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In utero treatment of myelomeningocele with placental mesenchymal stromal cells — Selection of an optimal cell line in preparation for clinical trials

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ABSTRACT

Background: We determined whether *in vitro* potency assays inform which placental mesenchymal stromal cell (PMSC) lines produce high rates of ambulation following *in utero* treatment of myelomeningocele in an ovine model.
Methods: PMSC lines were created following explant culture of three early-gestation human placentas. *In vitro* neuroprotection was assessed with a neuronal apoptosis model. *In vivo*, myelomeningocele defects were created in 28 fetuses and repaired with PMSCs at 3×10^5 cells/cm^2 of scaffold from Line A ($n = 6$), Line B ($n = 7$) and Line C ($n = 5$) and compared to no PMSCs ($n = 10$). Ambulation was scored as ≥13 on the Sheep Locomotor Rating Scale.
Results: In vitro, Line A and B had higher neuroprotective capability than no PMSCs (1.7 and 1.8 respectively *vs* 1, $p = 0.02$, ANOVA). *In vivo*, Line A and B had higher large neuron densities than no PMSCs (25.2 and 27.9 respectively *vs* 4.8, $p = 0.03$, ANOVA). Line C did not have higher neuroprotection or larger neuron density than no PMSCs. *In vivo*, Line A and B had ambulation rates of 83% and 71%, respectively, compared to 60% with Line C and 20% with no PMSCs.
Conclusion: The *in vitro* neuroprotection assay will facilitate selection of optimal PMSC lines for clinical use.
Level of evidence: n/a.
Type of study: Basic science.

Myelomeningocele is a congenital open neural tube defect that results in a constellation of symptoms including lower extremity paralysis, hindbrain herniation, and bowel and bladder dysfunction [1]. Historically, neonates were treated with postnatal skin closure to prevent infection. However, in 2011, the Management of Myelomeningocele Study (MOMS) resulted in a paradigm shift toward fetal surgical repair. The MOMS trial demonstrated a decreased need for ventriculoperitoneal shunts for hydrocephalus and some improvement in lower extremity function with fetal surgical repair [2]. Despite these improvements, 58% of children were still unable to walk independently, leading to a search for new therapies that could augment the fetal repair.

Ideally, a new therapy would be applied at the time of fetal repair to reverse the cellular apoptosis in the spinal cord seen in children with myelomeningocele (MMC) [3,4]. We developed a novel therapy that utilizes the neuroprotective and immunomodulatory capabilities of early gestation mesenchymal stromal cells (MSCs) to protect the developing spinal cord [5–13]. Early gestation human placental mesenchymal stromal cells (PMSCs) have superior neuroprotective and immunomodulatory properties compared to later gestation PMSCs and other types of MSCs [5,7–10,14–19]. In a large animal model, augmentation of the fetal repair with PMSCs leads to ambulation in otherwise paralyzed animals [11,20]. Further, we demonstrated that treatment with PMSCs results

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Abbreviations: MMC, myelomeningocele; MOMS, Management of Myelomeningocele Study; MSC, mesenchymal stromal cell; PMSC, placental mesenchymal stromal cell; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assays; LN, large neuron; SLR, Sheep Locomotor Rating Scale; BDNF, Brain-derived neurotrophic growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor.

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in preserved motor neurons in the spinal cord in a dose-dependent manner [12,20]. In order to translate this therapy to clinical use, clinical grade PMSCs must be manufactured from multiple placentas, and an optimal PMSC cell line must be selected for use in clinical trials.

Various *in vitro* characterization metrics and potency assays have been developed to compare the growth and immunomodulatory or trophic capabilities of MSCs in order to select the cell lines with the best clinical effects [21–24]. The ideal *in vitro* assays measure specific MSC functions that correlate with *in vivo* effects. The ultimate goal of our therapy is to restore neurons in the developing spinal cord, so we compared both the *in vitro* secretion of known neurotrophic and angiogenic growth factors and the antiapoptotic capability of three PMSC lines [25,26]. We hypothesized that the antiapoptotic capability of cell lines, measured with a neuroprotection assay, would correspond with *in vivo* outcomes in the ovine MMC model.

