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Human amniotic fluid stem cells attract osteoprogenitor cells in bone healing

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Abstract

Current treatments of large bone defects are based on autologous or allogenic bone transplantation. Human amniotic fluid stem cells (hAFSCs) were evaluated for their potential in bone regenerative medicine. In this study, hAFSCs were transduced with lentiviral vector harboring red fluorescent protein to investigate their role in the regeneration of critical-size bone defects in calvarial mouse model. To distinguish donor versus recipient cells, a transgenic mouse model carrying GFP fluorescent reporter was used as recipient to follow the fate of hAFSCs transplanted in vivo into Healos® scaffold. Our results showed that transduced hAFSCs can be tracked in vivo directly at the site of transplantation. The presence of GFP positive cells in the scaffold at 3 and 6 weeks after transplantation indicates that donor hAFSCs can recruit host cells during the repair process. These observations help clarify the role of hAFSCs in bone tissue repair.

Keywords

bone; mice; transgenic; regenerative medicine; stem cell transplantation; transgenic

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AUTHOR CONTRIBUTIONS

M. B. contributed to the design of the study, performed the experiments, analyzed data, and drafted the manuscript. F. M. obtained preliminary results. S. N. performed mice surgery, FACS analysis, and helped to draft the manuscript. I. K. designed the experiments, discussed results and implications, and commented on the manuscript over the stages. R. D. P. conceived the idea, discussed results, and critically revised the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also form part of an ongoing study.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

1 | INTRODUCTION

Currently, large bone defect treatments are based on autologous or allogenic bone grafts that still have limitations. Bone tissue engineering with cell transplantation could provide an alternative way to stimulate bone repair. In the past few years, numerous studies have been conducted to investigate the osteogenic potential of human amniotic fluid-derived stem cells (hAFSCs) in the repair of bone defects. hAFSCs can be also considered as a new class of pluripotent stem cells, representing an intermediate stage between embryonic and adult stem cells, due to their differences in both surface markers and in differentiation ability (De Coppi et al., 2007). In fact, hAFSCs are capable of differentiation toward multiple lineages of all three embryonic germ layers (Cananzi & De Coppi, 2012). Furthermore, the absence of ethic controversy and risk of teratoma formation (De Coppi et al., 2007) and the fact that these cells can be cultivated for long periods without significant alteration in their biological features compared to bone marrow stromal cells (BMSCs; Alessio et al., 2018) make hAFSCs particularly interesting in the field of regenerative medicine. Previous studies demonstrated the osteogenic differentiation of hAFSCs in vitro when cultured on 2D surface and in the presence of osteogenic medium (Maraldi, Riccio, et al., 2011). hAFSCs produce robust mineralization if treated in culture with dexamethasone, ascorbic acid and β -glycerophosphate (De Coppi et al., 2007; Peister, Deutsch, Kolambkar, Hutmacher, & Guldberg, 2009). Considering this, recent studies investigated the behavior of hAFSCs in vivo after their osteogenic commitment in vitro. Maraldi, Riccio, et al. (2011) demonstrated that mineralization occurs when hAFSCs are seeded in fibroin scaffold, cultured 1 week in osteogenic medium and implanted in a rat subcutaneous model. Other authors indicated that hAFSCs, once transplanted in vivo, can recruit host/progenitor cells to the lesion, suggesting a possible role of their releasing soluble factors in tissue repair (Mirabella et al., 2011). These studies indicate the importance of distinction of the donor versus recipient cells. Although hAFSCs might show limited osteogenicity in vivo, they can trigger a strong proangiogenic response (Mirabella, Cilli, Carlone, Cancedda, & Gentili, 2011; Mirabella, Gentili, Daga, & Cancedda, 2013). Balbi et al. (2016) also provided a description of the paracrine potential of hAFSCs-extracellular vesicles (EVs) through the horizontal transfer of specific miRNAs (Balbi et al., 2016), suggesting a role of hAFSCs secretome in tissue repair. In spite of these findings, at present there are no studies highlighting the potential chemotactic properties of hAFSCs seeded in collagen-hydroxyapatite scaffolds and transplanted in calvarial defect model. Thus, the aim of this study was to investigate the role of hAFSCs in the regeneration of critical-size large bone defects. For this purpose, we transduced hAFSCs with red fluorescent protein (RFP-cherry) and used a transgenic mouse model carrying green fluorescent reporter (GFP-topaz). This approach allowed us to track the fate of hAFSCs transplanted in vivo into Healos® scaffold construct and to distinguish donor and host cells at the implant site.

