Anim. Reprod, v.7, n.4, p.367-372, Oct./Dec. 2010

Isolation and characterization of mesenchymal cells isolated from caprine umbilical cord matrix

M. Moshrefi[1], H. Babaei[1,4], S.N. Nematollahi-Mahani[2,3]

[1]Theriogenology Section, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.
[2]Department of Anatomy, Afzalipour School of Medicine, Kerman, Iran.
[3]Neuroscience Research Center of Kerman, Kerman, Iran.

Abstract

To date, no investigations have been found about the isolation and *in vitro* cell culture characterization of Wharton's jelly mesenchymal cells (WJMC) from caprine umbilical cord (CUC). Therefore, in the present experiment the isolation protocol and growth kinetic of CUC matrix cells were studied. CUC were collected from an abattoir and pregnant uteri (late-gestation) and their Wharton's jellies (WJ) were cut into 2×2 mm^2 fragments for explanting. Explants (n = 8-10) were transferred to each 35 mm culture dish. WJ explants were removed 5 days after plating and the remaining adherent cells were cultured for another 5 days. CUC isolated cells were immunostained for Actin protein and histochemically were assayed for the presence of alkaline phosphatase (AP) activity. Besides, in this study growth kinetic and clonogenicity were evaluated for the isolated cells. CUC isolated cells displayed spindle-form and small round-shape with large nucleus. Confluent cells formed colonies that presented AP activity. Immunocytochemical analysis revealed expression of Actin protein. Initial seeding concentration of 2×10^4 cells resulted in smaller time for doubling the population compared to fetal fibroblasts (46.6 vs. 54.3 h, respectively). In conclusion, WJ of CUC contains an easily isolated and rich source of myofibroblast-like cells which exhibit some stem cell-like properties.

Keywords: actin, alkaline phosphatase, caprine, mesenchymal cells, umbilical cord.

Introduction

Adult stem cells are undifferentiated cells found in various tissues and are mostly multipotent cells (Cai *et al*., 2004). Postnatal sources of stem cells can be achieved from any tissue type including brain (Uchida *et al.*, 2000), bone marrow (Minguell *et al*., 2001), adipose tissue (Zuk *et al.*, 2001), amniotic fluid (De Coppi *et al.*, 2007) and umbilical cord blood (Lee *et al*., 2004). The umbilical cord is an extraembryonic structure essential to provide feeding for the fetus during the intrauterine development. The umbilical cord is formed early during gestation and encloses the yolk sac, which is the embryonic source of two different populations of mesenchymal stem cells. This structure contains mesenchymal stem cells or unrestricted somatic stem cells isolated from fresh umbilical cord blood at the time of birth (Fuchs *et al.*, 2005) and fibroblastoid mesenchymal stem cells isolated from umbilical cord matrix (Mitchell *et al.*, 2003) that can be collected and stored after birth for therapeutic uses or biotechnology purposes.

WJ is the gelatinous connective tissue from umbilical cord and it is composed of myofibroblast-like stromal cells, collagen fibers and proteoglycans (Kobayashi *et al*., 1998). Previous studies have shown that mesenchymal cells derived from human (Mitchell *et al.*, 2003) or porcine umbilical cord matrix are capable of expressing a variety of stem cell characteristics (Carlin *et al*., 2006). The stem cells in WJ of the umbilical cord have properties that make them of interest. For example, they are simple to harvest through non-invasive methods, provide large numbers of cells without risk to the donor, the stem cell population may be expanded *in vitro*, cryogenically stored, thawed, expanded, genetically manipulated and differentiated *in vitro* (Mitchell *et al*., 2003; Fu *et al*., 2004; Wang *et al*., 2004; Carlin *et al*., 2006). To date, no investigations have been found about the isolation and *in vitro* cell culture characterization of WJMC in CUC. Therefore, in the present experiment the isolation protocol, growth kinetic and some stem cell-like properties of CUC-WJMC were studied.

