ORIGINAL ARTICLE

Routine clonal expansion of mesenchymal stem cells derived from amniotic fluid for perinatal applications

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ABSTRACT

Introduction Stem cells (SCs) isolated from amniotic fluid (AF) are a promising source for autologous perinatal cell therapy. The aim of this study was to develop a routine isolation, selection, and expansion protocol of clonal SC lines from redundant clinical amniocentesis samples.

Materials and methods Amniotic fluids were collected between 15 and 22 weeks of gestation, and SCs were isolated by CD117-based and mechanical selection protocols. SCs were characterized by mesenchymal SC marker expression and differentiation protocols. Cells were manipulated with a lentiviral vector system expressing the β-galactosidase reporter gene and were injected into immunodeficient newborn mouse pups. Qualitative assessment was performed to detect the infused cells after 1 week.

Results A total of 78 clonal AF SC populations were successfully isolated by mechanical selection from 21 consecutive amniocentesis samples. They were positive for mesenchymal SC cluster of differentiation markers and could be differentiated into the different lineages. SCs were stably labeled using β-galactosidase and were detected in the lungs and hearts of the neonatal mice.

Conclusion We demonstrate that mesenchymal SCs can be routinely isolated and clonally expanded from mid-gestation human AF using mechanical isolation. They can easily be transduced and be tested for perinatal treatment in animal models. © 2013 John Wiley & Sons, Ltd.

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INTRODUCTION

Stem cell (SC) therapy is a medical therapeutic strategy that introduces SCs into an ill developed or damaged tissue in order to prevent or treat disease or injury. Current research investigates the potential of SC therapy to improve the clinical treatment of many human diseases. The ability of SCs to self-renew and give rise to subsequent generations with variable degrees of differentiation capacities can be exploited in several ways. On the one hand, they can be used for the *in vitro* generation of tissues that can potentially replace diseased and damaged areas in the body.[1] On the other hand, they can be used for direct *in vivo* modulation of pathophysiologic processes, such as disease progression, inflammatory responses, organ development, or developmental maturation.[1]

As SCs evolve from totipotent to multipotent, they gradually lose their differentiation potential, which limits their clinical applicability. Embryonic SCs (ESc) with their broad differentiation potential are theoretically most promising, but their use is limited by ethical concerns[2] and tumor formation when injected *in vivo*.[3] In contrast, adult SCs (ASC) can be readily obtained but often in small amounts and have limited propagation potential.[1] Thus, alternative sources of human SCs for clinical use are needed.

Amniotic fluid (AF) has been collected for decades as a source of cells for prenatal diagnosis. The origin of AF cells is still debated,[4,5] but it contains mainly cells that are terminally differentiated and have limited proliferative capacity.[6–9] Recent studies have demonstrated the presence of a subset of cells that can proliferate and differentiate,[5,10] which classifies them as SCs. A variety of different SC types have been isolated from AF.[11] These include cells expressing the hematopoietic marker CD34[12] as well as cells with mesenchymal characteristics [amniotic fluid-derived mesenchymal stem cell (AF-MSC)]. They are negative for CD45, CD34, and CD14[13–16]

and are able to proliferate *in vitro* more rapidly compared with ASC.[14,17–21] Despite their high proliferation rate, these cells display a normal karyotype when expanded *in vitro* and do not form tumors *in vivo*.[21] They exhibit a broad differentiation potential toward mesenchymal lineages[14,19,22–24] but could be easily reprogrammed to fully pluripotency.[25]

Potential application for regenerative medicine and tissue engineering strategies as well as the relative absence of ethical concerns make AF SCs ideal for regenerative medicine and autologous tissue engineering.[26] They could be used for perinatal applications and retrieved either at the time of birth, or, in case of congenital anomalies, by amniocentesis. Herein, we focused on finding a reliable and reproducible technique to isolate and expand SCs derived from mid-gestational amniocentesis samples for preclinical disease models.

MATERIAL AND METHODS

Isolation of amniotic fluid-derived mesenchymal stem cells

Five milliliters of 21 separate AF samples were obtained from 15 to 22-week pregnant women who underwent an amniocentesis to exclude Down syndrome. Patients gave informed consent, and the study protocol was approved by the Ethics Committee of the UZ Leuven.

