



Flow-perfusion interferes with chondrogenic and hypertrophic matrix production by mesenchymal stem cells



Linda M. Kock^a, Jos Malda^{a,b}, Wouter J.A. Dhert^{a,b}, Keita Ito^{a,c}, Debby Gawlitta^{a,*}

^a Department of Orthopaedics, University Medical Center Utrecht, Utrecht, The Netherlands

^b Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

^c Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands

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ABSTRACT

Flow-perfusion is being promoted as a way to grow tissue-engineered cartilage *in vitro*. Yet, there is a concern that flow-perfusion may induce unwanted mechanical effects on chondrogenesis and terminal differentiation. Therefore, the aim of this study is to evaluate the effect of fluid flow on chondrogenesis and chondrocyte hypertrophy of MSCs in a well-established pellet culture model.

Human MSC pellets were mounted into 3D-printed porous scaffolds in basic chondrogenic differentiation medium, containing TGF- β 2. Constructs were then allowed to form cartilaginous matrix for 18 days, before they were transferred to a custom-built flow-perfusion system. A continuous flow of 1.22 ml min⁻¹ was applied to the constructs for 10 days. Controls were maintained under static culture conditions. To evaluate chondrogenic and hypertrophic differentiation, RNA was isolated at day 20 and 28 and histology, immunohistochemistry and western blot analyses were performed after 28 days of culture.

Abundant matrix was formed in the constructs, but production of chondrogenic and hypertrophic matrix components was affected by flow-perfusion. Although gene expression levels of the (late) hypertrophic and osteogenic marker osteocalcin increased by flow-perfusion, this did not result in more collagen type X protein deposition. Decreased GAG release, in combination with diminished collagen II staining, indicates reduced chondrogenesis in response to flow-perfusion.

Caution should thus be taken when applying flow-perfusion to cultures to improve nutrient diffusion. Although we show that it is possible to influence the differentiation of chondrogenic differentiated MSCs by flow-perfusion, effects are inconsistent and strongly donor-dependent.

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1. Introduction

Articular cartilage is an avascular, low-friction, and wear-resistant weight-bearing tissue, which has a limited self-renewal capacity once it is damaged. Therefore, cartilage tissue engineering approaches have been introduced involving chondrocytes or mesenchymal stem cells (MSCs) embedded in a support material which are stimulated mechanically or chemically, to create a functional tissue replacement (Kock et al., 2012; Gardner et al., 2013; Huey et al., 2012).

It is generally known that MSCs can undergo *in vitro* chondrogenic differentiation in the presence of appropriate stimuli (Puetzner et al., 2010; Johnstone and Yoo, 1999; Johnstone et al., 1998). The advantage of using MSCs in cartilage tissue engineering

studies is that they have an extensive proliferation capacity, while maintaining their differentiation properties and they can be obtained in a less invasive manner than chondrocytes. Differentiation of MSCs into the chondrogenic lineage is a complex process, which is largely donor dependent (Gawlitta et al., 2012; Meyer et al., 2011). While clinical studies employing MSCs for treatment of cartilage defects are still in their infancy, MSCs hold great promise for future production of cartilaginous constructs. A major disadvantage of MSCs is that they exhibit a hypertrophic tendency when differentiated into the chondrogenic lineage. This can result in calcification of ectopically transplanted neocartilage matrix as was demonstrated in mouse models *in vivo* (Pelttari et al., 2006; Jukes et al., 2008). Thus, methods to better understand and control this unwanted hypertrophic differentiation are required (Zhang et al., 2012).

Mechanical cues are believed to influence hypertrophic differentiation of chondrocytes and MSCs *in vitro*, since growth plate and articular hypertrophy *in vivo* are regulated by mechanical loading (van Donkelaar and Huiskes, 2007). Different types of

* Correspondence to: Department of Orthopaedics, University Medical Center Utrecht, PO Box 85500, G05.228, 3508 GA Utrecht, The Netherlands.
Tel.: +31 88 755 8035; fax: +31 30 251 0638.

E-mail address: d.gawlitta@umcutrecht.nl (D. Gawlitta).

mechanical loading have been predicted to influence chondrogenic and hypertrophic differentiation of chondrocytes (van Donkelaar and Wilson, 2012). Cyclic tension, for example, induces expression of terminal hypertrophic differentiation markers in chondrocytes in vitro (Wong et al., 2003; Yang et al., 2006; Beaupre et al., 2000; Wu and Chen, 2000). In contrast, dynamic compressive loading enhances cartilage matrix synthesis and distribution and suppresses the expression of major hypertrophic genes and matrix mineralization in MSC-laden hyaluronic acid hydrogels (Bian et al., 2012).

