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Review

Epithelial and Mesenchymal Stem Cells From the Umbilical Cord Lining Membrane

Ivor J. Lim and Toan Thang Phan

Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
CellResearch Corporation Pte Ltd, Singapore

Intense scientific research over the past two decades has yielded much knowledge about embryonic stem cells, mesenchymal stem cells from bone marrow, as well as epithelial stem cells from the skin and cornea. However, the billions of dollars spent in this research have not overcome the fundamental difficulties intrinsic to these stem cell strains related to ethics (embryonic stem cells), as well as to technical issues such as accessibility, ease of cell selection and cultivation, and expansion/mass production, while maintaining consistency of cell stemness (all of the stem cell strains already mentioned). Overcoming these technical hurdles has made stem cell technology expensive and any potential translational products unaffordable for most patients. Commercialization efforts have been rendered unfeasible by this high cost. Advanced biomedical research is on the rise in Asia, and new innovations have started to overcome these challenges. The Nobel Prize-winning Japanese development of iPSCs has effectively introduced a possible replacement for embryonic stem cells. For non-embryonic stem cells, cord lining stem cells (CLSCs) have overcome the preexisting difficulties inherent to mesenchymal stem cells from the bone marrow as well as epithelial stem cells from the skin and cornea, offering a realistic, practical, and affordable alternative for tissue repair and regeneration. This novel CLSC technology was developed in Singapore in 2004 and has 22 international patents granted to date, including those from the US and UK. CLSCs are derived from the umbilical cord outer lining membrane (usually regarded as medical waste) and is therefore free from ethical dilemmas related to its collection. The large quantity of umbilical cord lining membrane that can be collected translates to billions of stem cells that can be grown in primary stem cell culture and therefore very rapid and inexpensive cell cultivation and expansion for clinical translational therapies. Both mesenchymal and epithelial stem cells can be isolated from the umbilical cord lining membrane, usefully regenerating not only mesenchymal tissue, such as bone, cartilage, and cardiac and striated muscle, but also epithelial tissue, such as skin, cornea, and liver. Both mesenchymal and epithelial CLSCs are immune privileged and resist rejection. Clinically, CLSCs have proved effective in the treatment of difficult-to-heal human wounds, such as diabetic ulcers, recalcitrant chronic wounds, and even persistent epithelial defects of the cornea. Heart and liver regeneration has been shown to be successful in animal studies and await human trials. CLSCs have also been shown to be an effective feeder layer for cord blood hematopoietic stem cells and, more recently, has been recognized as an abundant and high-quality source of cells for iPSC production. Banking of CLSCs by cord blood banks in both private and public settings is now available in many countries, so that individuals may have their personal stores of CLSCs for future translational applications for both themselves and their families. Cord lining stem cells are strongly positioned to be the future of cell therapy and regenerative medicine.

Key words: Cord lining; Stem cells; Regenerative medicine; Tissue repair and regeneration; Antiaging

INTRODUCTION

The umbilical cord has the distinction of being the first fetal tissue to be explored for the presence of stem cells. Broxmeyer first contemplated the use of hematopoietic stem cells in the umbilical cord blood in 1982, with the first transplantation performed in 1988 (1). This was the starting impetus to exploring the rest of the umbilical cord–placenta complex for novel and alternative sources of stem cells, which has now been the subject of intense stem cell investigation over the past decade or so (4,14,15,27).

The umbilical cord as a source of stem cells is an attractive one, as it is usually discarded as biowaste

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Address correspondence to Toan Thang Phan, Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Block MD11, 10 Medical Drive, Singapore 117597. E-mail: surptt@nus.edu.sg

accompanying the delivery of a newborn. The absence of morbidity to either mother or child makes it ethically very acceptable. Further, the supply of umbilical cords is sustainable with no conceivable shortage.

A structure derived solely from the fetus, the umbilical cord is well established as having different sources from which stem cells may be derived, namely, Wharton's Jelly (24) and the umbilical vein endothelial/subendothelial layer (37). In what was certainly a case of being hidden in plain sight, the bland-looking outer amniotic membrane lining of the umbilical cord remained largely ignored and uncharacterized.

Covering the umbilical cord's entire external surface, the umbilical cord amniotic membrane, which we term the cord lining, extends from the placenta to the fetal abdomen whereupon it transits directly to the fetal skin. It has a bilayer structure resembling the epidermal and dermal layers of skin. This microscopic appearance led us to utilize modified keratinocyte and fibroblast culture techniques to derive the cells using both explant and enzymatic digestion methods when we started our investigations in 2003. These two cell strains were subsequently found to be multipotent (as outlined below) and are termed cord lining stem cells (CLSCs).

