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Placental Mesenchymal Stromal Cells Rescue Ambulation in Ovine Myelomeningocele

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Key Words. Stem cell • Placenta • Mesenchymal stem cells • Fetal stem cells • Cellular therapy

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Received December 22, 2014; accepted for publication February 27, 2015; published Online First on April 24, 2015.

1066-5099/2015/$20.00/0

http://dx.doi.org/10.5966/sctm.2014-0296

ABSTRACT

Myelomeningocele (MMC)—commonly known as spina bifida—is a congenital birth defect that causes lifelong paralysis, incontinence, musculoskeletal deformities, and severe cognitive disabilities. The recent landmark Management of Myelomeningocele Study (MOMS) demonstrated for the first time in humans that in utero surgical repair of the MMC defect improves lower limb motor function, suggesting a capacity for improved neurologic outcomes in this disorder. However, functional recovery was incomplete, and 58% of the treated children were unable to walk independently at 30 months of age. In the present study, we demonstrate that using early gestation human placenta-derived mesenchymal stromal cells (PMSCs) to augment in utero repair of MMC results in significant and consistent improvement in neurologic function at birth in the rigorous fetal ovine model of MMC. In vitro, human PMSCs express characteristic MSC markers and trilineage differentiation potential. Protein array assays and enzyme-linked immunosorbent assay show that PMSCs secrete a variety of immunomodulatory and angiogenic cytokines. Compared with adult bone marrow MSCs, PMSCs secrete significantly higher levels of brain-derived neurotrophic factor and hepatocyte growth factor, both of which have known neuroprotective capabilities. In vivo, functional and histopathologic analysis demonstrated that human PMSCs mediate a significant, clinically relevant improvement in motor function in MMC lambs and increase the preservation of large neurons within the spinal cord. These preclinical results in the well-established fetal ovine model of MMC provide promising early support for translating in utero stem cell therapy for MMC into clinical application for patients. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:659–669*

SIGNIFICANCE

This study presents placenta-derived mesenchymal stromal cell (PMSC) treatment as a potential therapy for myelomeningocele (MMC). Application of PMSCs can augment current in utero surgical repair in the well-established and rigorously applied fetal lamb model of MMC. Treatment with human PMSCs significantly and dramatically improved neurologic function and preserved spinal cord neuron density in experimental animals. Sixty-seven percent of the PMSC-treated lambs were able to ambulate independently, with two exhibiting no motor deficits whatsoever. In contrast, none of the lambs treated with the vehicle alone were capable of ambulation. The locomotor rescue demonstrated in PMSC-treated lambs indicates great promise for future clinical trials to improve paralysis in children afflicted with MMC.

INTRODUCTION

Myelomeningocele (MMC)—commonly known as spina bifida—is caused by incomplete neural tube closure during development of the spinal cord. Intrauterine damage to the exposed cord leaves afflicted children with lifelong paralysis, incontinence, musculoskeletal deformities, and severe cognitive disabilities [1]. The healthcare costs for these children are 13 times as great as for children without MMC [2], and in the United States, 4 children a day are born with this devastating disease [3]. The recent Management of Myelomeningocele Study (MOMS), a multicenter randomized controlled clinical trial, demonstrated that in utero surgical repair of the MMC defect was safe, decreased the risk of hindbrain herniation, and decreased the need for cerebrospinal fluid shunting compared with postnatal repair. Most importantly, the MOMS trial showed that in utero surgical defect repair could improve distal motor function, suggesting the capacity for improved neurologic outcomes exists [4]. Although promising, these motor function improvements were limited and sporadic, and 58% of children who underwent in utero repair were still

unable to ambulate without assistance at 30 months of age. Although in utero closure of the spinal cord might prevent additional intrauterine damage, the existing surgical procedure does not completely restore neurologic function, and additional therapeutic options are needed.

In utero stem cell therapy has the potential to build on this surgical advance to mitigate the paralysis associated with MMC. The prenatal period is believed to be the ideal time to administer stem cell therapies, because the fetal environment is immunologically naïve and naturally receptive to stem cell-mediated remodeling [5, 6]. Mesenchymal stromal cells (MSCs) isolated from a range of tissues have generated substantial interest for use in cell therapy and tissue engineering, because of their ability to secrete paracrine factors that can improve endogenous repair of damaged tissues [7]. It has been shown that fetal MSCs express pluripotent stem cell markers, have greater expansion capacity than adult stem cells, and can be readily obtained from the placenta throughout pregnancy [8]. The placenta is a promising autologous cell source [9–11], and early gestation placental mesenchymal stromal cells (PMSCs) have been shown to be immunomodulatory [12], to be neuroprotective [13], and to improve wound healing [14]. Furthermore, PMSCs can be obtained reliably from either discarded placenta or via chorionic villus sampling [9, 15, 16] and can be expanded on a timeline suitable for autologous therapy [11].

We hypothesized that PMSCs could augment current in utero surgical repair in the well-established and rigorously applied fetal lamb model of MMC. We demonstrate that application of human PMSCs significantly and consistently improved neurologic function and preserved spinal cord neuron density in the ovine MMC model.

Materials and Methods

Isolation and Culture of PMSCs and Acquisition of Bone Marrow MSC Lines

Donated early gestation placental tissue (11–17 weeks) was collected at the University of California, Davis, Medical Center. The PMSCs were isolated using an explant culture method. Chorionic villus tissue was dissected into $<$5-mm pieces and washed in sterile PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin. Dissected tissue was spread across adherent culture dishes precoated with CELLStart xeno-free substrate for 1 hour at 37°C (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). The cells were harvested at 3–4 weeks and expanded as a monolayer until the third passage before being cryopreserved in liquid nitrogen in a solution of 10% dimethyl sulfoxide and 90% fetal bovine serum (FBS). Cryopreserved cell stock was used for all in vitro and in vivo experimentation.

The culture medium for all experiments was DMEM high glucose with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml recombinant human basic fibroblast growth factor 2 (PeproTech, Rocky Hill, NJ, http://www.peprotech.com), and 20 ng/ml recombinant human epidermal growth factor (PeproTech).

Adult human bone marrow MSCs (BM-MSCs) were a gracious gift from Claus Sondergaard, Ph.D. (University of California, Davis). The cells were thawed from cryopreservation at passage 2, and conditioned media were collected at passages 3–4 for each line. The culture media and precoated dishes were the same for both BM-MSC and PMSC cultures.

Flow Cytometry Analysis of PMSCs

We harvested PMSCs for flow cytometry using Accutase (Invitrogen) and counted PMSCs using trypan blue. Resuspended cells were split into fractions containing approximately 10^6 cells and stained with fluorescein isothiocyanate-CD44 (560977), PECy5-CD90 (555597), PE-CD73 (561014), Alexa Fluor 647-CD105 (561439), PE-CD29 (561795), PE-CD34 (560941), Alexa Fluor 647-CD31 (561654), or isotype-specific control (all from BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). In the case of CD90 and CD44, unstained cells were used as negative controls. The samples were counterstained using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes, Eugene, OR, http://probes.invitrogen.com) to detect dead cells and then fixed in 4% paraformaldehyde (PFA). The PMSCs were analyzed using a BD Fortessa LSR Cell Analyzer, and the data were prepared with FlowJo software (Tree Star, Inc., Ashland, OR, http://www.treestar.com).

