Musculoskeletal tissue engineering with human umbilical cord mesenchymal stromal cells

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Abstract

Multipotent mesenchymal stromal cells (MSCs) hold tremendous promise for tissue engineering and regenerative medicine, yet with so many sources of MSCs, what are the primary criteria for selecting leading candidates? Ideally, the cells will be multipotent, inexpensive, lack donor site morbidity, donor materials should be readily available in large numbers, immunocompatible, politically benign and expandable *in vitro* for several passages. Bone marrow MSCs do not meet all of these criteria and neither do embryonic stem cells. However, a promising new cell source is emerging in tissue engineering that appears to meet these criteria: MSCs derived from Wharton's jelly of umbilical cord MSCs. Exposed to appropriate conditions, umbilical cord MSCs can differentiate *in vitro* along several cell lineages such as the chondrocyte, osteoblast, adipocyte, myocyte, neuronal, pancreatic or hepatocyte lineages. In animal models, umbilical cord MSCs have demonstrated *in vivo* differentiation ability and promising immunocompatibility with host organs/tissues, even in xenotransplantation. In this article, we address their cellular characteristics, multipotent differentiation ability and potential for tissue engineering with an emphasis on musculoskeletal tissue engineering.

Keywords

differentiation; musculoskeletal; regenerative medicine; umbilical cord stroma

An ideal cell source for tissue engineering should satisfy requirements including ease of access, sufficient cell number and immunocompatibility. Mature autologous cells have commonly been used in previous studies, owing to the advantages of already being differentiated and immunocompatible. In contrast to mature autologous cells, embryonic stem cells (ESCs) are a major area of contemporary research interest, and offer significant

potential to treat or even cure a plethora of diseases [1,2]. They can differentiate into all cell types of the body including germ cells [3–5]. By contrast, bone marrow mesenchymal stromal cells (BMSCs) have a more limited differentiation potential. The self-renewal ability of ESCs also allows for a theoretically unlimited number of undifferentiated cells to be produced for tissue engineering applications. However, ESCs suffer from a series of constraints including ethical concerns, limited availability, potential for teratoma formation upon transplantation and immune rejection [6]. Induced pluripotent stem cells are an alternative to ESCs, and have been generated from many cell types including cells from umbilical cords [7]. Although the use of patient-specific induced pluripotent stem cells will overcome the immune rejection obstacle [8–11], a great deal of work remains before these cells can be developed into a safe therapy in humans.

Adult BMSCs are the most extensively investigated mesenchymal stromal cells (MSCs) for tissue engineering applications and are considered the 'gold standard' cell source in musculoskeletal tissue engineering. BMSCs have the ability to differentiate *in vitro* and *in vivo* into connective tissues such as cartilage, bone and adipose tissues. The main advantage of using BMSCs is to provide an autologous cell source for tissue engineers to avoid potential immune rejection. Autologous and allogeneic BMSCs also exhibit immunosuppressive capabilities [12–14]. BMSCs are relatively easy to access, and have limited self-renewal ability. The first tissue engineering studies to treat bone defects in clinical studies used human BMSCs (hBMSCs) [15,16]. In a 7-year clinical trial [15,16], this integration of implants into host bone was maintained and no further fractures were observed, thus demonstrating the feasibility of a tissue engineering approach in human bone regeneration with long-term durability. However, there are some limitations to the applications of BMSCs, including the low number of hBMSCs in marrow, their proliferation ability and the fact that their differentiation potential decreases significantly with age [17]. Moreover, the invasive harvesting procedure may lead to complications and morbidity [18].

There are a number of other sources of MSCs that are gaining popularity in the literature. However, for the sake of brevity, we refer readers to outstanding reviews on adipose-derived stem cells (ADSCs) [19–21] and dental pulp stem cells [22–24].

Human umbilical cord mesenchymal stromal cells

Great importance has been given to the identification of other cell sources that can be utilized for tissue engineering and regenerative medicine [25–27]. To date, other than BMSCs, MSCs have been identified in a variety of tissues, such as adipose tissue, blood, synovial fluid, dermis, muscle, dental pulp and umbilical cords, and can be differentiated along several mesenchymal lineages [21,24,28–33]. However, there are significant differences in their proliferation and differentiation abilities, and in harvesting procedures among these MSCs [34]. With the discovery of new MSC sources, a standard definition of these cells is needed. The International Society of Cellular Therapy proposes that cells must meet three criteria to be classified as MSCs [35]. The criteria are:

- Be plastic adherent

- Express CD105, CD73 and CD90 cell surface markers and not express hematopoietic stem cell markers

- Be capable of osteoblast, adipocyte and chondrocyte differentiation *in vitro*

In recent years, the Wharton's jelly of human umbilical cords has been discovered as a source of MSCs [36]. Human umbilical cord mesenchymal stromal cells (hUCMSCs) are a multipotent stromal cell population that are plastic adherent, share MSC surface markers such as CD73, CD90 and CD105, and are nonhematopoietic cells [37]. Nomenclature of

these cells varies among investigators, and names used for cells derived from the Wharton's jelly include umbilical cord matrix stem cells [33,38], human umbilical cord stroma cells [36], Wharton's Jelly cells [37], umbilical cord stem cells [39], umbilical cord stromal cells [40], UCMSCs [41], umbilical cord tissue-derived cells [42] and various other combinations. Cell naming can also be specific to the site of isolation within the Wharton's Jelly, for example human umbilical cord perivascular cells (hUCPVCs) [43,44]. As these cells are further characterized, a standard naming system for these cells may develop. Although the nomenclature of these cells varies among investigators, cells isolated from Wharton's jelly are referred to as UCMSCs later.