Flow perfusion is being promoted as a way to grow both tissue engineered cartilage and bone in vitro (Yeatts and Fisher, 2011; Schulz and Bader, 2007). Culturing chondrocytes under direct flow perfusion has been shown to stimulate proliferation and accumulation of cartilage markers (Beaupre et al., 2000; Pazzano et al., 2000; Freyria et al., 2005). However, fluid flow induces shear stress that may induce unwanted mechanical effects on chondrogenesis and terminal differentiation (Stevens et al., 1999; Carter and Wong, 2003; Garzon-Alvarado et al., 2011). A more thorough investigation of the effect of fluid flow on chondrogenic MSC differentiation is necessary to diminish potentially harmful effects of flow-perfusion in cartilage tissue engineering studies. Furthermore, these insights can be used to improve the development of bioreactors that give an optimal yield of cartilaginous tissue in a tissue engineering setting. Therefore, the aim of this study is to evaluate the effect of flow-perfusion on chondrogenic hypertrophy of MSCs in a well-established pellet culture model (Schon and Schrobback, 2012).

2. Methods

2.1. Preparation of PCL scaffolds

Polycaprolactone (PCL, Sigma) was printed (27G needle, 200 mm/min speed, 200 rpm spindle speed) into 2 × 2 cm layered, porous 3D square lattice-work

constructs with a height of 3.75 mm and a fiber spacing of 1.05 mm (Fig. 2). Scaffolds were cut into 5 × 5 mm pieces, sterilized in 70% ethanol and air-dried in a laminar airflow cabinet. Before cell pellets were inserted (Schon and Schrobback, 2012; Schuurman et al., 2013), scaffolds were incubated with MSC expansion medium without rhB-FGF2 (α MEM (22561, Minimum Essential Medium α ; Invitrogen) containing 10% fetal bovine serum (DE14-801F; BioWhittaker), 0.2 mM L-ascorbic acid 2-phosphate (A8960; Sigma), and 100 U/ml penicillin with 100 mg/ml streptomycin (PenStrep; Invitrogen)).

2.2. Flow-perfusion bioreactor system

The flow-perfusion bioreactor system consists of a custom-built polycarbonate flow chamber, a peristaltic continuous flow pump (7519-15; Masterflex L/S™) and a spinner flask as medium reservoir (Fig. 1). Protrusions of 0.5 mm from the flow chamber wall prevented the scaffold from moving. The construction was connected with flexible tubes using Luer locks (Fig. 1).

2.3. Isolation of Human MSCs

Human MSCs were isolated from bone marrow biopsies out of the iliac crest after obtaining written informed consent (Medical Ethical Committee, University Medical Center Utrecht) of patients undergoing hip replacement surgery (donor A: male, 56 years; donor B: male, 63 years; donor C: female, 32 years). Cells were harvested at passages 2–4 for pellet formation. After expansion, the MSCs were characterized as described previously (Gawlitza et al., 2012). The complete experiment was performed 3 times with MSCs from the individual donors.

2.4. Pellet culture system

MSCs were centrifuged in 96-well plates (7007; Corning) at 300 g for 5 min to form cell pellets of 250,000 cells each ($n=96$ per donor). Pellets were subsequently cultured in chondrogenic differentiation medium (CDM) containing high glucose Dulbecco's modified Eagle's medium (DMEM 31966; Invitrogen) with 1% ITS+ premix (354352; BD Biosciences), 10^{-7} M dexamethasone (D8893; Sigma), 0.2 mM L-ascorbic acid 2-phosphate (A8960; Sigma), PenStrep, and 10 ng/ml TGF β 2 (302-B2; R&D Systems) for 7 days. The CDM was refreshed at least twice weekly.

