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Influence of a dual-injection regimen, plerixafor, and CXCR4 on *in-utero* hematopoietic stem cell transplantation and engraftment using the sheep model

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Abstract

Background—Inadequate engraftment of hematopoietic stem cells (HSCs) following *in-utero* HSC transplantation (IUHSC) remains a major obstacle for the prenatal correction of numerous hereditary disorders. HSCs express CXCR4 receptors that allow homing and engraftment in response to SDF1 ligand present in the bone marrow (BM) stromal niche. Plerixafor, a mobilization drug, works through the interruption of the CXCR4-SDF1 axis.

Methods—We used the fetal sheep large animal model to test our hypotheses that: a) by administering plerixafor *in-utero* before performing IUHSC to release fetal HSCs and thus vacating recipient HSC niches, b) by using human mesenchymal stromal/stem cells (MSCs) to immunomodulate and humanize the fetal BM niches, and c) by increasing the CXCR4⁺ fraction of CD34⁺ HSCs, we could improve engraftment. Human cord blood-derived CD34⁺ cells and human bone marrow-derived MSCs were used for these studies.

Results—When MSCs were transplanted one week prior to CD34⁺ cells with plerixafor treatment, we observed 2.80% donor hematopoietic engraftment. Combination of this regimen with additional CD34⁺ cells at the time of MSC infusion increased engraftment levels to 8.77%. Next, increasing the fraction of CXCR4⁺ cells in the CD34⁺ population albeit transplanting at a late gestation age was not beneficial. Our results show engraftment of both lymphoid and myeloid lineages.

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Discloser of Interest

The authors have no conflict of interest to report.

Discussion—Prior MSC and HSC cotransplantation followed by manipulation of the CXCR4-SDF1 axis in IUHSCT provides an innovative conceptual approach for conferring competitive advantage to donor HSCs. Our novel approach could provide a clinically relevant approach for enhancing engraftment early in the fetus.

Keywords

hematopoietic stem cell transplantation; in utero transplantation; CXCR4; SDF1; plerixafor; sheep model

Introduction

In-utero hematopoietic stem cell transplantation (IUHSCT) provides the opportunity for transplanting cells from an allogeneic donor into the early fetus to correct numerous genetic disorders of hematological, immunological, and metabolic etiologies, that could be diagnosed prenatally (1). IUHSCT offers the promise of the delivery of a healthy baby and preventing the consequences of the disease at its earliest stages. Furthermore, this procedure provides therapeutic advantages of a fetal environment such as acceptance of unmatched allogeneic donor cells in the preimmune fetus and engraftment without the need for conditioning regimen in the rapidly expanding bone marrow (BM) niche. The fetal sheep is a relevant pre-clinical animal model for IUHSCT with a large body size and long gestation such that chronology of procedures and dosing of cells/cytokines/pharmaceuticals are easily translatable to the human clinical scenario (2). Rodent models of IUHSCT have also proved useful, especially with the availability of recipients lacking certain immune cells. As such, the murine anemic model and severe combined immunodeficient (SCID) model demonstrate better engraftment than normal mice following IUHSCT, similar to the observation with SCID patients where donor cells have an advantage over recipient HSC for populating the niche (3, 4). Unfortunately, the IUHSCT of human donor cells into immune competent models, mice (5) or sheep (6, 7), results in only low levels of engraftment in those recipients that do engraft, which is also a key reflection of limitations facing patients in actual clinical settings.

Immunological hurdles to achieving clinically relevant levels of engraftment that have recently been identified include maternal alloantibodies, maternal T cells, and recipient NK cells (8-10). Herein, we propose that access to the fetal BM HSC niche must also be of prominence, for engraftment in the absence of conditioning regimens is a competitive process between donor and recipient HSCs for populating limited niche space (11, 12). We therefore hypothesized that vacating the fetal HSC niche prior to IUHSCT would increase available niche spaces for incoming donor cells. Standard conditioning regimens for vacating BM niches are prohibitively toxic at the fetal stage of development. Plerixafor (AMD3100) is a drug that mobilizes HSCs out of the BM into the peripheral blood (PB) with no cytotoxicity so that HSCs return to the BM niche when drug effects subside (13, 14). BM stromal cells present stromal derived factor 1 (SDF1) (also known as C-X-C ligand 12 (CXCL12)), which functions as the ligand for the C-X-C receptor 4 (CXCR4) present on HSCs (15), whereas plerixafor, an antagonist for SDF1, disrupts this ligand-receptor axis. Plerixafor has been administered to pediatric patients as young as 2 months of age (16). In

this study we explored a novel use for this drug and administered plerixafor just prior to injecting donor HSCs in the fetus. We estimated that at 4-6 hours after dosing when the effects of plerixafor start to diminish (17), donor and recipient HSCs in circulation would home to the BM. In this manner, donor cells would have better access to the vacated recipient HSC niche and may have competitive advantage due to their high cell numbers in the bolus injection. In using the sheep model, we also proposed that transplanting human BM-derived mesenchymal stromal/stem cells (MSCs) would result in a “humanized” sheep HSC niche. MSCs are known to promote HSC engraftment and immune recovery after HSC transplantation, probably through the provision of hematopoietic supportive elements such as cytokines, matrix proteins, and cell-to-cell contacts in the BM niche, while also modulating the immune response thereby promoting tolerance (18-24). Lastly, we tested the transplantation of HSCs with a larger fraction of CXCR4⁺ cells in the CD34⁺ population to evaluate the effect of the CXCR4 receptors in enhancing engraftment.