Before the 21st century, umbilical cord research focused on the structure of umbilical cords and the characterization of extracellular matrix and stromal cells, going back as early as the 1940s [45,46]. In 1994, only two publications dealt with the subject of umbilical cord and MSCs (including blood derived), and that number increased to 349 publications in 2009 [47]. Following the recognition of their neural differentiation ability, demonstrated early in the current decade [48], it has been reported that hUCMSCs may differentiate along several cell lineages in all three germ layers including chondrogenic, osteogenic, adipogenic, myogenic, pancreatic, neurogenic and hepatogenic [36,38,48–50]. Additionally, hUCMSCs do not appear to form teratomas when transplanted [51]. *In vivo* transplantation of these cells has been demonstrated to prevent progressive deterioration with brain injury and to rescue the eye from retinal disease in a rodent model [52,53]. Recent tissue engineering studies with hUCMSCs have focused on cardiovascular tissue engineering [49,54–58] and musculoskeletal tissue engineering [59–61]. In this article, we have emphasized the mesenchymal differentiation of hUCMSCs and their potential for musculoskeletal tissue engineering. Cell biology and transplantation reviews of hUCMSCs can be found in detail elsewhere in the literature [37,39,40].

Cell characterization

Cell isolation & growth

The human umbilical cord includes one vein, two arteries and a surrounding connective tissue known as Wharton's jelly laying deep to the amnion (Figure 1). For more physiological information about the umbilical cord, see cited references [62,63]. The connective tissue may be divided into three zones: subamniotic, intervascular and perivascular stroma [36]. Various methods have been used to liberate cells from the umbilical cord tissue. Enzyme digestion of trypsin and/or collagenase has been recently utilized to extract hUCMSCs with fast isolation and high yield [33,36,64,65]. Moretti *et al.* provided a diagram mapping the many isolation methods, including steps such as removal of blood vessels, enzymatic digestion or explant culture [47]. Protocols can be modified to target the Wharton's jelly cells or specific zones within the stroma. For example, Fong *et al.* modified their protocol to obtain a population of Wharton's jelly cells using a specific enzymatic digestion process [51,66]. Currently there is no standard operating procedure for isolation of hUCMSCs from the umbilical cord. As a result, isolation methods may differ in their ability to release the MSCs from their niche, differ in the number of cells found at initial isolation or enrich one or more cells types found in the mixed MSC population. Thus, cell populations that are isolated may vary from group to group.

Human umbilical cord mesenchymal stromal cells resemble fibroblasts in their morphology (Figure 1) and have been defined as myofibroblasts based on their positive expression of vimentin, desmin and/or α-smooth muscle actin in native tissue or with *in vitro* cultured cells [48,67,68]. As an extraembryonic cell source, hUCMSCs have a rapid proliferation rate with a shorter doubling time than adult stem cells [37]. hUCMSCs also maintain their expansion and multipotent differentiation properties longer *in vitro*, compared with hMSCs

[69]. Following cell harvesting, hUCMSCs have been passaged for seven times to achieve a 300-fold increase in cell number while the differentiation potential was maintained [36]. However, the quality of the cells can be affected by the length of culture time and cell seeding density [70]. The rapid proliferation rate, accompanied by the wide availability of umbilical cords, is highly desirable for tissue engineering as it is possible to obtain a large number of cells in a short time.

Cell phenotype

Human umbilical cord mesenchymal stromal cells represent an adherent cell population with a nonhematopoietic and nonendothelial (CD34 negative) phenotype and share a similar set of surface markers with adult MSCs, including CD10 [33], CD13 [33,38,71,72], CD29 [33,65,71,72], CD44 [33,36,38,65,71,72], CD49e [33], CD51 [65], CD73/SH3 [36,38,65,71], CD90 (Thy-1) [33,38,72], CD105/SH2 [33,36,38,65,71], CD106 [71], CD117 [38,48], CD166 [38,71,72] and HLA-1/HLA-ABC [33,71,72], and are negative for hematopoietic markers (e.g., CD14, CD31, CD34, CD38, CD45 and HLA-DR) [33,36,38,48,[49],54,65,71–76]. Besides MSC surface markers, transcription factors such as Oct-4, Nanog and Sox-2 have been reported to exist in porcine umbilical cord cells, albeit at low levels of transcription [75]. Recent studies revealed that hUCMSCs also expressed these three markers [77,78] and other pluripotent markers including Rex-1, SSEA-3, SSEA-4, Tra-1–60 and Tra-1–81 [77]. More importantly, these pluripotent stem cell markers were retained for at least nine passages during subculture [77]. The presence of these pluripotent markers was also observed in transcriptome profiling of hUCMSCs using DNA microarrays [79]. It is important to note that the pluripotent markers (POUF1, NANOG, SOX2 and LIN28), in this study and in other studies, were expressed in low levels, generally orders of magnitude less than ESCs. Moreover, there is no demonstration that these transcription factors function in MSCs. The presence of low level gene expression of pluripotent transcription factors is insufficient to demonstrate pluripotency [80]. However, if a group would be able to demonstrate with blastocyst injection of a clonal cell line that the distribution of MSCs within all three cell types, including the germline, is observed, that would provide a much greater argument for pluripotency. Until such a demonstration is made, the most appropriate conclusion at this stage would be that UCMSCs most likely are not pluripotent cells.

