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Tissues Reborn: Fetal Membrane-Derived Matrices and Stem Cells in Orthopedic Regenerative Medicine

Renae Keeley, Natasha Topoluk, & Jeremy Mercuri

Laboratory of Orthopedic Tissue Regeneration & Orthobiologics, Department of Bioengineering, Clemson University, Clemson, South Carolina

All authors contributed equally to this work.
*Address all correspondence to: Jeremy Mercuri, PhD, 301 Rhodes Engineering Research Center, Department of Bioengineering, Clemson University, Clemson, SC, 29634; jmercur@clemson.edu.

ABSTRACT: The amniotic and chorionic membranes, as well as the stem cell populations contained within them, represent a widely available, versatile, and promising resource for use in numerous regenerative medicine applications. The primary focus of this review is to examine the use of the fetal membranes and/or their resident stem cell populations for regenerating orthopedic tissues. This discussion is prefaced by a brief synopsis of the structure, function, and biological properties of the extracellular matrix; embryological development; and a brief description of the distinct stem cell populations residing within the amniotic and chorionic membranes. Commercially available perinatal tissue allograft products available in the United States are reviewed, and a concise summary regarding the US Food and Drug Administration's current viewpoint on these technologies is provided. Concluding remarks regarding suggested future research directives for evaluating these tissues and stem cell sources in relation to orthopedic regenerative medicine applications also are presented.

KEY WORDS: extraembryonic membranes, orthopedics, regenerative medicine, stem cells, tissue engineering

ABBREVIATIONS

ADSC, adipose derived stem cell; **AM**, amniotic membrane; **BMP**, bone morphogenetic protein; **BMSC**, bone marrow derived stem cells; **CBER**, Center for Biologics Evaluation and Research; **CM**, chorionic membrane; **ECM**, extracellular matrix; **FDA**, US Food and Drug Administration, **hAEC**, human amniotic epithelial cell; **hAMSC**, human amniotic mesenchymal stem cell; **hCMSC**, human chorionic mesenchymal stem cell; **HCT/P**, human cells, tissues, cell- and tissue-based product; **HLA**, human leukocyte antigen; **IDO**, indoleamine 2,3-dioxygenase; **MHC**, major histocompatibility complex; **OA**, osteoarthritis; **oAEC**, ovine amniotic epithelial cell; **TRG**, Tissue Reference Group; **VEGF**, vascular endothelial growth factor.

I. INTRODUCTION

Debilitating orthopedic conditions, including osteoarthritis, degenerative disc disease, torn tendons, damaged cartilage, bone neoplasms, metabolic syndromes, fractures, and congenital defects, inflict significant socioeconomic burden in the United States and around the world. Nearly 4 million surgical operations to address musculoskeletal concerns were performed in the United States in 2011; this represented the largest proportion (24.2%) of all procedures performed.[1] The total estimated cost of treatment and lost wages associated with these disorders was $950 billion in 2008, amounting to nearly 7.4% of US gross domestic product.[2] Treatment options for these conditions are vast and often palliative, including physical therapy, pharmaceutical intervention, and/or implantation of inanimate biomedical materials.

Over the past 40 years, the field of tissue engineering and its potential as an alternative method or adjunct to established treatment regimens have garnered much attention. This specialized field falls under the broader scope of regenerative medicine and

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is primarily concerned with developing tissue-based implants composed of cells, soluble biochemical signals, and a delivery template (i.e., "biomaterial scaffolds") for the de novo development or *in vivo* regeneration of healthy tissue. The primary purpose of these implants is to replace tissues that have succumbed to a traumatic injury, congenital deformity, or disease processes. One of the many proposed advantages of this approach is that an individual's ailing tissue can be replaced with a healthy surrogate or can be influenced to regenerate toward a reinvigorated status as opposed to merely replacing it with a nonliving material. Thus the field of tissue engineering may represent the next frontier in clinical medicine.

For any tissue engineering approach to be successful, the cell populations used for implantation and regeneration must (1) be available in sufficient quantities, (2) be functionally viable and healthy, (3) exhibit a desired phenotype, and (4) not elicit an immune reaction from the recipient. For these reasons, over the past quarter century much attention has turned toward the use of stem cells as alternative cell sources for tissue engineering and regenerative medicine. Stem cells are found within many adult and embryonic tissues and possess the ability to differentiate into almost any cell type found in the human body.[3] Thus these cells have gained the attention of the clinical and scientific community for their potential therapeutic impact. To date, most researchers have focused on obtaining stem cells from various adult tissues; most often investigated are stem cells isolated from bone marrow (BMSCs) or adipose tissue (ADSCs). While these stem cell populations exhibit the ability to aid in the regenerative process and possess immunomodulatory and anti-inflammatory properties, they do suffer from drawbacks, including donor site morbidity, a limited ability to self-renew, and a reduced capacity for differentiation with age.[4–6] In terms of stem cell yields, BMSCs account for only 0.01–0.001% of the total 6×10^6 mononuclear cells typically isolated from human bone marrow, whereas $0.5–2 \times 10^6$ ADSCs are found in each gram of adipose tissue.[4,6] While this results in approximately 100–500 times more ADSCs per unit volume of tissue compared with BMSCs, significant *ex vivo* expansion is required to achieve clinically relevant cell numbers for therapeutic applications. In turn, requisite *in vitro* expansion of cells is accompanied by significant scrutiny and oversight by the US Food and Drug Administration (FDA), which ultimately impacts the clinical utility of these cells.

Many researchers, including those in our laboratory, have more recently begun investigating stem cell populations that reside within the fetal membranes, which surround the human fetus, as an alternative source of stem cells. The amniotic and chorionic membranes (AM and CM, respectively) typically remain intact following cesarean delivery, are routinely discarded following the birth of full-term babies, and contain orders of magnitude more stem cells than adult tissues. In 2012 a total of 3,952,841 births were registered in the United States alone, with nearly 33% of those by cesarean delivery,[1] making the AM and CM widely available sources for stem cells that do not pose significant ethical concern, as is the case for human embryonic stem cells.

Within this review we briefly provide a background describing the embryological origins, histological organization, and function of human fetal membranes. In addition, the distinct cell populations found within these membranes are described, with particular attention being paid to the characterization of AM- and CM-derived stem cells. The primary purpose of this review, however, is to highlight research conducted to date aimed at evaluating fetal membrane–derived extracellular matrices (ECMs) and stem cells and their application toward orthopedic regenerative medicine. A synopsis of commercially available fetal membrane–derived products and regulatory considerations provides insight into the translational potential and clinical utility of fetal membrane–derived stem cells for orthopedic applications. Finally, future research endeavors for evaluating the use of human fetal membrane–derived ECM and stem cells within the field of orthopedics are suggested.