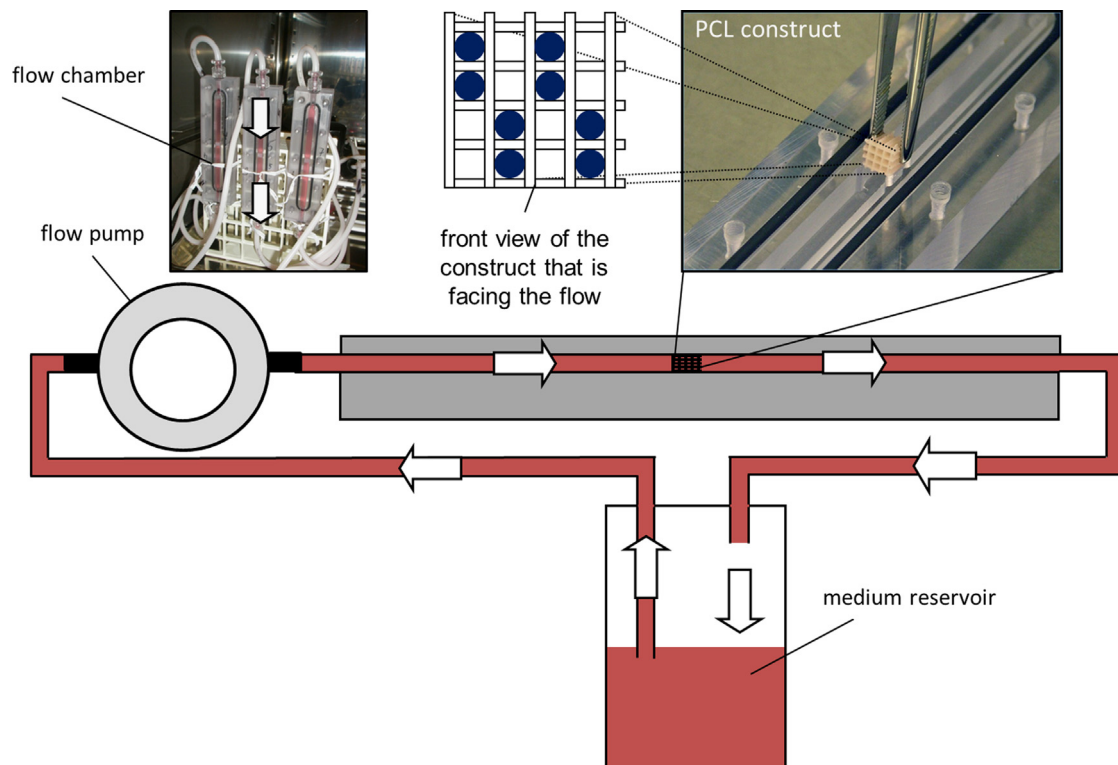


Fig. 1. Schematic representation of the flow-perfusion bioreactor system, consisting of a custom-built polycarbonate flow chamber, a peristaltic continuous flow pump and a spinner flask as medium reservoir.

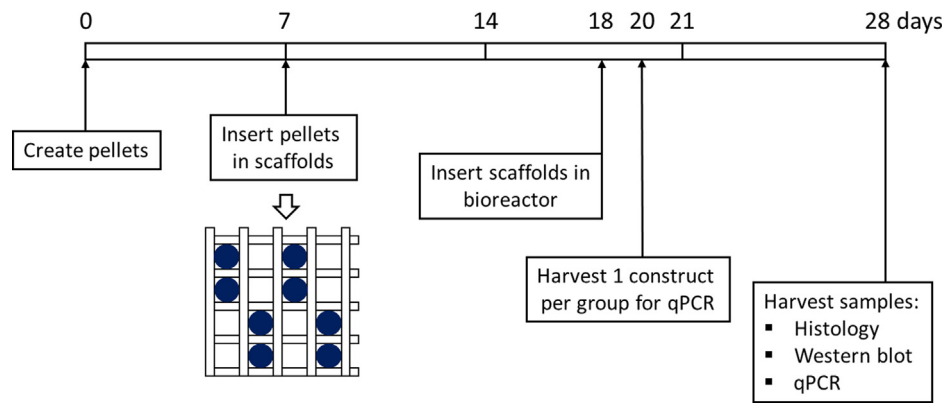


Fig. 2. Overview and timeline of experimental set-up. At day 0 pellets were created and after 7 days of culture in CDM they were transferred to the scaffold. At day 18, half of the scaffolds ($n=6$) was transferred to the flow-perfusion bioreactor system, while the other half served as static culture controls. At day 20, 1 construct per group was harvested for qPCR. The remaining constructs were harvested at day 28 for qPCR, histology, immunohistochemistry and quantification of collagen type X.

2.5. Experimental set-up

After 7 days, 8 pellets per scaffold were transferred into 8 separate pores of 12 separate PCL scaffolds (geometry shown in Fig. 2) with small sterile spoons and cultured in 24-wells plates in CDM that was replaced twice weekly. At day 18, half of the constructs ($n=6$) were placed into the flow chambers with sterile tweezers (Fig. 1). Cell pellets were located at the side of medium inflow in such a way that pellets were facing the medium flow (Fig. 1). To prevent dehydration, 1 ml of CDM was added to each scaffold in the flow chamber before closing it. After covers were secured, flow chambers were placed upright and connected to the medium reservoir, containing 9 ml of CDM. Medium was pumped around to check for leakages before the assembly was transferred to an incubator ($37\text{ }^{\circ}\text{C}$ and 5% CO_2). A continuous flow of 1.22 ml min^{-1} was applied for 10 days. Controls ($n=6$) were maintained in a 6-well plate with 1 sample and 10 ml CDM per well. Medium was not refreshed during these 10 days. At day 20, 1 construct per group was harvested for quantitative real-time polymerase chain reaction (qPCR).

2.6. Histology and immunohistochemistry

Pellet-containing constructs ($n=2$ per group) were fixed in 10% formalin, embedded in paraffin and subsequently $5\text{ }\mu\text{m}$ sections were cut. Before (immuno) histological stainings were performed, sections were deparaffinized and rehydrated. After staining, sections were dehydrated, mounted in depex and evaluated with light microscopy (Olympus).

2.6.1. Safranin-O staining

Sections were stained with Weigert's hematoxylin (640490; Klinipath BV) for 5 min and 0.4% fast green (1.04022.0025; Merck) for 4 min. After rinsing in 1% acetic acid, they were stained with 0.125% Safranin-O (1.15948.0025, Merck) for 5 min.

