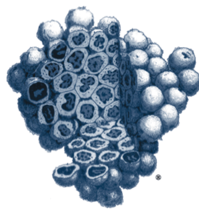

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MESENCHYMAL STEM CELLS

Concise Review: Human Umbilical Cord Stroma with Regard to the Source of Fetus-Derived Stem Cells

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Key Words. Umbilical cord • mesenchymal stem cell • differentiation • stroma • Wharton's jelly

ABSTRACT

Human umbilical cord (UC) has been a tissue of increasing interest in recent years. Many groups have shown the stem cell potency of stromal cells isolated from the human UC mesenchymal tissue, namely Wharton's jelly. Since UC is a postnatal organ discarded after birth, the collection of cells does not require an invasive procedure with ethical concerns. Stromal cells, as the dominant cells of this fetus-derived tissue possess multipotent properties between embryonic stem cells and adult stem cells. They bear a relatively higher proliferation rate and self-renewal capacity. While they share common surface markers with bone marrow-derived MSCs, they also express certain embryonic stem cell markers albeit in low levels. Without any spontaneous differentiation they can be successfully differentiated into mature adipocytes, osteoblasts, chondrocytes, skeletal myocytes, cardiomyocytes, neurons and endothelial cells. While causing no immunorejection reaction, they effectively function *in vivo* as dopaminergic neurons, myocytes and endothelial cells. Given these characteristics, particularly the plasticity and developmental flexibility, UC stromal cells are now considered an alternative source of stem cells and deserve to be examined in long-term clinical trials. This review first aims to document the published findings so far regarding the nature of human UC stroma with special emphasis on the spatial distribution and functional structure of stromal cells and matrix which serves as a niche for residing cells, and secondly, to assess the *in vitro* and *in vivo* experiments in which differential stem cell potencies were evaluated.

INTRODUCTION

The umbilical cord (UC) represents the link between mother and fetus during pregnancy. It is composed of a special embryonic mucous connective tissue called Wharton's jelly lying between the covering amniotic epithelium and the umbilical vessels. The main role of this jelly-like material is to prevent the compression, torsion and bending of the enclosed vessels, which provide bidirectional blood flow between fetal and maternal circulation.

The UC did not gain enough attention in the 70's and 80's, most probably because of being a discarded material after delivery. Two main questions drove scientists to re-examine the Wharton's jelly stromal cells and extracellular matrix (ECM) composition in the 90's. One was the search for a possible reason and consecutive structural alterations in pre-eclampsia cases. A series of ECM components were found altered in pre-eclamptic patients associated with the "premature ageing" of this tissue [1, 2]. The second reason was the cellular identification of UC stromal cells, which basically resemble mesenchymal

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fibroblasts found elsewhere during *in utero* development. Ultrastructural studies indicated that their intrinsic properties were also similar to smooth muscle cells [3-5] and they are therefore considered as myofibroblasts.

In recent years, in parallel to the enormous effort to explore novel and alternative sources of stem cells in the human body, the UC appeared as a promising reservoir of fetal cells that could be readily used as multipotent stem cells. Following the year 2003, a steep increase was noted in studies examining the stem cell potency of these myofibroblastic cells. The published articles in peer-reviewed journals written in English by July 2007 are listed in Table I. It is very likely that many more will come in the near future since *in vivo* tests of *in vitro* differentiated or undifferentiated UC-derived stem cells are just starting to be examined in several disease models and in regenerative medicine.

Cellular and Extracellular Matrix Components of Umbilical Cord

a. General overview

The human UC weighs around 40 g, its length reaches to approximately 60-65 cm having a mean diameter of 1.5 cm at term [6, 7]. It is covered by a single/multiple layers of squamous-cubic epithelial cells [8, 9] called umbilical epithelium, which is generally thought to be derived from amniotic epithelium. Those epithelial cells display ultrastructural and functional characteristics to those seen in keratinocytes [10] and were recently shown to possess stem cell characteristics [11].

The inner tissue architecture is composed of a set of two arteries and one vein, and a surrounding matrix of mucous connective tissue comprised of specialized fibroblast-like cells and occasional mast cells embedded in an amorphous ground substance rich in proteoglycans, mainly hyaluronic acid. Neither capillaries nor lymphatics are found in the UC. Vessels are normally organized as left spiral (counter clockwise) turns. In clinical practice, determining the "umbilical coiling index" (number of complete coils divided by the length of the cord; average 0.24 coils/cm) may identify the fetus at risk [12, 13].

Wharton's jelly appears to serve as the function of adventitia, which the UC vessels lack, binding and encasing the umbilical vessels. It has been speculated that the stromal cells of Wharton's jelly may participate in the regulation of UC blood flow and that, at least in some cases, the reduction in fetal growth could be the consequence of stromal diminution leading to hypoplasia of umbilical vessels [14, 15]. Due to many reports and careful examination of cellular and ECM components, human UC shows a tissue compartmentalization in which cellular characteristics and ECM elements differ from each other. At least six distinctive zones are now recognized based on the structural and functional studies; from outer to inner, i) *surface epithelium (amniotic epithelium, UC epithelium)*; ii) *subamniotic stroma*; iii) *clefts*; iv) *intervascular stroma (named classically as Wharton's jelly)*; v) *perivascular stroma*; and vi) *vessels*. Fine structural, immunohistochemical [3, 4, 16, 17] and *in vitro* functional studies [16, 18] represented that there are significant differences in the number and nature of cells between subamniotic, intervascular and perivascular regions that leads to hypothesize that those regions might be originating from different pre-existing formations. For instance, myofibroblastic cells of the intervascular stroma might have derived from adjacent vascular smooth muscle cells or alternatively from pre-existing fibroblasts.

b. Stromal cells and their spatial organization

Since structural features and main functions of UC stromal cells resemble those of fibroblasts, they were firstly recognized as "unusual fibroblasts" [19] and were demonstrated to be structurally responsive to the distension of cord [19]. Moderate quantities of intracytoplasmic glycogen, lipid droplets and procollagen secretion granules, the presence of a well developed endoplasmic reticulum with dilated cisternae and well developed Golgi complexes with numerous mitochondria [3, 20], directly indicated active protein synthesis and secretion. These findings together with the demonstration of prolyl 4-hydroxylase , an

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enzyme involved in collagen synthesis, eventually supported the idea that UC stromal cells are primarily responsible for the synthesis of collagen and other matrix components.

