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Sheep CD34+ amniotic fluid cells have haematopoietic potential and engraft after autologous *in utero* transplantation

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Running Title: Sheep CD34+ amniotic fluid cell haematopoiesis

Abstract:

Unmatched allogeneic *in utero* stem cell transplantation (IUSCT) produces poor engraftment unless the fetus has congenital immunodeficiency, probably because of maternal and fetal immune responses to injected cells. We studied the functional haematopoietic potential of transduced GFP+ sheep AF stem cells, before and after autologous IUSCT. CD34+ cells were selected from first trimester sheep AF, transduced overnight and injected intravenously into NOD-SCID-gamma (NSG) mice. At 3 months primary recipient bone marrow (BM) was injected into secondary NSG recipients. GFP+ cells were detected in the haematopoietic organs and peripheral blood of primary and secondary recipients at 3 months. Autologous IUSCT (transduced GFP+CD34+AF) was performed in fetal sheep. Six months postnatally, lamb BM was injected into secondary NSG recipients. GFP+ cells were detected in the peripheral blood of primary and secondary recipients. This confirms the haematopoietic potential of AF stem cells supporting the concept of autologous IUSCT to treat congenital haematopoietic disease.

Keywords: sheep CD34, amniotic fluid stem cells, autologous transplantation, cell therapy.

Introduction

In utero stem cell transplantation (IUSCT) aims to provide the fetus with therapeutic cells for to correct prenatal congenital diseases. Beside the obvious advantage of preventing the development of pathology *in utero* and thus avoiding postnatal complications, intervening during fetal life has a stoichiometric advantage, allowing the transplantation of much larger cell doses on a per kilogram basis than can be achieved postnatally [1]. Finally, the maturation of the immune system during the fetal life allows new antigens to be introduced early in gestation without rejection, and can induce immunologic tolerance to these new antigens [2].

Despite all the above, results of allogeneic IUSCT [3] in humans have been successful only for the treatment of congenital severe combined immunodeficiency (SCID) [4]. Allogeneic mesenchymal stem cells (MSCs) injected into fetuses affected with osteogenesis imperfecta successfully engrafted in only 2 out of 5 cases [4]. Attempts to treat other diseases such as sickle cell disease [5] or metabolic storage disorders for example, have been unsuccessful.

In the last few years work conducted by Flake and colleagues [6] has demonstrated that this may be at least partially explained by the migration of the *in utero* injected cells into the maternal circulation and mounting of a rejection response, which could diminish the engraftment. This is most likely due in mice to activated maternal T cells which can cross the placenta in mice and destroy engrafted allogeneic cells [7]. In order to avoid this response, stem cells matched to the fetus or even the mother could be used. Fetal mice injected with congenic haematopoietic stem cells (HSCs) for example, all maintained stable, long-term, multilineage chimerism compared with fewer than 20% of allogeneic recipients [8].

Alternatively, in monogenic disease, stem cells derived from the fetus itself could be used after genetic modification for treatment of a fetus affected by a congenital disease. Fetal stem cells can be easily derived during pregnancy from the amniotic fluid (AF) [9-11]. In particular, ckit+ amniotic fluid stem cells (AFSC) are characterized by long-term self-renewal, clonal properties and differentiation capabilities which defined them at a pre-pluripotent status [9, 12, 13]. They share with embryonic stem cells the expression of some stem markers (e.g. OCT4 and SSEA4), but they are not tumorigenic unless reprogrammed as induced pluripotent stem cells (iPS) [14].

Using a sheep model we have previously shown the potential of transduced AF mesenchymal stem cells (AFMSCs) for autologous IUSCT [15]. After first trimester amniocentesis, AFMSCs could be isolated, cultured, transduced and transplanted back into the donor fetus as an *in utero* autologous transplant. Engraftment of GFP transduced cells was detected two weeks after transplantation in various tissues and organs.

In this study we hypothesised that cells with haematopoietic potential were also present in sheep AF and could be used for autologous IUSCT. We used a recently developed novel sheep-specific CD34 antibody that identifies haematopoietic stem cells in the adult sheep bone marrow [16]. After selecting CD34+ cells from the AF, we transplanted them into NOD-SCID-gamma (NSG) immunocompromised mice to study their engraftment, and into donor fetal sheep after gene marking them using a viral vector for autologous IUSCT. For the first time, we have demonstrated that AF-derived cells from a large animal (sheep) possess haematopoietic potential in xenotransplanted mice, and can mediate long-term engraftment after autologous IUSCT in the sheep.

Methods

All procedures on animals were conducted in accordance with UK Home Office regulations and Guidance for the Operation of Animals (Scientific Procedures) Act (1986).

Sheep animal procedures

Time-mated Romney breed ewes were used. Eight fetuses from four ewes provided first trimester amniotic fluid (AF, 300-700 ml, 60 to 64 days of gestation) that was collected at post mortem examination under sterile conditions and frozen, for later use in transplantation experiments into NSG mice. Ten fetuses from five ewes were used for *in utero* autologous transplantation experiments. Here, general anesthesia was induced with Ketamine (4mg/kg, Merial, UK) intravenously, and after intubation, the ewes were maintained on Isoflurane-Vet 3% (Merial, UK) in oxygen. Fetal measurements were confirmed by ultrasound [17]. The first procedure, ultrasound-guided amniocentesis, was performed in the first trimester (60 - 64 days of gestation, term = 145 days) using a 22 Gauge, 15cm echo-tip needle (Cook Medical, USA). Amniotic fluid (AF, 10ml) was withdrawn from each amniotic sac. Two days later ewes were re-anaesthetized as above for the second procedure, an ultrasound-guided intraperitoneal injection of freshly isolated and transduced CD34+ amniotic fluid stem (AFS) cells ($2x10^4$, 1-2ml) into the donor fetus using a 22-gauge 15cm echo-tip disposable needle (Cook Medical, USA). To ensure correct needle placement within the peritoneal cavity, the needle was inserted through the anterior abdominal wall of the fetal sheep superior and lateral to the fetal bladder to avoid the umbilical arteries. Microbubbles observed moving within the peritoneal cavity as the cells were instilled confirmed correct needle placement. Following recovery of the

ewe, fetal wellbeing and standard measurements of growth (occipital snout length, biparietal diameter, femur length and abdominal circumference) were measured every 20 days until birth [17].

At birth, lambs were weighed and measured (girth at umbilicus, biparietal diameter, standing height to shoulder) and assessed for wellbeing (time to standing, heart rate, respiratory rate, temperature, and oxygen saturation). Lambs were fed naturally with ewe breast milk. Lamb peripheral blood was collected from the jugular vein (No. 18 needles with 20ml syringe) at birth and every 2 weeks thereafter for flow cytometry analysis. Data on lamb growth and wellbeing were collected regularly until scheduled post mortem examination at 6 months of age. This was performed under general anesthesia to allow sterile collection of lamb bone marrow (BM) from multiple sites (sternum, femur, humerus and iliac crest) using a trocar needle, and sampling from all internal organs. Ewes underwent a scheduled post mortem examination with extensive tissue sampling at 3 months after birth once weaning was completed.