CELL CULTURE CHARACTERISTICS

Umbilical cord amniotic membrane cultured in modified keratinocyte culture yields polyhedral epithelial cells, which we term cord lining epithelial cells (CLECs). A subtype of CLECs termed CLEC-muc is obtained when CLECs are grown in low serum [2.5% fetal bovine serum (FBS)] supplemented PTTe-1 medium (patented and proprietary medium of CellResearch Corp, Singapore) with components that preferentially support epithelial cell growth.

When cultured in modified fibroblast media, spindle-shaped fibroblast-like mesenchymal cells are obtained with plastic adherent properties, which we term cord lining mesenchymal cells (CLMCs). Grown to confluence, both cell strains form colonies, a gross morphological indicator of stem cell identity.

CORD LINING STEM CELL YIELD

Uniquely, the number of CLECs and CLMCs that can be explanted is enormous. Each square centimeter of umbilical cord amniotic membrane normally yields 20 million CLECs and 20 million CLMCs at passage 1. The average dimensions of the umbilical cord are a length of 55 cm and a diameter of 2 cm. If the entire length of umbilical cord amniotic membrane was unfurled, an area of 330 cm^2 ($\pi \times$ diameter $\times$ length) would be obtained. Extrapolating this to cell numbers, a fully unfurled length of umbilical cord could therefore yield 6 billion CLECs and 6 billion CLMCs at passage 1. Without considering the epithelial stem cells, the number of mesenchymal stem cells that can be obtained from one umbilical cord greatly exceeds the mesenchymal stem cells that can be derived from bone marrow, cord blood, and adipose tissue.

CORD LINING EPITHELIAL CELLS (CLECs)

CLECs are positive for the epithelial cell markers cluster of differentiation 44 (CD44), CD 54, and CD104; express embryonic stem cell markers octamer-binding transcription factor 4 (Oct-4), Nanog, stage-specific embryonic antigen 4 (SSEA-4), reduced expression protein 1 (Rex-1), Trafalgar antibody 1-60 (Tra-1-60), and sex-determining region Y box 2 (Sox-2), as well as epithelial stem cell (EpiSC) markers tumor protein p63, cytokeratin 18 (CK18), CK19, albumin, α-fetoprotein, and mucin-1 consistent with EpiSC morphology. They closely resemble primary neonatal epidermal keratinocytes (39), and initial studies have shown their ability to differentiate into fully stratified epithelium on a three-dimensional organotypic model, with excellent potential for clinical epidermal reconstitution (14). Their multipotent nature has further been demonstrated by differentiation into insulin-secreting cells (46) and metabolically active hepatoblasts (6) using the established differentiation protocols.

The CLEC subtype CLEC-muc expresses mucin-1 (CD227) and p63 and has been shown to be highly proliferative with significant clonogenic ability. CLEC-muc also expresses the mesenchymal stem cell (MSC) marker CD166 (35).

Human leukocyte antigen (HLA) analysis of CLECs shows them to be entirely fetal in origin with no maternal HLA present. Of note is that CLECs show the presence of nonclassical HLA forms HLA-E and all seven known isoforms of HLA-G, which are linked with placental mechanisms that modulate maternal immune rejection by inhibiting natural killer (NK) cell lysis (26) and confer upon them immunosuppressive qualities (46). The immunoprivileged environment within the pregnant uterus, and the temporary tolerance induced, functions to prevent the fetus from rejection as an allograft during its 9-month gestation.

Animal studies have shown that CLECs survive for extended periods when xenotransplanted on polyethylene phthalate (PET) membranes into immune-competent mice when compared to normal keratinocyte controls. Interestingly, mixed CLEC/keratinocyte cultures on membrane showed improved keratinocyte survival indicative of nonspecific protection by paracrine factors secreted by CLECs (46).

CLECs have been gene modified using phiC31 integrase to insert the factor VIII (FVIII) gene into the safe

8p22 site without incurring genotoxic or oncogenic risks. Implantation of FVIII-secreting CLECs partially corrected the bleeding phenotype of hemophiliac mice by raising plasma FVIII levels (42), which points strongly toward future human applications, including hemophilia A, for this single gene defect. This study also showed minimal inflammation and no teratoma formation when CLECs were injected into mice kidney capsules, suggesting that CLECs are nontumorigenic and nonimmunogenic.

Engineered stratified CLEC-muc sheets on human amniotic membrane have also been shown to regenerate a smooth and clear cornea epithelial surface on limbal stem cell-deficient rabbit eyes. The gene profile of the CLEC-muc-grafted eyes was found to be similar to normal control eyes (34).

The success of this animal study led to applications to conduct a human proof-of-concept case series for the treatment of persistent corneal epithelial defect (PED) at the Vietnam National Institute of Ophthalmology in Hanoi, Vietnam. Local Institutional Review Board approval was given to the study in 2010 and patient consent obtained on recruitment for the study. Allogeneic CLEC sheets cultured on contact lens-shaped plastic were applied onto chronic corneal ulcers of 16 eyes with good to excellent healing in 15. The cohort of this case series has since been extended, and to date, vision has been restored to more than 60 eyes using this technique with success rate of 95% (author's unpublished data).