2 | MATERIALS AND METHODS

2.1 | Cell cultures and conditioned medium

2.1.1 | Human amniotic fluid stem cell—Patient amniotic fluid (7 ml) was collected from 17-week pregnant women and kept at 4°C for 24 hr. Cells were pelleted by

centrifugation at 290g for 15 min at room temperature, then resuspended in 10 ml hAFSCs culture medium (Life Technology). Cells were cultured in 10-cm tissue culture dishes at 37°C in a humidified atmosphere of 5% CO₂. The medium was changed every other day for 7 days up to a cell density of 80–90%. Cells were harvested by means of Accutase (Innovative Cell Technologies, Inc.) digestion at 37°C for 5 min, and after expansion cryopreserved in “Banbanker” cell freezing medium (Wako Pure Chemical Ind., Ltd.). hAFSCs were cultured in AmnioMAX-II (Gibco). After viral transduction, cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/L glucose (Lonza), 10% fetal bovine serum (FBS; Atlanta Biologicals), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco) (complete medium) supplemented with 5 ng/ml fibroblast growth factor (FGF; Invitrogen). Adherent cells were detached from the plastic plate using a trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco). hAFSCs at 70% of confluence were used for the preparation of conditioned medium (hAFSCs CM) for in vitro studies. Fresh DMEM containing 4.5 g/L glucose (Lonza) was changed 48 hr before collection of CM, each ml of which being obtained from 350,000 hAFSCs.

Human AFSCs were plated at a density of 3,000 cells/cm². The passage used for the experiments was the 10th, since it is largely reported in literature that these cells maintain their self-renewal capacity up to late passages.

2.1.2 | Human mesenchymal stromal cells—Human MSCs (derived from human bone marrow) were purchased from Millipore®. Routine characterization of hMSCs includes testing for surface antigen expression and functional testing for in vitro differentiation into adipogenic, chondrogenic, and osteogenic lineages, as indicated by the manufacturer. The cells were grown in complete DMEM with 4.5 g/L glucose (Lonza). Adherent cells were harvested in 0.25% trypsin-EDTA (Gibco). Human MSCs were plated at a density of 5,000 cells/cm². The sixth passage was used for the experiments.

2.1.3 | Primary human fibroblasts—Human Fibroblasts (hFbs) were obtained from 4-year-old normal male subjects, were expanded in DMEM with 4.5 g/L glucose (Life Technology) and 10% FBS (Hyclone) and harvested in 0.125% trypsin-EDTA (Gibco). Primary human fibroblasts were plated at a density of 3,000 cells/cm². The 6th passage was used for the experiments.

2.1.4 | Mouse bone marrow stromal cells—Mouse BMSCs were prepared from 8- to 10-week old mice. Bone marrow was flushed from the femora and tibiae and cells were seeded at a density of $4 \times 10^5/\text{cm}^2$ and expanded in minimum essential medium α (α MEM) (Gibco), 10% FBS (Atlanta Biologicals) plus 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) (complete medium). Once plated, BMSCs were left for 4 days, then half medium was changed. At day 7 in culture, cells were harvested in 2.5% trypsin-EDTA (Gibco) and used for experiments (second passage). All cultures were kept at 37°C in a humidified atmosphere of 5% CO₂.