Materials and Methods

Materials

All chemicals except those otherwise indicated were purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

Umbilical cord acquisition and Wharton's jelly matrix cells isolation

All experimental protocols were approved by the Research Ethic Committee of the Kerman

[4]Corresponding author: Babaei_H@mail.uk.ac.ir
Phone: +98(341)320-2918; Fax: +98(341)322-2047
Received: December 1, 2009
Accepted: January 11, 2011

Neuroscience Research Center. Uteri of pregnant goats (n = 4) were collected from an abattoir and were transported within 2 h to the Cell Culture Research Laboratory of Afzalipour School of Medicine, Kerman, Iran. Umbilical cords were obtained from the late-gestation fetuses and placed in sterile phosphate buffer saline (PBS, composition in mM: 140 NaCl; 2 KCl; 1.5 KH_2PO_4; 15 Na_2HPO_4) supplemented with 2 µg/ml amphotericin B (Bristol-Myers Squibb), 200 IU/ml penicillin and 200 µg/ml streptomycin. Umbilical cord segments, 5 cm in length, were cut longitudinally and then the umbilical cord artery and veins were cleared off. The remaining umbilical cord tissue, including WJ, was cut into 2 × 2 mm^2 segments by using small scissors. Segments were (n = 8-10) transferred to each 35 mm disposable Falcon culture dish (Becton Dickinson & Company Franklin Lakes) containing 1 ml of cell culture medium (α-MEM; Alpha modification of Minimum Essential Medium Eagle) supplemented with 20% fetal bovine serum (FBS, Gibco), 2 µg/ml amphotericin B, 200 IU/ml penicillin and 200 µg/ml streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2. Adherent WJ of CUC were observed 24 h after plating and their cell culture media were filled to 3 ml. WJ explants were removed from dish cultures 5 days after plating, the remaining adherent cells were culture for at least another 5 days and the medium was refreshed every 72 h. Then adhered CUC cells were dissociated with 0.1% trypsin + 1.0 mM EDTA in PBS for 2 min and then FBS was added to stop trypsinization. Cells that had been detached at this time were subcultured in a new 250 ml Falcon flask (Becton Dickinson & Company Franklin Lakes) and denoted passage 1. For long term storage, CUC cells were cryopreserved in a freezing medium consisting of 20% FBS and 10% dimethyl sulfoxide (DMSO) in α-MEM.

Fetal fibroblast cells isolation

As a comparison with CUC cells, fetal fibroblasts were isolated from a 4-5 month old goat fetus collected from a slaughterhouse. The surface of uterine horns was thoroughly disinfected with 75% ethanol and fetus was obtained through an incision. About 1 cm^2 of subdermis connective tissue was placed in sterile PBS supplemented with 2 µg/ml amphotericin B, 200 IU/ml penicillin and 200 µg/ml streptomycin. Small pieces of subdermis were explanted in culture dishes with 2 ml culture medium and allowed to proliferate. Explants were removed at day 5 and the proliferating fibroblasts were allowed to reach desired confluency.

Immunocytochemistry

Isolated CUC cells were immunostained for α-SMA (mouse monoclonal Clone 1A4; Sigma, A2547) to identify and verify the presence of actin filaments in cultured cells. Approximately one million cells were obtained from the whole amount of fourth passage of cultured CUC cells and were seeded over a glass slide then allowed to grow up to 48 h. Afterwards, growth medium was removed and the cells on the slides were washed with PBS and were fixed in 4% paraformaldehyde for 5 min at 4°C. After a subsequent rinse with PBS, slides were blocked with 10% normal goat serum for 30 min in a humidified chamber at room temperature and washed with PBS. Then the slides were incubated with primary antibody for 60 min, washed three times with PBS and incubated with the secondary antibody (goat anti-mouse IgG) for 60 min at room temperature. Afterwards, the cells were stained with 3,3'-diaminobenzidine and observed by phase contrast microscope (Olympus, IX71, Tokyo, Japan).