Within 2 h, cells were passed through a 40-μm cell strainer (BD Falcon) and subsequently isolated from AF and debris by centrifugation at 1200 rpm for 5 min (Eppendorf 5810R, Ø 30 cm). The supernatant was removed, and the cell pellet was resuspended in growth medium[10] consisting of α-MEM (Invitrogen, Ghent, Belgium), 15% fetal bovine serum (FBS) (Invitrogen), 1% L-glutamine (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 18% Chang B and 2% Chang C (Irvine Scientific, Brussels, Belgium), plated in a petri dish and incubated at 37 °C in 5% CO_2. A fraction of cells directly isolated from the AF sample was immediately characterized for mesenchymal SCs markers CD117, CD24, and CD44 by flow cytometry.

For further selection of the mesenchymal SC population, two different protocols were explored. As reported by De Coppi *et al.*,[10] growth medium was replaced after 4 days when the first cells attached and every 4 days until the cell population became confluent. Subsequently, immunoselection for CD117 was performed using Magnetic cell sorting kit for CD117 (MACS h-CD117 MicroBead Kit, Leiden, The Netherlands). The CD117 positive cell fraction obtained was plated in a Petri dish and expanded to higher passages for further analysis.

In parallel, a mechanical isolation procedure was used.[20] About 5 mL of AF were passed through a 40 μm strainer and plated in a petri dish. The medium was replaced when the first attached cells were detected in the primary culture dish and studied under a light microscope the following days. The individual single cells, defined as starter cells, were observed for 2–3 days until they started to form colonies. To prevent confluence with neighboring colonies, cells growing too close to each other were mechanically removed, and the medium was replaced. The culture was maintained in the primary culture dish for 48 h. Next, each separate cell colony was mechanically isolated using an inverted microscope inspection and fine-tipped pipettes. The aspirated cells of each colony were re-seeded into an individual well of a 96-well plate. The culture was maintained at 37 °C under humidified 5% CO_2. When a clonal cell culture reached 70% confluence, sub-culturing was performed by trypsinization and re-plating into a 24-well plate and thereafter into a six-well plate and finally moved in a 10-cm Petri dish. The cells were allowed to expand to 70% confluence and then routinely sub-cultured with a dilution of 1 : 3.

Characterization of amniotic fluid-derived mesenchymal stem cells

CD marker expression

Amniotic fluid-derived mesenchymal stem cells from passage 4 were characterized using flow cytometry analysis. A total of 100 000 cells were harvested and incubated with either FITC or PE-conjugated antibodies against CD117, CD24, CD90, CD44, CD105, CD73, human leukocyte antigen A-B-C, HLA-DR, CD34 and CD45 (BD bioscience, Erembodegem, Belgium), and CD29 (Acris, Herford, Germany) mouse anti-human monoclonal antibodies and appropriate isotype control. Stained cells were then analyzed using a Beckton Dickinson flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA) using the Cell Quest software, and data were computed using the FlowJo software (Tree Star, Ashland, OR, USA).

Differentiation protocol

Furthermore, cells were analyzed for their capacity to differentiate into osteogenic, adipogenic, and chondrogenic lineages. Differentiation was performed in a monolayer as detailed by the manufacturer's protocol.

Osteogenic differentiation. Osteogenic differentiation was induced by culturing AF-MSC at 70% confluence for 4 weeks in osteogenic medium. This consisted of the commercially available 'osteogenic differentiation medium' (Invitrogen). Differentiation was assessed by Alizarin staining (Sigma Aldrich, Diegem, Belgium) of the calcified extracellular matrix deposition.

Adipogenic differentiation. To induce adipogenic differentiation, cells were cultured at 100% of confluence and subsequently differentiated with adipogenic differentiation medium for 14 days at 37 °C in a 5% CO_2 incubator. The medium was composed of 10% FBS, 10^{-6} M dexamethasone, 0.5 M 3-isobutyl-methylxanthine, 10 μg/mL insulin, and 200 μM indomethacin in Dulbecco's Modified Eagle Medium high glucose (Invitrogen). Differentiation into the adipogenic lineage was determined by Oil Red O staining (Sigma-Aldrich, MO, USA).

Chondrogenic differentiation. To induce chondrogenic differentiation, cells were cultured in high-density pellet mass cultures for 14 days. About 20 μL droplets of cells suspension (400 000 cells resuspended in phosphate buffered saline) were seeded into individual wells of a 24-well plate. Cells were allowed to attach without medium for 3 h at 37 °C in a 5% CO_2 incubator and then cultured for 24 h in growth medium. One day later, the medium was replaced with chondrogenic differentiation medium. This consisted of the commercially available 'chondrogenic differentiation medium' (Invitrogen). Chondrogenic differentiation was determined by Alcian Blue staining (Sigma-Aldrich).