2.2 | Lentivirus preparation

Lentiviral vector FUG W Ub cherry, which utilized ubiquitin promoter-driven cherry expression, was previously used (Xin et al., 2019). Briefly, the virus was assembled in hEK293 FT cells with three helper vectors encoding virus elements of REV, RRE, and VSV (G). hEK293 FT cells were cultured to 90% density and replated onto 100-mm dish with 0.05% trypsin/EDTA (Gibco) treatment before transfection. For each 100-mm dish transfection, 10 µg DNA (4.5 µg backbone vector, 1.5 µg of VSV(G), 3.5 µg of RRE, and 1 µg of REV) and 30 µl of Lipofectamine 2000 (Life Technology) were separately diluted in 2.5 ml of optimum medium (Life Technology), respectively. Diluted DNA and lipofectamine solutions were mixed before applying to the cells as transfection solution. Cells were harvested and resuspended in the transfection solution. After incubating in culture incubator for 30 min, cell suspension was transferred to 100-mm cell culture dish, and cultured overnight. Medium was changed into DMEM with 4.5 g/L glucose (Lonza) and 10% FBS (heat inactivated). Viral medium was harvested after 48 and 72 hr of transfection and the virus was concentrated through centrifugation at 200,000 rpm for 90 min. Supernatant was discarded, viral pellet was gently resuspended in serum-free DMEM basal medium. Aliquots of the concentrated virus were kept in -70°C freezer for use.

2.2.1 | hAFSC viral transduction—For viral transduction of hAFSCs with Ub-mCherry virus, cells at 70% of confluence and passage 2 were rinsed once with phosphate buffered saline (PBS) 1× (Lonza) and detached from the plate (Accutase®; Innovative Cell Technologies, Inc.). The pellet was suspended in DMEM with 4.5 g/L glucose (Lonza) and the cell suspension was incubated with Ub-mCherry virus (20 µl/ml) and polybrene (1 µl/ml) for 1 hr at 37°C within a 5% CO₂ atmosphere. After the first incubation, DMEM with 4.5 g/L glucose (Lonza) and polybrene (1 µl/ml) were added and the cell suspension was plated in a plastic dish and kept for 3 hr under the previous conditions. Then, new medium was added and the cells were maintained for 48 hr at 37°C in a humidified atmosphere containing 5% CO₂. The lentiviral expression in transduced hAFSCs was checked and analyzed with fluorescence microscopy using Zeiss Observer Z.1 inverted microscope to capture both red fluorescence and bright-field images. Cell sorting was performed using a FACS Aria II (BD Biosciences). Voltages and gates were set based on unstained sample from untransduced hAFSCs. FACS Diva 8 (BD Biosciences) software was used for cell analysis. Human AF cherry positive cells were then plated and expanded in complete DMEM with 4.5 g/L glucose (Lonza) added with FGF (Invitrogen) (5 ng/ml) for further experiments.

2.3 | Osteogenic precommitment of hAFSCs

hAFSCs were precommitted for in vivo and in vitro osteogenic differentiation studies. For osteogenic differentiation, cells were cultured for 7 days in complete αMEM supplemented with ascorbic acid (2 µl/ml) (Sigma) to stimulate extracellular matrix (ECM) synthesis. Medium was changed every other day. Flow cytometry (FACS Aria II, BD Biosciences) was used to determine expression of osteogenic markers. Reverse transcription polymerase chain reaction (RT-PCR) was performed to analyze the expression of osteogenic and stemness markers.

2.4 | Flow cytometry analysis of uncommitted and precommitted hAFSCs

Flow cytometry analysis was performed to detect the expression of CD90, CD73, CD166, CD44, and CD34 markers on the surface of uncommitted and precommitted hAFSCs to study the expression of mesenchymal stem cell markers. The cells were harvested after treatment with 0.2% collagenase P and 0.2% hyaluronidase in 1× PBS, and were washed and collected. Cells were then labeled with allophycocyanin-conjugated anti-human CD90 (Thy1) antibody (clone: 5E10; Biolegend), PE-conjugated anti-human CD73 antibody (clone: AD2; BD Pharmingen), PerCP-eFluor 710-conjugated anti-human CD166 antibody (clone: 3A6; eBioscience), Pacific Blue-conjugated anti-human CD44 antibody (clone: IM7; eBioscience) and fluorescein isothiocyanate-conjugated anti-human CD34 antibody (clone: 581; BD Pharmingen). The analysis was performed using FACS-LSR II (BD Biosciences). Voltages and gates were set based on unstained samples from untransduced cells and single stain controls. FACS Diva 8 (BD Biosciences) or FlowLogic (Inivai Technologies) software were used for cell analysis.

