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The effects of culture conditions on the functionality of efficiently obtained mesenchymal stromal cells from human cord blood

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Abstract

Background aims. Cord blood (CB) is an attractive source of mesenchymal stromal cells (MSCs) because of its abundant availability and ease of collection. However, the success rate of generating CB-MSCs is low. In this study, our aim was to demonstrate the efficiency of our previously described method to obtain MSCs from CB and further characterize them and to study the effects of different culture conditions on MSCs. *Methods.* CB-MSC cultures were established in low oxygen (3%) conditions on fibronectin in 10% fetal bovine serum containing culture medium supplemented with combinations of growth factors. Cells were characterized for their adipogenic, osteogenic and chondrogenic differentiation capacity; phenotype; and *HOX* gene expression profile. The functionality of the cells cultured in different media was tested *in vitro* with angiogenesis and T-cell proliferation assays. *Results.* We demonstrate 87% efficacy in generating MSCs from CB. The established cells had typical MSC characteristics with reduced adipogenic differentiation potential and a unique *HOX* gene fingerprint. Growth factor–rich medium and a 3% oxygen condition enhanced cell proliferation; however, the growth factor–rich medium had a negative effect on the expression of CD90. Dexamethasone-containing medium improved the capacity of the cells to suppress T-cell proliferation, whereas the cells grown without dexamethasone were more able to support angiogenesis. *Conclusions.* Our results demonstrate that the composition of expansion medium is critical for the functionality of MSCs and should always be appropriately defined for each purpose.

Key Words: *angiogenesis, CD90, cord blood, immunosuppression, low oxygen, mesenchymal stromal cell*

Introduction

Mesenchymal stromal cells (MSCs) regulate many important physiological events in our body and can be isolated and expanded from literally all tissues. Isolated MSCs are studied in regenerative therapy for many indications, such as steroid-resistant graft-versus-host disease [1], Crohn's disease [2], in tissue regeneration of bone [3], cartilage [4] and myocardium repair after infarction [5]. In tissue regeneration, to form functional tissues, it is necessary to have vascularization, a process enhanced by MSCs [6,7]. It is well known that MSCs are not a homogenous population; rather, each preparation contains cells sharing only a few common phenotypic markers: CD73, CD90 and CD105. Characteristically, MSCs have the ability to differentiate into mesodermal linages (bone, fat and cartilage) [8]. It has, however, long been debated that not every generated cell population is capable of differentiating into all lineages; instead, specific cell populations vary in their differentiation potential and gene expression profiles [9].

Generation of MSCs from cord blood (CB) is challenging compared with bone marrow (BM) or fat tissue. Many studies using CB report low yield of MSCs, if any, using standard protocols with 20% oxygen and basic medium with fetal bovine serum (FBS) and no additional growth factors [10–15]. CB-derived stromal cells seem to need higher serum content and/or additional growth factors compared

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with, for example, BM-derived MSCs (BM-MSCs) [16–18]. Generation of different cell populations from CB demonstrates that CB contains many cell types [19], and the generation of different populations may be a result of different culture conditions used during cell harvesting and expansion [20]. Despite the development in culture conditions in recent years, there are still difficulties in deriving clinically relevant amount of cells from CB.

Physiological oxygen pressure is much lower than the 20% that is routinely used in cell culture, and it is known that low oxygen is important in glycolytic energy metabolism of stem cells [21,22]. MSCs use glycolytic pathway in their energy metabolism, and this is known to be further enhanced in low oxygen conditions [23–25]. The oxygen pressure seems to be important factor at the beginning of the cell culture, affecting the subset characteristics of cells with typical *HOX* gene expression pattern [26,27]. Because *HOX* genes are transcription factors coordinately regulating genes involved in differentiation of tissues [28], the finding that *HOX* genes could serve as a specific fingerprint of cell types is intriguing.

Primary cells require specific growth conditions that mimic the original niche of the cells. Stem cells originating from different tissues seem to have their own specific growth factor preferences that are necessary for optimal establishment and growth of the cell lines [29,30]. It is difficult to estimate what characteristic is merely a consequence of different growth factors in the medium and which of the observed characteristics are typical for the original cells [31], as it has been shown how medium changes affect the cell characteristics [31,32].

We previously published a method to obtain CB-MSCs using specific combination of growth factors with 3% oxygen and fibronectin coating [33]. Our results here demonstrate the efficacy of the method to obtain adherent cell populations even from small CB units. In this study, we have further characterized the phenotype, *HOX* gene expression and differentiation capacity of the cells. The generated cell populations seem to vary, and thus further studies are needed to fully understand the nature of different cell populations obtained by our method. For clarity, we refer to all the populations obtained as CB-MSCs. Furthermore, we studied whether modifications in culture conditions affect the proliferation, immunomodulation and angiogenesis supporting potential of MSCs.

Methods

CB-MSC establishment

Human CB units were collected at the Helsinki University Central Hospital, Department of Obstetrics and Gynecology, and Helsinki Maternity Hospital. All donors gave informed consent, and the ethical review board of Helsinki University Central Hospital and the Finnish Red Cross Blood Service approved the study protocol. The CB-MSC cultures were established as described previously [33]. Briefly, human CB units were collected after delivery into blood collection bags and processed within 28 h. The mononuclear cells (MCs) were isolated from diluted CB by density-gradient centrifugation either manually (Ficoll-Paque Plus, GE Healthcare) or by Sepax cell separator (Biosafe). MCs were plated in density of $10^6/cm^2$ on fibronectin (FN, Sigma-Aldrich)-coated plates in standard growth medium (StdM) consisting of alpha-MEM Glutamax (Life Technologies, Thermo Fisher Scientific), 10% FBS (Life Technologies), 50 nmol/L dexamethasone (DX, Sigma-Aldrich), 10 ng/mL epidermal growth factor (EGF, Sigma-Aldrich), 10 ng/mL platelet derived growth factor–BB (PDGF-BB, R&D Systems, Inc.) and 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies). The cells were incubated at 37°C in 5% CO_2, 3% O_2 in a humidified atmosphere. The medium was replaced next day and twice a week thereafter until the first passage. The cells were detached with trypsin–ethylenediaminetetraacetic acid (EDTA) solution (0.25%, 1 mmol/L, Life Technologies) and replated for expansion.

Cell culture procedures

To study the effects of culture conditions, the cells were cultured in several media. StdM was modified as follows: Medium 1 (M1) as StdM but FBS level reduced to 5% and excluding DX and EGF and Medium 2 (M2) as StdM but FBS level reduced to 5% and excluding DX. Medium 3 (M3), which was used in conditioning purpose for angiogenesis assay, was as StdM but FBS level reduced to 5% and excluding PDGF-BB (Table I). When the cells were cultured in different conditions for the period of same time, higher seeding densities (1500–3000 cells/cm^2) were used for cells cultured in M1 and M2 compared with seeding density in StdM (700–1500 cells/cm^2) to reach similar confluency (<90%) at the day of harvesting. Studies were performed using at least two donor cell lines.

