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Age Does Matter: A Pilot Comparison of Placenta-Derived Stromal Cells for in utero Repair of Myelomeningocele Using a Lamb Model

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Key Words
Myelomeningocele · Spina bifida · Fetal surgery · Experimental surgery · Placental stromal cells · Cell therapy · Fetal amniotic membranes · Sheep

Abstract
Introduction: Fetal amniotic membranes (FM) have been shown to preserve spinal cord histology in the fetal sheep model of myelomeningocele (MMC). This study compares the effectiveness of placenta-derived mesenchymal stromal cells (PMSCs) from early-gestation versus term-gestation placenta to augment FM repair to improve distal motor function in a sheep model. ***Methods:*** Fetal lambs (n = 4) underwent surgical MMC creation followed by repair with FM patch with term-gestation PMSCs (n = 1), FM with early-gestation PMSCs (n = 1), FM only (n = 1), and skin closure only (n = 1). Histopathology and motor assessment was performed. ***Results:*** Histopathologic analysis demonstrated increased preservation of spinal cord architecture and large neurons in the lamb repaired with early-gestation cells compared to all others. Lambs repaired with skin closure only, FM alone, and term-gestation PMSCs exhibited extremely limited distal motor function; the lamb repaired with early-gestation PMSCs was capable of normal ambulation. ***Discussion:*** This pilot study is the first in vivo comparison of different gestational-age placenta-derived stromal cells for repair in the fetal sheep MMC model. The preservation of large neurons and markedly improved motor function in the lamb repaired with early-gestation cells suggest that early-gestation placental stromal cells may exhibit unique properties that augment in utero MMC repair to improve paralysis.

Introduction

Myelomeningocele (MMC), or spina bifida, is a common birth defect resulting from failed neural tube closure during fetal development. The ensuing spinal cord lesion is susceptible to intrauterine damage, leading to severe paralysis, skeletal deformities, bowel and bladder incontinence, and cognitive disabilities for children born with MMC. While traditionally repaired after birth by closing the dura and skin over the defect, surgery does not rescue children from the lifelong consequences of the disease. The NIH/NICHD Management of Myelomeningocele Study (MOMS) was the first trial to definitively demonstrate the superiority of in utero repair over postnatal closure of the defect [1]. However, while prenatal repair improved associated hindbrain abnormalities (such as the Chiari II malformation) for most and improved distal motor function in some, the majority of children repaired in utero were still unable to ambulate independently.

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In an effort to augment prenatal repair to improve MMC-associated paralysis, we focused on the placenta as a source of stem cells for tissue repair using the fetal sheep model. We previously demonstrated that use of a fetal amniotic membrane (FM) patch improved preservation of normal spinal cord histology in the fetal sheep model of MMC; however, the patch was also shown to interfere with wound healing [2]. Given the known anti-inflammatory and epithelialization-promoting properties of the fetal membranes [3–8], we continued to believe that there was promise for FM repair for MMC. Recent literature has shown that the placenta is also a source of cells that have remarkable immunomodulatory properties, can improve wound healing, and exhibit neuroprotective effects [9–13].

Based on our previous experience with the FM repair in the fetal sheep model and the widespread literature describing the unique properties of both the fetal membranes and placenta-derived mesenchymal stromal cells (PMSCs), we hypothesized that addition of PMSCs to augment the previous success of FM repair may yield distinctive regenerative potential for MMC repair. This pilot study compares the effectiveness of PMSCs from early-gestation placenta versus term-gestation placenta to augment FM repair in order to improve distal neurologic function in the fetal lamb model of MMC.

Materials and Methods

All protocols were approved by the University of California, Davis IACUC, and all animal care was in compliance with the Guide for the Care and Use of Laboratory Animals. All facilities are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International [14]. Donated placental tissue was collected at UC Davis Medical Center with approval from the University of California, Davis Institutional Review Board.

Isolation and Culture of PMSCs from Human Placenta

We isolated PMSCs using an explant culture method from one randomly selected sample of early-gestation (17 weeks) placenta and one term-gestation (approx. 40 weeks) placenta. Chorionic villus tissue was dissected into <5-mm pieces, washed vigorously in sterile 1× PBS containing 1% penicillin-streptomycin, and dissected further. Tissue was spread onto adherent culture dishes coated with CELLStart xeno-free substrate (Invitrogen) for 1 h at 37°C. Adherent cells were harvested at 3–4 weeks and passaged to a monolayer after removing residual tissue.

