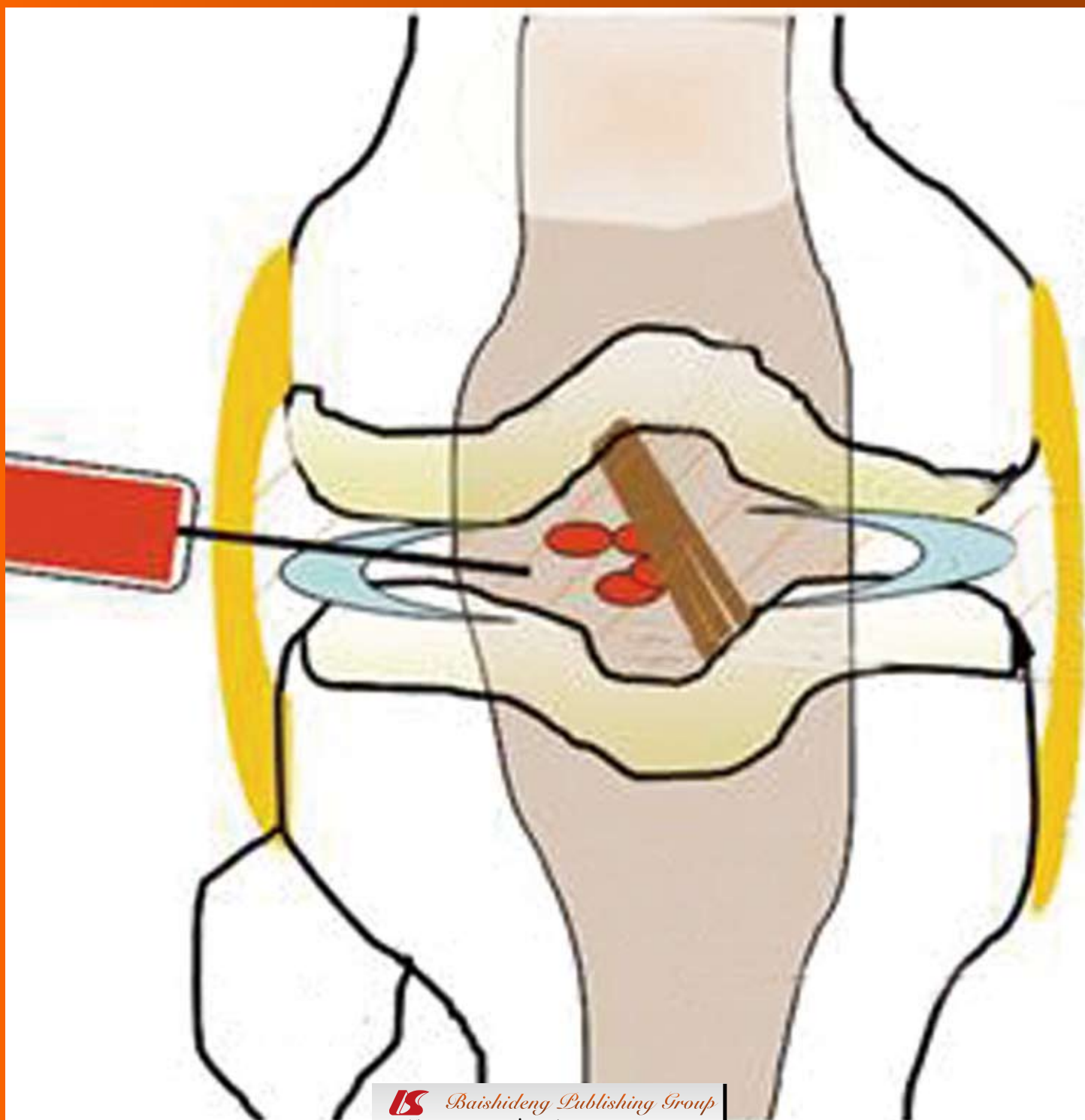


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Umbilical cord as a mesenchymal stem cell source for treating joint pathologies

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Abstract

Articular cartilage disorders and injuries often result in life-long chronic pain and compromised quality of life. Regrettably, the regeneration of articular cartilage is

a continuing challenge for biomedical research. One of the most promising therapeutic approaches is cell-based tissue engineering, which provides a healthy population of cells to the injured site but requires differentiated chondrocytes from an uninjured site. The use of healthy chondrocytes has been found to have limitations. A promising alternative cell population is mesenchymal stem cells (MSCs), known to possess excellent proliferation potential and proven capability for differentiation into chondrocytes. The "immunosuppressive" property of human MSCs makes them an important candidate for allogeneic cell therapy. The use of allogeneic MSCs to repair large defects may prove to be an alternative to current autologous and allogeneic tissue-grafting procedures. An allogeneic cell-based approach would enable MSCs to be isolated from any donor, expanded and cryopreserved in allogeneic MSC banks, providing a readily available source of progenitors for cell replacement therapy. These possibilities have spawned the current exponential growth in stem cell research in pharmaceutical and biotechnology communities. Our objective in this review is to summarize the knowledge about MSCs from umbilical cord stroma and focus mainly on their applications for joint pathologies.