1. Methods

1.1. Placenta-derived mesenchymal stromal cell isolation

We used three human PMSC lines (A, B and C) that were fully characterized following explant culture of three early gestation (14–21 weeks) placental donors as described in Lankford et al. as Donor 1, 2 and 3 [27]. Cells were cultured in Dulbecco's Modified Eagle Medium/High Glucose with the following additions: 5% fetal bovine serum (Hyclone, Thermo Fisher Scientific), 100 U/ml penicillin/100 μg/ml streptomycin (Thermo Fisher Scientific), 20 ng/ml recombinant human basic fibroblast growth factor (R & D Systems), and 20 ng/ml recombinant human epidermal growth factor (R & D Systems). Transduction of the PMSCs with green fluorescent protein (GFP)-containing lentiviral vector (University of California, Davis Stem Cell Center, California Institute of Regenerative Medicine, Sacramento, CA) was performed at passage 4 to aid in cell tracking and identification. Previous immunohistochemical evaluation has not shown long-term engraftment of PMSCs into the spinal cord or surrounding tissue [7].

The PMSCs were seeded at passage 6 onto a 6 cm × 2 cm piece of small intestine submucosa-derived extracellular matrix (ECM) (Biodesign® Dural graft, Cook Biotech, West Lafayette, IN) at a density of 300,000 cells/cm^2, which was determined to be optimal by both *in vitro* studies and *in vivo* rodent and ovine studies [11,12,27]. Both the combined product (PMSC-ECM) and control (ECM without PMSCs) were incubated in culture medium overnight. On the day of MMC repair, PMSC-ECMs were imaged using fluorescent microscopy, verifying adherence of the PMSCs to the ECM.

1.2. Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISAs) were performed to test for the levels of two neuroprotective growth factors: human brain-derived neurotrophic factor (BDNF) and hepatocyte growth factor (HGF), and one angiogenic growth factor: vascular endothelial growth factor (VEGF). Supernatant from PMSCs at passage 5 seeded on tissue culture-treated plastic at a density of 100,000 cells/cm^2 was collected at 24 h. BDNF, HGF and VEGF levels were detected with DuoSet ELISA kits (DY248, DY294, DY293B respectively, R&D Systems) and measured with a SpectraMaxi3 plate reader (Molecular Devices LLC). Protein levels were normalized to 500,000 cells. ELISAs were repeated in triplicate for each PMSC line.

1.3. In vitro neuroprotection assay by indirect coculture

The *in vitro* neuroprotection assay was performed by inducing apoptosis in a human neuroblastoma cell line followed by indirect coculture with PMSCs to rescue apoptosis as previously described [25]. The human neuroblastoma cell line SH-SY5Y (American Type Culture Collection, Manassas, VA) is a commonly used cell line in neurobiology because the cells are able to display a neuronal phenotype and propogate neurites, which can subsequently be quantified in the neuroprotection assay [8,28]. The SH-SY5Y cells were seeded in 12-well tissue culture-treated dishes at 100,000 cells/cm^2 for 24 h. Next, SH-SY5Y cells were treated with 1 μM staurosporine for 4 h to induce apoptosis. PMSCs were seeded onto 12-well hanging 0.4 μm Millicell inserts (MilliporeSigma) for 24 h at passage 5 before they were placed onto the apoptotic SH-SY5Y cells and incubated for 96 h at 37 °C, 5% CO$_2$. The inserts were removed, and the SH-SY5Y cells were stained with 2 μM calcein AM (Thermo Fisher Scientific) to identify living cells. Images of 5 random positions per well were processed by WimNeuron Image Analysis (Onimagin Technologies, Cordoba, Spain) for neurite outgrowth analysis. The neuroprotective capability of each line was calculated as the fold improvement in total neurite branch points in comparison to coculture without PMSCs. The neuroprotection assay was repeated in triplicate for each PMSC line.

1.4. Ovine myelomeningocele defect creation and repair

Fetal MMC defects were created at a median gestational age of 76 days (interquartile range (IQR) 72–79) in time-mated Dorper ewes as previously described [11]. The MMC defect was created by removing the skin, paraspinal muscles, vertebral lamina, and dorsal portion of the dura from L1 to L6. No myelotomy was performed because this study targeted motor function rather than consequences of hindbrain herniation. Lost amniotic fluid was replaced with normal saline, and antibiotics (1 million units of penicillin and 100 mg of gentamicin) were added to the amniotic fluid.

