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Review

Umbilical cord revisited: from Wharton's jelly myofibroblasts to mesenchymal stem cells

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Summary. The umbilical cord (UC) is an essential part of the placenta, contributing to foetal development by ensuring the blood flow between mother and foetus. The UC is formed within the first weeks of gestation by the enclosure of the vessels (one vein and two arteries) into a bulk of mucous connective tissue, named Wharton's jelly (WJ) and lined by the umbilical epithelium. Since their first identification, cells populating WJ were described as unusual fibroblasts (or myofibroblasts). Recent literature data further highlighted the functional interconnection between UC and the resident cells. The UC represents a reservoir of progenitor populations which are collectively grouped into MSCs (mesenchymal stem cells). Such cells have been sourced from each component of the cord, namely the sub-amnion layer, the WJ, the perivascular region, and the vessels. These cells mainly show adherence to the phenotype of adult MSCs (as bone marrow-derived ones) and can differentiate towards mature cell types belonging to all the three germ layers. In addition, cells from human UC are derived from an immunoprivileged organ, namely the placenta: in fact, its development and function depend on the elusion of the maternal immune response towards the semi-allogeneic embryo. This is reflected in the expression of immunomodulatory molecules by UC-derived MSCs. The present paper describes UC structural features and the cell types which can be derived, with a focus on their phenotype and the novel results which boosted the use of UC-derived cells for regenerative medicine applications.

Key words: Umbilical cord, Wharton's jelly, Mesenchymal stem cells, Extracellular matrix, Immunomodulatory markers, Stromal myofibroblasts

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Umbilical cord: development and morpho-functional features

The umbilical cord (UC) is an extraembryonic formation that originates at day 13 of embryonic development (Karahuseyinoglu et al., 2007 and refs therein) and that connects foetus and mother during pregnancy through the placenta. The UC is formed essentially by the closing in of the somatic stalk. The lateral tissue plates arise as a proliferation of the embryonic connective tissue between the ectoderm of the amnion and its mesothelial or endothelial lining. They connect the allantoic stalk to the septum transversum and represent the formation of the UC. By a proliferation of the mesoderm the tissue plates continue to grow in length and thickness. In the stages immediately succeeding the formation of the UC, there is an absolute and relative increase in the cranio-caudal length of its embryonic attachment. The obliteration of the umbilical cord coelom is determined by a proliferation of the fibrous tissue which forms a ring at the embryonic attachment of the cord. Some faults of the junctional mesoderm are responsible for congenital herniation which may result from incorrect development of the cord (Wyburn, 1939). After birth, closure of the UC is an important and yet poorly understood process

that safeguards against blood loss of the newborn. In this process, oxygen tension has been suggested to be one of the initiators of the umbilical closure through artery contraction, even if vasoconstrictor molecules, such as 5-hydroxytryptamine (serotonin) and thromboxan A_2, are the main players of the postpartum UC closure (Quan et al., 2003).

