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# Effect of Three-Dimensional Culture and Incubator Gas Concentration on Phenotype and Differentiation Capability of Human Mesenchymal Stem Cells

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## ABSTRACT

To obtain sufficient numbers of cells for tissue engineering applications, human bone marrow-derived mesenchymal stem cells (hBM-MSC) are commonly cultured as monolayers in incubators containing room air. In this study, we investigated whether three-dimensional (3D) culture conditions and incubator gas concentrations more similar to those observed in vivo impacted on cell expansion, differentiation capability, or phenotype of hBM-MSC. We found that 3D culture alone increased the expression of some molecules involved in osteogenic and adipogenic differentiation. In contrast, 3D culture did not induce chondrogenic differentiation, but enhanced the response to the chondrogenic differentiation medium. Changing the oxygen concentration to 6% and the carbon dioxide concentration to 7.5% did not impact on the results of any of our assays, showing that the hyperoxia of room air is not detrimental to hBM-MSC proliferation, differentiation, or phenotype. J. Cell. Biochem. 112: 684–693, 2011. © 2010 Wiley-Liss, Inc.

**KEY WORDS:** MESENCHYMAL STEM CELLS; OXYGEN; DIFFERENTIATION; THREE-DIMENSIONAL CULTURE

Human bone marrow-derived mesenchymal stem cells (hBM-MSCs) are multipotent stem cells that can differentiate into tissue-specific cells of several lineages, including osteocytes, chondrocytes, and adipocytes [Pittenger et al., 1999]. hBM-MSCs are found at very low frequencies in the bone marrow (BM) [Shahdadfar et al., 2005]. In order to use hBM-MSCs in cell therapy and tissue engineering, monolayer in vitro expansion of the cells is necessary. Because the frequency of hBM-MSCs in BM preparations is too low to allow characterization of these cells, the published properties of MSCs derive mainly from monolayer cultures [Dominici et al., 2006].

As hBM-MSC may have a role to play in many tissue engineering protocols, it is important to determine how the cells are best managed in vitro prior to clinical use. Traditionally, hBM-MSCs are cultured in vitro as adherent cells on plastic surfaces in incubators containing room air oxygen tension (approximately 20% $O_2$) and 5% carbon dioxide ($CO_2$). These conditions are very different from the environment surrounding hBM-MSCs in vivo [Ma et al., 2009]. In vivo, some hBM-MSC are found as pericytes surrounding the endothelial layer of the sinusoids, while other hBM-MSC are found away from the sinusoids, surrounded by extracellular matrix and other BM cells [Sacchetti et al., 2007; Guilak et al., 2009]. These are all three-dimensional (3D) environments, which induce cell polarity and attachment patterns very different from the monolayer culture system. The $O_2$ tension in these locations may range from approximately 6% surrounding the sinusoids to as low as 1–2% away from the sinusoids [Ma et al., 2009]. Thus, room air oxygen tension used in most in vitro culture strategies represents hyperoxic conditions for these recently isolated cells. Also, the $CO_2$ concentration provided during standard cell culture is lower than

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that surrounding the BM-MSC in the tissues [Tonnessen, 1997].

The present study was designed to determine if culture conditions more similar to the in vivo situation, i.e., autologous serum (AS), 3D rather than monolayer cell culture and incubator gas containing lower $O_2$ concentrations and higher $CO_2$ concentrations might impact on the rate of proliferation, functionality, and phenotype of BM-MSC. Alginate containing the tripeptide RGD (arginine–glycin–aspartic acid) was chosen as scaffold because RGD is a binding motif found in different extracellular matrix molecules, such as fibronectin, vitronectin, collagen, and fibrin [Ruoslahti and Pierschbacher, 1986]. It is well known that several integrins bind to the RGD motif, mediating different intracellular signals which may regulate cell differentiation, proliferation, survival, shape, and the cell cycle [Boudreau and Jones, 1999]. In the BM-MSC niche, it is likely that the cells bind to the RGD sequence within the matrix molecules. Therefore, the RGD-coupled alginate may provide some of the signals that the BM-MSC encounters in their niche in vivo. Thus, we compared the expression of integrins and several other cell surface molecules, and the ability to differentiate along the chondrogenic, osteogenic, and adipogenic lineages in cells derived from monolayer or 3D RGD alginate cultures, and under standard incubator gas concentrations as well as in incubators containing 6% $O_2$ and 7.5% $CO_2$.

## MATERIALS AND METHODS

All reagents were purchased from Sigma–Aldrich (St. Louis, MO; http://www.sigmaaldrich.com) unless otherwise stated.

### PREPARATION OF AUTOLOGOUS SERUM

From each BM donor, 400–500 ml of venous blood was drained into blood bags (Baxter, Deerfield, IL). The blood was then transferred quickly to 10 ml vacutainer tubes without anticoagulants (BD, Plymouth, UK) and allowed to clot for 1 h at room temperature. Subsequently, the blood was centrifuged at 1,800*g* for 20 min. The AS was collected and passed through a 0.45 μm and then a 0.20 μm pore size filter. Aliquots of the sterile AS were stored at −20°C.