Fetuses were assigned to repair with no PMSCs (ECM only, $n = 10$), Line A PMSC-ECM ($n = 6$), Line B PMSC-ECM ($n = 7$), or Line C PMSC-ECM ($n = 5$). Repairs were performed at a median gestational age of 102 days (IQR 99–106). The fibrinous scar overlying the spinal cord was removed as previously described [11,29]. The ECM was applied over the exposed spinal cord so that animals receiving the PMSC-ECM product had the cell-seeded side placed on the spinal cord. The corners of the ECM were sutured in place and the fetal skin was closed over the ECM. Lost amniotic fluid was replaced and antibiotics were added in the same manner as the defect creation.

Animal work was approved by the Institutional Animal Care and Use Committee (IACUC) and care was in compliance with the Guide for the Care and Use of Laboratory Animals. The facilities used to conduct this study were accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. Some animals included in this study (8 lambs treated with ECM only and 5 lambs treated with Line A) were reported in a previous publication regarding PMSC seeding density [20]. They have been included here for appropriate comparison among all tested lines.

1.5. Postnatal motor function

Following birth at median gestational age of 146 days (IQR 145–147) by spontaneous vaginal delivery or cesarean section (if delivery had not occurred by the due date), motor function was assessed on the first and second day of life as previously described using the sheep locomotor rating scale (SLR) [11,30]. Hindlimb motor function was rated on a scale of 0 to 15, with a score of 0 characterized by complete paraplegia and 15 characterized by spontaneous ambulation and the ability to clear an obstacle. A score of greater than or equal to 13 corresponded to the ability to ambulate.

1.6. Postmortem magnetic resonance imaging and histologic examination of the spinal cord

The study endpoint was two days after birth. Per the IACUC protocol, lambs that were unable to walk and nurse normally had to be euthanized by the second day of life, which was then established as the study endpoint for consistency across animals. Magnetic resonance imaging (MRI) of the lumbar spine was performed to assess the level and degree of spinal angulation as previously described [20,31]. Lambs with spinal angulation of greater than 60° were marked with circles in figures owing to potential confounding spinal deformation causing spinal cord compression (no PMSCs ($n = 2$), Line A ($n = 0$), Line B ($n = 1$) and Line C ($n = 2$)). Tissues were fixed and the lumbar spine was dissected as previously described [7]. The average number of large neurons (30–70 μm in diameter) in 9 sections of the lumbar spinal cord was counted to quantify surviving motor neurons at the area of greatest deformity, which was determined by dividing the height over the width of the spinal cord [20]. Quantification of neurons at the lumbar segment with greatest deformity allowed evaluation of the treatment effects at the most injured portion of the spinal cord. Large neuron (LN) density was calculated as the number of large neurons per mm^2 of gray matter.

1.7. Statistical analysis

ELISA and neuroprotection results are reported as mean ± standard deviation (SD). Comparisons among groups for normally distributed *in vitro* results were performed using one-way ANOVA with Tukey's post-hoc test. Data from animals are reported as median with IQR. Comparisons among treatment groups for *in vivo* results were performed using Kruskal–Wallis test with Dunn's post-hoc comparison. Spearman's correlation was calculated for SLR score and LN density. Analysis was performed using PRISM 8 (GraphPad Software Inc) with significance set at $p < 0.05$.

2. Results

2.1. In vitro neuroprotection

In vitro neuroprotective capability was significantly higher in Line A and B than no PMSCs. Compared to no PMSCs (1.0), the neuroprotection capability of Line A and B was 1.7 ± 0.2 and 1.8 ± 0.3, respectively ($p = 0.01$, Fig. 1). Line C was not significantly different from no PMSCs (1.3 ± 0.3). There was no significant difference among the lines.