Immunofluorescence of PMSCs

Before immunostaining, PMSCs were fixed in 4% PFA in PBS, followed by membrane permeabilization with 0.5% Triton X-100 in PBS. We incubated the PMSCs with one of the following primary antibodies overnight at 4°C: Sox9 (AB3697; Abcam, Cambridge, U.K., http://www.abcam.com), Sox10 (MAB2864; R&D Systems, Minneapolis, MN, http://www.rndsystems.com), Sox17 (MAB1924; R&D Systems), Nestin (MAB5326; EMD Millipore, Billerica, MA, http://www.emdmillipore.com), Snail (SC-28199; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), Slug (SC-166476; Santa Cruz Biotechnology), S100β (S2532; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), neurofilament heavy chain (NFH) (N4142; Sigma-Aldrich), Class III β-tubulin (TUJ1) (MRB435P; Covance Inc., Princeton, NJ, http://www.covance.com), or Twist (T6451; Sigma-Aldrich). Subsequently, the samples were incubated with Alexa Fluor 546–conjugated secondary antibodies (A10040; Molecular Probes) for 1 hour at room temperature. The negative controls consisted of staining with secondary antibody only. Cell nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI) (40011; Biotium, Hayward, CA, http://www.biotium.com). The images were collected using an Axio Observer D1 inverted microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

Multipotency Analysis of PMSCs

To induce osteogenic differentiation, the PMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 10 mM β-glycerol phosphate (Sigma-Aldrich), 0.1 μM dexamethasone (Sigma-Aldrich), and 200 μM ascorbic acid (Sigma-Aldrich) for 3–4 weeks. To confirm osteogenic differentiation, the cells were fixed in 4% PFA and stained with alizarin red (Sigma-Aldrich) to identify calcified matrix and immunostained for the osteogenic marker osteocalcin (ab76690; Abcam).

To induce chondrogenic differentiation, the PMSCs were cultured as cell pellets in suspension in DMEM containing 10% FBS, 10 ng/ml transforming growth factor β3 (PeproTech), and 200 μM ascorbic acid (Sigma-Aldrich) for 3–4 weeks. After chondrogenic differentiation, chondrogenic pellets were fixed in 4% PFA before subsequently being embedded in Optimal Cutting Temperature compound (Fisher Scientific, Waltham, MA, http://www.fisherscientific.com). Cross sections were immunostained for collagen II (ab53047; Abcam) and stained with alcian blue (Sigma-Aldrich) to detect glycosaminoglycans.

To induce adipogenic differentiation, PMSCs were cultured in DMEM containing 10% FBS, 1 μM dexamethasone (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), 5 μM isobutylxanthine (AdipoGen Life Sciences, San Diego, CA, http://www.adipogen.com), and 200 μM indomethacin (MP Biomedicals, Irvine CA, http://www.mpbio.com) for 3–4 weeks. After adipogenic differentiation, the cells were fixed in 4% PFA and stained with Oil Red O (Sigma-Aldrich) to identify lipid accumulation and immunostained for lipid-associated protein perilipin (MAB7634; R&D Systems).

Enzyme-Linked Immunosorbent Assays

The concentrations of secreted cytokines and growth factors were quantified using enzyme-linked immunosorbent assays (ELISAs). Supernatants were collected from confluent BM-MSC (n = 3 lines) and PMSC (n = 6 lines) cultures with an initial seeding density of 7.5×10^5 cells per 100-mm dish. Human brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), and tissue inhibitor of metalloproteinase 1 (TIMP-1) levels were detected using ELISA kits from R&D Systems. All ELISAs were performed according to the manufacturer's instructions, and absorbance was measured using a Molecular Devices plate reader instrument (Molecular Devices Corp., Union City, CA, http://www.moleculardevices.com). The negative control for the ELISAs consisted of complete media only, and no protein was detected. Statistical analysis for ELISAs was performed using an unpaired t test with Welch's correction.

Green Fluorescent Protein Transduction of PMSCs

To facilitate cell tracking in vivo, cells were transduced with a green fluorescent protein (GFP)-containing lentiviral vector (pCCLc-MNDU3-LUC-PGK-EGFP-WPRE construct from University of California, Davis/California Institute for Regenerative Medicine Institute, Sacramento, CA, http://www.cirm.ca.gov). The cells were plated at a density of 7.5×10^5 cells per 100-mm dish and allowed to adhere overnight. The next morning, the media were changed to transduction media (multiplicity of infection = 5) for 6 hours; the cultures were washed twice, and normal culture media were reintroduced. GFP expression was confirmed 72 hours later using fluorescent microscopy.

Seeding GFP-Tagged PMSCs Into a Hydrogel Delivery Vehicle

GFP-labeled PMSCs were harvested from adherent cultures, pelleted, and resuspended in complete media before being mixed at 4°C (to prevent premature crosslinking) into a solution of 2 mg/ml rat tail collagen (BD Biosciences), water, and PBS at a density of 5×10^5 cells per milliliter solution before subsequent neutralization with 0.1 N NaOH. After homogenization and introduction of GFP-PMSCs, 1 ml of cell or collagen solution was placed in a 35-mm suspension culture dish and allowed to gel for at least 45 minutes at 37°C. After gelation, 1 ml of complete culture media was added to the dish, covering the hydrogel layer. Control collagen gels were prepared identically, but no PMSCs were added to the mixture.

Cytokine Profile of GFP-Tagged PMSCs Suspended in Hydrogel Delivery Vehicle

Cytokine array assays were performed on culture supernatants collected at 24 hours from GFP-PMSC-containing and control collagen gels that were prepared in parallel with gels used for in vivo experiments. Human Cytokine Array Panel A (R&D Systems) and Angiogenesis Array (R&D Systems) assays were performed according to the manufacturer's instructions. The membranes were read using a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, http://www.bio-rad.com) and further analyzed using ImageJ software (NIH, Bethesda, MD, http://www.nih.gov.ij) and the DotBlot analysis plug-in. Collagen gel controls displayed no detectable levels of secreted cytokines.

In Vivo MMC Defect Creation and Repair

The University of California, Davis, institutional animal care and use committee (IACUC) approved all animal protocols, and all animal care was in compliance with the Guide for the Care and Use of Laboratory Animals. All facilities used during the study period were accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Domestic barn sheep were used for the present study. A Dorper ram was sire of all lambs assessed in the experiment. The sheep were obtained from the same breeder.