Pluripotency markers

To investigate pluripotency of AF-MSCs, Oct4 and Nanog gene expression was analyzed. RNA from each clone was extracted using the Tri-Pure reagent (Roche Diagnostics, Vilvoorde, Belgium) according to the manufacturer's instructions. cDNA was synthesized from 0.1 μg of RNA using Taq Man Reverse Transcription Reagents (Applied Biosytem, Ghent, Belgium). The Platinum SyBR Green qPCR supermix-UDG (Invitrogen) was used to detect Oct4 and Nanog expression in three random clones from every gestational week (from 15th to 22nd gestational age) ,and GAPDH gene was used as a housekeeping gene to normalize mRNA levels (Oct4 FW: 5′-GATGGCGTACTGTGGGCCC-3′, RV: 5′-TGGGACTCCTCCGGGTTTTG-3′; Nanog FW: 5′-CAGAAGG-CCTCAGCACCTAC-3′, RV: 5′-ATTGTTCCAGGTCTGGTTGC-3′, GAPDH FW: 5′-GCCTTCCGTGTCCCCACTGC-3′, RV: 5′-CAATGCCAGCCCCAGCGTCA-3′). RNA extracted from human ESC line H9 and differentiated human umbilical vein endothelial cells cell line were obtained from the SC Institute of Leuven and used as a positive and negative controls, respectively.

Lentiviral vector generation, production and transduction of AF-MSCs

We used a lentiviral vector platform to introduce the reporter gene β-Galactosidase (LacZ) into AF-MSCs to be able to detect cells *in vivo*. The lentiviral production is described elsewhere.[27] Lentiviral vector particles were added to the cell culture in serial dilution with an multiplicity of infection ranging between 1 and 10. To investigate the transduction efficiency of lentiviral vector in AF-MSCs, an X-gal staining was used. Cells were fixed in 25% glutaraldehyde and 37% of methanol for 2 min and incubated with an X-gal solution (Sigma Aldrich) O/N at 37 °C. LacZ-AF-MSCs were analyzed by flow cytometry for the complete mesenchymal CD marker expression panel to show that lentiviral manipulation did not affect the plasticity and functionality of SCs.

In vivo experiment

Five severe combined immunodeficient (SCID)/Beige mice were purchased from Charles River Laboratories (Wilmington, MA, USA). SCID/Beige mice (10 weeks old) were time-mated to obtain neonates of known gestational age. All animal procedures were carried out under 'Biosafety Level 2' conditions approved by the Biosafety Committee of the KU Leuven and conform to Directive 2010/63/EU of the European Parliament. The protocol was approved by the Local Ethics Committee for Animal Experimentation, and all animals were treated according to the current guidelines of animal care. All neonatal injections were performed within the first 24 h of life. The mother of each litter (singly housed) was removed from the cage and pups placed briefly on a bed of ice for anesthesia. A light source was used to visualize thoracic area, and the 20 μL volume containing 1×10^5 LacZ-AF-MSC was administered slowly intraventricularly using a 33-gauge needle by subxiphoid approach. For identification of injected animals from their non-injected littermates, specific areas of each injected pup was marked by subcutaneous injection of approximately 1 μL of colloidal carbon suspension immediately following SC administration. One week after injection, the animals were euthanized, and heart and lung tissues were embedded in tissue freezing medium OCT. Frozen lung and heart sections were stained to detect engraftment of β-gal positive cells by X-gal staining and counterstained with Paracarmine.

RESULTS

Isolation of amniotic fluid stem cells

Cell growth was observed in 18 out of 21 individual patient samples, in three samples of bacterial contamination that occurred. Cells derived from AF samples resulted in a mixed population of adherent cells with different types of morphology and size, with spindle and round shape cells predominantly present.

Initially, we focused on isolating the CD117+ fraction. Freshly isolated cells derived from five samples were found to be weakly positive for CD117 marker with flow cytometry showing an expression level of around 0.5–2%. However, after immunoselection protocol and passage in culture, the percentage of positive cells did not increase. Therefore, for this set of experiments, we proceed with isolation of AF-MSCs using a mechanical separation by seeding.