A study on the length of human UC showed that for normal UC at term, there is a wide range of physiological lengths, comprised between 61 and 129 cm. There is little relationship between UC length and parameters such as foetal or placenta weight (Malpas, 1964). The number of twists of the UC are not clearly related to the length, suggesting that the helical structure of the cord is established at a very early stage of the foetus development and that the cord gains in length not by an increase of the number of twists but by a progressive increase in pitch of the primary helix (Malpas and Symonds, 1966); the absence of direct concordance in monozygotic twins suggests a possible control by both genetic and environmental factors (Chaurasia and Agarwal, 1979). The direction of the helixes is dependent on spiral direction of the two arteries around one vein; about 81% of the cords present an anti-clockwise spiral, 10% a clockwise spiral, and 9% show a changed direction of the spiral one or more times (Malcom and Pound, 1971). UCs with a single artery, uncoiled cords and short umbilical cords have been described in cases with chromosomal defects and other genetic syndromes (Ghezzi et al., 2002 and refs therein). Other morphological alterations of UC structure and composition have been found at delivery in a variety of pathologic conditions, such as hypertensive disorders, gestational diabetes, foetal distress and growth restriction (Ghezzi et al., 2002 and refs therein; Tantbirojn et al., 2009), differences in blood flow (Skulstad et al., 2006) and congenital intestinal atresia (Ichinose et al., 2010). The water content, especially in the stroma, seems related to the occurrence of pathological conditions: the presence of oedema can be a warning signal of pending respiratory distress or transient respiratory distress (Scott and Wilkinson, 1978 and refs therein). Moreover, a relationship has been described between foetus growth retardation and the fatty acid content of both umbilical artery and vein, depending on enzymatic placental activity and the blood flowing through vessels (Felton et al., 1994). In addition, pre-eclampsia also affects the structural features of the umbilical cord, with variations in diameter and wall thickness. Kim et al recently demonstrated that pre-eclamptic cords featured reduced amounts of Wharton's jelly, which was also holed in the boundaries (Kim et al., 2012). Given these premises, prenatal morphometry analyses of UC substructures resulted in fundamental assessment of UC global features and performance (Di Naro et al., 2001).

The major vessels of UC are the only conduction organs in it: the surrounding mesenchyme does not present lower calibre vascular structures or neural elements (Hoyes, 1969). Endothelial cells from umbilical cord vessels display standard expression of key markers such as CD31 and vWF (von Willebrand Factor) (Anzalone et al, 2009; Eleuteri et al., 2009; La Rocca et al., 2009a). The control of vascular tone is thought to be mediated by humoral and local molecules, such as eicosanoids, endothelins and endothelium-derived relaxing factor, which act on arterial smooth muscle (Myatt, 1992), and by systems that control intracellular chloride accumulation (Davis et al., 2000). Biomolecular analyses showed the presence of vasoactive peptides in the stromal compartment of full-term UC, as demonstrated for orphanin, oxytocin, atrial natriuretic peptide (ANP), endothelial nitric oxide synthase (eNOS). Similar results were also obtained for the epithelial (Oxytocin, ANP, eNOS) and endothelial (iNOS, inducible nitric oxide synthase) compartments (Mauro et al., 2011). Other molecules involved in vasoconstriction, such as EGF (epidermal growth factor), TGF-alpha (transforming growth factor-alpha) and their receptors, were observed in different zones of the UC (Rao et al., 1995). The well-developed elastic laminae assist the contraction of walls of the umbilical vessels within 15-60 seconds after birth. This contraction is characterized by a change in vessel wall thickening, and an internal elastic lamina with fibres arranged in a roughly circular direction (Martin and Tudor, 1980 and refs therein). Some authors described the presence of large pore spaces which closely surround the cord vessels, hypothesizing their function as a compensatory extra-vascular space which acts facilitating the movement of vessels during pulsatile blood flow (Ferguson and Dodson, 2009).

Microscopic anatomy of human umbilical cord

The umbilical cord is layered by cubic epithelial cells forming the umbilical epithelium (Fig. 1A), an ectoderm-derived structure that continues with amniotic epithelial cells and the tegumentary epithelium of the fetus (Copland et al., 2002; Mizoguchi, 2004). As well described by Hoyes (1969), in human umbilical cord (HUC) the epithelium develops into a structure which resembles the early fetal epidermis. The morphology of its superficial layer is closely related to that of the periderm, a layer of cells for which ultrastructural investigations have suggested the involvement in the production of various constituents of the amniotic fluid (Hoyes, 1968a). At the first week of gestation, the cells show microvilli and cilia and, between the 8th and 10th week, they constitute the single layer of the cord epithelium. The epithelium becomes bilaminar at the end of the 3rd month (Hoyes, 1969). The functional activity of the periderm declines after the onset of differentiation in the intermediate layers of the epidermis. Following the appearance of keratinization and the formation of the umbilical stratum corneum, the periderm disappears from the surface (Hoyes, 1968b). Between 6th and 7th months the epithelium is composed by three or more cell

layers bordered by the condensation of collagen filaments immediately beneath the epithelial basement membrane. UC epithelium keratinization rarely occurs, except in the region close to the foetus. At term, this area is opaque and the remaining part consists of a simple squamous epithelium, until the sudden transition to the cubical amniotic epithelium at the junction of the cord with the placenta (Hoyes, 1969).