2.5 | Gene expression analysis of precommitted hAFSCs

RT-PCR analysis was carried out to study the expression of osterix (OSX), osteocalcin (OC), dentin matrix acidic phosphoprotein (DMP) and bone sialoprotein (BSP) osteogenic markers. Gene expression analysis of Nanog, Oct4, Sox2, and Rex1 was also performed. Precommitted hAFSCs were lysed with TRIzol reagent (Thermo Fisher Scientific), and RNA was extracted following the manufacturer's instructions. One microgram RNA was treated with DNase (Invitrogen, Carlsbad, CA), cDNA generated using the ImProm-II Reverse Transcription kit (Promega, San Luis Obispo, CA) and RT-PCR was carried out using SYBR green-based assay (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA; Life Technologies, Grand Island, NY). Uncommitted hAFSCs were used as control.

2.6 | Cell migration assay

Chemoattractant properties of hAFSCs were investigated using a six-well plate and a transwell system made by 8- μ m pore size Falcon® Cell Culture Inserts. Human AFSCs, hMSCs, mBMSCs, hFB, were cultured in complete DMEM with 4.5 g/L glucose (Lonza) in the bottom chamber to assay their chemoattractant properties. Control wells contained complete medium or hAFSCs CM. Fresh medium was added to each cell type 48 hr before the experiment. Human AFSCs were washed three times with PBS to remove FGF. Mouse BMSCs (obtained as previously described) were resuspended in serum-free DMEM with 4.5 g/L glucose, transferred onto the filter of the insert (2.5×10^5 cells/ml), and incubated at 37°C in 5% CO₂ for 24 hr. Nonmigratory cells were carefully scraped off with cotton swabs from the upper side of the filter. Migratory cells at the bottom of the membrane were fixed with 3.7% formalin, soaked with PBS, permeabilized with 100% methanol, stained with crystal violet, and counted under a light microscope with $\times 100$ magnification. Five randomly selected regions/well were quantified and the average number of migrated cells was considered. Three replicates for each group were performed.

2.7 | In vivo cell transplantation

2.7.1 | Transgenic mouse model—For this study, a transgenic immunodeficient mouse model was used in which the 3.6-kb fragment of collagen type I promoter directs the expression of fluorescent marker (green variant) to osteoblast lineage cells (Kalajzic et al., 2002; Villa, Wang, Huang, Rowe, & Wei, 2013). Immunodeficient NOD.Cg-PrkdcscidIl2rgtm1wj1/SzJ mice (NSG, The Jackson Laboratory, Bar Harbor, Maine) were purchased and maintained in our animal facility. To generate Col3.6GFPtpz reporter NSG mice, CD1-Col3.6GFPtpz mice were crossed to NSG mice and then backcrossed with NSG for 10 generations.

2.7.2 | Calvarial defect model—All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Connecticut Health Center. To evaluate the effects of hAFSCs in the reconstruction of large cranial bone defects, female immunodeficient mice NSG3.6tpz between 12 and 14 weeks aged were used as hosts.

A total of 4 animals were used for each experiment ($n = 4/\text{experiment}$). Each animal received 2 implants. The following repair procedures for bone defects were performed in 4 separate experiments: (a) implantation of Healos® scaffolds seeded with precommitted hAFSCs (1×10^6 cells each scaffold), (b) implantation of Healos® scaffolds seeded with hMSCs (1×10^6 cells each scaffold), (c) implantation of Healos® scaffolds seeded with mBMSCs (1×10^6 cells each scaffold), (d) implantation of Healos® scaffold alone as control. Mice were anesthetized with a combination of ketamine (135 mg/kg) and xylazine (15 mg/kg) administered with I.P. injection. A midline skin incision was made to expose the cranium and two symmetric full-thickness 3.5 mm critical size-defects were created on the parietal region using a drill. The underlying dura mater was not damaged. Healos® discs were used to load the cells to transplant and the constructs were then implanted in each surgical site to fill the entire defect area. After surgery, the incision was closed with resorbable sutures, and analgesics were administered to the animals. After 3 and 6 weeks of implantation, the animals were euthanized using carbon dioxide asphyxiation and the calvarial bone samples were rapidly collected and fixed in 4% paraformaldehyde in PBS for 2 days for further analysis.