Isolation and characterization of sheep CD34+ AFS cells and adult BM cells

Fresh sheep CD34+AFS cells were selected using a sheep-specific primary monoclonal IgG1 CD34 antibody [16] immediately after fluid collection using MACS system (Miltenyi Biotec, Germany). The primary CD34 antibody was incubated with sheep AF total cells for 15 minutes on ice (1:100 concentration). After washing out the primary antibody, the secondary antibody (rat anti-mouse IgG1 MicroBeads, Miltenyi Biotec, Germany) was incubated with the previous cell suspension for15 minutes on ice (1:100 concentration). Before sorting, the MS columns were rinsed with PBS with 0.5% BSA. The CD34+ fraction of cells conjugated with microbeads was collected after washing three times with PBS. Adult sheep BM collected from the

sternum was washed by passing through a cell strainer with PBS, and CD34+ cells were selected as described above for AFS. Sheep CD34+AFS or BM cells were prepared as single cell suspensions in PBS and counted. Fresh unsorted sheep AF cells were frozen down in FBS (90%) and DMSO (10%) in liquid nitrogen for 3 to 6 months before NSG mice transplantation experiments and thawed for CD34+ sorting and transplantation. Sorted CD34+ cells were characterized using sheep-specific surface antibodies including FITC or PE conjugated CD14, CD31, CD44, CD58, CD34, and CD45 (AbD Serotec, UK) by flow cytometry.

Culture and viral transduction of sheep CD34+AFS or BM cells

Frozen CD34+AFS or BM cells were used immediately after thawing. The cell suspension was centrifuged at 1500rpm for 5 minutes, the supernatant was discarded and the thawed cells were resuspended in culture medium for viral transduction. Fresh or thawed CD34+AFS or thawed CD34+BM cells were transduced for 48 hours with a lentivector encoding the HIV-1 central polypurine tract element, the Spleen Focus Forming Virus LTR promoter, and the marker gene eGFP, at MOI=50 as previously describe [15]. The culture medium contained basal IMEM, 10% FBS, and StemSpan CC100, which contains a combination of cytokines including Flt-3, Stem cell factor (SCF), IL3, and IL6 (Stemcell Technologies, UK). Fresh or frozen CD34+ AFS or frozen CD34+BM cells ($1x10^5$) were seeded in 24-well low attachment plates with culture medium for 48 hours (37^0C, 5% CO_2). The lentivector (10ul, MOI=50) was added while seeding the cells into each well. After 48 hours of viral transduction, the cell suspension was washed and re-suspended in PBS for *in utero* autologous injection or NSG mice injection.

Xenotransplantation of NOD-SCID gamma mice and collection of peripheral blood and organs

NSG mice, up to 12 weeks of age, were sublethally irradiated (3Gy) 1 hour prior to xenotransplantation. Sheep fresh or frozen CD34+AFS cells, or adult CD34+BM cells ($3x10^5$ in 0.2ml PBS using 0.5ml insulin syringe with 27½ gauge needle) (BD, UK) were injected into the tail vein of primary or secondary NSG mice.

At four and eight weeks after transplantation, NSG recipients were sedated using vaporised Isoflurane-Vet 5% in oxygen (VetTech Solutions Ltd, UK), and the peripheral blood was sampled from an incision on the tail made using a scalpel (No. 11 blade). Blood (100ul) was collected by pipette into a heparin-rinsed microcentrifuge tube. Red blood cells (RBC) lysis solution (1ml, 5-PRIME Inc, USA) was added to each blood sample for 5 minutes at room temperature. The sample was centrifuged (1500 rpm for 5 minutes), the supernatant was removed, and the procedure was repeated with RBC lysis solution until the pellet was clean. The pellet was resuspended in 300µl PBS for flow cytometry analysis of GFP+ cells.

At 12 weeks of age, all recipient mice were sedated (vaporised Isoflurane-Vet 5% in oxygen, VetTech Solutions Ltd, UK), and blood (0.5 to 1ml) was collected via intracardiac puncture using a 22 gauge needle. The mice were sacrificed by manual dislocation of cervical cord and underwent extensive post mortem examination and tissue harvest (liver, thymus, heart, striated muscle from thigh, lung, kidney, spleen, adrenal gland and gonad). Femoral bones were collected for retrieval of bone marrow. Liver and spleen samples were ground using a syringe plunger in a sterile dish, rinsed with PBS, then filtered through a cell strainer (40µm nylon mesh, BD, UK). The BM was flushed from the femoral bones with PBS using a 22 gauge needle with syringe and then strained. RBC lysis buffer (2ml) was added to each sample for

10 minutes at room temperature and then centrifuged (1500 rpm for 5 minutes). The supernatant was removed, the pellet was suspended in 300µl PBS, and washed and centrifuged again. The supernatant was discarded and the pellet was resuspended in 300µl PBS at 4°C for flow cytometry.

Flow cytometry for eGFP detection and analysis

Single cell suspensions of fetal or lamb spleen, liver and BM were prepared immediately following animal sacrifice by straining tissue through a 40µm cell strainer. After rinsing with PBS, red-blood cell lysis buffer was added for 5 minutes at 37°C. Mononuclear cells were isolated from the umbilical cord and maternal blood samples by density gradient centrifugation using Ficoll-Paque solution (Stem Cell Technology, Canada). Single cell suspensions from control uninjected ewes and their fetuses were used as negative controls for sheep samples. Uninjected CD1 mice were used as the negative control for mice experiments. The cells were acquired on Becton Dickinson FACSCalibur and LSR II machines (Becton Dickinson, San Jose, CA), and analyzed using FlowJo version 5.7.1 software (Tree Star, USA). Ten thousand events were collected per sample.

eGFP+ signal was detected using FITC gating in all the organs of NSG mice, sheep and lambs. The organs from YFP+ mice were used as a positive control. To study transduction efficiency, the same FITC channel was used to gate the eGFP+ population, untransduced AFS cells were used as negative control, and AFS cells of YFP transgenic mice were used for the positive control.

PCR for detection of eGFP DNA

PCR to detect eGFP DNA was performed as previously described (Shaw et al, 2011). DNA from sheep AFS cells transduced with eGFP was used as a positive control.

Colony forming unit (CFU) assay

MethoCult® GF H4434 kit and M3434 kit (Stem Cell Technologies, Canada) were used to perform CFU assays for sheep CD34+AFS or BM cells and NSG mice BM cells. $CD34^+$ cells that were isolated by MACS were added into methylcellulose cocktail containing cytokines and growth factors ($1x10^4$ cells per plate). The mixture was vortexed and plated into sterile 35mm plates that were then incubated for 14 days (37°C, 5% CO_2 in humidified atmosphere). Colonies were then scored using an inverted microscope and colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), colony forming unit-granulocyte, monocyte (CFU-G/M/GM) and burst-forming unit/CFU-erythroid (BFU/CFU-eE) were recorded.

Confocal microscopy

Fresh sheep and mouse tissues, embedded in OCT, were snap frozen in methyl-butane and liquid nitrogen and were cut into 10-15μm sections using a cryostat (OTF, Bright, UK). Blocking solution was prepared with 1% BSA, 0.15% Glycine and 0.1% Triton in PBS and preserved at 4°C. The liver sample slides were co-stained with mouse monoclonal anti-GFP antibody (A11120, Invitrogen, UK) and rabbit polyclonal anti-alpha fetoprotein (AFP) antibody (ab74663, abcam, UK) at 1:100 dilution with buffer solution. Slides were incubated for 2 hours at room temperature. The secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Flour 594 donkey anti-mouse IgG (A11008 and A21203, Invitrogen, UK) were used (1:100 dilution) for another two hours incubation at room temperature. The

sample slides were observed using confocal microscopy (Leica TCS SP5 II, Germany), and the images were analyzed with the software ImageJ (NIH, US). The green positive signal indicated the eGFP+ cells, and the red signal indicated hepatocytes staining with AFP.

