MESENCHYMAL STROMAL CELLS FROM HUMAN UMBILICAL CORDS DISPLAY POOR CHONDROGENIC POTENTIAL IN SCAFFOLD-FREE THREE DIMENSIONAL CULTURES

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Abstract

Many researchers world over are currently investigating the suitability of stromal cells harvested from foetal tissues for allogeneic cell transplantation therapies or for tissue engineering purposes. In this study, we have investigated the chondrogenic potential of mesenchymal stromal cells (MSCs) isolated from whole sections of human umbilical cord or mixed cord (UCSCs-MC), and compared them with cells isolated from synovial membrane (SMSCs), Hoffa’s fat pad (HFPSCs) and cartilage. All MSCs were positive for surface markers including CD73, CD90, CD105, CD44, CD146 and CD166, but negative for CD11b, CD19, CD34, CD45 and HLA-DR in addition to CD106 and CD271. Chondrogenic potential of all cell sources was studied using 3D pellet cultures incubated in the presence of different combinations of anabolic substances such as dexamethasone, IGF-1, TGF-β1, TGF-β3, BMP-2 and BMP-7. BMP-2 and dexamethasone in combination with TGF-β1 or TGF-β3 excelled at inducing chondrogenesis on SMSCs, HFPSCs and chondrocytes, as measured by glycosaminoglycans and collagen type II staining of pellets, quantitative glycosaminoglycan expression, quantitative PCR of cartilage signature genes and electron microscopy. In contrast, none of the tested growth factor combinations was sufficient to induce chondrogenesis on UCSCs-MC. Moreover, incubation of UCSCs-MC spheroids in the presence of cartilage pieces or synovial cells in co-cultures did not aid chondrogenic induction. In summary, we show that in comparison with MSCs harvested from adult joint tissues, UCSCs-MC display poor chondrogenic abilities. This observation should alert researchers at the time of considering UCSCs-MC as cartilage forming cells in tissue engineering or repair strategies.

Keywords: Chondrogenesis; 3D pellet culture; Mesenchymal stromal cells; Umbilical cord derived stromal cells; Hoffa’s fat pad derived stromal cells; Synovial membrane derived stromal cells.

Introduction

Articular cartilage is a specialised avascular and hypocellular load-bearing tissue that covers the terminal edges of adjacent bones and provides frictionless movement of the joints (Hunziker, 2002). Lesions in articular cartilage evolving from traumatic or pathological processes represent a very common clinical condition in both developed and developing countries, and show increasing prevalence due to the steady prolongation of life expectancy (Bers et al., 2005). During the last decades biological repair of cartilage has been attempted by both the direct implantation of autologous cells, (Brittberg, 2008) and by the ex-vivo generation of tissue engineering-based implants (Filardo et al., 2013). Despite promising achievements in the laboratory and in animal models, clinical translation of these techniques remains very limited.

One of the main obstacles encountered is the limited availability of donor tissue, along with some ethical constraints associated with painful harvesting and possible donor site morbidity (Horas et al., 2003). Furthermore, articular chondrocytes used in autologous chondrocyte implantation (ACI) or bone marrow-derived MSCs (bMSCs) have been shown to gradually lose proliferative and differentiation potential in vitro in relation to increasing patient age (Mueller and Glowacki, 2001; Roodrouck et al., 2008; Smeriglio et al., 2015). In the clinic, the procedures mentioned above are often associated with the formation of biomechanically inferior fibrocartilage in the repair zone (Horas et al., 2003; Wright et al., 2013). Hence, approximately 25% failure rate has been observed in both ACI and micro-fracture in a randomised control clinical study after 5 years (Knutsen et al., 2007), which is expected to increase in longer follow up studies.

To overcome the aforementioned constraints, researchers have started exploring the use of allogeneic cell sources such as foetal-derived stromal cells from placenta and umbilical cords which are young and immature, easily accessible, abundant and not associated to ethical concerns (Baksh et al., 2007; Fong et al., 2012). MSCs can be isolated from different compartments of the umbilical cord including Wharton’s jelly (WJ), the perivascular region (PV), cord lining (CL), artery, and from the whole cord (MC, without separating each compartment) (Mennan et al., 2013; Subramanian et al., 2015). Like other adult MSCs, umbilical cord-derived stromal cells (UCSCs) have been shown to display high proliferative rate and multilineage differentiation potential (Mennan et al., 2013; Nirmal and Nair, 2013). They also express markers typical for MSCs, whilst being negative for haematopoietic, ...
macrophage and endothelial cell markers. In addition, UCSCs are thought to have an immune privileged status and to exert immunosuppressive effects over different immune cell types in vitro, which makes them an attractive candidate for allogeneic based therapies (Subramanian et al., 2012; Troyer and Weiss, 2008).

The use of UCSCs for cartilage repair or cartilage tissue engineering have been studied to some extent in the past. Most available literature refers to the chondrogenic abilities displayed by MSCs collected from the blood compartment of cords; however, much less information is available on cells isolated from cord matrix or stroma (Park et al., 2015; Zhang et al., 2011). The intrinsic chondrogenic potential of MSCs isolated from the solid parts of umbilical cords has been studied mostly in vitro with divergent outcomes. While some studies are showing differentiation of UCSCs towards immature cartilage-forming cells (Wang et al., 2009a; Wang et al., 2009b), other studies argue poorer chondrogenic ability of UCSCs when compared with MSCs from other adult tissues (Bailey et al., 2007; Hildner et al., 2010; Mennan et al., 2013). In line with published original papers, many review articles also underscore several contradictory differences including multi-lineage potential and phenotypic profiles, which could be due to many factors, notably, isolation and handling techniques of MSCs in different laboratories, culture medium, scaffolds or cell carriers and use of various growth factors for chondrogenesis (El Omar et al., 2014; Troyer and Weiss, 2008). Of importance, many of the available studies are showing only qualitative outcomes and provide insufficient evidence for expression of cartilage tissue signature genes and proteins quantitatively, or the development of tissue-like structures that clearly resemble cartilage.