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Key words: Human; Mesenchymal stem cell; Umbilical cord; Cartilage degeneration

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INTRODUCTION AND JUSTIFICATION

Mesenchymal stromal cells or mesenchymal stem cells (MSCs) are a plastic-adherent cell population that expresses specific surface markers and have the capacity to self-renew and differentiate into bone, cartilage, adipose, muscle and neural cells in adequate conditions^[1-3]. MSCs are found in a variety of tissues, including the umbilical cord^[4-6], which is the focus of this review and provides a non invasive source of abundant stem cells. MSCs are a relatively new source for cell therapy for cartilage and surrounding joint tissues. Regenerative therapies for cartilage defects will profit greatly from the combined applications of available basic science knowledge of matrix biology, hormone and receptor biology, biomechanics, virology, scaffolds and transplantation medicine^[7]. Although the therapeutic potential of MSCs is being studied and has clear objectives, the requirements and conditions for their direct chondrogenic differentiation *in vitro* remains a challenge. This review is intended to summarize our knowledge of umbilical cord MSCs, including their derivation from Wharton's jelly or umbilical cord blood and their therapeutic application for joint pathologies.

UMBILICAL CORD MSCS

MSCs have been isolated from umbilical cord blood, umbilical vein subendothelium and Wharton's jelly cells. All these cells are a primitive stromal population that displays the characteristics of MSCs defined by the International Society for Cellular Therapy. Umbilical cord MSCs grow as adherent cells with mesenchymal morphology, are self-renewing, express cell surface markers typical of MSCs (CD14-, CD34- and CD45-negative while expressing high levels of CD73, CD90 and CD105), maintain their differentiation potential and can differentiate *in vitro* into various cell lineages, including bone, cartilage and adipose tissue^[8]. Umbilical cord MSCs also have the capacity to form neurons and glia *in vitro*^[9] and can be successfully trans-differentiated into cardiomyocytes *in vitro*^[10]. Like other stromal cells, MSCs derived from the umbilical cord support the expansion of other stem cells, such as hematopoietic stem cells, and are well-tolerated by the immune system^[11]. Human umbilical cord MSCs share similar *in vitro* immunosuppressive properties with MSCs obtained from bone marrow and blood. However, the mechanisms and cell interactions used by MSCs to control immune responses are not yet fully elucidated^[12]. Functional module analysis has shown different osteogenic potential and differentiation capacity for human MSCs from bone marrow and Wharton's jelly^[13]. There are no known limitations to using umbilical cord-derived MSCs. Wu *et al*^[14] published

the first report of umbilical cord MSCs in a human clinical application. They found that umbilical cord MSCs had superior proliferative potential and more suppressive effects on peripheral blood mononuclear cell proliferation than bone marrow MSC. The acute graft-versus-host disease improved dramatically after each of four infusions of umbilical cord MSCs into the two patients. No adverse effects were noted. Both patients are doing well now and this procedure seems both feasible and safe.

Umbilical cord blood

Several studies of MSCs derived from the umbilical cord blood of several species show them to be capable of being isolated and differentiated toward defined mesoderm lineages. Included in these species are equine^[15,16], canine^[17], porcine^[18], ovine^[19] and human^[20,21]. Studies have also focused on the ability of umbilical cord MSCs to support the *ex-vivo* expansion of other cell types and their differentiation into other lineages^[22,23]. Recent studies by Kim *et al*^[24] have examined the molecular mechanism of differentiation, such as the involvement of NOD1 and NOD2 for differentiation of MSCs derived from human umbilical cord blood, and analyzed the ability of these cells to function as alloantigens *in vitro*. Current results indicate that human umbilical cord blood-derived MSCs do not provoke allogeneic peripheral blood mononuclear cell proliferation even when their HLA-molecule expression was up-regulated by interferon-(IFN)- γ pre-treatment. This suppressive effect was mediated by soluble factors. Overall it seems that MSCs from umbilical cord blood can suppress the allogeneic response of lymphocytes and may prove useful in allogeneic cell therapies^[25]. Umbilical cord blood is a valuable source for hematopoietic progenitor cell therapy. It also contains another non-hematopoietic cell population, mesenchymal progenitor cells (MPCs), which can be expanded *ex vivo* and differentiated into osteoblasts, chondrocytes and adipocytes. Several studies^[4,26] indicate that two sub-populations of MSCs can be isolated from umbilical cord blood by using specific methods. These cells exhibit different morphological phenotypes, the majority of which resemble flattened fibroblasts and the remainder spindle-shaped fibroblasts. Both MSC types share similar cell surface markers, except for CD90, and have similar osteogenic and chondrogenic potential. The spindle-shaped population is positive for CD90 and shows more capability for adipogenesis, while the flattened cell type is CD90 negative and shows less capability for adipogenesis. The larger number of flattened umbilical cord blood MPCs might be linked to their lowered capability for adipogenic differentiation^[27].