2.7.3 | Radiological analysis—Digital radiographic images of the calvarial samples were obtained using an X-ray cabinet (Faxitron LX-60). The images were captured at $\times 43$ magnification (3 s at 26 kV).

2.8 | Histological evaluations

Fixed samples were decalcified in 14% ethylenediaminetetraacetic acid (Sigma Aldrich) for 2 days and then transferred to 30% sucrose solution in PBS (pH 7.4) for 24 hr. The samples were embedded in Shandon Cryomatrix™ placed in cryomolds by flash freezing on dry ice. Leica CM3050S cryostat (Leica, Wetzlar) was used to obtain 7- μm thick sections of the embedded tissues using the tape transfer technique (Cryofilm type IIC (10), Section-Lab Co. Ltd.). The sections were stained with 4',6-diamidino-2-phenylindole and fluorescent images were captured using fluorescent inverted microscope (Zeiss Oberver Z.1) to detect mCherry

signal (red) from donor cells (precommitted hAFSCs) and to detect GFP signal from host cells (green). Routine hematoxylin/eosin (H&E) as well as Masson trichrome staining was also performed on serial frozen sections to analyze morphological details. Light microscopy images were obtained using a Zeiss Axioskop 40 (Carl Zeiss) light microscope, equipped with a Coolsnap videocamera (Photometrics). MetaMorph Software System (Universal Imaging Inc., Bedford Hills, NY; Molecular Devices Sunnyvale, CA; Crisel Instruments, Rome, Italy) was used to acquire, visualize, and analyze digital images.

2.9 | Statistical analysis

Results of in vitro experiments are presented as mean $\pm$ standard deviation. Statistically significant differences were determined by one-way analysis of variance. Statistical significance was accepted at $p < .05$.

3 | RESULTS

3.1 | Transduction efficiency of hAFSCs

We transduced hAFSCs with Ub-mCherry lentivirus to obtain a stable cell-labeling. High level of transduction was detected with fluorescence microscopy (Figure 1a). Transduced hAFSCs were then gated and sorted with FACS Aria (BD Biosciences). We successfully obtained 96% cherry positive fluorescent cells in the total population under investigation (Figure 1b,c). The majority of the red fluorescent cells was within the log scale of 10^4 – 10^5 as defined population of positive cells. This approach allowed us tracing the donor cells once they were transplanted in vivo.

3.2 | Phenotypic characterization of uncommitted and precommitted hAFSCs

Flow cytometry was used to characterize the expression of mesenchymal stem cells-like surface markers (CD90, CD73, CD44, and CD166) of precommitted hAFSCs compared to uncommitted hAFSCs. As shown in Figure 2, precommitted hAFSCs showed a decrease in the expression of early mesenchymal markers CD90 and CD73 compared to uncommitted hAFSCs, suggesting a partial commitment of precommitted hAFSCs following treatment with osteogenic medium containing ascorbic acid. We did not observe any expression of CD34 (data not shown). In addition, no expression of osteogenic markers like OSX, OC, DMP, and BSP was detected with RT-PCR after 1 week of exposure to ascorbic acid (data not shown). As well, no changes in the expression of Nanog, Oct4, Sox2, Rex1 after 10 days in culture in the presence of ascorbic acid were detected (data not shown).

3.3 | In vivo transplantation of hAFSCs attract host cells in the calvarial bone defect

To follow the fate of hAFSCs transplanted in vivo, we transduced the cells with a lentiviral vector to obtain a stable labeling with cherry red fluorescent protein. Transduced hAFSCs were then exposed to osteogenic medium supplemented with ascorbic acid for 7 days to have a cell population partially committed to osteogenic lineage. Precommitted cherry positive hAFSCs were transferred into Healos® scaffolds, transplanted to calvarial bone defect and the ability for new bone deposition in a critical-size bone defect was determined. The NSG/Col3.6tpz transgenic reporter mouse was used to track and distinguish host cells versus implanted cherry positive hAFSCs (red). In this study, mice were killed at 3 and 6

weeks after the procedure and the presence of the host and donor cells in the defect was determined. We used frozen sectioning as a gold standard for GFP visualization. As shown in Figure 3, hAFSCs promote the attraction of host cells in the defect compared to mBMSCs at 3 weeks after surgery, as demonstrated by the presence of numerous green cells in the scaffold seeded with hAFSCs compared to the construct carrying mBMSCs (Figure 3, GFP positive cells indicated by the arrows).