Conditioned media from CLEC growth in maintenance media (which we term CALECIM®) has been collected and used for the treatment of chronic ulcers with good success. CALECIM® applied to intact skin has also been shown to have positive effects on skin renewal with cosmetic improvement.

CORD LINING MESENCHYMAL CELLS (CLMCs)

CLMCs have the immunophenotype $CD73^+$, $CD90^+$, and $CD105^+$ consistent with MSC morphology. They are negative for the hematopoietic stem cell markers CD 34, CD 45, and CD 117; mildly positive for the embryonic stem cell marker SSEA-4; and negative for the maturity marker SSEA-1 as well as human embryonic carcinoma marker TRA-1-60 (9). OCT-4 and Nanog are shown to be expressed by CLMCs, which was not lost after freeze–thawing (18). The same study also demonstrated the absence of typical embryonic stem cell-specific markers and no growth on soft agar suggestive of tumorigenicity in vitro. This has been confirmed in vivo by the absence of teratoma formation after the injection of CLMCs into the midthigh muscle mass of severe combined immunodeficient (SCID) mice (authors' unpublished data). CLMCs have been differentiated into osteoblasts, chondrocytes, neurons (including dopamine-secreting neurons), fibroblasts, and adipocytes (5), demonstrating their multipotent nature.

HLA analysis of CLMCs shows them to be entirely fetal in origin with no maternal HLA present. Although levels of CLMC HLA-E and HLA-G have been found to be insignificant (9), significant upregulation of indolamine 2,3-dioxygenase (IDO) expression, which mediates the suppressive effect of MSCs on T-cells (23), appears to contribute to the strong immunosuppressive characteristics of these cells.

Compared to bone marrow MSCs, CLMCs demonstrate faster cell proliferation in cell-specific media, lower expression of HLA class I molecules, and are more immunosuppressive by stronger expression of tolerogenic factors; these data strongly suggest that they may be superior to bone marrow MSCs for immunomodulation (9). Another study comparing extraembryonic tissue-derived MSCs from the cord lining, placenta, Wharton's Jelly, and cord blood showed CLMCs to have the lowest immunogenicity and highest rates of cellular proliferation and migratory potential (44).

A recent study used a CLMC-containing fibrin graft supplemented with an omental flap to induce cardiac revascularization in a rat chronic ischemic heart model. Cardiac dysfunction was ameliorated, and postischemic remodeling was attenuated. The authors attributed this to paracrine factor-mediated myocardial repair rather than cell differentiation-mediated replacement of the injured myocardium (21). This was the first study to use CLMCs for a cardiac application and has demonstrated the close similarities between CLMCs and other strains of MSCs in cardiac repair.

In 2005, with local Institutional Review Board approval, proof-of-concept studies were initiated at the National Burns Centre in Hanoi, Vietnam, to assess the use of CLMCs in improving the wound bed of indolent chronic wounds to facilitate skin grafting and closure in consenting patients. Interestingly, CLMCs cultured on various inert scaffolds (Tegaderm® and Biobrane®) applied onto surface wounds, such as chronic burn wounds, appeared not only to improve the bed but also to cause reepithelialization without requiring a skin graft. Cavity wounds such as pressure ulcers filled with hydrocolloid (Solosite®) seeded with CLMCs also induced healthy granulations, wound edge contraction, and closure without requiring a skin graft. These remarkable early results led to an extended case series, which now includes more than 2,000 patients in Vietnam. At least 80% of the wounds, ranging from chronic postirradiation burn ulcers to chronic venous and diabetic ulcers, were successfully closed with CLMC treatment (author's unpublished data). The remaining 20% of wounds were significantly improved to allow successful

partial thickness autologous skin grafting. We are currently in the process of starting clinical trials both in Singapore and in the US (FDA) using CLMCs to close chronic diabetic ulcers.

DISCUSSION

By virtue of their inbuilt capacity for self-renewal and capacity to differentiate, stem cells are rapidly getting established as the new direction for the treatment of human disease. A number of hematopoietic stem cell therapies already exist, the best known of which are bone marrow transplants and cord blood transplants for hematopoietic reconstitution (12).

The subsequent finding of other multipotent cells in the blood (comprising a heterogenous population of mesenchymal and hematopoietic stem cells with endothelial progenitor cells and immature immunological cells) led to the use of these mononuclear cells derived from bone marrow, peripheral blood, or cord blood for various applications. Mononuclear cells have been used to treat chronic stroke (13), acute spinal cord injury (13), acute myocardial infarction (20), chronic ischemic cardiomyopathy and heart failure (29), and critical limb ischemia (2). Interestingly, in the absence of myeloablation and immunosuppression, nonmatched allogeneic mononuclear cell therapy for a variety of neurological conditions has been reported to be free from immune-mediated adverse effects (45).

