

Engraftment Potential of Adipose Tissue-Derived Human Mesenchymal Stem Cells After Transplantation in the Fetal Rabbit

Itziar Martínez-González,^{1,*} Rafael Moreno,^{2,*} Jordi Petriz,³ Eduard Gratacós,⁴ and Josep M. Aran¹

Due to their favorable intrinsic features, including engraftment, differentiation, and immunomodulatory potential, adult mesenchymal stem cells (MSCs) have been proposed for therapeutic in utero intervention. Further improvement of such attributes for particular diseases might merely be achieved by ex vivo MSC genetic engineering previous to transplantation. Here, we evaluated for the first time the feasibility, biodistribution, long-term engraftment, and transgenic enhanced green fluorescent protein (EGFP) expression of genetically engineered human adipose tissue-derived MSCs (EGFP⁺-ASCs) after intra-amniotic xenotransplantation at E17 of gestation into our validated pregnant rabbit model. Overall, the procedure was safe (86.4% survival rate; absence of anatomical defects). Stable, low-level engraftment of EGFP⁺-ASCs was confirmed by assessing the presence of the pWTEGFP lentiviral provirus in the young transplanted rabbit tissues. Accordingly, similar frequencies of provirus-positive animals were found at both 8 weeks (60%) and 16 weeks (66.7%) after in utero intervention. The presence of EGFP⁺-ASCs was more frequent in respiratory epithelia (lung and trachea), according to the route of administration. However, we were unable to detect EGFP expression, neither by real-time polymerase chain reaction nor by immunohistochemistry, in the provirus-positive tissues, suggesting *EGFP* transgene silencing mediated by epigenetic events. Moreover, we noticed lack of both host cellular immune responses against xenogeneic ASCs and humoral immune responses against transgenic EGFP. Therefore, the fetal microchimerism achieved by the EGFP⁺-ASCs in the young rabbit hosts indicates induction of donor-specific tolerance after fetal rabbit xenotransplantation, which should boost postnatal transplantation for the early treatment/prevention of many devastating congenital disorders.

Introduction

TODATE, ONLY A FEW GENETIC disorders have been treated in utero with some success. However, the proof of principle for the early treatment of inherited diseases by prenatal gene therapy has been extensively demonstrated in several animal models [1]. Despite the great potential of prenatal gene therapy, safety problems particularly associated with the use of integrative vectors (risk of insertional mutagenesis, germline transmission, or abnormal organogenesis [2–4]) highlighted that new approaches are necessary to circumvent and/or minimize these adverse events before fetal gene therapy could be accepted for clinical application. An encouraging alternative to improve the efficacy of prenatal therapy is the use of stem cells as therapeutic agents, either unmodified or genetically engineered using viral or nonviral vectors.

In utero stem cell administration would share some of the advantages of prenatal gene therapy: (i) the early fetus presents a relative immature immune system, providing a favorable environment for allogeneic or xenogeneic grafts; (ii) the small size of the fetus facilitates increased donor-to-host-cell ratio; and (iii) prenatal administration may prevent many genetic disorders before a definitive organ damage has occurred. In addition, fetal cell therapy has also some advantages *per se* such as the possibility that stem cells could engraft at several stem cell niches, increasing their expansion; thanks to the high rate of proliferative and migratory events that occur during ontogeny [5]. Finally, whether a combination of cell and gene therapy with integrative vectors would be necessary, the risk associated with insertional mutagenesis might be minimized by performing ex vivo clonal analysis before cell expansion and administration,

¹Human Molecular Genetics Group, Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), Hospital Duran i Reynals, L'Hospitalet de Llobregat, Barcelona, Spain.

²IDIBELL-ICO, Translational Research Laboratory, Hospital Duran i Reynals, L'Hospitalet de Llobregat, Barcelona, Spain.

³Biomedical Research Unit, Vall d'Hebron Institut de Recerca (VHIR), Barcelona, Spain.

⁴Maternal-Fetal Medicine Department, Hospital Clinic, University of Barcelona, Barcelona, Spain.

*These two authors contributed equally to this work.

selecting those stem cell clones in which the vector has been inserted into favorable loci.

At present, prenatal hematopoietic stem cell (HSC) transplantation has been the most developed application for fetal stem cell therapy. Numerous groups have demonstrated diverse degrees of engraftment after in utero HSC administration in animal models. However, significant engraftment has been only documented when cell transplantation was made on stem cell-deficient recipients, where there is a competitive advantage for donor HSCs [6]. Interestingly, studies carried out by Zanjani and col. demonstrated that cotransplantation of mesenchymal stem cells (MSCs) enhanced the engraftment of HSCs after fetal administration [7], pointing out the adjuvant potential of MSCs.