II. DEVELOPMENTAL ORIGINS OF FETAL MEMBRANES

In-depth investigations into the embryological origins of fetal membranes has been previously de-

scribed in work by Luckett,[7,8] as well as Dobreva *et al*.[9] Briefly, the blastocyst separates into an inner cell mass and the trophoblast at embryonic day 5. The inner cell mass subsequently differentiates into the epiblast (which gives rise to the amniotic ectoderm and embryonic epiblast) and the hypoblast (Fig. 1). The amniotic ectoderm subsequently contributes to the formation of the human amniotic epithelium, whereas the embryonic epiblast eventually yields all 3 germ layers of the developing embryo and the extraembryonic mesoderm. Of note, the amniotic epithelium is derived from the amniotic ectoderm, which develops from the epiblast and appears approximately 1 week before gastrulation. Some suggest that the epiblastic origins of human amniotic epithelial cells (hAECs) and their potential segregation from organogenetic signals involved in gastrulation may account for the pluripotent nature of these cells.[9–11] The extraembryonic mesoderm subsequently develops and contributes to the mesodermal layer of the amniotic membrane, as well as the mesenchymal layer of the chorion. The remaining elements of the chorion are derived from the extraembryonic trophoblast.

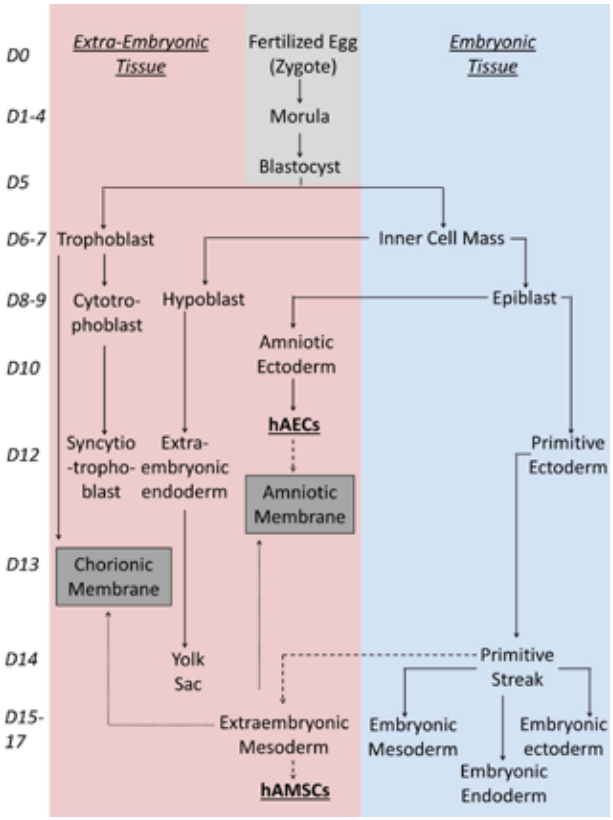

FIG. 1: Schematic representation of the temporal development of extraembryonic (pink) and embryonic tissues (light blue), illustrating the contributions of these tissues to the amniotic and chorionic membranes as well as the stem cell populations contained within. Approximate day of embryologic development is noted in italics at the far left.

III. STRUCTURE AND FUNCTION OF FETAL MEMBRANES

The 2 human fetal membranes that surround the developing fetus and segregate it from the maternal endometrium are the AM and CM. The AM, also referred to as the amnion, is the innermost membrane and is in direct contact with the fetus and amniotic fluid and is contiguous with the umbilical cord. The second fetal membrane, the CM, is weakly attached to and envelops the AM. The villous chorion comprises the fetal portion of the placenta, which interfaces with the maternal aspect of the placenta, known as the maternal decidua. Importantly, the amnion and chorion are both derived from extraembryonic tissue and are composed entirely of fetal tissue. For a schematic representation depicting the organization of these membranes, referred to the published work of Dobreva *et al*.[9]

Research has classified the AM via its histological microarchitecture, which has been reviewed succinctly by Niknejad *et al*.[12] Previously believed to be a simple epithelial lining for the uterine contents, it is now apparent that the AM is composed of multiple layers (Fig. 2). This translucent, thin (0.02–0.5 cm), avascular, and aneural membrane can be divided into 5 distinct strata based on differences in microarchitectural and biochemical constituents.[13] Beginning with the innermost layer of the AM (in order), there is a layer of cuboidal hAECs known as the amniotic epithelium. These cells reside on a basement membrane composed primarily type IV collagen as well as the glycoproteins laminin, fibronectin, and nidogen (entactin).[12] Furthermore, perlican—a large heparin sulfate proteoglycan that efficiently binds growth factors—as well as structural molecules including actin, vimentin, cytokeratin, and actinin

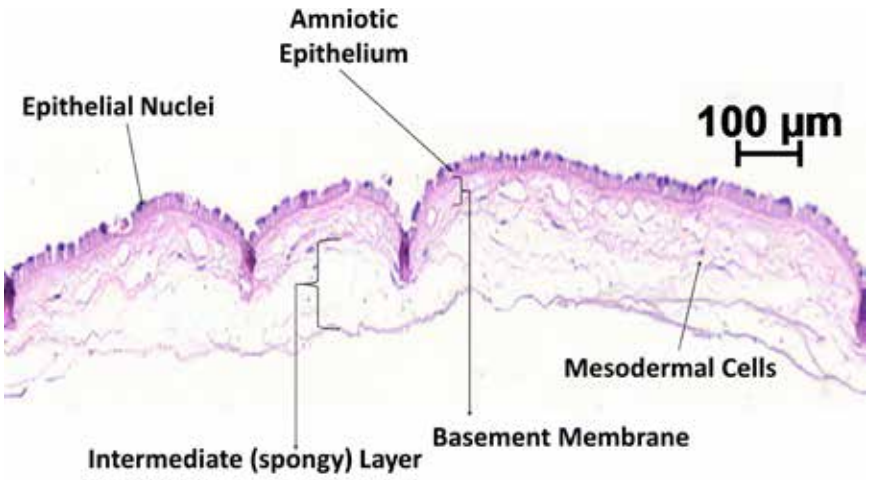

FIG. 2: Hematoxylin and eosin–stained section of the human amniotic membrane illustrating the multilayered microarchitecture and the presence of human amniotic epithelial cells and human amniotic mesenchymal stem cells. (original magnification, ×400).

have been reported to be present in the basement membrane of the AM as well. Immediately underlying the basement membrane is a mesodermal layer that can be further subdivided into a compact layer containing type I, III, VI, and VI collagens, a fibroblast/reticular layer, and a spongy stratum that is in direct contact with the CM and contains mostly proteoglycans, glycoproteins, and nonfibrillar type III collagen.[12–16,17] This spongy layer allows for a sliding movement to occur between the AM and the underlying CM. These 2 layers can be easily separated by mechanically peeling the AM away from the CM. In addition to hAECs, macrophages, fibroblasts, and mesenchymal stem cells (discussed in more detail within the following sections) also have been identified within the layers of the AM and CM.[18–20] The CM is predominantly comprised of type IV and V collagens and can also be subdivided into multiple layers, including an extraembryonic mesenchymal layer immediately adjacent to the mesodermal layer of the AM and a trophoblastic layer composed of cytotrophoblast and syncytiotrophoblast cells (Fig. 3).[12]

