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ORIGINAL ARTICLE

The amniotic fluid as a source of mesenchymal stem cells with lung-specific characteristics

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Abstract

The amniotic fluid is a clinically accessible source of mesenchymal stem cells (AF-MSC) during gestation, which enables autologous cellular therapy for perinatal disorders. The origin of AF-MSC remains elusive: renal and neuronal progenitors have been isolated from the AF-MSC pool, yet no cells with pulmonary characteristics.

We analyzed gene expression of pulmonary and renal markers of 212 clonal lines of AF-MSC isolated from amniocentesis samples. AF-MSC were cultured on dishes coated with extracellular matrix (ECM) proteins from decellularized fetal rabbit lungs. In vivo differentiation potential of AF-MSC that expressed markers suggestive of lung fate was tested by renal subcapsular injections in immunodeficient mice.

Of all the isolated AF-MSC lines, 26% were positive for lung endodermal markers FOXA2 and NKX2.1 and lacked expression of renal markers (KSP). This AF-MSC subpopulation expressed other lung-specific factors, including IRX1, P63, FOXP2, LGR6, SFTC, and PDPN. Pulmonary marker expression decreased over passages when AF-MSC were cultured under conventional conditions, yet remained more stable when culturing the cells on lung ECM-coated dishes. Renal subcapsular injection of AF-MSC expressing lung-specific markers resulted in engrafted cells that were SPTB positive.

These data suggest that FOXA2+/NKX2.1+/KSP- AF-MSC lines have lung characteristics which are supported by culture on lung ECM-coated dishes.

1 | INTRODUCTION

In recent years, stem cell-based therapy has been successfully implemented in clinics as a new branch of regenerative medicine.[1] Different cell sources have been employed for this experimental approach, ranging from mesenchymal stem cells (MSC) that influence disease progression by their anti-inflammatory effects, to tissue-specific stem or progenitor cells that can be used to engineer tissues to replace diseased or damaged structures.

The amniotic fluid (AF) is described as an alternative source for cell therapy and tissue engineering applications for several unique characteristics.[2] The use of adult stem cells is restricted because of the invasive harvesting methods and their limited proliferation and differentiation potential, while embryonic stem cells raise too many ethical restrictions despite their propensity for proliferation and differentiation. Fetal stem cells may overcome these limitations and can be clinically relevant to a large number of patients in an allogenic setting, provided that both biobanking and GMP-compliant isolation and culture procedures can be implemented on a large scale. Fetal stem cells can be derived from multiple tissues that are currently being considered as medical waste including the umbilical cord, the placenta, the amniotic membranes, and the AF. The AF has the unique feature that it not only becomes available at the time of birth, but it can also serve as a cell source during gestation as it can be safely collected via amniocentesis. This enables autologous perinatal cell therapeutic applications for congenital or acquired disorders.

The AF is mainly composed of water, nutrients, and cells derived from the fetal developing tissues and the amniotic membranes. Most of these cells are terminally differentiated and show limited proliferation potential. However, a minor fraction (around 1%) retains stemness characteristics.[2] These plastic-adherent amniotic fluid stem cells (AF-MSC) express mesenchymal markers (CD73, CD90, CD105) and have a broad differentiation potential towards adipogenic, osteogenic, and chondrogenic lineages. They have a high proliferative and clonogenic capacity and retain a normal karyotype during in vitro culture.[3,4] Their therapeutic applicability has already been extensively studied

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preclinically in the context of muscle, bone, kidney liver, and also lung regeneration.[5-10]

Additionally, AF-MSC expressing the pluripotency markers OCT4 (POU5F1) and CD117 (stem cell factor, C-KIT) have been described.[2,11] These cells are capable to differentiate into cell types from both mesenchymal and non-mesenchymal lineages, such as neuronal or cardiac cells, and can thus be defined as multipotent. However, unlike embryonic stem cells, they do not form teratomas when injected in vivo.[2] Full pluripotency is only realized upon reprogramming, which can be achieved by adjusting their culture conditions, without any genetic manipulation.[12] CD117-expressing AF-MSC have been successfully used in models of respiratory, cardiovascular, renal, musculo-skeletal, and nervous injury (as reviewed in Loukogeorgakis and De Coppi[13]).