For a complete table of all cell markers, see the review by Moretti *et al.* [47]. Regarding the 'stemness' of UCMSCs, UCMSCs have not been shown to be stem cells by the strict definition (permanently engraft and self-renew and produce differentiated progeny). However, recent work from Méndez-Ferrer *et al.*, in the mouse, indicates that progress is being made to better identify the stem cell populaton within MSCs [81]. It remains to be seen whether the identifiers for MSCs found in mouse bone marrow will be found in other regions or other species where MSC-like cells have been derived.

Cell populations from different zones

Human UCMSCs represent a heterogeneous cell population. Thus, it might be important to isolate cells from each zone and characterize them to better understand their respective properties. hUCPVCs have been isolated through a delicate enzyme digestion of stroma surrounding the vessels [44]. These cells have shown a surface phenotype and multipotency similar to BMSCs and hUCMSCs [64,82], including CD44, CD73, CD90, CD105 and HLA-ABC. However, CD146, a surface antigen widely expressed in endothelial cells, was expressed at a higher rate of 52% with hUCPVCs compared with hBMSCs (15%) [82]. Only one group has isolated cells from the subamniotic zone due to the difficulty of isolating these cells from the surrounding tissue [83]. *In situ* characterization indicated that the cell number in the subamniotic area was less than 10% of the population from the whole cord

and the cells had a stronger staining of α-smooth muscle actin and pancytokeratin [36]. After isolation, subamniotic cells expressed stem cell (SSEA-4, Oct-4 and Nanog) and MSC markers (CD73 and CD105), and were capable of adipogenic, osteogenic and chondrogenic differentiation [83]. However, more data are required to characterize this subpopulation.

Differentiation & tissue engineering

Human UCMSCs may have the ability to differentiate along several cell lineages of mesodermal or ectodermal origin (Figure 1) [36,38,48,49,84,85]. Furthermore, recent studies reported that hUCMSCs were able to differentiate both *in vivo* and *in vitro* into hepatocyte-like cells and pancreatic islet precursors of endodermal origin [38,50]. Exposed to a hepatogenic culture medium, hUCMSCs expressed hepatic markers including albumin, α-fetoprotein, cytokeratin-19, connexin-32 and dipeptidyl peptidase IV [38]. This study provided the foundation for the application of hUCMSCs for liver regeneration. Work from Chao *et al.* suggested that UCMSCs may be differentiated into pancreatic islet precursors, produce human insulin and respond to a glucose challenge for up to 12 weeks in a xenotransplantation Type I diabetes mellitus model [50]. In the following sections, we focus specifically on mesenchymal differentiation of hUCMSCs for the application of musculoskeletal tissue engineering.

Chondrogenic differentiation & cartilage tissue engineering

Cartilage, which plays a critical role in load bearing and distribution, has limited self-regeneration capabilities after injury. Compared with the current clinical approaches such as microfracture, artificial prosthesis and cell-based implantation, tissue engineering may be an ideal method to fully restore the cartilage function [86]. Cell dedifferentiation of mature chondrocytes during monolayer expansion makes it difficult to obtain enough cells for tissue engineering [87]. Thus, the application of MSCs in tissue engineering is promising mainly due to their self-renewal and differentiation ability. Chondrogenic differentiation of MSCs can be triggered by the TGF-β isoforms (TGF-β1, β2 and β3) and be enhanced by the addition of dexamethasone [88–90]. IGF-I, an important anabolic agent, remains controversial with its role in chondrogenic differentiation [90–92]. However, a recent study reported that IGF-I and TGF-β had equal chondroinductive ability with MSCs after the removal of insulin from insulin–transferrin–selenious acid Premix (1X serum substitute Premix), a substitute for fetal bovine serum in serum-free chondrogenic medium [92]. In addition, we showed that switching from a chondrogenic medium with TGF-β to an anabolic medium with IGF-I not only significantly increased collagen production relative to remaining in the chondrogenic medium, but also significantly increased the gene expression and the synthesis of collagen II [93].

Like other MSCs, hUCMSCs are able to differentiate along a chondrogenic lineage under the stimulation of TGF-βs in 3D cell pellets and polyglycolic acid (PGA) scaffolds [36,60,65,82,94]. In a more recent study, the chondrogenic ability of hUCMSCs was initiated at the mRNA level by the upregulation of *SOX-9*, a key regulatory gene for chondrogenesis [41]. At the extracellular protein level, evidence of chondrogenic differentiation was supported by Alcian blue staining for glycosaminoglycans (GAGs) [65,82], and immunohistochemical staining for type II collagen [36,65]. However, the chondrogenic differentiation studies of hUCMSCs have had inconsistent results [36,65]. In a study by Wang *et al.*, untreated cells in the control group (no TGF-β1) also demonstrated positive staining for GAGs and collagen type II [65]. By contrast, no type II collagen was observed with the control group in a study by Karahuseyinoglu *et al.* [36], although only a trace of type II collagen was detected with hBMSCs, in contrast to the formation of abundant type II collagen in previous hBMSC studies [95,96]. Type I collagen was also identified in this study in both hBMSCs and hUCMSCs. Nevertheless, larger pellets were

observed in the hUCMSC group with a better filamentous extracellular matrix than hBMSCs [36,82]. Moreover, the GAG quantification revealed that the perivascular cells, a subpopulation of hUCMSCs, produced a quantity of GAGs comparable to hBMSCs after 21 days, although the perivascular groups had fewer GAGs than hBMSC groups during the first week. Therefore, the better differentiation ability compared with hBMSCs and the coexistence of type I and II collagen in both cell pellets and biomaterial-based culture indicate that hUCMSCs may be considered as a MSC source for fibrocartilage tissue engineering applications such as for the temporomandibular (TMJ) disc, intervertebral disc and knee meniscus.