### ISOLATION AND CULTURE OF HUMAN MULTIPOTENT MESENCHYMAL STEM CELLS AND HUMAN ARTICULAR CHONDROCYTES IN MONOLAYER

BM aspirate (50 ml) was obtained from the superior posterior iliac crest of three voluntary donors after informed consent. The aspirate was immediately diluted 1:3 in DMEM-F12. Following density-gradient centrifugation (Lymphoprep, Axis Shield, Oslo, Norway) at 800*g* for 20 min, the mononuclear cell layer was collected, washed twice and allowed to adhere overnight in 175 $cm^2$ culture flasks (Nunc, Roskilde, Denmark) at 37°C. At the beginning of cell culture each experiment was split into two arms: One set of experiments were performed in incubators containing room air (approximately 20% $O_2$) and 5% $CO_2$ (20/5), while the other set of experiments were performed in incubators containing 6% $O_2$ and 7.5% $CO_2$ (6/7.5; Fig. 1). Each flask contained DMEM/F12 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 10% AS. For the 6/7.5 conditions the culture medium and trypsin used for these cells were always pre-incubated for 2 h in the 6/7.5 incubators before replating the cells. At day 1, non-adherent cells were discarded and adherent cells were washed three times with DMEM-F12, then fresh culture medium was added to the culture flasks. Culture medium was replaced every 3 or 4 days until 60–70% confluence was reached. Subsequently, cells were detached using trypsin-EDTA and replated at 3,000 cells/$cm^2$. After the first passage, amphotericin B was removed from the medium. Cells were counted at each passage.

Isolation and culture of human adipose-derived MSC (hAT-MSC) and human articular chondrocytes (hAC) were performed as previously described [Boquest et al., 2005; Shahdadfar et al., 2008]. These cells were used for pericellular measurements only (Supplemental Figure 1).

### PERICELLULAR MEASUREMENTS

A fiber optic probe system (Neurotrend Diametrics Medical Inc., Minneapolis, MN) was applied for continous pericellular monitoring of pH, $PCO_2$, and $PO_2$ [Peterson and Vurek, 1984]. The sensors were calibrated before and after the experiment, and the results were used to make a calibration line to correct any error due to the drift of the sensors. Measurements were recorded every hour. The results presented are from the same time every 24 h. These results were highly representative for the measurements made also for the 2–3 h preceding and following those shown in Supplemental Figure 1.

### ALGINATE PREPARATIONS AND CULTURE OF hBM-MSC IN ALGINATE GELS

GRGDSP coupled alginate (PRONOVA MVM, batch FP-510-01) was generously provided by Novamatrix/FMC Biopolymer (Sandvika, Norway). In this alginate the guluronic acid content is 68%, the alginate molecular weight is 226 kDa and the RGD content is 7.4:1, that is 7.4 RGD molecules/alginate molecule. The alginate powder was dissolved in 250 mM isotonic mannitol solution and stirred over night at room temperature to make a 2.25% alginate solution. The alginate solution was then sterile filtered through a 0.2 μm pore size filter. Cells from monolayer cultures at passage 3 (P3) were suspended using trypsin and pelleted at 300*g* for 10 min. Then, the cells were washed in 250 mM mannitol solution and centrifuged at 300*g* for 10 min. The cells were resuspended in mannitol and mixed into the alginate solution to make a final concentration of $2 \times 10^6$ cells/ml and 2% alginate. The alginate/cell solution was transferred into a syringe. A blunt end 25 gauge needle was connected to the syringe and the needle was submerged in a bath of isotonic 50 mM $SrCl_2$ solution. Subsequently, the alginate/cell suspension was gently pushed into the $SrCl_2$ bath. The alginate gelled immediately, and thread-like alginate structures with a diameter of ~300 μm were made. The alginate was left in the $SrCl_2$ for 10 min to ensure complete polymerization, and was then washed several times with DMEM-F12. The alginate threads containing cells was cultured as explained in Figure 1. For assays the cells were released from the alginate by washing in isotonic 50 mM Na-citrate solution for 20–25 min followed by centrifugation at 500*g* for 15 min.