2.2. ELISA

All lines secreted both neurotrophic growth factors BDNF and HGF and angiogenic growth factor VEGF; however, there were no differences among cell lines ($p = 0.99$, $p = 0.27$ and $p = 0.21$, respectively, Fig. 2A, B, C). Mean levels of BDNF and HGF for Line A were 290 ± 69.2 pg/ml and 86.8 ± 29.2 ng/ml, levels for Line B were 304.8 ± 194.2 pg/ml and 57.9 ± 14.2 ng/ml, and levels for Line C were 294.3 ± 186.8 pg/ml and 75.9 ± 11.2 ng/ml, respectively. Mean VEGF level for Line A was 2200 ± 373 pg/ml, level for Line B was 2170 ± 743 pg/ml, and level for Line C was 2980 ± 466 pg/ml.

2.3. Large animal motor function

The median motor score for lambs treated with any PMSC line was higher than no PMCSs (14.5 (IQR 8–15) *vs* 7.5 (IQR 4–12), $p = 0.03$). The median SLR score following treatment with Line A was 15 (IQR 12–15), Line B was 14 (IQR 8–15), and Line C was 14 (IQR 6–15, $p = 0.11$) (Fig. 3). A majority of lambs treated with cells from each line were able to ambulate independently (SLR score ≥ 13), but the percentage that ambulated varied by line. The rate of ambulation was 83% in lambs treated with Line A (5/6), 71% with Line B (5/7), 60% with Line C (3/5), and 20% with no PMSCs (2/10) ($p = 0.06$).

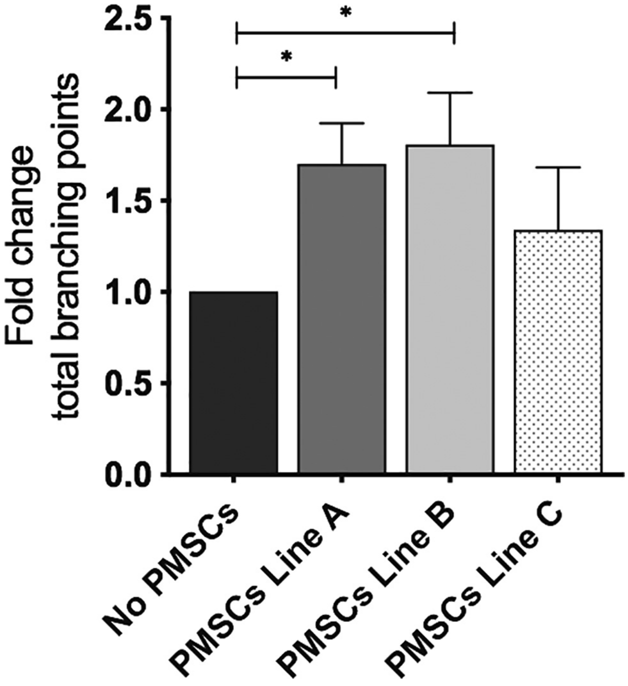

Fig. 1. Comparison of *in vitro* neuroprotective capability of PMSC lines. Compared to no PMSCs, Lines A and B had significantly higher neuroprotection than no PMSCs (1.7 ± 0.2 and 1.8 ± 0.3, respectively, $p = 0.01$). Mean neuroprotection for Line C was not significantly higher than no PMSCs (1.3 ± 0.3).

2.4. Large neuron density

Large neuron (LN) density in the spinal cord was significantly higher following treatment with PMSCs *versus* no PMSCs (ECM only). On subset analysis, Line A and B had significantly higher LN density compared to no PMSCs; however, Line C was not significantly different ($p = 0.04$, Fig. 4). Compared to an LN density of 4.7 (IQR 2.7–13.7) with no PMSCs, LN density in Line A was 25.2 (IQR 19.1–30.4), Line B was 27.6 (IQR 3.4–33.2), and Line C was 24.8 (IQR 12.3–28.1). There was no significant difference among the cell lines. There was a significant positive correlation between motor function score and large neuron density ($r = 0.79$, $p < 0.0001$, Fig. 5). All ambulatory animals had an LN density of at least 15.