Wharton's jelly

The number of studies of MSCs derived from stromal tissues has increased over the last 6 years. However, these cells have not been as widely studied as MSCs derived from umbilical cord blood. Can *et al*^[28] described the noninvasive isolation, culture and basic characterization of human umbilical cord stroma -MSCs. MSCs derived from Wharton's jelly were isolated using their adherence

to plastic and characterized by flow cytometry, looking for cells positive for STRO1, OCT3/4 and SSEA-4, as well as those positive for the classic MSC-markers, CD44, CD73, CD90, Ki67, CD105 and CD106, and negative for CD34 and CD45^[29-32] (Figure 1). These MSCs were differentiated towards musculoskeletal tissue^[33] and CD10 positive cells that displayed contractile properties^[34]. Wharton's jelly MSCs are also candidates for β cell regeneration, utilizing their immune response inhibiting benefits for treatment of type 1 diabetes^[35]. Wharton's jelly-derived MSCs may prove to be a better clinical alternative to bone marrow-derived MSCs because of their ease of access, higher expansion potential and low immunogenicity. The use of allogenic MSCs would be possible *in vivo* only if they retain their immune properties in an inflammatory environment. Thus, the focus of a study by Prasanna *et al*^[36] sought to understand and compare the immune properties of MSCs from Wharton's jelly and from bone marrow primed with the key pro-inflammatory cytokines, IFN- γ and tumor necrosis factor α . Importantly, these authors found that inflammation affects the immune properties of these MSCs differently and that MSCs derived from different tissues may utilize different unique mechanisms of immune-modulation.

Recent studies by Huang *et al*^[37] have demonstrated that MSCs can differentiate into germ cells under appropriate conditions. MSCs were induced to differentiate into germ cells in all-trans retinoic acid, testosterone and testicular-cell-conditioned medium prepared from newborn male mouse testes. These MSCs formed "tadpole-like" cells after induction with different reagents and showed both mRNA and protein expression of the germ-cell-specific markers, Oct4 (POUF5), Ckit, CD49 (f) (α 6), Stella (DDPA3) and Vasa (DDX4). These results could lead to a new reproductive therapy utilizing human umbilical cord MSCs as well as provide a novel *in vitro* model for investigating the molecular mechanisms regulating development of the mammalian germ cell lineage^[37]. Proteomic analyses of human MSCs from Wharton's jelly during *in vitro* expansion have demonstrated their potential capacity for self-renewal and *in vitro* expansion^[38]. More than 30% of 158 proteins identified by these analyses belong to the cytoskeleton complex. However, several proteins were no longer expressed after the 2nd *in vitro* passage, suggesting the proliferative potency of these cells was reduced after the initial *in vitro* stage. This important study provides an essential step for gaining an understanding of the molecular properties of Wharton's jelly cells^[38].

Obstetric parameters, including the gender of the baby, birth weight, the age of the mother at delivery, gestational stage at parturition and mode of delivery were examined by Penolazzi *et al*^[39] who found that osteoblastic potential was not influenced by the baby's gender and mode of delivery. The highest degree of osteoblastic potential was shown by MSCs from Wharton's jelly selected from the heaviest full-term babies and with high basal levels RUNX-2. The results of this study are helpful to select optimal umbilical cord donors for efficiently collecting high potential Wharton's jelly-derived osteoprogenitors.

Therefore, the isolation and culture of human umbilical cord-MSCs still need better clarification to ultimately build an optimal standard procedure among laboratories, tissue banks and clinics.

UMBILICAL CORD MSCS AND CHONDROGENESIS

MSCs from equine umbilical cord blood produced a tissue with a cartilage-like morphology that stained positive for proteoglycans and expressed typical cartilage markers^[40]. These authors found that MSCs from umbilical cord blood possessed more chondrogenic potential than bone marrow-derived MSCs, based on the cell populations tested and parameters measured^[40]. Another study using canine MSCs from umbilical cord blood employed osteogenic and chondrogenic differentiation detected by alizarin red and toluidine blue staining respectively^[17]. With osteogenic differentiation, the MSCs were shown to express osteoblastic differentiation genes by reverse transcriptase-polymerase chain reaction (RT-PCR). The results from this study suggest that canine MSCs are capable of multipotential differentiation^[17].

MSCs from porcine umbilical cord blood were isolated and their morphology, proliferation, cell cycle status, cell-surface antigen profile and expression of hematopoietic cytokines characterized by Kumar *et al*^[18]. The capacity of porcine MSCs to differentiate *in vitro* into osteocytes, adipocytes and chondrocytes was also evaluated. These investigators found that the expression of lineage-specific genes was gradually up-regulated during osteogenesis, adipogenesis and chondrogenesis. Porcine umbilical cord blood also contains a population of MPCs capable of self-renewal and of differentiating *in vitro* into three classical mesenchymal lineages.