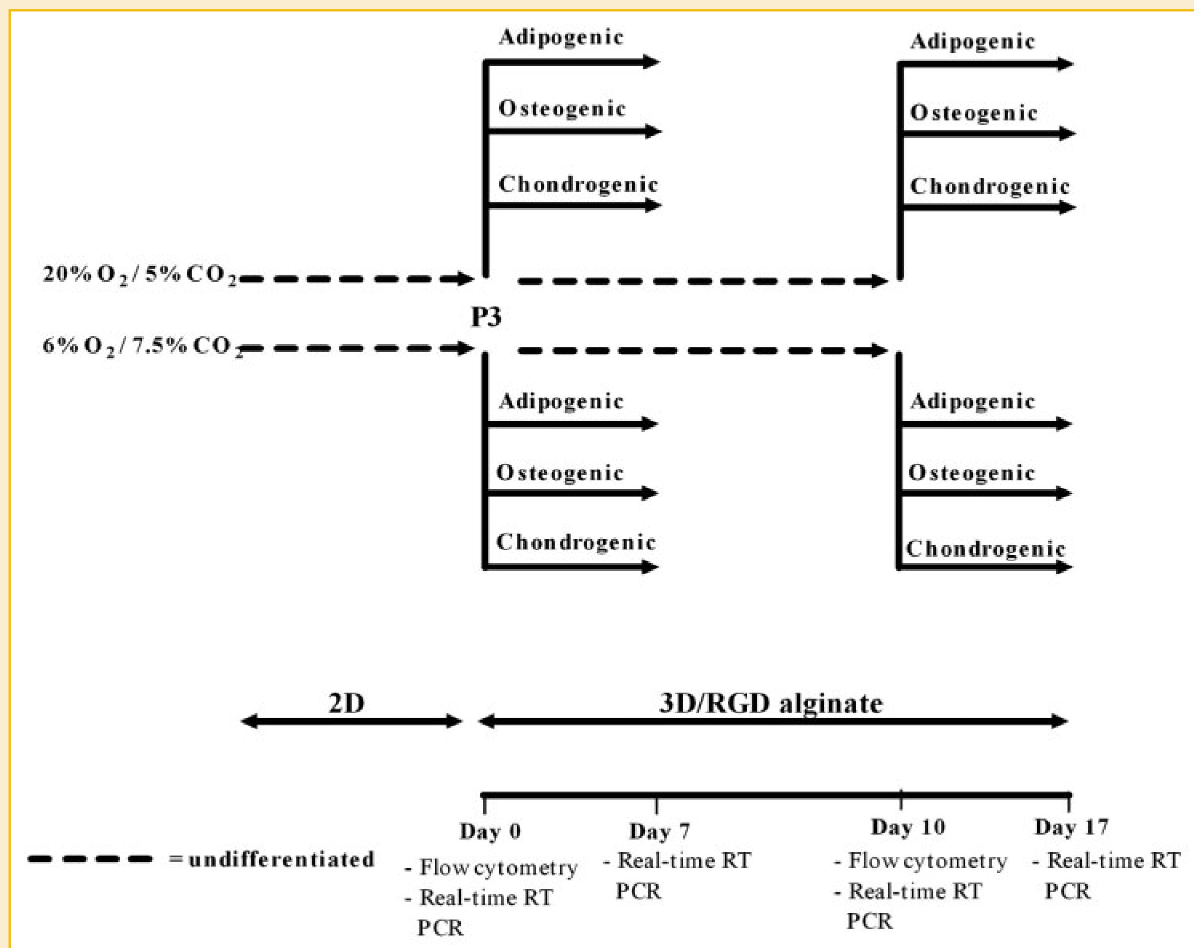


Fig. 1. Outline of the experiments. hBM-MSC were split in two and grown in 20% $O_2$ with 5% $CO_2$ or 6% $O_2$ with 7.5% $CO_2$. The cells were grown in monolayer until P3 when the cells were embedded in alginate containing RGD and either given differentiation medium (adipogenic, osteogenic, and chondrogenic) or cultured for 10 days in regular culture medium before changing to differentiation medium. Flow cytometry and real-time RT PCR was performed on monolayer cells at the end of P3 and on undifferentiated cells after 10 days in alginate cultures. Real-time RT PCR was also performed on monolayer and 3D cells after 7 days in differentiation medium (days 7 and 17).

## ADIPOGENIC, OSTEOGENIC, AND CHONDROGENIC DIFFERENTIATION

Differentiation along the mesodermal lineages was performed on hBM-MSC at $2 \times 10^6$ cells/ml encapsulated in alginate, in 3D cultures. As shown in Figure 1, cells grown in different gas tensions were differentiated in their respective gas tensions. In addition, to determine if prior culture in 3D would impact on the differentiation capability, the hBM-MSCs were differentiated either immediately after monolayer culture, or after a period of 10 days in alginate 3D culture with regular culture medium.

For adipogenic differentiation cultures were incubated in DMEM/F12 containing 10% AS, 0.5 mM 1-methyl-3 isobutylxanthine, 1 μM dexamethasone, 10 μg/ml insulin (Novo Nordisk, Copenhagen, Denmark), and 100 μM indomethacin [Pittenger et al., 1999]. For osteogenic differentiation cultures were incubated with DMEM/F12 containing 10% AS, 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM L-ascorbic acid-2-phosphate [Jaiswal et al., 1997]. For chondrogenic differentiation cultures were incubated with high glucose DMEM (4.5 g/L) containing 500 ng/ml bone morphogenic protein-2 (Wyeth Pharmaceuticals, Madison, NJ), 10 ng/ml recombinant human transforming growth factor-β1 (R&D systems, Abingdon, UK), 1 mM sodium pyruvate (Gibco, Paisley, UK, http//:www.invitrogen.com), 0.1 mM ascorbic acid-2-phosphate, 1% ITS (insulin 25 μg/ml, transferring 25 μg/ml, sodium selenite 25 ng/ml), 1.25 mg/ml bovine serum albumin, and 100 nM dexamethasone [Yoo et al., 1998]. Differentiation medium in all cultures was changed every 3–4 days. After 1-week samples from all three differentiation lineages were collected for RNA isolation and real-time PCR analyses.

## FLOW CYTOMETRIC ANALYSIS

For flow cytometric analyses of integrins and other cell surface markers the following directly conjugated monoclonal antibodies (Mab) were used: CD49a (integrin α1)/FITC, CD49b (α2)/PE, CD49f (α6)/PE, CD104 (β4)/FITC, CD90/PE, CD106/FITC, CD146/FITC (AbD Serotec, Kidlington, UK), CD34/FITC, CD45/FITC, CD49c (integrin α3)/PE, CD49e (α5)/PE, CD29 (β1)/APC, CD44/PE, CD73/PE, CD117/PE (BD Biosciences, San Diego, CA), CD19/APC, CD41

TABLE I. Taqman Assays

| Gene | Taqman assay no. |
|---|---|
| *COL2A1* | Hs00264051_m1 |
| *SOX9* | Hs00165814_m1 |
| *ACAN* | Hs00202971_m1 |
| *OMD* | Hs00192325_m1 |
| *BGLAP* (osteocalcin) | Hs01587813_g1 |
| *RUNX2* | Hs00231692_m1 |
| *PPARG* | Hs01115513_m1 |
| *CEBPA* | Hs00269972_s1 |
| *LPL* | Hs00173425_m1 |
| *ITGA2* | Hs00158148_m1 |
| *ITGA10* | Hs00174623_m1 |
| *GAPDH* | Hs99999905_m1 |

(integrin α2b)/PE, CD105/APC, HLA-DR/APC (Diatec, Oslo, Norway), CD144/PE, Integrin β5/FITC (eBioscience, San Diego, CA), CD51 (integrin αV)/PE (Chemicon Internationals, Temecula, CA), CD14/FITC (Sigma), CD133/APC (Miltenyi Biotec, Gladbach, Germany), CD49d (integrin α4)/FITC (Immunotec, Quebec, Canada).