MSCs are multipotent nonhematopoietic cells, easily isolated and expanded *ex vivo* from a great variety of sources and species, with the capability to differentiate into a variety of cell types of mesenchymal origin such as adipocytes, chondrocytes, osteocytes, and tenocytes, and into other cell types such as myocytes, astrocytes, and neurons [8]. Furthermore, MSCs present low inherent immunogenicity [9]. The main functions described for MSCs include hematopoiesis regulation, apoptosis prevention, cellular repair, and the control of immune-inflammatory reactions [10]. All these attributes make MSCs particularly appealing in tissue repair and regenerative medicine. Therefore, special efforts are being focused in the development of preclinical in utero MSC transplantation studies. In fact, several reports have confirmed that fetal administration of MSCs resulted in correct engraftment and differentiation of transplanted cells [11–13].

To advance toward clinical application, we have focused our efforts elucidating the capacity of MSCs as cell vehicles in prenatal studies, after *ex vivo* transduction with integrative vectors. We have recently evaluated the overall safety, biodistribution, engraftment capacity, and transgene expression of enhanced green fluorescent protein (EGFP)-expressing rabbit fetal liver MSCs (EGFP⁺-fl-MSCs) after allogeneic fetal transplantation into the pregnant rabbit model [14]. We highlighted the safety of fl-MSCs for fetal transplantation in the pregnant rabbit model, the efficiency, and persistence of fl-MSC engraftment and transgene expression in fetal organs, and the absence of immune reactions against both allogeneic fl-MSCs and transgenic EGFP after fetal transplantation.

Based on our results and once established the pregnant rabbit as a reliable middle-size animal model to evaluate prenatal stem cell approaches, we decided to evaluate the capability of a widely characterized MSC type with higher ethical and clinical relevance: adult human adipose tissue-derived MSCs (ASCs), transduced with an integrative vector for in utero combined gene and cell therapy through intra-amniotic administration. This approach might become particularly appropriate for the treatment of congenital respiratory diseases.

Materials and Methods

Isolation and characterization of human ASCs

According to the Institutional Ethics Committee guidelines, ASCs were isolated from discarded human adipose tissue obtained from a healthy adult female undergoing

elective lipoaspiration, as recently described [15]. Briefly, the raw lipoaspirate was washed extensively with an equal volume of phosphate-buffered saline (PBS) (Invitrogen) to remove blood cells, local anesthetic, and cellular debris. The washed tissue was then resuspended in PBS containing 0.075% type II collagenase (Invitrogen) and incubated at 37°C for 1 h. The enzymatic activity was neutralized by adding an equal volume of the Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS). The resulting suspension was filtered through a 70- μ m nylon filter and centrifuged at 1200 g for 10 min. The pellet was resuspended in an α -MEM (Invitrogen) containing 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 2.5 μ g/mL amphotericin (all from Invitrogen), and seeded overnight in culture flasks (Corning, Inc.) at 37°C in a 5% CO₂ atmosphere.

Next day, the cell monolayer was washed once more with PBS to remove nonadherent cells and cellular debris and replaced with a fresh medium. Finally, the ASCs were amplified for 10–12 days (passage 0) until 75%–90% confluent, substituting the culture medium every 3–4 days, and were passaged periodically by detachment [0.25% trypsin-1 mM EDTA in PBS (Invitrogen)] after achieving a subconfluent monolayer.

The isolated ASCs were further characterized by cell surface marker expression and differentiation capability [15].

Human ASC transduction and sorting

The replication-defective self-inactivating HIV-1-derived lentiviral vector pWPT-EGFP [16], expressing the EGFP, was produced and employed to generate the EGFP⁺-ASC population.

ASCs (passage 1) were transduced by spinoculation. About 5×10^5 ASCs and 5×10^4 viral vector particles (MOI = 0.1) were cocultured on a 6-well cell culture plate (Corning Inc.) with 2 mL/well α -MEM complete medium plus 8 μ g/mL of polybrene (Sigma), and centrifuged at 1000 g for 90 min at 32°C. Next day, the cell culture supernatant was replaced by a fresh medium, and the cells were cultured for another 24 h. The resulting EGFP⁺ fluorescent ASCs (Fig. 1A) were excited at 488 nm, sorted through a 530/40-band pass filter, recovered as bulk populations of 1.5×10^5 cells using a MoFlo[®] jet-in-air high-speed sorter (Beckman Coulter), and amplified *in vitro* to obtain enough transduced cells for *in vivo* administration.