Alkaline phosphatase assay

CUC cells were grown on a 35 mm culture dish for several days until colony formation and the medium was refreshed every 72 h. AP activity was detected by using an AP Kit (Sigma-Aldrich Chemie GmbH, Germany, Catalog No. 86-1) according to the manufacture's instruction. A red reaction product following 15 min of exposure to alkaline dye mixture confirmed AP activity. As a positive control, a blood smear from patient with pyogenic leukocytosis was prepared and stained.

Growth kinetic

CUC cells collected by trypsinization were diluted to 1 x 10^4 cells/ml and seeded in 35 mm Falcon culture dishes (n = 30). Cells were incubated for a period of 10 days when culture was terminated. Culture medium was refreshed every 72 h. Every 24 h, three culture dishes were removed from the incubator, and following trypsinization, cell concentration (cells per ml of culture medium) was counted by using a hemocytometer (Neubauer chamber). Semilog curve of the increase in cell concentration was plotted and PDT was calculated using the following equation:

PDT = t log2/log(N_t/N_0), where N_0 = initial cell number and N_t = cell number at culture period (Long *et al.*, 2008). Fetal fibroblast cells were also cultured and counted parallel to CUC cells.

Clonogenicity evaluation

The ability to generate clones (colony formation) is a formal demonstration of the self-renewal ability, which is a characteristic of stem cell populations. In order to give a formal demonstration of the self-renewal capability of CUC cells, the presence of clones was assessed into the 35 mm explant culture dishes under a light microscope (Nikon, TS100).

Results

Cellular isolation, morphology and clonogenicity

Two panels of representative inverted (Olympus IX71) micrographs of CUC cells at the different days have been shown in Fig. 1. Primary CUC cells were observed at the second (Fig. 1A) or third (Fig. 1B) days of culture of WJ explants. These cells reached a good confluency after about 10 days so they were subcultured and considered as passage 1.

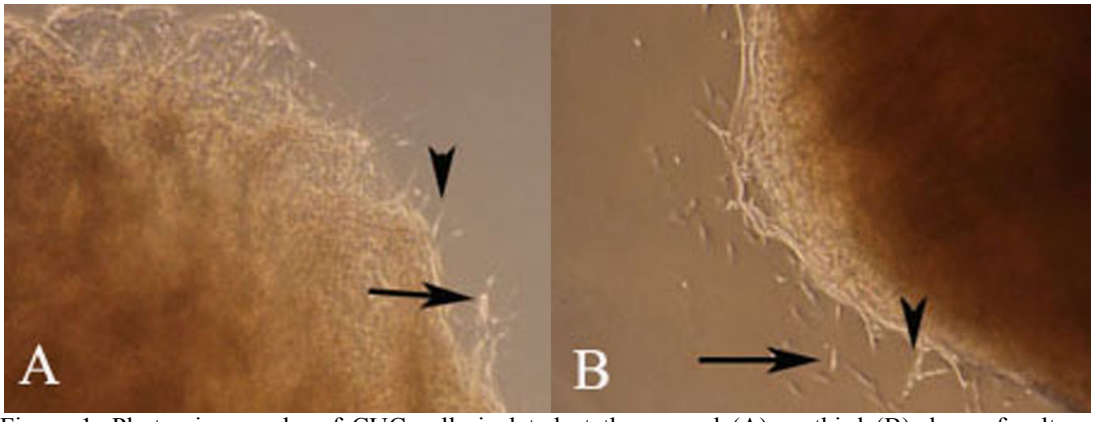

Figure 1. Photomicrographs of CUC cells isolated at the second (A) or third (B) days of culture. Magnification 100X.