One week before surgery, time-mated, pregnant ewes were delivered to the housing facility, where they had unrestricted access to food and water, except for the 24-hour period directly preceding surgery. The first operation was performed at a gestational age (GA) of approximately 75 days. All ewes underwent survival laparotomy and hysterotomy, followed by creation of the MMC defect, as previously described in detail [17, 18]. In brief, the MMC defect was surgically created in each fetal lamb by removing the skin, paraspinal muscles, lamina of six lumbar vertebrae, and dura overlying the spinal cord. The maternal hysterotomy and laparotomy incisions were closed in a routine fashion.

Approximately 25 days later, a second survival laparotomy and hysterotomy were performed to repair the MMC defect. Any overlying fibrinous exudate on the spinal cord was removed to allow for direct application of the treatment onto the open neural placode. At the repair, singleton lambs were divided into control and experimental groups on an alternating basis. With twin lambs, one lamb was given the cell treatment and the other served as the control. The treatment lambs were assigned randomly to the treatment groups. Three male and three female lambs received the cell treatment, and four male and two female lambs served as vehicle controls. The lambs in the control group were treated with 1 ml of collagen applied directly to the neural placode. To hold the collagen in place, a single-ply layer of Oasis (Cook Biotech, West Lafayette, IN, http://www.cookbiotech.com), a commercially available extracellular matrix patch, was secured over the defect with interrupted 5-0 monofilament sutures to serve as a dural replacement. The skin was then closed over the patch. The lambs in the experimental group were repaired with 1 ml of collagen gel seeded with 5×10^5 GFP-tagged PMSCs followed by an extracellular matrix patch and skin closure in an identical fashion.

Motor Function Analysis

The lambs were delivered at term (GA approximately 145 days). After birth, motor function was evaluated for all lambs, in addition to 3 normal, negative controls, using the sheep locomotor rating (SLR) scale, as previously described [19]. In brief, motor function in 7 categories was observed and scored on a scale of 0–15. A grade of 15 indicates completely normal function; grades of 0–4, 5–9, and 10–14 indicate severe, moderate, and mild motor deficits, respectively. Locomotor testing was performed and

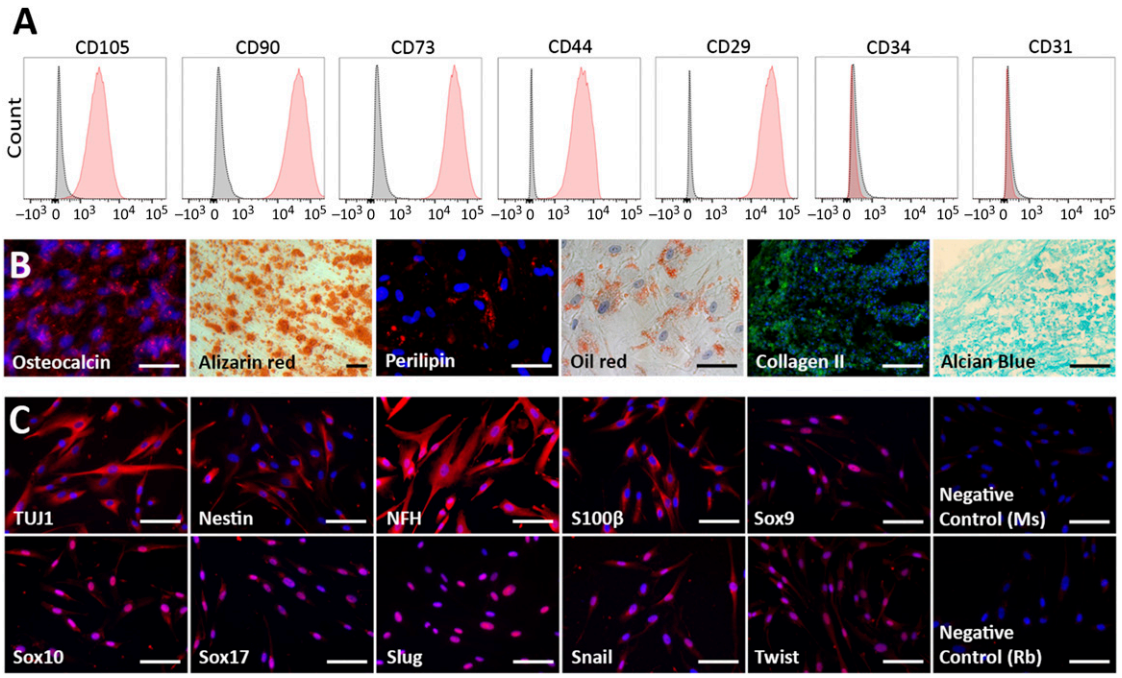

Figure 1. Early gestation human placenta-derived mesenchymal stromal cells (PMSCs) express a profile consistent with MSCs. Flow cytometry immunophenotyping indicated that PMSCs express the MSC markers CD105, CD90, CD73, CD44, and CD29. PMSCs were negative for hematopoietic and endothelial lineage markers CD34 and CD31, respectively **(A)**. Stained sample profiles are shown in red overlaid with the negative control in gray. Directed differentiation of PMSCs demonstrated multipotency toward mesodermal cell lineages **(B)**. The presence of intracellular proteins and transcription factors expressed by PMSCs was analyzed using immunocytochemistry. Cells displayed positivity for proteins commonly associated with neural lineage, such as TUJ1, nestin, NFH, and S100β, compared with control staining. Cells also expressed transcription factors, including Sox9, Sox10, Sox17, Slug, Snail, and Twist **(C)**. Scale bars = 100 μm.

filmed by two examiners, once at 2 hours after birth and once 24 hours after the initial assessment. Because the welfare of animals with spinal cord injury deteriorates rapidly after birth, the IACUC protocols required euthanasia of severely affected sheep within 48 hours. Thus, the best overall performance from both time points was used for analysis of the treated animals and vehicle controls. Two examiners, who were unaware of the treatment group and live locomotor score, evaluated and scored all videos independently. All examiners met as a group to discuss the scores, the videos were reviewed to reconcile any differences, and a consensus score was assigned to each lamb.

Histopathologic Analysis

After completion of the motor function analysis, all the lambs were euthanized and perfused. The perfusion circuit was primed with lidocaine and heparin, followed by 1 liter of 0.9% NaCl and 2 liters of 4% paraformaldehyde. The spinal cord of each lamb was dissected for analysis. After gross inspection, the cord was blocked into lumbar spinal segments and then embedded in Optimal Cutting Temperature compound for cryosectioning. A series of 20-μm sections were taken through each spinal segment. Nissl (cresyl violet) staining was performed on the tissue sections.

The full lumbar spinal cord was imaged for all lambs. A sample of six sections per lumbar segment for each lamb was analyzed using ImageJ and averaged to determine the height, width, and cross-sectional area of the gray matter, white matter, and entire spinal cord. The GNU Image Manipulation Program (available at http://www.gimp.com) was used to create the spinal cord tracings. The proportions of gray and white matter were calculated by dividing the respective tissue cross-sectional area by the total cross-sectional area of the spinal cord. The epicenter of the MMC lesion was defined as the lumbar segment with the greatest degree of deformation (defined by the cross-sectional height divided by the width). The entire gray matter of each section was imaged at $\times$20 magnification using an inverted bright field microscope (Keyence BZ-9000; Keyence, Osaka, Japan, http://www.keyence.com). Large neurons, cells with a diameter of 30–70-μm within the gray matter, as previously described [20], were counted using ImageJ software. All counts were performed by a single, blinded individual to maintain consistency. Large neuron density was calculated by dividing the average number of neurons by the total cross-sectional area of gray matter to normalize for variability in the amount of gray matter present.