These data illustrate that cells with varying characteristics and with high cellular plasticity can be derived from the AF. AF-MSC originate from different tissues at specific times during gestation, which results in a heterogeneous cell population especially during the last trimester of pregnancy. We have identified before cells with a renal phenotype by clonal isolation and expansion of AF-MSC.[7,14] In this project, we aim to unravel the origin of AF-MSC that are negative for renal markers by studying additional marker expression and differentiation potential.

WHAT IS ALREADY KNOWN ABOUT THIS TOPIC?

- Fetal tissues are interesting sources of stem cells for autologous therapy in a neonatal context.
- AF-MSC are a heterogeneous stem cell population of which the exact origin remains elusive.

WHAT DOES THIS STUDY ADD?

- This study identifies AF-MSC with lung-specific characteristics.
- This study provides a reliable culture method that supports the expansion of lung-specific AF-MSC.

2 | METHODS

2.1 | Isolation and standard culture of AF-MSC

The cell harvesting protocol was approved by the Ethics Committee of the University Hospitals Leuven, Belgium (license ML4149). The AF of 42 donors was obtained by amniocentesis at 15 to 26 weeks of gestation from which 212 MSC clones were isolated as previously reported.[15] Briefly, 5-mL AF was passed through a 40-μm cell strainer and centrifuged at 1200 rpm for 5′. The cell pellet was resuspended in standard growth medium consisting of α-MEM supplemented with 15% fetal bovine serum (FBS; Gibco, ThermoFisher Scientific, Ghent, Belgium), 1% L-glutamine (Gibco), 1% penicillin/streptomycin (Gibco), and 18% Chang B and 2% Chang C (Irvine Scientific, Brussels, Belgium), plated in a petri dish and incubated (37°C, 5% CO_2). After ±7 days colonies arising from individual single cells were mechanically removed and plated into a 96-well plate for further clonal expansion. The cells were allowed to reach 70% confluence and routinely split in a 1:5 dilution using 0.25% Trypsin-EDTA.

2.2 | Flow cytometric analysis

AF-MSC from passage 4 were characterized for CD marker expression using FITC or PE-conjugated mouse anti-human antibodies for CD117, CD90, CD73, CD44, CD45, CD29, CD105, CD34, HLA-ABC, and HLA-DR and appropriate isotype controls (Supplementary Table 1). Cells were analyzed using a BD FACSCANTO II (BD Biosciences, San Jose, CA, USA) with the BD FACSDiva™ software, and data were computed using the FlowJo software (Tree Star, Ashland, OR, USA).

2.3 | AF-MSC differentiation

Cells were analyzed for their capacity to differentiate in mesenchymal lineages as previously reported.[15]

2.4 | Immunofluorescent staining

AF-MSC were plated in Lab-Tek chambers (ThermoFisher Scientific) and were fixed at 70% confluence with 1% PFA for 30′ and washed with PBS. Blocking and permeabilization were performed with 10% FBS and 0.1% Triton-X-100 in PBS. Slides were incubated overnight at 4°C with primary goat anti-human FOXA2 (Santa Cruz, Heidelberg, Germany) and rabbit anti-human NKX2.1 (Abcam, Cambridge, UK) antibodies diluted in blocking buffer (w/o Triton; 1:50 and 1:100, respectively). Species-appropriate secondary antibodies were conjugated to AlexaFluor dyes (ThermoFisher Scientific). DAPI nuclear staining was performed prior to cover slipping with Moviol. Images were acquired using a Zeiss LSM700 confocal microscope.