The first full-length study of hUCMSCs for musculoskeletal tissue engineering was a comparison of hUCMSCs to TMJ condylar cartilage cells *in vitro* for TMJ regeneration [60]. Both types of cells were cultured in PGA scaffolds over a 4-week period. The hUCMSC constructs formed a fibrocartilage-like tissue similar to the native TMJ cartilage and outperformed TMJ condylar cartilage cells with higher cellularity and biosynthesis of collagen and GAGs. Based on the limited availability of TMJ cells relative to umbilical cord cells and the superior biosynthesis of hUCMSCs, hUCMSCs were suggested as a promising alternative to mature condylar cartilage cells for TMJ tissue engineering. In a 3D comparison of hUCMSCs and hBMSCs for cartilage tissue engineering, hUCMSCs also demonstrated superior extracellular matrix synthesis with more GAGs and collagen than hBMSCs, while hBMSCs appeared to progress further along the chondrogenic lineages with higher type II collagen at both the gene expression and protein levels [97]. This study further supported the suitability of hUCMSCs for fibrocartilage tissue engineering for tissues such as TMJ cartilages and the intervertebral disc. After the establishment of hUCMSCs for fibrocartilage tissue engineering, the effects of cell seeding densities on cell proliferation, differentiation and matrix synthesis were explored [59]. hUCMSCs were seeded in PGA scaffolds at three densities: 5 (low), 25 (middle) or 50 (high) million cells/ml of scaffold. After a 4-week culture period, the high and medium density groups possessed significantly higher cell numbers and extracellular matrix content per construct and per cell than the lower density group. More importantly, constructs in the high and medium density groups maintained their mechanical integrity, which was confirmed by unconfined compression testing. Therefore, a seeding density greater than 25 million cells/ml was recommended for related future fibrocartilage tissue engineering studies. In summary, the formation of fibrocartilage-like tissue with the coexistence of type I and II collagen and aggrecan indicates that hUCMSCs differentiate along a fibrocartilage lineage and may be an excellent cell source candidate for fibrocartilage regeneration. A further modified chondrogenic environment, perhaps including the investigation of factors such as bioactive chemical signals (e.g., growth factors and aggrecan), oxygen tension, coculture, mechanical stimulation and/or *in vivo* regulation, will be needed to enhance chondrogenesis to apply hUCMSCs in hyaline cartilage tissue engineering.

Osteogenic differentiation & bone tissue engineering

Unlike cartilage, bone is a vascularized tissue with an innate self-healing and remodeling ability [98]. Although bone defects can be treated by autologous bone grafts, the current gold standard, this method is limited by donor site morbidity, malformation and infection [99]. Moreover, there is no satisfactory treatment for some severe bone injuries [100]. As a tissue engineering approach, BMSCs can be incorporated into biomaterials for direct implantation, with or without prior *in vitro* culture in osteogenic media. A typical osteogenic medium includes osteogenic signals of dexamethasone and/or BMP-2, in addition to β-glycerophosphate and ascorbic acid to aid in matrix synthesis. Exposure of BMSCs to this medium before implantation allows the delivery of more mature osteogenic cells to defects, thus accelerating the *in vivo* bone regeneration [100].

Osteogenic induction of hUCMSCs in 2D monolayer has been accomplished by treatment with dexamethasone and β-glycerophosphate [36,64,65,71,72,82]. During the course of osteogenic differentiation, *Runx2*, a master gene of osteogenesis, and its transcriptional coactivator for osteocalcin expression, TAZ, were activated [41], osteocalcin and osteopontin genes were up-regulated [41,65,71], positive alkaline phosphatase (ALP) activity was observed [64,65,82], the bone-specific immunostaining of osteonectin, osteocalcin and bone sialoprotein-2 was visualized [36], and mineralization was verified by von Kossa staining and alizarin red staining [36,64,65,71,72,82]. Like hBMSCs, mineralization was observed with hUCMSCs after 2 weeks [36,65,71,72] and similar side-by-side gene expression was observed during this period [71]. However, it appeared that hUCMSCs had less mineralization with larger bone nodules than hBMSCs as evidenced by alizarin red and von Kossa staining [36,71]. Similarly, after exposure to dihydroxyvitamin D3 for 6 weeks, hUCMSCs exhibited weak osteogenesis, with few ALP-positive cells or little mineralization [70]. Exposed to osteogenic medium, perivascular cells differentiated faster along the osteogenic lineage with the formation of bone nodules after only 4–5 days [64]. In a direct comparison of hUCMSCs and hUCPVCs, the cell types were phenotypically similar, except for CD146 expression (37.9% of perivascular cells, 16.2% in hUCMSCs) [101]. After exposure to osteogenic medium, positive von Kossa staining was slightly stronger in perivascular cells than in hUCMSCs. It will be interesting to further evaluate a subpopulation excluding hUCPVCs from hUCMSCs, which may clarify how hUCPVCs contributed to the osteogenic activity of hUCMSCs. In BMP-2-induced differentiation, hUCMSCs had similar expression of osteogenic phenotypes and signal transduction pathways compared with hBMSCs [102]. The differentiation of hUCMSCs could be triggered by osteoinductive materials. Cells cultured on calcium phosphate cement were shown to proliferate and undergo osteogenic differentiation [103]. When hUCMSCs were cultured for 7 days in monolayer with a medium containing demineralized bone matrix [104], their proliferation was inhibited, their morphology appeared shortened and flattened, and ALP activity increased significantly. These phenomena indicated that hUCMSCs may differentiate along the osteogenic lineage [104], while long-term culture will be needed to verify the presence of late stage osteogenic markers.