2.6.2. Von Kossa staining

Sections were rinsed with distilled water and then incubated at room temperature in 1% silver nitrate (5/1240/46; Thermo Fisher) solution directly under a light bulb for 1 h. Sections were subsequently washed with 5% sodium thiosulphate (A17629; Alta Aesar) and counterstained with hematoxylin.

2.6.3. Collagen types II and X

Immunohistochemical detection of collagen types II and X was performed as described in detail previously (Gawliita et al., 2012).

2.7. Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted from the pellets of 1 construct at day 20 and 2 constructs at day 28 of each donor and treated with $10\times$ TURBOTM DNase (Ambion). Subsequently, cDNA was synthesized by using the iScriptTM cDNA Synthesis kit (Bio-Rad). qPCR was performed by applying iQ SYBR Green Supermix (Cat. No. 170-8885; Bio-Rad) on a MyIQ PCR system (Bio-Rad). Gene expression patterns were determined for GAPDH, alkaline phosphatase (ALPL), osteocalcin (BGLAP) and collagen types II (COL2A1) and X (COL10A1) (Table 1). The C_t values of the target genes were corrected for GAPDH expression levels. Data are represented as fold induction of genes of interest for flow-perfusion conditions versus static conditions using the delta-delta C_t method.

Table 1

Primer sequences for the human genes that were used in the polymerase chain reaction analyses. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; COL, collagen; BGLAP, osteocalcin; ALPL, alkaline phosphatase.

Gene	Forward primer sequence (3'→5')	Reverse primer sequence (3'→5')
GAPDH	ATGGGGAAAGGTGAAGGTCG	TAAAGCAGCCCTGGTGACC
COL2a1	AGGGCCAGGATGTCGGCA	GGGTCCAGGTTCTCCATCT
COL10a1	CACTACCCAACACCAAGACA	CTGGTTCCCTACAGCTGAT
ALPL	CCAAAGGCTTCTTCTTGCTG	CCACCAATGTGAAGACGTG
BGLAP	CGCAGCCACCCAGACCCAT	GGGCAAGGCAAGGGGAAGA

2.8. GAG release

GAG release in the culture medium from days 18 to 28 was determined spectrophotometrically after reaction with dimethylmethylene blue dye (DMMB, Sigma) by measuring absorbances at 540 and 595 nm, using a chondroitin sulfate C standard (Sigma-Aldrich).

2.9. Collagen type X quantification

Protein was extracted overnight from pellets of 1 construct per donor at $4\text{ }^{\circ}\text{C}$ in lysis buffer, containing 0.05 M Tris-HCl, 5 mM EDTA, 1 mM *N*-ethylmaleimide, 1 mM phenylmethanesulphonyl fluoride and 0.1% SDS. Extracts were centrifuged for 10 min at $10,600\text{ g}$ at $4\text{ }^{\circ}\text{C}$ and supernatant was stored at $-20\text{ }^{\circ}\text{C}$. Protein was separated on a 8% polyacrylamide gel, followed by transfer to a polyvinylidene fluoride membrane (Millipore) by electroblotting. Membranes were blocked for non-specific binding in Odyssey[®] Blocking Buffer (927-40000; LI-COR) followed by incubation with a 1:500 diluted murine anti-collagen type X antibody (X53; Quartett) and a 1:400 diluted anti- β -actin antibody (130656, Santa Cruz) overnight at $4\text{ }^{\circ}\text{C}$. After washing of the membranes, blots were incubated with an anti-mouse biotinylated IgG (1:400; RPN1001V; GE Healthcare) for collagen type X detection. Finally, membranes were incubated with streptavidin Dylight 800 conjugated (1:10,000, Thermo Fisher) for collagen type X and goat anti-rabbit IgG Dylight 680 (1:10,000, Thermo Fisher) for β -actin. Signals were visualized and quantified by an Odyssey[®] Infrared Imaging System (LI-COR).

2.10. Statistical analysis

All data are presented as means \pm standard deviation. For evaluation of gene expression levels, effects of the independent variables treatment and time as well as their interaction were examined for significant differences by two-way analysis of variance (ANOVA) in SPSS. If no significant interaction was found, a one-way ANOVA with Bonferroni post-hoc testing was used to search for significant differences within the other factor level. In case of significant interaction, an independent *t*-test with Bonferroni correction for a number of specific comparisons was used to test for significant differences. Significant differences in GAG release between static and perfused constructs were determined with a one-way ANOVA with Bonferroni post-hoc testing.