Results

Sheep CD34+ AF cells and CD34 BM cells both have haematopoietic potential

Amniotic fluid (mean volume 487ml, range 300-700ml) collected from first trimester time mated sheep (n=8, mean gestational age 62 days, range 60-64) at post mortem examination showed a mean total cell count of 6.25 $x10^6$ (range 6-8$x10^6$ cells), with a similar percentage of CD34+ cells for fresh (n=4) and frozen (n=4) AF samples (Figure 2A). The percentage of CD34+ cells from adult sheep BM was also similar to that in the AF (Figure 2A, 2B). CD34+ cells derived from fresh or frozen AF, and from BM were seeded into semi-solid culture *in vitro* and generated haematopoietic colonies with a similar percentage of CFU-GEMM, CFU-G/M/GM and BFU/CFU-E (Figure 2C, 2D). Both AF and BM derived CD34+ cells were positive for CD45 (96.5%, 95.1%, and 94.3% in fresh AF, frozen AF, and adult BM groups, respectively), but were negative for MSC markers including CD44 and CD58 (Figure 2E). Moreover, the cells were also homogenously negative for CD14 (monocyte marker) and CD31 (endothelial cell marker; Figure 2E).

Sheep CD34+ AF cells are effectively transduced with lentiviral vectors

Following selection of sheep CD34+ cells using MACS, cells from all three sources were transferred into ultra-low attachment 24-well plates at a seeding

density of 100000 cells/ml, and grown with conditioned? medium containing cytokines. Cells were transduced with a lentivector (HIV-SFFV vector encoding eGFP, $1x10^9$/ml, MOI=50) for 48 hours before transplantation. Mean transduction efficiency tested 72 hours after incubation using flow cytometry was 56.63% ± SD 11.0% (range 46.9 -73.2%, n=6, Figure 2F). There was no significant difference in transduction efficiency of cells between the three cell sources.

Transduced sheep eGFP+ CD34+ cells engraft haematopoietic organs in NSG mice

The experimental design is shown in Figure 1. For primary xenogeneic transplantation, sheep CD34+ containing eGFP+ and eGFP- cells from three sources ($3x10^5$ cells of fresh AF, frozen AF, and adult BM) were injected into NSG mice (n=14; fresh AF n=4, frozen AF n=6, adult BM n=4). Two mice that received injection of frozen AF cells died within 10 days due to poor tolerance to irradiation; the other animals survived long-term until scheduled post-mortem examination (12 out of 14, 85.7% survival). Blood samples collected from all primary transplanted NSG mice at 1 and 3 months were analysed using flow cytometry. Blood from YFP transgenic mice was used as a positive control, while blood from an uninjected NSG mouse was the negative control. eGFP+ cells were detected in the peripheral blood at 1 month after primary xenogeneic transplantation of sheep CD34+eGFP+ frozen AF, fresh AF, or adult BM cells (mean eGFP+ cells: 3.24%, 3.48%, and 4.9%, respectively) and at 3 months (mean eGFP+ cells: 3.12%, 3.4%, and 5.26%, respectively Figure 3A). There was a higher percentage of eGFP+ cells detected in the peripheral blood of NSG mice injected with sheep adult BM CD34+ cells compared to sheep CD34+ cells from AF sources at 3 months ($p<0.05$, ANOVA).

There was no significant difference in the percentage of eGFP+ cells in NSG mice injected with frozen or fresh sheep AF CD34+ cells. At sacrifice, eGFP+ cells were detectable in the BM of all animals transplanted with frozen AF, fresh AF, and adult BM cell sources, with no significant differences detected (n=3 per group, mean eGFP+ cells; 12.03%, 14.87%, and 15.87%, respectively, Figure 3B). After culture in semi-solid conditions, the number of eGFP+ colonies per high power field was significantly higher in BM cells of NSG mice that received transduced sheep CD34+ adult BM cells compared to those injected with transduced CD34+ sheep frozen, but not fresh, AF cells ($p<0.05$ Figure 3C). Besides blood and BM, eGFP DNA was detected by PCR in the liver (8 out of 9 transplanted NSG mice), spleen (5 out of 9), and the adrenal gland (2 out of 9; Figure 4A). There was no signal detectable in tissue samples from the heart, muscle, lung, kidney, thymus and gonad. PCR results were confirmed by FACS, and showed eGFP+ cells in the spleen and liver at 3 months after injection (Figure 4B and C). Transplantation of transduced sheep CD34+eGFP+ adult BM cells resulted in a slighter higher engraftment rate than cells from fresh AF and frozen AF sources, in the spleen (6.7%, 5.8%, and 5.2%), and in liver (15.6%, 14.3%, and 8.5%, Figure 4B), but this difference did not reach significance. Only in the liver, did NSG mice that received frozen AF cells show a significantly lower engraftment than the other two groups ($p<0.05$, ANOVA; Figure 4C). Immunofluorescence study showed the presence of eGFP+ cells in liver samples of all transplanted NSG mice, that were cuboid in shape, consistent with hepatocyte morphology (Figure 4D). Co-staining with alpha fetoprotein (AFP, Figure 4E) showed a few double-stained cells providing further evidence of hepatocyte differentiation. The percentage of eGFP+ cells per high power field in liver samples

was not significantly different among the three cell sources (1.2% to 1.8%) (Figure 4F).

Secondary transplantation into NSG mice

Secondary NSG mice were transplanted with BM cells collected from femoral bones of primary NSG mice recipients transplanted with frozen (n=3) and fresh (n=3) AF, and adult BM (n=2) CD34+ cells at 3 months time (Figure 2). Freshly isolated BM cells ($3x10^6$ per recipient) were injected into the tail vein of secondary NSG recipients. As for primary transplantation, these mice were treated with a sub-lethal dose of irradiation one hour before transplantation. Survival to scheduled post-mortem examination at three months was 100%. At one month after transplantation, eGFP+ cells could be detected in the peripheral blood of 6 out of 8 secondary transplanted animals (2 out of 3 frozen AF, 2 out of 3 fresh AF, and 2 out of 2 adult BM group, Figure 5A). There was a significantly higher level of engraftment in the peripheral blood of secondary NSG recipients that were transplanted with the BM from transduced sheep CD34+eGFP+ BM cell injected NSG primary recipients compared with frozen AF and fresh AF groups. The percentage of eGFP+ cells in the peripheral blood of secondary transplanted mice was around 2 to 4%, and these levels were maintained up to the scheduled post-mortem examination at 3 months post-transplantation (Figure 5B). Flow cytometric analysis of other haematopoietic organs three months after transplantation showed the presence of eGFP+ cells in the liver, spleen, and BM of the secondary NSG recipients that also had eGFP+ cells in the peripheral blood. Animals exhibiting no eGFP+ cells in their peripheral blood also showed no engraftment in liver, spleen and BM. There were no eGFP+ cells detected in the thymus of any animals at three months after transplantation (Figure 5B).

In utero autologous transplantation with sheep CD34+ AFS cells

Time-mated pregnant ewes (n=5, 10 twin fetuses) were studied (Figure 1). The overall survival to live-birth after the operative procedure was 50% (5 out of 10). In one twin pregnancy, both fetuses miscarried within a week of the amniocentesis procedure. In another twin pregnancy, both fetuses had reduced fetal growth velocity around 100 days and were found to have died *in utero* at 126 days of gestation. Bacterial cultures of endometrium showed evidence of endometritis with a coliform. In a second twin pregnancy, one twin demised at 90 days of gestation, but the other continued to term and was born healthy. Fetal growth velocity was normal in all cases that survived to birth. Five lambs of three ewes were delivered at term. All lambs had normal initial assessments at birth (time to stand, heart rate, respiratory rate, O2 saturation and temperature) and birthweights were within the normal range. In each of the born twin pairs, one twin lamb was rejected by the ewe and both lambs became dehydrated within 24 hours of birth. One ewe had obvious mastitis, but the other ewe did not. Despite artificial feeding, the lambs' condition deteriorated and an elective post-mortem examination was performed two days after birth on the advice of the veterinary surgeons. There was no evidence of structural abnormality or infection at post-mortem examination of these lambs. The remaining three lambs grew at a normal rate, and survived to scheduled post-mortem examination at 6 months of age.