In terms of function, the AM serves as a selectively permeable membrane, allowing diffusive nutrient uptake, waste elimination, and gas exchange to and from the fetus. This membrane, in conjunction with the amniotic fluid, also allows for physical protection of the developing fetus by (1) providing a pressure-absorbing cushion, (2) enabling free movement of the developing fetus, and (3) regulating pH via the production of bioactive factors. The CM, specifically the villous cytotrophoblast and syncytiotrophoblastic layers, in conjunction with the maternal decidua, contribute significantly to the development of maternal–fetal immunological tolerance. Proposed mechanisms of tolerance contributed by the outermost layers of the CM include (1) the expression of human leukocyte antigen (HLA)-G on trophoblastic cells, which induce apoptosis of cytotoxic T-cells and alters natural killer cell function; (2) the downregulation of HLA class Ia and II molecules by the trophoblast before implantation into the endometrium; (3) expression of Fas-L by trophoblastic cells; (4) the upregulation of indoleamine 2,3-dioxygenase, which degrades tryptophan and subsequently inhibits maternal T-cell activation; and (5) production of anti-inflammatory mediators inducing TH2 responses.[21–25]

IV. STEM CELL POPULATIONS WITHIN HUMAN FETAL MEMBRANES

One of the most promising aspects of the AM and CM, from a tissue engineering and regenerative medicine perspective, is the readily available and

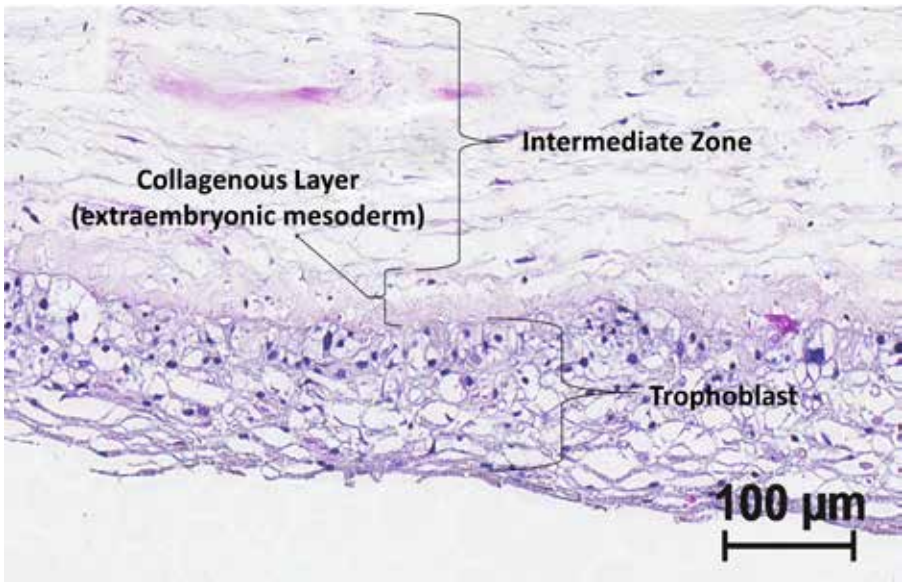

FIG. 3: Hematoxylin and eosin–stained section of the human chorionic membrane illustrating the intermediate zone, the extraembryonic mesenchymal layer, and the presence of trophoblastic cells (original magnification, ×400).

developmentally juvenile stem cells found within the ECM of these membranes. Research indicates that at least 2 distinct populations of stem cells reside within the AM: (1) a percentage of the hAECs display pluripotent markers, and (2) the mesenchymal layer of the AM contains an abundance of mesenchymal stem cells (hAMSCs). In addition, stem cells have been identified within the CM (hCMSCs). In terms of cell yields from the term human AM, reports suggest that nearly 60–200 × 10^6 hAECs and 20–60 × 10^6 hAMSCs can be isolated from each membrane.[20,26] Given the average surface area (1500 cm^2)[27,28] and mass (24 g)[27] of the term AM, normalized cell yields equate to approximately 2.5–10 × 10^6 hAECs and 1–2.5 × 10^6 hAMSCs per gram wet weight of AM. Furthermore, mass-normalized hCMSC yields have been reported to be similar to those of hAECs.[27] It should be noted, however, that variability between isolation methods, culture conditions, as well patient-to-patient variability may result in significant differences between primary stem cell yields and the differentiation capacity of the cells.[29–31]

A. Human Amniotic Epithelial Cells

Freshly isolated hAECs display a cobblestone morphology, and approximately 5–50% express pluripotent stem cell markers, including octomer-binding protein 4, SRY-related HMG-box gene 2 (*SOX-2*), NANOG, stage-specific embryonic antigens 3 and 4, and tumor rejection antigen 1-60 and 1-81, which are involved in maintaining pluripotency and self-renewal.[10] Expression of the hematopoietic marker CD34 and telomerase reverse transcriptase is notably absent, and these cells do not seem to promote tumorigenicity in SCID mice.[10] It does seem that hAECs are a heterogeneous population of cells exhibiting various stages of stemness.[11] In addition, hAECs stain positively for the epithelial marker cytokeratin but do seem to undergo an epithelial-to-mesenchymal transition following *in vitro* expansion, as indicated by a decrease in cytokeratin staining and a concomitant increase in mesenchymal markers, including CD90 and CD105, with an increasing passage number.[30] Fatimah and colleagues[32] illustrated that culture media supplementation with 10 ng/mL human recombinant epidermal growth factor is required to support proliferation of hAECs

in vitro; however, there seems to be a trade-off between increasing cell proliferation and reducing the expression of pluripotency markers. High-density culture expansion of hAECs does not seem to alter their major histocompatibility complex (MHC) molecule expression patterns; hAECs maintain low expression of HLA-A/B/C (MHC class I molecules) and a complete absence of HLA-DP/DQ/DR (MHC class II molecules) surface antigens. Conversely, these cells do exhibit a reduced capacity to suppress concanavelin-A-stimulated T-cell proliferation and a reduced expression of HLA-G molecules with increasing passage number.[31] Although the quantities of surface markers vary within hAEC populations, the ratio of positive stem cell markers is considerably higher than in other somatic or tissue stem cells of the human body. The ability of hAECs to differentiate into multiple phenotypes representing all 3 germ layers has been demonstrated by many investigators and is summarized succinctly by Parolini and colleagues.[20] Studies also have reported the secretion of various growth factors from hAECs, including epidermal growth factor, platelet-derived growth factor, noggin, activin, vascular endothelial growth factor (VEGF), transforming growth factor-β2, and tissue inhibitors of metalloproteinases.[33] The membrane ECM and isolated hAECs obtained from term AM were recently shown to decrease the viability and proliferation of cervical and breast cancer cells in a dose-dependent manner.[34] Furthermore, Niknejad *et al*.[34] illustrated the ability of hAECs to prevent angiogenesis in an *in vitro* model of blood vessel infiltration; however, when human AM was denuded of hAECs, angiogenesis was promoted. Taken together, these data suggest the discordant roles that cells and the ECM may play within the same fetal membrane.