Materials and Methods

Cells for IUHSCCT

Cord blood (CB) units deemed unfit for clinical use due to insufficient volume at Duke University Medical Center, and BM from donors at the University of Nevada-Reno, were collected at respective institutions after approval from their institutional review boards. All cells were cryopreserved until use. CB units were thawed and sorted prior to transplantation. CD34⁺ cells were isolated via magnetic activated cell sorting (MACS) using the CD34 MicroBead kit (Miltenyi Biotec, Auburn, CA) according to manufacturer instructions. MACS-sorted populations for sheep transplantation typically were ~97% pure for CD34⁺ by flow cytometry. MSCs used in these studies were generated from adult BM and met all criteria for MSC characteristics defined elsewhere (25). Cryopreserved MSCs were thawed ~2 weeks prior to use and expanded in culture. MSCs up to passage 7 were transplanted after digestion into single cells on day of transplantation according to standard protocols (26, 27).

Up-regulation of CXCR4 receptors on HSCs

The chemokine receptor, CXCR4, can be up-regulated by hypoxia on PB cells (28). We simulated hypoxic conditions in a normoxic incubator (20% O₂, 37°C, 5% CO₂, humidified) through the inclusion of deferoxamine (DFX) (Sigma, St Louis, MO) in cell culture media as demonstrated by others (29). DFX inhibits the hydroxylation of a prolyl residue that is essential for the ubiquitination of HIF-1 α , thereby mimicking hypoxia. A 60 mM stock of DFX was made in Dulbecco's phosphate buffered saline (D-PBS) (Invitrogen, Carlsbad, CA) and sterilized through a 0.22 micron filter. CB-derived cells were incubated in QBSF60 serum-free media (Atlanta Biologicals, Lawrenceville, GA) containing a final concentration of 600 μ M DFX. Cell samples were analyzed by flow cytometry at 0, 24, and 48 hours for the determination of cell surface expression of CD34 and CXCR4. Anti-human antibodies that were either FITC- or PE-conjugated were purchased from BD Biosciences (San Jose, CA).

Sheep transplantation procedures

Transplantation into fetal sheep was carried out at the University of Nevada-Reno Agriculture Experimental Station after receiving approval from our Institutional Animal Care and Use Committee (IACUC). While ultrasound-guided injections are considered minimally invasive, sheep must be anesthetized and immobilized to facilitate this procedure. Pregnant ewes on gestation days 53-75 after timed mating were fasted for 36 hours and water was also removed for the last 12 hours. Anesthesia was induced initially by Telazol (2.2 mg/kg, intramuscular) during surgical preparation of the dams that included shaving and sterilizing the abdominal area. This was followed by tracheal intubation, and then placement on isoflurane administered via an anesthetic machine. A transabdominal ALOKA SSD-1000 ultrasound with a 5-MHz probe was used to locate fetuses. A 22-gauge spinal needle was inserted through the skin and the uterine wall into the amniotic cavity and then into the liver of the fetus. While donor stem cells or the drug treatment (plerixafor) were injected into the liver, it exuded out and accumulated in the peritoneal cavity, confirmed by the development of an ultrasound echogenic focus in the peritoneal cavity. Injections were therefore considered “intra-peritoneal”. The presence of distress throughout the procedure was followed by monitoring heart rate, respiration and oxygen tension. Sheep returned to their normal activities after recovery from anesthesia.

Groups of up to five fetal sheep were injected with donor cells delivered in 0.5 mL of QBSF60 serum-free media. Fetuses received CD34⁺ cells, MSCs, or MSCs and CD34⁺ cells together, as indicated. When two transplantations were performed on the same recipient, they were done 1 or 2 weeks apart. Plerixafor (Sigma Aldrich, St. Louis, MO) was dissolved at 1 mg/ml in D-PBS, filter-sterilized through a 0.22 micron filter, and administered to fetal sheep at 5 minutes prior to injecting CD34⁺ cells through ultrasound-guided injections into the peritoneal cavity at a dose of 5 mg/kg, where indicated.

Mobilizing sheep for engraftment studies

Sheep were administered Banamine (Flunixin meglumine) at 0.5-1.1 mg/kg, intramuscular, to prevent/limit any possible pain due to stem cell mobilization. PB samples were collected at baseline and at 2, 4, 6, 8, and 24 hours after administering plerixafor at 5 mg/kg. Blood samples were processed for flow cytometry in order to determine levels of sheep CD34⁺ cells as described (30) and briefly outlined below.