The presence of extraordinary number of 10 nm-thick intracytoplasmic filaments and gap junction type intercellular communications as commonly observed at the interface of long cellular processes gave credence to understand their nature and be considered as "unusual" smooth muscle cells having some kind of contractile properties. Cell surfaces are partially covered by an external lamina [3-5] mainly composed of collagen type IV, laminin (Fig. 1) and heparan-sulfated proteoglycan [4]. They contain both subplasmalemmal and intracytoplasmic focal dense plaques as typically found in smooth muscle cells [17, 20], whereas no Z-discs or striated-muscle filaments were observed. Membranous caveolae [4], pinocytic vesicles beneath the plasma membrane [3] and numerous dense bodies differentially located in the peripheral cytoplasm intermingled with 10 nm-thick filaments [3-5, 21] were also reported. Those studies also demonstrated the expression of some muscle-specific cytoskeletal filaments in stromal cells, a finding which supports the notion that rather than the fibroblasts or smooth muscle cells, they are true "myofibroblasts", a term which was firstly used by Majno et al. [22] to define cells that exhibit some of the ultrastructural features of both smooth muscle cells and fibroblasts. Specifically, contractile proteins such as actin, non-muscle myosin, desmin and α-smooth muscle actin, a marker for myofibroblasts [23], are differentially expressed in stromal cells [3-5, 16, 21] while muscle-myosin is lacking [3]. Depending on the above findings it was suggested that these cells possibly display specific functions related to both fiber synthesis and organized cell communication and contraction. Supporting evidence was the fact that those cells also express vimentin [3, 4, 16, 24], an intermediate filament protein specifically expressed in mesenchyme-derived cells such as fibroblasts while not expressed in smooth muscle cells [25]. The co-existence of vimentin and desmin in these cells supports the hypothesis that they are intrinsically myofibroblasts. Another interesting intermediate filament expressed by the stromal cells is cytokeratin [4, 16, 24], which are basically expressed in endoderm and ectoderm originated epithelial cells [25]. While Nanaev et al. [4] did not find any significant expression of cytokeratins in stromal cells but in subamniotic epithelium, Karahuseyinoglu et al. [16] have demonstrated that cells of the perivascular region were strongly positive for cytokeratin filaments.

The next question was whether these myofibroblasts are derived from adjacent vascular smooth muscle cells based on their morphological similarity, contractile properties, similar responses to some reagents that contract or relax smooth muscle cells, and immunopositive reaction to anti-smooth muscle antibodies. Up to date, no direct evidence has been published to support this assumption. However, after recognizing that stromal cells vary in structure and display differential localization within the UC, a regional approach to study and explain the differences and origins of stromal cells would be mandatory to answer this question. A gradual change in morphological and probably functional properties of cells during the course of pregnancy was also noted [4, 5]. For instance, the number of α-smooth muscle actin increases with increasing weeks of pregnancy. In fact, the density of cells in a given section between subamniotic, intervascular and perivascular areas are different from each other. Cell frequency is considerably low in subamniotic regions whereas perivascular areas possess the highest cell density [3, 4, 16]. Intervascular cells possess longer and more numerous cytoplasmic processes than do perivascular cells [3]. Further analyses revealed a clearly defined spatial differentiation gradient with increasing cytoskeletal complexity in human UC stromal cells from the superficial layers towards the blood vessels [4, 5]. In parallel to this increasing differentiation status, a gradual decrease from outer to inner cells was noted in the proliferation rate of stromal cells [4]. Conclusively, the immature cells retaining the ability to proliferate are located close to the amniotic surface, whereas highly differentiated

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perivascular cells are found in closer proximity to the umbilical vessels.

c. Extracellular matrix (supplementary text)

Since the UC is the vital and exclusive connection providing the bi-directional blood flow between maternal and fetal sites, mechanical property of this tissue is extremely important. The consistency that prevents UC vessels to bend and occlude is mainly associated with the structure of the ECM which has a unique tissue structure in terms of the compositions of connective tissue fibers and related soluble proteins. The first observations of the ECM in the 50's and 60's reporting the collagen skeleton in close association with the amorphous ground substance [19, 26, 27] was confirmed by later studies. The most abundant fibrillar component of the intercellular stroma as arranged in wavy thick bundles is definitely collagen fibers while elastic fibers are absent [28]. Although it is a minor collagen form, the first type to be demonstrated by both immunohistochemistry and PCR was type VII collagen, found predominantly in the basal portion of the umbilical epithelium and in cultured stromal cells [29]. The extremely low solubility of collagen by chemical or organic substances [1, 30] implied that they build strong ionic bonds with glycosaminoglycans and proteoglycans, therefore providing the stroma with an extraordinary strength [30]. The spongy network of interlacing collagen fibers in a continuous soft skeleton enclosing a wide system of interconnected cavities and canalicule-like structures, collectively defined as clefts, were thought to have a mechanical role that allowed consistency of the ground substance during twisting or compression as reported by Vizza et al. [31]. A regional distribution of collagen fibers was firstly noted by Nanaev et al. [4], who showed collagen types I, III, VI in the subamniotic and intervascular regions as well as in the vessel walls. Type IV was found in both the umbilical epithelium basement membrane and external lamina of subamniotic stromal cells, whereas type VII was restricted to the epithelial basement membrane in term cord. More quantitative studies by Sobolewski et al. [30] regarding the amount of collagen showed that collagen comprised 50% of defatted, dry tissue of UC. Type I and III collagens were found to be the most abundant forms in the intervascular region and arterial wall, i.e. the amount of collagen in the stroma was reported as four times higher than in the artery wall [30]. Moreover, the ratio of type I/III was reported to be almost twice as much in arterial wall as compared to intervascular stroma [30]. Therefore, it was concluded that the resistance of collagen in the intervascular stroma to the action of collagen-solubilizing solutions may be related to the high amount of type III collagen. Another constituent of the microfibrillar network was found as typical 100-nm periodic filaments of type VI collagen [32].