Three weeks after transplantation cherry protein is still present in the scaffold, as highlighted by the red spots retrieved in the construct (Figure S1). Six weeks after surgery hAFSCs can still recruit recipient mouse cells in the defect compared to mBMSCs (Figure 4, indicated by arrows). X-ray images showed more mineralized tissue in the constructs implanted with mBMSCs compared to hAFSCs after 3 and 6 weeks. This finding was confirmed by H&E as well as Masson trichrome staining performed on serial frozen sections. After 3 weeks, the presence of fibrotic tissue was detected in both mice transplanted with hAFSCs and mBMSCs (Figure 5), with the addition, in the latter case, of areas of bone-like tissue formation. Furthermore, after 6 weeks, when Healos® scaffold was almost completely resorbed, calvarial defects of both mice presented bone-like tissue formation with different degrees of mineralization (Figure 6). Unlike other mice, control mice transplanted with Healos® scaffold alone in the calvarial defect did not show the presence of host green cells in the defect at 3 weeks after surgery (Figure S2).

3.4 | Effect of hAFSCs on cell migration in vitro

We utilized a migration assay to evaluate chemoattractant effects of hAFSCs. Mouse BMSCs were seeded on the upper side of a transwell membrane for 24 hr and used for evaluation of chemoattractant properties. We observed that hAFSCs significantly increased migration of mBMSCs through the membrane compared to the other cell types and conditions (** $p < .01$; *** $p < .001$; Figure 7). As shown in Figure 7, CM obtained from cultured hAFSCs is much less effective than the cells themselves. This apparent discrepancy could be due to the use of fresh hAFSCs versus CM collected from hAFSCs and stored at -80°C . The storage of the medium could have affected the efficacy of the soluble factors.

4 | DISCUSSION

Regeneration of Cranio-Maxillo facial skeleton defects, due to traumas, congenital malformations or tumor resection, represents one of the major challenges of bone tissue engineering research (Szpalski, Barr, Wetterau, Saadeh, & Warren, 2010). Cranial defects are not able to repair spontaneously in the adult, as it is well known that successful calvarial bone defects are able to repair only in infants younger than 2 years of age (Fong et al., 2003). Current treatments of large bone defects are based on autologous and allogeneic transplantations using autografts and allografts and metal or ceramic implants that still have several limitations (Amini, Cato, Laurencin, & Nukavarapu, 2012; Soucacos, Johnson, & Babis, 2008; Younger & Chapman, 1989). Autogenous bone grafts are considered the gold standard of treatment because of their nonimmunogenic properties, but can lead to morbidity at the donor sites (Banwart, Asher, & Hassanein, 1995; Dimitriou, Jones, McGonagle, & Giannoudis, 2011) while allogeneic transplantation has a higher cost and risk of infections

(Dominici et al., 2006). Cell transplantation is an alternative to the autologous bone grafting to stimulate bone repair process. For this purpose, bone tissue engineering needs a source of cells with suitable characteristics for medical application. These cells should be available in copious amount and should be easily obtained and differentiate into multiple cell lineages (Moradi, Golchin, Hajishafieha, Khani, & Ardeshirylajimi, 2018).

In recent years, the potential of hAFSCs in bone regenerative medicine has been investigated. Maraldi et al. (2013) demonstrated that hAFSCs can promote bone regeneration once transplanted with a collagen scaffold into calvarial mouse model and that human cells are still present in the implant 8 weeks after surgery. Mirabella et al. (2011a) showed that hAFSCs, once transplanted in vivo, can recruit host/progenitor cells, and are detectable in the implant after 2 weeks.