2.5 | qRT-PCR

RNA was isolated from AF-MSC using Tri-Pure (Sigma-Aldrich, Brussels, Belgium) in accordance with the manufacturer's instructions. cDNA was synthesized with Taq Man® Reverse Transcription Reagents (Applied Biosystem, Carlsbad, CA, USA). qRT-PCR was performed on a StepOnePlus Real-Time PCR instrument (ThermoFisher Scientific) using the Platinum® SYBR® Green qPCR Supermix-UDG with ROX (ThermoFisher Scientific) to detect in a first instance the expression of KSP. AF-MSC colonies that lacked the expression of KSP were further characterized for SIX2, PAX2, NKX2.1, FOXA2, SOX2, LGR6, IRX1, P63, PDPN, SFTC, OCT4, and NANOG expression. GAPDH was used as a stable housekeeping gene as identified by the geNorm algorithm.[16] Quantitation relative to housekeeping genes was determined with the comparative Ct method. Primers were synthesized by Integrated DNA Technologies (Haasrode, Belgium). Primer sequences can be found in Supplementary Table 2.

2.6 | Kidney capsule allograft and SFTB staining

Eight-week-old BALB/c-RAG-$2^{-/-}$ $\gamma C^{-/-}$ mice were provided by the animalium at KU Leuven. The experiments were approved by the Ethical Committee for Animal Experimentation (084/2014) and performed according to current guidelines of animal welfare. Mice were anaesthetized with isoflurane (0.5–2%). AF-MSC were dissociated with trypsin to generate a single cell suspension ($5{\cdot}10^5$ in 20–40 μL PBS) that was injected under the kidney capsule as published before.[17] Analgesia was performed with buprenorphine (0.1 mg/kg). Mice were killed 4 weeks later; the kidneys were collected, fixed with 4% PFA, and paraffin-embedded. Samples were sectioned at 4 μm. Following deparaffinization, antigen retrieval was performed in citrate buffer (pH 6; 1 h, 90°C). The slides were then allowed to cool down and were washed prior to incubation with blocking solution (TBS with 1% BSA, 2% nfdm and 0.1% Tween 80) for 1 hour to inhibit non-specific binding of the primary antibody. Primary goat anti-mouse SFTB (Santa Cruz) was diluted 1:75 in TBS and added onto the slides for 2 hours at 37°C. The secondary antibody was biotinylated and combined with an AP-labeled streptavidin (Dako, Leuven, Belgium). They were dissolved in TBS with 4% normal human serum. After washing, the reaction was visualized with nitro blue tetrazolium chloride.

2.7 | AF-MSC culture in Nutristem

AF was processed identically as described earlier. After centrifugation, the pellet was resuspended in Nutristem XF/FF (Miltenyi Biotec, Leiden, The Netherlands) and plated onto Matrigel-coated dishes (BD Biosciences). Colonies arising from individual single cells were mechanically removed and plated into a 96-well plate for further clonal expansion. The cells were allowed to reach 70% confluence and then routinely split in a 1:5 dilution using dispase (1 U/mL; Stemcell Technologies, Grenoble, France).

2.8 | Lung extracellular matrix (ECM) production and AF-MSC culture on lung ECM-coated plates