Animals and transplantation procedures

Timed pregnant White New Zealand rabbits (13–15 days of gestation) were housed for 1 week before surgery. The housing was quiet, at constant room temperature and humidity. Laparotomies were performed at 17 days of gestation (E17) (gestation = 31–32 days), as described previously [16]. The fetus was located and fixed by external palpation of the semitransparent uterine wall, and intra-amniotic injections were performed using 26-gauge needles directly in the amniotic fluid of the fetal sac in view of the common anatomical landmarks of the E16–18 embryo [16].

Approximately 20 min before the intervention, the EGFP⁺-ASCs were trypsinized and resuspended in PBS. Fetuses received a unique dose of 2×10^5 EGFP⁺-ASCs in a volume

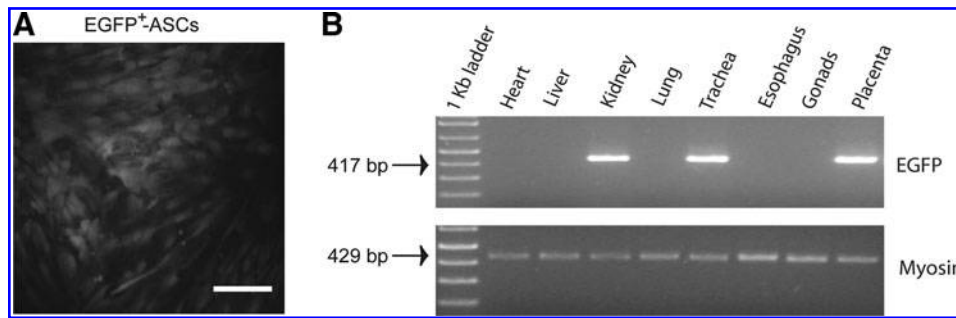


FIG. 1. Long-term assessment of xenogeneic EGFP⁺-ASC engraftment into young rabbit tissues after fetal transplantation. **(A)** Efficient lentiviral vector-mediated transduction of adipose tissue-derived mesenchymal stem cells (ASCs). Lentiviral vector-transduced ASCs were visualized by enhanced green fluorescent protein (EGFP) fluorescence (magnification: 400×; scale bar = 25 μm). **(B)** Polymerase chain reaction-mediated detection of integrated, EGFP⁺-ASC-derived, pWPT-EGFP provirus into genomic DNA isolated from a representative EGFP⁺-ASC-treated young rabbit at 16 weeks after transplantation. EGFP-specific primers amplified a 417-bp fragment. Lane 1, 1 kb DNA ladder; lanes 2–9 analysis performed in the indicated tissues.

of 100 μL/fetus. Control fetuses were injected with an identical volume of PBS. No more than 50% of the fetuses were treated per uterine horn (the vaginal-end sacs were always excluded). Whenever possible, injection of adjacent fetuses was avoided. Once the injections were completed, the uterus was returned to the abdominal cavity and the abdomen was closed in layers. Does were kept under a warming blanket until awake and active, and received subcutaneous injections of meloxicam (Metacam; Abbott Laboratories) (0.4 mL per rabbit/24 h) over 48 h as postoperative analgesia.

All procedures were conducted in compliance with applicable regulations and guidelines, and were reviewed and approved by the Animal Care Committee.

Tissue collection and processing

On day 31 of gestation (14 days after intervention), pregnant rabbits were sacrificed with a euthanizing dose of pentobarbital (150 mg/kg, intravenous). Samples of gonads and uterus from the does and placenta from the pups were frozen for DNA extraction. Experimental pups were retrieved by visual identification after caesarean section, re-animated, and weighted. The pups were marked with a subcutaneous microchip (AVID Microchip ID Systems) for further identification, kept in an incubator C100 (Air Shields) at 32°C for ~1 h, and finally allocated to foster rabbits. At the end of study (8 or 16 weeks after intervention), the rabbits were sacrificed with a euthanizing dose of pentobarbital, as described above, and tissue samples were harvested (gonads, kidney, liver, heart, lung, trachea, and esophagus for 16-week-old pups). A sample fraction was quickly frozen in liquid nitrogen for DNA extraction, and the remaining fraction was fixed overnight in formalin and embedded in paraffin for immunohistochemical evaluation.