Table I. Media supplement compositions.

Supplement	StdM	M1	M2	M3
FBS	10%	5 %	5 %	5 %
DX	50 nmol/L	—	—	50 nmol/L
EGF	10 ng/mL	—	10 ng/mL	10 ng/ml
PDGF-BB	10 ng/mL	10 ng/mL	10 ng/mL	—

Each media was alpha-MEM Glutamax–based medium with penicillin-streptomycin and the supplements listed in each column.

To investigate the long-term effects of culture conditions on the growth of CB-MSCs, the cells from passage 2 on were cultured in StdM and M1 medium in two oxygen conditions (3% O_2 and 20% O_2). The cells were detached before reaching 80% confluency and replated 1000 cells/cm^2.

Differentiation assays

Differentiation potential was tested with all cell batches at early passage (p2–p5) and with some batches also additional differentiation tests were performed at later passages (up to p14). The traditional adipogenic differentiation was performed differentiating the cells plated at 10 000 cells/cm^2 in Dulbecco's Modified Eagle's Medium high glucose (Lonza), 10% FBS (Life Technologies), 100 U/mL penicillin/streptomycin, 2 mmol/L L-glutamine (both from Lonza) 1 μmol/L DX (Sigma-Aldrich), 0.2 mmol/L indomethacin (Fluka), 0.1 mg/mL insulin (Sigma-Aldrich) and 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX, Fluka) as previously described [26].

To assess the osteogenic and adipogenic potential of the CB-MSCs with an enhanced adipogenic differentiation method, the cells were cultured in 12-well plates in StdM at 37°C in 5% CO_2 in a humidified atmosphere in incubator until the cultures reached confluency. For enhanced adipogenic differentiation, the medium was changed into adipogenic induction medium for 2–3 days, after which the cells were incubated in terminal adipogenic medium for additional 10–26 days with medium change twice a week. The induction and terminal differentiation media consisted of the same basal medium containing alpha-MEM Glutamax, 10% FBS, 20 mmol/L HEPES, penicillin-streptomycin (all from Life Technologies), 0.5 μg/mL insulin (Promocell) and 0.1 mmol/L indomethacin (Sigma-Aldrich). The induction medium contained also 0.2 mmol/L IBMX and 0.4 μg/mL DX (both from PromoCell). In terminal differentiation medium, the basal medium was supplemented with 3 μg/mL of ciglitazone (PromoCell).

For osteogenic differentiation, the medium was changed into osteogenic medium for 12–31 days, with medium change twice a week. The osteogenic media consisted of α-MEM supplemented with 10% FBS, 20 mmol/L HEPES, 2 mmol/L L-glutamine (all four from Life Technologies), 0.1 μmol/L DX, 10 mmol/L β-glycerophosphate, 0.05 mmol/L L-ascorbic acid-2-phosphate (all three from Sigma-Aldrich) and penicillin-streptomycin (Life Technologies).

After differentiation period the cells were fixed with 4% paraformaldehyde and stained with Sudan III to detect adipogenic differentiation and to demonstrate the osteogenic differentiation the accumulated mineralized calcium phosphate was stained with silver nitrate by method of von Kossa.

For chondrogenic differentiation, approximately 200 000 cells were placed in 15 mL polypropylene tube and pelleted into micro-masses by centrifugation at 400*g* for 5 min. The pellets were cultured for 2 weeks in incubator in 20% oxygen conditions in chondrogenic media that consisted of Dulbecco's Modified Eagle's Medium–HG (containing 0.1 mmol/L pyruvate, Life Technologies), supplemented with 10 ng/mL transforming growth factor (TGF)-β, 100 nmol/L DX, 0.1 mmol/L L-ascorbic acid-2-phosphate, 40 μg/mL L-proline (all from Sigma-Aldrich), 1 × ITS + premix (BD Biosciences) and penicillin-streptomycin (Life Technologies). The medium was changed twice a week. For staining, the cell pellets were fixed with 10% formalin, embedded in paraffin and cut into sections that were stained with Alcian blue (Sigma-Aldrich) and with Nuclear fast red (Merck).

Immunophenotypic characterization

For phenotypic characterization by flow cytometry (FACSAria, Becton Dickinson), the cells were briefly trypsinized with trypsin-EDTA or with Tryple-Express (Life Technologies) solution and suspended in staining buffer, 0.3% BSA (Sigma-Aldrich) in 2 mmol/L EDTA-PBS. Fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) conjugated antibodies against CD13, CD14, CD19, CD29, CD31, CD44, CD45, CD49e, CD73, CD106, CD166, HLA-DR, HLA-ABC (all from BD Pharmingen), CD34 (Miltenyi Biotec GmbH or BD Pharmingen), CD90 (StemCell Technologies or BD Pharmingen) and CD105 (Abcam) were used for direct labeling. Appropriate FITC-, PE- and APC-conjugated isotypic controls (BD Pharmingen) were used. The characterization was performed for all cell batches at early passage (p2–5) and in long-term cultures also at later passages (up to p14).

HOX *polymerase chain reaction*

To study the *HOX* gene expression of the obtained cell populations, total RNA was extracted from cells in a 40-μL volume applying the RNeasy Kit (Qiagen) according to the manufacturer's instructions. Determination of RNA concentrations was carried out by applying a NanoDrop device (NanoDrop Technologies). Reverse transcription was performed for 1h at 50°C using the First-strand cDNA Synthesis Kit (Life Technologies) and the enclosed oligo(dT)20 primer. Approximately 1000 ng total RNA was converted into first-strand cDNA in a 20-μL reaction. All control reactions provided with this system were carried out to monitor the efficiency of cDNA-synthesis. Before polymerase chain reaction (PCR), the completed first-strand

reaction was heat-inactivated at 85°C for at least 10 min. Finally, cDNA was treated with 1 µL RNaseH according to the manufacturer's protocol.

Reverse transcriptase (RT)-PCR was carried out with intron-spanning primers (Thermo Scientific) specific for each *HOX* gene and *GAPDH* as reference gene [27]. Approximately 50 ng of cDNA were used for subsequent RT-PCR analysis in a total volume of 25 µL containing 1 × PCR buffer, 0.2 µmol/L of each primer, 0.75 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP and 1 U *Taq* DNA Polymerase (Life Technologies) at the following conditions: (i) 2 min at 95°C for initial denaturation and *Taq* Polymerase activation, (ii) 30 sec at 95°C, 30 sec at 56°C, (iii) 30 sec at 72°C for 35 cycles, 5 min at 72°C for final extension of PCR products. PCR was performed on a Mastercycler ep gradient S (Eppendorf). Subsequently, aliquots of the RT-PCR products and related controls were analyzed on a 2% agarose/TBE gel by electrophoresis. The *HOX* gene expression was determined from passage 3–7 cells.