Statistical analyses were performed for the entire lumbar cord; a subset analysis was performed to analyze the epicenters. In order to normalize the variability in cord size in the different spinal cord segments, all calculations, including determining the lesion epicenter, were normalized by dividing by the average cross-sectional area for the corresponding lumbar segment in normal newborn lambs (n = 3). Subsequently, the large neuron density was also normalized by dividing by the average large neuron density for the corresponding lumbar segment in normal newborn lambs. Statistical analyses were performed using Student's t test for continuous variables and the Mann-Whitney U test for ordinal variables. Linear regression analysis was performed to evaluate the relationship between large neuron density and SLR score. We included 3 normal controls in the regression analysis, in addition to the 12 experimental animals. The controls have no defined epicenter; thus, the L3 lumbar segment was chosen, because this

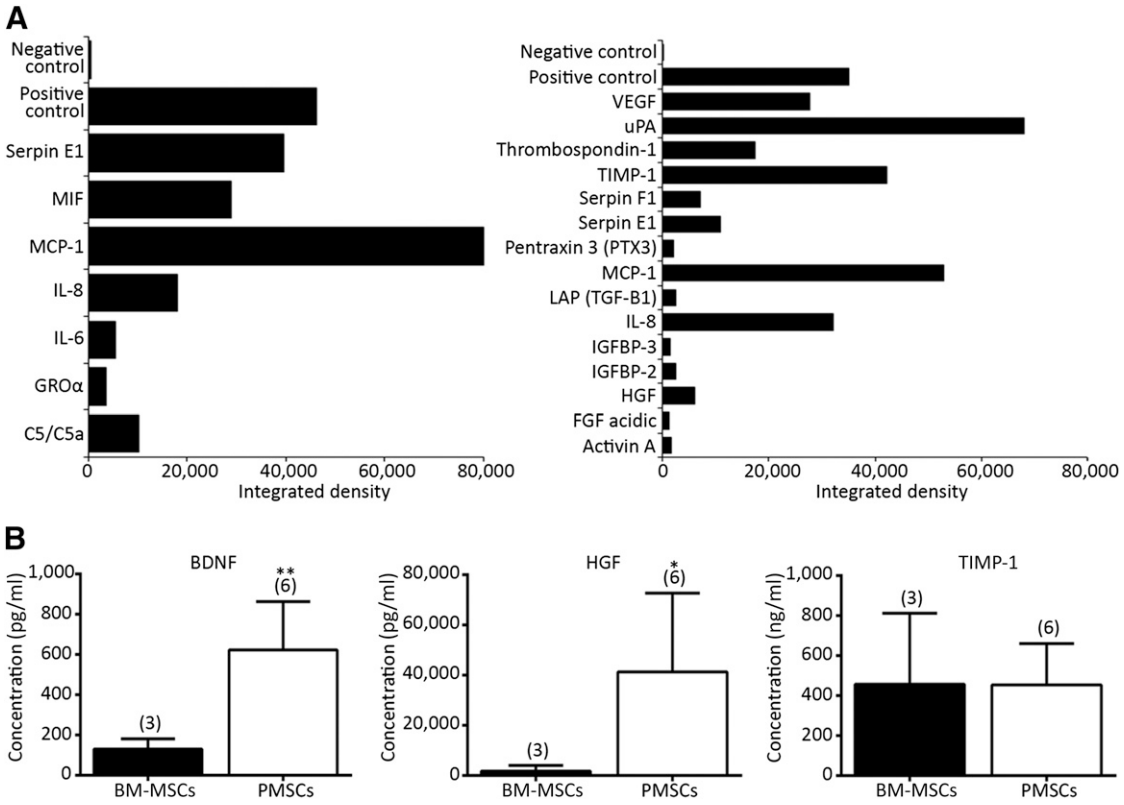

Figure 2. Early gestation human PMSCs display distinct paracrine properties. Cytokine array analysis revealed that PMSCs secrete a diverse array of immunomodulatory and chemotactic cytokines, including serpin E1, MIF, MCP-1, IL-8, IL-6, and C5/C5a. PMSCs secrete VEGF, thrombospondin-1, and HGF (angiogenic cytokines), uPA, and TIMP-1 (matrix remodeling proteins), and MCP-1 and IL-8 (immunomodulatory factors). Positive and negative controls spots are internal controls built into the assay and are shown as reference points **(A)**. Enzyme-linked immunosorbent assays were used to compare PMSC and BM-MSC secretion of factors with known neuroprotective function. PMSCs secreted significantly higher levels of BDNF and HGF in culture supernatants compared with the average levels secreted by BM-MSCs ($p = .0032$ and $p = .0272$, respectively). However, PMSCs did not display a significant difference in the quantity of secreted TIMP-1 compared with the average levels of BM-MSCs **(B)**. Error bars represent the standard deviation from the mean of all BM-MSC lines ($n = 3$) and all PMSC lines ($n = 6$) tested. Statistical analysis was performed using an unpaired *t* test with Welch's correction. *, $p < .05$; **, $p < .01$. Abbreviations: BDNF, brain-derived neurotrophic factor; BM-MSCs, bone marrow mesenchymal stem cells; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGFBP, insulin-like growth factor-binding protein; IL, interleukin; LAP, latency associated protein; MCP-1, monocyte chemoattractant protein 1; MIF, macrophage migratory inhibitory factor; PMSCs, placenta-derived mesenchymal stromal cells; TGF-β1, transforming growth factor-β1; TIMP-1, tissue inhibitor of metalloproteinase 1; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

was the most common level for the disease epicenter using both mean and median in the MMC lambs.

Immunohistochemical Analysis of Cellular Retention

To evaluate the presence of transplanted PMSCs in the sheep spinal cord tissue, immunohistochemical staining with anti-GFP antibody (A-11122; Invitrogen) was performed, followed by bright field/fluorescence microscopy. Immunohistochemical analysis was performed at the lesion epicenter for all animals included in the histopathologic analysis (n = 12). Next, 500,000 GFP-tagged cells were directly injected into the spinal cord of one lamb immediately after euthanization to serve as a positive control. Twelve cross-sections per lamb were analyzed: six for GFP analysis, three for secondary antibody only, and three for isotype control. In brief, the samples were washed in PBS for 10 minutes, followed by permeabilization of cells with 0.5% Triton X-100 in PBS for 10 minutes. For primary antibody incubation, the samples were incubated with anti-GFP antibody (1% bovine serum albumin [BSA]), IgG isotype control (A10040; Invitrogen), and 1% BSA for 24 hours at 4°C. All samples were then incubated with Alexa Fluor 546-conjugated secondary antibody (A10040; Molecular Probes) for 1 hour at 22°C. Finally, the cell nuclei were counterstained with DAPI (40011; Biotium) for 5 minutes at 22°C. In addition to immunofluorescence staining, 3,3′-diaminobenzidine (DAB) staining was used to validate the results. For DAB staining, the tissue sections were incubated with ImmPRESS Reagent (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) for 30 minutes at 22°C, washed with PBS for 10 minutes, and incubated with a peroxidase substrate solution for 2 minutes. The nuclei were counterstained with hematoxylin for 1 minute. GFP analysis was performed by examining all sections using an inverted fluorescence microscope (Carl Zeiss Axio Observer D1) at ×20 magnification.