In our study, we aim to shed some light on this still uncertain topic by studying the chondrogenic potential of UCSCs-MC isolated from whole cords. Here we have used multiple quantitative approaches for measuring cartilage genes and proteins. Chondrogenic potency has been investigated by exposing cells to multiple combinations of growth factors, including co-culture trials with cartilage explants, and the outcomes have been compared with MSCs collected from different sources. Our data revealed intriguing differences in chondrogenic potential among different MSCs. Of note, UCSCs-MC are found to have poor differentiation ability towards the chondrogenic lineage in vitro when compared to Hoffa’s fat pad derived stromal cells (HFPSCs), synovial membrane derived stromal cells (SMSCs) and articular chondrocytes (ACs).

Materials and Methods

**Human Material**

All human samples were collected from the University Hospital Northern Norway (UNN) with patients’ informed consent, and the Regional Ethical Committee (REK Nord) at the University of Tromsø approved the study. Human ACs, SMSCs and HFPSCs were isolated from the knee joints of three patients aged 45 to 60 years undergoing total knee replacement. UCSCs-MC were isolated from three umbilical cords collected immediately after birth and processed within 3 h of collection.

**Isolation and culture of UCSCs-MC, HFPSCs, SMSCs and ACs**

All cell types were isolated using a mixed enzymatic-explant method in which tissue sections were minced and digested in collagenase XI solution (Cat. no. C9407; Sigma Aldrich) at a final concentration of 1.25 mg/mL on a shaker at 37°C. Isolation of UCSCs-MC was conducted as previously described with minor modifications (Mennan et al., 2013). Briefly, whole cord was washed three times with sterile Dulbecco’s phosphate buffered saline (DPBS; Cat. no. D8537; Sigma-Aldrich) to remove blood, followed by immersion in 90 % ethanol for 30 s and immediately washed and stored in DPBS for immediate use. Approximately 2 cm sections of whole cord were cut into small pieces, and further subjected to enzymic digestion for 1.5 h at a concentration previously indicated. During knee replacement operations, pieces of synovium were collected and the membrane fraction (synovial membrane) was carefully separated from the fat tissues (Hoffa’s fat pad). HFP and SM tissues were washed three times with DPBS, followed by cutting into small pieces prior to enzymatic digestion for 1.5 h. On the other hand, cartilage biopsies were washed in DPBS and minced carefully into 1-1.5 mm³ pieces to avoid any bone fraction with the biopsies, prior to enzymatic digestion for 4 h at a concentration previously indicated.

Partially digested tissues were centrifuged for 10 min at 800 × g and re-suspended in high glucose Dulbecco’s Modified Eagle Medium (DMEM; Cat. no. D5796; Sigma-Aldrich) supplemented with L-ascorbic acid (62 mg/L) (Cat. no.103033E; BDH Laboratory), penicillin and streptomycin (1 %) (P/S; Cat. no. P4333; Sigma-Aldrich) and 20 % foetal bovine serum (FBS; Cat. no. S0115; Biochrom) and subsequently, plated in 75 cm² culture flasks (Cat. no. 156499; Thermo Scientific) at 37 °C in humidified atmosphere containing 5 % CO₂. Partially digested cartilage allowed more cells to attach to the culture flask. For isolation of HFPSCs, only the pelleted fraction of cells were collected after centrifugation, whereas the fat layer on top was discarded. After cell attachment, cultures of UCSCs-MC, HFPSCs and SMSCs were expanded in high glucose DMEM supplemented with 10 % FBS and basic fibroblast growth factor (25 ng/mL) (bFGF; Cat. no. 100-18C; Peprotech), whereas de-differentiated ACs were expanded with only 10 % FBS as medium supplement. Primary cultures were further expanded in monolayers, followed by dissociation from culture flasks using PBS based enzyme-free dissociation solution (Cat. no. S-014-B; Merck Millipore) and plated in 175 cm² culture flasks (Cat. no. 159910; Thermo Scientific). Medium was changed every 3 to 4 d, and the cells were used for experimentation at passage 3-4, which correspond to 6-8 cumulative population doublings, measured by direct cell counting.

**Immunoprofiling of stromal cells by flow cytometry**

Expression of cell surface molecules from cultured UCSCs-MC, HFPSCs and SMSCs on the third passages
Poor chondrogenesis of umbilical cord stromal cells

Chondrogenesis of UCSCs-MC in co-culture with cartilage explants and cultured synovial cells

Histology and immunohistochemistry

Chondrogenic media

3D cell culture and induction of chondrogenesis

Confluent cell cultures at passage 3–4 were harvested and pelleted at 400 × g for 3 min at 4 °C. The pellet was washed three times with cold stain buffer (Cat. no. 554656; BD Biosciences), filtered through a cell strainer (70 μm) and re-suspended in cold stain buffer to a concentration of 5 × 10⁶ cells/mL. Cells were incubated with directly conjugated antibodies against classical MSCs characterization markers described by the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). In addition, antibodies for CD44, CD106, CD146, and CD271 cell surface markers and appropriate isotype control antibodies (BD Biosciences, USA) were used in the analysis. Samples were analysed using a BD FACS Aria flow cytometer and FlowJo software (Tree Star Inc., USA).