PMSCs Seeding onto Hydrogel

PMSCs were harvested from adherent cultures, pelleted, and resuspended in complete media before being mixed at 4°C into 2 mg/ml rat tail collagen (BD Biosciences), water, and PBS before pH neutralization with 0.1 N NaOH. A total of 5×10^5 early-gestation cells and 1×10^6 term-gestation cells were available for seeding. Following homogenization and introduction of PMSCs, 1 ml of cell/collagen solution was placed into a 35-mm suspension culture dish and allowed to gel for at least 45 min at 37°C. After gelation was complete, 1 ml of complete culture media was added to the dish to cover the hydrogel layer.

PMSC Characterization with Flow Cytometry

PMSCs were harvested for flow cytometry using Accutase (Invitrogen) and counted using trypan blue. Cells were resuspended, split into fractions containing approximately 1×10^6 cells, and stained with: FITC-CD44 (560977), PECy5-CD90 (555597), PE-CD73 (561014), Alexa Fluor 647-CD105 (561439), PE-CD29 (561795), PE-CD34 (560941), and Alexa Fluor 647-CD31 (561654) (all BD Biosciences). Samples were counterstained using LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Molecular Probes) to detect dead cells, and fixed in 4% paraformaldehyde (PFA) after staining. Flow cytometry experiments were performed with a BD Fortessa LSR Cell Analyzer and analyzed with FlowJo (Treestar Inc.).

PMSC Immunocytochemical Staining

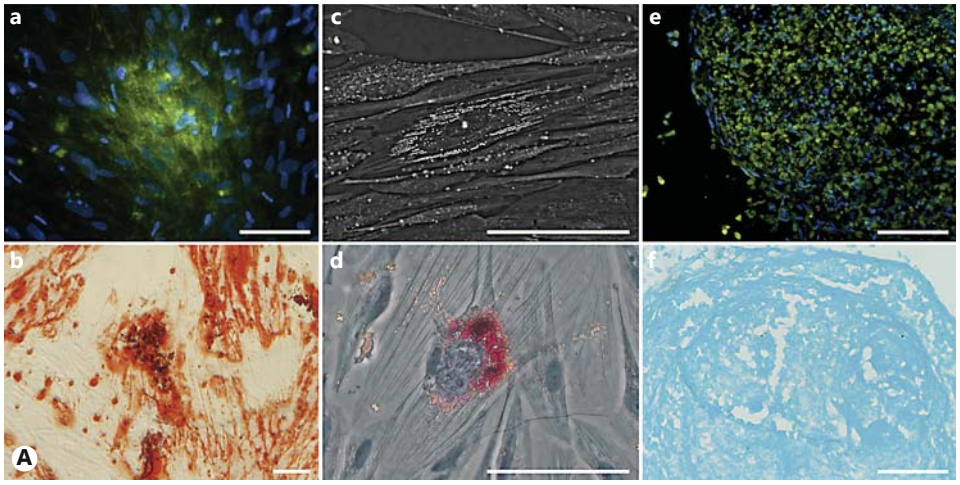
PMSCs were fixed in 4% PFA in PBS prior to immunostaining, followed by membrane permeabilization with 0.5% Triton X-100 in PBS. Fixed cells were then incubated with monoclonal antibody for Sox2 (R&D Systems, MAB2018) overnight at 4°C. Cells were subsequently incubated with Alexa Fluor 488- or 546-conjugated secondary antibodies (Molecular Probes) for 1 h at room temperature. Cell nuclei were counterstained with DAPI, and images collected using a Carl Zeiss Axio Observer D1 inverted microscope.

PMSC Multipotency Assays

PMSCs were assessed for their ability to differentiate into traditional mesodermal lineages. For osteogenic differentiation, PMSCs were cultured in 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. Cells were fixed in 4% PFA and stained with alizarin red (Sigma-Aldrich) and alkaline phosphatase (BD Biosciences). For chondrogenic differentiation, PMSCs were cultured as cell pellets in suspension in DMEM containing 10% FBS, 10 ng/ml transforming growth factor-β_3 (Peprotech), and 200 μM ascorbic acid (Sigma-Aldrich) for 3–4 weeks. Chondrogenic pellets were then fixed in 4% PFA, embedded in Optimal Cutting Temperature compound (Fisher Scientific), cross-sectioned, and immunostained for collagen II (Abcam) and Alcian blue (Sigma-Aldrich). For adipogenic differentiation, PMSCs were cultured in DMEM containing 10% FBS, 1 μM dexamethasone (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), and 5 μM isobutylxanthine (AdipoGen) for 3–4 weeks. Cells were then imaged with phase contrast microscopy before fixation in 4% PFA and staining with Oil Red O (Sigma-Aldrich).