From the time of characterization of multipotent EpiSCs in the bulge stem cell niche (3) similar to those of intestinal epithelium (36), EpiSCs have been the subject of active investigation for translational applications. To date, however, significant clinical applications have been limited largely to autologous limbic EpiSCs for human corneal repair (16,28) with no trials for allogeneic EpiSCs to date.

MSCs from the bone marrow were first described and characterized in 1999 (31). Knowledge of the embryology of these cells and differentiation along a variety of mesenchymal lineages pointed to their use for solid organ repair and regeneration. Numerous studies have since been conducted with preclinical studies rapidly progressing to clinical trials. MSCs have been utilized for direct repair of organs such as the kidney in acute renal failure (25) and chronic renal failure (7), and in patients with acute-on-chronic liver failure (41). With cardiovascular disease being a leading cause of morbidity and mortality in many parts of the world (38), it is not surprising that applications for the heart have been especially active, and MSCs for cardiac diseases (chronic myocardial ischemia, advanced chronic heart failure, congestive heart failure, acute myocardial infarction, dilated cardiomyopathy) have now reached phase II and phase III clinical trials (40).

With their acknowledged immunosuppressive properties, MSCs have also been administered in patients receiving solid organ transplants to decrease rejection and promote engraftment (11). MSCs have also been shown to suppress graft-versus-host disease (GVHD) in steroid-refractory patients in a phase II study (19). Interestingly, cord blood expanded ex vivo in coculture with allogeneic MSCs appeared to have improved engraftment compared to unmanipulated cord blood only (8).

One of the interesting properties of stem cells is their ability for directed migration to sites of inflammation or wound healing. MSCs have been well characterized for this (10), which predisposes these cells for targeted delivery of therapeutic substances. Phase I mouse studies have demonstrated MSC effectiveness as oncolytic virus carriers in the treatment of ovarian cancer (22).

Ultimately, there still remains a bottleneck for EpiSC and MSC therapy obtained from traditional sources. For example, the use of bone marrow-derived MSCs for regenerative medicine is hampered by i) the limited amount of bone marrow that can be extracted, which involves ii) a painful, invasive procedure with iii) the attendant risks required for bone marrow extraction (2), iv) low number of cells extracted (MSCs comprise only 0.001% to 0.01% of all nucleated cells extracted) (32), hampered by v) age-dependent decrease in cell numbers and viability (33), and vi) low proliferation of extracted cells (43). Downstream, the MSC derived first needs to be specifically selected and identified before enrichment and clonal expansion to obtain sufficient numbers for treatment. To complicate matters, MSCs possess a limited life span, and repeated in vitro culture results in MSCs undergoing replicative senescence with a change in morphology and loss of proliferation (17); however, this has been challenged by others (32). This degree of cellular manipulation also increases the overall fiscal cost of obtaining the stem cells.

MSCs in cord blood are newborn stem cells, which have excellent proliferation and differentiation abilities; they are also immediately available when required. However, there is still a limitation in that there is only a one-time supply (13) of limited volume, although expansion of these cells with allogeneic MSCs is a possibility (8). The number of MSCs contained in cord blood is also extremely small at 0.00003% of all nucleated cells extracted (30).

Studies on CLMCs have shown them to possess MSC characteristics and CLECs to have EpiSC characteristics. Both are multipotent and have been differentiated to cells of their respective lineages.

Overall, the optimal source of stem cells should:

1. Be derived from a source that is ethically acceptable.
2. Be free of morbidity to the patient.

3. Contain juvenile stem cells with excellent viability and proliferation.
4. Contain stem cells in large numbers to minimize downstream cellular manipulation.
5. Be cost-effective and easy to process.

The umbilical cord lining satisfies all the criteria above, as

1. It is derived from the umbilical cord, which is biowaste accompanying the delivery of a child.
2. Does not cause any mortality or morbidity to either mother or infant.
3. Comprises newborn stem cells, all of 9 months old.
4. Has the largest yield of stem cells, to date, at passage zero of any source in the human body.
5. Is economical to process by virtue of its smooth, uniform structure, and cost is contained by the sheer number of cells that can be explanted in the first instance, minimizing the need for cell manipulation and expansion to obtain sufficient numbers for clinical translational applications.
6. As an added bonus, the cord lining yields not just MSCs, but EpiSCs as well.

Preclinical studies have proven the efficacy of CLECs and CLMCs as practical and realistic alternatives to EpiSCs and MSCs from traditional stem cell sources. It leaves us to further explore the utility of these cells in the treatment of human diseases.

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