The *in vitro* growth of human umbilical cord blood MSCs has been achieved; their differentiation into chondrocytes and osteoblasts demonstrates their multipotentiality and predisposition towards a mesodermal fate^[41]. Wharton's jelly contains mucoid connective tissue and fibroblast-like cells. Using flow cytometric analysis, Wang *et al*^[29] found that mesenchymal cells isolated from human Wharton's jelly express the matrix receptors CD44 and CD105, plus integrin markers CD29 and CD51, but not the hematopoietic lineage markers CD34 and CD45 (Figure 2). Interestingly, these cells also express significant amounts of the MSC markers SH2 and SH3^[29]. Isolated and characterized MSCs from human umbilical cord stroma and quantitative RT-PCR analysis of the genes ALP, MEF2C, MyoD, LPL, FAB4 and AMP, characteristic for the differentiated lineages, were used to evaluate early and late differentiation of three germ lines^[30]. Direct chondrogenic differentiation was achieved by utilizing spheroid formation by MSCs in a chondrogenic medium; testing for the presence of chondrogenic markers was done at 4, 7, 14, 28 and 46 d of culture. Immunohistochemistry and quantitative RT-PCR analyses were employed to assess the expression of collagen type 1 (COL1), collagen type 2 (COL2) and collagen type X (COLX) at the times

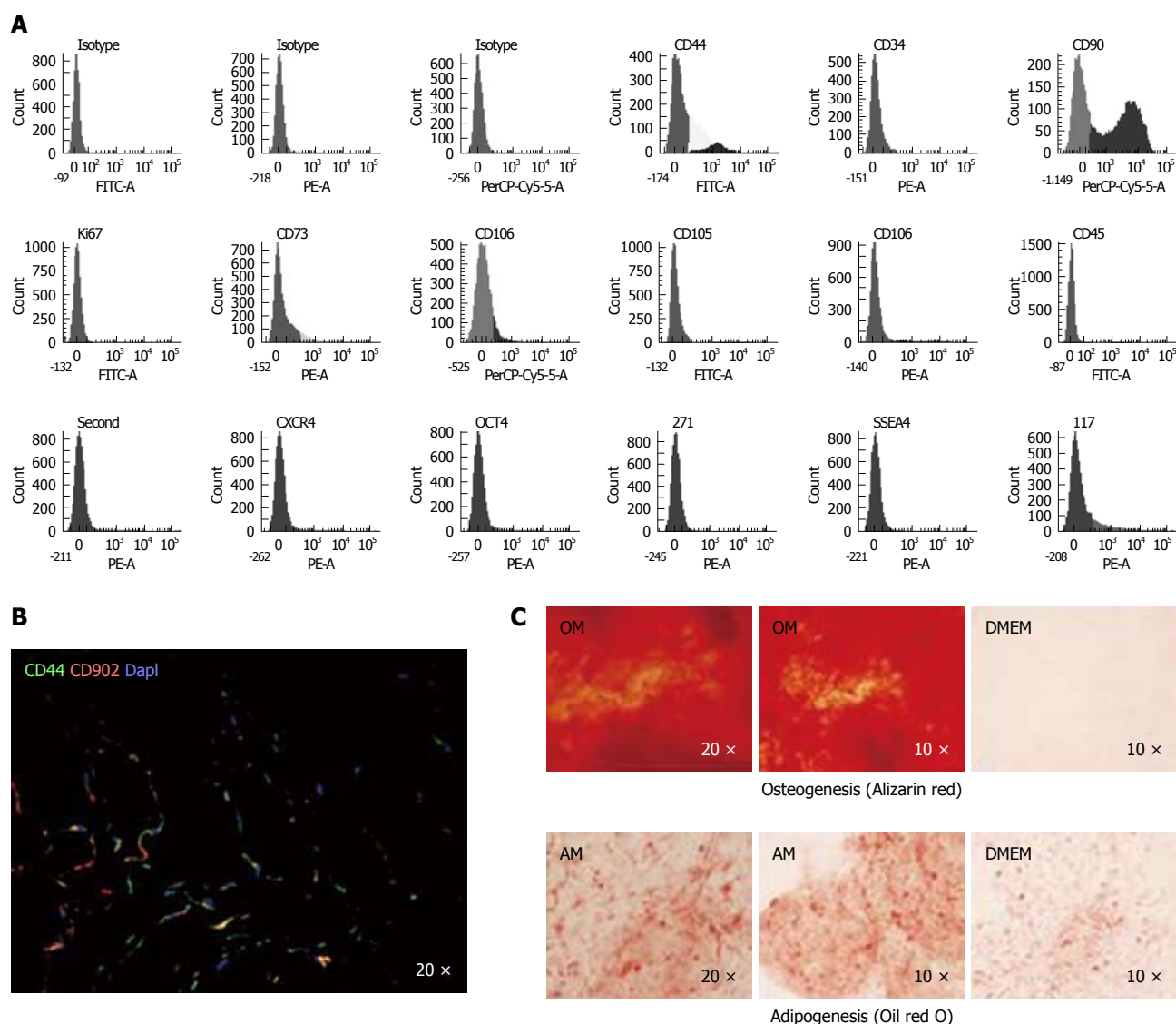


Figure 1 Characterization of the mesenchymal stem cell population from human umbilical cord stroma. A: Flow cytometry of the principal mesenchymal stem cell (MSC) markers. In each diagram, at the top is the name of the marker, at the bottom the fluorochrome used and at the top right the percentage of positive cells; B: Immunofluorescence analysis of CD44 and CD90 in a human umbilical cord cryosection; DAPI was used to label cell nuclei. (Magnification 20 ×); C: Alizarin red stain (upper) and Oil red O stain (lower) of spheroids engineered from MSCs differentiated in two defined media, proving their pluripotency. OM: Osteogenic medium; AM: Adipogenic medium; DMEM: Control medium with no defined cytokines to promote differentiation.

