

THE CHONDROGENIC DIFFERENTIATION POTENTIAL OF DENTAL PULP STEM CELLS

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Abstract

Dental pulp stem cells (DPSCs) are particularly promising for tissue engineering (TE) due to the ease of their isolation procedure, great expansion potential and capability to differentiate towards several cell types of the mesodermal, ectodermal and endodermal lineages. Although several studies hint that DPSCs exhibit potential for cartilage tissue formation, the chondrogenic potential of DPSCs has only been marginally explored. Thus, the aim of the present study was to closely investigate the chondrogenic differentiation capacity of DPSCs for TE applications. More specifically, the potential of DPSCs for engineering hyaline and fibrous cartilage was determined.

DPSCs obtained from 7 human molars were expanded and chondrogenically differentiated in a 3D pellet culture model. After 21 d of differentiation with chondrogenic stimuli, DPSCs displayed glycosaminoglycan, aggrecan and limited collagen type II deposition. Cells presented an elongated morphology and produced a collagen-rich extracellular matrix, with a predominance of collagen type I in most of the samples, a characteristic of fibrous cartilage tissue. Variations in the administration periods of several chondro-inductive growth factors, including transforming growth factor beta 3, bone morphogenetic protein-2, -6, -7 and insulin-like growth factor-1, did not increase glycosaminoglycan or collagen type II deposition, typical markers of hyaline cartilage tissue. Furthermore, DPSCs could not be stimulated to go into hypertrophic chondrogenesis.

These results indicated that under a large variety of chondro-inductive culture conditions, DPSCs could form fibrocartilaginous tissues but not hyaline cartilage. Thus, DPSCs represent a valuable cell source for the regeneration of fibrocartilage in joints.

Keywords: Cartilage regeneration, hyaline, fibrous, neural-crest-derived stem cells, adult stem cell differentiation.

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List of Abbreviations		CDM	chondrogenic differentiation medium
3D	three dimensional	<i>COL1A1</i>	collagen type I
ACAN	aggrecan	<i>COL2A1</i>	collagen type II
ALP	alkaline phosphatase	<i>COL10A1</i>	collagen type X
α MEM	minimum essential medium α	<i>COMP</i>	cartilage oligomeric matrix protein
APC	allophycocyanin	DMMB	1,9-dimethyl-methylene blue
BMP	bone morphogenetic protein	DMEM	Dulbecco's modified Eagle medium
BSA	bovine serum albumin	DPSCs	dental-pulp-derived stem cells
CD	cluster of differentiation	EDTA	ethylenediaminetetraacetic acid

FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
GAGs	glycosaminoglycans
<i>GAPDH</i>	glyceraldehyde 3-phosphate dehydrogenase
GFs	growth factors
IGF-1	insulin-like growth factor-1
ITS	insulin-transferrin-selenium
MSCs	mesenchymal stem cells
NCSCs	neural crest stem cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDT	population doubling time
PE	phycoerythrin
Sox9	SRY-Box 9
TE	tissue engineering
TGF- β 1	transforming growth factor beta 1
TMJ	temporomandibular joint

Introduction

Since their first reported isolation (Gronthos *et al.*, 2000), DPSCs have emerged as a particularly promising cell type for TE for several reasons (Anitua *et al.*, 2018; Graziano *et al.*, 2008). One of these reasons is the accessibility of the surgical site, as DPSCs can be isolated from routinely extracted third molars and potentially stored for further use, without additional donor site morbidity (Graziano *et al.*, 2008; Kruijt Spanjer *et al.*, 2017; Tirino *et al.*, 2012). After extraction, DPSCs can be obtained from the pulp through enzymatic digestion or direct plating of minced pulp tissue (Hilkens *et al.*, 2013). Previous work has demonstrated that these cells display a higher proliferation rate and higher colony forming efficiency *in vitro* than other types of multipotent stem cells in both humans (Gronthos *et al.*, 2000; Ponnaiyan and Jegadeesan, 2014; Tamaki *et al.*, 2013) and rats (Alge *et al.*, 2010). Additionally, due to their neural crest origin, DPSCs have the potential to differentiate towards cell types belonging to the mesodermal, ectodermal and endodermal lineages, such as osteoblasts (Takebe *et al.*, 2017; Zhang *et al.*, 2006), odontoblasts (Gronthos *et al.*, 2000), adipocytes (Takebe *et al.*, 2017; Zhang *et al.*, 2006), chondrocytes (Takebe *et al.*, 2017; Zhang *et al.*, 2006), myoblasts (Zhang *et al.*, 2006), neurons (Zhang *et al.*, 2006) and hepatocytes (Graziano *et al.*, 2008; Nuti *et al.*, 2016). In particular, their osteogenic and odontoblastic differentiation potential for intramembranous bone TE and dentistry applications is the most extensively studied (Anitua *et al.*, 2018; Gronthos *et al.*, 2000; La Noce *et al.*, 2019; Morad *et al.*, 2013; Nuti *et al.*, 2016; Paino *et al.*, 2017). In contrast, the chondrogenic potential of DPSCs for TE applications remains underexplored as most studies investigating DPSCs have only addressed their chondrogenesis as part of their multilineage differentiation potential for human (Hilkens *et al.*, 2013; Koyama *et al.*, 2009; Mokry *et al.*, 2010; Takebe *et al.*, 2017; Zhang *et al.*,