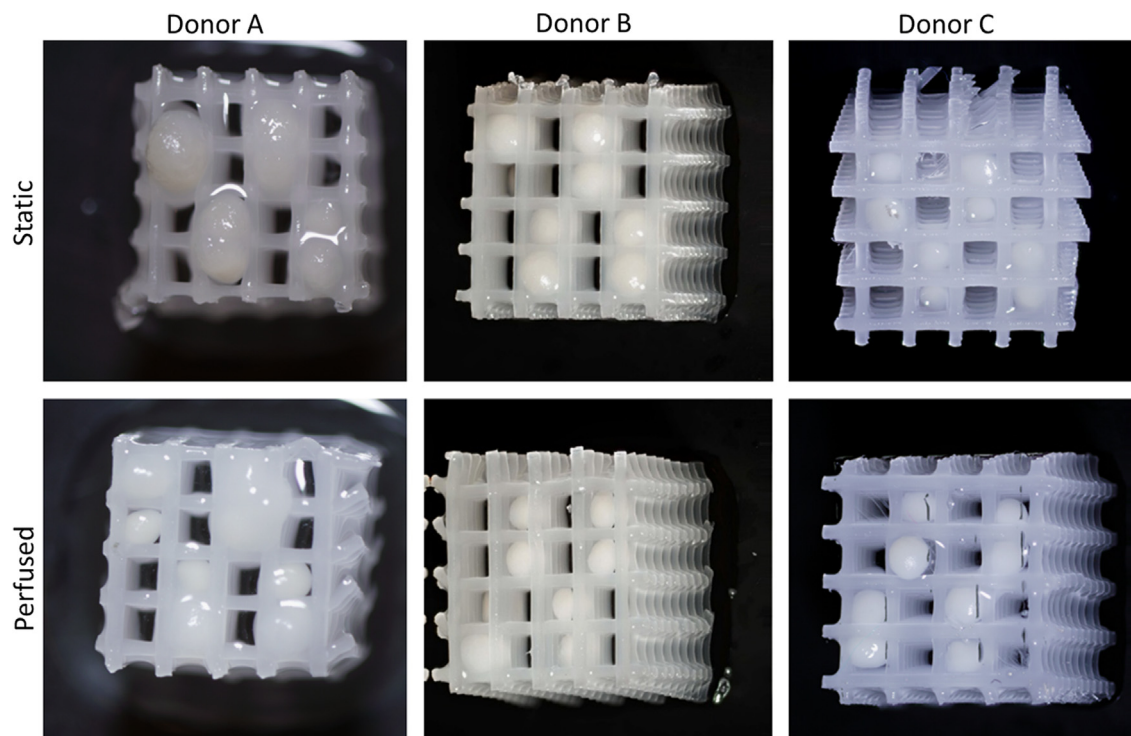


Fig. 3. Overview of gross macroscopic appearance of MSC pellets from 3 different donors in PCL scaffolds at day 28 of static (upper row) and perfused (lower row) constructs.

3. Results

3.1. Macroscopic appearance of scaffolds and MSC pellets at day 28

The MSCs of all 3 human donors successfully formed spherical aggregates during the first 2 weeks of culture and aggregates remained in the PCL mesh for the remaining culture period. Pellets of donors B and C demonstrated a similar spherical appearance and single aggregates could be distinguished. Pellets of donor A were larger and had fused together within the pores in some cases (Fig. 3). In general, pellets of static cultures appeared to be larger compared to pellets cultured under perfusion.

3.2. Histology and immunohistochemistry

Intensity of Safranin-O staining on cultures of donor A was very homogeneous and no obvious differences between static and perfused samples were observed (Fig. 4a). Heterogeneous and less intense staining was seen in cultures from donors B and C. The presence of flow-perfusion resulted in absence of GAG deposition in samples of donor B and in reduced localized deposition of GAGs in the periphery of pellets from donor C. Collagen type II immunostaining confirmed the results of the Safranin-O staining, except for donor A (Fig. 4b). Deposition of collagen type II in pellets from donor A was less homogenous and mostly located in the periphery as compared to proteoglycan deposition in pellets from the same donor. In cell pellets from donor B and C, collagen type II deposition was reduced in perfused samples and contained only local areas with collagen type II. Hypertrophic differentiation was evaluated with collagen type X immunohistochemistry and Von Kossa staining. Von Kossa staining was not observed in any of the cultures (data not shown), indicating absence of mineralization. In contrast, collagen type X was detected locally in most of the constructs and appeared primarily in the periphery of the pellets (Fig. 5). Although differences were not very clear, collagen type X staining appeared to be more pronounced in static cultures.

Notably, cell morphology in these areas did not resemble the typical hypertrophic geometry in large lacunae.

3.3. Expression of chondrogenic and hypertrophic genes

Collagen type II expression was decreased significantly in flow-perfusion versus static cultures at day 28 (Fig. 6). In general, genes of (early) hypertrophic markers ALP and collagen type X were expressed at similar or lower levels in the flow-perfusion cultures compared with their static counterparts, albeit not statistically significant. In contrast, gene expression of osteocalcin was increased in flow-perfusion cultures at day 28 compared to static controls.

3.4. GAG release in culture medium

Fewer GAGs were detected in the culture medium of perfused constructs of donor A and C, compared to static controls (only significant for donor C) (Fig. 7). In culture medium of donor B, an overall lower release of GAGs was detected and no differences between control and flow-perfusion were observed. These results confirm the reduced chondrogenic potential, shown by gene expression levels, of constructs that were subjected to flow-perfusion.