Irrelevant Mabs were used as controls for all fluorochromes. Cells were coated with Mabs at room temperature for 20 min, washed with PBS, and fixed in 1% paraformaldehyde.

Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, http://www.bd.com). Median fluorescence index (MFI) was calculated as follows: Median fluorescence intensity of target molecule – median fluorescence intensity of isotype control.

### REAL-TIME RT PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA). Following DNase treatment (Ambion, Austin, TX), RNA was quantified by spectrophotometry (Nanodrop, Wilmington, DE). Reverse transcription (RT) was performed using the High Capacity cDNA Archive Kit (Applied Biosystems, Abingdon, UK) with 200 ng total RNA per 20 μl RT reaction. Relative quantification (RQ) was performed using the 7300 real-time RT PCR system (Applied Biosystems) and Taqman® Gene Expression assays following protocols from the manufacturer (Applied Biosystems). The genes analyzed by RQ are listed in Table I. All samples were run in triplicates (each reaction: 1.0 μl cDNA, total volume 25 μl). The thermo cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were scaled relative to the expression level of *GAPDH*, which was used as endogenous control. The validity of *GAPDH* as endogenous control was demonstrated by ensuring that its expression was unaffected by the different gas concentrations and the differentiation treatment.

## RESULTS

The experimental design for hBM-MSC is outlined in Figure 1. To obtain enough cells for these experiments, the BM-MSC were cultured in monolayer for three passages either under 20/5 or 6/7.5 gas concentrations. At P3 all the cell cultures were in log phase of growth. The different incubator gas concentrations did not affect the proliferation rate under monolayer conditions (data not shown).

To determine the relationship between the gas concentrations in the incubator air and those in the immediate surroundings of adherent cells cultured under monolayer conditions, we performed measurements of pericellular gas tension and pH using hAT-MSC and dedifferentiated hAC with 4 mm of medium. Under 20/5 conditions, we found the pericellular $pO_2$ to be 16.5–18%, and the $pCO_2$ to be 4.5–5.5%. Under the 6/7.5 conditions, the pericellular $pO_2$ decreased to 2.5–4% and the $pCO_2$ increased to 7.5–8.5%. pH was slightly reduced from approximately 7.4 to 7.25 (Supplemental Figure 1).

Expression of genes involved in differentiation may reach a plateau after long-lasting differentiation culture. For this reason we chose to evaluate expression of key genes at an early time point, day 7, in order to better observe differences between cells differentiated after monolayer or 3D culture, or under different incubator gas concentrations. We have previously shown that most changes in mRNA levels during chondrogenic differentiation of BM-MSCs have already occurred at day 7 of differentiation (Herlofsen et al., 2010), and others have shown similar observations for adipogenic and osteogenic differentiation [Sekiya et al., 2004; Guillot et al., 2008]. The results for all real-time RT PCR analyses for three donors (two donors only for the 6/7.5 differentiation) are shown in Figure 2.

### CHONDROGENIC DIFFERENTIATION

To assess chondrogenic differentiation we used the expression of *COL2A1*, *ACAN* and *SOX9* as markers [Wu et al., 2010]. *COL2A1* mRNA was not detected in undifferentiated monolayer cells or in undifferentiated cells embedded in alginate for 10 days. After monolayer culture, *COL2A1* was expressed at high levels in cultures from all donors at day 7 of differentiation. However, BM-MSC pre-cultured in 3D expressed even higher *COL2A1* mRNA compared with cells differentiated from monolayer cultures (Fig. 2A). *SOX9* and *ACAN* levels also increased in the differentiating cells, but there were no differences between cells differentiated from 2D or 3D cultures. There was no consistent impact by changing incubator gas concentrations on any of the markers examined.

### OSTEOGENIC DIFFERENTIATION

For osteogenic differentiation we measured osteocalcin (*BGLAP*), *RUNX2* and osteomodulin (*OMD*) mRNA levels (Fig. 2B) [Balint et al., 2003; Wang et al., 2010]. Surprisingly, *BGLAP* mRNA levels were found to be consistently up-regulated in 3D culture without differentiation medium. Addition of osteogenic differentiation medium actually decreased *BGLAP* mRNA levels in 3D cultured cells, while the *BGLAP* level remained largely unchanged in cells differentiated after monolayer culture. Changing the incubator environment did not induce consistent changes in *BGLAP* mRNA expression.

In nearly all the cultures (except donor 2 6/7.5 conditions) there was an increase in the *OMD* mRNA level as a consequence of 3D culture. A more profound and consistent increase was seen as a result of addition of differentiation medium. There was a small decrease in the *OMD* expression in the monolayer and 3D undifferentiated cells exposed to 6/7.5 conditions.