Time-mated pregnant rabbits (New Zealand White and Dendermonde hybrid) were provided by the animalium at KU Leuven. The experiments were approved by the Ethical Committee for Animal Experimentation and performed according to current guidelines of animal welfare. Pups were delivered by C-section at 28 days of gestation and euthanized by intracardiac injection of a solution of embutramide 200 mg, mebezonium 50 mg, and tetracaine hydrochloride 5 mg (T61®, Hoechst GmbH, Munich, Germany). Lungs were excised and perfusion decellularized by tracheal cannulation. Lungs were perfused at 30 mmHg sequentially with heparinized PBS for 15′, 0.1% SDS in deionized water (DIW) for 3 hours, DIW for 1 hour, 1% Triton-X-100 in DIW for 1 hour, DIW for 72 hours, PBS containing 100 U/mL penicillin G, and 100 U/mL streptomycin and amphotericin B for 1 hour (protocol adapted from Ott et al[18]). A sample of decellularized matrix was used to analyze the remaining DNA content of decellularization by DNA quantification using Quant-iT™ dsDNA Assay Kit (ThermoFisher Scientific) and by hematoxylin and eosin staining of the paraffin embedded tissue. The rest of the matrix was minced into pieces of 1 mm^3, concentrated with Savant™ SpeedVac™ (ThermoFisher Scientific), frozen to −80°C, and manually milled to create a powder. The powder was solubilized as previously described.[19] Briefly, approximately 50 mg of the ECM was digested in 5 mL of pepsin (1 mg/mL in 0.1 M HCl) under constant shaking for 48 hours. Acetic acid (0.1 M) was added to dilute the ECM to a concentration of 1 mg/mL, and this solution was used to coat tissue culture plates with 20 ng/cm^2 for 1 hour at 37°C, followed by rinsing with PBS. AF-MSC were cultured as described earlier, where uncoated plates were replaced by ECM-coated plates from p1 onwards. A graphical display of the production of fetal lung ECM-coated dishes for cell culture is shown in Supplementary Figure A.

2.9 | Chromosomal microarray analysis

Clonal AF-MSC were divided for extended culture under separate conditions: either Nutristem/hM or Chang/lung ECM, and standard culture. Cells were trypsinized and centrifuged, and the pellets were washed twice in PBS. Genomic DNA was extracted using the DNA mini kit (QIAGEN, Venlo, The Netherlands) following the manufacturer's recommendations. Array analysis was performed using the 8 × 60 K CytoSure ISCA v3 microarray (Oxford Gene Technology, OGT, Oxford, UK). Genomic DNA was labeled with Cy5 for 2 hours using the CytoSure Labelling Kit (Oxford Gene Technology) and hybridized versus Cy3-labeled sex-matched reference DNA. Hybridization was performed for minimum 16 hours in a rotator oven (SciGene, CA, USA) at 65°C. Washing of arrays was performed using Agilent wash solutions with a Little Dipper Microarray Processor (SciGene). Arrays were scanned using an Agilent microarray scanner at 2-μm resolution, followed by calculation of signal intensities using Feature Extraction software (Agilent Technologies). Visualization of results and data analysis were performed using the CytoSure Interpret Software (Oxford Gene Technology) and the circular binary segmentation algorithm. Quality control metrics are monitored with CytoSure Interpret software (Oxford Gene Technology).

2.10 | Statistical analysis

Normal distribution of the data was verified with D'Agostino and Pearson omnibus normality test. Significance was determined using the Mann-Whitney test. A *P*-value less than 0.05 was considered as significant. Data are represented as mean ± SEM.

3 | RESULTS

3.1 | Isolation and characterization of amniotic fluid derived mesenchymal stem cells

Plastic-adherent clonal AF-MSC with a spindle-shaped morphology were successfully isolated and expanded in standard culture conditions (Figure 1A). Cell surface antigen expression was analyzed by flow cytometry (Figure 1B). The AF-MSC colonies were negative for hematopoietic markers CD34, CD45, and Class I major histocompatibility cell surface receptor HLA-DR. They were positive for the mesenchymal markers CD105, CD73, and CD90, for the adhesion molecules CD44 and CD29, and for the Class I major histocompatibility antigens