3.5. Collagen X quantification

Production of collagen type X differed between donors (Fig. 8). Statically cultured constructs of donor A and C contained clearly more collagen type X, compared to the perfused constructs. However, absolute amounts of collagen type X in samples of donor A were almost one order of magnitude higher than in constructs of donor C. For donor B, no obvious differences between perfused and static constructs were observed.

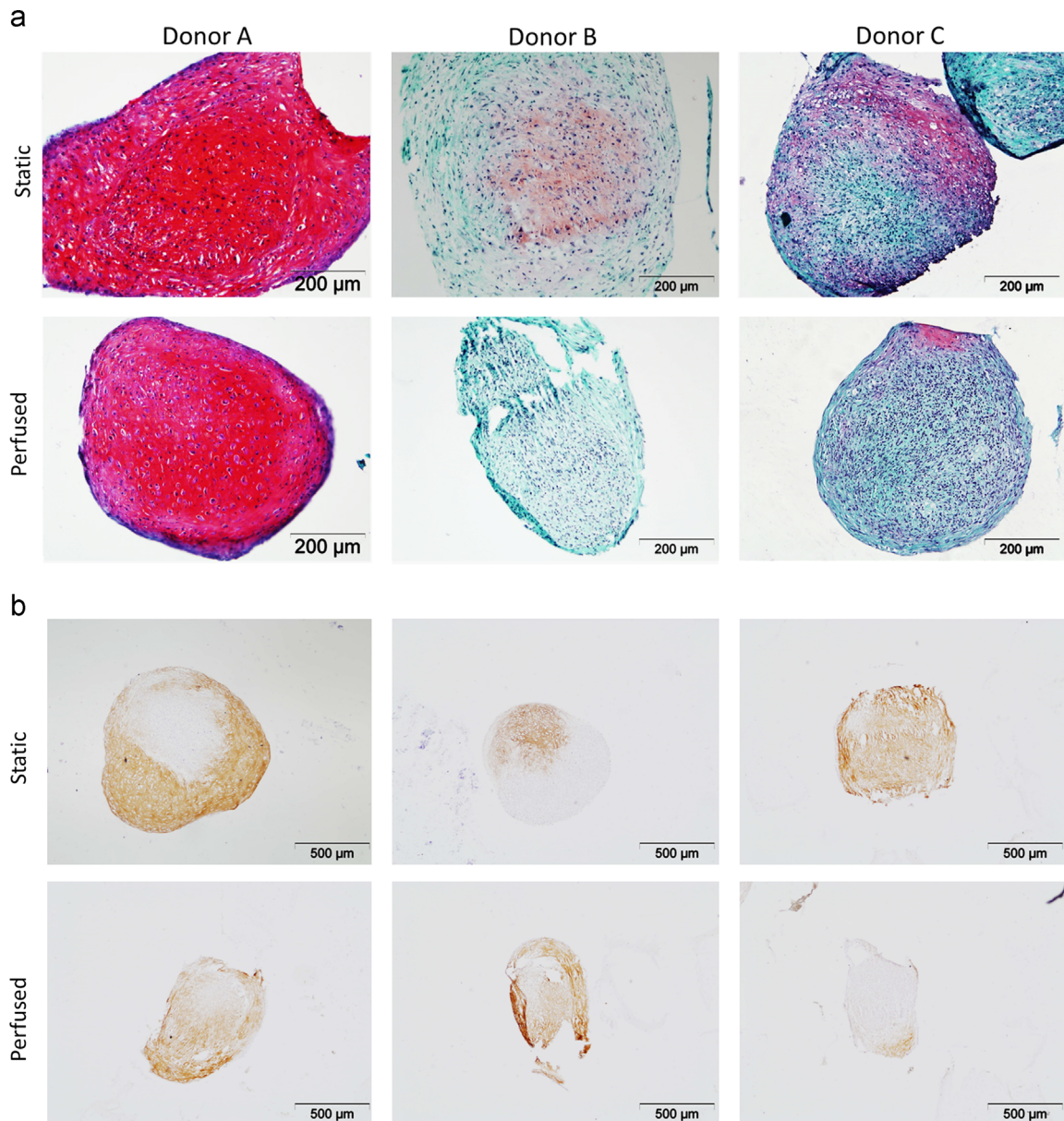


Fig. 4. Chondrogenic differentiation of MSC pellets, isolated from 3 human donors (A–C) and cultured under static or flow-perfusion conditions. (a) Safranin-O/fast green-stained paraffin sections reveal the presence of proteoglycans (red). (b) Immunohistochemical detection of collagen type II (brown) in the sections. Scale bars in (a) denote 200 μm and in (b) 500 μm . (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

4. Discussion

Flow-perfusion caused decreased production of chondrogenic and hypertrophic matrix components. Although gene expression levels of the (late) hypertrophic and osteogenic marker osteocalcin increased by flow-perfusion, this did not result in more collagen type X protein deposition. Decreased GAG release, in combination with diminished GAG and collagen II staining, indicates reduced chondrogenesis in response to flow-perfusion.