studied^[30]. These investigators found expression of all chondrogenic markers as early as 4 d of chondrogenic differentiation culture, with their expression increasing with time except for COL1 which decreased in expression in the formed spheroids after 4 d of differentiation. A secretome study to validate this model for *in vitro* chondrogenic differentiation was performed using the spheroids formed during the chondrogenesis process^[30]. The results summarized in Figures 1 and 2 indicate the multipotential capacity of human MSCs from this source; their chondrogenic capacity may prove useful for future cell therapies for articular diseases^[30]. It would also be interesting to see if the chondro-type tissues being differentiated from these MSCs can be modified *in vitro* using biomaterials or other factors to mimic the 5 different layers observed in normal cartilage but it is too early in the research to know this and there is no report of it in the literature.

THERAPEUTIC APPLICATION OF UMBILICAL CORD MSCS FOR JOINT PATHOLOGIES

Human MSCs, with particular reference to Wharton's jelly MSCs, have an important role as immunomodulators and their multilineage differentiation potential makes their use in tissue regeneration and repair possible^[42].

Although bone marrow represents the primary available source of MSCs, the use of bone marrow-derived cells is not always acceptable because of their high degree of viral infection and the significant drop in cell number and both proliferative and differentiation capacity with ageing. Romanov *et al.*^[43] isolated MSCs from the subendothelial layer of the umbilical cord and found that cord vasculature contains many MSC-like elements forming

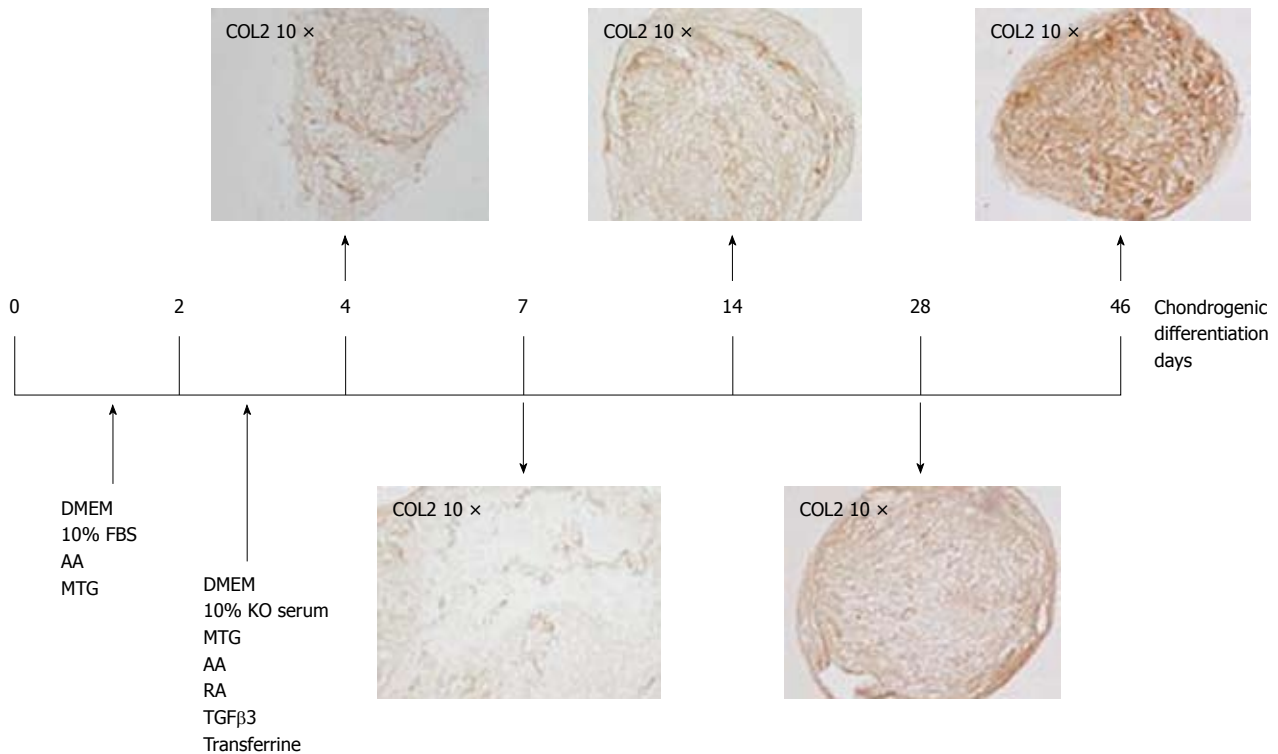


Figure 2 Representative diagrams of the methodology for spheroid chondrogenesis. Diagram showing culture times used for a new method for the induction of chondrogenesis. Representative sections of immunohistochemical analyses for collagen type 2 (COL2) expression from spheroids engineered from Wharton's jelly mesenchymal stem cells at different times of chondrogenesis are shown. (Magnification 10x). AA: Ascorbic acid; MTG: Monothiolglycerol; RA: Retinoic acid; KO serum: Knock out serum (GIBCO™); TGFβ-3: Recombinant human transforming growth factor-β3; TRANS: Transferrine; DEX: Dexamethasone.