DNA isolation and polymerase chain reaction analysis

Genomic DNA was obtained from does and fetal tissues by standard phenol–chloroform extraction and isopropanol precipitation. Briefly, samples were washed twice in 1× SSC and 10 mM EDTA and incubated overnight at 56°C in a sterile lysis buffer [10 mM Tris-Cl, pH 10.5; 1 mM EDTA;

0.15 mM NaCl; 0.5% SDS; 0.3 mg/sample proteinase K (Roche)] before phenol extraction. Vector presence in the harvested organs was determined by polymerase chain reaction (PCR) amplification of a provirus-specific fragment carried out in a 25-μL volume containing 50 ng of genomic DNA, 100 μM dNTPs, 2.5 μL 10× Biotaq DNA polymerase buffer, 1.50 mM MgCl₂, 0.15 μL Biotaq DNA polymerase (5 U/μL) (Bioline), and 20 pmol of the following primers: WPT-EGFP-F (5'-ACC CCG ACC ACA TGA AGC AGC-3') and WPT-EGFP-R (5'-CGT TGG GGT CTT TGC TCA GGG-3'), localized within the EGFP sequence, in a GeneAmp[®] PCR system 2400 DNA thermocycler (Perkin-Elmer). A control PCR reaction containing no DNA to monitor for contamination of PCR reagents and a positive control-containing vector DNA were included in each PCR analysis. Amplification conditions involved an initial 10-min denaturation step at 94°C, followed by 42 cycles (94°C, 1 min; 60°C, 30 s; and 72°C, 45 s), and a final 7-min extension at 72°C, producing a 417-base-pair DNA fragment. Rabbit skeletal muscle myosin heavy-chain gene (MHC) primers, RMMHC-F (5'-AAAGAAGATGGATGTTGAGGC-3'), and RMMHC-R (5'-TCACCGTCACTTTCCTGCT-3'), were used to amplify a 429-base-pair-specific DNA fragment as described previously [17], which served as a control for genomic DNA isolation and loading. All samples were evaluated through 2% agarose gel electrophoresis.

Immunohistochemistry

Formalin-fixed tissues were dehydrated with an ethanol gradient, cut, and embedded in paraffin. Sliced 5-μm sections were mounted on poly-L-lysine-coated glass slides. Before immunohistochemistry, sections were deparaffinized and rehydrated. Immunogenic retrieval was performed, incubating the slides in 10 mM citrate buffer pH 6.0 for 20 min in a microwave oven. After performing several TBS-T (TBS plus 0.3% Triton X-100) washes, tissue sections were blocked by 2-h incubation in 10% FBS diluted in a washing buffer [TBS plus 1% bovine serum albumin (BSA) and 0.2% Tween 20] at room temperature by gentle agitation. Further, TBS-T-washed sections were incubated overnight at 4°C in a humid chamber with a biotinylated goat polyclonal anti-GFP

antibody (Abcam) diluted 1/100 in a washing buffer. Next day, the endogenous peroxidase was blocked with 3% H₂O₂ for 10 min in the dark. Sections were subsequently washed with PBS and incubated with a biotinylated horseradish peroxidase–avidin complex (ImmunoPure® ABC Peroxidase Staining Kit; Pierce). Finally, sections were stained with the peroxidase substrate diaminobenzidine tetrahydrochloride (DAB; Pierce) and counterstained with hematoxylin. Moreover, each specimen was routinely stained with hematoxylin/eosin to evaluate tissue morphology and the presence of inflammatory cell infiltrates.

Enzyme-linked immunosorbent assay

Rabbit sera were obtained at 8 and 16 weeks of age. Blood was taken from the lateral saphenous vein and left coagulating for ~30 min. Samples were centrifuged twice at 3000 g for 10 min and kept at –80°C until use.

The generation of a rabbit humoral immune response against the EGFP reporter protein was assessed by enzyme-linked immunosorbent assay (ELISA). Maxisorb ELISA 96-well plates (Nunc) were coated overnight at 4°C with 0.1 µg/well of recombinant EGFP (BioVision) diluted in 50 µL of 50 mM carbonate buffer (pH 9.6). After several washes with PBS-T (PBS plus 0.05% Tween 20), plates were blocked with 300 µL of 3% fish gelatin (Sigma-Aldrich) diluted in PBS for 2 h at room temperature. Serum samples diluted 1: 500 with a dilution buffer (PBS-T + 3% BSA) were incubated in triplicate wells for 2 h at room temperature. The plates were then washed again with PBS-T, and 100 µL of secondary antibody (biotin-conjugated goat anti-rabbit IgG, diluted 1:200,000) (Sigma-Aldrich) was further incubated for 2 h at 37°C. Subsequently, plates were washed with PBS, incubated for 30 min with biotinylated horseradish peroxidase–avidin complex (ImmunoPure ABC Peroxidase Staining Kit; Pierce), and developed using 1-Step Slow TMB (Pierce) for 30 min. Finally, the reaction was stopped with 2 M H₂SO₄, and the corresponding absorbances were measured at 450 nm on an ELISA plate reader. A standard curve was prepared by serial dilutions of a polyclonal anti-GFP antibody (MBL) in rabbit serum (prediluted 1: 500 with dilution buffer) to establish the range of sensitivity.