Histology and immunohistochemistry

Spheroids from all cell sources were harvested after three weeks incubation, washed three times with ice cold DPBS and fixed in 4 % formalin overnight. Fixed spheroids were washed with DPBS embedded in agarose blocks (1 %) and transferred into paraffin. Paraflin embedded sections (4 μm) were heated at 60 °C for 30-60 min prior to de-waxing and immersing in xylene twice for 10 min and rehydrated in a series of ethanol washes followed by washing twice in water for 3 min each. Sections were stained with Alcian blue solution (Cat. no. A5268; Sigma-Aldrich) for 30 min followed by washing in distilled water for 2 min and counter stained with nuclear fast red solution (Cat. no. N3020; Sigma-Aldrich) for 5 min, ending by washing in distilled water for 2 min. The sections were dehydrated in a series of ethanol wash followed by cleaning in xylene twice and a cover slip with Histokit (Cat. no. 1025/500; Glaswarentiefabrik Karl Hecht) mounted over stained sections. The image was analysed using bright field light microscopy for metachromatic staining of proteoglycans with Alcian blue.

For immunostaining of collagen type II and type I, rehydrated paraffin sections were treated with hyaluronidase (2 mg/mL) (Cat. no. H3506; Sigma-Aldrich) and pronase (1 mg/mL) (Cat. no. 10165921001; Roche) for 15 min and 30 min, respectively at 37 °C for antigen retrieval. The slides were then rinsed in distilled water followed by blocking with 2 % BSA for 10 min, prior to overnight incubation at 4 °C using rabbit antihuman collagen type II antibody (Cat. no. TS59104R; Meridian Life Science) and rabbit antihuman collagen type I antibody (Cat. no. LS-B3653; LifeSpan Biosciences) at a dilution of 1:100 and 1:500 respectively. These sections were treated with peroxidase block solution for 5 min, followed by rinsing with distilled water. The slides were then wiped gently.
and incubated with peroxidase labelled polymer-HRP (Cat. no. K4010; Dako) for 30 min followed by washing three times with PBS. The sections were incubated with diaminobenzidine (DAB) chromogen and substrate-buffer solution (1 drop of DAB + 1 mL of substrate-buffer) (Cat. no. K4010; Dako) for 5-10 min, followed by rinsing off with distilled water. Sections were then counterstained with haematoxylin (Cat. no. RBA-4213-00A; Cell Path) for 45 s followed by washing with distilled water and incubation in Scotts solution for 20 s. Finally, sections were dehydrated and mounted with Histokitt.

Quantitative analysis of histology

We have used “The Bern Score” with a few modifications as a semi-quantitative scoring method to evaluate the chondrogenicity of 3D spheroids, based on the staining of matrix proteoglycans with Alcian blue (Grogan et al., 2006). The Bern score evaluates the cartilage formation by three categories and each of the categories have a scoring range from 0 to 3 with an overall score of 9 for each spheroid (Table 1).

Biochemical analysis of GAGs

Spheroids from ACs and UCSCs-MC were subjected to biochemical analysis to determine the GAG (glycosaminoglycan) and DNA content. Spheroids were harvested after three weeks in chondrogenic conditions and washed with PBS three times followed by digestion with papain (125 μg/mL) (Cat. no. P3125; Sigma-Aldrich) buffer for 18-24 h at 65 °C. Papain digested samples were centrifuged at 16,000 ×g for 10 min and supernatants were used immediately or stored at −70 °C for biochemical analysis. Sulphated-GAG content was measured spectrophotometrically at 655 nm by 1, 9 dimethylmethylene blue dye assay using the Blyscan s-GAG assay kit (Cat. no. B1000; Biocolor). In addition, DNA was measured using Quanti-IT PicoGreen dsDNA Assay Kit (Cat. no. P7589; Life Technologies) as described previously (Toh and Cao, 2014). Fluorescence readings were taken using the CLARIOstar microplate reader (BMG LABTECH, Germany) with excitation and emission at 485 nm and 535 nm, respectively. The amount of s-GAG content was normalised to DNA content for each sample.

Transmission electron microscopy

Spheroids were harvested after three weeks and processed as previously described (Meknas et al., 2012). Briefly, spheroids were fixed in McDowell’s fixative (McDowell and Trump, 1976) overnight followed by post-fixation in 1 % OsO₄ for 1 h. Samples were stained en bloc in 2 % uranyl acetate prior to dehydration in a graded series of ethanol washes and embedded in Epon according to standard procedure. Sections were cut on a Leica Ultracut S (Vienna, Austria) with a diamond knife from Diatome (Switzerland). Micrographs were taken using a JEOL 1010 (Tokyo, Japan) electron microscope with a Morada camera system (Olympus Soft Imaging Systems, Germany).

Stemness-related transcriptional factors (SRTF) gene expression analysis

RNA from monolayer cultures was extracted using the Perfect Pure RNA Cultured Cell Kit (Cat. no. 2900319; 5′ prime) according to the manufacturer’s instructions. RNA from spheroid cultures was extracted using the RNeasy Micro Kit (Cat. no. 74004; Qiagen). In brief, the spheroids were harvested into 2 mL PCR clean tubes containing one stainless steel ball (Cat. no. 69989; Qiagen), washed once with PBS before disruption in buffer RLT in a Qiagen TissueLyser machine (Retch) and homogenisation using QiaShredder columns (Cat. no. 79654; Qiagen). RNA was cleaned using MinElute columns including on-column DNase digestion. Concentration was measured using spectrophotometry (Nano Drop ND-1000), and samples were diluted to a final concentration of 8.25 ng/L before reverse transcription using the qScript cDNA Synthesis Kit (Cat. no. 95047; Quanta Biosciences) according to the manufacturer’s protocol. Specific primers for stemness-related transcriptional factors (Drela et al.,