colonies of fibroblastoid cells that express several mesenchymal cell markers and can be successfully expanded in culture. They proposed that umbilical cord/placenta stroma may become an alternative source of MSCs for experimental and clinical needs^[43]. Scaffolds provide a template for cell distribution, growth, differentiation and extracellular matrix accumulation in three dimensions. Recent studies^[44,45] have demonstrated the potential of scaffolding to enhance articular cartilage repair both *in vitro* and *in vivo*. Kao *et al*^[46] demonstrated that chemically synthesized thermoreversible gelation polymer provided a competent three-dimensional culture environment for cord blood MSCs to differentiate into chondrocytes and may prove to be clinically useful to induce chondrogenic differentiation of MSCs from cord blood for cartilage repair. Although bone marrow was the first reported source to contain MSCs, obtaining and utilizing these MSCs is not always practical due to the very invasive technique required for drawing them and the decline in cell number and differentiation capability with increasing age.

Mononuclear cells derived from human umbilical cord blood and obtained by a negative immunoselection technique exhibited either osteoclast-like or mesenchymal-like phenotypes^[47]. However, these cells were able to produce homogeneous populations of MSCs displaying a fibroblast-like morphology while expressing mesenchyme-related antigens and showing differentiation capability for osteoblastic and early chondroblastic lineages. This study by Barachini *et al*^[47] is one of the few investi-

gating human umbilical cord blood-derived MSC growth and differentiation on three-dimensional scaffolds. The potential applications in regenerative medicine and tissue engineering of umbilical cord blood-derived MSCs were illustrated by their ability to grow on biodegradable microfiber meshes and their capability to differentiate into mature osteoblasts when cultured inside human plasma clots; this suggests their potential applications in orthopedic surgery. In a 6 wk study by Wang *et al*^[48], a comparison of chondrogenic differentiation of human bone marrow MSCs and human umbilical cord MSCs in a three-dimensional scaffold was conducted for the first time. Cells were seeded on polyglycolic acid scaffolds at 25 M cells/mL and cultured in identical conditions. Cell proliferation, biosynthesis and chondrogenic differentiation were assessed at weeks 0, 3 and 6 after seeding. The authors concluded that human umbilical cord MSCs might provide a desirable option for use as a mesenchymal cell source for fibrocartilage tissue engineering, based on the abundant COL1 and aggrecan production of human umbilical cord MSCs in a three-dimensional matrix. Further investigations of the optimal signals to promote COL2 production from human umbilical cord MSCs are necessary for their use in hyaline cartilage engineering.

MSCs from Wharton's jelly have been shown to be therapeutic in several pre-clinical animal models for neurodegenerative disease, cancer, heart disease, *etc.* The preclinical work suggested that MSCs from Wharton's jelly are therapeutic by mechanisms of trophic rescue and