Quantitative real-time PCR of VEGF

For vascular endothelial growth factor (VEGF) gene expression studies RNA was extracted from passage 6 cells by RNeasy Mini Kit (Qiagen). Dnase-I treatment was performed during RNA extraction with Rnase-Free DNA set (Qiagen) according to manufacturer's instructions. Quality and concentration of RNA was verified by Nanodrop 1000 (NanoDrop Technologies). For all samples, equal amount of RNA was transcribed with Multiscribe reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Life Technologies). Quantitative PCR analysis was performed with 50 ng of cDNA in 25-µL reaction by using *VEGF-A* Hs00900054_m1 Taqman Gene expression assay probe set (Life Technologies) *according* to manufacturer's recommended protocol with Bio-Rad CFX9 real-time PCR detection system (Bio-Rad Laboratories). Reactions omitting transcriptase were used as negative controls demonstrating absence of genomic DNA contamination.

Data were analyzed with a Bio-Rad CFX manager gene expression analysis software (Bio-Rad Laboratories) using relative quantification of target genes versus TATA-box binding protein as reference gene with normalized calibrator and with efficiency correction method using Pfaffl method, a variation of Livak and Schmittgen's 2(-Delta Delta C(T)) method [34]. PCR efficiency corrections were determined for target and reference genes by establishing of standard PCR curve using dilution series of pooled cDNA samples.

VEGF ELISA

The VEGF levels were measured with Human VEGF Quantikine ELISA Kit (R&D Systems). Cells initially cultured in StdM and M1 were transferred into M3 (p6) at a density of 40 000 cells/cm^2. The media for VEGF quantification was collected after 21-h incubation and assayed for VEGF according to manufacturer's protocol.

Angiogenesis assay

An *in vitro* tubule formation assay of endothelial cells was performed to investigate whether cells primed under different conditions would support angiogenesis differently. The assay was performed by exposing the angiogenesis assay with conditioned media (CM) of the p6 cells that were initially cultured in StdM or M1 for 6 days and then in M3 for 21 h.

For tubule formation, co-culture assay of fibroblasts and human umbilical vein endothelial cells (HUVECs) was established. The assay was set up as described by Sarkanen et al. [35]. Briefly, BJ fibroblasts (American Type Culture Collection, CRL-2522; ATCC) were seeded at a density of 20 000 cells/cm^2 into 48-well plates and grown for 3 days. Next, the HUVECs were seeded on the top of fibroblast cultures at a density of 4000 cells/cm^2 in EGM-2 bullet kit medium (Lonza). The day after plating HUVECs, co-cultures were induced with different CM. Basic test medium (BTM), which consisted of endothelial cell basal medium (EBM-2, Lonza) supplemented with 0.1% gentamicin (GA-1000, Lonza), 2% FBS and 1 mmol/L L-glutamine and BTM supplemented with 10 ng/mL VEGF (R&D Systems) and 1 ng/mL basic fibroblast growth factor (bFGF, R&D Systems) were used as negative and positive controls, respectively. The CM samples as well as positive and negative controls and M3 medium control were added to the angiogenesis assay in 500-µL volume. The media was changed twice during the culture before immunocytochemical staining at the day 6 of co-culture. The assay was performed in 20% oxygen conditions. The immunostaining with anti–von Willebrand factor (anti-vWF) was performed as previously described [35].

The number and length of the endothelial tubules were quantified microscopically and by using a predetermined scale [35] and compared with negative (value 0, no tubule formation) and/or positive control (VEGF/bFGF induction, average value of at least 5.75 tubule formation seen throughout the wells) and scaled accordingly.

T-cell proliferation assay

To test the immunosuppressive capacity of differently primed MSCs, the cells (p4–p6) were cultured before the assay in StdM, M1 or M2 conditions. MSCs were detached with trypsin, and 0.75×10^5 cells were suspended in RPMI 1640 medium supplemented with

5% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Life Technologies) and plated in 48-well plates.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by Ficoll-Paque Plus (GE Healthcare) gradient centrifugation and labelled with 2.5 µmol/L CFSE [5(6)-carboxy-fluorescein diacetate N-succinimidyl ester] solution (Molecular Probes) in 0.1% HSA-PBS (human serum albumin, Sanquin) for 5 min at room temperature. 1.5×10^6 labeled PBMCs were then added to the co-culture. For T-cell activation 0.1 µg/mL of CD3 antibody (clone Hit3a, BioLegend) was added to the wells. T-cell proliferation was recorded after 4 days of co-culture as dilution of fluorescent dye by flow cytometry (FACSAria, BD) and data were analyzed using the FlowJo software (7.6.5 Treestar). The proliferation of PBMCs without MSCs was designated as 100%.

Statistical analysis

The statistical analysis was performed with GraphPad Prism 5.0 software (GraphPad). Data from angiogenesis experiments is shown as mean + SD. Statistical significance was analyzed with one-way analysis of variance and Tukey's post hoc test ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Data from T-cell proliferation experiments is presented as mean ± SEM. Statistical significance was calculated by two-tailed Student's *t*-test ($*P < 0.05$, $**P < 0.01$).

Results

CB-MSCs were established efficiently in 3% oxygen conditions with rich growth factor combination

MSCs were produced from 87.5% of the CB units studied (14 of 16 units). From some CB units, different populations were established and kept apart. These populations were marked with a suffix at the end of CB-unit code, such as 454T(6) and 454T(7). The volumes of collected CB units were from 40 to 101 mL and the MC number varied from 162×10^6 to 714×10^6. There was no difference to obtain MSCs from MC separated manually versus Sepax separation. The number of MSCs after passage 1 was from 0.1×10^6 to 21.4×10^6 (Table II). No correlation was observed between CB unit volume or MC number and MSC amount in passage 1 ($R^2 = 0.0374$ and $R^2 = 0.0023$, respectively).

The cells obtained had typical spindle shaped morphology. Cells cultured in M1 medium had more elongated morphology compared with cells cultured in StdM. Oxygen condition did not remarkably affect the morphology of the cells (Figure 1). The cells had typical MSC phenotype (negative for CD14, CD19, CD31, CD34, CD45, CD106 and HLA-DR and positive for CD13, CD29, CD49e, CD73, CD90 CD105, CD166 and HLA-ABC), and they showed the capacity to differentiate into adipocytes, osteoblasts and chondroblasts (Figure 1). With standard method [26] adipocyte differentiation was low or undetectable, but with the enhanced differentiation method in the

Table II. CB unit characterization and MSC gain from different units.