Results

Early Gestation Human PMSCs Display Distinct Paracrine Properties

Flow cytometric and immunocytochemical analyses were performed to characterize PMSCs cultured in neurotropic media

[21]. PMSCs were positive for typical MSC markers CD105, CD90, CD73, CD44, and CD29 and negative for hematopoietic marker CD34 and endothelial marker CD31 (Fig. 1A). Using differentiation induction protocols, PMSCs were capable of trilineage differentiation into osteogenic, adipogenic, and chondrogenic lineages, which is characteristic of MSCs [22] (Fig. 1B). Immunocytochemistry demonstrated that PMSCs express the intracellular neural and stem cell-related markers Nestin, TUJ1, NFH, and S100β and transcription factors Sox9, Sox10, Sox17, Slug, Snail, and Twist [23–27] (Fig. 1C).

To improve PMSC viability and therapeutic potential for in vivo transplantation, PMSCs were cultured in a three-dimensional collagen hydrogel. The characterization of PMSCs in the collagen hydrogel revealed that PMSCs secrete a wide array of paracrine factors within the first 24 hours after seeding. The most highly detected factors were urokinase plasminogen activator (uPA), vascular endothelial growth factor, TIMP-1, HGF, thrombospondin-1, monocyte chemoattractant protein 1, interleukin-8, serpin E1, serpin F1, and macrophage migratory inhibitory factor (Fig. 2A), which have been previously implicated in angiogenesis, chemotaxis, extracellular matrix remodeling, and the innate immune response.

We further analyzed the paracrine activity of PMSCs by quantifying the secretion of factors previously recognized as critical for neurogenesis, neuroprotection, and angiogenesis. ELISAs revealed that compared with adult BM-MSCs, PMSCs secreted significantly higher levels of BDNF (p = .0032) and HGF (p = .0272), and no statistically significant difference was found in TIMP-1 secretion between the BM-MSCs and PMSCs (Fig. 2B).

Applying PMSCs In Utero Significantly Improves Motor Function in the MMC Lamb Model

We surgically created a MMC defect in 12 fetal lambs at an average GA of 77.3 days (range, 73–81), as previously described [17] (Fig. 3A–3D). The average length of the resultant spinal column defect was 3.1 $\pm$ 0.2 cm. We noted direct spinal cord damage at the time of defect creation in one lamb that was included in the analyses; no other complications were noted during the defect creation surgeries. The second operation for defect repair was performed at mean GA of 103.5 days (range, 97–107), and no complications were noted during the repair operations. During the repair, the lambs were randomly chosen for treatment with PMSCs plus delivery vehicle or the delivery vehicle without PMSCs as a control.

All experimental lambs surviving to term (n = 12) were analyzed. The average GA at birth was 145.7 days (range, 138–152), and within 24 hours of birth, motor function was assessed using the SLR scale [19]. Three untreated newborn lambs (no MMC defect) exhibited normal motor function (SLR score of 15) and served as positive controls. The lambs in the PMSC-treated group received significantly higher neurologic scores compared with the lambs in the vehicle-only group (p = .0108; Fig. 4A). Of the 6 PMSC-treated lambs, 4 (67%) were able to ambulate independently, with 2 exhibiting no motor deficits (SLR score of 15), 2 exhibiting mild deficits (SLR score of 10 and 13), and 2 exhibiting moderate deficits (SLR score of 5 and 8). No lambs in the vehicle-only group were able to ambulate; 2 displayed moderate deficits (SLR score of 6 and 8) and 4 displayed severe deficits (SLR score of 2, 2, 4, and 4). Two sets of twin lambs with the same genetic parentage and intrauterine environments were studied; one twin per set received PMSCs and the other vehicle only. Both twin lambs treated with PMSCs ambulated normally, and the vehicle-only treated twins exhibited moderate or severe deficits and were unable to ambulate (Fig. 4B, 4C; supplemental online Movie 1).

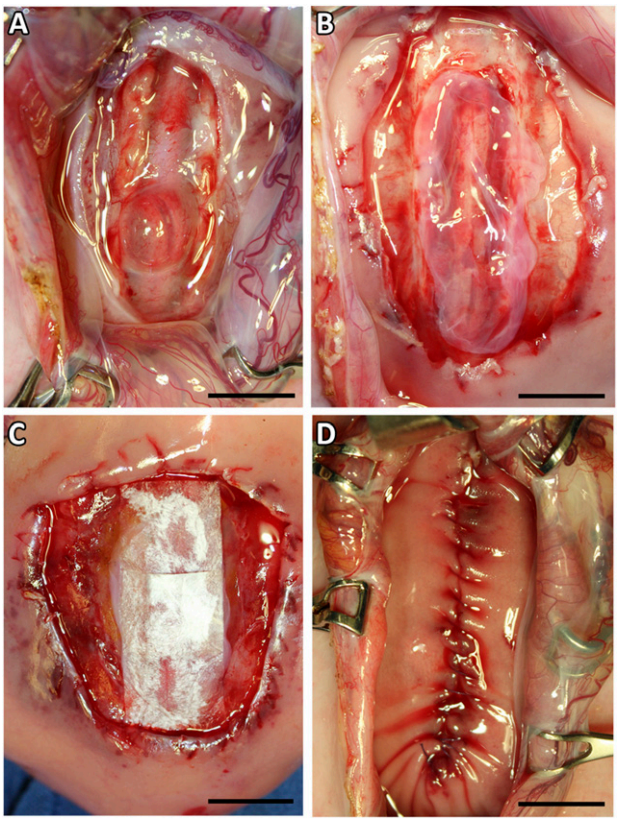

Figure 3. Spina bifida defect and in utero repair. **(A–D):** Representative images taken from each major step of the spina bifida repair process in the fetal lamb model. At myelomeningocele (MMC) repair (gestational age, 100 days), the MMC defect is evaluated and debrided of all overlying fibrinous and inflammatory tissue **(A)**. Collagen containing placenta-derived mesenchymal stromal cells or collagen alone is then applied directly to the neural placode **(B)**. To hold the collagen in place, a single-ply layer of Oasis extracellular matrix (Cook Biotech) was secured over the defect as a dural replacement **(C)**. Finally, the skin was closed over the defect **(D)**. Scale bars = 1 cm.