B. Human Amniotic Mesenchymal Stem Cells

hAMSCs reside in the mesodermal layer of the AM and have been shown to express the mesenchymal stem cell markers CD105, CD90, and CD73.[20,35–37] These cells have exhibited a fibroblast-like morphology and are typically 3 times larger than hAECs in terms of a single cell's surface area.[29] Similar to hAECs, these cells exhibit a low expression of HLA-A/B/C; they lack HLA-DR and the hematopoietic markers CD34, CD45, and CD14.[20,35–37] hAMSCs have proliferated well in culture and can be expanded through 15 passages without the use of exogenous growth factor supplementation, resulting in an idealized (theoretical) yield of 5×10^8 cells/amnion.[28] Furthermore these cells have illustrated the capacity to differentiate down an osteogenic, chondrogenic, adipogenic, and endothelial lineage; however, affinity for differentiation toward a specific lineage seems to be dependent on the isolation method used.[39,40,49] In addition, these cells have differentiated toward hepatocytes, pancreatic cells, and cardiomyocytes.[41–44] Interestingly, in comparative cultures with CM-derived stem cells, hAMSCs secrete significantly higher concentrations of hepatocyte growth factor and basic fibroblast growth factor.[27] Furthermore, in response to coculture with activated T-lymphocytes, hAMSCs produce significantly higher concentrations of prostaglandin-E2; a well-known immunomodulator, compared with CM-derived stem cells.

C. Human Chorion Mesenchymal Stem Cells

Stem cells derived from the human CM have been less extensively characterized compared with the stem cell populations obtained from the AM. They are reported as being more primitive and metabolically quiescent, as indicated by a more simplistic ultrastructure.[45] Koo *et al*.[46] recently performed a series of studies to characterize this population of cells. Their findings illustrated that hCMSCs display a typical fibroblast-like morphology and express the mesenchymal stem cell markers CD73, CD105, and CD90. Furthermore, these cells have the capacity to differentiate toward the classical mesodermal lineages and produce anti-inflammatory cytokines, including interlukin-1 receptor agonist as well as interlukins-6, -8, and -10.[47] Similar to hAECs and hAMSCs, hCMSCs lack HLA-DR and hematopoietic markers; however, a small percentage of cells did express the leukocyte marker CD11b.[46] In comparative studies with their AM-derived stem cell counterparts, hCMSCs seem to have an increased affinity

for osteogenic and chondrogenic differentiation, as indicated by higher proportions of hCMSCs staining positively for collagen type II, alkaline phosphatase, osteocalcin, and collagen type I during directed differentiation *in vitro*.[36] Of note, a larger proportion of hCMSCs stained positive for MHC class I molecules compared with hAECs and hAMSCs obtained from third-trimester placentas, potentially indicating a reduced capacity for immune tolerance. Most recently, stem cells derived from the human CM have been shown prevent activated macrophages from polarizing into an M1 phenotype; rather, the presence of hCMSCs shifted macrophages into an anti-inflammatory M2 phenotype, which may have positive implications for their utility in tissue regeneration applications.[48,49]

V. APPLICATION OF FETAL MEMBRANE ECM AND STEM CELLS FOR ORTHOPEDIC APPLICATIONS

A. Cartilage Regeneration/Osteoarthritis

The American Academy of Orthopedic Surgeons has estimated that approximately 4 million knee arthroscopies are performed annually worldwide (nearly 1 million of which are performed in the United States) and that 20–60% of these procedures reveal focal chondral or osteochondral defects.[50] These lesions, which can be caused by wear and tear over time, traumatic injury, or metabolic disorders, result in persistent pain and functional disability and, if left untreated, may progress to osteoarthritis (OA). Current biologic repair strategies for cartilage restoration, including microfracture, osteochondral autograft transfer/mosaicplasty, and matrix-assisted chondrocyte implantation, have shown promise; however, shortcomings with these approaches remain. Furthermore, if debilitating OA develops, pharmacological interventions to quell associated inflammation, viscosupplementation, and joint resurfacing or arthroplasty with metallic and polymeric implants may be warranted. OA was recently ranked as the 11th highest contributor to global disability.[51] To date, preclinical studies using stem cell therapy in conjunction with the use of scaffolds have illustrated promise for the treatment of chondral and osteochondral defects[52]; however, an ideal stem cell source and delivery vehicle has yet to be determined. Furthermore, intra-articular injections of stem cells have improved pain and disability scores in patients with OA; however, further clinical investigations are needed.[53] Thus, fetal membrane ECM allografts and/or its resident stem cells may represent excellent alternatives in cartilage regeneration applications and for use in disease-modifying approaches targeting OA.

As mentioned previously, the stem cell populations found within the AM and CM have demonstrated the capacity to differentiate into a chondrogenic phenotype. Nogami and colleagues[54] describe a subpopulation of hAMSCs with the ability to proliferate for 50 population doublings over the course of approximately 250 days in culture. Chondrogenic differentiation of hAMSCs (as indicated by the production of the mature isoform of collagen type IIB) required media supplementation with bone morphogenetic protein (BMP)-2.[54] With respect to the feasibility of using these stem cells *in vivo*, hAMSCs survived for up to 8 weeks following seeding on poly(lactic-co-glycolic) acid scaffolds and implantation into 5-mm-diameter defects in the knees of New Zealand White rabbits. Tumorigenicity and excessive inflammatory infiltrate was not elicited by the implants.[54] Predifferentiated hCMSCs seeded onto atelocollagen scaffolds have also maintained chondrogenic differentiation, generated cartilaginous constructs in a subdermal implantation model, and promoted lacunae formation when implanted into 2-mm-diameter osteochondral defects in the patellar groove of nude rats.[55] Taken together, this research indicates the potential safety and efficacy of implanting stem cells derived from fetal membranes for the regeneration of cartilage.