CB Unit code	Time before processing (h)	CB unit volume (mL)	MC number (10^6)	MSCs Acquired (yes/no)	Duration of culture at p0 (days)	Cell count at p1 10^6
391P	23.5	90	376.7	yes	9	1.5
392T	27.5	75	360.2	yes	13	ND
397P	19.5	75	360	yes	22	ND
452T	43	46	334.5	no	12	ND
454T	22	70	422	yes	10	0.3
457P	28	68	320	yes	10	0.1
553T	28.5	60	162	yes	15	1.2
582T	24	101	386	yes	13	6.3
585T	21	40	360	no	21	ND
588P	22	42	714	yes	15	2.3
594P	21	43	430	yes	19	7.3
606P	21.5	42	567	yes	16	15.2
609P	21.5	43	344	yes	19	6.3
611T	19.5	45	337.5	yes	15	2.6
613T	19	60	228	yes	12	21.4
618P	22	42	420	yes	20	4.3
Mean	23.97	58.88	382.61		15.06	5.72
SD	5.88	19.23	124.61		4.12	6.49

ND, no data. The cells were passaged on new plates without cell counting because of the low cell numbers observed by microscopy.

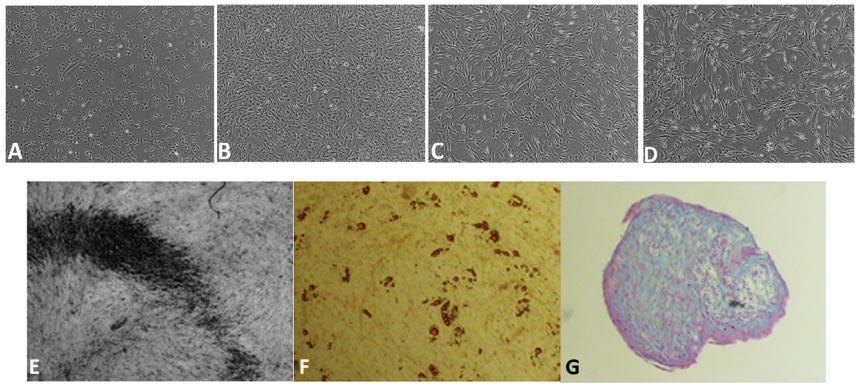

Figure 1. Representative photographs of cell morphology in different conditions and the tri-lineage differentiation of CB-MSCs. Passage 6 cells in (A) StdM 3% O_2, (B) StdM 20% O_2, (C) M1 3% O_2, (D) M1 20% O_2, 40 × magnification. (E) von Kossa staining of osteogenic differentiated cells, 20 × magnification. (F) Sudan III staining of cells differentiated with enhanced adipogenic differentiation method, 100 × magnification. (G) Alcian blue staining of chondrocyte differentiated cells, 100 × magnification. Differentiation results shown are from passage 9 cells.

presence of 3 µg/mL ciglitazone, adipogenic differentiation was detected with 11 of 13 MSCs. Osteogenic and chondrogenic differentiation was detected with 10 of 12 and 5 of 10, respectively (Table III).

Distinct HOX *expression profiles of CB-derived cell lines*

The *HOX* code has been introduced as a marker to distinguish CB-derived cell types, such as *HOX*-positive CB-MSC and *HOX*-negative USSC [27]. To characterize the cell lines applied here, the expression of 39 *HOX* genes distributed among four clusters (ABCD) was tested by PCR. Examples of individual cell lines are depicted in Figure 2, revealing specific *HOX* codes. On the basis of the typical expression pattern, four main characteristics could be observed: individual cell lines show either expression in all four *HOX* clusters ABCD (CB-MSC 582T and CB-MSC 454T), *HOX* cluster ACD (CB-MSC 391P and CB-MSC 392T), *HOX* cluster BCD (CB-MSC 397P), or little expression in any cluster (CB-MSC 457P(2) and CB-MSC 588P(1)). To define whether individual cell lines change their inherent *HOX* code, cells were cultivated over two passages and tested for *HOX* gene expression again exemplarily. The results given in Figure 2B confirm the stability of the inherent *HOX* code. Only marginal differences on single gene level are detectable, for example, the gain of *HOXA13* expression in CB-MSC 391P. However, if expression was detected within a single cluster, genes of the same cluster were still expressed after two passages.

Rich growth factor composition and 3% oxygen support MSC growth

The cells were cultured in StdM and in M1 in two oxygen concentrations (3% and 20%) to see the long-term proliferation capacity of the cells. The cells were capable of proliferating up to 17 passages. Although there were differences in the proliferation capacity of

Table III. Differentiation results of CB-MSCs.

CB-MSCs	Osteogenic differentiation	Enhanced adipogenic differentiation	Traditional adipogenic differentiation	Chondrogenic differentiation
391P	+	+	–	+
392T	+	–	–	+
397P	–	+	–	+
454T(6)	+	+	+	–
454T(7)	+	+	–	+
457P(1)	+	+	ND	–
457P(2)	+	+	–	–
553T	–	+	ND	+
582T	+	+	–	ND
588P(1)	+	+	–	ND
588P(2)	ND	+	–	–
594P(1)	+	–	ND	ND
594P(2)	+	+	ND	–

ND, no data.