2006), murine (Grottkau *et al.*, 2010) and porcine cells (Iohara *et al.*, 2006). Nevertheless, investigating DPSC chondrogenic potential might be appealing, as the dental pulp and its stem cells originate from the NCSCs, which are also involved in the development of the TMJ disc (Chai *et al.*, 2000) and Meckel's cartilage – the supportive, transient hyaline cartilage tissue present during embryonic development of the mammalian mandible (Chai *et al.*, 2000). Thus, DPSCs might represent a promising cell source for cartilage TE. In particular, it is interesting to explore the suitability of DPSCs for the regeneration of hyaline cartilage and fibrocartilage. These two types of cartilage have different characteristics. Hyaline cartilage is typically found on the articulating surfaces of synovial joints such as knees and elbows, in the connection between ribs and sternum and in the respiratory system. It is composed of small numbers of chondrocytes dispersed in a matrix rich in GAGs and collagen type II (Sophia Fox *et al.*, 2009). On the other hand, fibrocartilage is found in the TMJ, the intervertebral discs and the menisci. It is composed of a heterogeneous cell population, with most cells presenting a fibroblastic phenotype and the remainder being chondrocyte-like cells, which express chondrocyte markers but lack the typical hyaline pericellular matrix (Allen and Athanasiou, 2006). The smaller amount of GAGs and the predominance of collagen type I characterise the fibrocartilage matrix (Allen and Athanasiou, 2006).

By applying differentiation protocols developed for bone-marrow-derived MSCs, a variable chondrogenic potential of DPSCs is observed. In particular, depending on the donor and the culture conditions, DPSCs either fail to differentiate into chondrocytes (Pierdomenico *et al.*, 2005; Sternberg *et al.*, 2012) or are able to express typical chondrogenic differentiation markers, such as aggrecan (Spath *et al.*, 2010; Takebe *et al.*, 2017) and collagen type II (Koyama *et al.*, 2009; Zhang *et al.*, 2006), as well as to secrete GAGs *in vitro* (Nemeth *et al.*, 2014; Spath *et al.*, 2010; Takebe *et al.*, 2017; Zhang *et al.*, 2006). Discrepancies in the applied culture methods could partially explain these differences as, in a few studies, chondrogenesis was induced in a monolayer culture system (Alge *et al.*, 2010; Alvarez *et al.*, 2015; Pereira *et al.*, 2012). Despite this, the *in vitro* model presents limitations when compared to 3D culture methods (e.g. pellet culture) (Caron *et al.*, 2012; Yoo *et al.*, 1998). Nevertheless, during chondrogenesis, DPSCs present a different gene expression profile from MSCs (Sternberg *et al.*, 2012). Thus, similar to other types of MSC-like populations such as adipose-derived stem cells (Puetzer *et al.*, 2010), DPSCs might respond differently to chondrogenic stimuli. Therefore, differentiation protocols developed for MSCs might need to be further optimised to reveal the full chondrogenic potential of DPSCs.

It is uncertain whether differentiated DPSCs have the capability, as MSCs, to acquire a hypertrophic phenotype. This is particularly relevant when aiming

Table 1. DPSC donor demographics and molar origin. - indicates unknown element number.