Alternatively, others have investigated the use of AM ECM to promote cartilage regeneration and/or serve as a delivery vehicle for chondrocytes. Tan *et al*.[56] illustrated successful chondrogenic differentiation of rabbit BMSCs seeded on scaffolds derived from human AM that had been devitalized via a series of wash steps and processing via freeze- or air-drying coupled with gamma-irradiation. Similar studies were undertaken by Krishnamurithy and colleagues,[57] with similar outcomes. Jin *et al*.[58] denuded

the amniotic epithelial layer of the human AM ECM before seeding articular chondrocytes on either the apical surface (basement membrane) or the stromal surface (layer immediately adjacent to the chorion). They found that seeding chondrocytes on the stromal surface resulted in improved cell attachment, infiltration into the AM ECM, and collagen type II expression.[58] Alternatively, chondrocytes seeded on the basement membrane surface of the AM ECM resulted in increased proliferation compared with those seeded on the stromal surface.[58] It was suggested that this differential effect is the result of differences in ECM components and growth factors found within the membrane. Upon implantation into osteochondral defects in rabbits, Jin *et al*. noted improved International Cartilage Repair Society histological scoring and the regeneration of hyaline cartilage in defects treated with chondrocyte-seeded AM compared with cell-free AM ECM. Díaz-Prado *et al*.[39] studied the efficacy of using cryopreserved human AM ECM as a delivery vehicle for chondrocytes that could be used as a restorative covering for the surface of hyaline cartilage afflicted by OA. They seeded human chondrocytes on the stromal layer of AM ECM and applied the constructs to 6-mm-diameter cartilage discs *in vitro* that had been obtained from patients with OA. Results from this study indicated limited collagen type II staining, proteoglycan production, integration with adjacent osteoarthritic cartilage, and repair tissue with a fibrous appearance.[39] Of note, the authors attempted to culture chondrocytes on the epithelial side of the membrane; however, they were unable to infiltrate past the hAECs.[39] An alternative approach was used by Lindenmair *et al*.[59] in which the investigators evaluated the ability of intact AM containing hAECs and hAMSCs to undergo chondrogenic differentiation using various formulations of induction media. The primary findings suggest that standard chondrogenic media, as well as chondrogenic media supplemented with basic fibroblast growth factor could induce differentiation of the stem cell populations found within the intact AM. Moreover, they suggested that this induction methodology predominantly supported hAMSC chondrogenic differentiation compared with that of hAECs within the membrane, suggesting that hAMSCs preferentially undergo chondrogenic differentiation.[59] Alternatively, Ma *et al*.[60] found that effective chondrogenic differentiation of hAECs requires the presence of BMP-7, again indicating a difference between the 2 cell types in the AM.

With respect to the application of fetal membrane ECM to ameliorate arthritic conditions, one of the earliest publications describing the use of AM to treat OA was published in 1965.[61] This study evaluated the use of human AM as an interpositional matrix graft implanted within arthritic hips of dogs, a procedure termed *amniotic arthroplasty*. During this study, regeneration of synovium was observed 2 months postoperatively and range of motion was reestablished. Furthermore, no signs of rejection or inflammation were observed in the animals. Subsequently, in the 1980s patients with tuberculosis infections of the hip were treated via amniotic arthroplasty in which multilayered constructs composed of AM were overlaid on arthritic femoral heads.[62] At 24–30 months of follow-up, 25 of the 28 patients reported a significant reduction in pain and regained functional performance. The investigators observed the formation of fibrocartilage and a pseudo-synovial membrane in joints receiving treatment.[62] Tuncel *et al*.[63] more recently used amniotic arthroplasty to treat experimentally induced fibrous ankylosis formation in the temporomandibular joints of New Zealand white rabbits. Mandibular range of motion in rabbits receiving amniotic arthroplasty was significantly increased and no fibrous adhesions were observed compared with rabbits that were treated with gap arthroplasty alone.[63] Willett and colleagues[64] intra-articularly administered a micronized form of dehydrated and devitalized human AM/CM ECM as a prophylactic treatment for OA in a rat meniscus transection model. Despite observing a moderate inflammatory response and foreign body reaction to the material, histological and biochemical assessment illustrated a significant reduction in the number of erosion sites and reduced proteoglycan loss within the cartilage of joints that had been treated with micronized fetal membranes. Furthermore, an initial preclinical safety study evaluating the inflammatory effects of intra-articular injection of allogeneic and autologous placenta-derived stem cells was examined in healthy horses.[65] Findings in-

cluded variable lameness and marked inflammatory infiltrate in all injected joints, regardless of the cell source, illustrating the possibility of using allogeneic placental stem cells for orthopedic applications in horses.

B. Bone/Dental Regeneration

It has been estimated that nearly 500,000 bone grafting procedures are performed annually in the United States, accounting for $700 million in bone graft and bone graft substitute sales.[66] Furthermore, it has been noted that bone is the second most common transplanted tissue, eclipsed only by blood.[67] Common procedures that use bone grafts include resection of bone tumors, revision hip or knee arthroplasties, spinal fusions, trauma procedures, and a limited number of dental procedures. Therefore, the development of bone tissue engineering strategies using synthetic bone substitutes and stem cells may be an effective alternative or, at the very least, may lessen the burden of having to harvest significant numbers of autografts and allografts to keep pace with yearly demands. Mohr *et al*.[68] illustrated that hCMSCs derived from the first-trimester chorionic villi or term CM can be induced toward an osteogenic lineage following seeding onto cell-free human CM–derived ECM. Similarly, hCMSCs seeded onto polyurethane foams show similar promise and exhibit the ability to deposit mineral *in vitro*.[69] Lindenmair *et al*.[70] placed intact human AM in osteogenic differentiation media in an attempt to drive differentiation and bone formation. Their findings suggest the preferential differentiation of hAECs toward an osteoblast-like phenotype, as was indicated by positive staining for calcium deposition and osteocalcin staining in the epithelial layer. Furthermore, the ability of ovine amniotic epithelial cells (oAECs) to contribute directly to bone formation was investigated by Barboni and colleagues[71] following seeding onto a hydroxyapatite/β-tricalcium phosphate matrix and implantation into a maxillary sinus augmentation in sheep. The oAECs stimulated osteogenesis within the scaffold by directly participating in the bone formation process. Compared with porous implants without oAECs, those seeded with fetal stem cells illustrated enhanced boney matrix deposition and significantly higher expression of VEGF at early study time points while exhibiting a reduction in inflammation and in-growth of fibrous tissue.[71] Rosen[72] reported a case study in which a composite allograft of mesenchymal stem cells and human AM/CM ECM matrices were fabricated into a barrier used to fill an osseous void created by significant bone loss under the molars of a 62-year-old human patient. Radiographs 6 months postoperatively indicated that the class III furcation was filled with new bone. Similarly, Wallace and Cobb[73] used commercially available AM/CM ECM matrices as a covering for particles of cancellous and cortical bone graft placed into an alveolar bone defect following tooth extraction in a cohort of 7 patients. On average, approximately 54% of the defect areas were filled with new bone at an average of 13 weeks following the intial procedure. In addition, no inflammatory cell infiltrate was present.[73] The authors stated that the rapid maturation of bone observed may have been due in part to the presence of the biological factors derived from the AM/CM ECM. Although conclusive evidence was not provided in this study, research by others has shown that the AM does in fact contain BMPs.