Histopathologic Analysis Demonstrates Increase in Large Neuron Density

Cross-sectional tracings through the length of the lumbar spinal cord displayed prominent cord compression in all 12 experimental lambs in contrast to the normal lumbar cords of the negative controls. However, the degree of deformation varied throughout the lumbar cord and by animal. At the lesion epicenter or the level of the greatest spinal cord deformation, determined by the height/width normalized by lumbar segment, no significant differences in spinal cord cross-sectional area (p = .711), degree of deformation (p = .245), or proportional area composed of gray or white matter (p = .969 and p = .571, respectively) was observed between the two treatment groups (Fig. 5A, 5B).

The density of the large neurons (defined as the number of neurons 30–70 μm in diameter normalized to the

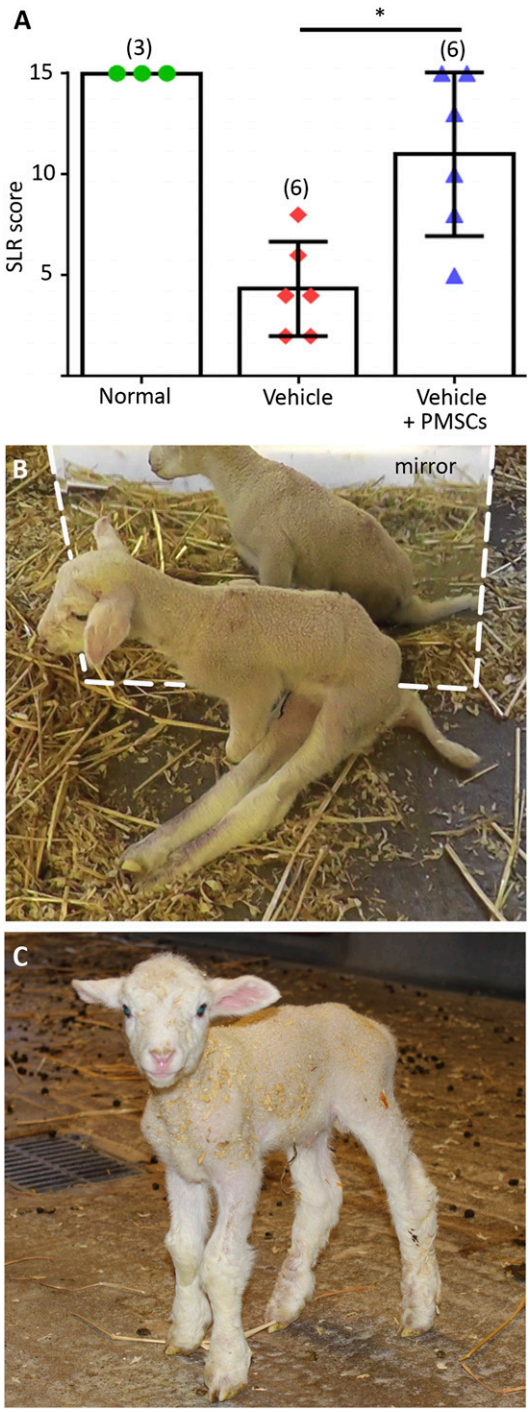

Figure 4. PMSC treatment improves locomotor outcome. Lambs were scored using SLR. All normal lambs (n = 3) scored 15 on the SLR scale, indicating normal motor function. Vehicle-treated MMC lambs scored from 2 to 8 on the SLR scale (2, 2, 4, 4, 6, and 8). Vehicle plus PMSC-treated lambs scored from 5 to 15 on the SLR scale (5, 8, 10, 13, 15, and 15). Lambs in the vehicle plus PMSC treated group had significantly higher SLR scores than did the vehicle-only lambs (p = .0108) **(A)**. Photographs of the twin lambs are shown. A lamb treated with vehicle only was unable to bear weight on its hind limbs and is pictured with the fully extended legs typical of lambs with lower extremity paralysis **(B)**. Its twin was treated with vehicle plus PMSCs and is shown standing independently **(C)**. Abbreviations: MMC, myelomeningocele; PMSC, placenta-derived mesenchymal stromal cell; SLR, sheep locomotor rating.

cross-sectional area of gray matter) was significantly greater in the PMSC-treated lambs than in the vehicle controls (p = .0125; Fig. 6A–6D). Linear regression confirmed a significant positive association between large neuron density and SLR scores (r^2 = .5108, p = .0028; Fig. 6E).

The immunohistochemical evaluation did not reveal any evidence of engraftment of the GFP-labeled PMSCs in the spinal cords or surrounding tissues in any of the PMSC-treated animals, indicating that the PMSCs did not migrate or integrate into the spinal cord or surrounding tissue. No histologic evidence of tumor formation was observed.

Discussion

Spina bifida is the most common cause of lifelong childhood paralysis in the United States. In the past two decades, several groups have been involved with developing new treatments for spinal bifida [17, 28, 29]. The potential treatments that have been investigated include the use of biologic and synthetic patches to cover the defect and the application of various stem cells to supplement traditional surgical repair [30–32]. The present study is the first to use stromal cells derived from early gestation chorionic villus tissue to augment in utero repair of MMC in a rigorously defined large animal model. The placenta is a well-known source of progenitor cells [33, 34], and we selected PMSCs as our therapeutic cells because of the advantageous characteristics of early gestation fetal stem cells and potential clinically feasible time points for autologous therapy. Human MMC is typically diagnosed at approximately 12 weeks' gestation, and after diagnosis, placental tissue can be obtained via chorionic villus sampling [35]. In utero surgical repair of MMC is performed at approximately 24–28 weeks' gestation; therefore, a 12-week window exists during which autologous PMSCs can be acquired and expanded for therapy. Although previous studies have suggested that placental stem cells are immunoprivileged [12, 36] and thus could be amenable to allogeneic therapies, additional studies must be performed to determine whether this therapy could be applied in an allogeneic context. Regardless of the ultimate conclusion regarding allogeneic versus autologous therapy, the impressive locomotor rescue demonstrated by PMSC-treated lambs holds great potential for future clinical trials to improve paralysis in children affected by MMC.