*RUNX2* mRNA was not consistent between the donors. An increase in *RUNX2* was only observed for two of the donors in

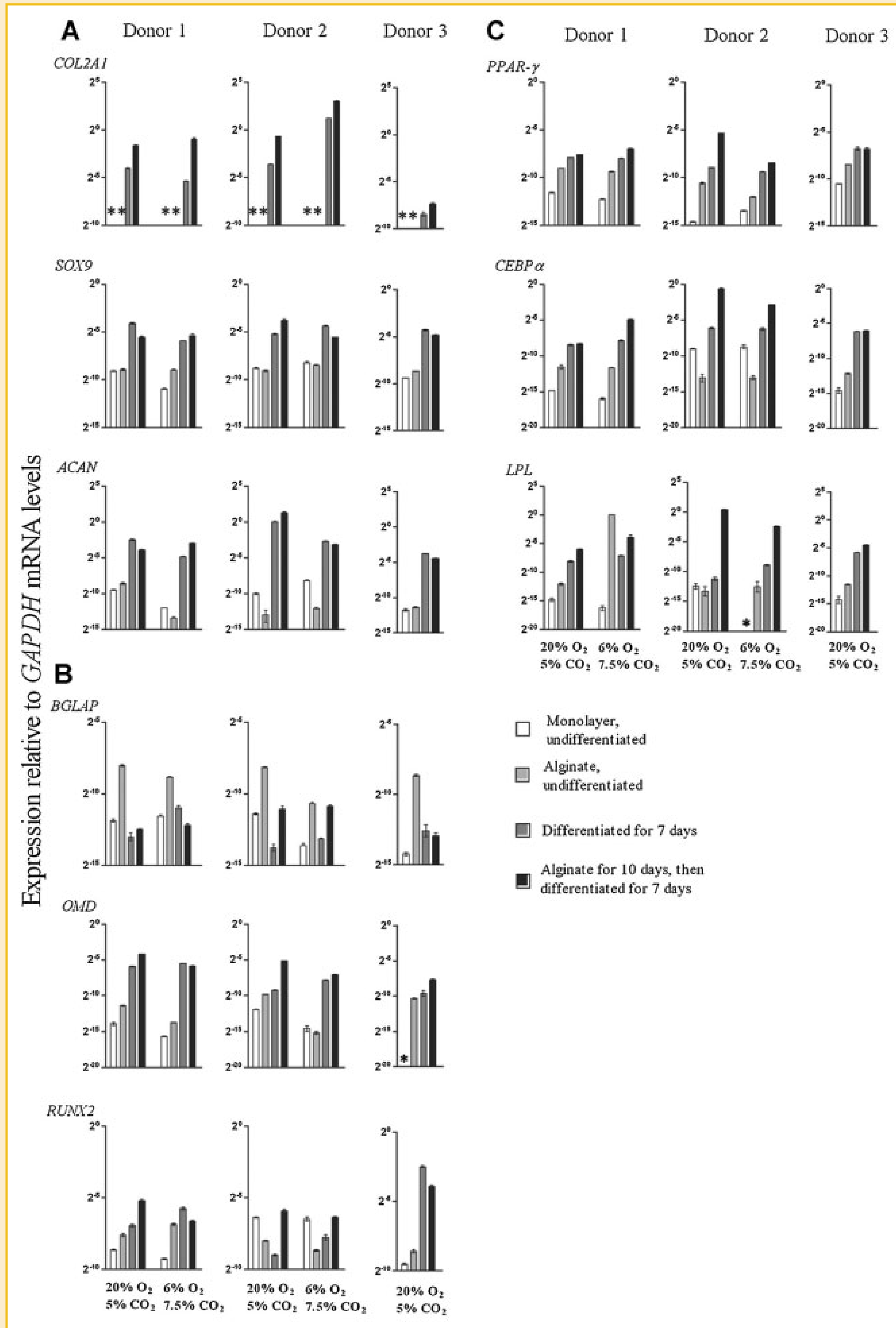


Fig. 2. RQ by real-time RT PCR of lineage-specific genes. RQ of (A) mRNA levels of COL2A1, SOX9, and ACAN (chondrogenic differentiation); (B) BGLAP, OMD, and RUNX2 (osteogenic differentiation), and (C) PPARG, CEBPA, and LPL (adipogenic differentiation) scaled relative to GAPDH expression levels. The columns represent results from undifferentiated monolayer cells (white), monolayer cells cultured in 3D in alginate for 10 days using regular medium (light gray), monolayer cells differentiated for 7 days in 3D (dark gray), and 3D undifferentiated cells exposed to differentiation medium for 7 days (black). The incubator gas concentrations are shown under the columns. Data are shown as mean ± SD (n = 3). *Not detected.

cultures with differentiation medium. No effect on *RUNX2* was seen as a consequence of changing the incubator gas concentrations.

### ADIPOGENIC DIFFERENTIATION

*PPARG*, *LPL*, and *CEBPA* mRNA were used to assess adipogenic differentiation (Fig. 2C) [Pittenger et al., 1999; Xiao et al., 2010]. Also for *PPARG* mRNA, we observed an up-regulation just by culturing the cells in 3D for 10 days. There was an increase in *PPARG* mRNA levels both in monolayer and 3D cultured cells by adding differentiation factors, and the highest levels were found in cells that were grown for 10 days in 3D with culture medium prior to differentiation.

*CEBPA* and *LPL* levels increased in almost all of the cultures containing differentiation medium. However, expression was not consistently affected by 3D. Altering the incubator gas concentrations did not consistently change mRNA expression of these molecules.