HUC epithelium covers the sub-amnion and a special embryonic connective tissue, the so-called Wharton's jelly (WJ) which surrounds the adventitia and media of the fetal vessels and is thought to prevent their compression, torsion and bending (Fig. 1B,C) (Ghosh et al., 1984). It is composed of cells which are dispersed in an amorphous ground substance composed of water (about 90%), sulphated glycosaminoglycans (GAGs), such as hyaluronic acid, and proteoglycans, such as decorin and biglycan (Yamada et al., 1983; Gogiel et al., 2003). The extracellular matrix of the three zones of the stroma (the subamniotic stroma, the Wharton's jelly, and the vessels' adventitia) showed immunoreactivity for collagen types I, III and VI and for basement membrane molecules such as collagen type IV, laminin and heparan sulphate proteoglycan (Nanaev et al., 1997; Can and Karahuseyinoglu, 2007). Hyaluronic acid represents the most abundant (almost 70%) of total GAGs, whereas little amounts of other sulphated GAGs, such as keratan sulphate, heparan sulphate, chondroitin-4-sulphate, chondroitin-6-sulphate and dermatan sulphate, were observed (Bańkowski et al., 1996). Collagen filaments have a wide distribution, with various directions in the mesenchyme and an increased amount beneath the epithelium and in the deeper part of the cord, especially near the large umbilical vessels, where they are oriented circularly to the vessels (Hoyes, 1969; Bankowski et al., 1996). In WJ, collagen fibrils create a three-dimensional network that runs from the amniotic membrane to the umbilical vessels: the fibril network is softer in the inner part, characterized by canalicular structures, while it has a dense, sponge-like structure in the outer part (Vizza et al., 1995, 1996). Collagen fibrils in the ECM form striated small diameter structures, ranging between 30 and 60 nm and organized in spiral bundles (Franc et al., 1998). Type I and type III collagens were found to be the most abundant with an unexpected resistance to solubilization (Bańkowski et al., 1996; Sobolewski et al., 1997). Type VII collagen is expressed in the epithelium and in the endothelial cells, but it was found as predominately expressed by fibroblast-like WJ cells (Ryynänen et al., 1993). The fibrillar network system seems to be maintained by coupling with glycoprotein microfibrils (Meyer et al. 1983; Franc et al., 1998). Special distribution of the various collagen types has been suggested to be responsible for the mechanical properties of the UC (Takechi et al., 1993). Extracellular matrix (ECM) components can act as a storage of growth factors that substain stromal cells (Sobolewski et al., 2005): an increasing number of growth factors such as IGFs (insulin-like growth factors), FGFs (fibroblast growth factors) and TGF-ß (transfroming growth factor-beta), have been found to be associated with ECM proteins or with heparan sulphate. These growth factors, in turn, control cell proliferation, differentiation, synthesis and remodelling of the ECM. IGF-1 is known as a stimulator for the biosynthesis of the main components of ECM, such as collagen and sulphated glycosaminoglycans (Palka et al., 2000; Bańkowski et al., 2000). IGF-1 also has a role in cartilage biosynthesis and repair in animal models (Martin et al., 1997; Loeser et al., 2000; Messai et al., 2000). Early reports on microscopic features of ECM revealed presence of elastic fibres. As reported by Parry (1970), full-term HUC showed the staining properties of mature elastic fibres. The WJ showed very fine and scanty fibres, while they were abundant in vessels walls. At the fine structure, the fibres were composed entirely of tubular 10-nm diameter filaments (Parry, 1970). ECM homeostasis is regulated by balanced secretion and degradation of collagens, proteoglycans, elastin and structural glycoproteins, suggesting that any imbalance