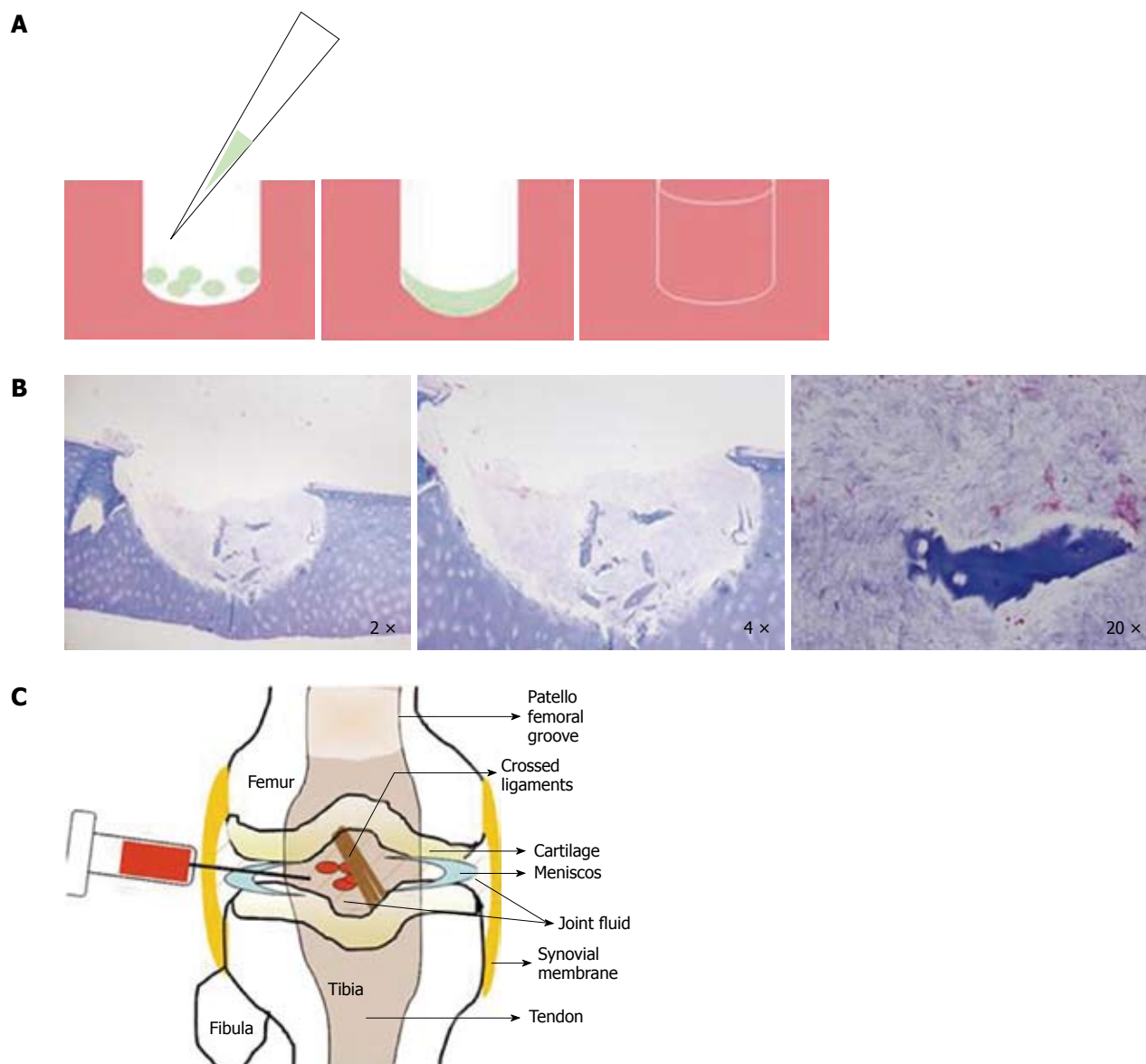


Figure 3 Cellular therapy. A: Graphic illustrating the regeneration of a chondral lesion *in vitro*; B: Wharton's jelly mesenchymal stem cells (MSCs) growing *in vitro* in a human osteochondral punch lesion over 90 d in chondrogenic medium with added transforming growth factor β 3. Modified Masson staining shows the MSCs differentiating in the lesion (Magnification 2 \times , 4 \times and 20 \times); C: Graphic portraying the technique of intra-articular injection of MSCs into an injured joint to treat lesions *in vivo*.

immune modulation. MSCs from human Wharton's jelly represent a promising source for progenitor cells with the potential to repair and regenerate solid tissues^[49]. Collagen-embedded MSCs from Wharton's jelly have a homogenous growth pattern as well as a constant expression of growth factors and extracellular matrix proteins without any negative effects on the epidermal layer, as shown by histology, electron microscopy, immunohistochemistry and real time-RT-PCR. These results indicate the necessity for an organizing a biomaterial-based scaffold to direct stem cell differentiation, proliferation and paracrine activity, as well as regulation of extracellular matrix deposition^[50,51].

Preliminary *in vitro* studies done in our group have shown some degree of differentiation and integration of MSCs from Wharton's jelly into a human osteochondral punch (Figure 3). Further work is needed to determine whether the Wharton's jelly MSCs can engraft *in vivo* over

the long term and can self-renew cartilage. There are several studies^[52] using acellular umbilical veins seeded with MSCs from bone marrow to engineer tendon but umbilical cord-derived MSCs have not yet been tested for this purpose.

New research studies^[53,54] involving the use of new scaffold materials made from hyaluronic acid, collagen and other extracellular matrix proteins should be mentioned. These extracellular matrix scaffolds have been used to create three-dimensional structures for supporting *in vitro* growth and chondrogenesis of MSCs and their possible clinical use for the replacement of damaged joints^[51,55-57]. To date, all these studies have utilized only MSCs from bone marrow and none of them have considered the dangerous aspects of such tissue-like inducing malignancies.

In summary, umbilical cord MSCs are multipotent stem cells that may serve many therapeutic and biotech-

nological roles in cartilage repair opportunities. Considering that acquiring umbilical cord derived-MSCs is a non invasive technique, these cells would appear to be the ideal candidates for clinical cell-based therapies. It would appear to be promising news for use in future *in vivo* studies and consequently clinical trials for cartilage repair therapies. Successful *in vitro* and *in vivo* differentiation to several lineages makes these cells an invaluable stem cell source, deserving further testing as a cellular therapy or other applications in regenerative medicine.