C. Tendon Regeneration

Damaged tendons account for nearly 4.5 million physician visits and 300,000 surgical procedures annually in the United States, resulting in an estimated total cost of $3 billion.[74,75] A significant number of studies have illustrated the ability of the AM and the stem cells it contains to promote the regeneration of healthy tendon tissue. Yang *et al*.[76] created a partial tendon laceration model in the Achilles tendon of rabbits and wrapped the defect with commercially available bovine-derived AM ECM. At 2 weeks the investigators noted a significant reduction in the number of inflammatory macrophages and neutrophils within the treatment group. Furthermore, by 4 weeks tendon modulus was significantly greater in the treatment group compared with lacerated controls, suggesting an advantageous contribution of the AM to early tendon healing.[76] Zelen and colleagues[77] performed a prospective, randomized clinical trial to study the effects of administering

micronized dehydrated AM/CM ECM composite in 45 patients with refractory plantar fasciitis. They observed significant improvement in pain, function, and alignment scores in patients receiving AM/CM ECM injections versus saline-injected controls. Zelen *et al*. did not observe any adverse events in the treatment group. AM ECM has also reduced the formation of peritendinous adhesions following flexor tendon surgery without inhibiting the healing process in chickens.[78] The use of AM-derived stem cells have the capacity to aid in tendon regeneration and healing via multiple mechanisms. Barboni *et al*.[79] illustrated that oAECs can, in fact, undergo tenogenic differentiation via indirect coculture with fetal and adult tenocytes/tendon explant tissues and directly contribute to tendon matrix formation. The oAECs were able to maintain low HLA-A, -B, and -C expression and no HLA-DR throughout the coculture period and exhibited improved differentiation when cultured in the presence of fetal tendon tenocytes/explants. In addition, studies using undifferentiated allogeneic oAECs injected under ultrasound guidance into sheep Achilles tendons defects have shown that oAECs can survive up to 30 days without causing any adverse events.[80] Furthermore, histological examination indicated an increase in the number of reparative cells at the injection site, suggesting that the fetal stem cells can also play an orchestrating role in the healing process via paracrine signaling.[80] Philip *et al*.[81] performed a similar study using a rat Achilles tendon defect model to assess the therapeutic efficacy of injecting hAMSCs or their cytokine extracts. The primary findings illustrated that the mechanical properties of tendons receiving hAMSCs exhibited a significantly larger cross-sectional area, Young's modulus, and yield strength 4 weeks after implantation compared with tendons receiving hAMSCs condition media.[81] Results suggest that implanting hAMSCs compared with hAMSC condition media yield improved healing likely due to ECM production and/or sustained release of hAMSC-derived growth factors. Allogeneic AECs also have been injected into digital flexor tendon lesions in a small population of horses.[82] Treatment was tolerated well, and no tendon failures were observed 6 months after injection. Similar studies were carried out in horses exhibiting superficial digital flexor tendon injury; however, oAECs were injected.[83] The authors found improved clinical ultrasound healing scores and the presence of oAECs 60 days after injection. Furthermore, injected oAECs produced collagen type I and seemed to be aiding in blood vessel formation and the proliferation of neighboring reparative cells.[83] No evidence of a severe immune or inflammatory response was noted; however, a few membrane-labeled oAECs were phagocytosed within macrophages. oAECs also were allotransplanted into Achilles tendon defects of sheep using a fibrin-based delivery vehicle.[84] Cell labeling indicated that oAECs were found at the injury site 28 days after injection. In addition, the cells seemed to reside initially in healthy tendon tissue adjacent to the injury site and eventually migrated into the wound. Proliferation of the oAECs was observed, along with an early increase in collagen type III, which was expeditiously replaced by aligned collagen type I by 28 days.[84] The authors also observed an increase in blood vessel infiltration as well as VEGF and transforming growth factor-β expression within the oAEC-treated group compared with untreated controls. Taken together, these data suggest that these cells, which exhibit stem cell–like characteristics, can directly produce tendon matrix as well as influence healing and adjacent cells via the production of soluble signals.

VI. CLINICAL USE AND COMMERCIAL AVAILABILITY OF FETAL MEMBRANE–DERIVED ECM

Over the past century allogeneic matrices derived from fetal membrane ECM have found clinical utility in human patients for treating ocular wounds, skin ulcers, burns, wounds, and mucopolysaccharidosis.[61,85–88] These matrices possess antibacterial, anti-inflammatory, antiadhesive, antiangiogenic, and immunomodulatory properties, which make them ideal candidates for use in tissue regeneration therapies.[89–95] A query of the National Institute of Health's clinicaltrials.gov website for the terms *amnion and allograft* or *amnion and stem cells* returned approximately 15 enrolling, ongoing, or completed clinical studies using fetal membrane–derived ECM matrices. The purpose of the majority of these investigations is to evaluate the efficacy of using fetal

membrane matrices for wound healing, preventing intrauterine adhesion, skin grafting, treating diabetic ulcers and glaucoma, as well in using them as an absorbable hemostatic agent. There are, however, a handful of studies enrolling or underway to establish the safety and efficacy of AM ECM in patients with orthopedic conditions including hallux rigidus due to OA, plantar fasciitis, peroneal tendon repair, lateral epicondylitis, bone augmentation following tooth extraction, and spinal fusion (i.e., evaluating AM to enhance spinal fusion or its effectiveness in preventing soft-tissue adhesions following posterior lumbar instrumentation removal).

A wide variety of AM-based products are currently commercially available. Most of these products are classified as allografts and are thus controlled through the FDA's 361 HCT/P regulations (discussed in detail in the next section). The one notable exception to this is PROKERA, which is regulated as a class II medical device. The primary differences between these products have been outlined succinctly in Table 1, which includes information regarding their components, processing method, and intended use.