In 1995, Meuli et al. [37] conducted the first study using the fetal sheep model of MMC. Their study demonstrated that in utero repair with a fetal muscle flap resulted in improved distal motor function [37]. Although this surgical treatment is not feasible in human patients and current in utero repair did not recapitulate the fetal lamb results, we speculate that these original and encouraging findings could have resulted from the delivery of fetal stem cells within the muscle flap to the MMC defect. Fauza et al. reported improved motor function after directly injecting murine neural stem cells into the ovine spinal cord after MMC defect creation [38]. Significant differences were found between the model used in the study by Fauza et al. and that used in the present study, including the timing of defect creation and repair and the severity of the surgically created defects. The Fauza group reported no significant differences in motor function between the acellular dermis-treated control animals and the cell-treated animals. Most importantly, the Fauza group reported 25% of their untreated MMC lambs were capable of

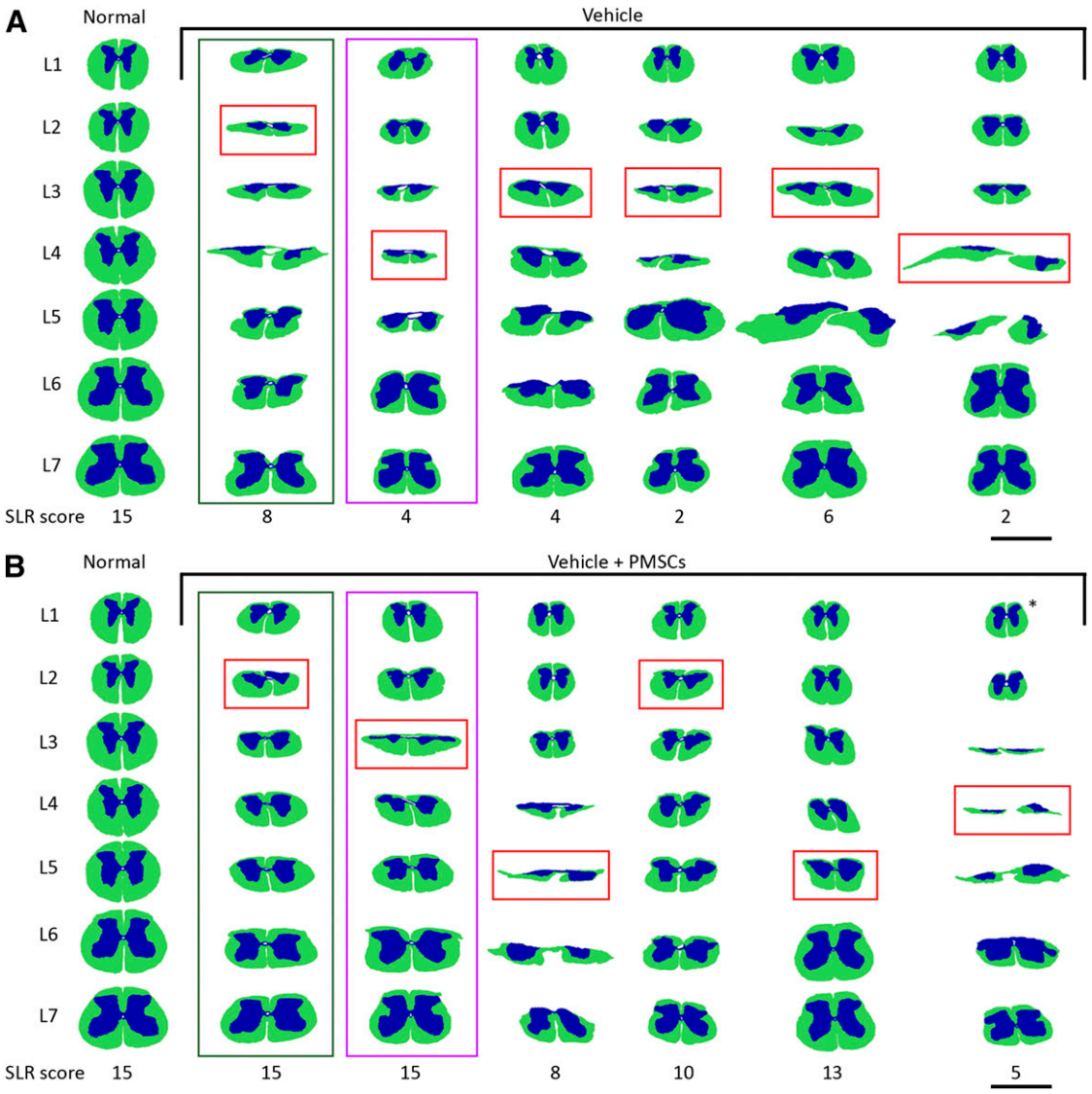

Figure 5. Histopathologic analysis of spinal cord anatomy. Cross-sectional histologic analysis was completed for the 15 lambs (normal $n = 3$, vehicle $n = 6$, vehicle plus PMSCs $n = 6$). Cross-sectional tracings from each lumbar segment from L1 to L7 are shown. The gray matter is shown in blue and the white matter in green. The red box for each lamb designates the lesion epicenter. The six animals treated with vehicle are compared with a normal cord, with the SLR score for each lamb listed below the lumbar segments **(A)**. The 6 animals treated with vehicle plus PMSCs are shown in the lower panel with their corresponding SLR scores listed below **(B)**. *, Spinal cord that was noted to have direct cord damage during defect creation. The green and purple boxes highlight the lumbar segments for the two sets of twins, with one twin lamb treated with vehicle plus PMSCs and the corresponding twin lamb treated with vehicle in each set of twins. Scale bars = 5 mm. Abbreviations: PMSC, placenta-derived mesenchymal stromal cell; SLR, sheep locomotor rating.

ambulation [38]. In our study, no lambs repaired with the vehicle could ambulate, and PMSC treatment significantly improved the motor outcomes compared with vehicle repair. The lack of ambulation in the control lambs from our study more accurately reflects the limited function observed in humans with MMC.

The rationale for in utero repair of MMC is based on the "two-hit hypothesis," which states that incomplete closure of the neural tube is followed by secondary damage from amniotic fluid toxicity, direct trauma, and hydrodynamic pressure [39, 40]. It is this secondary damage that can be ameliorated and is the target of surgical repair [18]. Previous studies have shown that neurologic function appears to be lost during the course of gestation; thus, early intervention can prevent additional spinal cord damage, highlighting the importance of neural tissue protection [41]. The PMSCs used in the present study exhibited a comparable in vitro profile to other MSCs described in published studies [22, 42, 43]. However, analysis of PMSC paracrine secretion demonstrated that PMSCs secreted significantly more BDNF and HGF than adult BM-MSCs, which are currently undergoing clinical trials as treatments for spinal cord injury [44]. HGF is a potent angiogenic factor shown to activate endothelial cell migration and proliferation and might contribute to wound healing in vivo by promoting rapid neovascularization [45–47]. Additionally, HGF has been shown to function in nervous system development and act synergistically with neurotrophic factors in signaling developing neurons [48]. HGF has also been shown to promote the development of oligodendrocytes and neurons and to mediate