Derivation of a clonal population using the mechanical selection protocol was successful in 78 out of 104 clones. A total of 25 clones ceased to grow after less than three passages in culture. At day 3 of the primary culture of each AF sample, we detected on average three to eight of single cells adhering to the culture dish (starter cells), which gave rise to a colony of 100–300 cells on average [Figure 1(A)].

The selected colonies were expanded and with this method, 800 000 cells from a clonal AF-MSCs population could be derived within 2 weeks. After expansion, clones consisted of a mixed population where both round-shaped and spindle-shaped cells were present, but after several passages in culture, spindle-shaped cells became more abundant [Figure 1(B, C)]. A survival analysis was performed and showed that the isolated cells could be expanded in culture to a median of 13 passages (range from 5 to 36) [Figure 1(D, E)].

Characterization of amniotic fluid-derived mesenchymal stem cells

CD marker expression

Cell surface antigenic characteristics were analyzed by flow cytometry (Figure 2). A total of 76 out of 78 clones were negative for hematopoietic markers CD34, CD45, and Class II major histocompatibility cell surface receptor HLA-DR. The cells were positive for the mesenchymal markers CD105, CD73, and CD90, for the adhesion molecule CD44, CD29, and for Class I major histocompatibility antigens (HLA-ABC). CD117 (c-Kit) expression was low in all samples from the different gestational ages (median of 2.19%, range from 0.16% to 43.4%).

Differentiation protocol

Amniotic fluid-derived mesenchymal stem cells were able to differentiate into different mesenchymal lineages *in vitro* when cultured in appropriate conditioning medium (Figure 3). Clones from all gestational weeks could be differentiated into the adipogenic lineage as