Donor	Age	Sex	Molar origin	Element number
A	21	Female	Maxilla	-
B	25	Female	Mandible	-
C	20	Male	Maxilla	-
D	20	Female	Maxilla	18
			Maxilla	28
			Mandible	38
E	21	Female	Mandible	38

at cartilage regeneration. If chondrocytes acquire a hypertrophic phenotype, they start secreting proangiogenic factors and metalloproteinases that promote blood vessel invasion and matrix remodelling upon *in vivo* implantation. Consequently, the cartilaginous TE construct could be eventually remodelled into bone, by following the endochondral ossification pathway (Collignon *et al.*, 2019; Gawlitta *et al.*, 2010).

A more systematic approach is required for exploring the chondrogenic potential of DPSCs. Thus, the aim of the present study was to closely investigate the chondrogenic differentiation potential of human DPSCs to evaluate the feasibility of their use for hyaline cartilage and fibrocartilage TE applications. To do so, the differentiation potential of DPSCs was characterised from 7 third molars. Furthermore, the differentiation protocol was tailored by comparing the effect of several chondro-inductive GFs. Finally, the potential of DPSCs to pursue hypertrophic chondrogenic differentiation was investigated.

Materials and Methods

Isolation and expansion of human DPSCs

DPSCs were obtained from the dental pulp of 7 extracted third molars from 5 patients at the University Medical Centrum Utrecht after acquiring their written informed consent (UMCU biobank, protocol number 15-149) (Table 1). Directly after extraction, the molars were sterilised in 70 % ethanol and stored temporarily in sterile PBS containing 100 units/mL penicillin and 100 µg/mL streptomycin (pen/strep; Invitrogen). Subsequently, a slit was created in the enamel along the buccal and lingual grooves using a sterile dental aerator drill with a diamond burr. Next, the teeth were sterilised in 10 % povidone-iodine (Fresenius-Kabi, Bad Homburg vor der Höhe, Germany) for 5 min and, subsequently, bifurcated with a dental elevator. Then, the dental pulp was removed from the pulp chamber, cut into pieces of approximately 1 mm³ and incubated for 3 h at 37 °C and 5 % CO₂ in 3 mg/mL collagenase type II (Worthington). A single cell suspension was obtained from the digests using a 70 µm cell strainer. Cells were plated and cultured at 37 °C and 5 % CO₂ in expansion medium consisting of αMEM (Gibco) supplemented

with 10 % heat-inactivated FBS (HyClone), 0.2 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich), 1 % pen/strep and 1 ng/mL basic fibroblast growth factor (R&D Systems). Medium was gently refreshed after 24 h to remove debris and every 3-4 d thereafter. DPSCs were cultured until 80 % confluency and passaged at a density of 1,500 cells/cm². Human MSCs used as control were isolated and characterised as previously described (Gawlitta *et al.*, 2012).

Marker expression profile

At passage 4, DPSCs were characterised for their surface and internal marker expression by flow cytometry using a FACS Canto II system (BD Biosciences). The presence of the MSC markers CD73 (PE-conjugated), CD90 (FITC-conjugated), CD105 (APC-conjugated) and CD271 (FITC-conjugated) along with the absence of the haematopoietic stem cell markers CD34 (APC-conjugated) and CD45 (PE-conjugated), the monocyte marker CD14 (FITC-conjugated) and the B cell marker CD79α (PE-conjugated) were determined. All antibodies were purchased from Abcam. Additionally, the level of internally expressed neural crest marker nestin (PE-conjugated; R&D Systems) was examined in fixed and permeabilised cells. DPSCs were stained for 15 min at room temperature, shielded from direct light. Dilutions of the antibodies were used according to the manufacturer's instructions. Isotype-matched controls (Abcam) and unstained cells were included as negative controls for the gating strategy. For nestin, an additional control consisting of unstained, fixed and permeabilised cells was included. Data were analysed using FlowJo (version 10, Ashland, OR, USA). Gating of single, viable cells for the groups that were stained for the extracellular CD markers was based on the forward and sideward scatter dot plots of the unstained cell controls. Since the number of recorded events per sample varied, the flow cytometry data are presented normalised to the peak height (number of events), resulting in relative percentages. A minimum of 10,000 events were recorded for each flow cytometry measurement.