<table>
<thead>
<tr>
<th>Scoring Categories</th>
<th>Score</th>
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<tbody>
<tr>
<td>1. Uniformity and intensity of Alcian blue stain</td>
<td></td>
</tr>
<tr>
<td>No stain</td>
<td>0</td>
</tr>
<tr>
<td>Weak stain of matrix</td>
<td>1</td>
</tr>
<tr>
<td>Moderate stain</td>
<td>2</td>
</tr>
<tr>
<td>Strong stain of matrix</td>
<td>3</td>
</tr>
<tr>
<td>2. Matrix formation based on proteoglycan staining</td>
<td></td>
</tr>
<tr>
<td>No matrix formation</td>
<td>0</td>
</tr>
<tr>
<td>Little matrix formation with high cell density</td>
<td>1</td>
</tr>
<tr>
<td>Moderate matrix formation with relatively low cell density</td>
<td>2</td>
</tr>
<tr>
<td>High matrix formation with low cell density</td>
<td>3</td>
</tr>
<tr>
<td>3. Cell morphology</td>
<td></td>
</tr>
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<tr>
<td>Less condensed and elongated/rounded cells</td>
<td>1</td>
</tr>
<tr>
<td>Mixed elongated/rounded cells with lacunae</td>
<td>2</td>
</tr>
<tr>
<td>Rounded Cells with Lacunae/cartilage morphology</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 1. Scoring categories for histological evaluation of chondrogenesis of Alcian blue stained spheroids.
Adenine phosphoribosyltransferase (APRT) was included for RNA quality control as an 800 bp product would yield in the case of contaminating DNA. The RT-PCR reaction mix comprised of 12.5 µL JumpStart™ REDTaq® ReadyMix® Reaction Mix, 0.5 µL forward primer (20 nM), 0.5 µL reverse primer (20 nM), 9.5 µL H₂O and 2 µL cDNA. The 25 µL reaction was run on a MJ Research PTC-200 thermal cycler using the following conditions: initial denaturation for 2 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 2 min at 72 °C; final extension for 5 min at 72 °C. Products were separated on a FlashGel (Cat. no. 57023; Lonza) and photographed using the ImageQuant LAS4000 system.

**Cartilage specific gene expression analysis**

Quantitative (qPCR) reactions were performed for spheroids culture on StepOnePlus Real-Time PCR detection system (Applied Biosystems). The relative quantification experiment was run with hydrolysis probes targeting cartilage signature genes (Table 3, Life Technologies). Based on validation experiments YWHAZ alone proved as the most stable reference gene. The reaction volume of 10 µL included 5 µL TaqMan Fast Universal PCR Mastermix No AmpErase UNG (Cat. no. 4366072; Applied Biosystems), 2.5 µL water, 2 µL cDNA and 0.5 µL of the respective assays. Three technical replicates of each sample were applied to a MicroAmp Fast Optical 96-well reaction plate (Cat. no. 4346906; Applied Biosystems), and water and no-RT samples were added for the negative control. The plates were run on a StepOnePlus (Applied Biosystems), using the following cycling conditions: hold at 95 °C for 20 s, 40 cycles at 95 °C for 1 s and then at 60 °C for 20 s. Expression relative to chondrocyte spheroids was calculated using the delta delta Cq method and 95 % confidence intervals were generated in Excel.

**Statistical analysis**

For the biochemical analysis, the Student’s t-test was used to compare the mean differences between experimental and control groups (three different biological replicates per group). The result was expressed as mean ± SD and values of p < 0.05 were considered as statistically significant. In addition, for histological scoring, the Kruskal-Wallis was used followed by post hoc Bonferroni correction with Mann-Whitney U comparisons for statistical analysis using IBM SPSS statistics 22 (Chicago, USA). Values of p < 0.05 were considered as statistically significant. Last, for gene expression analysis, relative gene expression normalised to ACs was shown as 95 % confidence intervals using Microsoft Excel.

**Results**

**Outcomes of cell isolation and features of cell growth in monolayers**

Cells with fibroblastic appearance were successfully isolated from all four tissues sources. Isolated cells from umbilical cords, Hofa’s fat pads and synovial membranes were adherent to plastic and had colony-forming abilities. From umbilical cord tissue, cells normally took 5-10 d to attach to the culture plate, and the yield of cells after initial cell seeding was relatively low. In primary cultures, UCSCs-MC cultures presented a heterogeneous morphology and had a slow growing rate, taking 15-20 d to reach confluence (Fig. 1b). However, after first sub-culturing, UCSCs-MC acquired a more homogeneous...
morphology and higher proliferation rate (Fig. 1f). In contrast, isolated HFPSCs and SMSCs contained a high number of adherent MSC-like cells, which attached to the culture plate during the first 3-5 d (Fig. 1c,d). In addition, HFPSCs and SMSCs grew initially faster than UCSCs-MC, reaching 100% confluence in 10-14 d in 75 cm² culture flasks (Fig. 1g,h). Unlike stromal cells, chondrocytes attached to plastic faster (2-4 d) but grew at a slower rate (18-25 d to reach confluence). In addition, the de-differentiated chondrocytes looked less elongated and more polygonal than the mesenchymal cells from the other sources (Fig. 1a). In 3D conditions, one week after cell aggregation, spheroids from all cell sources looked similar, displaying a perfectly round and compact morphology with sharp edges (Fig. 1j-l). However, sometimes spheroids from all cell sources had fused with others after 2-3 weeks and turned into a larger structure (Fig. 1i-l).