Cells isolated from caprine umbilical cord matrix explants displayed a heterogeneous morphology including fusiform or spindle-form cells and small round cells with a large nucleus. Confluent cells were arranged in parallel arrays. As CUC cells reached considerable confluency (near 90%), colonies of cells began to form (Fig. 2). In the 35 mm culture dishes, the first colonies were observed at day 9 of explant cultures. The mean size of colony diameter was 169 ± 6.3 µm.

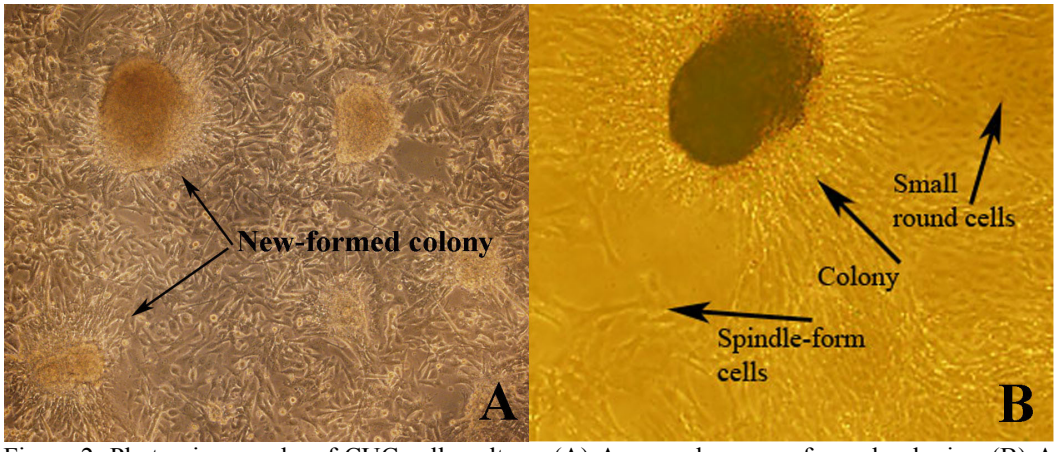

Figure 2. Photomicrographs of CUC cells culture. (A) Arrows show new-formed colonies. (B) A compact colony with two different cell types isolated from Wharton's jelly explants (spindle-form cells and small round cells) based on morphology. Magnification 100X.

Immunocytochemistry of isolated cells

Immunocytochemical analysis was performed to test the expression of Actin protein; a positive labeling (Fig. 3) strongly confirmed our isolated caprine mesenchymal cells.

Growth kinetic

Figure 4 shows semilog plot of the increase in CUC cell concentration. After 2 days of culture, the subsequent cell concentration in CUC cells was significantly higher than that of the fibroblast cells (16666 ± 833 vs. 10833 ± 833, $P < 0.008$). Initial seeding concentration of 2 x 10^4 cells resulted in mean doubling times of 46.6 and 54.3 h for CUC and fibroblast cells, respectively. However, nearly on the day 10 of culture, both CUC and fibroblast cells were entered to the plateau or stationary phase.

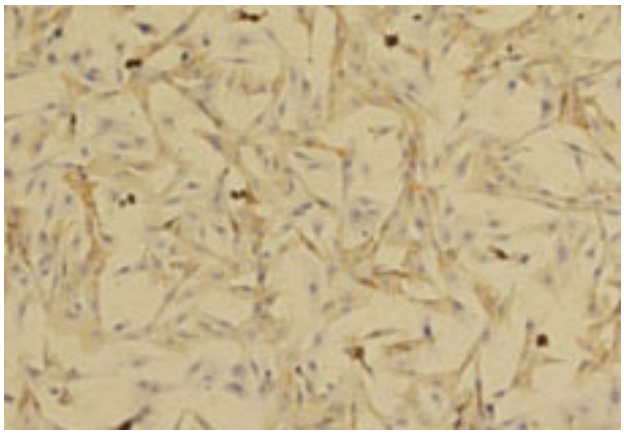

Figure 3. Immunocytochemical staining against Actin protein in CUC cells. Magnification 100X.