3. Discussion

In utero treatment of MMC augmented with PMSCs rescued ambulation in an ovine model following treatment with cell lines generated from three different placental donors. Though rates of ambulation varied by cell line, each line was capable of curing the paralysis associated with the MMC model. The two lines (A and B) that had significantly better neuroprotection than no PMSCs had high rates of ambulation (83% and 71%) and significantly higher large neuron density than treatment without PMSCs.

We identified an *in vitro* neuroprotection potency assay that corresponds with the *in vivo* presence of large neurons, which correlates with motor function in both this study and previous studies [20]. While the true mechanism of action of our PMSC treatment remains unknown, it is likely a combination of neuroprotection and immunomodulation that exerts its effect through the reversal of existing observed apoptosis. The *in vitro* neuroprotection assay appears to be a reasonable adjunct for the screening of donor cell lines for future clinical trials.

The goal of comparing *in vitro* and *in vivo* outcomes was to identify which assays could help reliably select donors that will generate a functional improvement *in vivo*. Lines A and B were superior to line C based on the higher motor neuron density they produced *in vivo*. We believe this is a result of the superior paracrine secretion of cytokines, growth

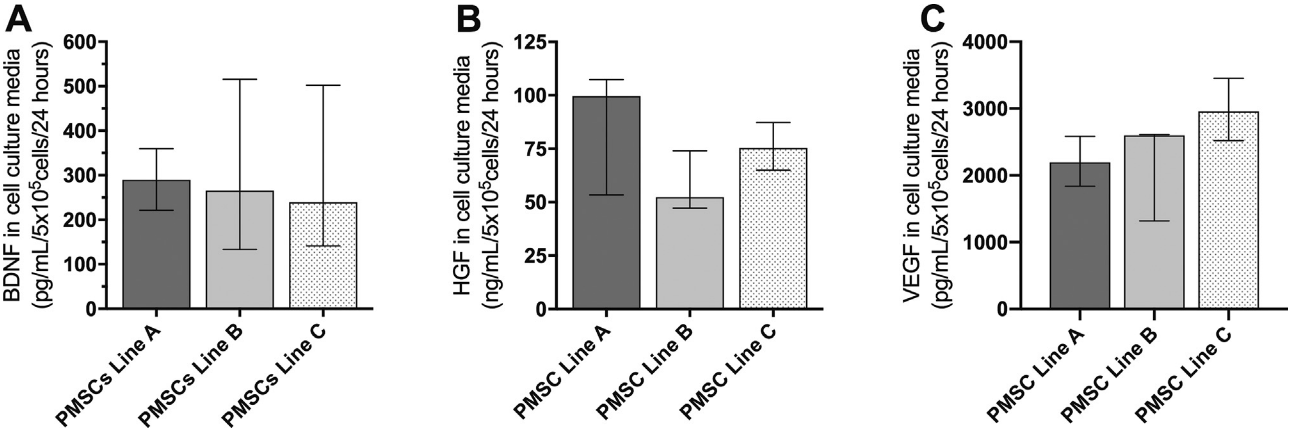

Fig. 2. Growth factor secretion. There was no difference in BDNF (**A**) HGF (**B**) or VEGF (**C**) secretion among cell lines ($p = 0.99$, $p = 0.27$ and $p = 0.21$, respectively).

factors, and exosomes by Line A and B. This superior secretion correlates to superior performance in the neuroprotection assay, which was designed to test *in vitro* function of the entire paracrine secretory milieu rather than individual growth factors. While the motor function outcomes (SLR score and percent ambulation) did not significantly differ among cell lines, the study was not powered to detect this difference, and the cost of using large animals as a screening mechanism is cost prohibitive. Higher density of large neurons *in vivo*, which we have repeatedly shown to correlate with motor function, corresponds to improved *in vitro* neuroprotection seen with Line A and B. Thus, the *in vitro* neuroprotection assay provides some evidence of superiority among the cell lines.

MSCs are promising regenerative therapeutic agents owing to their trophic, angiogenic and immunomodulatory capabilities although clinical outcomes have been variable and exact mechanisms of action are unknown [32–34]. Minimal criteria for defining MSCs have been proposed by the International Society for Cellular Therapy [35]; however, better and more rigorous criteria are needed to predict clinical therapeutic outcomes [22]. Other groups have correlated growth capacity, expression of certain growth factors, and/or gene expression with functional outcomes in small animal models, but these are specific to their therapeutic purposes and not widely generalizable [23,26]. The ideal *in vitro* assays for a therapeutic agent must measure specific MSC functions that match intended *in vivo* effects, which for our therapy are neuroprotection and neuroregeneration.