MMC Defect Creation and Repair

Four time-mated, pregnant ewes underwent laparotomy and hysterotomy at a gestational age of approximately 75 days. Fetuses underwent surgical creation of the MMC defect as previously described in detail [15, 16]. Briefly, the skin, paraspinal muscles, and 6 lumbar vertebrae were removed, followed by removal of the dura



	Early PMSCs	Term PMSCs
CD105	+	+
CD90	+	+
CD73	+	+
CD44	+	+
CD29	+	+
CD34	–	–
CD31	–	–
Oct 4	+	–

B

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Fig. 1. A Trilineage differentiation potential of the term-gestation PMSCs demonstrating osteogenic differentiation characterized by immunostaining for alkaline phosphatase (**a**) and alizarin red staining (**b**), adipogenic differentiation by phase contrast imaging (**c**) and Oil Red O staining (**d**) for lipid deposition, and chondrogenic differentiation by collagen II immunostaining (**e**) and Alcian blue staining for glycosaminoglycans (**f**). Identical differentiation potential was noted in early-gestation PMSCs but is not shown. Scale bars = 100 μm. **B** Summary of cell surface marker and Sox2 expression profiles of early- and term-gestation PMSCs.

overlying the exposed spinal cord. Approximately 25 days later, a second survival laparotomy and hysterotomy was performed to repair the MMC defect. Any overlying fibrinous exudate on the spinal cord was removed, and the fetal defects were repaired with the following: FM repair with term-gestation placenta-derived stromal cells (n = 1), FM repair with early-gestation placenta-derived stromal cells (n = 1), FM only (n = 1), and skin closure only (n = 1). Lambs treated with PMSCs were treated with cells suspended in 1 ml of collagen and applied directly to the open neural placode. For lambs repaired with FM, the patch was harvested from the uterus at the time of the second operation, placed chorion side down, and secured over the defect with interrupted 6-0 monofilament sutures. The skin was then closed over the patch or over the open defect in the lamb repaired with skin only.

Motor Function Analysis

Lambs were delivered at term (approx. 145 days' GA) via spontaneous vaginal delivery. Motor function was evaluated for all lambs with the Sheep Locomotor Rating (SLR) scale as previously described [17]. The scale assesses motor function in seven categories and results in a score from 1 to 15, with normal function scoring 15 and complete hind limb paralysis scoring 0. Grades of 0–4, 5–9, and 10–14 indicate severe, moderate, and mild motor deficits, respectively. Live locomotor testing was performed by 2 examiners within 24 h of birth; the best performance was used for analysis. Motor testing was recorded by video camera and scored by 2 blinded examiners independently. All scores were compared, and videos were reviewed to reconcile differences and assign a consensus score.

Histopathologic Analysis

After completion of the motor function analysis, all lambs were euthanized and perfused with PFA. Brains and spinal cords were dissected for analysis and grossly inspected. The area of greatest deformation within the lumbar spine was identified and labeled as the lesion epicenter. This segment was cut, embedded in Optimal Cutting Temperature compound, and 20-μm sections were taken through each epicenter. Following cryosectioning, Nissl (cresyl violet) staining was performed, and 6 cross sections per epicenter were analyzed. Total cross-sectional area, gray matter surface area, and white matter surface area were determined using ImageJ software. The area of gray and white matter was calculated and averaged. Large neurons, cells with a diameter of 30–70 μm as previously described [18], within the gray matter were counted with an inverted bright field microscope (Carl Zeiss Axio Observer D1) at ×10 magnification. All counts were performed by a single, blinded individual.

Results

In vitro Characterization of PMSCs

Multipotency assays demonstrated that both early and term-gestation PMSCs were capable of differentiating into osteogenic, chondrogenic, and adipogenic lineages, as is characteristic of traditional mesenchymal stromal cells (MSCs; fig. 1A). Flow cytometry confirmed that both early- and term-gestation cells were positive for CD105, CD90, CD73, CD44, and CD29, traditional MSC markers (fig. 1B). PMSCs of both lineages were also negative for the hematopoietic stromal cell marker CD34 and endothelial cell marker CD31. Early-gestation PMSCs stained positive for Sox2, a transcription factor normally seen in pluripotent or more primitive cells; however, term-gestation cells did not.