In monolayer culture, hUCMSCs demonstrated osteogenic differentiation ability after exposure to chemical signals and osteoinductive biomaterials. In 3D biomaterials, hUC-MSCs demonstrated their osteogenic differentiation, such as the upregulation of Runx2 and osteonectin and evidence of mineralization [61,105,106], while hBMSCs had higher osteogenic activity with 4.1-times more mineral content than hUCMSCs when cells were cultured in polycaprolactone–tricalcium–phosphate scaffolds [105]. hUCMSCs are capable of *in vitro* osteogenic differentiation when seeded on polycaprolactone–collagen–hydroxyapatite nanofibrous scaffolds [107], and when encapsulated in alginate microbeads [108], injectable calcium phosphate–alginate hydrogel paste [109] and fast-resorbable calcium phosphate bone cement [103]. In a pair of bone tissue engineering studies, hUCMSC seeding densities were compared on PGA scaffolds, and the effect of switching from osteogenic medium (dexamethasone and vitamin D3) to anabolic medium (IGF-I) with poly(L-lactic acid) scaffolds was investigated. It was discovered that with these nonwoven meshes a seeding density of 25 million cells/ml or higher was preferred and that changing to anabolic medium under the prescribed conditions was not beneficial [106,110]. *In vivo* osteogenic differentiation has been examined in a mouse model. Subcutaneous implantation of hUCMSCs demonstrated ectopic bone formation by von Kossa staining in 2 months and micro-CT and inferior osteogenesis of hUCMSCs compared with hBMSCs [105]. In another study, a porous biomimetic bone scaffold material, nano-hydroxyapatite–collagen–poly(L-lactic acid) composite, was seeded with hUCMSCs and then implanted into nude mice subcutaneously. Osteoblasts were detected by transmission electron microscopy after 12

weeks [61]. However, there was no additional technique used to investigate if these cells were host mouse cells or differentiated hUCMSCs.

Our group has evaluated hUCMSCs in osteochondral tissue engineering, where chondrogenesis and osteogenesis were regionally promoted within a given construct. One approach seeded hUCMSCs into poly(L-lactic acid) scaffolds with either chondrogenic or osteogenic medium for 3 weeks [111]. These constructs were then sutured together, with or without a thin layer of undifferentiated hUCMSCs in between. Although differentiation was limited, the primary finding was that the inclusion of hUCMSCs in this 'sandwich' approach noticeably improved the integration of the two constructs, evidenced by histology. In another approach, microsphere-based scaffolds were constructed to release TGF-β from one side of a cylindrical scaffold and BMP-2 from the other side, with a gradual and continuous transition in release from one side to the other [112]. While differentiation was again limited, and there was evidence of calcium deposition throughout the scaffold, it was encouraging to see regionalized Safranin-O staining on the TGF-β side of the construct, demonstrating that regionalized chondrogenesis may be possible in an osteochondral approach. Although these two studies have provided two alternative approaches for the inclusion of hUCMSCs in osteochondral tissue engineering, the high priority at this stage is to evaluate the *in vivo* performance of hUCMSCs in osteochondral tissue engineering. If hUCMSCs are able to respond to inductive signals from surrounding native tissues in osteochondral defects, they may hold significant promise in regenerating an integrated osteochondral tissue.

In summary, hUCMSCs may share an osteogenic differentiation pathway similar to hBMSCs, namely the activation of the *Runx2* gene and TAZ and the upregulation of osteocalcin and osteopontin, leading to mineralization. However, the osteogenic capability of hUCMSCs seems inferior to hBMSCs based on strategies employed to date. It will be valuable to make a side-by-side comparison among hUCMSCs, hUCPVCs and hBMSCs. The success of *in vivo* bone tissue engineering is promising using subcutaneous mouse models and further segment grafts in a larger animal model will be necessary to evaluate the functionality of engraftment when exposed to physiological mechanical and chemical cues.

Side-by-side comparisons of BMSCs & UCMSCs for bone & cartilage tissue engineering

In 2009, hBMSCs and hUCMSCs were passaged, seeded onto PGA scaffolds and cultured in chondrogenic medium, all under virtually identical conditions [97]. The major findings were that the hUCMSCs were far superior in terms of the quantity of collagen produced (per construct and on a per cell basis), although they were inferior in terms of collagen II gene expression and synthesis. The following year, hBMSCs and hUCMSCs were again compared under virtually identical conditions, seeded in the microsphere-based gradient scaffolds described in the previous section [112]. In this osteochondral application, the hUCMSCs were superior in terms of collagen synthesis and ALP activity, but were inferior in terms of *Sox9* and *Runx2* gene expression.