A second major component of the ECM of Wharton's jelly is the ground substance, mainly composed of glycosaminoglycans (GAGs). The total amount of GAGs in Wharton's jelly is twice as high as that contained in the arteries [30]. A certain amount of GAGs is derived from the mast cells that are predominantly localized in close proximity to blood vessels [33]. Hyaluronic acid (HA), being 70% of all the GAGs, is the most abundant form; the amount of sulfated GAGs (keratin, heparan, dermatan, chondroitin-4 and chondroitin-6 sulfate) is very low, whereas heparin is found only in traceable amounts [30]. The extracellular matrix of the UC is one of the highest hyaluronic acid-containing tissues in humans [34]. However, abnormal amounts of HA in the UC is also linked to the pathogenesis of some structural defects. HA was shown to be synthesized higher than normal levels in the UCs of Down Syndrome cases than in euploid UCs [34]. The mobility of HA in the intercellular stroma is reduced both by the collagen network and by the presence of proteoglycan(s) and/or micro fibrils [35]. Therefore, reduction in HA mobility due to physical entanglements may play a major role in UC tissue remodeling since a certain degree of HA mobility is vital to permeate the extracellular space.

Fibrillar component of artificially formed tissue pellets and constructs using UC stroma-derived cells showed that under certain cultures conditioned cells tend to accumulate and secrete reasonable amount of collagen I and II

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[16, 36] when induced for chondrogenic purposes. Heparin, heparan sulfate and aggrecan have also been found to synthesize by cultured stromal cells [36].

Isolation and Culture of Stromal Cells

After delivery, freshly obliterated 15-20 cm long UC obtained in the operating room should be immediately transported to the laboratory in a sterile and cooled transfer media such as HBSS or EBSS. If the aim is to retrieve the stroma cells, arteries and vein should be removed under sterile conditions prior to tissue processing (Fig. 2). Removal of vessels is usually accomplished by simply stripping them off the surrounding tissue. Mechanical chopping of the cord is an essential step before enzymatic digestion since the highly woven intercellular fiber pattern obscures the stromal cells and increases the interacting surface area of digestion reagents. It must be noted that the isolation of cells other than stromal ones generally requires different approaches as described elsewhere [9, 10, 37, 38].

The common point of isolation-digestion protocols applied by different investigators is the use of collagenase-containing solutions, which contain strong collagenase activity as well as caseinase, clostripain and tryptic activities. Type I collagenase is widely used for the isolation of stromal cells [18, 36, 39-41]. A combination of collagenase with hyaluronidase facilitates the degradation of matrix ground substance and shortens the time required for isolation process [36, 39, 41]. However, duration of collagenase treatment is critically important especially if collagenase/hyaluronidase cocktails are used (Can A et al. , unpublished observations) since there is always a risk of degradation of cellular external lamina, a phenomenon preventing cells from adhering to the culture substrate after isolation and even causing severe cellular damage. Type II collagenase, which is stronger for its clostripain activity or collagenase type B, which is more efficient in solubilizing the UC micro fibrils than other types of collagenases [28], are also preferable [16, 42]. The time required to reach a tissue homogenate ranges from 30 minutes [39] to 16 hours [43] depending on the dose and duration of treatment with digesting reagents. Filtering the digested material through 70-100 µm pore-sized sieves usually facilitates the removal of any unwanted tissue debris [44]. Enzymatic digestion may be skipped if obtaining an explant culture is targeted [45-47].

Cell harvesting by a tissue digestion procedure should follow a further isolation of stromal cells that would characterize stromal subpopulations depending on their specific cell surface markers. Fluorescence-activated cell sorting (FACS) or magnetic immunobead separation techniques are generally used to sort and count the interested cells in a relatively short period of time. UC stromal cells have some genetic and surface markers that are also common in MSCs. Table II summarizes the entire cell surface markers tested so far by means of PCR, real-time PCR, microarray and immunocytochemistry techniques. Among these, CD105, CD73 and CD90, which are known to characterize MSCs [48], were consistently found to be positive in UC stromal cells. They do not express hematopoietic stem cell markers such as CD45, CD34 and HLA-DR, which are also lacking in MSCs [48]. However, some discrepancies among groups were reported, i.e. Sarugaser et al. [18] found that HLA-1 expression is only stable up to five passages and lost after cryopreservation while Weiss et al. [41] did not find any change throughout the passages. These and similar findings (*see*, Table II) occur most likely due to the epigenetic factors caused by varying culture conditions. Nonetheless, UC stromal cells are generally considered as MSC-like cells, a hypothesis that is supported by many studies regarding the *in vitro* and *in vivo* differentiation potentials (*see* below). Stromal cells were also found to express low levels of some transcriptional factors which are mainly expressed in embryonic stem cells such as Oct-4 [41] and Nanog [46] both at the mRNA and protein level. Additionally, these cells were found to express a low amount of Wnt-signaling pathway molecules; however they do possess the canonical Wnt transduction mechanisms [49]. Given that these factors are among the key regulators of self-renewal and pluripotency in stem cells [50, 51], it is therefore possible to assume that UC stromal

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cells use similar regulatory mechanisms as embryonic stem cells, especially when dissociated from their microenvironment that can be considered as their niche. Further characterization of these MSC-like cells and their niche is inevitable to fully estimate their responses due to varying differentiation recipes.