Long-term engraftment in lambs after in utero autologous transplantation

eGFP+ cells were detected in the peripheral blood of all lambs that survived to birth (mean = 2.72%, range 1.6% to 4.5%, Figure 6B). In those lambs that survived long-term, the peripheral blood remained eGFP+ up to scheduled post-mortem

examination at 6 months of age (M1-A, M2-B, and M3-A, Figure 6B). The strength of the eGFP+ signal started to decrease after 4 weeks of age (4 months after *in utero* injection), but the level of eGFP+ cells in the blood remained steady at around 2% up to 6 months of age. Samples of haematopoietic organs were collected from lambs at birth (n=2) and eGFP+ cells were detected by flow cytometry in the liver (2.9%, 4.3%) and the BM (2.2%, 2.5%). The spleen and thymus were negative for eGFP+ cell engraftment in both lambs. At 6 months of age, BM was collected from the surviving lambs (n=3) from multiple sites (femur, humerus, iliac crest and sternum) under terminal anaesthesia as part of the scheduled post-mortem examination. eGFP+ cells were detected in the BM (mean = 4.05%, range 2.43 to 5.47%, Figure 7A), and also in the peripheral blood and liver (data not shown).

Secondary xenogeneic transplantation of AF-derived sheep haematopoietic-repopulating cells into NSG recipient mice

In order to further confirm the haematopoietic potential of CD34+ AF cells, whole unfractionated BM isolated from the transplanted lambs was transplanted into NSG secondary recipient mice ($3X10^5$ cells, n=9, with BM from one lamb being transplanted into three NSG recipients) with 100% survival to scheduled post-mortem examination at three months post-transplantation. eGFP+ cells could be detected in the peripheral blood at 1, 2, and 3 months after secondary transplantation (2 out of 9 mice). At three months after secondary transplantation, eGFP+ cells were detected by flow cytometry in the liver, blood, and BM of secondary NSG recipient mice (Figure 7B, 7C). PCR analysis of haematopoietic

tissues also demonstrated eGFP+ DNA in the liver, blood, BM, spleen and thymus (Figure 7C).

Discussion

In this study we have demonstrated that CD34+ cells can be isolated from sheep amniotic fluid, transduced, and reconstitute haematopoiesis in primary and secondary NSG recipient mice, with evidence of engraftment in multiple haematopoietic organs. In addition, autologous IUSCT of these cells into donor sheep fetuses results in long-term engraftment in the peripheral blood and haematopoietic organs up to the last sampling time of 6 months after birth (9 months post-IUSCT). Finally, transduced cells derived from those animals maintained their *in vivo* haematopoietic potential and showed haematopoietic engraftment when transplanted into NSG recipients.

Our results showed that CD34+ cells isolated from sheep AF and BM possess a similar haematopoietic potential. AF and BM sheep cells isolated using a novel sheep-specific CD34 antibody were also positive for CD45, but negative for CD31, CD14, CD44, CD58, a haematopoietic phenotype which is in keeping with their potential to form colonies, further proving that the presence of cells in the AF with haematopoietic potential is not limited to mice and humans [13]. After being transduced, cells were transplanted without sorting for two reasons. Firstly, we focused on maximising cell number since the efficiency of gene transfer achieved in the CD34+ sheep AF or BM cells was relatively high at around 60%. This is higher that what has been previously reported in human bone marrow-derived MSC, HSC, and embryonic stem cells, in which gene transfer efficiency was 40% [18], 50% [19],

and 14-48% [20], respectively. We have previously shown that human AFS cells can be efficiently transduced using adenoviral vectors [21], and we achieved a similar transduction efficiency using a lentivector in sheep AF-derived MSCs [15]. Lentivectors also have the advantage of allowing sustained transgenic protein expression [22]. Secondly, we wished to avoid clonal selection of the cells and rather to observe what could happen with minimal cell manipulation.

After primary transplantation into NSG recipients, the haematopoietic potential of these cells was demonstrated using flow cytometry, PCR, and immunostaining, which confirmed that these cells engrafted in most of the haematopoietic organs. CD34+ BM cells tended to have a slightly higher rate of engraftment compared to CD34+ cells derived from frozen AF, but a similar rate to cells derived from fresh AF. This is in keeping with the finding that haematopoietic stem cells could be more sensitive to cryopreservation, as described before for other cell sources such as peripheral blood stem cells [23].

Having demonstrated their haematopoietic potential in NSG mice, we tested whether AF-derived CD34+ stem cells could engraft following autologous IUSCT in sheep. Transduced autologous cells readily migrated from the peritoneal cavity injection site to engraft all of the major haematopoietic organs, providing continued transgenic protein (eGFP) expression in multiple fetal organs, the BM, and the peripheral blood. Tissue analysis by PCR, immunofluorescence, and flow cytometry revealed that CD34+ AF or BM cells preferentially engrafted, as expected, in the fetal liver and other haematopoietic organs [24]. Admittedly, the level of tissue engraftment was low, which is likely due to the small number of autologous cells transplanted. For some congenital diseases however, such as severe haemophilia for example, only 1% level of protein expression is required to ameliorate the severe

phenotype [25]. Thus, for many congenital diseases the levels of engraftment we achieved may be sufficient to improve the phenotype or even effect a cure. Increasing the number of cells delivered is likely to lead to improved levels of engraftment. Currently however, expansion of AF stem cells results in the production of committed MSC progenitors, and expansion while maintaining haematopoietic progenitors has not yet been achieved. Other options for increasing the level of engraftment rate could include targeting the liver with direct injection, resulting in a degree of liver damage that could potentially enhance liver engraftment. Indeed, prior work demonstrated that intrahepatic injection of human MSC into first trimester fetal sheep gave a higher level of engraftment (12.5%), particularly in the parenchyma, when compared with intraperitoneal injection (2.6%), which resulted in higher periportal generation of hepatocytes [26]. A combined injection approach might therefore result in the highest level and broadest distribution of donor-derived hepatocytes. In current clinical practice, however, intraperitoneal injection is the route of choice for fetal transfusion in the first trimester, due to its inherent safety, but improvements in fetal imaging may make first trimester intravascular injection more feasible. Damaging an organ such as the liver or muscle provides a proliferation stimulus for the transplanted stem cells and may create a donor stem cell advantage for engraftment. For example, protocols such as postnatal boosting after irradiation, donor lymphocyte infusion [27, 28] or co-transplantation of MHC-matched purified facilitating cells [29] have been used successfully in the fetal mouse to improve engraftment.

A limitation of this study was the lack of data on the fetal and maternal immune response, since we were concentrating on demonstrating haematopoiesis and engraftment. Detailed studies on the immune response comparing autologous

with allogeneic and maternal HLA-matched IUSCT will be necessary to study immune rejection of *in utero* transplanted cells.