Collectively, these studies told us that under the prescribed conditions *in vitro*, hUCMSCs were inferior in terms of their differentiation capacity, but had a much greater capacity for tissue formation. However, it must be noted that these findings apply to a specific set of *in vitro* conditions, and that if hUCMSCs are indeed able to respond favorably to inductive signals *in vivo*, hUCMSCs may hold the potential for superior osteochondral regeneration in terms of the rate and quality of tissue regeneration.

Myogenic differentiation & muscle tissue engineering

Skeletal muscle provides voluntary motor control. Muscular disease and atrophy can permanently damage the muscle, and one category of muscular disease is progressive muscular dystrophy. With no effective treatment for progressive muscular dystrophy, stem cell therapy and tissue engineering could potentially be treatment options. To date, there has been limited investigation of UCMSCs differentiation into skeletal myocytes. Current techniques for myogenic differentiation of UCMSCs are exposure to myogenic medium [113], gene transfection [114], coculture [114], and injection into injured or dystrophic *in vivo* models [113,115]. After exposure to myogenic medium containing 5-azacytidine, a genomic DNA demethylating agent, Myf5 and MyoD were expressed after 7–11 days [113]. UCMSCs were also injected into an injured muscle, and skeletal muscle differentiation was observed by immunofluorescence of HLA-1 and sarcomeric tropomyosin antigens [113]. UCMSCs have been transfected with MyoD transcription factor. After 5 days, the cells exhibited functional markers of fusion machinery, muscle cell-specific structural proteins and muscle cell-specific enzymes [114]. Lastly, a dystrophic murine model was used to evaluate systemic exposure to hUCMSCs, and the cells' ability to engraft into the damaged muscle [115]. While hUCMSCs were able to reach the muscle, they possessed a lesser degree of differentiation than human ADSCs, as analyzed by dysferlin and human-dystrophin expression. The authors hypothesized that the niche difference between hUCMSCs and human ADSCs may have been the reason for the difference in *in vivo* differentiation. Further research is needed to determine the best differentiation technique for these cells, and if *in vivo* differentiation is possible.

Adipose differentiation & tissue engineering

Traditional autologous fat tissue transplantation suffers from the loss of graft volume with time [116]. When tissue engineering is applied to adipose tissue regeneration, the usage of mature adipocytes is limited by the inferior *in vitro* proliferation and expansion ability of these cells, and stem cells have been proposed as substitute cells [117]. Like hBMSCs, adipose differentiation of hUCMSCs has been induced in monolayer culture by dexamethasone, insulin, indomethacin and isobutylmethylxan-thine, and has been verified by lipoid deposits that were detected by Oil Red O staining [36,65,71–72,82]. Adipogenic differentiation has also been observed in hUCPVCs [64,82]; however, cyclic stretch-induced TGF-β1/Smad signaling inhibited expression of adipocyte markers [118]. Following adipogenic differentiation, adipose-related genes, such as peroxisome proliferator-activated receptor-γ2 [65], lipoprotein lipase [71] and plasminogen activator inhibitor-1 [36], were detected in hUCMSCs by reverse transcription-PCR. Compared with hBMSCs, hUCMSCs took a longer period to achieve adipose differentiation (40 vs 21 days, respectively) [36]. A similar observation was made between fetal MSCs and adult MSCs. In addition, hBMSCs formed more homogeneous lipid droplets and had a more round morphology than the hUCMSCs [36]. However, in a 2-week study by Lu *et al.*, no significant difference was found in the percentage of adipogenic positive cells between hUCMSCs and hBMSCs (69.4 vs 57.3%, respectively) [71]. After long-term exposure to adipogenic medium, 90% of hUCMSCs differentiated toward the adipogenic lineage and formed mature adipocytes [119]. Therefore, the adipose differentiation ability of hUCMSCs makes them a promising cell source for regenerative medicine, despite the fact that hUCMSCs have not yet been used for adipose tissue engineering.

Undifferentiated hUCMSCs & cardiovascular tissue engineering

Detailed reviews regarding the application of hUCMSCs in cardiovascular tissue engineering have appeared [120,121], so a brief overview is provided. hUCMSCs were utilized for cardiovascular tissue engineering prior to the identification of multipotent differentiation [49], and they have been shown to be a suitable cell source for this

application [54–58]. In monolayer culture, undifferentiated hUCMSCs expressed positive staining of α-smooth muscle actin and vimentin with the deposition of type I and III collagen after 2 weeks in a culture medium containing only Dulbecco's modified Eagle's medium and 10% fetal bovine serum. After exposure to the culture medium, the hUCMSCs were phenotypically similar to the native cells in heart valve leaflets [49]. This similarity provided the rationale for using these cells for cardiovascular regeneration [49]. The culture of hUCMSCs on 3D PGA/poly-4-hydroxybutyrate [49,54,56–58] or poly-4-hydroxybutyrate [55] scaffolds produced a layered tissue with type I and III collagen, GAGs, elastin and a mechanical stiffness similar to native cardiovascular tissue. These tissue engineered constructs were endothelialized, under cyclic strain and growth factor perfusion, with human umbilical cord blood endothelial progenitor cells (UC-EPCs) to provide a functional endothelial layer [57]. Hence, the approach using hUCMSCs for cardiovascular tissue engineering is attractive, since UC-EPCs can also be obtained at birth along with hUCMSCs. This alone will not solve the problem of tissue rejection. However, if the umbilical cord is collected from children who have a congenital heart defect, then UC-EPCs and UCMSCs may be used to construct autologous bilaminar constructs.