Freshly isolated and plated cells principally display a fibroblast-like appearance over the first culture period and lasts around 10-15 days until the first passage. However, some groups reported that there are more than one phenotype of cells grown in cultures and continued up to later passages [16, 18]. Population doubling time which was found to be approximately 60-85 hours in early passages [16, 18] dramatically declines as the passage number proceeds [16, 18] and the number of population doubling varies among different groups [16, 41, 44, 47] ranging from 20-60 until cells reach a replicative senescence without any sign of abnormal karyotype. Upon these relatively high variations among laboratories, it is realistic to suppose that isolated UC stromal cells contain more than one set of stem cell population or more likely they contain a subset of primitive stem cells, a hypothesis which is our current task to unravel.

An important issue of interest in adult stem cell studies is the availability of the source and efficacy of isolation techniques to yield a reasonable amount of viable cells to expand. Therefore, the expansion efficiency of UC stromal cells without spontaneous differentiation is critical before they are induced to differentiate. While the frequency of bone marrow stromal cells is often reported to range from 1-10 MSCs in a total of 10^6 bone marrow mononuclear cells [52, 53], the harvesting ratio of stromal cells in cord stroma is found to be relatively high ($4x10^5$ cells/sample, $10x10^{3-5}$/cm of cord) [16, 41, 42].

UC stromal cells were successfully served as a feeder layer during expansion of equine embryonic stem cells derived from the inner cell mass for more than 350 divisions without ant supplementation of leukemia inhibitory factor [54].

Telomerase activity (TA) in proliferating cells is an active area of research since unlike embryonic stem cells and tumorigenic cells somatic cells display low levels of TA [55]. Few studies dealing with the TA of human UC stromal cells revealed that TA in those cells is higher than in somatic cells of the body. Mitchell et al. [45] showed that TA in isolated porcine UC stroma cells is 10% of carcinoma cells lines, while Weiss et al. [41] found that TERT (telomerase reverse transcriptase) gene expression is elevated in cultured human UC stromal cells. Karahuseyinoglu et al. [16] demonstrated a stable but higher than normal TA in those cells during early passages, which then decreased and reached a level below control HeLa cell lines. Since the transplanted UC stromal cells did not develop any tumorigenic formation [47], it is possible to conclude that UC-derived stem cells have a certain limit of TA that provide cells with the ability to proliferate up to 30-60 divisions but never to a level of tumorigenic state.

In view of the fact that the therapeutic use of stem cells would require freezing of cells in order to store them until thawing whenever needed, freezing conditions of isolated UC stromal cells and viability rates after thawing have to be evaluated. Most of the researchers prefer to use defined culture media supplemented with high amounts of FBS and 7-10% DMSO [16, 41] or glycerol [41] and freeze the cells gradually and keep them between -135 and -196 °C [16, 18, 41]. After rapid thawing at 37 °C, both viability and expansion of cells were found to be satisfactory, with viability rates over 50% [18]. The use of higher levels of fetal bovine serum especially during the first week after the thaw substantially increased growth rates [16]. Certainly, more controlled studies are needed to maximize the freeze-thaw efficiency, especially when their routine use is concerned in clinical trials.

In vitro Differentiation Potential of UC Stromal Cells

Since a growing body of evidence suggests that human UC stromal cells contain a substantial amount of common properties with MSCs (*see* above) it would be reasonable to examine their

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differentiation potency in conditioned cultures specifically designed to differentiate MSCs and many other embryonic and adult stem cells into certain lineages. Table III summarizes the successfully differentiated lineages using a variety of cell culture techniques and reagents which will not be given here in detail.

As the UC stromal cells are originated from extraembryonic mesoderm, adipogenic, chondrogenic, osteogenic, cardiomyogenic and skeletal myogenic inductions have been the most studied cell lineages. Compared to bone marrow MSCs, Karahuseyinoglu et al. [16] demonstrated that UC stromal cells are capable of forming premature adipocytes bearing smaller multilocular lipid droplets. In contrast, Baksh et al. [49] found that UC stromal cells generated significantly more fat containing cells than bone-marrow MSCs by day 21, while Lu et al. [42] did not report any significant adipogenic differences between these two cell types. Adipogenically-induced cells specifically express adipocyte-specific genes, lipoprotein lipase [42] and plasminogen activator inhibitor-1 (PAI-1) [16], which together suggest that the culture conditions used could mimic the *in vivo* differentiation and initiate the adipogenic pathways.

Chondrogenic induction was investigated in a few studies using pellet cultures in conditioned media to form 3-dimensional cell spheres [16, 43] or polyglycolic (PGA) scaffolds [36], in which collagen fiber formation, GAG accumulation and chondrocyte differentiation was examined. One-two millimeter shiny-surfaced cell spheres resembling articular cartilage were formed within 3 weeks containing many chondrocytes embedded in a mucopolysaccharide-rich stroma [16]. Type-II collagen was found to be most abundant [16, 36] as detected by hydroxyproline assays [36] or by immunohistochemistry [16] while type-I collagen fibers were also detected especially in the peripheral area of cell spheres implying a capsule formation [16]. *De novo* synthesis of type-II collagen fibers, which are normally synthesized by chondroblasts [56], indicates the functional differentiation of UC stromal cells in a cohort behavior.