For clinical translation, demonstration that autologous IUSCT can cure congenital haematopoietic disease long term is required in a large animal model of disease. Our future work aims to test this in a haemophilia A sheep model [30]. Recent data in this model showed that postnatal transplantation with paternal MSCs transduced with a porcine FVIII-encoding lentivector and transplanted via the intraperitoneal route without preconditioning resulted in a phenotypic correction for up to a year after transplantation, but long-term cure was not possible due to the development of antibodies to the porcine FVIII protein [31]. The use of autologous cells and ovine FVIII may avoid this problem. The immune response and safety of IUSCT using transduced autologous AF cells for the fetus and mother will also need to be explored, and suitable vectors developed that produce regulated long term expression. Ultimately repeated postnatal infusion of transduced autologous AF cells may be required. Prenatal screening and diagnosis for haematological diseases such as thalassaemia and sickle cell already exists in many countries with the current aim of offering couples termination of pregnancy if an affected fetus is detected. Non-invasive prenatal diagnosis using circulating free fetal DNA from the mother's blood is now a clinical reality for some congenital disease and will allow the earlier detection of fetuses affected with haematological disease. Autologous IUSCT with gene corrected cells may provide a treatment option.

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Authorship Contributions

SWSS designed and performed the research, collected data, analysed and interpreted data, performed statistical analysis and wrote the manuscript.

MB designed and performed the research, collected data, analysed and interpreted data

CP collected data, analysed and interpreted data

PS collected data, analysed and interpreted data

JL collected data, analysed and interpreted data

PM collected data, analysed and interpreted data

MB performed the research and collected data

AT designed the research and interpreted the data

CDP contributed vital new reagents and interpreted the data

GA-P contributed vital new reagents and interpreted the data

AP analysed and interpreted the data

PJC analysed and interpreted the data

ALD designed and performed the research, collected data, analysed and interpreted data, and co-wrote the manuscript.

PdeC designed and performed the research, collected data, analysed and interpreted data, and co-wrote the manuscript.

Conflict of Interest Disclosures

SWSS has no conflict of interest to declare

MB has no conflict of interest to declare

CP has no conflict of interest to declare

PS has no conflict of interest to declare

JL has no conflict of interest to declare

PM has no conflict of interest to declare

MB has no conflict of interest to declare

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ALD has no conflict of interest to declare

PdeC has no conflict of interest to declare

References

1. Burt RK. Clinical utility in maximizing CD34+ cell count in stem cell grafts. Stem Cells. 1999;17:373-376.
2. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. Nature. 1953;172:603-606.
3. Surbek D, Schoeberlein A, Wagner A. Perinatal stem-cell and gene therapy for hemoglobinopathies. Semin Fetal Neonatal Med. 2008;13:282-290.
4. Tiblad E, Westgren M. Fetal stem-cell transplantation. Best Pract Res Clin Obstet Gynaecol. 2008;22:189-201.
5. Westgren M, Ringden O, Eik-Nes S et al. Lack of evidence of permanent engraftment after in utero fetal stem cell transplantation in congenital hemoglobinopathies. Transplantation. 1996;61:1176-1179.
6. Merianos DJ, Tiblad E, Santore MT et al. Maternal alloantibodies induce a postnatal immune response that limits engraftment following in utero hematopoietic cell transplantation in mice. The Journal of clinical investigation. 2009;119:2590-2600.
7. Nijagal A, Wegorzewska M, Le T et al. The maternal immune response inhibits the success of in utero hematopoietic cell transplantation. Chimerism. 2011;2:55-57.
8. Peranteau WH, Endo M, Adibe OO et al. Evidence for an immune barrier after in utero hematopoietic-cell transplantation. Blood. 2007;109:1331-1333.
9. De Coppi P, Bartsch G, Jr., Siddiqui MM et al. Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol. 2007;25:100-106.

10. Prusa A, Hengstschlager M. Amniotic fluid cells and human stem cell research–a new connection. Signature. 2002;8:253-257.

11. Fauza D. Amniotic fluid and placental stem cells. Best Pract Res Clin Obstet Gynaecol. 2004;18:877-891.

12. Piccoli M, Franzin C, Bertin E et al. Amniotic fluid stem cells restore the muscle cell niche in a HSA-Cre, Smn(F7/F7) mouse model. Stem Cells. 2012;30:1675-1684.

13. Ditadi A, de Coppi P, Picone O et al. Human and murine amniotic fluid c-Kit+Lin- cells display hematopoietic activity. Blood. 2009;113:3953-3960.

14. Galende E, Karakikes I, Edelmann L et al. Amniotic fluid cells are more efficiently reprogrammed to pluripotency than adult cells. Cell Reprogram. 2010;12:117-125.

15. Shaw SW, Bollini S, Nader KA et al. Autologous transplantation of amniotic fluid-derived mesenchymal stem cells into sheep fetuses. cell transplantation. 2011;20:1015-1031.

16. Porada CD, Harrison-Findik DD, Sanada C et al. Development and characterization of a novel CD34 monoclonal antibody that identifies sheep hematopoietic stem/progenitor cells. Experimental hematology. 2008;36:1739-1749.

17. David A, Cook T, Waddington S et al. Ultrasound-guided percutaneous delivery of adenoviral vectors encoding the beta-galactosidase and human factor IX genes to early gestation fetal sheep in utero. Hum Gene Ther. 2003;14:353-364.

18. Zhang XY, La Russa VF, Reiser J. Transduction of bone-marrow-derived mesenchymal stem cells by using lentivirus vectors pseudotyped with

modified RD114 envelope glycoproteins. Journal of virology. 2004;78:1219-1229.

19. Sutton RE, Wu HT, Rigg R et al. Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. Journal of virology. 1998;72:5781-5788.

20. Gharwan H, Hirata RK, Wang P et al. Transduction of human embryonic stem cells by foamy virus vectors. Molecular therapy : the journal of the American Society of Gene Therapy. 2007;15:1827-1833.

21. Grisafi D, Piccoli M, Pozzobon M et al. High transduction efficiency of human amniotic fluid stem cells mediated by adenovirus vectors. Stem Cells Dev. 2008;17:953-962.

22. Philippe S, Sarkis C, Barkats M et al. Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. Proc Natl Acad Sci U S A. 2006;103:17684-17689.

23. Lioznov M, Dellbrugger C, Sputtek A et al. Transportation and cryopreservation may impair haematopoietic stem cell function and engraftment of allogeneic PBSCs, but not BM. Bone Marrow Transplant. 2008;42:121-128.

24. Almeida-Porada G, Porada C, Gupta N et al. The human-sheep chimeras as a model for human stem cell mobilization and evaluation of hematopoietic grafts' potential. Exp Hematol. 2007;35:1594-1600.

25. Waddington SN, Nivsarkar MS, Mistry AR et al. Permanent phenotypic correction of hemophilia B in immunocompetent mice by prenatal gene therapy. Blood. 2004;104:2714-2721.

26. Chamberlain J, Yamagami T, Colletti E et al. Efficient generation of human hepatocytes by the intrahepatic delivery of clonal human mesenchymal stem cells in fetal sheep. Hepatology. 2007;46:1935-1945.

27. Hayashi S, Peranteau WH, Shaaban AF et al. Complete allogeneic hematopoietic chimerism achieved by a combined strategy of in utero hematopoietic stem cell transplantation and postnatal donor lymphocyte infusion. Blood. 2002;100:804-812.

28. Peranteau WH, Hayashi S, Hsieh M et al. High-level allogeneic chimerism achieved by prenatal tolerance induction and postnatal nonmyeloablative bone marrow transplantation. Blood. 2002;100:2225-2234.

29. Gaines BA, Colson YL, Kaufman CL et al. Facilitating cells enable engraftment of purified fetal liver stem cells in allogeneic recipients. Exp Hematol. 1996;24:902-913.

30. Porada CD, Sanada C, Long CR et al. Clinical and molecular characterization of a re-established line of sheep exhibiting hemophilia A. J Thromb Haemost. 2010;8:276-285.