Analysis of peripheral blood samples

Peripheral blood (PB) samples were collected from sheep at 8-11 weeks after transplantation (except for 3 animals in Group 1, at 5 weeks after transplantation), and analyzed by flow cytometry for levels of human hematopoietic cell engraftment. All antibodies were purchased from BD BioSciences (San Jose, CA). PB samples were also collected from plerixafor-dosed adult sheep to obtain CD34⁺ mobilization kinetics data. Anti-sheep CD34 antibody was purchased from Genovac AG (Freiburg, Germany) and used as described previously (30). Briefly, one hundred μ L aliquots of PB samples were added to tubes containing 5 μ L each of a FITC- and PE-conjugated antibody and incubated in the dark for 10 minutes. Two mL of BD FACS lysing solution (BD Bioscience) was added per tube and further incubated for 5 minutes in the dark. Cells were pelleted at 1,500 RPM on a Dupont

Sorvall RT7 tabletop centrifuge with a RT-H250 swinging bucket rotor for 10 minutes. The supernatant was decanted and cells were washed with 1 mL PBS/0.1% sodium azide, and then resuspended in 0.5 mL PBS. Cell suspensions were analyzed on a FACScan flow cytometry instrument with CellQuest software. Cells were gated for lymphocytes and monocytes, and then PE and FITC stained cells were enumerated. Non-transplanted control sheep PB samples were analyzed with corresponding antibodies or with isotype controls in order to gate for events in the test sheep PB samples. Any reactivity of antibodies against human markers with control sheep blood was subtracted from data from chimeric sheep. Levels of engraftment in chimeric sheep were calculated by summing up data for different hematopoietic lineages.

Immunohistochemistry Analysis of tissue samples

Bone tissue samples were placed into cassettes, preserved in buffered formaldehyde (Fisher, Kalamazoo, MI), and embedded in paraffin wax. Five micron-thick sections were cut on a microtome after incubating embedded paraffin blocks in decalcification solution (Decal Stat) (Decal Chemical Corp, Tallman, NY) to dissolve mineralized bone. Tissue sections were mounted and baked onto slides. Target retrieval using citrate buffer was done as described previously (31). Immunohistochemistry (IHC) was carried out using rabbit anti-SDF1 antibody (clone RB32982) which reacted with both human and sheep tissue sections (Abgent, San Diego, CA), and/or mouse anti-human nuclei antibody (clone 235-1) (PhosphoSolutions, Aurora, CO) which only reacted with human cells. Secondary antibodies included donkey-anti-rabbit Alexa Fluor 647 (red) and donkey-anti-mouse Alexa Fluor 488 (green) (Jackson ImmunoResearch Laboratories West Grove, PA). Nuclei were stained using slide mounting media (Prolong Gold antifade with DAPI) (Invitrogen). Photomicrographs were taken on an Olympus Fluoview FV1000 confocal microscope with UPlanFLN 40x1.30 numeric aperture oil objective lens, using FV10-ASW version 01.05.00.14 software (Olympus America Inc., Melville, NY, USA). Images were processed using Adobe Photoshop, version CS5.

Calculation of fetal weight and cell dosage for recipients

We collected fetal weight data at necropsy at various gestational ages (data not shown). This data correlated with a more comprehensive data set published recently (32). Therefore we chose to use the published data to graph gestational age vs. fetal weight in order to extrapolate and approximate fetal weights on any given day between days 25 and 80. The cell dosage for each recipient was calculated at the second transplantation day while also incorporating the number of HSCs infused during the first transplantation.

Statistical tests

For each transplantation group, engraftment levels were analyzed and reported as the median score for the group. Several parameters were varied in each group such that comparisons between groups were comparisons between clusters of parameters in order to gauge a set of favorable conditions. In this manner, future experiments could be pursued to fine-tune transplantation regimens based on our preliminary results. The difference in the levels of engraftment between groups was compared for statistical significance using the Mann-Whitney U-test (significance: $p < 0.05$). This test is not affected by outliers as it is

dependent on data ranking, or whether a data point is larger than another but not how much larger. The Mann-Whitney U-test does not assume a normal distribution of data points and is applicable to small data sets with at least five data points, as was obtained with our large animal model study. Group 4 data was not analyzed due to a smaller data set.

Results

Human MSCs engraft sheep BM

The engraftment of human MSCs in the sheep model has already been studied in much detail elsewhere (33). We confirmed engraftment in the BM by transplanting six fetal recipients with MSCs on gestation day 69 (term is 147 days). Bone sections were collected on days 94, 115, and 121, and analyzed by IHC staining with anti-human nuclei primary antibody and a fluorescently tagged secondary antibody. We found human donor cells in transplanted recipients (a representative picture is shown in Figures 1A-B). Therefore, as shown by others, human MSCs are capable of homing and engraftment in sheep BM following intra-peritoneal injection. Ten non-transplanted control animals were negative for human nuclei staining (data not shown).