Osteogenic potency was first demonstrated in 2004 by Wang et al. [43] as the formation of alkaline phosphatase-positive aggregates and von Kossa stained nodules with an associated expression of osteopontin, an osteo-specific matricellular protein. Later, Karahuseyinoglu et al. [16] showed that calcium deposition and osteoid formation gradually increased up to four weeks during which bone sialoprotein-2, osteonectin and osteocalcin appeared during the second, third and fourth weeks respectively. More specifically, Sarugaser et al. [18] used perivascular cells to build mineralized bone nodules of 300-800 μm in diameter containing a collagenous ECM in their inner structure. Baksh et al. [49] showed that perivascular cells generate a greater extent of mineralization than bone marrow-derived MSCs.

Differentiation into cardiomyocytes or skeletal myocytes of UC stromal cells was less examined. 5-azacytidine, a chemical analogue of the cytosine nucleoside in the DNA and RNA helix, is currently used as a key chemical initiator of myogenic differentiation. Rat cardiac myocyte culture supernatant was added to culture media to differentiate UC stromal cells to cardiac myocytes [43]. Although, cardiomyogenically-induced cells synthesize α-sarcomeric actinin, troponin I and T, all of which are cardiac muscle-specific proteins, and N-cadherin, a calcium dependent cell adhesion molecule [43], they did not form any sarcomeres although Z-discs between cells did form as naturally found in mature cardiac myocytes. In many studies examining the regenerative potency of transplanted MSCs it has been directly or indirectly demonstrated that undifferentiated cells could incorporate into the myocardium and could somehow repair the injured tissue [57]. Therefore, these partly differentiated cells may hold the potency to differentiate into cardiomyocytes when appropriate *in vivo* signals are received. Recently, differentiation of UC stromal cells into skeletal myocytes has been successfully achieved both *in vitro* and *in vivo* (*see* below) [47].

Obviously, after the pioneering studies published in the late 90's and early 2000's [58,

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59], differentiation of MSCs and MSC-like cells into neuronal lineages has been an interesting experimental phenomenon. Initial findings relevant to the *in vitro* neuron formation of porcine UC matrix cells were published in 2003 [45] in that a relatively complex and multistep neuronal induction procedure, as previously defined by Woodbury et al. [59] was used to transform the cells into a neuronal phenotype within few days. Consequently, differentiated cells expressed tuj-1 (β-III tubulin), neurofilament (NF), neuron-specific enolase, tyrosine hydroxylase (TH) and GAP-43. Mitchell et al. [45] also demonstrated that some of cells in the same cultures exhibited GFAP and CNPase positivity, indicating that a portion of cells are capable of differentiating into glial cells. Similarly, human UC cells were partially differentiated *in vitro* into neurons and glia in later studies [16, 41, 42, 60] in which neuronal formation was confirmed by early expressions of NeuN, NF and GFAP and then later by the expression of functional mRNA responsible for the synthesis of subunits of the kainate receptor and glutamate decarboxylase [60]. More recently, Karahuseyinoglu et al. [16] demonstrated that only a portion of stromal cells are responsive to neuronal induction and suggested that this may be due to the presence of heterogeneous cells populations derived from the UC stroma. Supporting evidence for this came from the study of Sarugaser et al. [18] who showed that perivascular cells could not be differentiated into neurons. Recently, Baksh et al. [49] demonstrated that perivascular cells express elevated levels of CD146. Interestingly, a subpopulation of perivascular cells with no CD146 expression could not be differentiated into mesenchymal lineages.

Ma et al. [61] used a different reagent, a medicinal herb, to induce human stromal cells into neuronal lineages. Whether related or not, they found that when treated with the herb extract, human UC stromal cells were found to express pleiotrophin, a secreted growth factor that induces neurite outgrowth and is mitogenic for fibroblasts, epithelial and endothelial cells [62]. In addition to pleiotrophin, human UC stromal cells were found to secrete several neurotrophic factors, like FGF 2 and BDNF [44]. Therefore, the tendency of UC-derived stromal cells to differentiate into neuronal cells may be explained by these intrinsic properties, a hypothesis which is also consistent with the idea that these cells might function by delivery of neurotrophic agents.

In vivo Transplantation Experiments

One of the main aims lying behind all studies concerning stem cells is to achieve the capability to use them in regenerative treatment of cell-based diseases. Since UC is one of the most easily reached stem cell sources both ethically and technically, *in vivo* studies would be definitely worth trying. Compared to other sources of stem cells, especially hematopoietic and non-hematopoietic ones in bone marrow, *in vivo* studies concerning UC matrix cells are very few in number, though all can be regarded to be quite promising (Table III). The first attempts were the xenotransplantation of porcine UC stroma cells into rat brain in which donor cells were tracked 2-8 weeks after transplantation [63, 64]. It was interesting to note that neither any immune rejection nor any teratoma formation due to transplanted material was reported. Secondly, transplanted undifferentiated stromal cells were stained positively with a neurofilament antibody [63] and showed TH-positivity [64], which together indicated that donor cells could transform into neurons or neuron precursors *in vivo*.