### EFFECT ON CELL SURFACE MARKER EXPRESSION OF 3D CULTURE AND CHANGES IN INCUBATOR GAS CONCENTRATIONS

Figure 3 shows the expression levels and the impact of 3D culture on the expression of a number of integrins and some other cell surface molecules. The data are from 20/5 cell culture conditions, but the cells cultured under 6/7.5 conditions showed the same expression levels. Integrins CD49c (α3), CD49e (α5), and CD29 (β1) were highly expressed in cells cultured in monolayer, but had consistently lower MFI after 3D culture. The expression of CD61 (β3) showed the same expression pattern but was expressed at much lower levels. The only integrin subunit that had a dramatic increase in MFI when cultured in 3D was the integrin β5 subunit. CD90 (Thy1) and CD105 (endoglin) had a much lower MFI in 3D cultures. However, most of the cells in 3D were still positive for these two cell surface markers. We also analyzed other cell surface markers that are not shown in Figure 3. CD14, CD19, CD34, and CD45 were analyzed to ensure that there was no contamination of hematopoietic cells in our cultures, and these markers were not found on any of the cells. Also CD117, CD133, CD144, and HLA-DR were not detected. CD44 and CD73 were highly expressed, but no consistent differences were found between monolayer and 3D. Expression of CD146 was low and there were also no differences between monolayer and 3D cultures.

### INTEGRINS KNOWN TO BIND TYPE II COLLAGEN

Two integrin α chains, α2 and α10 are known to bind type II collagen, both in association with the β1 chain [McCall-Culbreath and Zutter, 2008]. As Mab specific for α10 was not available to us, we examined changes induced in the mRNA expression of these α chains in 3D and 6/7.5 conditions (Fig. 4). For both we observed a consistent increase in mRNA expression as a result of 3D culture, while no consistent change was induced by altering incubator gas concentrations.

## DISCUSSION

Tissue engineering based on the use of in vitro expanded cells is a new treatment modality. For each cell type to be used, we need to define the best in vitro cell expansion protocol. This means not only determining the right composition of the culture medium and whether or not to use growth factors, but also includes decisions about the external milieu for the cell cultures: Incubator gas concentrations, monolayer versus 3D culture and whether or not external pressure or shear forces should be applied [Shahdadfar et al., 2005; Guilak et al., 2009; Ma et al., 2009]. To guide our decisions we should use not only cell numbers, but also gene expression studies, phenotype, functional studies, and eventually animal models resembling the human treatment situation.

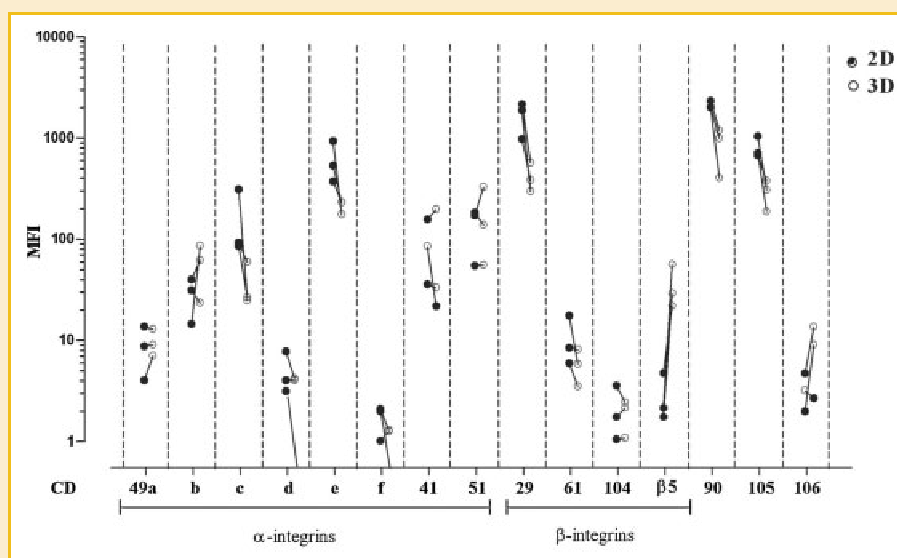


Fig. 3. Cell surface marker expression during monolayer and 3D alginate cultures. Black circles show expression by MFI during 2D (monolayer) culture and white circles shows expression after 10 days in 3D culture (RGD alginate). Results from all the donors are shown for each molecule and the line between the circles joins results from the same donor.