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Events Calendar 2011

January 16-20, 2011
Combined 4th International
Conference of the Saudi Orthopaedic
Association & SICOT Trainee Day,
Abha, Saudi Arabia

January 24-27, 2011
7th Middle East Orthopaedics
Conference 2011, Dubai International
Convention Centre, Dubai,
Saudi Arabia

January 28-30, 2011
National Orthopedic Conference
2011, San Francisco, California,
United States

February 15-19, 2011
American Academy of Orthopaedic
Surgeons, San Diego, CA,
United States

February 16-20, 2011
2011 Annual Meeting of the American
Academy of Orthopaedic Surgeons,
San Diego, CA, United States

February 19, 2011
Pediatric Orthopaedic Society of
North America Specialty Day, San
Diego, CA, United States

March 09-11, 2011
Annual London Imperial Spine
Course, London, United Kingdom

March 21-25, 2011
31st Caribbean Orthopaedic
Meeting, Anse Marcel, Saint Martin

March 28-April 02, 2011
The Association of Children's
Prosthetic-Orthotic Clinics 2011
Annual Meeting, Park City, UT,
United States

April 01-04, 2011
Ain Shams 2nd Orthopaedic
intensive course (Orthopaedics from
A to Z), Cairo, Egypt

April 20-22, 2011
IMUKA 2011: Masterclass in
Arthroscopy and Related Surgery,
Maastricht, Netherlands

May 11-14, 2011
2011 POSNA Annual Meeting,
Montreal, Quebec, Canada

May 12-15, 2011
84th Annual Meeting of the
Japanese Orthopaedic Association,
Yokohama, Japan

May 15-19, 2011
8th Biennial ISAKOS Congress
(International Society of
Arthroscopy, Knee Surgery and
Orthopaedic Sports Medicine), Rio
de Janeiro, Brazil

May 25-28, 2011
16th Pan Arab Orthopedic
Association Congress & 27th
SOTCOT Congress, Tunis, Tunisia

June 01-04, 2011
12th EFORT Congress in cooperation
with the Danish Orthopaedic
Association (European Federation

of National Associations of
Orthopaedics and Traumatology),
Copenhagen, Denmark

June 08-12, 2011
2011 ABJS Annual Meeting
(Association of Bone and Joint
Surgeons), Dublin, Ireland

June 15-18, 2011
11th Annual Meeting of the
International Society for Computer
Assisted Orthopaedic Surgery,
London, United Kingdom

July 07-09, 2011
66th Annual Meeting of the
Canadian Orthopaedic Association,
St. John's, Newfoundland and
Labrador, Canada

July 13-16, 2011
18th International Meeting on
Advanced Spine Techniques,
Copenhagen, Denmark

July 22-24, 2011
Sri Sathya Sai International
Orthopaedic Conference- 2011
On Pelvis And Lower Extremity
Trauma", Sri Sathya Sai Institute
of Higher Medical Sciences,
Prasanthigram, Puttaparthi, Andhra
Pradesh, India

July 25-28, 2011
2011 Update in Orthopaedics, Grand
Wailea Hotel Resort & Spa, Wailea,
Maui, Hawaii, United States

September 06-09, 2011

SICOT 2011 XXV Triennial World
Congress, Prague, Czech Republic

September 13-16, 2011
BOA/IOA Combined
Meeting(British Orthopaedic
Association & Irish Orthopaedic
Association), Dublin, Ireland

September 14-17, 2011
23rd SECEC-ESSSE Congress
(European Society for Surgery of
the Shoulder and the Elbow), Lyon,
France

September 14-17, 2011
46th SRS Annual Meeting &
Course (Scoliosis Research Society),
Louisville, Kentucky, United States

September 15-18, 2011
2011 World Congress on
Osteoarthritis, San Diego, California
92167, United States

September 21-23, 2011
HIP IMPROVEMENTS AND
PROCEEDINGS, Toulouse, France

October 25-28, 2011
DKOU 2011-Deutscher Kongress
für Orthopädie und Unfallchirurgie,
Berlin, Germany

November 7-11, 2011
86ème Réunion Annuelle SOFCOT,
Paris, France

December 12-15, 2011
EOA 63rd Annual International
Conference, Cairo, Egypt



INSTRUCTIONS TO AUTHORS

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The aim of *WJO* is to report rapidly new theories, methods and techniques for prevention, diagnosis, treatment, rehabilitation and nursing in the field of orthopedics. *WJO* covers diagnostic imaging, arthroscopy, evidence-based medicine, epidemiology, nursing, sports medicine, therapy of bone and spinal diseases, bone trauma, osteoarthritis, bone tumors and osteoporosis, minimally invasive therapy, traditional medicine, and integrated Chinese and Western medicine. The journal also publishes original articles and reviews that report the results of applied and basic research in fields related to orthopedics, such as immunology, physiopathology, cell biology, pharmacology, medical genetics, and pharmacology of Chinese herbs.

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The columns in the issues of *WJO* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide Guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Articles: To report innovative and original findings in orthopedics; (9) Brief Articles: To briefly report the novel and innovative findings in orthopedics; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJO*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of orthopedics; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on the research orthopedics.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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