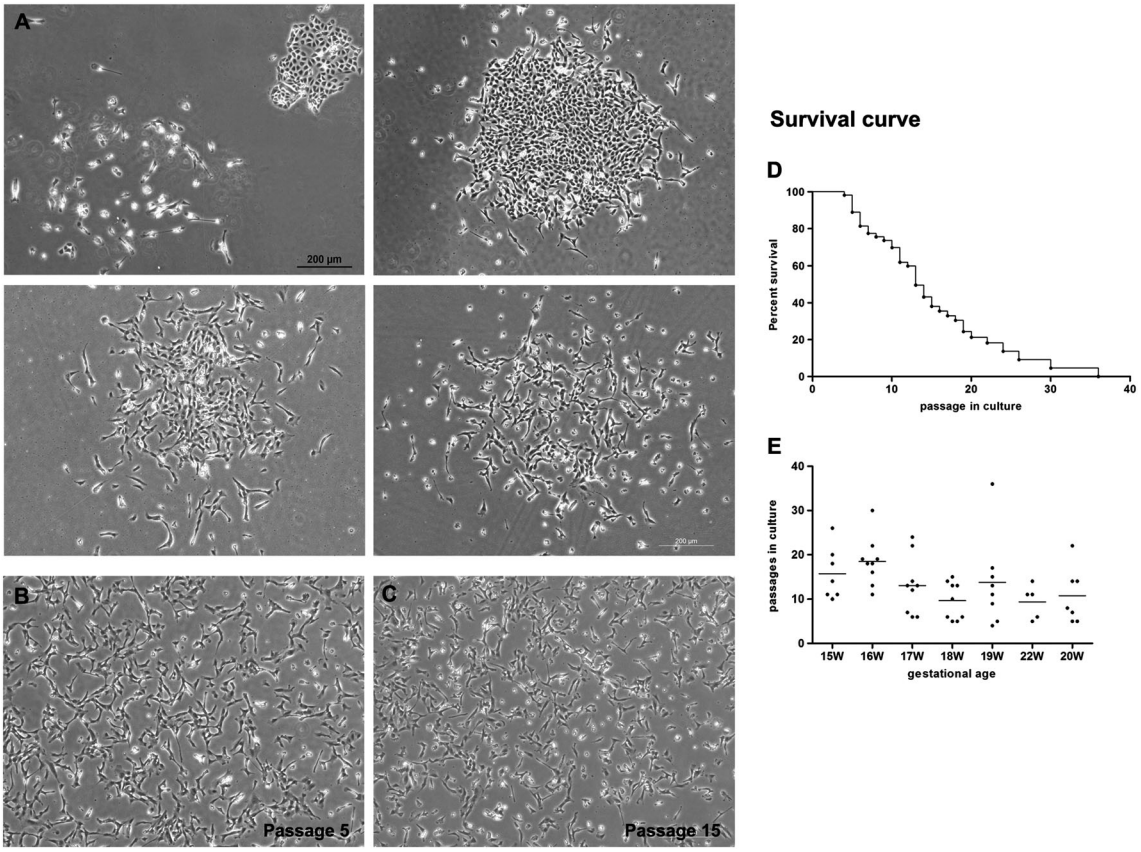

Figure 1 Derivation of amniotic fluid-derived mesenchymal stem cell (AF-MSC) by mechanical selection. (A) Representative image of AF-MSC colonies derived from fresh amniotic fluid sample. Top left of the panel, an example of spindle and round shaped cells forming a colony. Morphology of AF-MSC at passage 5 (B) and passage 15 (C) in culture. Proliferative ability of AF-MSC. Cell Survival curve (D) for all clones derived from different gestational time point. (E) Passages in culture reached by every single clone grouped for gestational age

determined by oil red staining. The calcium mineralization of AF-MSC-derived osteogenic cells was verified by Alizarin staining. Glycosaminoglycan's matrix was detected by Alcian blue staining in AFSc cultures under chondrogenic condition.

Pluripotency markers

The AF-MSC clones from different gestational weeks were evaluated by qRT-PCR for expression of the Oct-4 and Nanog. In our samples, no marker could be detected at a level above the negative control. This was probably related to the fact that analyses were conducted in the whole population and not in the CD117 selected cells.

Lentivirus-mediated transduction of amniotic fluid-derived mesenchymal stem cell

Amniotic fluid-derived mesenchymal stem cells were efficiently transduced by lentiviral vectors as shown by the X-gal staining, which detected the nuclear activity of β-galactosidase in 94.5% cells [Figure 4(A)]. Manipulated AF-MSCs maintained their mesenchymal profile as shown by flow cytometry analysis (data not shown) and were still positive for nuclear X-gal staining after 20 passages in culture.

In vivo engraftment of transduced amniotic fluid-derived mesenchymal stem cell

In vivo engraftment of LacZ positive AF-MSCs intracardially injected was investigated in five newborn SCID/beige mice. Seven days after injection, mice were euthanized and X-gal staining was performed to detect the distribution of LacZ positive cells in different tissues. Microscopic analysis identified individual AF SCs in the conducting airways [Figure 4 (B)], lung parenchyma, and myocardium. No individual cells or aggregates were found in the blood vessels. Qualitative assessment of the slides did not show local inflammation around AF-MSCs.

DISCUSSION

In recent years, AF-derived SCs have been proposed as a promising source of cells for regenerative medicine. AF-MSCs are a heterogeneous population of cells with different origin.[28] Cells present in the urinary, respiratory, and gastrointestinal tract can in theory be shed into the AF.[29–32] The varied origins of the cells can result in different degrees of cell differentiation depending on the stage of organ development. Only 1% of cells in AF demonstrate SC characteristics.

We aimed to develop a reproducible method to isolate AF SCs of different gestational weeks to be used in perinatal therapy.