Mixed lymphocyte culture

After mechanical disaggregation on a culture dish, rabbit spleens were homogenized by gently flushing with a 21G needle. The cellular splenocyte suspension was filtered through a 70-µm nylon filter to remove cellular debris, incubated for 30 min with a lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) at 4°C, and centrifuged at 300 g for 5 min.

Splenocytes were washed twice with PBS and finally cultured in a complete DMEM at 37°C and 5% CO₂ atmosphere. Cell viability was assessed by trypan blue exclusion. To study the effect of ASCs on lymphocyte reactivity, a 2-way mixed lymphocyte culture (MLC) was performed. Lymphoid cells were seeded in triplicate at a concentration of 2 × 10⁵ cells/well in 96-well, U-bottom microtiter cell plates (Corning), in the presence or absence of 1 × 10⁵ ASCs (passage 2) preplated overnight before the coculture experiment. After a 5-day incubation period, cell proliferation was de-

termined using the Amersham cell proliferation Biotrack ELISA system (GE Healthcare Amersham Biosciences).

Results

We intra-amniotically administered a unique dose of 2 × 10⁵ EGFP⁺-ASCs (100 µl/fetus) into fetal rabbits at day E17 (control fetuses were injected with an identical volume of PBS) [14]. At E31 (14 days after intervention), pregnant rabbits were sacrificed (samples of gonads and uterus from the does and placenta from the pups were frozen for DNA extraction), and experimental pups were retrieved by visual identification after caesarean section, reanimated, and weighted. Regarding survival, 22 fetuses from 4 pregnant does were intervened (18 were injected with EGFP⁺-ASCs, and 4 were injected with PBS), and 3 abortions were documented: 2 abortions among the ASC-treated fetuses and 1 abortion among the PBS-treated fetuses. Thus, the overall survival rate at birth was 86.4%. Furthermore, no anatomical defects were detected in any of the treated fetuses. They maintained a correct weight at the termination of pregnancy according to a normal development pattern. No significant weight differences were found between EGFP⁺-ASC- and PBS-treated young rabbits (mean weight of EGFP⁺-ASC-treated fetuses: 56.49 ± 8.24 g, *n* = 16, vs. control PBS-treated fetuses: 57.43 ± 12.59 g, *n* = 3) (*P* = 0.8670, Student's *t*-test). Therefore, neither the intrinsic features of the EGFP⁺-ASCs nor the delivery procedure generated toxicity to the in utero-treated fetuses beyond the standard risk from the surgical intervention.

After reanimation, 19 treated pups were placed to the care of a foster mother and 17 survived to adulthood (14 EGFP⁺-ASC-treated fetuses and 3 PBS-treated fetuses). Analogously to that recently reported for in utero EGFP⁺-fl-MSC transplantation in the fetal rabbits [14], 2 casualties were documented among the EGFP⁺-ASC-treated pups due to nurturer rejection. The corresponding necropsies did not reveal any pathological sign in the deceased animals.

To assess engraftment of transplanted EGFP⁺-ASCs, young rabbits were sacrificed at 8 weeks (5 EGFP⁺-ASC-treated and 2 PBS-treated) and 16 weeks (9 EGFP⁺-ASC-treated and 1 PBS-treated) after in utero intervention, and tissues were collected. Genomic DNA isolated from the harvested tissues was employed to detect integrated proviral sequences (Fig. 1B and Table 1). PCR analysis of the EGFP sequence at 8 weeks after transplantation showed the presence of donor cells in several tissues from 3 of 5 recipients (60%), similar to the frequency observed at 16 weeks [6 of 9 recipients (66.7%)]. Each recipient exhibited a different distribution pattern of donor cells, but their presence in the lung and trachea was most frequent, accordingly to the intra-amniotic route of administration. To note, we also detected the presence of donor cells in some placentas (frequency: 2/14, 14.3%). In contrast, gonadal tissue was PCR negative. Moreover, all PBS-treated control animals were completely negative for the presence of EGFP⁺-ASC proviral sequences (data not shown).