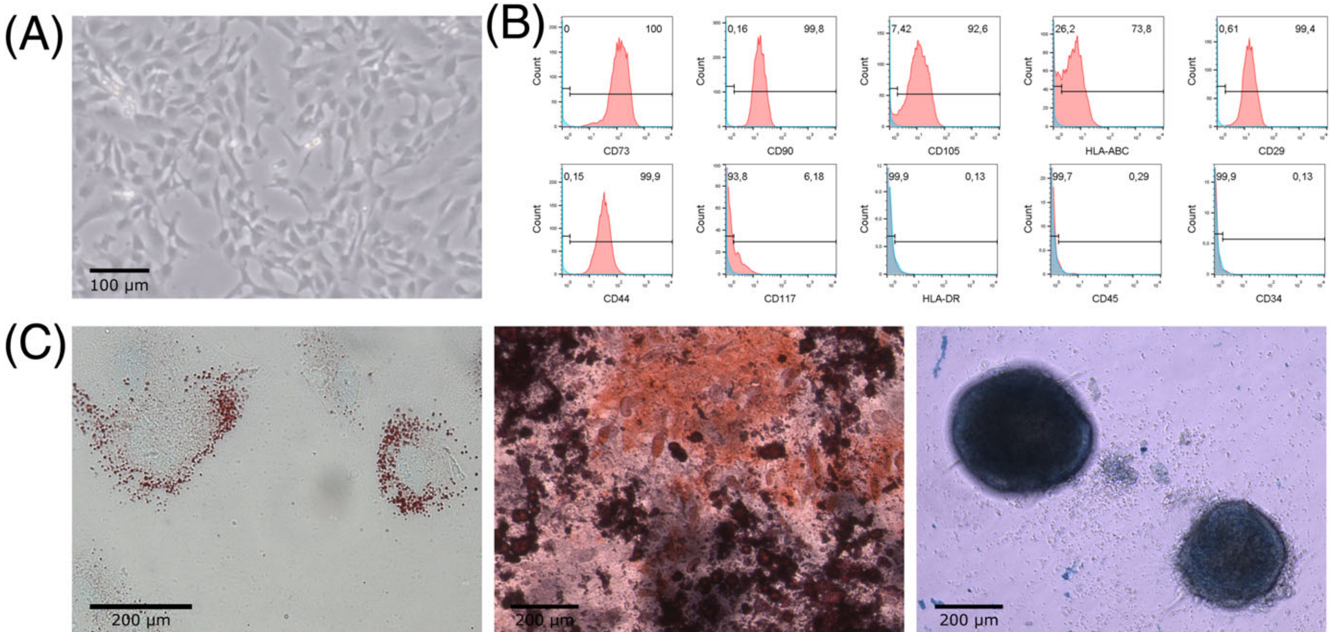

FIGURE 1 Mesenchymal stem cells from the amniotic fluid isolated by mechanical selection 353. (A) Plastic-adherent AF-MSC with a typical spindle-shaped morphology; (B) expression pattern 354 of AF-MSC as determined by flow cytometry; (C) AF-MSC differentiate into adipogenic (Oil red 355 staining; left), osteogenic (Alizarin staining; middle), and chondrogenic cells (Alcian blue; right)

(HLA-ABC). Stem cell factor (c-kit; CD117) expression was very low. Stemness characteristics of different AF-MSC colonies were proven by differentiation into adipogenic, osteogenic, and chondrogenic lineages (Figure 1C). For the adipogenic differentiation, the lipid vacuoles stained by Oil Red are not on the large size. In addition, the adipogenic markers PPAR-γ and FABP4 had undetectable mRNA expression levels as was analyzed by qRT-PCR. As such—and in accordance with previous reports—these cells resemble more the pre-adipocyte phenotype rather than the mature adipocyte with large confluent vacuoles.[3-6] It is unclear if this is a unique feature of these cell lines or if it is secondary to the culture conditions.