Discussion

In this review, hUCMSCs were introduced as an alternative to ESCs and BMSCs, with clear advantages over each (Table 1). hUCMSCs are noncontroversial, in contrast to ESCs. In terms of availability and harvesting efficiency, hUCMSCs offer a major advantage over ESCs, as there is an endless supply of umbilical cords with 4.1 million births in the USA alone in 2005 [122]. The ample supply of the cords and the cryopreservation of UCMSCs will allow cells to be banked for future use as an autologous or allogeneic (with tissue typing) cell source. Practically speaking, hUCMSCs are much easier and less expensive to harvest and culture than ESCs. The umbilical cord stroma can be digested by trypsin, type I and II collagenase, and hyaluronidase in 4 h [33,36,64,65]. Among these enzymes, type II collagenase has been proved to be the strongest and most efficient with all hUCMSCs released [36], while the cocktail of type I collagen and hyaluronidase [33] may only release a part of the cells from the stroma, which might isolate a different cell type. It must also be noted that the hUCPVCs reported by Sarugaser *et al.* represented a subpopulation with great intrinsic osteogenic ability [64], although they share most of the surface markers with hUCMSCs, and should be distinguished from the whole population of hUCMSCs when comparing hUCMSCs to other types of stem cells.

As for their differentiation ability, hUCMSCs differentiate along mesenchymal lineages and perhaps across germ layer boundaries, which translates to a broad potential for curing a range of diseases such as diabetes and Parkinson's disease. Currently, most of the differentiation efforts were conducted *in vitro* in monolayer culture, which is different from the 3D architecture and microenvironment of scaffolds and native tissues. The differences between 2D and 3D environments are certain to affect cellular migration, proliferation and differentiation. For example, Zhang *et al.* reported that hUCMSCs had slightly better proliferative and osteogenic potential than adult hBMSCs in 2D monolayer culture; however, when these cells were cultured in 3D scaffolds both in *in vitro* culture and *in vivo* implantation, the adult hBMSCs outperformed the hUCMSCs [105]. Another feature of the contemporary literature is that all differentiation studies evaluated extracellular protein markers biochemically, and only examined several genes for a specific lineage at the mRNA level. An examination of gene expression over the time course of differentiation may reveal both the expression of genes associated with the initial lineage commitment at early time points and, later, the expression of genes associated with the fully differentiated cell [5]. These cell products, which indicate a fully differentiated cell, are critically important to produce the biomechanical and physiological properties required in many tissue engineering

applications. For example, a couple of studies used osteopontin as a differentiation marker to confirm osteogenic differentiation, although osteopontin is not a bone-specific marker, also being expressed in other tissues such as kidney and placenta [65,71]. Based on the current limitations with gene expression data, it appears that hUCMSCs share a gene activation at the initial commitments similar to other MSCs. For instance, the *SOX9*, *Runx2* and *PPARγ* genes play the key role in chondrogenic, osteogenic and adipogenic differentiation, respectively, and all of these genes were upregulated after exposure to differentiation signals at the earlier stage of differentiation and their expression continued to increase with time. At the late stage, lower type II collagen at both the mRNA and protein levels with chondrogenesis in hUCMSCs relative to hBMSCs, along with the inferior mineralization with osteogenesis, and no presence of mature adipocytes with adipogenesis, indicate that hUCMSCs may follow a different differentiation pathway from other MSCs or require a longer time course to reach mature phenotypes. These puzzles can be further clarified by side-by-side comparison with other MSCs using cDNA microarray and reverse transcription-PCR analysis.

In addition to the differentiation ability, the self-renewal ability is the other key characteristic of stromal cells. The self-renewal capability of stromal cells not only plays a key role in tissue homeostasis *in vivo,* but also has particular implications for tissue engineering, requiring a large amount of undifferentiated cells for large defects. hUCMSCs, as fetus-derived MSCs, have a better *in vitro* expansion capability without the loss of differentiation ability than adult stem cells, perhaps due to their longer telomeres [105]. The original phenotypic markers of hUCMSCs were positive throughout a nine-passage period, including MSC surface markers (e.g., CD73, CD90 and CD105) and transcription factors (e.g., Nanog, Oct-4 and Sox2), and the differentiation ability showed no significant differences among these passages [77]. Therefore, great self-replenishing activity of hUCMSCs and their extensive availability make it possible to obtain a tremendous number of cells in a short time (orders of magnitude larger than BMSCs) to meet the urgent need of tissue engineering applications.

With regard to their immunocompatibility, hUCMSCs suppress the proliferation of activated splenocytes and T cells appear to be immune suppressive like other MSCs and more immunocompatible than ESCs [43,123–127]. *In vitro* immune characterization of hUCMSCs revealed that the immunosuppression properties can be elucidated by evidence from the literature [123]. First, hUCMSCs suppress the proliferation of immune cells such as Con-A-stimulated rat splenocytes, human peripheral blood mononuclear cells and purified T cells. Second, hUCMSCs synthesize HLA-G6, an immunosuppressive isoform of HLAs; while hUCMSCs do not express HLA-G5, a critical isoform to the immunosuppressive function of adult hBMSCs [128]. Third, immune response-related costimulatory molecules including CD40, CD80 and CD86 are not detected on hUCMSCs. Finally, hUCMSCs produce IL-6, which is involved with MSC immune regulation [129]. The *in vitro* results support the earlier finding that hUCMSCs can be tolerated in animal models [33,42,43,64,123,130]. In an allogeneic study by Cho *et al.*, the immunogenicity of UCMSCs was only observed after repeated injection of MHC-mismatched unactivated porcine UCMSCs into the same inflamed region [42]. In xenotransplantation studies, UCMSCs survived without immune response and displayed a rescue phenomenon in retinal and Parkinson's disease [53,130]. Moreover, hUCMSCs can also be used as an autologous MSC source, which can be cryogenically frozen, and thus it is possible to avoid immunorejection. Even if evidence of immunogenicity is discovered after differentiation [131], allogeneic hUCMSCs would be a viable option with the availability of cord cell banks, as routine tissue-typing (HLA/MHC matching) with a tremendous donor selection would be straightforward and would thus reduce any potential immunogenicity along with immuno-suppressants, as is done for transplant patients (but without waiting indefinitely for