The primary mechanism of action by which PMSCs exert their effects on tissue regeneration is thought to be paracrine secretion of neuroprotective, angiogenic and immunomodulatory cytokines, growth factors, and exosomes [14,25]. Other studies of MSCs have demonstrated that trophic factors such as VEGF, HGF, insulin-like growth factor (IGF-1) and epidermal growth factor (EGF) play important roles in wound healing and diseases such as multiple sclerosis and therefore represent therapeutic targets [36–38]. We evaluated two known neurotrophic growth factors BDNF and HGF

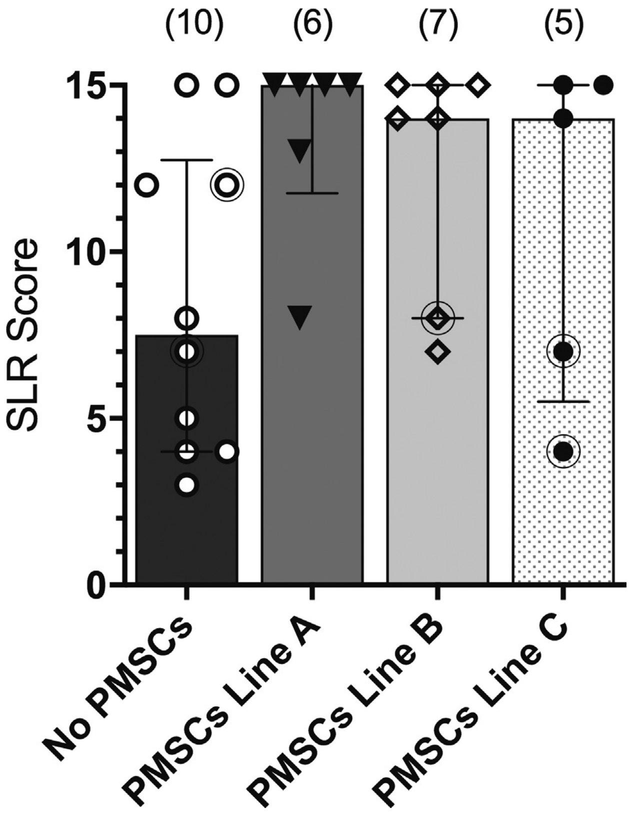

Fig. 3. Comparison of *in vivo* motor function. Median SLR score without PMSCs was 7.5 (IQR 4–13), score with Line A was 15 (IQR 12–15), score with Line B was 14 (IQR 8–15), and score with Line C was 14 (IQR 6–15) ($p = 0.10$). Circled data points represent animals with severe spinal angulation.

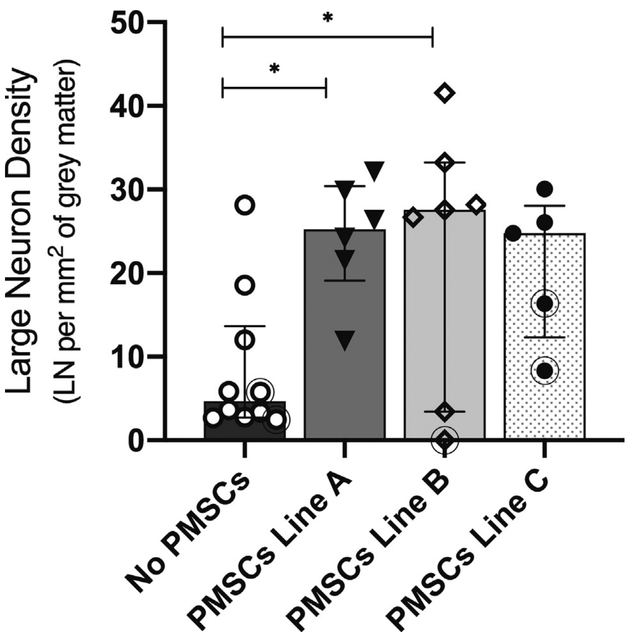

Fig. 4. Large neuron density. LN density was significantly higher with treatment using Lines A and B (25.2 [IQR 19.1–30.4] and 27.6 [IQR 3.4–33.2], respectively) compared to no PMSCs (4.7 [IQR 2.7–13.7], $p = 0.04$). LN density in Line C was not significantly higher than no PMSCs (24.8 [IQR 12.3–28.1]).