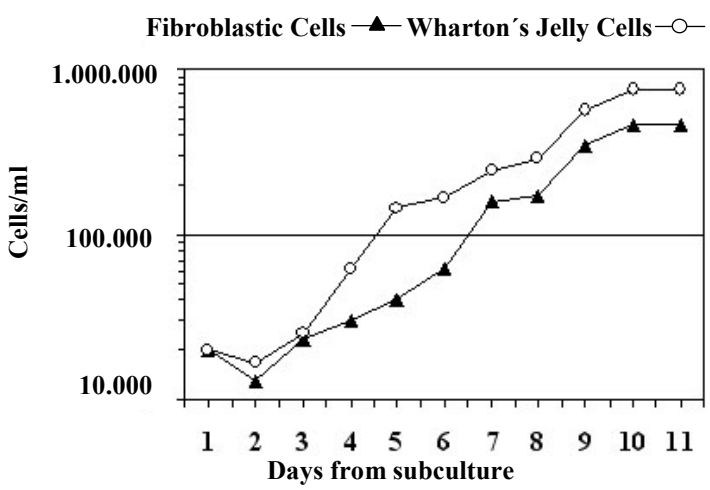

Figure 4. Semilog plot of the increase in Wharton's jelly cells in comparison with fibroblast cells concentration.

Alkaline phosphatase

In our study, pyogenic leukocytosis specimen from human patient was used as a positive control. According to the alkaline phosphatase kit used in our study a red colored reaction should be observed into the cytoplasmic granules of active leukocytes (Fig. 5A). In this regard, CUC cells colonies formed in culture exhibited positive AP activity. The reaction produced into the colonies formed by CUC cells was more intense at the border of colonies (Fig. 5B).

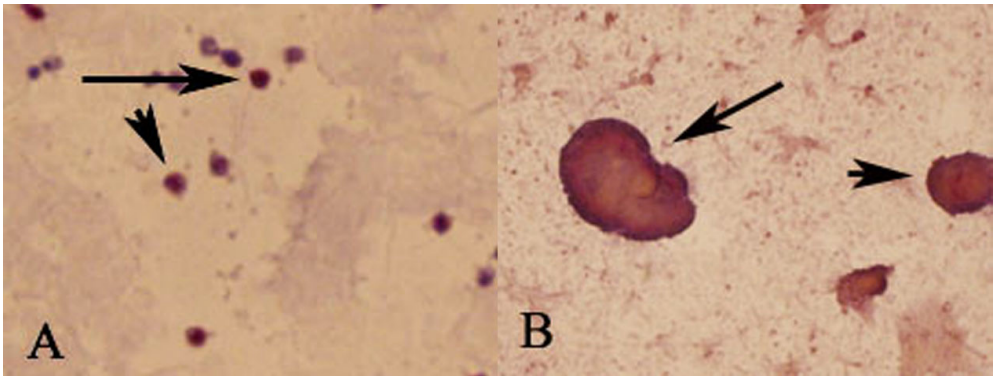

Figure 5. (A) Alkaline phosphatase assay in pyogenic leukocytosis specimen as a positive control (Magnification 400X) and (B) in CUC cells colonies (Magnification 40X).

Discussion

In recent years, parallel to the great efforts for exploring the novel and alternative sources of stem cells in the human and animal body, the umbilical cord appeared to be a promising reservoir of fetal cells that could be easily used as multipotent stem cells. Many reports have shown the characterization and stem cell potency of mesenchymal cells isolated from the human umbilical cord (Can and Karahuseyinoglu, 2007), but only a few reports were observed in animals (Zucconi *et al*., 2010; Raoufi *et al*., 2011). Here we report some characteristics of the isolated WJMC and its AP activity. To the best of our knowledge, this is the first report in goat.