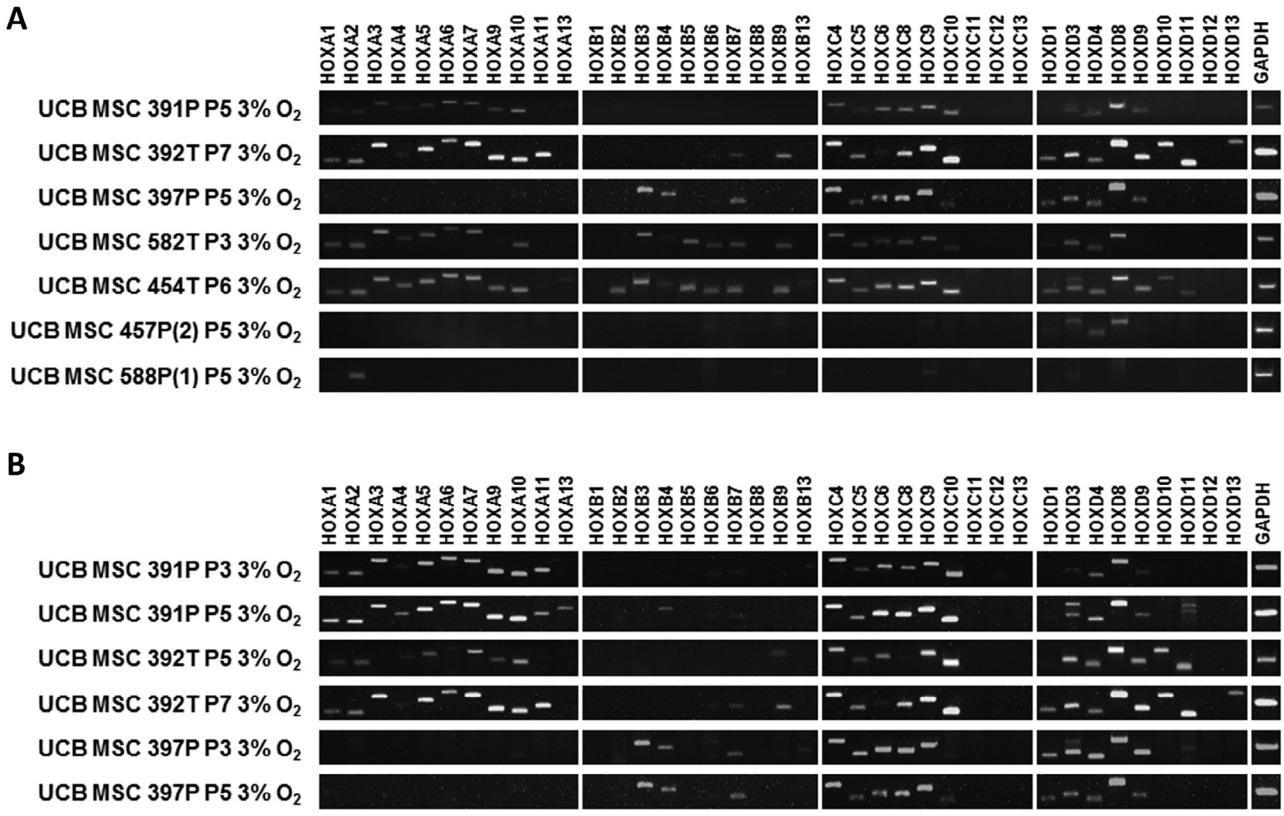

Figure 2. Representative *HOX* expression signatures of CB-derived cell lines (n = 7). (A) RT-PCR results show *HOX* gene expression within either HOXABCD (n = 2), HOXACD (n = 2), HOXBCD (n = 1) clusters, or no or marginal expression, respectively, in all *HOX* clusters (n = 2). (B) Stability of *HOX* expression signatures over two passages. With representative cell lines (n = 3), no relevant change of the typical inherent signature was observed.

the cells from different CB units, the cells reached higher population doubling (PD) numbers when cultured in StdM and in 3% oxygen conditions compared to cells cultured in M1 in 20% O_2 condition (Figure 3). The beneficial effect of the growth factor rich medium was more obvious than the effect of 3% oxygen on cell proliferation. On the contrary the cells in growth factor rich medium had a negative impact for the expression of CD90 as it decreased on StdM-cultured cells during passaging (Figure 4).

CB-MSCs cultured without DX produce more VEGF, supporting angiogenesis

The expression of VEGF mRNA was studied in cells cultured in various media for 5 days. The VEGF mRNA expression was up-regulated in cells cultured in media without DX, thus in M1 and M2 (fold-change difference values 3.7 and 4.3, respectively; Figure 5A). The media with the richest and poorest growth factor content (StdM and M1) were chosen for additional assays as the difference between M1 and M2 was minimal. The VEGF production analyzed by ELISA from cells initially cultured in two media (StdM and M1) and then transferred into same M3 medium for 21 h showed that the cells primed in M1 secreted more VEGF than the cells primed in StdM (Figure 5B).

Accordingly the CM of M1 primed cells induced significantly more tubule formation than the CM of StdM-primed cells ($P < 0.001$, Figure 5C) in the *in vitro* tube formation assay. CM of StdM primed MSCs from CB unit 454T(7) induced significantly more tubule formation than M3 control medium ($P < 0.01$), whereas the MSCs from CB unit 391P in the respective media did not. This was the only difference in the results between MSCs from different CB units. CM of M1-primed cells induced significantly more tubule formation than the M3 control medium ($P < 0.001$), induced 80% of the tubule formation of positive control, and the induction was 50% higher than the M3 control (Figure 5C). The M3 control induced significantly less tubule formation than positive control ($P < 0.001$).

StdM-primed cells have higher capacity to suppress T-cell proliferation

To determine the effect of various priming media on immunosuppressive capacity of MSCs, co-culture of

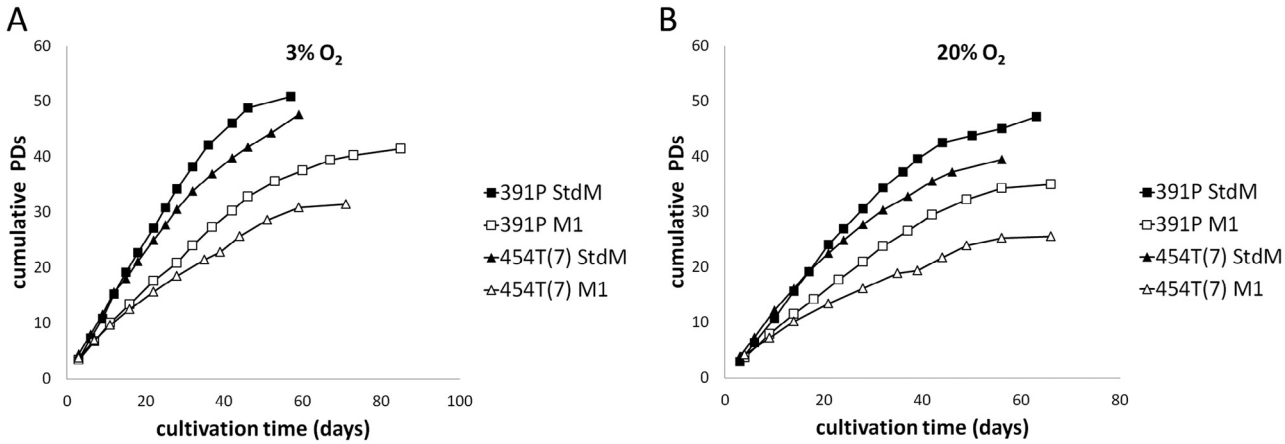

Figure 3. Comparative long-term culture of cells from two CB units cultured in 3% and 20% O_2 conditions in StdM and M1 (n = 2). Cells cultured in 3% O_2 reached higher PD numbers in less time than cells cultured at 20% O_2 conditions. (A) In 3% O_2, cells in StdM from CB unit 391P reached 51 PDs within 57 days, and cells from CB unit 454T(7) reached 48 PDs within 59 days. In M1, the cells from CB unit 391P reached 42 PDs within 85 days, and cells from CB unit 454T(7) reached 32 PDs within 71 days. (B) In 20% O_2, cells in StdM from CB unit 391P reached 47 PDs within 63 days, and cells from unit 454T(7) reached 40 PDs within 56 days. In M1, the cells from CB unit 391P reached 35 PDs within 66 days and cells from CB unit 454T(7) reached 26 PDs within 66 days. The comparative long-term culture was performed after passage 2.