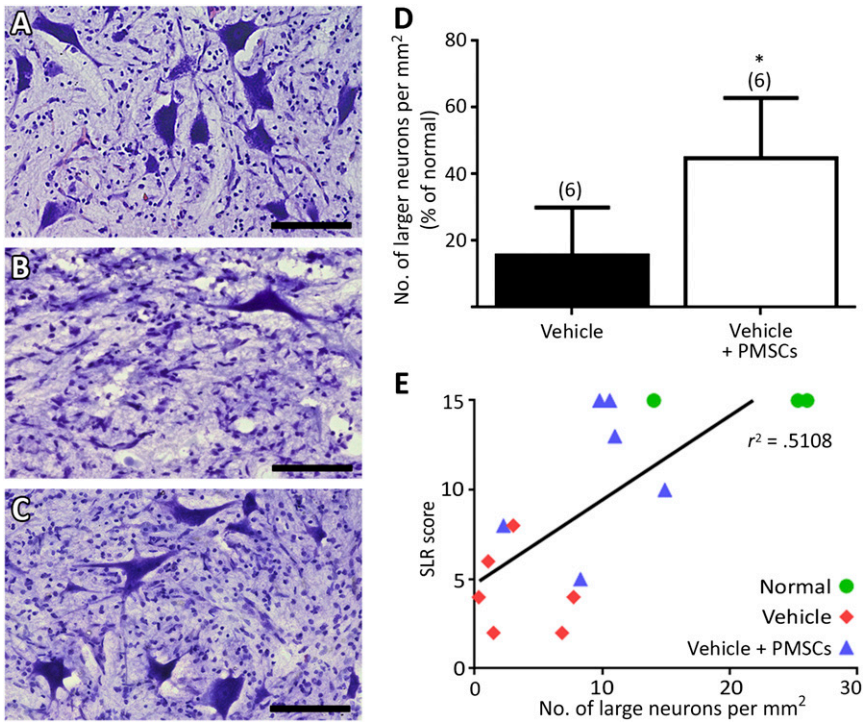

Figure 6. Improvements in motor function and histopathologic analysis. The number of large neurons per mm^2 was obtained for all 15 lambs included in the study. Representative ×20 images of the gray matter are shown: normal **(A)**, vehicle **(B)**, and vehicle plus PMSCs **(C)**. Lambs treated with the vehicle plus PMSCs show more preserved large neurons than lambs treated with vehicle alone. The number of large neurons per mm^2 of those lambs treated with vehicle plus PMSCs was significantly higher than that of those treated with vehicle ($p = .0125$) **(D)**. Linear regression analysis of the SLR scores to the number of large neurons per mm^2 of all lambs demonstrated a positive and significant trend ($r^2 = .5108$, $p = .0028$) **(E)**. Scale bars = 100 μm. Abbreviations: PMSC, placenta-derived mesenchymal stromal cell; SLR, sheep locomotor rating.

functional recovery in animal models of multiple sclerosis [49]. BDNF, a powerful neurotrophin, has widespread effects on the nervous system and affects synaptic plasticity, nerve fiber regrowth, and inflammation after injury [50, 51]. Heightened secretion of BDNF and HGF might play a role in the in vivo neuroprotective effects of PMSCs. TIMP-1 was detected from PMSC cultures at levels similar to that of BM-MSCs and is known to have powerful neuroprotective effects in in vivo models for traumatic and ischemic brain injury [52, 53]. Cumulatively, these studies indicate that PMSCs secrete a complex mix of paracrine factors that are capable of angiogenesis, neurogenesis, and neuroprotection and could be responsible for the observed locomotor improvements in vivo.

Although an exact mechanism of action of PMSCs in the context of MMC has not been identified, our in vivo experiments demonstrate a clear and impressive improvement in neurologic outcomes for lambs treated with PMSCs compared with those treated with the delivery vehicle alone. Although none of the lambs in the vehicle control group were capable of ambulation, most of those repaired with PMSCs were able to ambulate normally or with minimal deficits. Overall, the PMSC-treated lambs received significantly higher SLR scores than did the vehicle-treated lambs. Perhaps most tellingly, remarkable differences in motor function were seen in the two sets of twins included in our study (supplemental online Movie 1). The dramatic contrast between experimental lambs' normal motor function and their twins' significant paralysis highlights the powerful prohealing effect of the PMSCs when other gestational variables are controlled.

Supporting our locomotor findings, treatment with PMSCs significantly increased the density of large neurons in the spinal cord gray matter. Our previous experience in this model has shown that little spinal cord tissue and few large neurons remain at the level of the lesion in unrepaired lambs [32]. The vehicle-treated lambs exhibited some degree of tissue and large neuron preservation, indicating that physical protection alone bestows some benefit compared with no treatment. However, treatment with PMSCs significantly increased the large neuron density at the lesion epicenter compared with that in the vehicle control, a critical potential therapeutic benefit, given the correlation between increased large neuron density and improved SLR scores on regression analysis.

PMSCs exhibit distinctive neuroprotective properties and mediate significant rescue of distal motor function. PMSCs appear to act by a transient, paracrine mechanism to mediate improvements in motor function, consistent with research on the mechanism of action of other MSCs [54, 55]. The present study reports the first evidence of a stem cell therapy that dramatically improves the functional outcomes in a well-established large animal MMC model that consistently produces severe motor deficits.

Conclusion

Our study presents PMSC treatment as a potential therapy for MMC. PMSCs secrete a variety of immunomodulatory and angiogenic cytokines and secrete significantly higher levels of the neuroprotective factors BDNF and HGF than do BM-MSCs. Application of PMSCs can augment current in utero surgical repair in the well-established and rigorously applied fetal lamb model of MMC. Treatment with human PMSCs significantly and

dramatically improves neurologic function and preserves spinal cord neuron density in experimental animals. In our study, 67% of the PMSC-treated lambs were able to ambulate independently, with two exhibiting no motor deficits whatsoever. In contrast, none of the lambs treated with the vehicle alone were capable of ambulation. The locomotor rescue demonstrated in PMSC-treated lambs indicates promise for future clinical trials to improve paralysis in children afflicted with MMC.

Acknowledgments

We thank the staff of the University of California, Davis, Surgical Bioengineering Laboratory for support with this project; C. Long for assistance in editing; T. Selby, Z. Saenz, J. Compton, R. Abbott, and T. You for technical assistance; C. Sondergaard for gracious gift of BM-MSC samples; J. Nolta for scientific support; and W. Ferrier and L. Talken for assistance in the perioperative care and management for our research animals. All the funds used were institutional start-up funds provided by the Department of Surgery, University of California, Davis, Medical Center.

Author Contributions

A.W. and L.L.: in vitro and in vivo experimental design, in vitro experiments, data analysis and interpretation, editorial revisions; E.G.B.: in vitro and in vivo experimental design; animal surgery; motor function analysis, histopathologic analyses, data analysis and interpretation, manuscript writing; B.A.K. and C.D.P.: in vitro and in vivo experimental design; animal surgery; motor function analysis, histopathologic analyses, data analysis and interpretation, editorial revisions; N.A.S.: in vitro and in vivo experimental design, motor function analysis, manuscript writing; M.S.B.: in vitro and in vivo experimental design, editorial revisions; J.C.B.: in vitro and in vivo experimental design, motor function analysis, editorial revisions; D.L.F.: in vitro and in vivo experimental design, animal surgery, data analysis and interpretation, editorial revisions.

Disclosure of Potential Conflicts of Interest

A.W. and D.L.F. have applied for a patent for this work. The other authors indicated no potential conflicts of interest.

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