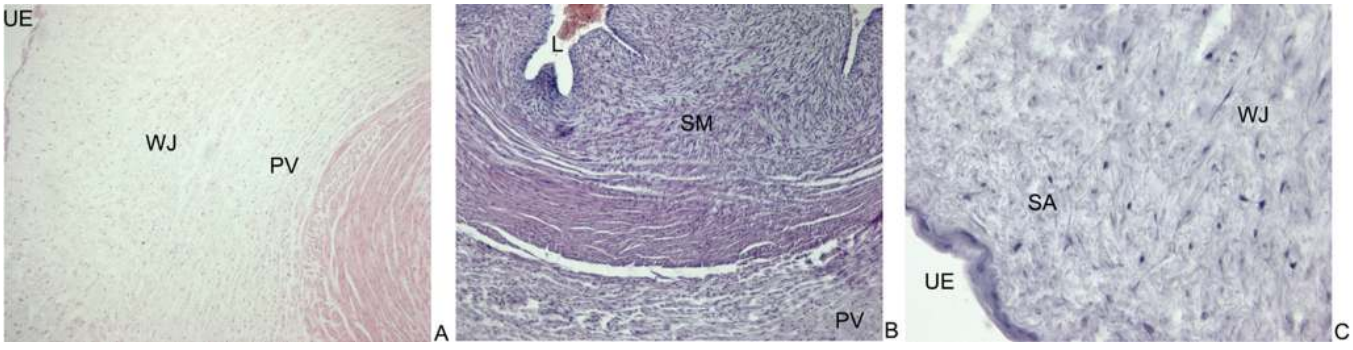

Fig. 1. Micrographs depicting umbilical cord tissue and zones. **A.** Wharton's jelly (WJ) represents the main bulk of tissue between the umbilical epithelium (UE) and the perivascular zone (PV). **B.** Higher magnification panel depicting part of a transverse section of umbilical vein enclosing its lumen (L), and with the smooth muscle layers of tunica media (SM). **C.** Higher magnification panel depicting the umbilical epithelium with the sub-amnion (SA) which is continuous with Wharton's jelly. A, x 10; B, x 20; C, x 40

of these molecules can affect the normal function of the tissue. ECM remodelling is thus a crucial point in the onset of diseases, where the proteolytic activities of enzymes, such as matrix metalloproteinases (MMPs), play a pivotal role (La Rocca et al., 2004, 2007; Galewska et al., 2008; Mauro et al., 2010; Romanowicz and Galewska, 2011).

Umbilical cord stromal stem cells: from myofibroblast to mesenchymal stem cells

The abundant ECM of umbilical cord stroma contain dispersed stromal cells, now referred to as mesechymal stem cells (MSC). Studies by Takechi and colleagues suggested that the majority of stromal cells were myofibroblasts (Takechi et al., 1993). The term 'myofibroblast' was first described by Majno and colleagues (1971), since fibroblasts from different tissues presented features typical of smooth muscle cells: they showed contractile systems, as well as bundles of fibrils, desmosomes, and cell-to-cell and cell-to- stroma attachments (Majno et al., 1971; Gabbiani et al., 1972). These observations confirmed data previously described after electron microscopy studies (Parry, 1970). Even if the stroma can be divided into three different zones (subamnion, Wharton's jelly, and perivascular zone) and there are some differences between cells dispersed in these zones (as discussed below), the term 'Wharton's jelly cells'(WJCs) is often extended to all umbilical stromal cells. Immunogold techniques showed that stromal WJCs are characterized by cytoplasmic α-smooth muscle actin microfilaments after second trimester, suggesting a maturation of these cells towards myofibroblasts (Kobayashi et al., 1998). WJCs are positive to vimentin, desmin and α-smooth muscle actin, therefore showing similarity to smooth muscle cells of umbilical vessels. Only the stromal cells were positive for prolyl 4-hydroxylase, and electron microscopy revealed the presence of rough endoplasmic reticulum, bundles of smooth-muscle type filaments with focal densities, a large Golgi apparatus and granules containing collagen, lipids and glycogen (Eyden et al., 1994). The presence of a wide rough endoplasmic reticulum and of a well-developed Golgi apparatus in most of the cells indicates a capacity for protein synthesis and secretion. Thus, they may be thought as the source of the cord collagen (as also suggested by prolyl-4-hydroxylase positivity) and, although showing some superficial resemblance to smooth muscle cells, WJCs were described as a population of unusual fibroblasts (Parry, 1970).