MSCs with PBMCs was performed. MSCs primed in StdM medium (DX-containing medium) before the assay had considerable immunosuppressive capacity (~70% decrease in T-cell proliferation compared with proliferation control). However, when MSCs were primed in M1 or in M2 (media without DX), the immunosuppressive capacity was significantly decreased ($P < 0.01$ and $P < 0.05$, ~50% and ~35% decrease in T-cell proliferation compared with proliferation control, respectively; Figure 6).

Discussion

MSCs can be isolated from many tissues [16,36–38], and there has been an interest in CB as an MSC source because this material is abundantly available and generally discarded. However, obtaining MSCs from CB is problematic [15] because the number of MSCs is not high. The success rate of generating MSCs from CB has turned out to be low or non-existent [12,15,39–41]. The inefficiency to obtain MSCs from CB affects its suitability for clinical applications. With our method, MSCs were received from 87.5% of processed units. This is much higher proportion compared with previously published methods, with success rates of 10–63% [10,14,16,42–46]. It has been claimed that the rate of generating MSCs from CB could be higher if the units were of optimal quality and the storage time before handling were short enough [10,40]. Zhang et al. reported that MSCs could be obtained from 90% of CB units if the units were processed within 2 h and the volume of the unit was at least 90 mL [47]. Similar limits with storage time and CB volume have been noticed by many others (e.g., MSCs were not obtained from units stored >6 h after collection) [40,42], and suggestions for optimal volume has been >80 mL [40]. With our method, the rate of generating MSCs from CB was almost 90%, although all CB units were processed at least 19 h after collection (mean 23.97 ± 5.88 h), and the mean volume was 58.88 ± 19.23 mL. A higher success rate in obtaining MSCs from CB has been reliably reported only with equine CB [48].

The methods of isolating and culturing CB-MSCs vary. Some methods are based on distinct separation procedures, such as hematopoietic lineage–negative, glycophorin A–negative or CD133-positive cell selection [11,18,49], but most of the methods rely on traditional MC separation. Also, various basal media with variable supplements and concentrations are used. FBS is used in concentrations from 10 to 30% [10,16,17]. Other supplements that have been used to support MSC growth are bFGF, PDPG-BB, DX, EGF, TGF-β, IL3, SCF, ascorbic acid, Wnt3, transferrin and interleukin-6 [18,47,50–52]. Coating plastic ware with various molecules has also been used to enhance MSC gain. FBS coating has shown to decrease the adherence of monocytes on the substrate [10] and thus reduce the possible inhibitory effects of monocytes on MSC proliferation. FN coating has shown to support MSC adherence to the substrate and the gain of MSC progenitors [50,53,54]. Also DX as a medium supplement inhibits the adhesion of monocytes on culture plates [55], and thus it has been used in the beginning of CB-MSC cultures [17,47]. Low concentrations of corticosteroids (e.g., hydrocortisone and DX) as medium supplements have also

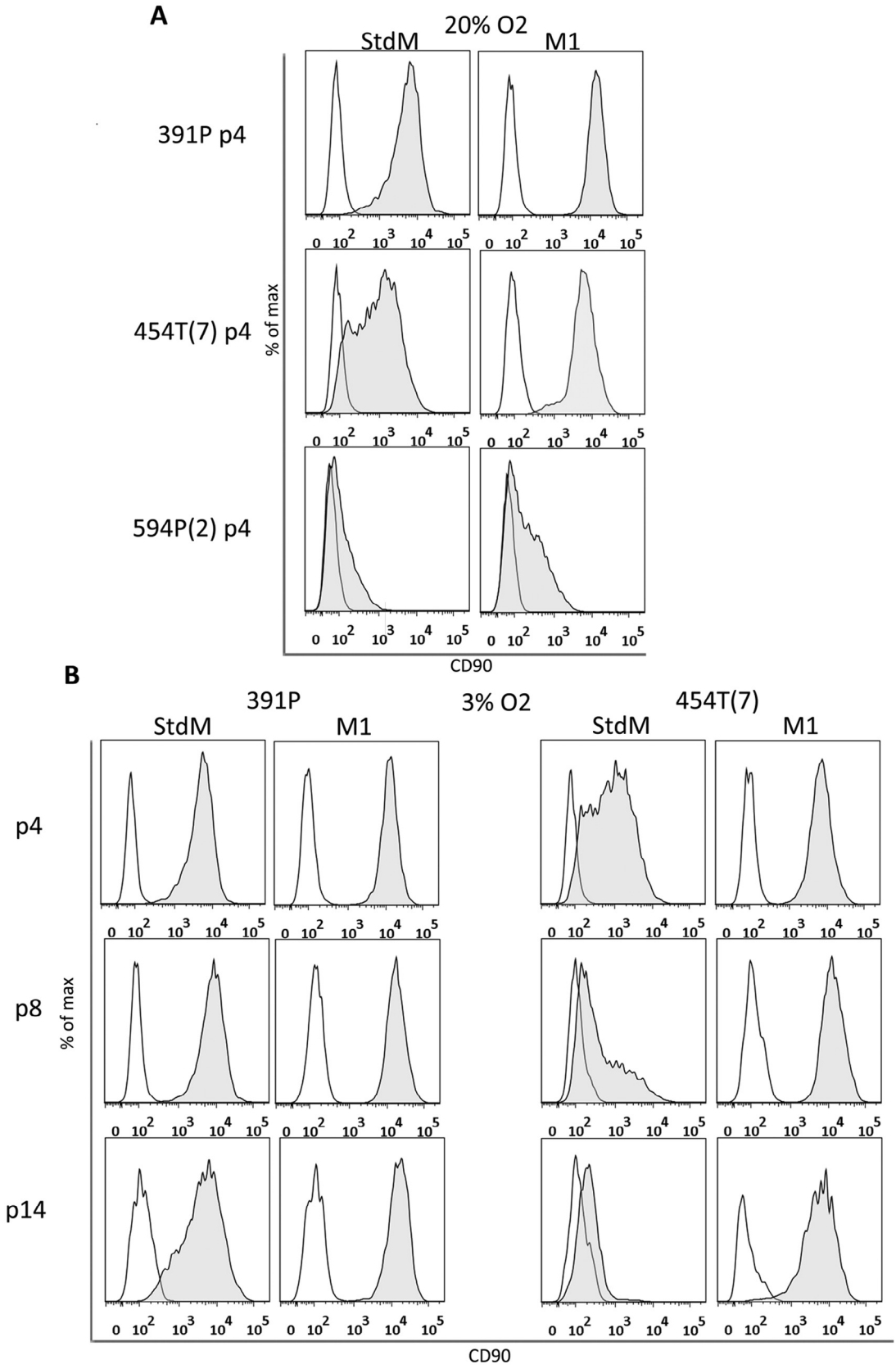

Figure 4. The representative histograms of CD90 expression (n = 3). (A) The expression level of CD90 varied among umbilical-MSCs. StdM-cultured cells expressed CD90 at lower levels compared to M1-cultured cells. (B) The expression of CD90 declined during passaging on StdM-cultured cells. Filled histograms represent the specific staining with FITC labeled anti-CD90 antibody, and the empty histograms represent the unspecific/isotype control staining.