Colony count and proliferation assay

To assess the colony forming potential, primary isolated DPSC colonies containing more than 25 cells were counted under a microscope (DMi1; Leica) in

duplicate. Data are presented as the mean of the two individual counts.

At passage 3, DPSCs were seeded at 1,500 cells/cm² and cultured in expansion medium for 7 d at 37 °C in 5 % CO₂. Afterwards, cells were harvested and counted in triplicate on at least three time points (ranging from 1 to 7 d after seeding). In order to determine the time points in between which the cells displayed exponential growth, the logarithmic average cell yield values were plotted against time. In this graph, the lag phase, log phase and stationary phase of cell growth were identified. The log phase (*i.e.* the linear region of this graph) was selected to calculate the *PDT* by applying the equation:

$$PDT = \frac{(Ty - Ti) * \log(2)}{\log\left(\frac{Y}{I}\right)}$$

where *Ty* = incubation time at end of log phase (h); *Ti* = incubation time at the start of log phase (h); *Y* = cell number at the end of the log phase; *I* = cell number at the start of the log phase.

Three-lineage differentiation potential

For osteogenic differentiation, DPSCs were cultured at passage 3 in monolayers in osteogenic differentiation medium [α MEM supplemented with 10 % heat-inactivated FBS, 0.2 mM L-ascorbic acid-2-phosphate, 1 % pen/strep, 10 mM β -glycerophosphate (Sigma-Aldrich) and 10 nM dexamethasone (Sigma-Aldrich)] that was refreshed twice per week. After 7 d, cells were fixed with 4 % formaldehyde, permeabilised with 0.2 % Triton-X in Tris-buffered saline and stained for ALP activity using the Fuchsin+ Substrate-Chromogen System (Dako) according to the manufacturer's instructions. Mineralisation was assessed at day 14 by staining the fixed monolayers with 2 % alizarin red S (pH 4; Sigma-Aldrich) for 2 min. Images were acquired using a DMi1 microscope (Leica).

For adipogenic differentiation, DPSCs were cultured in monolayers in adipogenic differentiation medium [α MEM supplemented with 10 % heat-inactivated FBS, 1 % pen/strep, 1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 0.2 mM indomethacin (Sigma-Aldrich) and 1.72 μ M insulin (Sigma-Aldrich)] that was changed twice a

week. After 21 d, cells were fixed in 10 % formalin, washed with 60 % isopropanol and stained with oil red O (Sigma-Aldrich) for 20 min. Images were acquired using a DMi1 microscope.

For chondrogenic differentiation, DPSCs were centrifuged into pellets (200,000 cells/pellet) in polypropylene tubes (Greiner) and cultured in 500 μ L CDM consisting of high-glucose DMEM (Thermo Fisher Scientific) supplemented with 1 % ITS + Premix (Corning), 0.1 μ M dexamethasone, 0.2 mM L-ascorbic acid-2-phosphate, 1 % pen/strep as previously described (Gawlitta *et al.*, 2012). 10 ng/mL TGF- β 1 (PeproTech) was freshly added to the CDM. The medium was refreshed daily during the first 4 d after pellet formation, thereafter twice a week. After 21 d of culture, the pellets were fixed in 10 % formalin, dehydrated, cleared in xylene and embedded in paraffin wax. 5 μ m-thick sections (cut using a Microm HM340E microtome; Thermo Fisher Scientific) were deparaffinised with xylene and rehydrated. To assess GAG production, sections were stained with 0.04 % toluidine blue solution (Sigma-Aldrich) in 0.1 M sodium acetate (pH 4; Sigma-Aldrich) and counterstained with 0.2 % fast green (Sigma-Aldrich). To evaluate collagen deposition, sections were stained with alcian blue (pH 2.5; Sigma-Aldrich) and picosirius red (Klinipath, Duiven, the Netherlands). Images were acquired using an Olympus BX51 microscope and Olympus DP70 camera.