Following the above findings, engraftment of human or rat UC cells into rodents were tested to assess whether donor cells could adapt to the recipient organism and function. Two fundamental approaches were applied after isolation and expansion of cells: transplantation of cells into animal tissues in undifferentiated [41, 47] or, very rarely, in differentiated forms [40]. Experimental Parkinsonian models were used to examine the recovery effects of transplanted cells in the 6-hydroxydopamine-induced brains. Weiss et al. [41] and Fu et al. [40] demonstrated that a significant decrease was noted in amphetamine-induced rotation of Parkinsonian rats by transplantation of undifferentiated UC stroma cells. Both groups also managed to induce human UC stromal cells into TH (+)

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dopaminergic neurons *in vitro*. Prior to transplantation, Fu et al. [40] induced them by a combination of sonic hedgehog protein and FGF 8 and developed TH (+), glutamate decarboxylase (+) and dopamine β-hydroxylase (+) neurons. *In vitro* differentiated dopaminergic cells were then transplanted into rat striatum, which had previously been damaged by 6-hydroxydopamine.Weiss et al. [41] used a similar method to transplant undifferentiated human UC stromal cells to rat striatum. Detailed work for characterization of human UC stroma cells before transplantation revealed the expression of genes related to morphogenesis, extracellular adhesion molecules, neurotrophic factors and three germ layer derivatives. An interesting finding was that although cells were recovered two days following transplantation, none were found at 6 or 12 weeks after transplantation [41]. In contrast, in Fu et al.'s study [40] transplanted TH (+) cells were detected 20 weeks after transplantation and were found to have migrated from the engraftment site. The efficacy of the stem cell treatment was determined by a decrease in the rotation behavior of transplanted animals; therefore both groups reported a significant improvement in the behavioral recovery in a relatively long-term post-transplantation period. Although rat UC stroma was used as a stem cell source, it would be reasonable to mention the study by Jomura et al. [39] who demonstrated a decrease in neuronal loss in global cerebral ischemia when pre-treated with UC stromal cells. Therefore, besides the catecholamine-synthesizing neuronal recovery attempts, studies concerning the rescue of ischemic areas in animal models seem also promising. Lund et al. [44], on the other hand, used human UC cells and human placenta-derived stromal cells to treat retinal nerve denervation in rats. Comparative results revealed a higher rescue of photoreceptor neurons obtained by UC stroma cells relative to placenta-derived cells.

In a recent study by Wu et al. [65], human UC stromal cells were differentiated into endothelial cells both *in vitro* and *in vivo*. They were induced to form endothelial cells by basic FGF and VEGF and transplanted into the hind limb ischemia model of nude mice where they differentiated into endothelial-lineage cells *in vivo* and subsequently became involved in the vessel network.

Cells with limited regenerative capacity, such as skeletal myocytes, are also of interest since musculoskeletal injuries are very common. Conconi et al. [47] showed the *in vitro* differentiation of CD105 (+) human UC cells into skeletal myocytes, which were used to treat the bupivacaine-injured *tibialis anterior* muscle of rats.

A striking common aspect emerging from *in vivo* studies is the absence of any graft rejection, which strongly gives credence to the use of those cells safely for treatment purposes even in xenotransplantation trials.

Conclusion and Future Perspectives

Over the past five years we have witnessed a growing number of evidences clearly indicating that the microenvironment of mammalian UC and the intrinsic properties of residing cells have to be considered as a valuable source of stem cells to be used in the future for both autologous and allogeneic transplantations. They can be harvested after birth with low cost, cryogenically stored, thawed and efficiently expanded for therapeutic purposes. In many lineages tested so far, they seem to give promising results in regenerative therapeutic applications, especially in orthopedic and cartilaginous interventions. It was recently demonstrated that they display a tremendous cell growth rate on bioabsorbable polymer constructs [66]. They were seeded onto pulmonary blood vessel conduits fabricated from rapidly bioabsorbable polymers and were successfully grown *in vitro* in a pulse duplicator bioreactor [67]. Recently, living patches engineered from UC-derived stromal cells and cord-derived endothelial precursor cells have been tested for potential use in human pediatric cardiovascular tissue engineering [68].

It was also shown by several experiments that UC-derived stromal cells are more efficient in terms of stem cell potency as compared to bone-marrow derived MSCs. Additionally, the shorter doubling time of cultured UC-derived

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stem cells compared to bone marrow MSCs [69] would give an easier and rapid propagation of these cells. Also, they do not require feeder layers or high serum concentrations to be expanded. Together with the distinct advantages of the UC, such as accessibility with little or no ethical concerns and painless procedures to donors with lower risk of viral contamination, it is generally accepted that the UC should be considered as an alternative to bone marrow. The ability to introduce exogenous DNA into the perivascular cells of UC stroma [49] makes them potent stem cells for gene therapies similar to bone marrow derived MSCs [70]. Most recently, interferon-β expressing UC stromal cells were targeted to experimentally developed lung tumors by intravenous and subcutaneous injections and were found to significantly reduce the tumor burden [71] which indicated the usability of UC stromal cells in cancer gene therapy.

There has been a great interest in the search for novel and potent stem cell sources in the human body. As a result, a huge number of publications and meeting abstracts emerged presented by scientists, physicians and all professionals of the relevant sectors in recent years. However, it is becoming clear that all of us need to be more cautious than ever in elucidating and interpolating our results and hopes for the future, especially for therapeutic solutions. Looking at ongoing clinical trials, it is too soon to tell whether all therapies based on stem cells will prove to be clinically effective. Nevertheless, following sufficient number of animal experiments and clinical studies using UC-derived stromal cells should be technically and ethically approved to test their ultimate potency in such diverse disease models. All we need to do as researchers in the field of UC-derived stem cells is to organize and reach consensus on the characterization, freezing/thawing, and expansion of clinical grade cells for therapies and tissue engineering as also suggested by Weiss and Troyer [72].