4.1. Flow-perfusion and chondrogenic hypertrophy

Gene expression levels of osteocalcin, a late hypertrophic marker, were significantly elevated after 10 days of flow perfusion compared to the static control. We hypothesize that a cartilage mold was created during the first 18 days of culture in chondrogenic medium and that increased osteocalcin gene expression

levels in response to flow-perfusion indicate the onset of hypertrophic differentiation. However, no significant effect of flow-perfusion on collagen type X or ALP gene expression levels was observed, although the latter seemed to be increased at day 28, albeit not statistically significant. Surprisingly, increased osteocalcin gene expression levels did not translate into more collagen type X protein deposition after 28 days of culture. Although differences were not very clear, collagen type X staining appeared to be more pronounced in static cultures. Interestingly, cell morphology in these areas did not resemble the typical hypertrophic geometry in large lacunae. The elevated levels of osteocalcin could also have indicated differentiation towards the osteogenic lineage (Yu et al., 2012; Gardel et al., 2013). It is possible that there were still undifferentiated MSCs present in the pellets after 18 days of chondrogenic culture, which were subsequently forced into the osteogenic pathway by flow-perfusion.

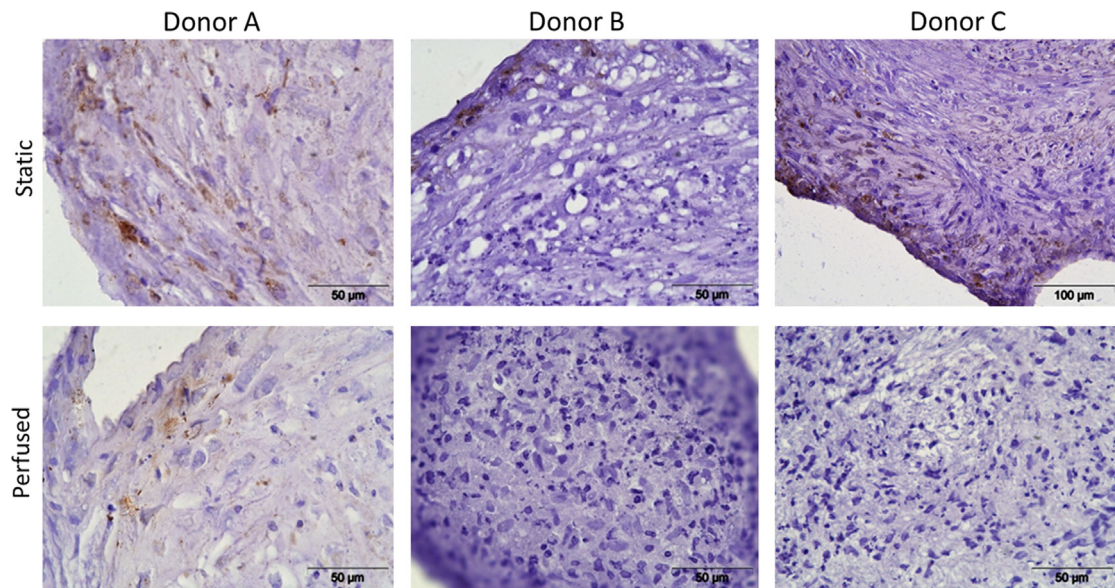


Fig. 5. Immunohistochemical detection of collagen type X (brown) in sections of MSC pellets, isolated from 3 different human donors (A–C) and cultured under static or flow-perfusion conditions. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

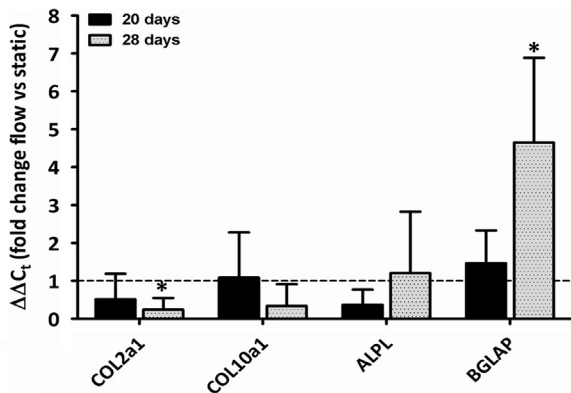


Fig. 6. The fold change of gene expression in perfused versus static cultures, normalized to housekeeping gene GAPDH at 20 and 28 days. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; COL2a1, collagen type II; COL10a1, collagen type X; BGLAP, osteocalcin; ALPL, alkaline phosphatase. * $p < 0.05$ versus static culture at the same time point.