As well summarized and analyzed by different groups, MSCs derived from HUC and other foetal/neonatal tissues share common features with MSCs derived from adult tissues (bone marrow, adipose tissue, peripheral blood) as well as self-renewal capability and differentiative potential towards different types of tissue cells, such as adipocytes (Fig. 2), osteoblasts and chondroblasts (Huang et al., 2012). The main differences from BM-MSCs reside in the number of cells obtainable from tissue, the feature of properties of true stem cells (which WJCs retain even after extended *in vitro* culture passages), and the surface markers involved in immune tolerance (Troyer and Weiss, 2008; La Rocca et al., 2009b; Anzalone et al., 2010, 2011a; Nekanti et al., 2010; Hass et al., 2011; Jeschke et al., 2011; Lo Iacono et al., 2011a; Prasanna and Jahnavi, 2011). Studies carried out by Miki and colleagues demonstrated that amniotic epithelial cells (AECs) from placenta possess a differentiative potential towards mature cell types derived from the three germ layers: endoderm (liver and pancreas), mesoderm (cardiomyocyte), and ectoderm (neural cells) *in vitro*. AECs did not express telomerase, and were non-tumorigenic when transplanted into immunodeficient SCID/beige or Rag2-/- mice (Miki et al., 2005). Similarly, primary cells with an epithelioid morphology, known as cord-lining epithelial cells (CLECs), were derived from cord lining membrane. These cells expressed classical pluripotency markers, as well as Oct-4 and Nanog (Kita et al., 2010) and they showed chromosomal stability after vectors integration, a parameter which may be of importance for their use in clinical applications in gene therapy (Sivalingam et al., 2010).

We and others described in WJCs the presence of markers responsible for the maintenance of an undifferentiated state and self-renewal, such as Nanog and Oct-4, and for the immune tolerance, such as the non-canonical class I MHC HLA-G. These reports suggest that one of the effects of WJCs administration may be the instauration of tolerogenic responses in the host, avoiding transplant rejection (Weiss et al., 2008; La Rocca et al., 2009b). In addition, apart from classical MSCs markers, *in vitro* expanded WJCs do express mesodermal markers such as vimentin and α-smooth muscle actin; endodermal markers as Gata-4, Gata-5, Gata-6, HNF4-α; and neuro-ectodermal markers as nestin, neuron specific enolase (NSE) and glial fibrillary acid protein (GFAP) (Romanov et al., 2003; La Rocca et al., 2009b). These findings support the hypothesis that these cells can differentiate towards different mature cell types derived from all three germ layers (La Rocca et al., 2009b). WJCs also expressed CD68at both the protein and RNA level. CD68 is a marker whose expression is not restricted to the macrophage lineage, as suggested by other recent reports (Gottfried et al., 2008; La Rocca et al., 2009c). Further recent reports from us and others allowed to better define the immune properties and immunomodulatory markers expressed by placenta-derived cells. Tee et al recently demonstrated that hepatocyte-differentiated hAECs also maintained the expression of key immunomodulatory molecules (Tee et al., 2013). In another report, also WJ-MSCs, subjected to the standard three-lineage differentiation experiments, have been demonstrated to maintain the expression of immunomodulatory molecules such as HLA-E and B7-H3 (CD276) (La Rocca et al., 2013). These molecules

have also been reported to be expressed in other adult MSCs populations (Anzalone et al., 2013). Recently, CD 271, an immunomodulatory molecule originally described in BM-MSCs, has also been shown to be expressed in fresh umbilical cord specimens (Margossian et al, 2012).