3.2 | A subpopulation of clonal AF-MSC shows lung-specific characteristics

It has been shown previously that MSC with renal characteristics are present in the AF.[7] Indeed, 61% of the isolated AF-MSC colonies (n = 212) expressed the renal marker KSP (Figure 2A) and the renal progenitor markers SIX2 and PAX2 (these cells will be abbreviated as KSP + AF-MSC below). When analyzing the AF-MSC colonies that were negative for kidney-specific markers, we identified 66% (ie, 26% of the total amount of analyzed colonies) that expressed lung embryonic markers FOXA2 and NKX2.1 (Figure 2A–C, Supplementary Figure 1A). In addition, these colonies expressed SOX2 and IRX1 (embryonic lung), P63 (basal lung stem cells), LGR6 (distal lung stem cells), SFTC (alveolar type 2 cells), and PDPN (alveolar type 1 cells) (Supplementary Figure 1C). This lung marker expression was absent in KSP+ AF-MSC colonies (Figure 2B). In vivo transplantation of cells expressing lung-specific markers under the kidney capsule of immunodeficient mice resulted in the growth of a cell collection, while transplantation of KSP+ AF-MSC did not. This cell collection was highly positive for SFTB (Supplementary Figure 1B). When AF-MSC were expanded for 10 passages on uncoated tissue culture plastic dishes in Chang-supplemented growth medium (ie, standard culture conditions for AF-MSC), a significant loss of the lung-specific marker expression was observed (Figure 2D, Supplementary Figure 1D).

3.3 | Alternative culture methods to support AF-MSC with lung characteristics

The culture of AF-MSC expressing FOXA2 and NKX2.1 in Nutristem on human Matrigel-coated dishes (Nutristem/hM) resulted in the retention of lung endodermal marker expression. In addition, these cells activated the expression of the pluripotency genes OCT4, NANOG, and SOX2 (Figure 3A). ArrayCGH analysis confirmed that Nutristem/hM culture of AF-MSC did not lead to significant chromosomal abnormalities as compared with the standard culture method (Supplementary Figure 2A). However, these cells ceased proliferation by passage 6.

In order to mimic the in vivo extracellular milieu of the lung, we coated tissue culture plastic dishes with lung ECM proteins derived from fetal rabbit pups. The lung ECM proteins were obtained from decellularized lung tissue that had no visible nuclear material left (Supplementary Figure 3B) and with a remaining DNA content of 9.6809 ± 0.0711 ng/mg dry weight. When FOXA2/NKX2.1-expressing AF-MSC were cultured on lung ECM-coated dishes in Chang-supplemented growth medium (Chang/lung ECM), they retained their lung-specific marker expression (Figure 3B, Supplementary Figure 3C) and did not cease proliferation before passage 15. Also, this novel culture method of AF-MSC did not lead to significant chromosomal abnormalities as compared with the standard culture method, as was analyzed by array CGH.

FIGURE 2 A subpopulation of clonal AF-MSC expresses lung endodermal markers FOXA2 and 358 NKX2.1 359. (A) mRNA expression analysis of 212 AF-MSC clones for the renal marker KSP and the lung 360 endodermal marker FOXA2 and NKX2.1; (B) relative mRNA expression analysis of FOXA2 and 361 NKX2.1 in KSP-positive versus KSP-negative AF-MSC clones (n = 12 for both groups); (C) 362 immunofluorescent staining of one representative clone; (D) relative mRNA expression 363 analysis of FOXA2 and NKX2.1 at passage 4 and 10 (n = 12 for both groups). Data: mean ± SEM. 364 Scale bars correspond to 20 μm