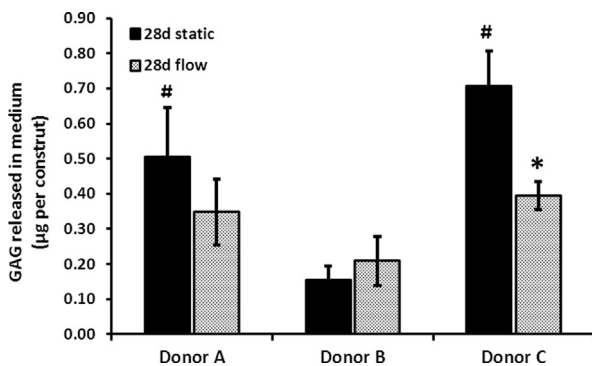


Fig. 7. GAG released per construct in culture medium in static and flow-perfused constructs at day 28, after 10 days of bioreactor culture. # $p < 0.05$ versus static culture of donor B and * $p < 0.05$ versus static culture of the same donor.

4.2. Flow-perfusion and chondrogenesis

Collagen type II gene expression levels were decreased in flow-perfused cultures. Safranin-O staining confirms the diminished chondrogenic potential of MSCs that were exposed to flow-perfusion.

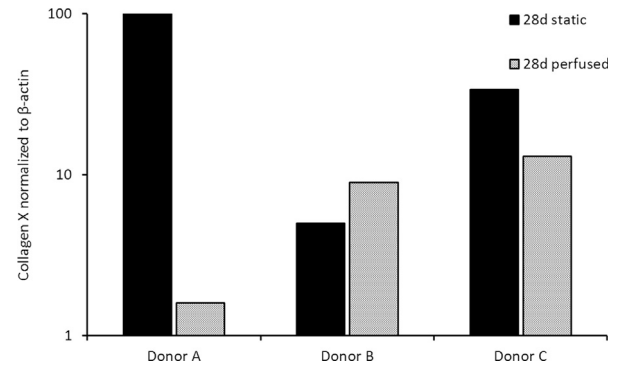


Fig. 8. The amount of collagen type X protein present in constructs cultured under static and flow-perfusion conditions after 28 days of culture.

Interestingly, previous studies demonstrated increased gene expression levels of collagen type II by goat bone marrow cells (Goncalves et al., 2011) and enhanced anabolic and catabolic responses by human articular chondrocytes (Grogan et al., 2012) and porcine chondrocytes (Tran et al., 2011) in response to a flow rate of 0.1 ml min^{-1} . The flow rate of 1.22 ml min^{-1} used in the present study is more than 10 times higher compared to aforementioned studies, which may explain the differential effects. This could be in agreement with the hypothesis that exposure of chondrocytes to low shear stress maintains chondrogenesis, whereas high shear stress induces hypertrophy (Stevens et al., 1999; Carter and Wong, 2003). However, exact amounts of shear stress applied are unknown and modeling of computational shear stress may be necessary to optimize flow-perfusion culture conditions.

4.3. Flow perfusion and distribution of matrix components

Limited mass transport may be a reason for the heterogeneous tissue distribution due to the characteristic fluid-flow environment that is created (Lappa, 2003; Radisic et al., 2006). During the 18 days of culture in chondrogenic medium prior to flow-perfusion, cells will have deposited abundant extracellular matrix components, which may have limited fluid flow and consequently nutrient supply within the pellets. One could hypothesize that application of flow-perfusion would stimulate a more homogenous tissue distribution. However,

the opposite was observed in our study (except for constructs of donor A), with cartilage deposition mostly in the periphery of flow-perfused constructs and more distributed matrix under static conditions. We suspect that the cell-tissue aggregates were very dense with low porosity, and flow-perfusion will therefore have predominantly caused shear stress at the outside of the aggregates instead of improving diffusion within the pellet cultures, which could also explain effects observed mostly at the periphery of perfused cultures.

4.4. Donor-dependent variations strongly influence treatment-dependent effects

Since inter-donor variations were mostly larger than inter-experimental differences, it is impossible to pinpoint the effect of flow-perfusion. Heterogeneity of MSCs is a well-known phenomenon that can explain success or failure of tissue regeneration (Siegel et al., 2013; Torensma et al., 2013). It was shown before that there are donor-dependent differences in MSC differentiation toward chondrogenic and hypertrophic phenotypes (Farrell et al., 2009; Gawlitta et al., 2012). Also, it was demonstrated that the isolation site of MSCs can affect the chondrogenic potential of the derived cells (Sakaguchi et al., 2005) and that tissue source has a greater influence on gene expression profiles than different culture methods (Torensma et al., 2013). To minimize variations due to site-specific characteristics, MSCs used in the present study were isolated from human bone marrow from the iliac crest. These and previously obtained results stress the necessity for a screening method to select MSCs with properties most suitable for their application.

5. Conclusion

In conclusion, our findings indicate that caution should be taken when applying flow-perfusion to cultures to improve nutrient diffusion. Although we show that it is possible to influence the differentiation of chondrogenic differentiated MSCs by flow-perfusion, effects are inconsistent and strongly donor-dependent.

Conflict of interest statement

The authors have no professional or financial conflicts of interest to disclose.

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