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Figure 1. Laminin distribution (*green signal*) in human UC stromal cells (*nuclei in red*). (A) Laminin is predominantly found in the basal lamina underlying the amnion epithelium. Few punctate staining corresponds to loosely arranged subamniotic stromal cells. (B) A stronger laminin expression is observed around the cells of the intervascular stroma in which many cross-sectional cellular processes were also viewed. (C) Laminin is predominantly found in the perivascular stroma ensheathing the entire cells. (D) Smooth muscle cells of vascular wall are intensively positive for laminin, scale bar= 50 µm. Human cord samples were obtained from term deliveries (n=26), frozen and cut 8 µm in thickness, labeled with anti-laminin mouse monoclonal antibody (Sigma-Aldrich, St. Louis, http://www.sigma-aldrich.com) (1:100, 90 min at 37° C) and FITC-conjugated IgG. Nuclei were labeled with anti-human nuclei antibody (Chemicon-Millipore, Billerica, MA, http://www.millipore.com/) (1:200, 60 min at 37° C) and Cy3-conjugated IgG.

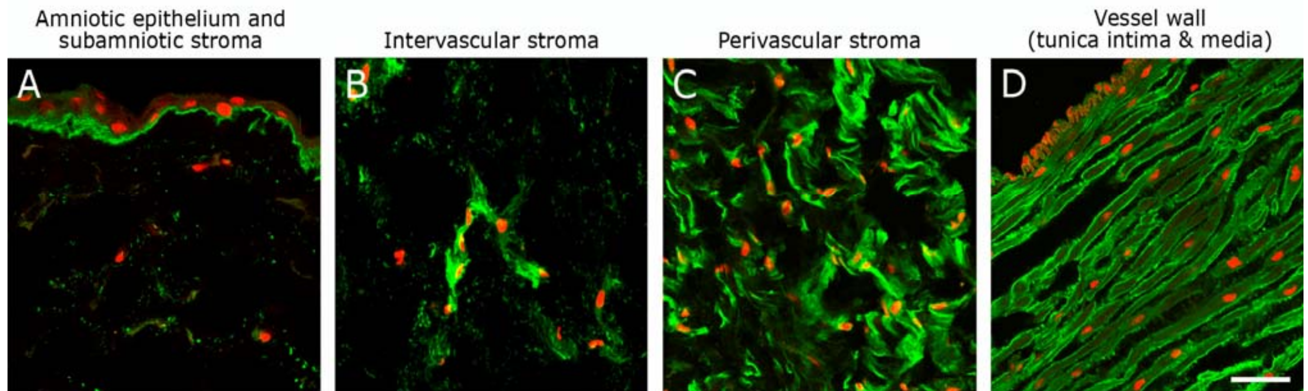

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Figure 2. Isolation of UC stromal cells is achieved by a severe digestion of intercellular collagen fibers and glycosaminoglycan molecules after removal of blood vessels by a blunt dissection. The entire procedure takes approximately 2-4 hours.

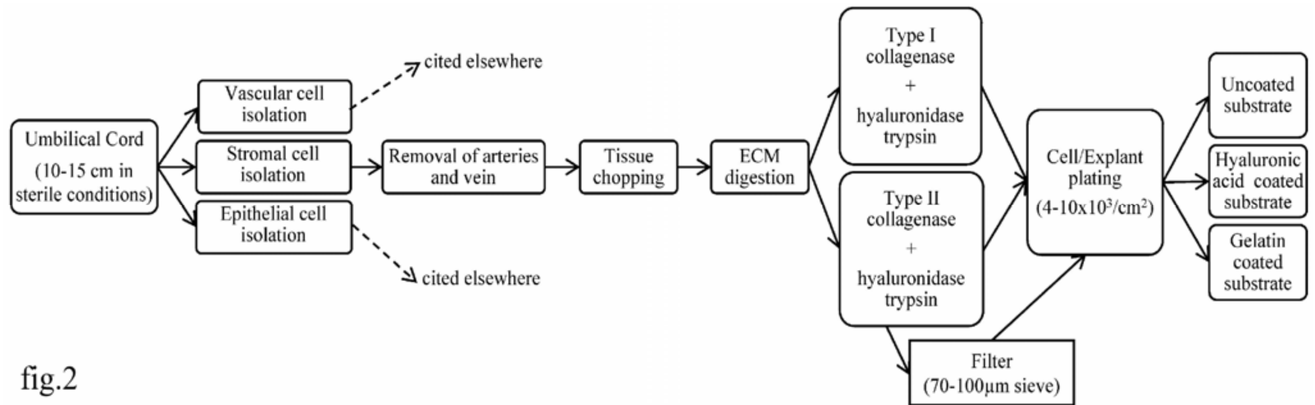

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Table I. Cumulated studies related to microscopic anatomy, functional structure and stem cell potential of human umbilical cord (UC) stromal cells and extracellular matrix published in the last five decades showing that descriptions of human UC and its components first appeared in the 50's and 60's. More detailed studies were published particularly in non-English journals in those years (not cited below), which pioneered later works published during the 70s' and 80's. In the 90's, with the aid of immunocytochemistry and protein biochemistry, studies began to highlight the functional structure and the nature of UC stromal cells and the surrounding microenvironment. However, until 2003, no study has been published depicting the stem cell potential of stromal cells. Interestingly, a burst of publications was noticed starting from 2003 till now (July 2007) after the stem cell potency of stromal cells was acknowledged.