Sheep HSCs can be mobilized with plerixafor

Plerixafor causes rapid and reversible mobilization of HSCs into the peripheral circulation and has been shown to be effective in mice (5 mg/kg, peak mobilization at 1 hour), non-human primates (1 mg/kg, mobilization between 3-6 hours), and dogs (4 mg/kg, mobilization between 2-10 hours) (13, 17, 34). In humans, plerixafor is typically used in lower doses in combination with cytokine therapy (240 µg/kg, peak mobilization at 6 hours) (35). To launch its effect on sheep, we first demonstrated the presence of SDF1 in sheep BM stroma. Bone samples collected from non-transplanted control sheep during the third trimester were analyzed by IHC staining with anti-SDF1 antibody. We demonstrate the presence of SDF1 in sheep bone (Figures 1C-D) and determined the specificity of the assay through obtaining negative results when the primary antibody was left out (data not shown). We also analyzed transplanted recipients and demonstrate the presence of SDF1-positive cells of human donor origin in animal #2738 (Table 1) on gestation day 146 (Figures 1E-F). Therefore, endogenous SDF1 is present in sheep BM while SDF1-positive cells may also arise from donor cells. To specifically demonstrate the activity of plerixafor in mobilizing sheep HSCs, an adult was dosed at 5 mg/kg and PB samples were collected. The levels of sheep CD34⁺ cells in PB demonstrated that the kinetics of HSC mobilization in sheep (Figure 1G) were comparable to that in the canine model (17), with mobilization peaking a few hours after drug administration followed by a disappearance of HSCs from PB by 24 hours.

Plerixafor enhances IUHSCT engraftment after prior MSC transplantation

The homing, engraftment, self-renewal, and differentiation of HSCs require the cooperation of HSCs and several cell types in the BM stroma. MSCs are a major component of stromal cells that encompass the BM niche (33). We reviewed historical data of sheep transplantation experiments with CD34⁺ cells, with CD34⁺ cells cotransplanted with MSCs, and with CD34⁺ cells transplanted one week after MSCs. Analysis of this data indicated

better engraftment when CD34⁺ cells were transplanted one week after MSCs (data not shown). Hence we adopted this latter regimen as the constant parameter in our current studies (Figure 2).

Plerixafor antagonizes the binding of SDF-1 to its cognate receptor, CXCR4. We hypothesized that this selective but reversible antagonist could be administered to a fetus *in-utero* to vacate the stem cell niche prior to performing IUHSCT. Five recipients (Group 1) were transplanted with MSCs one week prior to receiving CD34⁺ cells after plerixafor treatment (Table 1) (Figure 2). We report the detection of unambiguously visible, multi-lineage donor activity in Group 1 recipients (Figure 3A), which was used to calculate engraftment levels (Table 1). We confirmed the presence of B-cells (CD20), T-cells (CD4 and CD8), NK cells (CD16), neutrophils (CD15), and monocytes (CD14), at 11 weeks post-transplantation. There was no observed correlation between cell dosage and engraftment levels when all fetuses received at least of 10⁵ CD34⁺ cells (Tables 1 and 3). The median level of human hematopoietic activity in Group 1 was 2.80%.

Group 2 recipients were transplanted using a regimen similar to Group 1 except that low numbers of HSCs (from the same CB unit that was used for transplantation a week later) were cotransplanted with the MSCs in the first injection (Figure 2). The cotransplantation of MSCs has been used in various cellular therapy applications and shown to modulate the immune response of recipients (23). Our hypothesis was that cotransplantations of CD34⁺ cells and MSCs will provide not only a humanized BM niche but also modulate fetal immunity so that the second CD34⁺ transplantation one week later from the same CB donor would be better received. Our data for Group 2 demonstrates a median of 8.77% human hematopoietic engraftment was observed at 11 weeks post-transplantation using this strategy (Figure 3B and Table I). Similar to Group 1 recipients the group 2 recipients were analyzed at 11 weeks post-transplantation (animal #2738, #2739). Three animals that were analyzed sooner (animal #2740, #2741, #2742) yielded lower levels of engraftment (Table I) in accordance with the general observation that donor graft increases over time during gestation (whereas donor graft decreases over time after birth). The difference in the levels of engraftment between Groups 1 and 2 was statistically significant (Mann-Whitney U-test, p-value = 0.00604). Parameters common to Groups 1 and 2 were: 1) MSC was transplanted on day 59; 2) HSC was transplanted using plerixafor on day 66. Parameters that were different included transplanting Group 2 with a small number of HSC on day 59. In addition, the HSC dosage (Table III) was between 3 - 9.5 million HSC/kg for Group 1 and 1.5 - 2.8 million HSC/kg for Group 2, and the MSC dosage was 1.8 million for Group 1 and 1 million for Group 2).