VII. REGULATORY INFORMATION ON AMNION- AND CHORION-DERIVED MATRICES AND STEM CELLS FOR COMMERCIAL USE

ECM matrices derived from human fetal membranes are currently considered allografts and thus are typically screened, processed, and prepared at facilities that have been accredited by the American Association of Tissue Banks, which must register with the FDA. The American Association of Tissue Banks standards provide comprehensive guidance for tissue banking and are used as a model for state and federal regulations. The FDA currently regulates human cells or tissues "intended for implantation, transplantation, infusion, or transfer into a human recipient" as a human cell, tissue, and cellular- and tissue-based product (HCT/P).[96] These HCT/Ps are regulated by the Center for Biologics Evaluation and Research (CBER) under the Code of Federal Regulations Parts 1270 and 1271. These regulations essentially require those who are collecting, processing, and distributing allografts to follow established procedures to minimize/prevent the transmission of communicable diseases via the establishment of donor screening criteria and good tissue practices. It should be noted that CBER does not regulate the transplantation of vascularized human organs (e.g., liver, lungs, heart, kidney), nor does it regulate autografts that are transplanted during the same surgical procedure from which the tissue was originally obtained. CBER does, however, regulate AM when used alone (or without added cells), bone, cartilage, cornea, fascia, tendon, heart valve, ligament, pericardium, and so on. Furthermore, the FDA states that HCT/Ps can be regulated solely under Section 361 of the Public Health Service Act if the HCT/Ps meet certain criteria, including HCT/Ps that have undergone only minimal manipulation (i.e., processing of the HCT/P must not alter the tissue's original relevant characteristics), are for homologous use only (i.e., the HCT/P must perform the same basic function(s) in the recipient as it did in the donor), and the manufacture of the HCT/P does not involve combining the cells or tissue with another article). Thus establishments that manufacture these "361 HCT/Ps" do not have to undergo a regulatory approval process before marketing their products. If the aforementioned criteria are not met, however, manufacturers of HCT/Ps not only have to fulfill requirements set forth in 21CFR1271, they must also gain regulatory approval via the traditional device, drug, and/or biologic pathways (premarket notification, premarket approval, investigational device exemption, investigational new drug application, biologic license application, and so on) before marketing their products.

So what does this mean with regard to the clinical application of fetal membrane–derived ECM and AM- or CM-derived stem cells? What are the current thoughts of FDA regulators? In 2012 the FDA's Tissue Reference Group (TRG) revised its regulations on the subject of considering the AM as a 361 HCT/P when used as a wound covering.[97] The group states that if the AM contains viable cells to support tissue repair, that the function of the membrane itself is dependent on the metabolic activities of cells, which would designate it as a biologic. Furthermore, the TRG made the statement that if the AM is used for bone tissue regeneration it does not satisfy the 361 HCT/P requirement of homologous

TABLE 1: of Commercially Available Fetal Membrane–Derived Extracellular Matrix Products

Company	Product	Key Characteristics	Indications for Use	Acknowledged Use(s)	Known Orthopedic Applications
AmnioGenix	AmnioM™	Cryopreserved	Wound covering; localized soft tissue filler	Tissue voids; tissue defects; localized inflammation	Yes
	AmnioDryFlex®™	Membrane; dehydrated	Resorbable adhesion barrier	Dura and interspinal muscle protection protection	Yes
	AmnioExCel	Membrane; non-crosslinked; dried	Resorbable, natural scaffold	Wound covering; soft tissue repair; periodontal defects; boney defects; sinus coverage	Yes
Amniox Medical, Inc.	NEOX®Cord 1k™ Wound Matrix	Membrane and umbilical cord; CRYOTEK™ processed (deep freezing)	Wound covering	Dermal ulcers; dermal defects	No
	NEOX®100 Wound Matrix	Membrane; CRYOTEK™ processed (deep freezing)	Wound covering	Not described	No
	NEOX®100 Quick-Peel Wound Matrix	Membrane and umbilical cord; CRYOTEK™ processed (deep freezing)	Wound covering	Minor and superficial dermal wounds	No
	CLARIX®Cord 1k Regenerative Matrix	Membrane and umbilical cord; CRYOTEK™ processed (deep freezing)	Surgical covering, wrap	Bilateral MTP Cheilectomy; Lapidus Bunionectomy; Peroneus Brevis Tendon Repair	Yes
	CLARIX®100 Regenerative Matrix	Membrane; CRYOTEK™ processed (deep freezing)	Surgical covering, wrap	Not described	No
	CLARIX®100 Quick-Peel Regenerative Matrix	Membrane and umbilical cord; CRYOTEK™ processed (deep freezing)	Surgical covering, wrap	Not described	No

TABLE 1: Continued

Company	Product	Key Characteristics	Indications for Use	Acknowledged Use(s)	Known Orthopedic Applications
Amniox Medical, Inc. (Continued)	NEOX®FLO Wound Matrix	Dry-powder (injectable) form of NEOX® Wound Matrix	Wound covering	Dermal ulcers; dermal defects	No
	CLARIX®FLO Regenerative Matrix	Dry-powder (injectable) form of CLARIX®Cord Regenerative Matrix	Surgical covering	Not described	No
Amnio Technology / Arthrosurface	Nanofactor™	Membrane	Therapeutic for augmentation and repair	Tendonitis; joint pain; arthritis; cartilage damage	Yes
	Nanofactor™ Flow	Membrane; contains cells	Therapeutic for augmentation and repair	Biceps tendonitis; hip labral repairs; plantar fasciitis; patellar tendonitis; rotator cuff repair; epicondylitis; bursitis	Yes
Applied Biologics	Xwrap® ECM	Membrane (chorion-free); non-cross-linked	Resorbable, soft-tissue wound covering	Not Described	Not Described
	XWrap® Dry	Membrane (chorion-free); dry-packaged; acellular	Fibrosis minimization, soft-tissue wound covering	Not Described	No
	Xwrap® Hydro Plus	Membrane (chorion-free); saline-packaged; acellular	Adhesion minimization, soft tissue wound covering	Carpal tunnel; rotator cuff and achilles tendon repair; bone fracture; nerve repair	Yes
	FlōGraft®	Membrane and fluid (chorion-free); cryopreserved	Soft tissue defect filler	Tendonopothy; enthesopothy; wound closure	Yes
	FlōGraft® Freedom	Membrane and fluid (chorion-free); injectable; cryopreserved; non-steroidal	Injectable pain management allograft	Muscle strains/ partial tears; epichondylitis; facet-based pain; joint pain	Yes

TABLE 1: Continued

Company	Product	Key Characteristics	Indications for Use	Acknowledged Use(s)	Known Orthopedic Applications
Bio-Tissue	Amniograft®	Membrane; cryopreserved;	Ocular tissue replacement; wound repair	Ptergium; conjuntivochalasis; corneal defects; trabeculectomies; leaking glaucoma; chemical burns; Stevens-Johnson syndrome; strabismus	No
	AmnioGuard®	Membrane; cryopreserved;	Biologic glaucoma shunt grant	Not described	No
	ProKera®	Membrane in a thermoplastic ring; cryopreserved	Ocular surface and corneal wound healing	Superficial corneal erosion; neurotrophic corneal epithelial defects; recalcitrant corneal inflamm; acute burns; Stevens -Johnson syndrome	No
BioD, LLC	BIODEFENCE®	Membrane; saline-packaged	Resorbable adhesion barrier	Dura protection (laminectomy, craniotomy, discectomy); intraspinal muscle protection;	Yes
	BIODFACTOR®	Placental tissues; cryopreserved	Wound covering	Tissue voids and defects; localized areas of inflammation	No
	BIORESTORE™	Membrane; morselized, flowable	Resorbable adhesion barrier	Not described	No
	BIODRYFLEX®	Membrane; DryFlex® processed (dehydrated)	Resorbable adhesion barrier	Dura protection (laminectomy, craniotomy, discectomy, miscodiscectomy); intraspinal muscle protection; nerve bundle protection	Yes