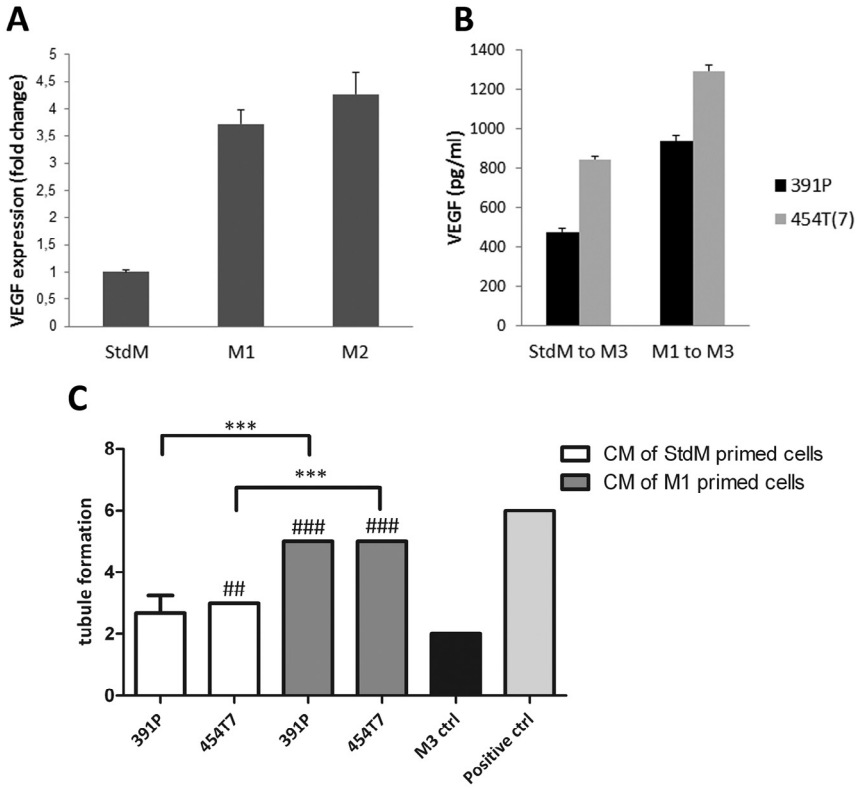

Figure 5. Angiogenic supporting capacity of differently cultured passage 6 cells. (A) Quantitative RT-PCR results on VEGF expression of differently cultured cells. (B) VEGF production of differently cultured cells (in either StdM or in M1) transferred into same medium (M3) for 21 h. (C) The angiogenic supporting potential of CM from the cells primed with StdM or M1 and then transferred into M3 condition (n = 2). Results are shown as mean ± SD. ***$P < 0.001$, indicating the difference between CM of StdM and M1 primed cells, ###$P < 0.001$, ##$P < 0.01$, indicating the difference between treatments compared to M3 medium control.

shown to increase the proliferation of MSCs, particularly at low cell densities [50,56,57]. In our current method, we used MC separation by density-gradient centrifugation and FN coating of the culture plates. We concluded, on the basis of the literature, to use a combination of growth factors (EGF and PDGF) and glucocorticoid (DX) in addition to 10% FBS in culture medium at the beginning of the culture because the traditional MSC medium supplemented only with FBS does not seem optimal for obtaining MSCs from CB. Veevers-Lowe et al. [58] have demonstrated the positive effect of the combination of FN coating and

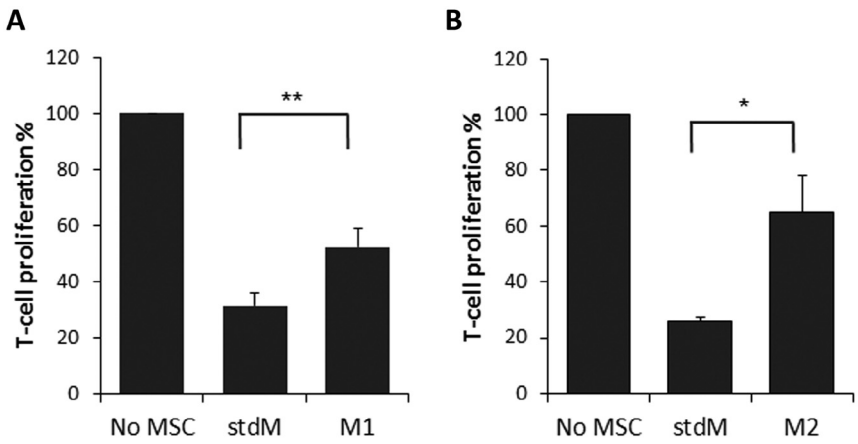

Figure 6. (A) MSCs cultured in M1 have decreased capacity to suppress T-cell proliferation compared with MSCs cultured in StdM. Results are mean ± SEM (n = 15). **P < 0.01. (B). MSCs cultured in M2 have decreased capacity to suppress T-cell proliferation compared with MSCs cultured in StdM. Results are mean ± SEM (n = 3). *P < 0.05.

PDGF and EGF has been shown to maintain the colony-forming capacity of the cells, thus helping to preserve early progenitors of MSC populations [59]. This attachment factor and growth factor combination together with 3% oxygen condition used in our current study proved out to be very efficient method to obtain MSCs from CB.

Because the oxygen concentration in incubators in normal atmosphere is considerably higher than the physiologic oxygen condition (20% vs 2–13%) [60,61] and because the gain of MSCs from CB had been low in 20% oxygen conditions in our previous attempts, we concluded to use 3% O_2 condition in the beginning of culture. Low oxygen condition improves the growth of many cell types, especially stem cells [62–65]. Although the gain of MSCs from CB at 3% O_2 was good and it decreased the PD time, the clear benefit of 3% oxygen for cell proliferation was seen only at late passages. Similar observations have also been noticed by others [66].

The long-term culture of cells indicated the capacity of growth factors to support the proliferation of cells because StdM-cultured cells proliferated more rapidly and reached higher PD numbers compared with cells cultured in M1. However, culturing the cells in StdM for extended passages had a negative impact on the phenotype of the cells as the expression of CD90 decreased on these cells. This was seen in all cells studied, although individual variation was observed. Phenotype changes in long-term culture in the expression of CD105 and CD146, but not with CD90, have been detected in the presence of growth factors [51]. The decrease or low expression of CD90 has been associated to differentiation process of CB-MSCs [40] and to diminished immunosuppressive activity of MSCs on T-cell proliferation. Thus, CD90 has been suggested to act as a marker for undifferentiated cells [40,67]. In our studies, we did not test the immunosuppressive capacity of the cells at late passages when the expression of CD90 was detected to be low. Low expression of CD90 on CB-MSCs has been reported by several groups [14,68,69], and Montesinos et al. [14]. suggested that CD90 is not a specific "mesenchymal" marker. The loss of CD90 expression has also been associated with the transformation of the cells [12], although this has in many cases turned out to be a false result due to contamination of cell cultures with tumor cell lines [70,71]. In the current study, we show that culture medium with high content of growth-supporting factors has a negative effect on CD90 expression.