UC-MSCs are also thought to be a promising tool in cancer therapy, because of their preferential homing to the site of the tumor. This feature may be due to the cellular response induced by release of chemotactic factors from the primary lesion site. In fact, *in vivo* and *in vitro* studies supported this hypothesis, demonstrating that WJCs administration may result in reduced tumor growth (Ayuzawa et al., 2009; Tamura et al., 2011). Recent data from our group also suggested the expression of heat shock protein 10 (Hsp10) in WJ-MSCs (Lo Iacono et al., 2011b). As described before by us and others, Hsp10 (known also as EPF, early pregnancy factor) is centrally involved in the modulation of the immune response during pregnancy, apart from its roles in tumour immunology and developmental processes (Cappello et al., 2006, 2007; Corrao et al., 2010). Immunohistochemical (Takechi et al., 1993; Nanaev et al., 1997) and *in vitro* (Karahuseyinoglu et al., 2007; Sarugaser et al., 2005) studies have suggested differences in the number and features of UC-MSCs. In fact, as well described in the literature, different types of stromal cells are dispersed in different zones of the

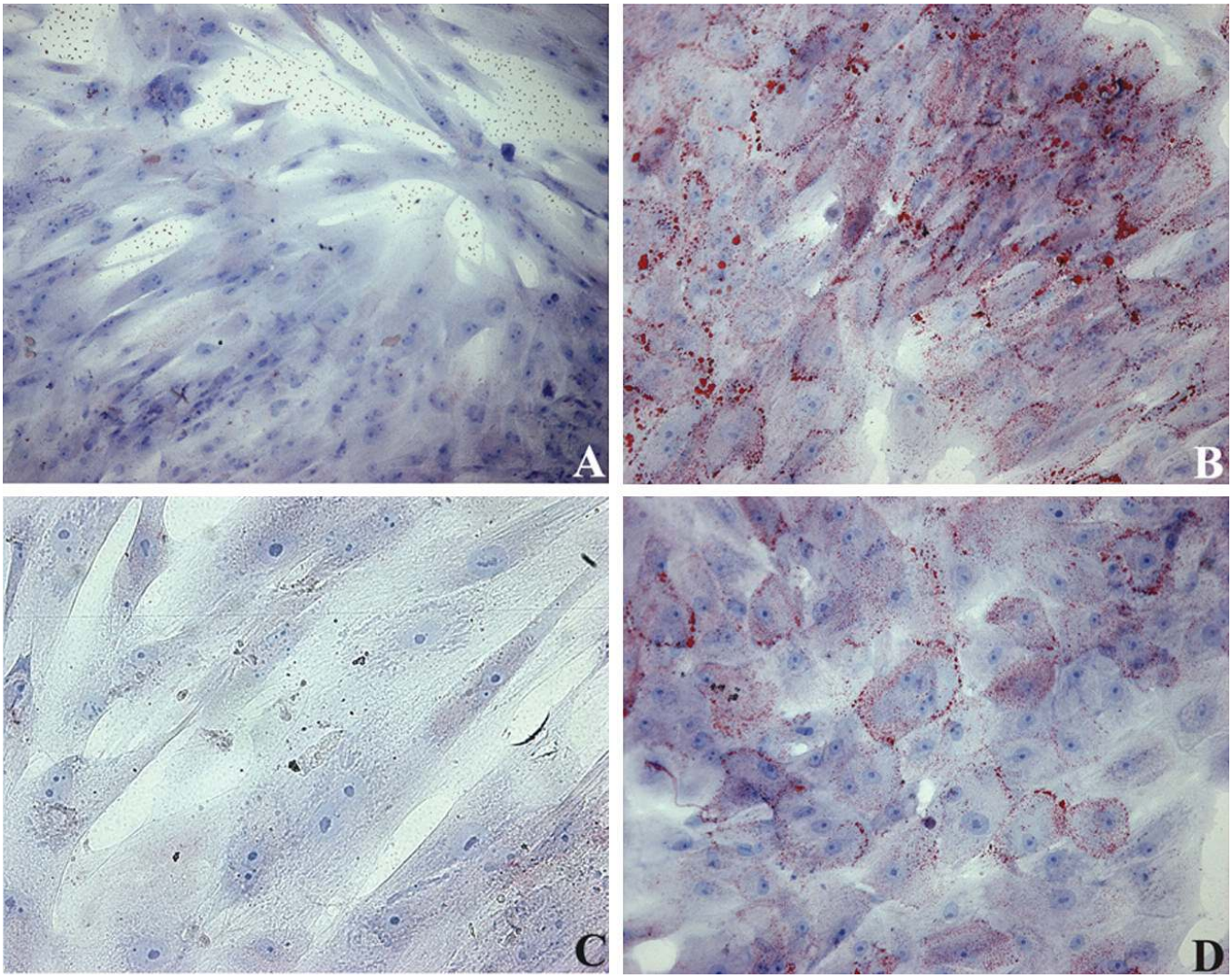

Fig. 2. Light microscopic demonstration of adipocyte differentiation of WJ-MSC with Oil Red O staining. WJ-MSC cultured for 3 weeks in adipogenic medium, showed variations in cellular morphology **(B, D)** and accumulation of neutral lipid vacuoles (demonstrated by Oil Red O staining) with respect to control cells. The latter **(A, C)** were cultured for the same time in standard culture medium, and retained the normal fibroblast-like morphology, without any positivity for the lipid-specific staining procedure. A, B, x 20; C, D, x 40