The up-regulation of CXCR4 receptor does not enhance engraftment when IUHSCT is performed late in gestation

The SDF1-CXCR4 ligand-receptor axis can be manipulated either by moieties that antagonize the binding of SDF1 in order to disrupt the axis, or by up-regulating CXCR4 receptor levels to encourage formation of the axis. CB-derived CD34⁺ cells were incubated overnight in serum-free media with the addition of an iron chelator, deferoxamine (DFX), in order to mimic hypoxic conditions. Under such conditions, the percentage of the CXCR4⁺

cells in the CD34⁺ population increased from 33.70% on day 0, to 50.74% at 24 hours, and 72.98% at 48 hours (Figure 4). Transplantation with 24 hour DFX-treated CD34⁺ cells resulted in engraftment levels with a median of 2.03% in Group 3 (without plerixafor) and with a median of 3.44% in Group 4 (with plerixafor) (Table II) (Figure 3C), when transplantation was performed late in gestation (days 62 and 76). Differences in engraftment levels between Groups 1 and 3 were not significant (Mann-Whitney U-test, p-value = 0.14917). Therefore, transplantation levels observed for Group 1 (day 59 with MSC, day 66 with plerixafor and HSC, HSC dosage between 3-9.5 million) is not significantly different from those for Group 4 (day 62 with MSC + HSC, day 76 with plerixafor and HSC-DXF, HSC dosage between 0.9-5.4 million).

Discussion

Clinical experience with IUHSCT has been successful for severe combined immunodeficiency (SCID) patients while engraftment in non-SCID patients has been low, with a recent study accounting success in 11/12 SCID cases and only 7/20 non-SCID cases (36). Translational research towards achieving chimerism levels of therapeutic value following IUHSCT have indicated that the receiving fetal environment, with a few disease-specific exceptions such as SCID, is highly competitive, necessitating strategies to improve the competitive advantage of transplanted donor cells to achieve clinically meaningful levels of engraftment (37). Adopting conditioning regimens for depletion of resident HSCs as done in the post-natal patient is prohibitively toxic to the fetus. The major goal of our research is to develop novel approaches to improve IUHSCT using the fetal sheep, a clinically relevant animal model. The availability of ultra-sound guided technology provides relative ease in locating and injecting fetuses following timed mating in this large animal. In the current studies, first, we utilized MSCs to humanize the BM niche; second, we assessed the value of dual HSC injections incorporating HSCs with MSCs in the first injection followed by HSCs 1-2 weeks later; and third, we evaluated two ways of manipulating the CXCR4-SDF1 axis with the ultimate goal of optimizing a clinically applicable strategy to bestow competitive advantage to donor cells.

In a xenogeneic model, donor HSCs rapidly diminish following IUHSCT due to lack of cross-species reactivity from self-renewal and differentiation cues in the host's environment (38). Others have demonstrated that transplanted human MSCs differentiate in the BM niche in mice into pericytes, myofibroblasts, BM stromal cells, osteocytes in bone, bone-lining osteoblasts, and endothelial cells, which resulted in enhanced human HSC engraftment in adult recipients (20). The cotransplantation of MSCs and HSCs has also proven useful in allogeneic settings where MSCs enhance tolerance (39, 40) or accelerate recovery from BM failure and induce hematopoietic tissue reconstitution (41), although it must be noted that higher passage MSCs were reported to be ineffective (42). Furthermore, the *in-utero* transplantation of MSCs has been demonstrated to be safe in a case of human fetal transplantation (43). In the current studies we confirmed human MSC engraftment in the BM of sheep (Figure 1A-B) whereas others previously demonstrated differentiation as well (33). When MSC-engrafted recipients were transplanted with HSCs with plerixafor treatment (Group 1), the engraftment data was noteworthy for several reasons. First, all recipients (100%) demonstrated engraftment, measured at 1.45% to 4.65%, when

transplanted with 250,000 to 800,000 CD34⁺ cells (3 to 9.5 million HSC/kg). Second, the transplantation regimen did not employ any harsh conditioning treatments, in contrast to the most recent improvement in IUHSCT where up to 3.3% engraftment was observed after transplanting 720,000 to 2.4 million CD34⁺ cells following conditioning with Busulfan – which was administered to the pregnant dam and crossed the placenta barrier (44). And third, the achievement of 2% donor cell engraftment after IUHSCT is considered to be clinically significant as it bestows tolerance to the recipient (10, 45). Historically, mice, sheep, and man have undergone IUHSCT in the absence of MSCs or plerixafor, which resulted in low levels of engraftment (46). We recently utilized the transplantation regimen of Group 1 in studies to evaluate human embryonic stem cell derived CD34⁺ cell transplantation and reported engraftment in all of the recipients (47).