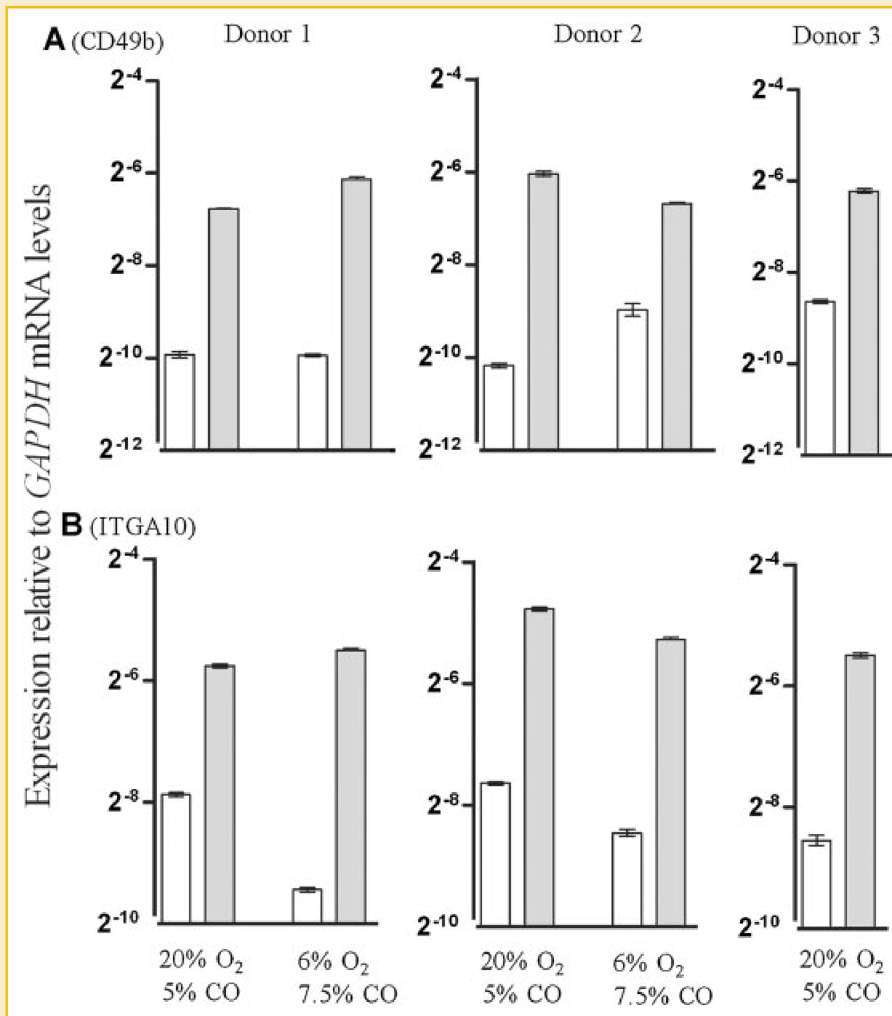


Fig. 4. RQ by real-time RT PCR of type II collagen receptors. (A) CD49b (integrin $\alpha2$) and (B) integrin $\alpha10$ during undifferentiated monolayer (white columns) and 3D (alginate, gray columns) cultures. mRNA levels were scaled relative to GAPDH expression levels. Data are shown as mean $\pm$ SD ($n = 3$).

We are in the process of performing such a series of studies for hBM-MSC. Using global gene expression arrays, we have previously shown that gene expression changes considerably when primary uncultured MSCs are placed in monolayer culture [Boquest et al., 2005]. When MSC were placed in 3D alginate culture following expansion in monolayer the gene expression changed again, but still retained more of the pattern observed during monolayer culture than that observed for the uncultured cells [Duggal et al., 2009]. The functional importance of these observations has not yet been determined. Here, using tri-lineage differentiation as our main assay, we have determined the impact of using the cells immediately after monolayer culture versus first expanding the cells in monolayer and then keeping them in 3D culture prior to differentiation. At the same time we wanted to determine the effect of different incubator gas concentrations on the outcome of the differentiation assays. For these studies we decided to compare the most commonly used 20/5 conditions with the gas concentrations that most closely resemble those observed in vivo. For chondrogenesis, our reasoning goes as follows: The oxygen tension in adult hyaline cartilage is 0.5–5%, depending on the depth from the surface [Brighton and Heppenstall, 1971]. However, adult cartilage is an extremely inert tissue, with little evidence of tissue turnover and very poor capacity for tissue regeneration [Brittberg et al., 1994]. The cells are not proliferating, and practically no new matrix is being generated [Verzijl et al., 2000]. It seems more relevant to the tissue engineering situation, therefore, to use gas concentrations that resemble those observed during embryogenesis, where active chondrogenesis is taking place. Here, the oxygen concentration has been found to be 4–5% [Ma et al., 2009].

We show that the 6/7.5 conditions gave pericellular oxygen concentrations of 2.5–4%. Thus, by using the 6/7.5 conditions we obtained oxygen tensions in the pericellular milieu which resemble those observed both during embryogenesis, and in some regions of the BM from where the MSCs were harvested [Simon and Keith, 2008; Ma et al., 2009]. The comparison between these two incubator gas concentrations will primarily give an indication as to whether the 20/5 conditions are unsuitable for the culture of primary cells not yet adapted to the hyperoxia of room air.

According to the assays used in this study the hyperoxia of 20/5 condition does not influence hBM-MSC behavior. After examining monolayer proliferation rate, impact of 3D culture on the expression of some genes and a number of cell surface markers and differentiation assays we observed no consistent differences between the two gas concentrations. One confounding issue is the possibility that the exposure to room air during the change of medium might reverse changes induced by the exposure to the 6/7.5 conditions in the incubator. We have tried to reduce the impact of the room air exposure by pre-incubating the new media in 6/7.5 incubators, and by making the medium change as swift as possible. A similar strategy was used in a recently published study which aimed to examine the effect of incubator gas concentrations on the in vitro longevity of human MSC [Moussavi-Harami et al., 2004]. These authors found that cells cultured under 20/5 conditions reached senescence much earlier than cells cultured under 5/5 conditions. This shows that the impact of the 5/5 conditions on the factors regulating longevity in vitro were not abrogated by brief exposures to room air. If we can presume that the same applies to the assays used in this study, then we can conclude that the hyperoxia of 20/5 condition does not influence hBM-MSC proliferation rate, trilineage differentiation, and cell surface markers.