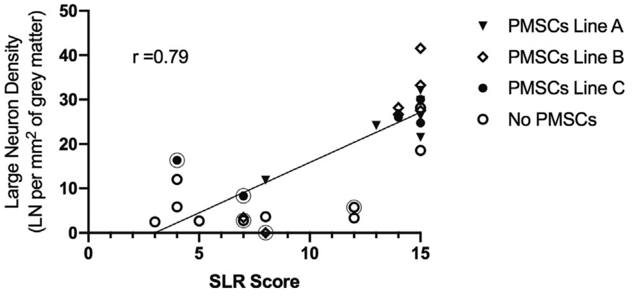

Fig. 5. Correlation of motor function and large neuron density. Motor function score (SLR) was significantly correlated with large neuron density (r = 0.79, solid line, p < 0.0001).

and one angiogenic growth factor VEGF as possible predictors of *in vivo* outcomes. HGF is neuroprotective, is involved in motor neuron development, and promotes neuron survival [39]. BDNF plays a significant role in neurogenesis and has shown potent survival effects on motor neurons [26,40]. VEGF is involved in cellular differentiation and angiogenesis pathways [41]. Despite their potential role in our therapy, we did not find differences in the secretion level of these factors among the three lines and cannot confirm that they are responsible for our therapeutic effect. However, we noted all lines demonstrated robust secretion. As BDNF, HGF, and VEGF are just a small part of the entire PMSC secretory milieu, it is not surprising that these individual factors did not directly correlate with *in vivo* results. We plan to use the BDNF, HGF, and VEGF ELISAs for screening during manufacturing to confirm that the cells lines have robust general paracrine secretion capability.

The neuroprotection assay was designed to test *in vitro* function of the entire secretory milieu rather than individual growth factors [25]. The assay tests the antiapoptotic effects of PMSCs to mimic the neuroprotective effect we see *in vivo*. Previous studies demonstrated that treatment with PMSCs results in preservation of large (motor) neurons of the spinal cord, which directly correlates with the motor function score [7,20].

One limitation of the study is the narrow scope of assays we tested to determine correspondence with *in vivo* outcomes. The growth factors and neuroprotection assay both target the desired outcome of preserving motor neurons and the spinal cord; however, the immunomodulatory role of the PMSCs should not be dismissed. In the immune-privileged environment of the fetus, PMSCs may play an important role in the preservation and regeneration of supportive cells and neurons in the spinal cord through immunomodulation [17–19]. This is an area of ongoing investigation.

It is our intention to screen potential donor cell lines by using a combination of cellular growth and proliferation kinetics, cytokine secretory profiles, and performance in the *in vitro* neuroprotection assay.

Clinical-grade PMSC manufacturing is currently ongoing in our on-site Good Manufacturing Process (GMP) facility in preparation for clinical trials of the PMSC-ECM product. We plan to seed our selected cells on the Cook Biodesign® Dural graft (ECM), which is an FDA-approved dural graft replacement already utilized in MMC repairs in which the dura cannot be closed primarily or requires reinforcement. The combined PMSC-ECM product will be applied directly to the exposed spinal placode during human MMC repairs with incorporation into the watertight dural closure. A successful pre-Investigational New Device (IND) meeting with the Food and Drug Administration (FDA) has already occurred. Following final approval of the FDA IND application, a Phase I/II safety and efficacy trial is planned.

4. Conclusion

In conclusion, *in utero* treatment of myelomeningocele with PMSCs rescued ambulation in the ovine model following treatment with multiple different cell lines. The *in vitro* neuroprotection assay, in combination with other assays, will facilitate selection of optimal PMSC lines for clinical use.

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Declarations of interest

None.

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