umbilical matrix (the sub-amnion, Wharton's jelly and the perivascular stroma), sharing common features in terms of marker molecules (generally expressed by MSCs from other tissues), such as CD73, CD105, CD90, and CD44, α-smooth muscle actin, and vimentin (Conconi et al., 2011; Jeschke et al., 2011; De Kock et al, 2012), while desmin was not expressed in sub-amniotic cells (Jeschke et al., 2011). On the other hand, sub-amniotic cells did express CD14 (which has not yet been detected in other UC-MSCs) and STRO-1 molecules; CD133 and CD235a molecules are expressed in the whole UC, but not in cells derived from the different zones (Conconi et al., 2011 and refs therein). As demonstrated for WJCs, sub-amniotic MSCs also showed the expression of Oct-4 and Nanog (Kita et al., 2010). Cells obtained from perivascular stroma feature a non-hematopoietic myofibroblastic mesenchymal phenotype (CD45-, CD34-, CD105+, CD73+, CD90+, CD44+, CD106+, 3G5+, CD146+), they are non-alloreactive, possess immunosuppressive activity, and significantly reduce lymphocyte activation *in vitro* (Sarugaser et al., 2009). They lack expression of Oct-4 marker and in prolonged *in vitro* culture (after the first five passages), these cells have been demonstrated to lose expression of both type I and II MHC molecules (Sarugaser et al., 2005). Perivascular cells have also been shown to contribute to both musculo-skeletal and dermal wound healing *in vivo* (Sarugaser et al., 2009). In literature, the features of MSCs from the sub-endothelial layer (vessel wall) were also described: they expressed molecules such as CD29 (integrin ß-1), CD44 (H-CAM), CD49e (integrin α5), CD13, while being negative for classical endothelial markers, such as vWF and CD31 (Romanov et al., 2003; Conconi et al., 2011 and refs therein). During the first passages of *in vitro* culture, these cells resulted positive for α-smooth muscle actin, fibronectin, type I collagen, and VCAM, showing also a differentiation potential towards adipocytes and osteoblasts (Romanov et al., 2003).