TABLE 1: Continued

Company	Product	Key Characteristics	Indications for Use	Acknowledged Use(s)	Known Orthopedic Applications
BioD, LLC (Continued)	BIODOPTIX®	Extracellular membrane; DryFlex® processed (dehydrated)	Scaffold for ocular tissue repair and regeneration	Corneal epithelial defects; corneal ulcers; pterygium; band keratopathy; bullous keratopathy; ocular surface burns	No
MiMedx	AmnioFix®	Composite tissue; PURION® processed	Tendon and soft tissue injuries	Patellar tendon inflammation; tendonitis; tendonosis; plantar fasciitis; tennis elbow	Yes
	EpiFix®	Composite tissue (epithelial cell layer, basement membrane, avascular connective tissue); PURION® processed	Acute and chronic wound repair	Diabetic foot ulcers; venous leg ulcers; pressure ulcers; arterial ulcers; inflammatory ulcers; acute and chronic burns; Mohs; scar revision	No
NUTECH Medical	NuCel®	Bioactive amniotic suspension	Tissue growth and reapair	Not described	No
	NuShield™ Spine	Membrane and fluid; dry-packaged	Wound patch	Dura protection	Yes
	NuShield™ Orthopaedics	Membrane and fluid; dry-packaged	Wound patch	Surgical protection of tendons and nerves	Yes
SNOASIS Medical	BioXclude™	Membrane and chorion; PURION® processed	Wound covering	Wound site preservation; extraction and ridge augmentation; periodontal intrabony defect; hard-soft tissue deformities; mandibular furcation	Yes

use.[97] In addition, the TRG states that allogeneic, cryopreserved, AM-derived powder is more than minimally manipulated and thus is not considered a 361 HCT/P. In general, it seems that the prevailing regulatory winds will require manufactures of fetal membrane–based products to follow the regulatory approval process of a new drug or biologic. Furthermore, to the best of our knowledge, to date there are currently no FDA-approved stem cell treatments for orthopedic regenerative medicine applications.

Those that are currently being investigated in clinical trials must undergo a regulatory pathway similar to that of most drug companies; thus the regulatory hurdles are significant.

VIII. SUGGESTIONS FOR FUTURE RESEARCH

To date, research aimed at investigating the utility of fetal membrane–derived ECM matrices and stem cells for orthopedic applications have centered on completing basic characterizations. Of the more than 20 articles reviewed that specifically focused on evaluating the utility of these matrices and stem cells for musculoskeletal applications, only 6 articles used cells and tissue from human fetal membranes; of these, only 3 evaluated the efficacy of human fetal membrane–derived stem cells alone (isolated from the fetal membrane ECM matrices) within *in vivo* models of orthopedic conditions. The remaining articles evaluated animal-derived fetal membrane ECM and/or their resident stem cell populations. Thus the first suggested research directive for further investigation would be to focus on evaluating human-derived AM and CM ECMs and stem cells. Differences between species certainly exist, and thus results obtained from nonhuman cells and tissues should be cautiously interpreted because direct extrapolation to the human equivalent may not be possible. Second, studying the effects of human AM-and CM-derived ECMs and resident stem cells independent of each other would be advantageous, such that their contributions to the therapeutic mechanism of action for orthopedic applications can be elucidated. It is becoming clear that both the fetal membrane–derived ECM and resident stem cells both possess therapeutic potential and can contribute to musculoskeletal tissue regeneration. The ECM contains soluble growth factors, cytokines, chemoattractants, and structural proteins that together may promote tissue regeneration.[64,98,99] Fetal membrane–derived stem cells have the ability to differentiate into various musculoskeletal tissue lineages and produce appropriate ECM, which contributes directly to tissue formation and strength, while producing growth factors and other paracrine signals.[33,81,100] Thus careful study design and subsequent evaluations should help determine the individual and cumulative therapeutic effects of AM and CM ECMs and stem cells. A third area of suggested research is to compare the efficacy of fetal membrane–derived stem cell populations with that of more traditional sources of stem cells, including adipose- and bone marrow–derived cells. Direct comparisons should be made under identical experimental conditions, such that the potential advantages of using one particular cell type over the other can be determined.

From a translational perspective, the clinical utility of fetal membrane–derived stem cells would likely require that they be cryopreserved and banked for autogenic or allogeneic transplant. Therefore understanding the effects of this process on the immunomodulatory and immunological profile of these cells with respect to time would be essential. Likewise, similar profiling studies should be completed following *in vitro* expansion and differentiation toward musculoskeletal phenotypes. Furthermore, considering that these stem cells will be implanted into musculoskeletal tissue and thus would interface (directly or indirectly) with local somatic cells, obtaining an understanding of the potential reciprocal influences of fetal membrane–derived stem cells with other musculoskeletal cell types in a coculture environment would be advantageous. Finally, with respect to the therapeutic use of AM- and/or CM-derived ECMs and stem cells, clinically relevant dosing time points should be used in *in vivo* models of human musculoskeletal conditions. For example, if the effects of fetal membrane–derived stem cells on OA are being studied, inducing OA in an *in vivo* model (e.g., via medial meniscectomy and/or anterior cruciate ligament transection) and awaiting disease progression until a clinically relevant time point before applying the stem cells would be most appropriate. Conversely, inducing OA and immediately (at time 0) apply the therapeutic agent without allowing progression (i.e., evaluating a prophylactic effect) would be less clinically relevant because this would not likely represent the clinical reality for targeting this pathologic process.

IX. CONCLUSION

The human AM and CM are abundant sources of epithelial and mesenchymal cells that possess stem cell characteristics. These cells express pluripotency

markers and are found in quantities that are significantly greater than their bone marrow– and adipose-derived stem cell counterparts. They also exhibit the capacity to differentiate toward musculoskeletal cell lineages (including cartilage, bone, and tendon) under various *in vitro* culture conditions and have produced musculoskeletal tissue–specific ECM components. Notably, hAECs, hAMSCs, and hCMSCs may each exhibit a preference or predisposition to differentiate toward a particular musculoskeletal lineage (hAECs toward bone, hAMSCs and hCMSCs toward cartilage), which is dependent on the cell type and the fetal membrane from which they originate. The ECM of the fetal membranes is also of value for musculoskeletal tissue regeneration applications via the delivery of growth factors, cells, and immunomodulatory molecules, while concurrently supporting stem cell viability and differentiation toward various musculoskeletal phenotypes. The potential clinical utility of the fetal membrane–derived ECMs and stem cells in orthopedic applications is great; however, so too are the imminent regulatory hurdles required to prove the safety and efficacy of such products.

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