According to our observations, cells isolated from caprine umbilical cord matrix explants displayed fusiform or spindle-form cells and confluent cells were arranged in parallel arrays (Fig. 2). Since structural features and main functions of umbilical cord mesenchymal cells resemble those of fibroblast cells, they were firstly recognized as unusual fibroblasts (Parry, 1970). The presence of an extraordinary number of 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 considered as unusual smooth muscle cells that have some kind of contractile properties (Kobayashi *et al*., 1998). The term myofibroblast was used first by Majno *et al*. (1971) to define cells that exhibit some of the ultrastructural features of both smooth muscle cells and fibroblast cells. Specifically, contractile proteins such as actin, non-muscle myosin, desmin and α-smooth muscle actin, markers for myofibroblasts (Chou *et al*., 1997) are differentially expressed in umbilical cord stromal cells (Kobayashi *et al*., 1998) while muscle-myosin is lacking (Takechi *et al*., 1993). Above mentioned reports are in agreement with our present immunocytochemical assay against actin protein in the isolated caprine mesenchymal umbilical cord cells.

In the present investigation, the doubling time of CUC-WJMC was shorter than fetal fibroblast cells. Shorter doubling time is a common feature for mesenchymal stromal cells derived from fetal blood (Campagnoli *et al*., 2001). Also, in the previous reports, it was mentioned that the doubling time of WJ cells and umbilical cord blood mesenchymal stromal cells is shorter than adult bone marrow-derived mesenchymal stromal cells (Baksh *et al*., 2007; Karahuseyinoglu *et al*., 2007). This common feature was thought to reflect the relatively primitive nature of mesenchymal stromal cells compared to the adult stromal cells (Troyer and Weiss, 2008).

The ability to generate clones, e.g., cellular colony derived from a single cell, is a formal demonstration of the self-renewal ability, a characteristic of stem cell populations (La Rocca *et al*., 2009). We demonstrated that CUC-WJMC have clonogenic properties, therefore a formal demonstration of self-renewal was provided for our isolated umbilical cord cells. However, still further studies are necessary to determine the expression of some transcription genes such as Oct-4. The Oct-4 gene has been proposed as a master regulator of the pluripotency of cells (Campbell *et al*., 2007). Another nonspecific test for approving stem cells is expression of AP enzyme. AP is an enzyme that is long-known to be expressed in embryonic stem cells as well as primordial germ cells (MacGregor *et al*., 1995). In agreement with the previous report in pig (Carlin *et al*., 2006), in the present study AP activity was observed very intense in CUC-WJMC colonies that was similar to the pattern observed in embryonic stem cells or other colony forming stem cells.

In summary, we demonstrated that WJ of caprine umbilical cord contains a large number of myofibroblast-like cells, which according to our tests exhibit some stem cells-like properties. In fact, we observed that these cells are clonogenic and AP positive. However, still additional researches are essential to determine other stem cells properties and pluripotential genes as well as their abilities to differentiate into adult mesenchymal cells.

Acknowledgments

This work was supported by Grant for Scientific Research from Vice Chancellor of Shahid Bahonar University of Kerman, Iran and financial support of Neuroscience Research Center of Kerman, Iran.

References

Baksh D, Yao R, Tuan RS. 2007. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells*, 25:1384-1392.

Cai J, Weiss ML, Rao MS. 2004. In search of "stemness". *Exp Hematol*, 32:585-598.

Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. 2001. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver and bone marrow. *Blood*, 98:2396-2402.

Campbell PA, Perez-Iratxeta C, Andrade-Navarro MA, Rudnicki MA. 2007. Oct-4 targets regulatory nodes to modulate stem cell function. *PLoS One*, 2:e533.

Can A, Karahuseyinoglu S. 2007. Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. *Stem Cells*, 25:2886-2895.

Carlin R, Davis D, Weiss M, Schultz B, Troyer D. 2006. Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells. *Reprod Biol Endocrinol*, 4:1-13.