We next wanted to confirm fetal homing and engraftment of EGFP⁺-ASCs into the different rabbit tissues. Therefore, we screened transgenic EGFP expression of tissue sections positive for the presence of pWPT-EGFP provirus sequences from EGFP⁺-ASC-treated rabbits, as previously described

TABLE 1. EFFICACY OF PRENATAL XENOGENEIC EGFP⁺-ASC TRANSPLANTATION IN THE RABBIT

Gestational Period	Time of analysis after in utero intervention (weeks)	Delivery route (N) ^a	Provirus-positive animals	EGFP ⁺ -ASC Biodistribution ^b							
				Heart	Liver	Kidney	Lung	Trachea	Esophagus	Gonads	Placenta
E17	8	IA (5)	3/5 (60%)	0/5 (0%)	1/5 (20%)	1/5 (20%)	0/5 (0%)	2/5 (40%)	1/5 (20%)	0/5 (0%)	0/5 (0%)
	16	IA (9)	6/9 (66.7%)	0/9 (0%)	1/9 (11.1%)	5/9 (55.6%)	1/9 (11.1%)	0/9 (0%)	0/9 (0%)	2/9 (22.2%)	

^aIA, intraamniotic. (N), number of EGFP⁺-ASC-treated fetuses.

^bThe presence of proviral DNA in the different tissues from the in utero-treated fetuses was analyzed after genomic DNA extraction and polymerase chain reaction amplification using EGFP-specific primers. For each tissue, frequencies are displayed as number of provirus-positive animals/total number of EGFP⁺-ASC-treated animals analyzed. ASC, adipose tissue-derived mesenchymal stem cell; EGFP, enhanced green fluorescent protein.

[14]. However, we were not able to substantiate engraftment of xenogeneic EGFP⁺-ASCs into any postnatal in utero-treated rabbit tissue by immunohistochemistry. We were also unsuccessful determining *EGFP* expression at the transcriptional level by real-time quantitative-PCR (RT-qPCR) (data not shown). Additional examination of the different tissue sections did not reveal the presence of cellular infiltrates in any of the analyzed samples, suggesting absence of immune-inflammatory activity.

Subsequently, we wished to determine the immunogenicity of human ASCs after prenatal transplantation. For this purpose, we first analyzed whether young rabbits that underwent prenatal transplantation of EGFP⁺-ASCs overexpressing the reporter EGFP protein were able to develop a humoral immune response against the ASC-derived *EGFP* transgene product. A specific ELISA assay detecting the presence of antibodies against EGFP [14] was performed on sera harvested from young rabbits after in utero EGFP⁺-ASC delivery (Fig. 2A). There was no evidence of anti-EGFP antibodies in any of the analyzed animals at both 8 and 16 weeks after in utero transplantation.

Finally, through an MLC assay [14], we analyzed whether young rabbits that underwent prenatal transplantation of EGFP⁺-ASCs were able to induce a cellular immune response against these xenogeneic cells at 16 weeks after transplantation. Mononuclear cells isolated from the spleen of in utero EGFP⁺-ASC-transplanted young rabbits were unable to proliferate when exposed to xenogeneic human ASCs (Fig. 2B), analogously to that observed from mononuclear cells isolated from an in utero PBS-treated rabbit spleen. This suggests absence of an immune reaction against the transplanted human ASCs.

Discussion

Although the most common source of human MSCs is the adult bone marrow (aBM-MSCs), the invasive methods used for their obtention and the low number of MSCs present in the bone marrow (<0.01% of the bone marrow mononuclear cell population [18]) stress the need to identify alternative MSC sources. Over the last decade, ASCs have arisen as a highly attractive alternative source of MSCs. They are easily obtained from adipose tissue lipoaspirates using a straightforward collagenase-based isolation protocol. Furthermore, the abundance of ASCs in the original tissue and their in vitro expansion capacity outperform that from BM-MSCs [19].