Mesenchymal stromal cell phenotypic characterisation

Immunophenotypic characterisation of cells using flow cytometry demonstrated that UCSCs-MC, HFPSCs and SMSCs remained positive for classical mesenchymal markers such as CD73, CD90 and CD105, with expression levels above 99%. In contrast, the same cells lacked expression for haematopoietic, macrophage and endothelial markers i.e. CD11b, CD19, CD34, CD45 and HLA-DR (Fig. 2a). In this study, we wanted to further investigate the phenotype of isolated MSCs for additional cell surface markers related to cell differentiation potential (Fig. 2b). Hence, it has been shown that subpopulations of MSCs may express cell surface markers which could predict differentiation potential towards a chondrogenic lineage (Arufe et al., 2010; Baksh et al., 2007; Maleki et al., 2014; Pretzel et al., 2011) including CD44, also called homing cell adhesion molecule (hyaluronate receptor), CD106 or vascular cell adhesion molecule (VCAM-1), CD146 or melanoma cell adhesion molecule (MCAM), CD166 or activated leukocyte cell adhesion molecule (ALCAM), and CD271 also named low-affinity nerve growth factor receptor. In this study, CD44 was expressed in 99% of the population in the three MSCs, whereas none of the cell types expressed CD106 and CD271. Furthermore, UCSCs-MC showed positivity for both CD146 and CD166 with expression levels above 90%. However, a subtle difference in expression of CD146 was observed among the three cell types. Whereas UCSCs-MC cultures showed a uniform positivity for CD146, HFPSCs and SMSCs showed two distinct populations, indicating the existence of a subgroup of cells in the cultures that were negative for this marker (Fig. 2b).

Chondrogenic potential of ACs, UCSCs-MC, HFPSCs and SMSCs in 3D cultures

Chondrogenic potential of UCSCs-MC and other cell sources was tested in scaffold-free 3D cultures originated by cell condensation or pellets. To take a more unbiased approach, chondrogenesis of all cell types was tested under the influence of different mixtures of growth factors (GF). Metachromatic staining with Alcian blue was used for early screening of spheroids in different chondrogenic medium, and the best growth factors combinations were used to

![Fig. 1. Light microscopy images of different stages of cell culture and in 3D spheroids. (a-d) Monolayer culture of (a) ACs, (b) UCSCs-MC, (c) HFPSCs and (d) SMSCs after first plating. Scale bar: 5 µm. (e-h) Confluent culture of (e) ACs, (f) UCSCs-MC, (g) HFPSCs and (h) SMSCs. Scale bar: 5 µm. In the lower row (i-l), 3D Cell culture of UCSCs-MC in micro-aggregates to induce chondrogenesis: (i) 50,000 cells after initial centrifugation in conical bottom wells, (j) single UCSCs-MC spheroid after 1 week, (k) UCSCs-MC spheroids prior to fuse after 1 week and (l) fused spheroids after 2 weeks in 3D culture. Scale bar: 500 µm.](https://www.ecmjournal.org)
Fig. 2a. Characterisation for stromal cell-like phenotype by flow cytometry. Cells in passage 3 were prepared from the three different tissue sources, stained with specific cell surface markers and analysed by flow cytometry. The classical set of positive and negative markers characteristic for mesenchymal stromal cells following requirements from the International Society for Cellular Therapy (ISCT). Red peaks represent the isotype control and blue peaks represent the tested markers.
Fig. 2b. Characterisation for stromal cell-like phenotype by flow cytometry. Cells in passage 3 were prepared from the three different tissue sources, stained with specific cell surface markers and analysed by flow cytometry. Additional cell surface markers related to stromal cells differentiation potential. Red peaks represent the isotype control and blue peaks represent the tested markers.
further check for collagen type II and type I staining and gene expression analysis. Histomorphological outcomes with Alcian blue demonstrated that chondrogenic medium containing TGF-β3+BMP-2 was the best chondrogenic medium for HFPSCs, whereas the combination TGF-β1 and BMP-2 was the best alternative for SMSCs (Fig. 4d,e). ACs spheroids demonstrated good cartilage-like features with all combinations of growth factors (Fig. 3 and 4). None of the chondrogenic media tested was sufficient to differentiate UCSCs-MC into cartilage-forming cells (Fig. 3 and 4). Chondrogenic medium containing TGF-β3 and BMP-2 was successful at inducing chondrogenesis with all three types of adult tissue cells and therefore this growth factor combination was used in further analyses, also with UCSCs-MC. All UCSCs-MC spheroids were characterised by low Alcian blue staining, low collagen type II staining, scant extracellular matrix and irregular cell shape. Semi-quantitative scoring for cartilage-like features

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**Fig. 3.** Chondrogenesis of ACs and UCSCs-MC in 3D spheroids exposed to different cocktails of growth factors. Images show bright light microscopy of thin sections from UCSCs-MC spheroids stained for proteoglycans with Alcian blue, and the nuclei counterstained with Sirius red. (a) Condition TGF-β1+BMP-2, (b) condition TGF-β3+BMP-2, (c) condition TGF-β1+IGF-1, (d) condition TGF-β3+IGF-1, (e) Condition TGF-β1+BMP-7 and (f) Condition TGF-β3+BMP-7. Scale bar: 200 µm and n = 3 donors.
### Fig. 4a-e

Bright light microscopy images of thin sections (4 µm) of 3D spheroids stained with Alcian blue, collagen-II and collagen-I. (a) Cartilage, (b) ACs in the presence of TGF-β3+BMP-2, (c) UCSCs-MC in the presence of TGF-β3+BMP-2, (d) HFPSCs in the presence of TGF-β3+BMP-2 and (e) SMSCs in the presence of TGF-β1+BMP-2. Scale bar: 200 µm.
after incubation with the best chondrogenic medium for each cell type showed no differences among ACs, HFPSCs and SMSCs (Table 4), but significant lower values for UCSCs-MC compared with the rest (Fig. 4f). Furthermore, immunostaining of spheroids against collagen type II and type I also demonstrated high expression of collagen type II in spheroids from ACs, HFPSCs and SMSCs, which resembles the collagen type II staining of native cartilage, and low expression in UCSCs-MC spheroids (Fig. 4). In contrast, collagen type I staining was relatively weak in spheroids from ACs, HFPSCs and SMSCs. For UCSCs-MC, due to low matrix formation in general, both collagen type II and type I staining were weak (Fig. 4).

