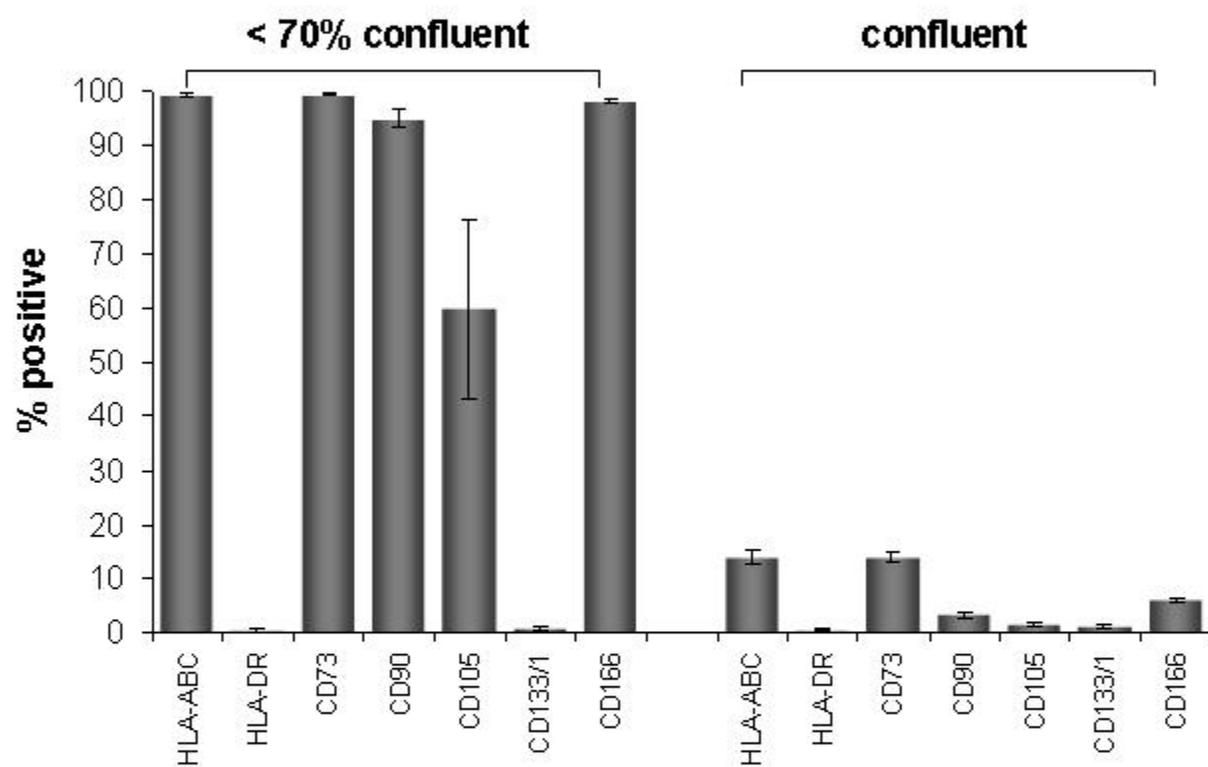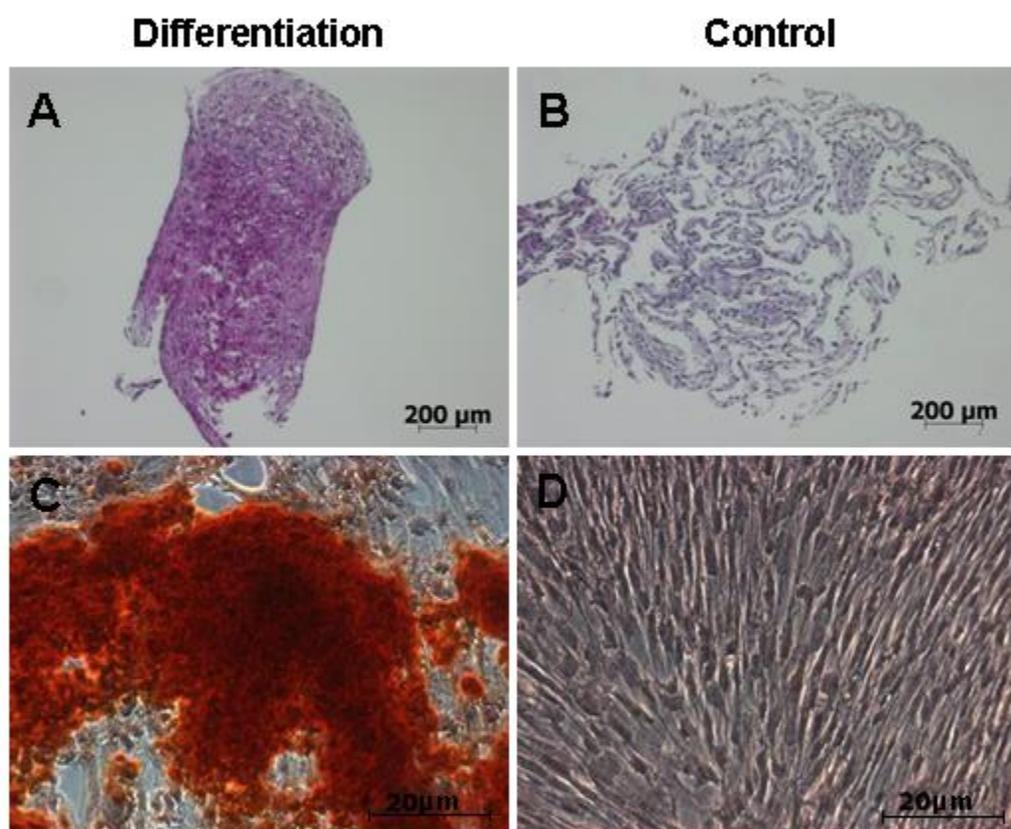
Table 1. Isolation methods for MSCs from human placenta