Here, we have presented evidence of the overall safety, biodistribution, and limited engraftment capacity of genetically engineered EGFP-expressing adult human ASCs after xenogeneic fetal transplantation into the pregnant rabbit model. We have demonstrated that xenogeneic in utero transplantation of lentiviral vector-transduced human ASCs is safe both for the recipient dams and for the intervened offspring. Concerning the overall engraftment frequency of xenogeneic EGFP⁺-ASCs into the transplanted fetuses, 60% and 66.7% of intra-amniotically administered E17 animals had detectable pWPT-EGFP provirus at 8 and 16 weeks after EGFP⁺-ASC delivery, respectively. These frequencies are analogous to those observed in our previous study using rabbit fetal liver MSCs transduced with a retrovirus vector expressing the EGFP reporter (EGFP⁺-fMSCs) through the

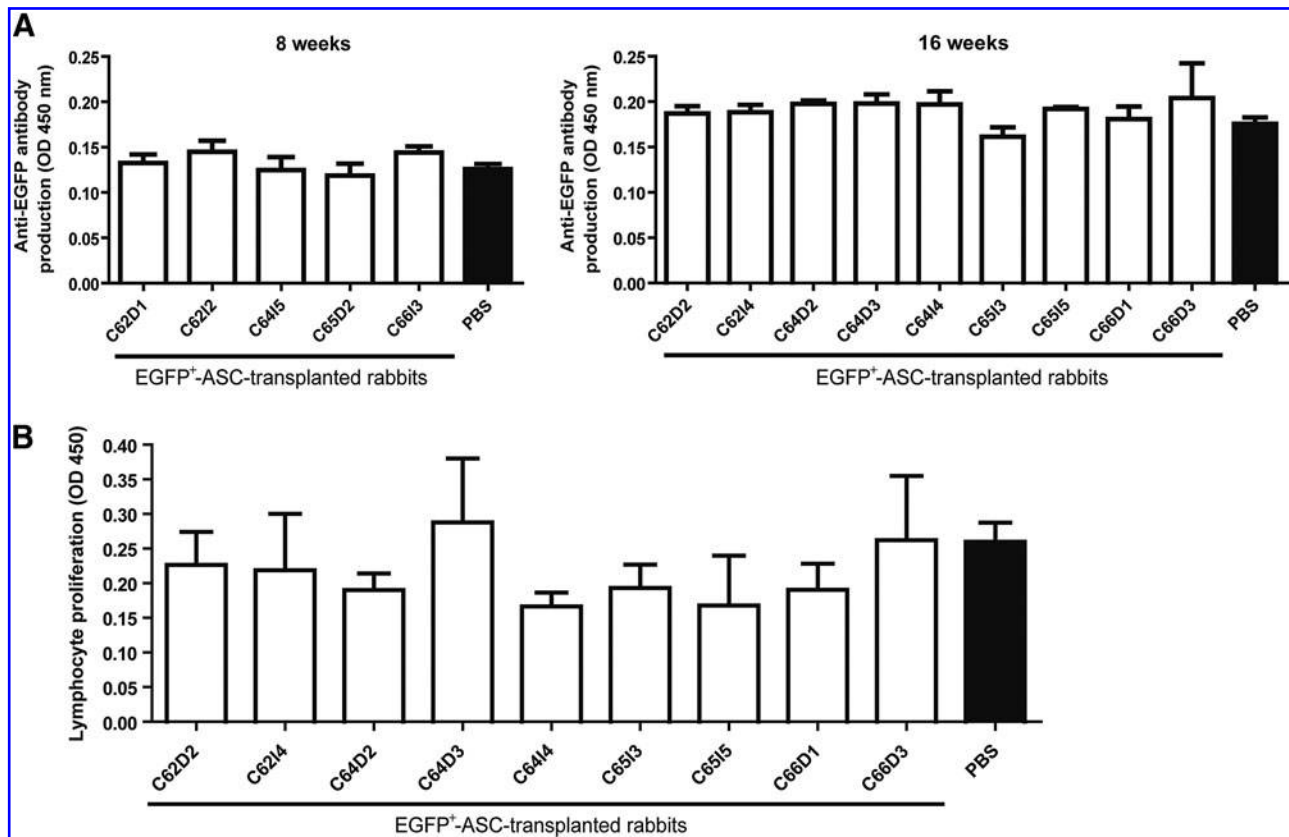


FIG. 2. Lack of immunogenicity of EGFP⁺-ASCs in the rabbit host. **(A)** Specific enzyme-linked immunosorbent assay (ELISA) to detect the presence of antibodies against EGFP. Serum samples from the in utero EGFP⁺-ASC-transplanted young rabbits were collected at sacrifice, and the presence of antibodies against the transgenic EGFP protein was analyzed by ELISA. **(B)** Xenogeneic lymphocyte proliferation assessed by mixed lymphocyte culture (MLC). About 2×10^5 splenocytes from the in utero EGFP⁺-ASC-transplanted young rabbits were cocultured with 1×10^5 xenogeneic ASCs during 5 days in triplicate wells, and cell proliferation was assessed by ELISA. *White column* labels refer to the rabbit identification, analyzed 16 weeks after transplantation. Individual values represent the mean \pm SD from technical replicates ($n=3$). Phosphate-buffered saline (PBS) (*black columns*) refer to the mean \pm SD values obtained from control, in utero PBS-treated young rabbits. Statistically significant differences among EGFP⁺-ASC-transplanted and control PBS-treated rabbits in both the ELISA, and the MLC assays were estimated using the nonparametric Mann-Whitney *U* test.

same route of administration [14]. Moreover, in both studies, the MSC biodistribution pattern appeared essentially restricted to the primary target organs according to the route of administration (lung and trachea for intra-amniotic administration). Both the frequency and depth of fetal breathing movements where the amniotic fluid is taken in would facilitate direct contact and further homing and engraftment of EGFP⁺-ASCs into respiratory tissues, as we and others have proven using viral vectors [16,20].

The limited engraftment of EGFP⁺-ASCs in our immunocompetent fetal rabbits indicates the lack of competitive advantage of these cells in prenatal recipient niches, similarly to that reported for in utero hematopoietic cell transplantation [21]. However, the rather stable levels of microchimerism achieved by the EGFP⁺-ASCs in the transplanted young rabbit tissues at both 8 and 16 weeks suggest that the engrafted cells were able to overcome the fetal immune barrier to transplantation. In that sense, it has been recently shown that in addition of being nonimmunogenic human ASCs display an even lower susceptibility to natural killer (NK) cell-mediated lysis than bone marrow-derived MSCs [22]. Indeed, it has been shown that in addition to durable donor-