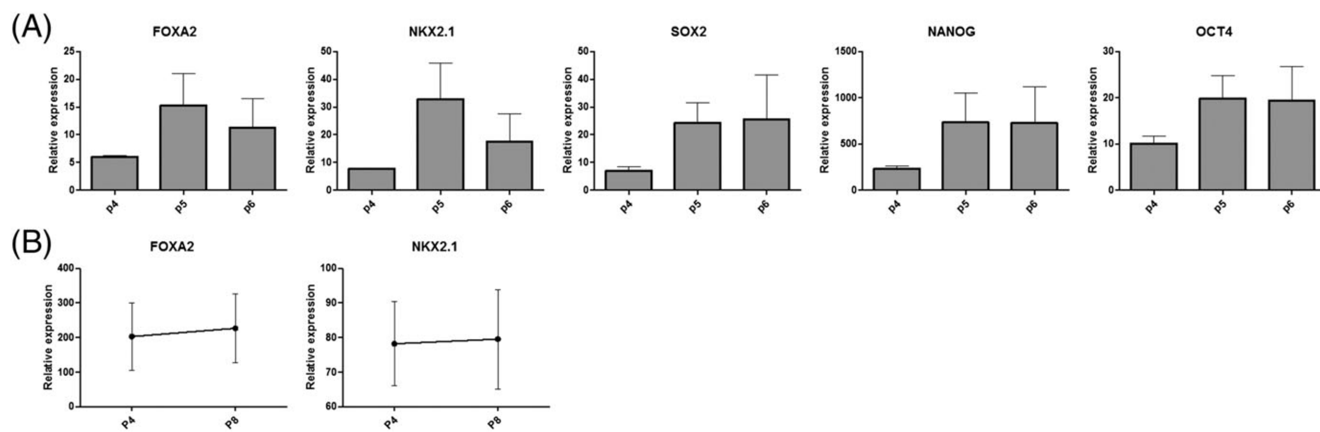

FIGURE 3 Alternative culture methods to support AF-MSC with lung characteristics 367. (A) Relative mRNA expression of lung endodermal and pluripotency markers of AF-MSC 368 cultured on human Matrigel in Nutristem medium at passage 4–6 (n = 12); (B) relative mRNA 369 expression of FOXA2 and NKX2.1 of AF-MSC cultured on lung ECM-coated dishes in Chang 370 medium at passage 4 and 8 (n = 12). Data: mean ± SEM

4 | DISCUSSION

Fetal tissues, and in particular the AF, are promising sources of stem cells for regenerative medicine purposes, both in an autologous or allogenic setting. AF-MSC are a heterogeneous population containing cells that originate from the developing fetal tissues (the urinary, respiratory, and gastrointestinal tract, or even the skin) and from the amniotic membranes.[20] Several factors will influence the exact composition of the AF-MSC population and hence their potency for downstream in vivo applications. Firstly, the gestational age—and thus the stadium of organ development and cellular maturation—affects the composition of the AF-MSC pool. AF-MSC collected early in gestation have a higher expression of generic mesodermal and endodermal markers, while samples collected later in gestation express more organ-specific markers.[21] Secondly, depending on the isolation procedure, different stem cell populations will be retrieved from the AF. These procedures are mostly based on plastic adherence and expansion in serum-rich conditions of unselected populations or on immuno-selection for the CD marker CD117. The first method results in the culture of AF-MSC with a clear mesenchymal phenotype, while for the latter method cells with a broadly multipotent differentiation potential are retrieved. Thirdly, a wide range of media for the culture of AF-MSC has been described, including Amniomax and Chang's medium, both specifically produced for the culture of AF-MSC. Chang's medium allows the expansion of AF-MSC up to 50 passages with the retention of cellular morphology.[21]

In this research, we isolated AF-MSC from clinical amniocentesis samples by plastic adherence and subsequent mechanical selection using the starter cell method.[15,22] This allows the clonal expansion of pure stem cell populations derived from 1 single cell within a short period of time (as opposed to the CD117 immuno-selection method). In addition, it enables the in vitro characterization of the clonal origin of organ-specific AF-MSC and efficient in vivo preclinical screening of their potency in selected disease models. If an organ-specific cell type would thus be characterized and be found to express a specific set of markers, then a tailored immuno-selection method could be devised for the purpose of selective isolation.