Stem cell source	Gestational age	Isolation method	Reference
Whole placental tissue	1st trimester	Collagenase + Trypsin	Genbachev et al. 2005
Whole placental tissue	1st trimester	Trypsin + Collagenase II + Dispase II	Portmann-Lanz et al. 2006
Whole placental tissue	1st trimester	Chorionic villi sampling	Poloni et al. 2008
Whole placental tissue	term	Explant culture method	Igura et al. 2004 Zhang et al. 2006a Zhang et al. 2006b
Central placental lobules	term	Trypsin	Fukuchi et al. 2004
Whole placental tissue	term	Trypsin-EDTA	Yen et al. 2005 Miao et al. 2006 Chien et al. 2006
Whole placental tissue	term	Collagenase I	Barlow et al. 2008 Brooke et al. 2009
Whole placental tissue	term	Collagenase P	Chang et al. 2007
Placental tissue without stem villi and amniotic fetal membranes	term	Collagenase + Dispase II	Battula et al. 2007 Battula et al. 2008
Decidua basalis	2nd trimester	Mechanical mincing (no enzymatic digestion)	In't Anker et al. 2004
Decidua parietalis	2nd trimester	Mechanical mincing (no enzymatic digestion)	In't Anker et al. 2004
Decidua parietalis	term	Collagenase + Hyaluronidase + Pronase	Strakova et al. 2008