Comparative analysis of GAGs and gene expression

To add further support to the observations gathered by histology and immunohistochemistry, chondrogenesis was assessed by measuring GAGs content in spheroids, and the expression of cartilage signature genes in best growth factors combination. In line with results from histological scoring, similar differences in GAGs production were observed between ACs and UCSCs-MC ($p < 0.05$) after normalising content of GAGs to the amount of DNA (Fig. 5). Spheroids from ACs produced $28.1 \pm 6.2$ GAGs/DNA ($\mu$g), whereas spheroids from UCSCs-MC produced $15.1 \pm 2.1$ GAGs/DNA ($\mu$g). Furthermore, gene expression analysis by qPCR demonstrated that the expression of COL1A1 was similar for all cell types. However, COL2A1, aggrecan and the transcription factor SOX9 were significantly downregulated in UCSCs-MC compared to spheroids from ACs and the other two MSCs types (Fig. 6). Gene expression analyses revealed also that UCSCs-MC spheroids did not express COL9A1 and little or no COL10A1. Of note, HFPSCs had significantly higher expression levels of the proteoglycan versican and the hypertrophic chondrocyte marker COL10A than ACs spheroids, which could reveal a tendency of this MSCs source to form deep zone-type/hypertrophic cartilage. To check the differentiation status of UCSCs-MC before and after 3D culture, we checked the expression of several SRTF genes including OCT4A, NANOG and SOX2 in monolayers and spheroids at different oxygen tensions. SRTF gene expression revealed that UCSCs-MC express these SRTF genes during monolayer cultures at both high and low oxygen (Fig. 7). Of note, expression of SRTF genes was downregulated in UCSCs-MC upon 3D culture, even when this incubation was carried out at low oxygen tensions.

Ultrastructural examination of 3D constructs

Transmission electron microscopy was used for ultrastructural evaluation of spheroids from ACs, UCSCs-MC, HFPSCs and SMSCs. This technique permits the visualisation of cellular and extracellular matrix characteristics that is not achievable with other microscopic techniques. Hence, spheroids from ACs, HFPSCs and SMSCs showed abundant inter-territorial matrix between cells, where randomly oriented collagen fibrils were easily identifiable (small arrows in images, Fig. 8). Of note, cells in spheroids from all cell sources had a round-shape morphology, had well organised cytoplasm with numerous organelles, and presented no signs of cell

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### Table 4. Bern Score of Alcian blue stained spheroids.

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Category 1</th>
<th>Category 2</th>
<th>Category 3</th>
<th>Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACs</td>
<td>1  A/B</td>
<td>3/2</td>
<td>3/3</td>
<td>9/8</td>
</tr>
<tr>
<td></td>
<td>2  C/D</td>
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<td>1/2</td>
<td>5/7</td>
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<tr>
<td></td>
<td>3  E/F</td>
<td>2/3</td>
<td>2/3</td>
<td>6/9</td>
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<td>UCSCs-MC</td>
<td>1  A/B</td>
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Fig. 4f. Semi-quantitative analysis representing histological scoring of Alcian blue stained 3D spheroids. (*) Shows levels of significance with $p < 0.005$ and $n = 3$ donors (per donor 2 samples were analysed).

Fig. 5. Comparison of GAGs/DNA ($\mu$g) between ACs and UCSCs-MC of three weeks old pellets in the presence of TGF-3+β3+BMP-2. Data shown are Mean ± Standard Deviation from three different donors and (*) shows levels of significance with $p < 0.05$. 

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death. On the other hand, cells in UCSCs-MC spheroids were more densely packed, had more irregular and less matrix, characterised by some electron dense structures, vacuole-like structures and few collagen fibrils (Fig. 8b and 9). In addition, HFPSCs and UCSCs-MC were rich in fat droplets (Fig. 8).

UCSCs-MCs chondrogenesis in co-culture conditions
Previous studies have suggested that the anabolic factor BMP-7, also known as osteogenic protein 1 (OP-1), may act synergistically with TGF-β1 inducing chondrogenesis of MSCs from different sources in pellet culture systems (Kim and Im, 2009; Kurth et al., 2007). Based on these previous observations, and considering that our earlier attempts to induce chondrogenesis in UCSCs-MC were unsuccessful, we also studied the response of UCSCs-MC to alternative combinations of growth factors that included BMP-7 (Fig. 3). Once again, none of the new GFs combinations was able to significantly affect chondrogenesis of UCSCs-MCs, as all spheroids were characterised by high cell density, little extracellular matrix and weak Alcian blue staining (Fig. 3).

In a last attempt to induce chondrogenesis from UCSCs-MCs, spheroids were put into co-culture with either cartilage explants or freshly isolated synovial cells (Fig. 10a,b). Results from Alcian blue staining (Fig. 4f) again reveal poor chondrogenic capacity of UCSCs-MCs even in the presence of natural elements of the joint (Fig. 10c,d).

Fig. 6. Relative expression of cartilage signature genes in 3D cultures of UCSCs-MC, HFPSCs and SMSCs compared to 3D cultures of ACs in the presence of best chondrogenic condition. Error bars represent 95 % confidence intervals. The error bar for UCSCs-MC in COL10A1 is not included as only one out of three biological replicate expressed this gene.

Fig. 7. Gene expression of stemness-related transcriptional factors OCT3/4, SOX2 and NANOG in 3D spheroids (3 % O2) and monolayer cultures at both atmospheric (21 %) O2 levels and low (3 %) O2 level. APRT is included for RNA quality control.

Discussion
The main goal in this study was to check the bona fide capacity of stromal cells isolated from umbilical cord to make cartilage in vitro, and to compare it with adult stromal cells harvested from other sources. By the use of different quantitative approaches and different culture conditions, we show here that, in contrast to what is observed with
stromal cells collected from synovial compartments or with chondrocytes, UCSCs-MC display low chondrogenic potential in scaffold-free 3D cultures, even under the influence of differentiated cells from the joint.