In this study, we investigated the role and the effect of hAFSCs in the repair of a calvarial bone defect using a model that allowed us to visualize the presence of donor and host cells directly in the transplantation site. Human AFSCs were permanently labeled with cherry red fluorescent protein, seeded on Healos® scaffold and transplanted in a transgenic mouse model carrying green fluorescent protein reporter in Col3.6 preosteoblastic cells. Before transplantation, hAFSCs were cultured for 10 days in the presence of osteogenic medium supplemented with ascorbic acid to obtain a partially committed population still able to proliferate. Our results showed that the transduction of hAFSCs with Ub-mCherry virus produce a population with a permanent label and is useful to track the fate of the cells in vivo directly in the site of transplantation. Different fluorescent intensities detected in cultured cells were related to different cell sizes and shapes (Figure 1a). To report a more measurable parameter in addition to the fluorescent imaging, we provided FACS analysis, which showed a clearly positive population in comparison to the negative control (Figure 1b,c). We also characterized hAFSCs phenotype showing that uncommitted hAFSCs highly express mesenchymal markers such as CD73, CD90, CD166, and CD44, but no hematopoietic markers like CD45 and CD34 (data not shown). Unlike uncommitted cells, hAFSCs precommitted by a 10 day-culture with osteogenic medium and ascorbic acid displayed a lower expression of CD73 and CD90 and the absence of osteogenic markers, indicating that this treatment is not enough to induce an osteogenic differentiation, but only a partial commitment of the cells that could be useful in transplantation studies. Cell migration assay showed that hAFSCs can better attract mBMSCs in vitro compared to other cell types and conditions. To evaluate these effects in vivo we transplanted cherry positive precommitted hAFSCs into mouse calvarial bone defects. Murine BMSCs were used in parallel as technical control for the calvarial transplantation procedure since several studies have demonstrated the ability of BMSCs to promote critical-size Cranio-Maxillo facial defects healing in small animal models being BMSCs the most common population of stem cells with osteogenic differentiation capability (Chen et al., 2013; Zhao et al., 2013). In our study, murine BMSCs seem to contribute to fill the bone gap indicating that the transplantation procedure was correct and comparable to hAFSCs transplantation. Since we observed that the effects of hAFSCs in the repair of calvarial bone defect were due to the attraction of host cells in the defect, and not to the osteoblastic differentiation of hAFSCs, we supposed that the only difference, in this particular model, could be that the regeneration induced by human cells is slower compared with the regeneration obtained following mouse

BMSCs transplantation. In accordance with previous results reported by other authors (Mirabella et al., 2011), our data suggest that hAFSCs, once transplanted in vivo, are not present in the implant after long periods, although cherry protein can still be detected. This is likely due to an incomplete immune response of immunodeficient NOD mice, which are deficient in natural killer (NK), macrophage, antigen presenting cell and complement activity, but still capable of a minimal immune reactivity. A slower immune response to human cells can justify the detection of the cherry protein signal in the scaffold after 3 weeks, when transplanted cherry positive hAFSCs are not present in the scaffold. Beside this, donor hAFSCs can recruit host cells as demonstrated by the presence of green cells in the scaffold at 3 and 6 weeks after transplantation. The host cells recruitment is likely triggered by hAFSCs paracrine releasing factors, as demonstrated by some authors. Among these, Yoon et al. (2010) showed that various chemokines and cytokines secreted are able to recruit host progenitor cells, whereas Balbi et al. (2016) provided a description of the paracrine potential of hAFSCs-EVs by means of the horizontal transfer of specific miRNAs, suggesting a role of hAFSCs secretome in tissue repair. In addition, although hAFSCs might show limited osteogenicity in vivo, they can trigger a strong proangiogenic response (Mirabella, Cilli, et al., 2011, Mirabella et al., 2013). All these effects are due to the presence of hAFSCs since the scaffold transplanted without hAFSCs is devoid of host progenitor cells already at 3 weeks after transplantation (Figure 2).

5 | CONCLUSIONS

We have demonstrated the chemoattractant activity of hAFSCs in the recruitment of host cells once transplanted in vivo in a transgenic mouse model of calvarial healing.