Table 2. Antibodies used for flow-cytometry and immunocytochemistry

Antigen	# Clone	Conjugate	Host	Company
CD11b	ICRF44	PE	M	BioLegend
CD14	H5E2	-	M	BioLegend
CD19	HIB19	PE	M	BioLegend
CD 34	AC136	FITC	M	Miltenyi Biotec GmbH
CD 44	G44-26	FITC	M	BD Pharmingen
CD 45	5B1.	FITC	M	Miltenyi Biotec GmbH
CD54	HA58	PE	M	BD Pharmingen
CD 73	AD2	PE	M	BD Pharmingen
CD79a	HM47	PE	M	BioLegend
CD 90*	5E10	-	M	BD Pharmingen
CD 105*	266	-	M	BD Pharmingen
CD 117	YB5.B8	PE	M	BD Pharmingen
CD 133/1	AC133	APC	M	Miltenyi Biotec GmbH
CD 163	GHI/61	PE	M	BD Pharmingen
CD 166	3A6	PE	M	BD Pharmingen
CD 271	ME20.4-1H4	PE	M	Miltenyi Biotec GmbH
HLA-ABC	G46-2.6	FITC	M	BD Pharmingen
HLA-DR	G46-6	FITC	M	BD Pharmingen
Cytokeratin-7 (CK-7)	CAM 5.2	FITC	M	BD Pharmingen
Cytokeratin-18 (CK-18)	CY-90	FITC	M	SIGMA - ALDRICH AG
Alpha smooth muscle actin (α-SMA)	1A4	PE	M	R&D Systems
Von Willebrandt factor (vWF)	sheep polyclonal	FITC	S	Abcam plc
KDR / VEGF receptor-2	89106	PE	M	R&D Systems
Placental alkaline phosphatase (PLAP)*	H17E2	-	M	AbD Serotec
Hepatocyte specific antigen (HSA)*	OCH1E5	-	M	Abcam plc
SSEA-3**	MC-631	-	R	R&D Systems
SSEA-4	MC813-70	PE	M	R&D Systems
Oct-3/4**	240408	-	R	R&D Systems
Vimentin	V9	PE	M	SIGMA - ALDRICH AG
Nestin*	10C2	-	M	Abcam plc
E-Cadherin	36	PE	M	BD Pharmingen
Stro-1*	STRO-1	-	M	R&D Systems
Embryonic stem cell marker Tra-1-60*	Tra-1-60	-	M	Abcam plc
Embryonic stem cell marker Tra-1-81*	Tra-1-60	-	M	Abcam plc
ZO-1	ZO-1-1A12	FITC	M	Zymed Laboratories

*   Polyclonal goat anti mouse Ig (BD Pharmingen)
**   Polyclonal rabbit anti rat Ig (DAKO Cytomation)

Table 3a. Cytogenetic analysis of PD-MSCs. Data represents karyotyping of passage 3 cell isolates obtained from 3 female and 3 male deliveries.

Donor	Gender of newborn	Normality	Karyotyp
2	female	normal	46 XX
4	female	normal	46 XX
7	female	normal	46 XX
9	male	normal	46 XX
11	male	normal	46 XX
13	male	normal	46 XX

Table 3b. FISH analysis of PD-MSCs. Data represents sex determining region analysis of passage 3 cell isolates obtained from 3 male deliveries

Donor	Gender of newborn	Normality	Karyotyp
9	male	normal	46 XX
11	male	normal	46 XX
13	male	normal	46 XX

Table 4. Phenotypic analysis of PD-MSCs by flow-cytometry and immunocytochemistry. Data represent flow cytometric analysis of passage 1 PD-MSCs and immunocytochemical analysis of passage 6 PD-MSCs.

Antigen	FACS	IC
CD11b	-	n.d.
CD14	-	n.d.
CD19	-	n.d.
CD34	-	-
CD44	+	+
CD45	-	-
CD54	n.d.	20%
CD73	+	+
CD79a	-	n.d.
CD90	+	+
CD105	+	30%
CD117	-	-
CD133/1	+	33%
CD 163	-	n.d.
CD166	+	+
CD271	-	-
Cytokeratin -7	-	-
Cytokeratin -18	n.d.	40%
KDR	-	-
Placental alkaline phosphatase	-	-
α-SMA	+	+
vWF	+	+
Hepatocyte	n.d.	-
HLA-ABC	+	+
HLA-DR	-	-
SSEA-3	+	+
SSEA-4	-	-
Oct-3/4	n.d.	-
Vimentin	n.d.	+
Nestin	n.d.	50%
E-Cadherin	n.d.	40%
Stro-1	-	-
Tra-1-60	n.d.	-
Tra-1-81	n.d.	-
ZO-1	n.d.	-