specific T-cell tolerance [23], the presence of microchimerism induces NK cell hyporesponsiveness [24], both necessary to overcome rejection.

Intriguingly, although human ASCs could engraft into the fetal tissues, being detectable by whole-tissue analysis of the proviral sequence through PCR, we were unable to detect EGFP transgene expression in any of the PCR-positive tissue samples, analyzed both by immunohistochemistry and by RT-qPCR. Because only a very small fraction of the ASCs administered in utero could undergo sustained engraftment, their detection at the cellular level would be complicated. However, with a comparable low-level engraftment, in our previously study using rabbit EGFP⁺-fl-MSCs, we could identify a few patchy areas expressing EGFP through immunohistochemistry. More likely, the lack of transgene expression detection might reflect the outcome of epigenetic events acting over the integrated EGFP-containing provirus. We have recently shown that the EGFP⁺-ASCs gradually downregulated EGFP transgene expression from integrated gamma-retroviral vectors. Transgene extinction occurred both in undifferentiated ASCs and in ASCs differentiated into 2 different cell lineages (adipocytes and osteocytes), and

correlated with increases in H3 histone deacetylation and CpG dinucleotide methylation within the proviral region [15]. Furthermore, epigenetic regulation has also been able to silence lentiviral vector-mediated transgene expression during stem cell differentiation *in vivo*, in transgenic mice [25,26]. This could limit the efficacy of genetically engineered ASCs for tissue repair/regeneration applications. In that case, the optimization of integrative vectors introducing insulators or other *cis*-regulatory elements allowing the maintenance of high-level transgene expression [27] should be a priority.

In summary, the results from this report support the potential of intrauterine allogeneic transplantation of ASCs, either unmodified or genetically engineered using optimized integrative vectors, leading to limited while sustained engraftment after prenatal application. Therefore, due to their intrinsic immunoregulatory features, ASCs might become an alternative to fetal MSCs, the latter more susceptible to ethical considerations, attaining microchimerism and leading to prenatal tolerance induction. This would selectively enhance donor cell competition, which may become an essential step to boost successful postnatal ASC transplantation [28,29] for the early treatment/prevention of many devastating hereditary disorders.

Acknowledgments

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Author Disclosure Statement

The authors declare no potential conflicts of interest.

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Address correspondence to:

Dr. Josep M. Aran
Human Molecular Genetics Group
Institut d'Investigació Biomèdica de Bellvitge (IDIBELL)
Hospital Duran i Reynals
Gran Via de L'Hospitalet, 199
L'Hospitalet de Llobregat 08908
Barcelona
Spain

E-mail: jaran@idibell.cat

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