31. Porada CD, Sanada C, Kuo CJ et al. Phenotypic correction of hemophilia A in sheep by postnatal intraperitoneal transplantation of FVIII-expressing MSC. Experimental hematology. 2011;39:1124-1135 e1124.

Figure Legends

Figure 1. *Experimental design.*

Transduced sheep eGFP+ CD34+ selected fresh or frozen amniotic fluid (AF) and adult bone marrow (BM) cells were transplanted into immunocompromised NSG mice (primary and secondary xenogeneic transplantation). Transduced sheep eGFP+CD34+ fresh AF cells were also injected into donor sheep fetuses (*in utero* autologous transplantation) that were subsequently delivered and followed for up to 3 months of age. Bone marrow from these primary sheep recipients was then used to perform xenogeneic secondary transplantation into NSG mice.

Figure 2. Characteristics of sheep CD34+ cells from frozen and fresh AF, and from adult BM.

(A) Proportion and (B) characteristics of CD34+ cells isolated from three sheep cell sources. There was no significant difference in the proportion of CD34+ cells isolated from the three cell sources. AF: amniotic fluid (frozen or fresh); BM: bone marrow. Error bar: standard deviation. (C) The morphology and percentage (D) of the three different types of haematopoietic colonies that grew in semi-solid culture of sheep CD34+ AF or adult BM cells. CFU-GEMM: colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte; CFU-G/M/GM: colony forming unit-granulocyte/monocyte/granulomonocyte; BFU/CFU-E: burst-forming unit/colony forming unit-erythroid. (E) Example of a flow cytometry study to characterise sheep CD34+ cells from fresh AF. Cells are positive for CD45, but negative for CD14, CD31, CD44, and CD58. Red line: negative control, blue line: cells tested. (F) Flow cytometry showing eGFP+ cells 48 hours after lentivector (HIV.SFFV.eGFP) gene transfer to CD34+ sheep AF cells. X-axis: GFP channel, Y-axis: cell number count,

the number on the gate represents the percentage of eGFP+ positive cells out of total population.

Figure 3. Analysis for engraftment of transduced sheep CD34+eGFP+ AF or adult BM cell sources in the peripheral blood and BM of primary transplanted NSG mice.

(A) Peripheral blood engraftment. Left panel shows a representative flow cytometric dot plot of peripheral blood from NSG mice injected with frozen or fresh AF or adult BM cells respectively. The distinct population of cells in the upper right quadrant of each graph exhibit a positive signal for eGFP. X-axis: GFP channel, Y-axis: PE channel. The histogram (right panel) compares blood engraftment after injection of transduced CD34+eGFP+ sheep cells from the three sources (n=4, each group) at 1 and 3 months after transplantation. There was significantly higher engraftment when the cell source was adult sheep BM (black bar) compared with cells sourced from frozen AF (light grey bar) or fresh AF (dark grey bar). Error bar: standard deviation; *: $p<0.05$; AF: amniotic fluid. Blood from YFP transgenic mice was used as a positive control while blood from uninjected NSG mice served as the negative control. (B) NSG mice BM engraftment. Left panel shows an example of flow cytometry to analyse engraftment of eGFP+ cells in the BM of primary NSG mouse recipients three months after transplantation. X-axis: eGFP+ cells, Y-axis: PE channel. The distinct population of cells in the upper right quadrant of each graph exhibit a positive signal for eGFP. Negative control: BM from uninjected mice; positive control: YFP transgenic mice. The histogram (right panel) compares the percentage of eGFP+ cells in the BM of primary NSG mouse recipients of transduced sheep CD34+eGFP+ cells from the three sources (n=4, each group) at 3 months after transplantation.

There was no significant difference in the percentage of engraftment in the BM. The BM cells from transgenic YFP mice were used as the positive control, and bone BM cells from uninjected mice were used for the negative control. Error bar: standard deviation. (C) Engrafted cells characteristics. The left panel shows the colony-forming assay to study eGFP+ cells in the BM of primary mouse NSG recipients. Positive green colonies showing characteristics of all three major progenitor cell types could be detected. Positive control: BM from a YFP mouse, Negative control: BM from uninjected mice. Upper panel: phase contrast field; lower panel: green fluorescence signal. Bar: 100μm. Histogram (right panel) shows the percentage of eGFP+ colonies in the BM of primary NSG recipient mice three months after transplantation with transduced sheep CD34+ eGFP+ cells from three cell sources. BM from NSG mice transplanted with cells from adult sheep BM had a significantly higher percentage of eGFP positive colonies than the other two groups (*p<0.05, ANOVA). Error bar: standard deviation.

Figure 4. Engraftment analysis of other organs of primary transplanted NSG mice.

(A) The table shows PCR detection of eGFP+ DNA in the tissues of NSG mice transplanted with sheep CD34+eGFP+ cells. Eight out of 9 animals showed an eGFP+ signal in the liver, while 5 out of 9 and 2 out of 9 were positive in the spleen and adrenal gland respectively. Sheep-specific beta-actin was used for the internal control primer. (B) Analysis for engraftment in other haematopoietic tissues. The left panel shows the flow cytometry results. The bar in the graphs show the percentage of eGFP+ cells in tissues as presented by the small peak located to the right. The right panel shows the summary graphs. Cell sources were frozen AF (light grey),

fresh AF (dark grey) and adult BM (black). In the liver, there was a significantly lower percentage of eGFP+ cells after transplantation with the frozen AF cell source when compared to the other two cell sources. There was no difference in spleen engraftment. Error bar: standard deviation, *: $p<0.05$. (C) Confocal microscopy shows eGFP+ cells in the liver of transplanted NSG mice. Green: eGFP in the cytoplasm. Blue: DAPI for the nucleus. Bar: 10μm. (D) Immunofluorescence study on a liver section from an NSG mouse transplanted with fresh CD34+eGFP+ AF cells. In the liver, rare cells that co-stained with eGFP and alpha-fetoprotein (AFP) were found. Bar: 10μm. (E) Level of liver engraftment: eGFP+ cells per high power field (HPF) were counted by three individuals blinded to the study. There was no statistically significant difference between the groups (ANOVA).

Figure 5. Analysis of NSG mice after secondary transplantation.

(A) Flow cytometry analysis of the peripheral blood of secondary NSG mice recipients at 1, 2, and 3 months after transplantation. The bone marrow (BM) cell source group (NSG21, NSG22, blue lines) showed higher level of eGFP+ cells in the blood than the frozen amniotic fluid (AF; red lines) and fresh AF (green lines) cell source groups. No cells were detected in the blood of two animals injected with frozen or fresh AF (NSG16 and NSG20). Control: uninjected animal (grey line). (B) Flow cytometry of the haematopoietic organs of NSG mice recipients showing the percentage of eGFP+ cells three months after secondary transplantation.

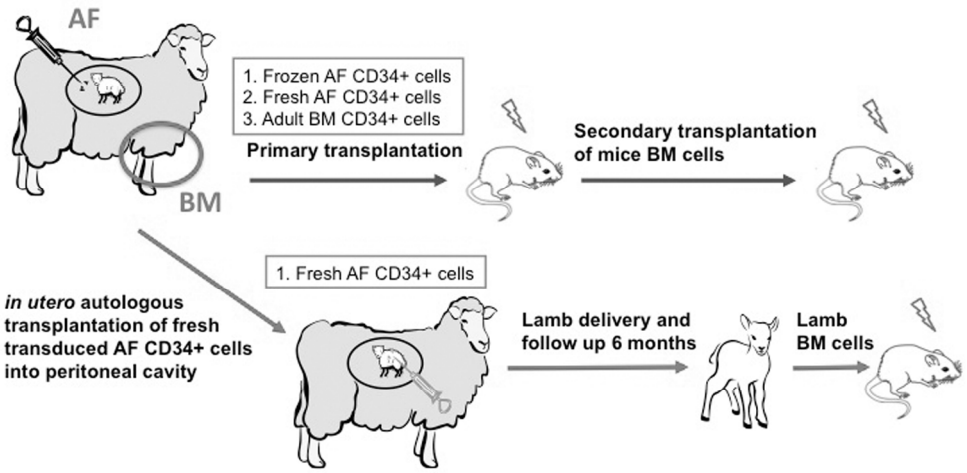
Figure 6. In utero autologous intraperitoneal transplantation of sheep amniotic fluid CD34+ cells in fetal sheep with long-term follow up.