Year	Subject-Findings	Authors
1952	Proportion of Wharton's jelly in distended vessels	[73]
1954	Mast cells in UC	[74]
1956-58	Mast cells and polysaccharides in UC	[75]; [76, 77]
1957-58	Polysaccharides in extracellular matrix of UC	[26, 78]
1960	Extracellular matrix fibers of UC	[27]
1970	Ultrastructure of stromal cells and extracellular matrix components	[19]
1988	Intermediate filaments in UC stromal cells	[24]
1991	Isolation, culture and characterization of UC stromal cells	[79]
1991	Intermediate filaments in UC stromal cells	[80]
1993	Immunohistochemical identification and ultrastructure of UC stromal cells	[3]
1993	Type VII collagen gene expression in UC stroma	[29]
1994	Evaluation of UC stromal cells with special emphasis to myofibroblasts	[20]
1995	EGF and TGFα expression in UC	[81]
1996	Collagen cytoskeleton of UC extracellular matrix	[31]
1997	Intermediate filament expression and collagen distribution during UC development	[4]
1997	Collagen and glycosaminoglycans of UC extracellular matrix	[30]
1998	Myofibroblastic character of UC stromal cells	[5]
1998	Characterization of fibrillin, collagen VI and intact collagen V in UC	[32]
2000	IGF-1 and IGF-BPs in UC	[82]
2002	TGF-β3 expression during UC development and pre-eclampsia	[8]
2002	Leptin and its receptors in UC components	[17]
2002	The use of UC stromal cell for tissue-engineered artery conduits	[67]
2003	Lectins in normal and intra-uterine growth retarded cases	[83]
2003	*In vitro* differentiation of human and porcine UC stromal cells into neurons/glia	[45]
2004	ECM protein compositions in UC	[84]
2004	IGF-binding proteins in normal and pre-eclamptic UC serum and tissues	[2]
2004	*In vitro* osteogenic, chondrogenic, adipogenic and cardiomyogenic differentiation of UC stromal cells	[43]
2004	*In vitro* differentiation of human UC stromal cells into neuron/glia	[60]

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2005	*In vitro* osteogenic differentiation of UC perivascular cells	[18]
2005	Use of UC stromal cells for engineered pediatric cardiovascular patches	[68]
2005	Differentiation of UC stromal cells into nerve-like cells	[61]
2005	Peptide growth factors in Wharton's jelly	[85]
2006	Hematopoietic supportive function of *in vitro* cultured UC stromal cells	[42]
2006	Effect of transplanted human UC stromal cells in a rodent Parkinson model	[41]
2006	*In vitro* and *in vivo* myogenic potential of UC stromal cells	[47]
2006	Cytofluorometric analysis of umbilical fibroblast-like cells	[86]
2006	Treatment potential of human UC stromal cells in Parkinsonism in rats	[40]
2007	*In situ* characterization of UC stromal cells compared with *in vitro* differentiation potentials	[16]
2007	Photoreceptor cell formation of transplanted UC stromal cells	[44]
2007	*In vitro* and *in vivo* differentiation of UC stromal cells to endothelial cells	[65]
2007	Comparing the differentiation of UC perivascular cells with bone marrow stromal cells	[49]
2007	3-D condylar cartilage formation using UC stromal cells on PGA scaffolds	[36]
2007	UC stromal cell-based gene therapy for experimental lung tumors	[71]

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Table II. Cell surface markers of stromal cells in the human[a] UC detected by either protein, mRNA or gene expression assays

Marker	Availability	Reference(s)
CD10	+	[41, 44]
CD13	+	[41, 42, 44, 65, 86]
CD14	-	[16, 41, 42, 44]
CD29 (integrin β1)	+	[37, 40-43, 65]
CD31 (PECAM)	-	[37, 41, 42, 44, 49, 65]
CD33	-	[41]
CD34	-	[16, 18, 37, 40-44, 47, 49, 65, 86]
CD38	-	[42, 47]
CD44 (HCAM)	+	[16, 18, 37, 40-44, 65, 86]
CD45	-	[16, 18, 37, 40-44, 47, 49, 65, 86]
CD49b (integrin α2)	+	[41, 86]
CD49c (integrin α4)	+	[41]
CD49d (integrin α3)	+	[41]
CD49e	+	[41, 49]
CD51 (integrin α5)	+	[40, 41, 43]
CD54 (ICAM-1)	-/+ [b]	[37, 47, 86]
CD56	-	[41]
CD73 (SH3)	+	[16, 18, 37, 40, 42-44]
CD90 (Thy-1)	+	[18, 41, 42, 44, 47, 49, 65, 86]
CD105 (endoglin,SH2)	+	[16, 18, 37, 40-43, 47, 86]
CD106 (VCAM-1)	-/+ [b]	[18, 42, 65, 86]
CD117 (c-kit)	-/+ [b]	[18, 44, 45, 49, 65, 86]
CD123 (IL-3 receptor)	-	[18]
CD133	-	[41]
CD146	+	[49]
CD166 (ALCAM)	+	[37, 42]
CD235a (glycophorin A)	-	[18]
HLA-1	+	[41, 47]
HLA-DR (MHC class II)	-	[18, 41, 42, 44, 65, 66, 86]
HLA-DP (MHC class II)	-	[18, 44]
HLA-DQ (MHC class II)	-	[26, 44]
HLA-A,B,C (MHC class I)	+	[18, 42, 44, 65, 86]
HLA-G (MHC class I)	-	[18]
STRO-1	-	[18, 49]
Oct-4	-/+	[18, 41]
Nanog	+	[41, 46]
SSEA-4	-	[18]
Sox-2	+	[41, 46]

[a]reference number 55 in porcine
[b]contradictory results between references
reference numbers 29 and 61 tested specifically in perivascular cells

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Table III. *In vitro* and *in vivo* [a] differentiation competence of the human UC stromal cells

Differentiation type	**Cell type**	**Reference(s)**
In vitro	Adipocyte	[16, 42, 47, 49]
	Chondrocyte	[16, 36, 43, 49]
	Osteocyte	[16, 18, 42, 43, 47, 49]
	Cardiomyocyte	[37, 43]
	Skeletal myocyte	[47]
	Neuronal/Glial precursor	[16, 42, 60, 61]
	Dopaminergic neuron	[40, 41]
	Endothelial cell	[65]
In vivo	Dopaminergic neuron	[40, 41]
	Photoreceptory neuron	[44]
	Endothelial cell	[65]
	Skeletal myocyte	[47]

[a]differentiation experiments were carried out in rodents

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