These findings are novel and pave the way for further investigations to determine cell mechanisms, to characterize the contribution of hAFSCs in bone healing, and improve host cell differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Transduction efficiency of human amniotic fluid stem cells (hAFSCs) with Ub-mCherry virus. (a) hAFSCs transduced with Ub-mCherry virus efficiently express cherry red fluorescent protein as demonstrated by fluorescent images at 48 hr after transduction. (b) Flow cytometry sorting of hAFSCs transduced with Ub-mCherry virus. The percentage of cherry positive cells before sorting was 49%. (c) Reanalysis of transduced cells after two passages in culture. The percentage of cherry positive cells after sorting was 96%. DIC, differential interference contrast; mCherry, fluorophore

FIGURE 2.

Flow cytometry analysis of surface marker expression in control and precommitted hAFSCs revealed that the expression of surface antigens such as CD90, CD73, CD166, and CD44 was positive in uncommitted hAFSCs (orange) compared to precommitted cells (blue), but CD34 was negative (data not shown). Unstained cells were used as control (gray).

Uncommitted and precommitted populations overlapped (purple). Data are representative of three independent experiments. APC, allophycocyanin; hAFSCs, human amniotic fluid stem cells; Pac Blue, pacific blue dye; PerCP, peridinin chlorophyll protein complex; PE, phycoerythrin

FIGURE 3.

Analysis of hAFSCs and mouse bone marrow stromal cells (mBMSCs) transplanted in NSG/Col3.6tpz reporter-mouse at 3 weeks after surgery. (a) The image shows the presence of numerous recipient mouse cells (arrows) in the implanted scaffold compared to (b) the scaffold implanted with mBMSCs. mCal: mouse calvaria. Sc: not resorbed scaffold. Both panels: X-ray analysis (A', B') showed the presence of mineralized tissue in the constructs implanted with mBMSCs compared to hAFSCs after 3 weeks. hAFSCs, human amniotic fluid stem cells

FIGURE 4.

Analysis of hAFSCs and mBMSCs transplanted in NSG/Col3.6tpz reporter-mouse at 6 weeks after surgery. (a) The image shows the presence of numerous recipient mouse cells (green) in the implanted scaffold compared to (b) the scaffold implanted with mBMSCs. Both panels: X-ray analysis (A', B') showed the presence of mineralized tissue in the constructs implanted with both mBMSCs and hAFSCs after 6 weeks. hAFSCs, human amniotic fluid stem cells; mBMSCs, mouse bone marrow stromal cells

FIGURE 5.

Comparative histological analysis of calvarial defect transplanted with hAFSCs and mBMSCs at 3 weeks after surgery. The image shows hematoxylin/eosin (H&E) staining (a, c) and Masson trichrome staining (b, d) of the scaffold seeded with hAFSCs (left panels) and mBMSCs (right panels). mCal: mouse calvaria. Sc: not resorbed scaffold. (*) fibrotic tissue; (#) bone-like tissue; 2D reconstructions magnification: $\times 100$; boxes magnification: $\times 200$. hAFSCs, human amniotic fluid stem cells; mBMSCs, mouse bone marrow stromal cells

FIGURE 6.

Comparative histological analysis of calvarial defect transplanted with hAFSCs and mBMSCs at 6 weeks after surgery. The image shows hematoxylin/eosin (H&E) staining (a, c) and Masson trichrome staining (b, d) of the scaffold seeded with hAFSCs (left panels) and mBMSCs (right panels). mCal: mouse calvaria; (*) fibrotic tissue; (#) bone-like tissue; 2D reconstructions magnification: $\times 100$; boxes magnification: $\times 200$. hAFSCs, human amniotic fluid stem cells; H&E, hematoxylin/eosin; mBMSCs, mouse bone marrow stromal cells

FIGURE 7.

Human AFSCs promote mBMSCs migration in vitro. (a) Light microscope images of mBMSCs migrated in the presence of different chemoattractant agents. Cells are dyed with crystal violet. Pictures were taken at $\times 100$ magnification. (b) Evaluation of migrated cells number. Quantitative results are presented as mean $\pm$ standard deviation (** $p < .01$; *** $p < .001$). These experiments were repeated three times. hAFSCs, human amniotic fluid stem cells; hAFSCs CM, hAFSCs conditioned medium; hFbs: human fibroblasts; hMSCs, human mesenchymal stromal cells; mBMSCs, mouse bone marrow stromal cells