a donor). With all of these characteristics, we believe that hUCMSCs will be a suitable, attractive and potentially preferred cell source for tissue engineering. Although hUCMSCs possess a number of advantages over other cell sources, there are limitations that warrant further investigation. The greatest limitation to date is the relative paucity of characterization data relative to the current gold standard, BMSCs. For example, methods to differentiate BMSCs are established and well-documented, whereas we have only begun to scratch the surface with regard to identifying the signaling strategies tailored to hUCMSC differentiation. Although there is evidence that hUCMSCs proliferate faster, can be passaged further and synthesize matrix at a higher rate, the key limitation is that the current literature is currently not able to provide compelling and agreed-upon evidence that hUCMSCs can match the differentiation capacity of BMSCs in a 3D *in vitro* or *in vivo* environment. The burden will be on the growing number of investigators joining the field of hUCMSC biology and tissue engineering to identify the set of conditions specific to hUCMSCs that will signal differentiation along desired lineages in a 3D environment.

En route to the application of hUCMSCs in tissue engineering, a number of other concerns remain to be addressed, including cell–biomaterial surface interaction, cell culture environment and safety. Tissue engineering strategies require hUCMSCs to adhere efficiently to biomaterials. Indeed, high seeding efficiency with hUCMSCs, coupled with fast proliferation, can reduce the quantity of cords and the cost of culture reagents significantly. Tissue engineering strategies also require an optimal culture environment that can maintain cell growth, differentiation potential, phenotype and genomic stability [132]. Currently, the most common medium for hUCMSCs involves the use of bovine serum, which may cause disease transmission and immune reaction. As tissue engineering using hUCMSCs is translated into clinical practice, a serum-free medium may be preferred to avoid these problems. In fact, a low-serum medium has been successfully used to culture hUCMSCs. It includes only 2% bovine serum, 1X serum substitute, and growth factors containing EGF and PDGF [33]. In this medium, cells grew robustly and most of the initial surface markers were maintained after four passages. In the future, human autologous serum may be an option to replace this 2% animal serum, although clearly a serum-free medium would be ideal. Moreover, to engineer large-scale tissues, typical scale-up problems need to be addressed as the diffusion of nutrients and wastes is hindered in large constructs [133].

Future perspective

Umbilical cord mesenchymal stromal cells represent an exciting MSC source for tissue engineering applications with clear advantages over other cell sources including, but not limited to, the ease of access, extensive availability, noninvasive harvesting and *in vitro* expansion ability. Although their degree of 'stemness' is still unclear due to the lack of data on long-term engraftment and self-renewal in the literature [37], the *in vivo* differentiation of these cells including osteogenic, endothelial, neurogenic, hepatic and pancreatic lineages, accompanied by their great *in vitro* expansion ability, certainly supports the contention that these cells may indeed act as true therapeutic cells. Investigations of hUCMSCs in musculoskeletal tissue engineering have shown encouraging results based on the *in vitro* and *in vivo* evaluations while more *in vivo* tissue engineering studies with large animal models are warranted en route to clinical trials. Following these initial current successes with these cells, we believe that hUCMSCs have the potential to be successfully used in other tissue engineering areas such as pancreas and neural tissue regeneration. Future work should include the optimization of cell culture environment, scaffold design and evaluation of *in vivo* regenerative medicine performance, all of which can be integrated in stem cell-based tissue engineering strategies toward clinical application.

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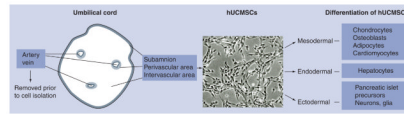
Figure 1. Structure of umbilical cord and differentiation schematic diagram of human mesenchymal stromal cells
hUCMSC: Human umbilical cord mesenchymal stromal cell.

Table 1

Criteria for cell source selection in tissue engineering.

Factor	Mature cells	ADSCs	BMSCs	ESCs[†]	UCMSCs	Ref.
Highly available deciduous tissue source					X	[122]
Politically noncontroversial	X	X	X		X	[48]
Easy to obtain and to use	X	X	X		X	[134]
No donor site morbidity				X	X	[134]
Multiple passaging				X	X	[33,36]
Multipotent		X	X	X	X	[36,65,135]
Low immunogenicity	X	X	X	X	X	[33,42,43,53,123]

[†]Includes embryonic germ cells.

ADSC: Adipose-derived stromal cell; BMSC: Bone marrow mesenchymal stromal cell; ESC: Embryonic stem cell; Mature: Fully differentiated cells from their host tissue; UCMSC: Umbilical cord mesenchymal stromal cell.