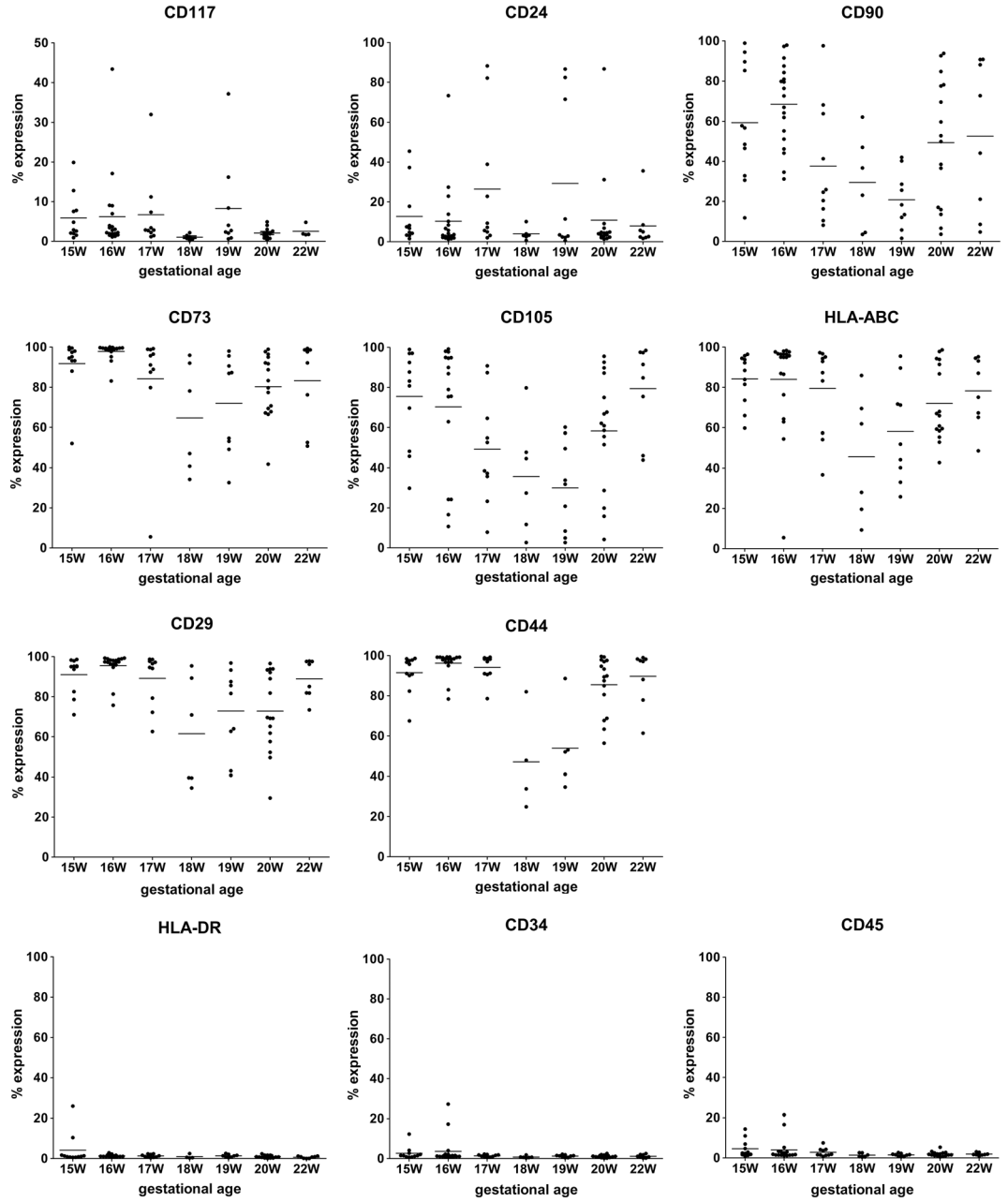

Figure 2 Expression pattern of amniotic fluid-derived mesenchymal stem cell clonal populations for CD117, CD24, CD73, CD90, HLA-ABC, CD44, CD105, CD29, HLA-DR, CD34, and CD45 markers. Every single graph shows the specific maker expression for every clonal population. The average is shown for every gestational week

The first procedure explored was the immunoselection method described by De Coppi *et al.*,[10] which first demonstrated the isolation AF SCs from confluent back-up human amniocentesis cultures. Cells were harvested and either expanded once or immediately subjected to immunoselection for CD117 marker. AF-MSCs were subcultured routinely and clonal lines were generated by the limiting dilution method in 96-well plates. We reproduced the procedure but did not succeed in obtaining a 100% $CD117^+$ population. Cells were analyzed before and after immunoselection and the percentage of $CD117^+$ after expansion of the selected cells did not change (0.5–2%). There are several reasons that could describe this discrepancy. First, there is the heterogeneity of these cells. In fact, AF contains a heterogeneous population of cells, derived mainly from the fetal skin, fetal digestive, respiratory, and urinary tracts, and placental membranes.[29] Another reason could be the internalization of CD117 receptor after immunoselection resulting in a detection failure by flow cytometry in the following passages. A third explanation can be that in our protocol, the cells were kept in culture for a longer period prior to the CD117 selection, at which time this specific CD marker expression could have decreased.