Chou YH, Skalli O, Goldman RD. 1997. Intermediate filaments and cytoplasmic networking: new connections and more functions. *Curr Opin Cell Biol*, 9:49-53.

De Coppi P, Bartsch G, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, Yoo JJ, Furth ME, Soker S, Atala A. 2007. Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotech*, 25:100-106.

Fu YS, Shih YT, Cheng YC, Min MY. 2004. Transformation of human umbilical mesenchymal cells into neurons in vitro. *J Biomed Sci*, 11:652-660.

Fuchs JR, Hannouche D, Terada S, Zand S, Vacanti JP, Fauza DO. 2005. Cartilage engineering from ovine umbilical cord blood mesenchymal progenitor cells. *Stem Cells*, 23:958-964.

Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, Tukun A, Uckan D, Can A. 2007. Biology of the stem cells in human umbilical cord stroma: in situ and in vitro surveys. *Stem Cells*, 25:319-331.

Kobayashi K, Kubota T, Aso T. 1998. Study on myofibroblast differentiation in the stromal cells of Wharton's jelly: expression and localization of alpha-smooth muscle actin. *Early Hum Dev*, 51:223-233.

La Rocca G, Anzalone R, Corrao S, Magno F, Loria T, Lo Iacono M, Di Stefano A, **Giannuzzi P, Marasa L, Cappello F, Zummo G, Farina G**. 2009. Isolation and characterization of Oct-4^+/HLA-G^+ mesenchymal stem cells from human umbilical cord matrix: differentiation potential and detection of new markers. *Histochem Cell Biol*, 131:267-282.

Lee SW, Wang X, Chowdhury NR, Roy-Chowdhury J. 2004. Hepatocyte transplantation: state of the art and strategies for overcoming existing hurdles. *Ann Hepatol*, 3:48-53.

Long A, Agler A, Colitz C, Zhang J, Hayek M, Failla M, Bomser J. 2008. Isolation and characterization of primary canine lens epithelial cells. *Vet Ophthalmol*, 11:38-42.

MacGregor G, Zambrowicz BP, Soriano P. 1995. Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells. *Development*, 121:1487-1496.

Majno G, Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR. 1971. Contraction of granulation tissue in vitro: similarity to smooth muscle. *Science*, 173:548-550.

Minguell JJ, Erices A, Conget P. 2001. Mesenchymal stem cells. *Exp Biol Med*, 226:507-520.

Mitchell KE, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T, Troyer D. 2003. Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells*, 21:50-60.

Parry EW. 1970. Some electron microscope observations on the mesenchymal structures of full-term umbilical cord. *J Anat*, 107:505-518.

Raoufi M, Tajik P, Dehghan M, Eini F, Barin A. 2011. Isolation and differentiation of mesenchymal stem cells from bovine umbilical cord blood. *Reprod Domest Anim*, 46:95-99.

Takechi K, Kuwabara Y, Mizuno M. 1993. Ultrastructural and immunohistochemical studies of Wharton's jelly umbilical cord cells. *Placenta*, 14:235-245.

Troyer D, Weiss M. 2008. Concise review: Wharton's jelly-derived cells are a primitive stromal cell population. *Stem Cells*, 26:591-599.

Uchida N, Buck DW, He D, Reitsmma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL. 2000. Direct isolation of human central nervous system stem cell. *Proc Natl Acad Sci*, 97:14720-14725.

Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, Fu YS, Lai MC, Chen CC. 2004. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells*, 22:1330-1337.

Zucconi E, Vieira NM, Bueno DF, Secco M, Jazedje T, Ambrosio CE, Passos-Bueno MR, Miglino MA, Zatz M. 2010. Mesenchymal stem cells derived from canine umbilical cord vein - a novel source for cell therapy studies. *Stem Cells Dev*, 19:395-402.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng*, 7:211-228.