In our study, we have used stromal cells harvested from whole cord. It has been shown that MSCs with multipotent abilities may be isolated from different regions of the cord. However, in comparative studies it has been demonstrated that cells from all regions display very similar growth kinetics and immunoprofiles (Mennan et al., 2013; Subramanian et al., 2015). Additionally, these studies reveal that differentiation potential towards adipogenic, osteogenic and to some extent chondrogenic lineages are better achieved by cells from Wharton’s jelly and whole cord. At our laboratory, we have also tested and compared chondrogenic potential of MSCs from Wharton’s jelly and whole cord, both cell sources exhibiting equally low chondrogenic differentiation potential (data not shown). Due to the easier processing, we decided to use stromal cells from whole cord in this study.

Different methods including enzymatic and non-enzymatic have been reported for successful isolation of MSCs from umbilical cords (Choudhery et al., 2013; Mennan et al., 2013). Here we have used a mixed enzymatic-explant method in which tissue biopsies were briefly treated with enzyme to disrupt the membrane followed by explant culture. This method helps to avoid excessive enzymatic digestion and to reduce the time for explant culture. In line with other studies, isolated MSCs demonstrated fibroblast-like characteristics and plastic adherence in \textit{in vitro} culture (Baksh et al., 2007; Vinardell et al., 2012). Unlike most published literatures, we observed UCSCs-MC grow slowly in early passages compared to HFPSCs and SMSCs and their proliferation rate increased after first sub-culture, which was also observed previously from stromal cells isolated from Wharton’s jelly (Chen et al., 2012).

Phenotypic comparison by surface markers of isolated MSCs also demonstrated that UCSCs-MC, HFPSCs and SMSCs positively expressed the classical stromal cell markers CD73, CD90, and CD105, and lack expression of CD11b, CD19, CD34, CD45 and HLA-DR as previously shown in the literature (Dominici et al., 2006). Furthermore, we studied additional markers including CD44, CD106, CD146, CD166 and CD271 to investigate chondrogenic specific lineages. Arufe et al. reported that cell populations collected from human synovial membranes with high expression of CD271 and low expression of

\textbf{Fig. 8.} Transmission Electron Microscopy (TEM) images of ultra-thin sections of 3D spheroids in the best chondrogenic conditions for each cell type. (a) ACs in the presence of TGF-β3+BMP-2, (b) UCSCs-MC in the presence of TGF-β3+BMP-2, (c) HFPSCs in the presence of TGF-β3+BMP-2 and (d) SMSCs in the presence of TGF-β1+BMP-2. Symbols: N = Cell nuclei, (ECM) = Extracellular matrix, (→) = Collagen fibres and (◄) = Fat droplets. Scale bar: 10 µm.
**Fig. 9.** Comparative Alcian blue and TEM images of UCSCs-MC spheroids at low and high magnification. (a) Alcian blue image at lower magnification (Scale bar: 200 µm), (b) Alcian blue image at higher magnification (Scale bar: 50 µm), (c) TEM image at low magnification (Scale bar: 10 µm) and (d) TEM image at high magnification (Scale bar: 5 µm). Symbols: N = Cell nuclei and (→) = Collagen fibres.

**Fig. 10.** Chondrogenesis of UCSCs-MC in co-culture with cartilage explants or cultured synovial cells in the presence of TGF-β3+BMP-2. Schematic images of experimental set up during co-culture (a) with cartilage explants; and (b) with mixed HFPSCs and SMSCs in monolayers. (c) and (d) bright light microscopy images of thin sections of UCSCs-MC spheroids stained with Alcian blue after co-culture. Scale bar: 200 µm and n = 3 donors.
CD106 possess higher chondrogenic potential (Arufe et al., 2010). Additionally, other studies also revealed that expression of CD44, CD146 and CD166 in cell populations from umbilical cord showed differentiation ability towards chondrogenic lineage (Ali et al., 2015; Baksh et al., 2007; Lu et al., 2006). Hence, we checked expression of these additional markers in all MSC cell types. Our results show uniform expression of CD44 and CD166 in all three cell sources, and absolute negative expression of CD106 and CD271. However, we have seen clear differences in the chondrogenic potential between the cells sources, altogether indicating that the set of markers included in this study were not good for predicting chondrogenic differentiation abilities. An exception could perhaps be done with CD146. We observed that over 99% of UCSCS-MCs express this receptor; however, HFPSCs and SMSCs show a subpopulation of cells that are negative for CD146. The predictive value for chondrogenesis of this cell surface marker could be further investigated by sorting positive and negative cells within the HFPSCs or SMSCs populations followed by chondro-induction in 3D cultures.

For chondrogenesis, in our study we have used the pellet culture method in conical-bottom multowell plates, which is relatively easy to perform and permits reproducible outcomes. Additionally, we have used different combinations of growth factors to test chondrogenic potential in vitro in hypoxic environment. The panel of anabolic factors chosen in this study are in line with the most widely used growth factors for chondrogenesis. In our hands, the best combination for chondro-induction of HFPSCs and SMSCs were TGF-β3 and TGF-β1 respectively, in combination with BMP-2 and dexamethasone. These outcomes are also in line with previous studies showing chondrogenic differentiation of HFPSCs and SMSCs (Ding et al., 2015; Vinardell et al., 2012).

In this study, UCSCs-MC showed poor differentiation potential toward the chondrogenic lineage compared with other adult stromal cells, irrespective of the presence of growth factors in the chondrogenic medium. Of note, we have deliberately used quantitative approaches to measure chondrogenesis in different ways, and we have used de-differentiated articular chondrocytes (ACs) as the “gold standard”. When compared to spheroids from ACs, UCSCs-MC demonstrated a lack of matrix production, densely packed cells and poor cartilage like morphology, which was confirmed by Alcian blue staining, collagen type II immunostaining. GAG content and electron microscopy. Furthermore, we studied gene expression analysis of seven cartilage related genes in which favourable articular cartilage gene expression profile includes low levels of COL1A1, COL10A1 and VCAN, while upregulation of ACAN, SOX9, COL2A1 and COL9A1. Compared to ACs, spheroids from UCSCs-MC showed a significant downregulation of COL2A1, ACAN and SOX9, which in turn demonstrate their poor differentiation ability towards a chondrogenic lineage.