One disadvantage of an isolation procedure based on CD marker expression is the possible exclusion of other multipotent cells. Therefore, for this set of experiments, we

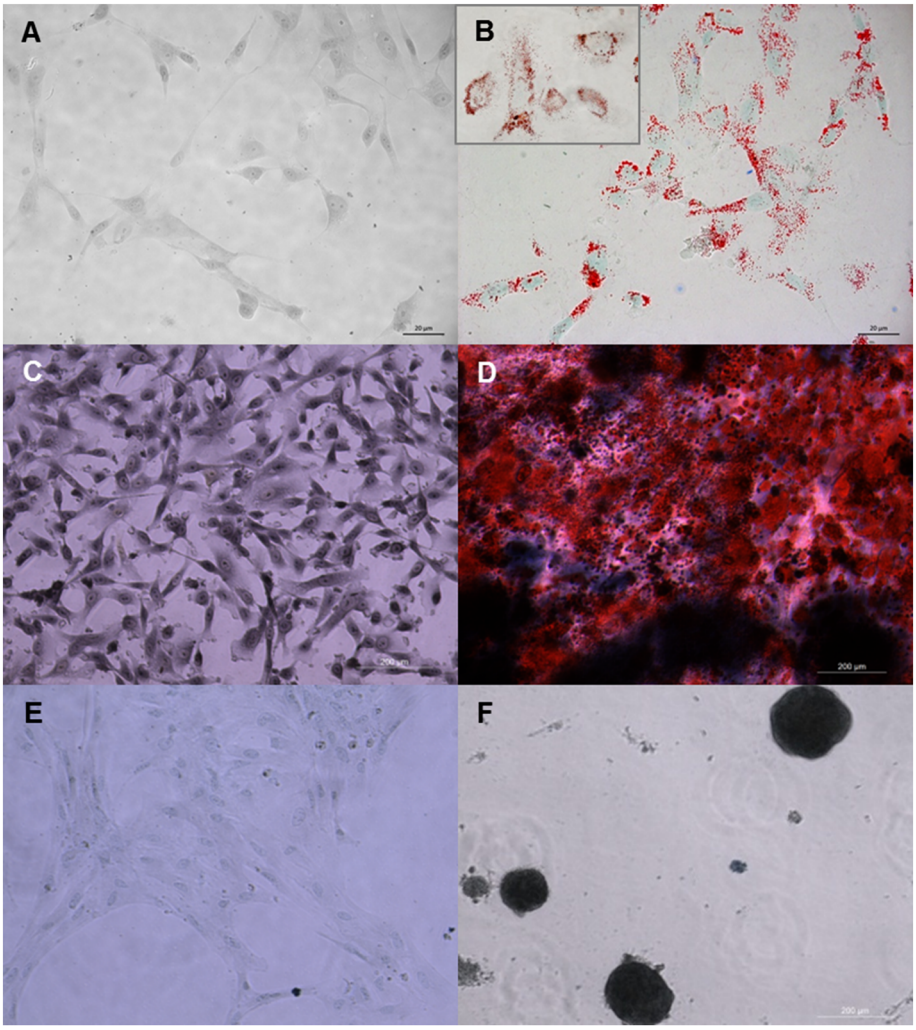

Figure 3 Differentiation potential of clonal populations of amniotic fluid-derived mesenchymal stem cell (AF-MSC). Representative figure of differentiation potential of AF-MSC. Oil Red O staining for adipogenesis differentiation in (A) control counterstained with Haematoxylin and (B) differentiated AF-MSC. Alizarin Red staining for osteogenic differentiation in (C) control, and (D) differentiated AF-MSC. Alcian Blue staining for control and differentiated AF-MSC under chondrogenic conditions in pellet mass cultures

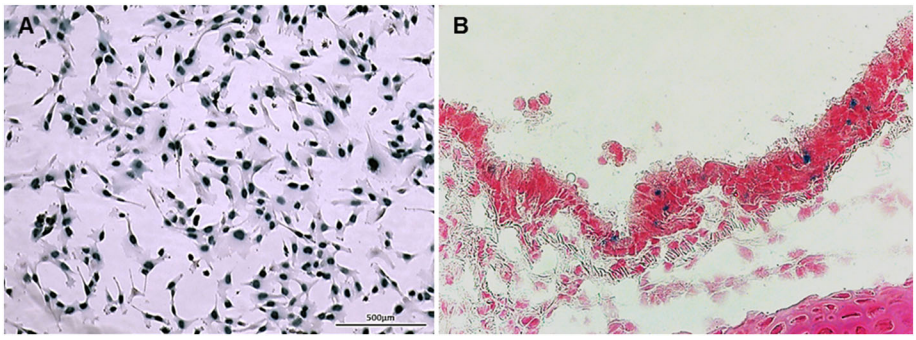

Figure 4 Manipulation of amniotic fluid-derived mesenchymal stem cell (AF-MSC) with a lentiviral vector expressing LacZ. (A) X-gal staining of AF-MSC shows highly positive nuclear signal for LacZ. (B) *In vivo* engraftment of LacZ-AF-MSC with a representative figure of tracheal tissue. Positive cells are localized by nuclear X-gal staining