In a previous study, limited engraftment after IUHSCT in an immune competent allogeneic mouse model was significantly improved by post-natal booster injections, where 5 million cells increased engraftment from 0.69% to 3.30% in newborn pups after 6 weeks (5). We mimicked this two-injection approach, *in-utero*. When recipients were injected first with HSCs and MSCs, then HSCs alone one week later (Group 2), engraftment levels were up to 3-fold higher than when HSCs were left out of the first injection (Group 1), in recipients analyzed at 11 weeks post-transplantation (Table 1) (Figure 2), with a lower HSC cell dosage (Table III). Plerixafor was utilized in the second injection for both groups. Therefore, when HSCs are included in the MSC injection, the second HSC injection behaves as a booster injection. The *in utero* booster injection can effectively be administered with dosage that requires fewer HSCs for the smaller sized fetus (Table III) and with relative ease using ultrasound-guidance.

Fetal sheep acquire the capacity to reject allogeneic skin grafts by day 75 in gestation (term=147 days) (48). The optimal age for IUHSCT in the sheep model is between 55-65 days in gestation and engraftment dwindles after day 75 (6, 49). The engraftment of MSCs, however, has shown to occur as late in gestation as day 85, likely due to their immunomodulatory characteristics (33). Group 3 and 4 recipients were transplanted with HSCs on gestation day 76, although the first MSC/HSC cotransplantation occurred on day 62. Engraftment here confirms that the day 62 injections occurred within the window of opportunity that bestowed immune tolerance towards donor cells during the preimmune status of the fetus such that the later HSC injection was tolerated.

The number of HSCs and MSCs transplanted into Groups 1-4 were variable due to our objective of transplanting each fetus with the maximum number of stem cells available. With HSCs, a single unit of cord blood-derived HSCs went to all the fetuses in a single ewe. With MSCs, all the cells harvested from culture flasks on surgery day were divided into all fetuses available on that day. However, despite the varying cell dosages, there were no correlations between HSC dosage (Table III) and engraftment levels (Tables I and II) within each group for Groups 1, 2, and 3. For Group 4, there was a correlation between cell dosage and engraftment level with an R² value of 0.98 calculated in a linear regression analysis. The number of samples in each group was n=5 except for Group 3 with n=2. The use of large animals as well as the sample size must be rigorously justified when obtaining approval

from institutional review boards, and pursuing full data sets for every parameter being tested is not always feasible due to the nature of such studies (50).

The combination of each of the four sets of parameters in our studies demonstrated engraftment in 100% of the recipients, and median engraftment levels above 2% in each group. The cluster of parameters in Group 2 supported the highest levels of engraftment whereby MSC and HSC were transplanted on day 59, a high dose of HSC was transplanted after plerixafor treatment on day 66, and the total HSC dosage was 1.5 to 2.8 million HSC/kg (Table III). In embracing a dual approach to manipulate the CXCR4-SDF1 axis in Group 4, plerixafor treatment was used to disrupt the recipient CXCR4-SDF1 axis and a larger fraction of CXCR4⁺ cells in the donor HSC population was used to promote donor HSC CXCR4-SDF1 axis formation in the BM niche. This dual approach when combined with other parameters in Group 4 (transplantation on days 62, 76, HSC dosage of 0.9 to 5.4 million HSC/kg) did not result in higher engraftment levels, and will have to be tested with group 3 transplantation timelines to determine whether there is merit in up-regulating CXCR4 on donor cells. It is curious that the highest cell dosage in Group 4 resulted in the highest engraftment level in the entire study. One explanation would be that the higher cell dose was useful in overcoming NK cell barriers to engraftment when transplantation was performed at a later day in gestation with a better developed immune system in the fetus. High cell dosage to overcome NK cell barrier during transplantation has been widely reported (9, 10, 51, 52). The up-regulation of CXCR4 on HSCs as well as MSCs to enhance in vivo engraftment has previously been reported (29, 53, 54). In addition, there are other ways of exploiting the CXCR4-SDF1 axis, such as utilization of prostaglandin and sitagliptin as recently demonstrated in pre-clinical and clinical studies (55-57).

In summary, the current studies provide proof of principle evidence in support of strategies to improve HSC engraftment via manipulating BM niche in utero. First, we show that MSCs could engraft and provide species-specific BM niche in the xenogeneic setting, and thus may be beneficial in the allogeneic settings too by promoting tolerance. Second, HSCs should be transplanted with a dual injection scheme in both the xenogeneic and allogeneic settings to presumably prime the recipient immunity and BM niche spaces so that it becomes more receptive towards the booster injection. Third, effects of the booster injection may be enhanced through manipulating the CXCR4-SDF1 ligand-receptor axis: By plerixafor treatment to antagonize SDF1 and gain access to limited niche space without cytotoxicity. Further experiments are necessary to decipher whether using HSCs with a larger fraction of CXCR4⁺ cells is beneficial.