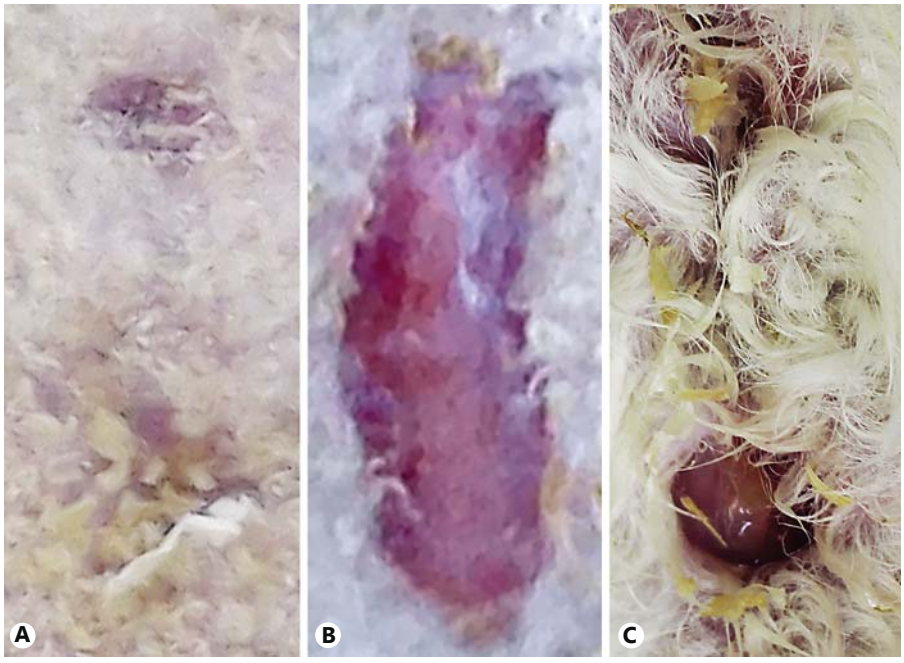

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Fig. 2. Surgical incisions in FM-repaired animals at birth: FM only (**A**), FM with term PMSCs (**B**), FM with early-gestation PMSCs (**C**).

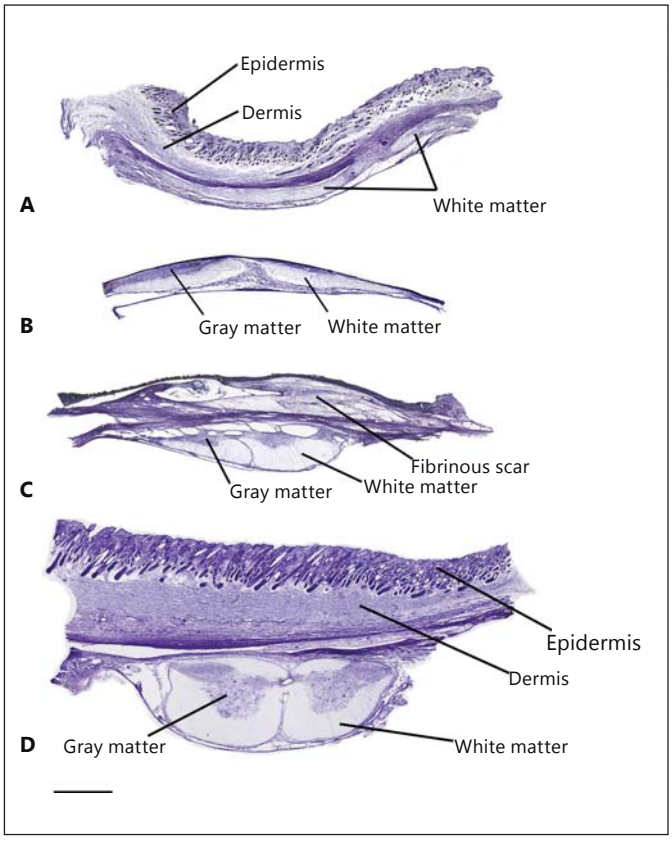

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Fig. 3. Cross-sectional images of the MMC lesion epicenters at ×4 magnification. Scale bar = 2 mm. **A** Skin-only repair. **B** FM only. **C** FM with term PMSCs. **D** FM with early PMSCs.