(A) Summary of fetal sheep experiments. Ultrasound-guided amniocentesis was used to collect 20ml of AF from each fetus, and CD34+ cells were selected. After 48 hours of lentivector transduction *in vitro* the transduced CD34+AF-derived cells were injected back into the donor fetus by ultrasound-guided intraperitoneal injection. (B) Engraftment in the peripheral blood after *in utero* transplantation of autologous sheep CD34+eGFP+ AF cells. All five born lambs showed eGFP+ cells in the peripheral blood at birth, and all three survivors revealed persistent levels of engraftment of around 2% up to the last sampling point at 6 months of age. Negative control: peripheral blood from uninjected sheep. M3, M4 and M5: the three ewes that showed negativity for eGFP signal.

Figure 7. Secondary xenogeneic transplantation of the BM cells from in utero autologous transplanted lambs into NSG mice.

(A) Following *in utero* transplantation of transduced autologous sheep CD34+eGFP+ AF cells, lamb BM (n=3) was collected at 6 months of age and was confirmed to be positive for eGFP by flow cytometry. (B) These lamb BM samples were transplanted into secondary NSG recipients (xenogeneic transplantation) and flow cytometry of peripheral blood, liver and BM of these secondary recipients demonstrated eGFP+ cells. Negative control: uninjected lamb BM cells; Positive control: BM cells of YFP transgenic mice. (C) Summary table showing results of secondary xenotransplantation of BM from *in utero* transplanted lambs into NSG recipients. +: positive for eGFP band by PCR; -: negative for eGFP; n/a: not available. Positive

control: samples from YFP mice, Negative control: samples from un-injected NSG mouse.



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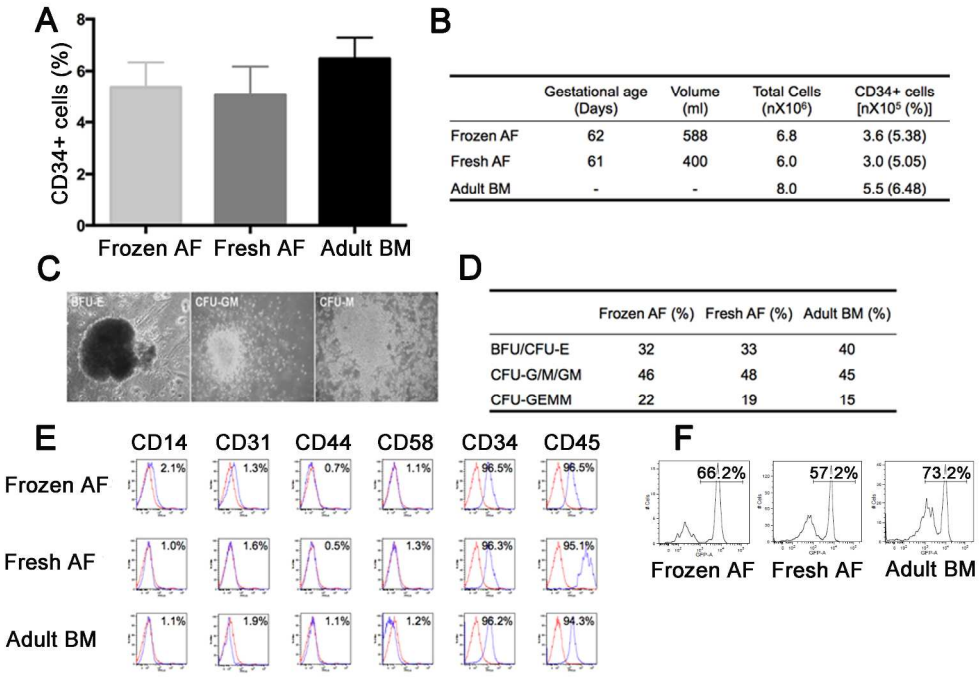

	Gestational age (Days)	Volume (ml)	Total Cells ($nX10^6$)	CD34+ cells [$nX10^5$ (%)]
Frozen AF	62	588	6.8	3.6 (5.38)
Fresh AF	61	400	6.0	3.0 (5.05)
Adult BM	-	-	8.0	5.5 (6.48)

	Frozen AF (%)	Fresh AF (%)	Adult BM (%)
BFU/CFU-E	32	33	40
CFU-G/M/GM	46	48	45
CFU-GEMM	22	19	15

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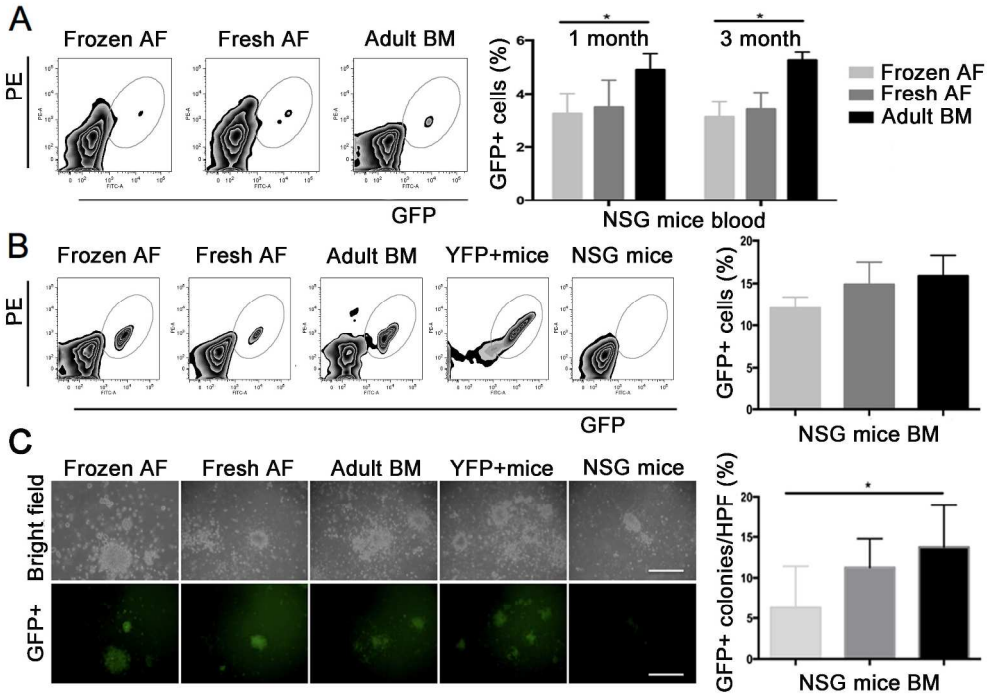