The concepts investigated here are for boosting engraftment during gestation and must be combined with other studies that have highlighted hurdles to be overcome for graft persistence after birth. The fetal sheep model has previously served as a preclinical model on which cellular therapy for X-linked SCID was developed and successfully translated to the clinical setting (6). The current studies present a protocol that is adaptable with a doubling of gestation time from sheep to man to translate timelines, and cell dosing translated as cell number per kg fetal weight. Nonetheless, challenges to translation of protocols to the clinical setting must not be trivialized, including overcoming effects of maternal alloantibodies, maternal T cells, and recipient NK cells (8-10). Our studies highlight techniques for

boosting initial engraftment during gestation; long-term post-natal engraftment will be dependent on HLA-matching donor cells to the mother of the fetus to overcome the maternal immune response implicated in rejection (58), a study suited for allogeneic animal models. Whereas we have implicated that the effect of plerixafor was on vacating the stem cell niche, these studies do not rule out the effect of plerixafor on the immune system of the recipient (59, 60).

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Abbreviations

BM	bone marrow
CB	cord blood
DFX	deferoxamine
DPBS	Dulbecco's phosphate buffered saline
HSC	hematopoietic stem cell
IHC	immunohistochemistry
IUHSCT	in utero hematopoietic stem cell transplantation
MSC	mesenchymal stromal/stem cell
MPB	mobilized peripheral blood
SCID	severe combined immunodeficiency

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Figure 1. Engraftment of human MSCs in sheep bone marrow; presence of SDF1 in sheep bone marrow; and plerixafor-responsive HSCs in sheep bone marrow

(A) Bone samples collected from fetal sheep during gestation after transplanting human MSCs on day 69 were stained with anti-human nuclei antibody. Human cells (green) are dispersed throughout this cross section with numerous sheep cells (blue: DAPI nuclei staining). (B) Transmitted display channel of same field of view as (A) depicting cells within the mineralized trabecular bone. (C) SDF1-secreting cells (red) are in association with trabecular bone structures (merged transmitted display with color channels) in bone sample from fetal sheep collected during gestation, and (D) a magnification (3x) of cells (white arrow) from (C) without transmitted display. (E) The presence of SDF1-secreting cells (red) in bone sample from animal # 2738 (Table 1) transplanted with MSC and HSC. Most of the SDF1-positive cells are of sheep origin. A double-positive cell (white arrow) for SDF1 (red) and human nuclei (green) is magnified (10x) in (F). Photomicrographs were taken on an Olympus Fluoview FV1000 confocal microscope with UPlanFLN 40x1.30 numeric aperture oil objective lens, using FV10-ASW version 01.05.00.14 software (Olympus America Inc., Melville, NY, USA). Images were processed using Adobe Photoshop, version CS5. (G) Kinetics of sheep HSCs in peripheral blood after plerixafor treatment assessed by flow cytometry for sheep CD34⁺ cells in peripheral blood collected at time points indicated. Plerixafor administered at 0 hour to an adult sheep reversibly mobilized sheep HSCs.

Figure 2. Schematics for IUH SCT regimens that resulted in notable engraftment levels
(A) Fetal sheep recipients in Groups 1, 2, 3, and 4 underwent ultrasound-guided two-injection transplantation of human cord blood-derived CD34⁺ HSCs. The first transplantation included MSCs (purple) with or without HSCs (blue) from the same cord blood unit as for the second HSC injection. The second transplantation of HSCs (blue) was with or without Plerixafor injection (yellow) 5 minutes before HSC injections. In two groups, the HSCs were incubated with deferoxamine (DFX) (orange) overnight to increase the fraction of CXCR4⁺ cells in the CD34⁺ population. All injections (HSC, MSC, plerixafor) were into the intra-peritoneal cavity of the fetuses, as described in methods. **(B)** Mapping of engraftment levels for recipients in each group.

Figure 3. Assessing donor cell engraftment in the peripheral blood of transplant recipients after IUHST

Human hematopoietic engraftment in fetal sheep transplant recipients in (A) Group 1, (B) Group 2, and (C) Group 4 was assessed using flow cytometry by summing up percentages of lineage-committed human cells in the PB of each recipient. The lymphoid lineage with B cells (CD20), T cells (CD4 and CD8), and NK cells (CD16), and the myeloid lineage with neutrophils (CD15) and monocytes (CD14) were tallied for each recipient (Tables I and II) after subtracting any non-specific staining observed in age-matched, non-transplanted control animals. CD16 was not determined for animals from Group 1.

Figure 4. Up-regulation of cell surface CXCR4 in culture

Cord blood-derived cells were cultured for 48 hours in serum-free media containing deferoxamine (DFX) to mimic hypoxic conditions. Cells were stained for flow cytometry as described in methods. The percentage of CXCR4⁺ cells in the CD34⁺ population was 33.70%, 50.74%, and 72.98% at 0, 24, and 48 hours, respectively.