In line with our studies, Hildner et al. and Mennan et al. demonstrated poor chondrogenesis UCSCs-MC using pellet culture system, while Wang et al. showed similar results in scaffold-based cultures (Hildner et al., 2010; Mennan et al., 2013; Wang et al., 2009b). Both Hildner et al. and Wang et al. question the hyaline cartilage transformation ability of umbilical cord stromal cells and rather emphasising on their fibrocartilage formation ability. Unlike these studies, chondrogenesis of umbilical cord stromal cells has been demonstrated in other studies using similar culturing conditions (Choudhery et al., 2013; Nirmal and Naïr, 2013; Subramanian et al., 2015). Choudhery et al. demonstrated chondrogenesis of UCSCs isolated by explant method, in pellet culture condition using commercial chondrogenic medium, while Nirmal and Naïr showed chondrogenesis of UCSCs isolated by enzymatic digestion, in scaffold-based culture using different combinations of TGF-β2 and BMP-2. Particular isolation or culturing procedures supporting chondrogenesis of stromal cells from UCSCs cannot be withdrawn from these studies.

Of relevance, none of these studies includes hypoxia during chondrogenesis. A recent study by Reppel et al. demonstrated better chondrogenesis of stromal cells from Wharton’s Jelly in hypoxic condition (Reppel et al., 2014). On the contrary, cell expansion in low oxygen environments has been suggested to maintain UCSCs in undifferentiated state (Drela et al., 2014). In our study, expression of OCT4A, NANOG and SOX2 is elevated in UCSCs-MC during monolayer expansion in both normoxic and hypoxic environments (albeit at higher levels in low oxygen conditions), which evidently shows that cells are kept in undifferentiated state during monolayer growth. Intriguingly, there was no expression of SRTF genes in UCSCs-MC spheroids at low oxygen levels, suggesting a transition of cells towards a differentiated phenotype, which certainly was not cartilage. Taking together, these observations indicate that hypoxia is not responsible for restraining chondrogenesis of UCSCs-MC.

A potential rationale behind the poor chondrogenesis displayed by UCSCs-MC in vitro might rely on low TGF receptor expression induced by BMP-2 stimulation, as demonstrated in adipose tissue derived stromal cells (Hennig et al., 2007). However, changing BMP-2 by BMP-7 as chondrogenic factor had no major effects (Fig. 3). On the other hand, CD105 (endoglin), a member of the TGF receptor complex which binds with high affinity to TGF-β1 and TGF-β3 (Robledo et al., 1996), was found to be expressed by nearly all undifferentiated cell types at both high oxygen and low oxygen conditions (data not shown). Further studies must be undertaken to explore expression of other receptors of the TGFβ family in UCSCs-MC before conclusions can be reached.

Spheroids from HFPSCs and SMSCs displayed an overall favourable cartilage gene expression pattern, much in line with spheroids from ACs. Of relevance, in our gene expression analyses we observe upregulation of genes such as COL10A1 and VCAN in differentiated HFPSCs. Higher expression of COL10A1 and VCAN was reported in earlier studies associated with cartilage hypertrophy and bone development, respectively (Nakamura et al., 2005; Pettari et al., 2006). Such observations should be taken into consideration at the time of choosing HFPSCs as a cell source for cartilage tissue engineering.

In earlier studies, chondrogenesis of MSCs have been enhanced by co-culturing the stromal cells with...
chondrocytes (Fischer et al., 2010). Thus, in a new attempt to induce chondrogenesis from UCSCs-MC, we developed a co-culture system to mimic in vitro intra-articular microenvironments. Once again, UCSCs-MCs spheroids did not develop histo-morphological characteristics of articular cartilage as revealed by metachromasia. Poor chondrogenic potential of UCSCs-MC in our co-culture study could be due to the use of UCSCs-MC and ACs separately rather than using in the same spheroids as previous studies shown (Bian et al., 2011).

Conclusions

Here we disclose poor chondrogenic potential of UCSCs-MC, at least under the conditions tested in the present study. However, it is becoming increasingly accepted that undifferentiated stromal cells might act as adjuvant elements during tissue healing, not by building new tissue themselves but rather by modulating the local environment, making it more favourable for anabolic processes (Liu et al., 2012). Thus, despite their low chondrogenic differentiation potential, stromal cells from the umbilical cord might still be considered for transplantation strategies as facilitators of tissue repair. The precise mechanism of action and the potential therapeutic effects of these cells is yet to be elucidated.

Acknowledgements

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**Discussion with Reviewers**

**Reviewer II:** What do you think are the major differences between your work and the work in other groups that have already shown robust chondrogenic potential of umbilical cord-derived stromal cells?

**Authors:** We have made several allusions to this point throughout the manuscript. In our view, no previous publications are showing “robust” chondrogenic potential of UCSCs. Most of these reports focus primarily on showing multi-lineage differentiation potential of cells, do not focus on chondrogenesis, and the evidences provided are only qualitative, and at its best questionable. The few other reports that provide more quantitative approaches do not compare expression of cartilage signature molecules with native cartilage or other cell sources, so it is not possible to ascertain the true degree of differentiation. For the rest of the reports, the conclusions are that at best UCSCs they are able to differentiate into fibrocartilage but not into hyaline cartilage.

**Editor’s Note:** Scientific Editor in charge of the paper: Brian Johnstone