Out of all the individual colonies isolated from AF samples obtained between 15 and 26 weeks of gestation, 61% were positive for KSP and the renal progenitor markers SIX2 and PAX2. This can be expected as a large part of the AF consists of fetal urine and is in line with our previous studies where we report on the isolation and therapeutic potential of AF-MSC with renal characteristics.[7,20] At this stage of pregnancy, the continuous breathing movements of the fetus ensure that a large part of the amniotic fluid circulates in and out of the lungs and is supplemented with fluid that is produced by the pulmonary tissue itself. Therefore, we hypothesized that also cells with pulmonary origin could be retrieved from the amniotic fluid. We found that 26% of all clonal AF-MSC express the lung embryonic markers NKX2.1 and FOXA2, as well as other lung-specific markers. We focused on these 2 transcription factors as both NKX2.1 and FOXA2 are indispensable for embryonic lung development and postnatal lung homeostasis.[14] Interestingly, cell lines with a high expression of early embryonic markers (FOXA2, NKX2.1, SOX2) could be isolated, but also cell lines with a low expression of embryonic markers and a high expression of more mature lung markers such as PDPN and SFTC. This could suggest that cells may originate from the fetal lung at different maturation states (progenitor or differentiated cells), from different areas of the organs (conducting airways or deeper parenchyma), and at different gestational ages (when the actual AF samples were retrieved). To test the ability of these cells to differentiate in vivo, together with their proliferation potential, we performed kidney capsule engraftments in immunodeficient mice.[23] Transplantation of FOXA2/NKX2.1-expressing AF-MSC to this niche environment resulted in the formation of a cellular mass highly positive for the alveolar type 2 marker SFTB. Possibly, due to the short incubation period (4 weeks instead of 8 weeks), we could not distinguish a pulmonary micro-architecture or morphologic populations reminiscent1 of epithelial type 1/2 cells, goblet cells, or ciliated cells.[24]

Any translation of AF-MSC therapy towards clinical application necessitates a level of in vitro expansion that extends beyond a few passages in culture. AF-MSC have a high proliferative potential; however, upon extended culture, FOXA2/NKX2.1-expressing AF-MSC decreased their lung-specific marker expression. This prompted us to explore alternative culture methods. Initially, we tested different commercially available culture media produced to support pluripotency (eg, E8) or lung-specific characteristics (eg, bronch-epithelial growth medium). None of these methods enabled the long-term proliferation of FOXA2/NKX2.1-expressing AF-MSC. Culturing them in the pluripotency medium Nutristem XF/FF on Matrigel-coated dishes allowed their expansion up to 6 passages. Interestingly, all of the cells cultured in these conditions had an upregulated expression of pluripotency proteins, including OCT-4, SOX2, and NANOG. Expression of these markers in conventional AF-MSC conditions is usually low. However, culture of AF-MSC in Nutristem/hM up to passage 6 results in a total amount of 5 to $25 \cdot 10^6$ cells, which is still relatively low for clinical applications.

In order to mimic the extracellular microenvironment of the developing lung, we used fetal lung ECM protein-coated dishes as a growth platform for FOXA2/NKX2.1-expressing AF-MSC. As the ECM plays an important role during organ development and influences cell growth and survival, we hypothesized that these ECM cues might be fundamental to support the growth of AF-MSC with lung characteristics.[19,25] Fetal lung ECM-coated dishes proved to be a suitable platform for the culture of FOXA2/NKX2.1-expressing cells with the maintenance of the expression of both embryonic and more mature lung markers up to 15 passages.

These data suggest that FOXA2/NKX2.1-expressing AF-MSC with lung-specific characteristics can be retrieved from the AF and that their culture is supported by combining Chang-supplemented medium with fetal lung ECM-coated dishes. However, a number of limitations of this research have to be recognized. Firstly, the characterization of clonal AF-MSC in this study largely depends on marker expression analysis. This can give an indication about the developmental origin of the cells; however, their true potency can only be established by in vitro differentiation assays, lung ECM recellularization assays, in vivo transplantation studies (with engraftment of the used cells in functional lung tissue), or chimera formation. Secondly, the extraction of ECM from lung tissue is associated with batch to batch variation, and the nature of remaining contaminants is mostly unknown. Also, the use of xenogenic compounds for cell culture is not compatible with clinical translation.

In conclusion, the AF is a source of clonal AF-MSC with lung characteristics which are supported by culturing them on lung ECM-coated dishes. However, further investigations into their in vitro pulmonary differentiation capacity and therapeutic potential in animal models for perinatal lung diseases is required.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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