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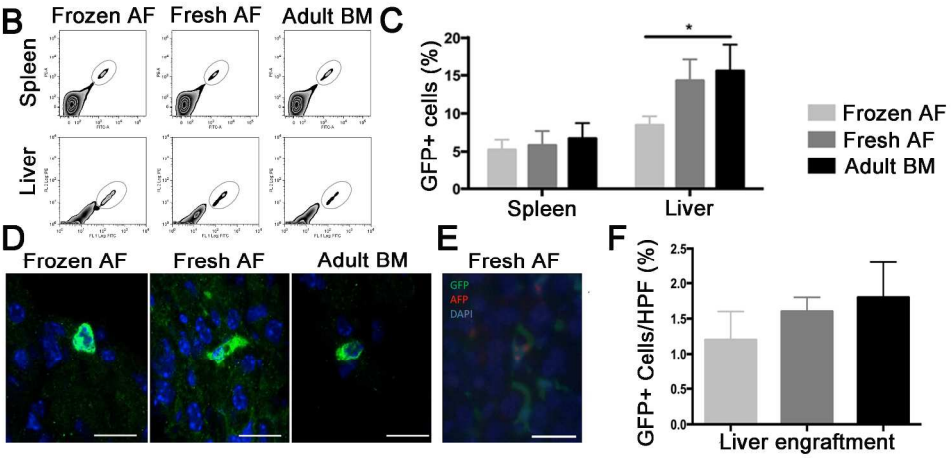
A

Animal	CD34+ Cells	Liver	Heart	Muscle	Lung	Kidney	Spleen	Adrenal	Gonad
NSG3	Frozen AF	+	-	-	-	-	-	-	-
NSG4	Frozen AF	+	-	-	-	-	+	-	-
NSG5	Frozen AF	-	-	-	-	-	-	-	-
NSG7	Fresh AF	+	-	-	-	-	+	+	-
NSG8	Fresh AF	+	-	-	-	-	+	-	-
NSG9	Fresh AF	+	-	-	-	-	-	-	-
NSG11	Adult BM	+	-	-	-	-	+	+	-
NSG12	Adult BM	+	-	-	-	-	-	-	-
NSG13	Adult BM	+	-	-	-	-	+	-	-



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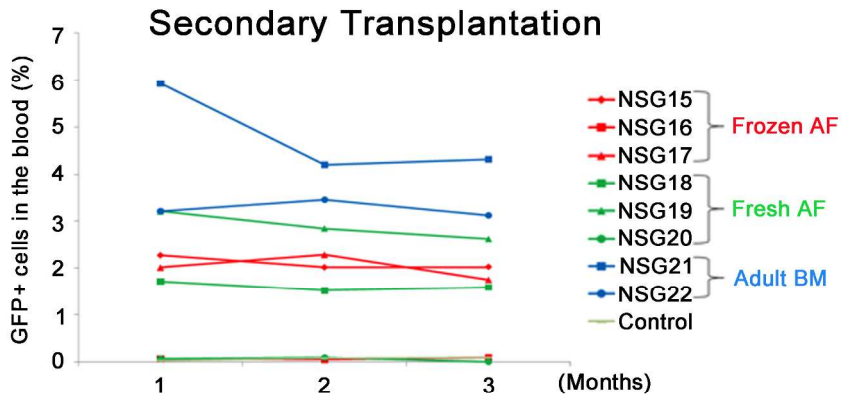
A



B

Cells injected	Animal	Blood, 1M	Blood, 2M	Blood, 3M	Liver	Spleen	BM	Thymus
Frozen AF	NSG15	2.27	2.01	2.02	3.15	1.31	2.34	0.43
Frozen AF	NSG16	0.07	0.05	0.09	0.27	0.31	0.41	0.13
Frozen AF	NSG17	2.01	2.28	1.75	2.45	2.12	2.54	0.01
Fresh AF	NSG18	1.71	1.51	1.58	2.69	1.12	1.98	0.32
Fresh AF	NSG19	3.2	2.84	2.62	4.12	2.74	3.12	0.14
Fresh AF	NSG20	0.07	0.09	0.01	0.47	0.21	0.35	0.34
BM	NSG21	5.92	4.21	4.32	4.76	2.55	4.12	0.21
BM	NSG22	3.21	3.45	3.12	4.49	2.34	3.23	0.18
uninjected	Control	0.03	0.08	0.09	0.16	0.24	0.22	0.45

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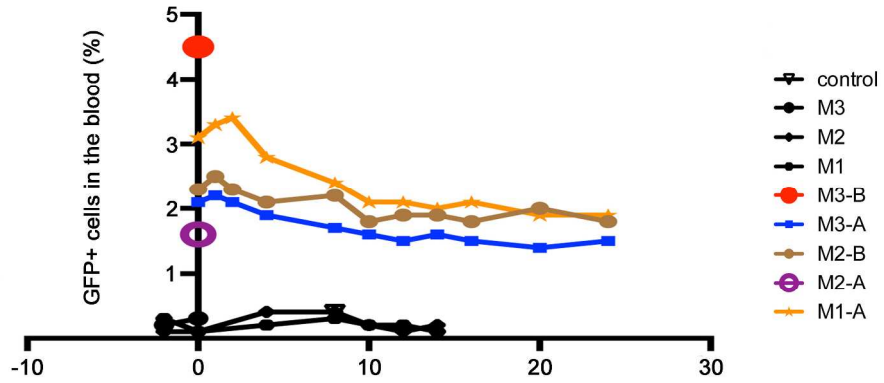
A

Ewe	Lamb	Gestational age (Days) Amniocentesis	Transplantation	Delivery	Postmortem	CD34+ cells injected	injected volume (ml)	MOI
M1	M1-A	60	62	145	6 months	20000	2	50
	M1-B	60	62	*	-	20000	2	50
M2	M2-A	63	65	145	1 day PD	20000	2	50
	M2-B	63	65	145	6 months	20000	2	50
M3	M3-A	63	65	145	6 months	20000	2	50
	M3-B	63	65	145	1 day PD	20000	2	50

B



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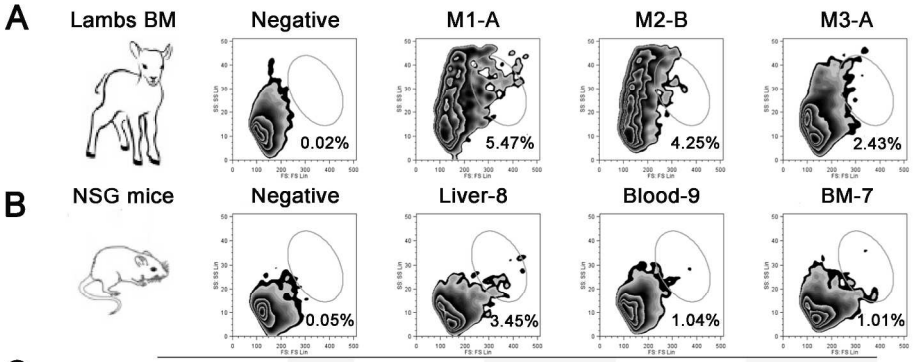

C

		Blood	Liver		BM		Spleen		Thymus	
Lamb	mice ID	flow	PCR	flow	PCR	flow	PCR	flow	PCR	flow
M1-A	1	-	-	3.72	n/a	-	-	-	n/a	-
M1-A	2	-	-	2.78	n/a	-	-	-	-	2.13
M1-A	3	3.74	+	1.37	n/a	-	-	-	+	-
M2-B	4	-	+	8.64	n/a	-	-	-	+	-
M2-B	5	-	+	-	+	-	+	4.15	+	-
M2-B	6	-	n/a	-	n/a	-	+	-	-	-
M3-A	7	-	+	1.05	+	1.01	-	3.69	+	-
M3-A	8	-	+	3.45	+	-	+	7.04	+	-
M3-A	9	1.04	-	2.45	-	-	-	-	n/a	-

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