The range of potential clinical indications for UC-derived MSCs in cellular therapy is constantly growing. On one hand, these cells are able to differentiate towards a number of mature cell types belonging to the three germ layers, as demonstrated for neural cells (Mitchell et al., 2003), cardiomyocytes (Hollweck et al., 2011; Corrao et al., 2013), endothelial cells (Alaminos et al., 2010) and hepatocytes (Campard et al., 2008; Anzalone et al., 2010). Recent data indicated WJCs as potential candidates for musculoskeletal tissue engineering (reviewed in Wang et al., 2011). HUC-MSCs differentiated towards muscle tissue as described by Kocaefe and colleagues. In their experiments, WJCs were used in gene transfection and/or co-culture with muscle cell lines: when genetically reprogrammed, these cells exhibited many cellular signs of myogenic conversion and became capable of forming multinucleated myofibers. Differentiated WJCs featured the expression of functional markers (ß-catenin, neural cell adhesion molecule and M-cadherin), as well as muscle cell-specific structural proteins (desmin, α-actinin, dystrophin, myosin heavy chain, and myoglobin) and muscle-specific enzymes (such as creatinine phosphokinase) (Kocaefe et al., 2010). Cartilage tissue engineering is another therapeutic option which is being actively explored for WJCs (Wang et al., 2009; Lo Iacono et al., 2011a). The possibility to apply WJCs to type I diabetes treatment has recently emerged. These cells may play a role either by direct differentiation towards ß cells, or by favouring organ repair processes, due to their anti-inflammatory and immunomodulatory roles (Anzalone et al., 2011b). The proposed ability of UC-derived MSCs, and in particular WJ-MSCs, as immune modulators attracted great interest for their application in a number of diseases, apart from their differentiative capacity (La Rocca, 2011; La Rocca et al., 2012). As a further example, recent data indicated that WJ-MSCs may be effectively used in the management of GVHD (graft versus host disease) (McGuirk and Weiss, 2011).

Conclusions and future perspectives

More than 40 years of research on umbilical cord and its resident cells have provided a great amount of information on the biological features of these extra-embryonic populations. UC matrix and cell types cooperate to maintain structure and functional performance of the organ throughout pregnancy. *In situ* and *in vitro* analyses demonstrated that cells from WJ and the other zones of UC do express stem cells markers, together with markers of mature cell types derived from all of the three germ layers. *In vitro* experiments, also confirmed by *in vivo* readouts in animal models, clearly highlighted the ability of UC-derived stem cells to differentiate towards several cell types, acquiring their marker expression and functional activities. This attracted great interest in the use of these cells in cellular therapy of various human diseases, and also for their peculiar ease of sourcing, the high cell numbers obtainable, and the lack of any ethical concerns.

In addition, in parallel to the classic repopulation-type approach of regenerative medicine, recent data indicated that UC-MSCs administration may favour organ repair independently from their differentiative capacity, e.g. by re-activating proliferative and differentiative mechanisms of local precursors (La Rocca and Anzalone, 2013). This may be achieved thanks to the expression of a number of immuno-modulatory molecules in UC-MSCs, which derive from an organ which is clearly immunoprivileged during pregnancy, and may therefore keep this "positional memory" also when cultured *in vitro* and when administered *in vivo*.

Due to the great interest and hopes for the use of these cell types, in our opinion more research is needed, in that universally accepted isolation procedures, subculture and cryopreservation are still far from being

established. In addition, basic research aimed to the discovery of new markers expressed by these cells must be encouraged to clearly define their phenotype and potentials and increase safety in patients receiving these cells.

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