moved to a strategy based solely on the capacity of the cells to adhere to plastic in order to isolate a monoclonal SC line as described by Roubelakis *et al.*[20] We used a method where AF-MSC colonies in primary culture were mechanically selected to derive a monoclonal population. Cells derived by this starter cell method initially appeared to have a

heterogeneous morphology in culture, with a mix of spindle and round-shaped cells. But after few passages, the spindle-shaped cells became more abundant.

We were able to isolate mesenchymal SCs from AF in 85% of the samples, and 75% of the clones derived from each sample were successfully expanded and characterized to have a mesenchymal phenotype.

All clones that grew beyond four passages in culture were analyzed by flow cytometry and expressed the specific mesenchymal SC marker according to standard criteria.[33] FACS analysis documented the presence of CD44, CD90, CD73, CD29, CD24, HLA-ABC, and CD105 and the absence of hematopoietic-related CD-antigens (CD34 and CD45) in 76 out of 78 lines.

Oct4 and Nanog expression levels were analyzed to assess pluripotency of the AF-MSC. The first is a key marker in ESCs and responsible for the maintenance of pluripotency and the latter is a transcription factor involved in the process of self-renewal of ESCs. AF-MSCs probably represent an intermediate stage between pluripotent ES cells and lineage-restricted ASCs.[10] Cells isolated from early gestational weeks have been shown to express Oct4 by western blot[15] and quantitative RT-PC.[10,12] In our study, we were not able to detect any significant expression compared with the negative control. Variations in the methodology could explain the difference between the published and obtained results. Previous studies detected Oct4 expression in AF-MSC derived by CD117 immunoselection (4, 16, 20), whereas in this study, the mechanical isolation of AF-MSC was used. A second explanation could be the higher passage number of the cells that were used to detect the Oct4 transcript. RNA was isolated from cells at 8–10 passages in culture at which stage Oct4 expression could already have decreased.

In order to define a cell as an MSC, the cell must be plastic-adherent, express specific surface markers, and be able to differentiate in to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiation conditions.[33] AF-MSC derived with our protocol satisfied all these criteria.

Amniotic fluid-derived mesenchymal stem cells are considered as a potentially useful source in the perinatal period. With the advent of prenatal screening, it has become possible to identify diseases or conditions in an embryo or fetus long time before birth. This means that AF-MSCs could be isolated from the patient and used for autologous SC therapy. Mesenchymal SCs are known to possess immunomodulatory properties,[11] which has also been shown for AF-MSCs.[34] SCs could further be considered as a vehicle for therapeutic genes such as growth factor or a reporter gene (GFP, LacZ or luciferase) to track manipulated SCs in animal models. Previous work showed that AF-MSCs could be manipulated with adenoviral[35] and lentiviral vector systems.[20,36]

Our data confirm that AF-MSCs are indeed permissive to lentiviral transduction, with no silencing effects over the multiple culture passages. Moreover, mesenchymal marker expression was not altered after transduction. After intracardiac injection, LacZ-AF-MSCs could be detected in the lungs and heart of immunodeficient mouse pups after 7 days. This suggests that these cells are ideal candidates for cell, combined cell and gene therapy and nanomedicine applications.

CONCLUSION

Amniotic fluid SCs are an interesting source of SCs for both preclinical and clinical researches. We were able to routinely isolate human SCs expressing mesenchymal characteristics and differentiation potential. Moreover, we proved that these cells can be genetically modified with a lentiviral vector and be used for *in vivo* cell therapy experiments.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Amniotic fluid-derived mesenchymal stem cells have the unique advantage of extensive self-renewal *in vitro* allowing for long-term expansion, full characterization and cryopreservation.

WHAT DOES THIS STUDY ADD?

- Isolation procedures were possible in samples derived from mid-gestation amniocentesis samples.
- The isolation procedure was reproducible and applicable in preclinical research.
- Amniotic fluid-derived mesenchymal stem cell can be genetically modified with a lentiviral vector and be used for *in vivo* cell therapy experiments.

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