Table I
Comparison of two novel *in-utero* hematopoietic stem cell transplantation regimens using human donor cells in the sheep model

Group 1. CD34 ⁺ engraftment using plerixa for and prior MSC transplantation				Group 2. CD34 ⁺ engraftment using plerixa for and prior MSC and CD34 ⁺ transplantation				
Sheep no.	Surgery 1 Day 59	Surgery 2 Day 66	Human Cells in PB (%)	Sheep no.	Surgery 1 Day 59	Surgery 2 Day 66	Human Cells in PB (%)	Avg.
2757	1,800,000 MSC	250,000 CD34 ⁺	2.80	2738	1,000,000 MSC 50,000 CD34 ⁺	80,000 CD34 ⁺	19.54	20.96
2758	1,800,000 MSC	250,000 CD34 ⁺	3.03	2739	1,000,000 MSC 50,000 CD34 ⁺	80,000 CD34 ⁺	22.37	
2759	1,800,000 MSC	800,000 CD34 ⁺	2.32	2740 [*]	1,000,000 MSC 67,000 CD34 ⁺	167,000 CD34 ⁺	8.77 [*]	8.27 [*]
2761	1,800,000 MSC	750,000 CD34 ⁺	1.45	2741 [*]	1,000,000 MSC 67,000 CD34 ⁺	167,000 CD34 ⁺	7.62 [*]	
2762	1,800,000 MSC	750,000 CD34 ⁺	4.65	2742 [*]	1,000,000 MSC 67,000 CD34 ⁺	167,000 CD34 ⁺	8.41 [*]	
Median			2.80	Median			8.77	
Standard deviation			1.17	Standard deviation			7.12	

Multi-lineage human hematopoietic engraftment in the peripheral blood was assayed as described in methods.

^{*} Fetal sheep were transplanted with cord blood-derived CD34⁺ cells with plerixa for treatment one week after transplanting bone marrow-derived MSCs with or without accompanying CD34⁺ cells from the same cord blood unit. Peripheral blood was collected for analysis at 11 weeks post-transplantation (during gestation) except animals 2740, 2741, and 2742 at 5 weeks post transplantation.

Table II
Up-regulation of CXCR4 receptors on HSCs prior to IUHST

Group 3. DFX-treated CD34 ⁺ engraftment without plerixafor, with prior MSC and CD34 ⁺ transplantation				Group 4. DFX-treated CD34 ⁺ engraftment with plerixafor, with prior MSC and CD34 ⁺ transplantation			
	Surgery 1 Day 62	Surgery 2 Day 76	Human Cells in PB (%)	Sheep no.	Surgery 1 Day 62	Surgery 2 Day 76	Human Cells in PB (%)
2795	4,000,000 MSC 120,000 CD34 ⁺	170,000 CD34 ⁺	2.35	2797	4,000,000 MSC 300,000 CD34 ⁺	750,000 CD34 ⁺	27.53
2796	4,000,000 MSC 120,000 CD34 ⁺	170,000 CD34	1.70	2798	4,000,000 MSC 70,000 CD34 ⁺	110,000 CD34 ⁺	3.44
				2799	4,000,000 MSC 70,000 CD34 ⁺	110,000 CD34 ⁺	4.90
				2800	4,000,000 MSC 89,000 CD34 ⁺	120,000 CD34 ⁺	2.35
				2801	4,000,000 MSC 89,000 CD34 ⁺	120,000 CD34 ⁺	2.50
Median			2.03	Median			3.44
Standard deviation			0.42	Standard deviation			10.88

Fetal sheep were transplanted with cord blood-derived CD34⁺ cells with or without plerixafor treatment two weeks after cotransplanting bone marrow-derived MSCs and CD34⁺ cells from the same cord blood unit. Cells for surgery 2 were incubated with deferoxamine overnight prior to transplantation. Peripheral blood was collected for analysis at 8 weeks post-transplantation for Group 3 and at 10 weeks post-transplantation (during gestation) for Group 4. Multi-lineage human hematopoietic engraftment in the peripheral blood was assayed as described in methods.

Table III
Calculation of cell dosage (number of cells per kg) per fetus in Groups 1, 2, 3, and 4

Group 1		Group 2		Group 3		Group 4	
Animal#	Total HSC/kg	Animal#	Total HSC/kg	Animal#	Total HSC/kg	Animal#	Total HSC/kg
2757	3.0×10^6	2738	1.5×10^6	2795	1.5×10^6	2797	5.4×10^6
2758	3.0×10^6	2739	1.5×10^6	2796	1.5×10^6	2798	0.9×10^6
2759	9.5×10^6	2740	2.8×10^6			2799	0.9×10^6
2761	8.9×10^6	2741	2.8×10^6			2780	1.1×10^6
2762	8.9×10^6	2742	2.8×10^6			2801	1.1×10^6

The total of CD34⁺ cells transplanted during the first and second surgeries were divided into the fetal weight on the second day. Fetal weight was estimated from a chart of fetal weight vs. gestation day generated using published data (32).