Motor Function Assessment

All lambs were born via spontaneous vaginal delivery at term. The lamb repaired with skin only was noted to have near-complete hind limb paralysis and severe motor deficits with an SLR score of 1; this was the lowest score among all 4 animals. Two lambs were noted to have moderate motor deficits: the lamb repaired with FM only scored a 7, and the lamb repaired with FM with term PMSCs scored an 8. These lambs could support their weight with their hind limbs but were unable to stand even when assisted. The lamb repaired with FM with early PMSCs was noted to have entirely normal locomotion (SLR score of 15).

Gross Necropsy and Histopathologic Analysis

Gross necropsy revealed open wounds on the back of all 3 lambs receiving FM repair as well as the skin-only repair lamb (fig. 2). Dissection of the spinal cords revealed varying degrees of spinal cord deformation throughout the lumbar cord. This deformation varied by animal and within the lumbar cord. The area of greatest compression, or the gross lesion epicenter, was at the L4 segment in all 4 animals. There were no other gross abnormalities of the spinal cord or complications of stromal cell application, such as tumor formation.

Cross-sectional imaging of the lesion epicenters displayed prominent cord compression in all 4 lambs (fig. 3). However, the degree of deformation varied by animal. Histopathologic analysis demonstrated a similar cross-

Table 1. Histopathologic analysis of MMC lesion epicenters and motor function scores for each animal

	Spinal cord cross-sectional area, mm^2	Gray matter area, mm^2	White matter area, mm^2	Large neurons, n	Motor function score
Skin only	5.34	0.0	5.36	0.2	1
FM only	5.63	1.81	3.83	9	7
FM + term PMSCs	5.56	1.31	4.25	16	8
FM + early PMSCs	18.67	6.89	11.78	74	15

sectional spinal cord area in the lambs repaired with FM with term-gestation stromal cells, FM alone, and skin closure only (table 1). The lamb repaired with FM with early-gestation stromal cells had three times the epicenter cross-sectional area than the other lambs. When looking specifically at the amount of gray matter preserved, the skin closure-only lamb had no residual gray matter within the lesion epicenter. The lambs repaired with FM only and FM plus term-gestation stromal cells both had some gray matter preservation at the epicenter, but, again, the lamb repaired with early-gestation PMSCs had a 4-fold increase in the area of gray matter present. Finally, when looking at the number of large neurons within the gray matter, the lamb repaired with skin only had no visible large neurons (average count 0.2), while the lambs repaired with FM only and FM plus term PMSCs had increased preservation of neurons (9.2 and 16.3 neurons, respectively). The lamb repaired with early-gestation cells had a dramatic increase in the number of large neurons (74.2) present in comparison to all others.

Discussion

Three decades ago, Meuli et al. [19] launched the notion of treating spina bifida before birth. He obtained remarkable results with the use of a fetal muscle flap in a fetal lamb model. When reflecting on this work today, it is possible that the muscle flap served as a delivery vehicle for fetal stem cells. The technical challenges of the muscle flap precluded replication of these results in human fetuses; however, this served as the starting point for stem cell therapy for in utero repair of MMC. The pilot study reported here is the first in vivo comparison of placenta-derived stromal cells of different gestational ages for prenatal repair in a fetal sheep model of MMC. Histopathology demonstrated an increased preservation of spinal cord tissue, gray matter, and neurons with the use of early PMSCs for repair. In addition to our pathologic analysis, we saw markedly improved motor function in the lamb repaired with early-gestation cells. This pilot study suggests that stromal cells derived from early-gestation placenta may exhibit unique properties to augment in utero repair of MMC and improve paralysis.

As the field of stem cell therapy continues to expand, multipotent stromal cells derived from placental tissues and amniotic fluid have piqued the interest of researchers in the field of fetal surgery. Researchers have shown that stromal cells can be reliably harvested from both sources [9, 20], and because these are fetus-derived tissues, they represent a potential form of autologous stromal cell therapy for patients undergoing fetal surgery. We have chosen to focus on placenta-derived tissues as they can be harvested via chorionic villous sampling, the procedure of choice at a gestational age of 12 weeks (when MMC is typically diagnosed), with minimal risk to the mother or fetus, and can be expanded in culture for autologous MMC repair during the second trimester [21].