Several other studies have investigated the effects of low $O_2$ tensions on MSC with very contrasting results. In two studies, where hBM-MSC were expanded in 2% and 1.5% $O_2$, respectively, increased proliferation was observed [Grayson et al., 2007; Martin-Rendon et al., 2007]. In addition, several genes known to be regulated by $O_2$ tension were up-regulated [Martin-Rendon et al., 2007]. Rat BM-MSC (rBM-MSC) enhanced transcription of genes involved in cell proliferation and survival when cultured in 1% $O_2$ [Ohnishi et al., 2007]. Lennon et al. [2001] showed that rBM-MSC increased proliferation during monolayer expansion and enhanced osteoblast differentiation in a porous ceramic vehicle in vivo and in vitro in 5% $O_2$. Krinner et al. [2009] cultured ovine BM-MSC in 5% $O_2$ and reported increased chondrogenesis and proliferation. Also Zscharnack et al. [2009] showed enhanced chondrogenesis of ovine BM-MSC when cultured in collagen I hydrogel in 5% $O_2$. On the other hand, murine AT-MSC showed enhanced proliferation, but reduced osteogenesis and chondrogenesis in 2% $O_2$ [Malladi et al., 2006], while hAT-MSC showed reduced proliferation and reduced osteogenesis when differentiated in 2D and in PLG scaffolds in both 1% and 5% $O_2$ [He et al., 2010]. $O_2$ (5%) inhibited proliferation and enhanced chondrogenic differentiation in hAT-MSC [Wang et al., 2005]. Several studies have shown reduced adipogenic and osteogenic differentiation in human MSCs when cultured in low $O_2$ [Fehrer et al., 2007; Hung et al., 2007; Holzwarth et al., 2010]. However, there are also articles showing increased [Fink et al., 2004; Grayson et al., 2006] or no effect [Grayson et al., 2007; Carrancio et al., 2008] by low $O_2$ on adipogenic differentiation. Finally, data from a previous study in our lab, using global mRNA arrays, showed that there were no significant differences between the transcriptome of hBM-MSC expanded in 2D in 20/5, 6/7.5, and 9/7.5 conditions [Karlsen, 2007].

At first sight it may be difficult to reconcile all these different results. A first separation may be made based on the incubator $O_2$ levels. When the cells are cultured at incubator $O_2$ levels of 2% or less, the pericellular $O_2$ concentration is presumably sufficiently low for Hypoxia inducible factor (HIF-1α) to escape degradation and be active as a transcription factor [Jiang et al., 1996]. HIF-1α is known to induce expression of genes involved in many cellular processes [Manalo et al., 2005]. Conditions leading to up-regulation of HIF1-α are frequently termed truly hypoxic, and studies employing such conditions are examining a different aspect of biology from that investigated in the present study. While truly hypoxic conditions may be found in vivo, they are unusual at sites where MSCs reside. In this respect, truly hypoxic conditions may not be biologically relevant for MSCs. More important to the discussion of in vitro culture conditions, the safety of culturing stem cells in very low $O_2$ may be compromised. Rodriguez-Jimenez et al. [2008] reported genomic instability in MSCs and neural stem cells cultured in 1% $O_2$. Also, Li and Marban [2010] reported reduced karyotypic abnormalities in human cardiac stem cells cultured in 5% $O_2$ compared with cells cultured in room air. Consistent with this observation, addition of antioxidants to room air cell cultures suppressed DNA damage at low concentrations, but potentiated such damage at high concentarions, suggesting that very low concentrations of $O_2$ may be harmful [Li and Marban, 2010]. As cell culture under truly hypoxic conditions also requires specialized enclosed incubators, we believe that this strategy is unlikely to be used for the culture of MSCs for therapeutic purposes. As for the other diverging results discussed above, the most likely explanations must be differences in MSCs from different species, differences in tissue of origin of the MSCs, the use of different scaffolds and other technical factors. The effect of embedding MSCs in different scaffolds is a particularly interesting and large research area, which has been very well reviewed by Guilak et al. [2009]. For the present experiments we chose alginate, which is thought to be a bioinert material, to which an RGD-containing peptide was biochemically coupled. This sequence, found in many ECM proteins, would allow binding between the biomaterial and cell surface integrins [Ruoslahti and Pierschbacher, 1986; Hersel et al., 2003]. However, a large number of different scaffold systems are available, the choice of which depends on the application, the cells used, biodegradability and other factors [Yang et al., 2006; Guilak et al., 2009].

Interestingly, the transfer of hBM-MSC from monolayer to 3D culture induced a number of changes, most notably in the mRNA levels of the osteogenesis markers osteocalcin and osteomodulin and in the adipogenesis marker PPARγ. Chondrogenesis could only be induced with differentiation factors, but also here 3D culture prior to chondrogenic differentiation induced higher expression of *COL2A1* mRNA. We have previously shown that MSC cease to proliferate following establishment in alginate cultures [Duggal et al., 2009]. This profound change in metabolism, plus the change in shape and polarization occurring when the cells are no longer adherent to plastic may be responsible for these changes in differentiation markers. The 3D culture also induced some changes in the expression of cell surface molecules. Most, but not all surface molecules examined were down-regulated in 3D. For CD49c and e, CD29, and CD61 the same changes were previously found in BM-MSC from other donors and in AT-MSC [Duggal et al., 2009]. However, RGD receptors such as α5β1 and αVβ5 [Takagi, 2004] were still expressed at high levels, and β5 was actually considerably

up-regulated. The surface expression of the type II collagen-binding integrin CD49b was not greatly altered by 3D culture. However, the mRNA encoding this molecule was consistently up-regulated in all donors. This discrepancy cannot be explained by available data, but the same mRNA changes were observed both by mRNA array analysis and real-time RT PCR for adipose tissue-derived MSC in our previous study, and a similar discrepancy between surface expression and mRNA was observed for CD61 [Duggal et al., 2009].

## CONCLUSION

In this study the effect of 3D culture and incubator gas concentration on phenotype and differentiation capability of hBM-MSC were examined. Gas concentration did not seem to impact on any of these parameters, demonstrating that the standard incubator settings are acceptable for proliferation and differentiation of hBM-MSC. On the other hand, the establishment of hBM-MSC in 3D cultures induced up-regulation of the osteogensis markers osteocalcin and osteomodulin, the adipogenic marker PPAR-γ and the chondrogenesis marker type II collagen and induced some changes in phenotypic markers.

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