MSCs are a heterogenic cell population [20]. Because CB units are variable in their cell content, different MSC enrichment conditions are likely to produce populations with different characteristics depending on which cells are present in the starting material and capable of proliferating. The frequency of MSCs in CB is much lower than in BM (0.002 and 83 colony-forming unit fibroblasts/10^6 MC) [72], and thus the CB-MSC cultures may differ from unit to unit more than BM-MSC cultures with regard to their differentiation capacity and phenotypic characteristics [19]. In our studies, the MSCs from a few CB units had complete tri-lineage differentiation capacity, whereas there were MSCs from some CB units showing more prone differentiation toward one or two of the differentiation lineages. The adipogenic differentiation was clearly enhanced in the presence of PPARγ ligand (ciglitazone) during terminal differentiation. With the traditional differentiation method, adipogenesis was poor, which is typical for CB-MSCs [12,14,72,73].

HOX-gene expression profile also indicated the heterogeneity of populations received from different CB units. Cells from different CB units had different *HOX* gene expression profiles, and this did not considerably change during passaging. However, the heterogeneity regarding individual *HOX* expression patterns does not depend on individual units but seems to be related to specific cell subtypes within a single unit because different *HOX* signatures can be generated from a single unit on clonal level [9]. *HOX* genes are set on and expressed when cells/tissues are directed into their specialized terminal stage. They are turned on in a coordinated manner, and there are specific *HOX* genes regulating differentiation of tissues and organs. Interestingly, the typical *HOX* expression pattern of murine MSC refers to the topographic origin of the cells analyzed [74]. Therefore, typical *HOX* codes determined here for CB-derived cell lines may as well account for their developmental origin or define individual subsets of cells. It has already been indicated that different subpopulations of CB stem cells can be distinguished from each other by their differentiation capacity and their differential expression of *DLK-1* and *HOX* genes [9,26]. For example *HOX* genes like *HOXA9*, *HOXB7*, *HOXC10*, *HOXD8* are expressed typically in CB-MSCs [26,27]. All of our cell lines studied expressed *HOXC10* and *HOXD8* but had variation in their *HOXA9* and *HOXB7* expression. In addition, they expressed several other *HOX* genes depending from the cell line. This indicates that these cells may not be CB-MSCs but that we obtained different subpopulations of the MSC-like cells, yet to be characterized. More detailed studies are needed to clarify whether the differently named cells from CB are related or they are truly of different origin and should thus be named more precisely than simply MSCs.

Different processing protocols have been suggested to modify MSCs to be particularly suited for a specific clinical indication [32]. In our study, we

showed that CM of differently cultured cells had distinct potential to support angiogenesis. Several culture conditions have been demonstrated to induce the angiogenic capacity of MSCs, for example, hypoxia, the addition of TGF-α or tumor necrosis factor (TNF)-α into culture media [75–77] and aggregate culture of MSCs [78]. It has been suggested that MSCs can support angiogenesis via paracrine mechanisms and through direct endothelial differentiation [79,80]. In our studies, the expression and secretion of VEGF, one of the angiogenesis supporting factors, was higher in cells cultured in media without DX (M1 and M2) compared with cells cultured in the presence of DX (StdM). Accordingly, the cells primed in medium without DX (M1) had superior angiogenic potential in an *in vitro* assay compared with the cells primed in the presence of DX (StdM), even though the assay medium was the same. The angiogenic potential of MSCs makes these cells interesting candidates in restoration of cardiac function after myocardial infarction [5,81–85]. The capacity of MSCs to support angiogenesis has been improved by making them overexpress VEGF through genetic engineering [83]. Because the genetic manipulation of MSCs is demanding and controversial, altering the culture conditions is more a straightforward method to improve the capacity of cells to support angiogenesis.

In our studies, MSCs primed in the presence of DX had greater capacity to suppress T-cell proliferation in an *in vitro* assay. Our findings are supported by results from studies by Ankrum et al. [86], who showed that glucocorticoid steroids, such as budesonide and DX, significantly boost the indoleamine-2,3-dioxygenase (IDO) activity of MSCs. IDO activity has been presented as a major factor contributing to the suppressive effect of MSCs in an *in vitro* MSC-PBMC co-culture assay [87]. On the other hand, Wang et al. [88] indicated that MSCs cultured in the presence of DX before co-culture with stimulated T cells impairs the immunosuppressive capacity of MSCs. Their *in vitro* assay was considerably different from ours, using phytohemagglutinin (PHA) as T-cell stimulant instead of anti-CD3 antibody, and their results relayed only on secretion of interferon-γ and TNF-α without testing the capacity of MSCs to suppress T-cell proliferation. In our study, we observed repeatedly that MSCs cultured in the presence of DX (StdM) have a higher capacity to suppress T-cell proliferation than cells cultured without DX (M1 and M2). Others have also been suggested that certain types of preconditioning of MSCs, such as interferon-γ and TNF-α treatment, may be required to enable the full suppression capacity of MSCs [89,90].

In vitro culturing of MSCs is an unavoidable step to reach cell numbers that are sufficient for clinical needs. As shown in our current study and by others, culture conditions affect cell properties [31,91,92]. Even though some effects of culture conditions are reversible [51,93], the conditions in which cells are cultured for clinical use may have an effect to prime the cells to act more effectively in certain indications. Thus, this is important to keep in mind when establishing the conditions to produce MSCs for clinical use. Also, tests demonstrating the functional capacity of MSCs rely mainly on *in vitro* assays, which are always restricted to measure only a few parameters; thus the artificial conditions of the assays may not indicate the true potential of the cells *in vivo*. Potency assays need to be improved to better evaluate the quality of cells produced for clinical use.

We have presented an effective method to generate MSCs from CB. The frequency of generating MSCs from human CB with our method was almost 90%, which is much higher than presented shown. Supportive conditions included the 3% oxygen, the growth factors EGF and PDGF, the glucocorticoid DX and fibronectin coating of culture plates. We also demonstrate that culture conditions containing DX prime MSCs to act more efficiently in suppressing T-cell proliferation. On the other hand, cells cultured without DX are more prone to support angiogenesis. Culture conditions may have remarkable effects not only on cell proliferation but also on their functionality and thus should always be appropriately defined for specific purposes.

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