Cells harvested from placental tissue have been characterized as MSCs [9], but have also been shown to display more unique properties. Lee et al. [10] compared MSCs derived from placenta with standard bone marrow- and adipose tissue-derived stromal cells and found that placenta-derived MSCs express higher levels of immunomodulatory cytokines. Portmann-Lanz et al. [11] compared the differentiation potential of stem cells harvested from various fetal and placental tissues. They found that chorion-derived cells exhibited better neurogenic differentiation capacity than amnion-derived cells and postulated that these properties may confer a neuroprotective effect. In our study, as well as others, early-gestation cells were found to express Sox2, a transcription factor often associated with a more primordial state which has also been shown to be expressed by neural progenitor cells [9, 22, 23]. Interestingly, term-gestation PMSCs did not express this in our study. The unique properties of early-gestation placenta-derived cells seen in our study and others' support the hypothesis that use of early

PMSCs for fetal therapy may successfully augment the ongoing remodeling of the fetal environment by enhancing immunomodulation and providing neuroprotection.

We are limited by the small sample size inherent in a pilot study. Despite the inability to perform a statistical analysis, we demonstrate an obvious benefit to treatment with early-gestation PMSCs compared to all other groups, as evidenced by the dramatic increase in cross-sectional area, gray matter area, neuron preservation, and the remarkable preservation of motor function in this lamb. While the lambs repaired with FM only and FM with term PMSCs did show both histologic and motor benefits over the skin-repaired lamb, these improvements were greatly overshadowed by the more dramatic findings seen with early PMSC treatment. Interestingly, there was little, if any, benefit of treatment with term-gestation cells over the FM repair alone. It is likely that the more primordial nature of the early-gestation cells is a critical component in promoting a regenerative state that is conducive to rescuing neurologic function in the developing fetus.

The significant variability in defect appearance associated with the fetal sheep model of MMC is another potential limitation worth discussing. We previously characterized this variability and demonstrated that despite uniform defect creation at the initial surgery and minimal variation in size, the phenotypic appearance of the resulting defect remains highly variable [24]. While the significance of this variability in terms of correlation with injury severity is unknown, it does emphasize the need for a larger study to corroborate these findings. However, given the promising results in this small pilot study, a larger study is warranted to determine whether treatment with early PMSCs may be a solution to the devastating paralysis affecting children with MMC.

In addition to expanding this pilot study to a large-scale animal study, there are several future directions of study for our work with PMSCs. First, research investigating the optimal cell dose and delivery vehicle is needed. The current study is limited by the differing number of early and term cells used for treatment, and it is possible that the difference in cell dose may have had an impact on our results. Furthermore, the potential interaction of the cells with the patch is unclear. We plan to investigate alternative patch materials as well as stem cell application without the use of the collagen gel. Lastly, an important aspect of investigation for any stem cell therapy is the potential for teratogenesis. Based on the existing literature, MSCs are proposed to enact their effects via transient, paracrine mechanisms [25, 26]. Therefore, MSCs are not expected to integrate into native tissues and theoretically pose less risk for tumor formation. However, we plan to address these risks with more long-term survival studies.

Our previous work using an autologous FM patch for in utero repair of MMC showed potential promise over the traditional skin closure repair technique [2]. In this study, FM repair resulted in an increased preservation of spinal cord tissue in addition to increased large neuron preservation. However, the FM seemed to retard healing of the surgical incision. This deficit in wound healing was met with great concern given that the inability to create a watertight seal may negate the benefits of prenatal repair by failing to prevent the Chiari II malformation [27]. However, given the promising results and known anti-inflammatory effects of FM, we conducted this pilot study to investigate whether the addition of placenta-derived stromal cells might enhance the neuroprotective effects of the fetal membranes and possibly overcome the wound healing deficits. Unfortunately, we again saw a failure of surgical wound healing in all lambs repaired with FM. Combining our prior study with the current pilot results, 6 out of 7 lambs repaired with FM had an open wound at birth. While the lamb with the skin repair in this pilot study also had an open wound, we know this to be an extremely uncommon occurrence that is difficult to explain and further emphasizes the limitations of this small pilot study. The phenomenon of wound healing abnormalities noted with FM repair must be strongly considered prior to use in humans as the consequences of these wound-healing deficiencies may allow for Chiari II malformation and preclude one of the major benefits of prenatal repair.

In summary, this pilot study details the first reported use of placenta-derived stromal cells to augment FM patch repair in a fetal model of MMC. Our in vivo comparison of early- and term-gestation placental cells demonstrated an increased preservation of spinal cord tissue and large neurons in the animal repaired with early-gestation cells. Most importantly, we saw a remarkable complete recovery of distal motor function in the lamb repaired with early-gestation PMSCs. This pilot study suggests that stromal cells derived from early-gestation placenta may exhibit unique properties to augment in utero repair of MMC and improve paralysis. Furthermore, it supports the need for a larger study investigating the potential benefits of early-gestation PMSCs to cure paralysis associated with MMC.

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