Gene expression analysis

DPSCs [*n* = 3, donor A, D(28) and E] were expanded and harvested at passage 4 to form pellets as described above. After 21 d of chondrogenic induction, pellets were mechanically crushed, using pestle and mortar, in TRIzol reagent (Thermo Fisher Scientific) and mRNA was isolated from the aqueous phase obtained, according to the manufacturer's instructions. DNA contaminations were removed by a DNase treatment (Turbo DNase; Thermo Fisher Scientific), according to the manufacturer's instructions. mRNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) at 260/280 nm. cDNA was synthesised using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Gene expression analysis of *SOX9*, *COMP*, *ACAN*, *COL1A1*, *COL2A1* and *COL10A1* was done on a Bio-Rad CFX96 Real-

Table 2. List of primers used.

Gene	Primer forward (5'-3')	Primer reverse (5'-3')
<i>SOX9</i>	CAACGCCGAGCTCAGCA	TCCACGAAGGGCCGC
<i>COMP</i>	CCCCAATGAAAAGGACAACCTGC	GTCCTTTTGGTCGTCGTTCTTC
<i>ACAN</i>	ATGTTCCCTGCAATTACCACCT	TTGATCTCATAACCGGTCCTTCTTC
<i>COL1A1</i>	AAGAGGAAGGCCAAGTCGAG	GTTTCCACACGTCTCGGTCA
<i>COL2A1</i>	GCCTCAAGGATTTCAAGGCAAT	GCTTTTCCAGGTTTTCCAGCTT
<i>COL10A1</i>	CAAGGCACCATCTCCAGGAA	AAAGGGTATTGTGGCAGCATATT
<i>GAPDH</i>	ATGGGGAAGGTGAAGGTCTG	TAAAGCAGCCCTGGTGACC

Time PCR Detection System (Bio-Rad) using FastStart SYBR Green Master mix (Sigma-Aldrich). *GAPDH* was used as a housekeeping gene and the relative expression was determined by the $2^{-\Delta CT}$ method. Values above cycle 40 were considered undetectable. Primers used are listed in Table 2.

Tailoring the DPSC chondrogenic differentiation protocol for hyaline cartilage

DPSCs [$n = 3$, donor A, D(28) and E] were expanded and harvested at passage 4 to form pellets (500,000 cells/pellet) in round-bottom, Corning® Costar® Ultra-Low Attachment 96-well plates. Different GFs associated with chondrogenesis were evaluated for their potential to stimulate GAG and collagen deposition when supplemented to the CDM, either singularly or in combinations. The screened GFs and their respective concentrations were: 10 ng/mL TGF- β 3 (PeproTech), 100 ng/mL BMP-2 (InductOS, Wyeth/Pfizer, New York, NY, USA), 100 ng/mL BMP-6 (PeproTech), 100 ng/mL BMP-7 (PeproTech) and 100 ng/mL IGF-1 (PeproTech). Pellets of each donor were divided into three groups. In the first group, pellets received only one single GF during the whole culture period (*i.e.* TGF- β 3, BMP-2, BMP-6, BMP-7 or IGF-1). In the second group, pellets received a

continuous combination of TGF- β 3 and one of the BMP isotypes or IGF-1 (*i.e.* TGF- β 3 + BMP-2, TGF- β 3 + BMP-6, TGF- β 3 + BMP-7, TGF- β 3 + IGF-1). The third group of pellets was stimulated with a cyclic alternating pattern of TGF- β 3 and one of the BMP isotypes or IGF-1 (*i.e.* TGF- β 3 and BMP-2, TGF- β 3 and BMP-6, TGF- β 3 and BMP-7, TGF- β 3 and IGF-1). A schematic representation of the different medium changes is available in Fig. 1. Pellets cultured in CDM without any additional GF were included as negative controls. After 28 d, 2 pellets per group were processed for histological analysis as described in the previous paragraph. 3 other pellets were digested overnight at 60 °C in papain digestion buffer [250 μ g/mL papain, 0.2 M NaH₂PO₄, 0.1 EDTA and 0.01 M DL-cysteine hydrochloride (all Sigma-Aldrich)]. The total amount of GAGs was determined using the DMMB (pH 3.0; Sigma-Aldrich) assay (Farndale *et al.*, 1986). Chondroitin sulphate C (Sigma-Aldrich) was used as standard and the absorbance was measured. The sulphated GAG content was expressed as the ratio between the 525 and 595 nm absorbance values. To normalise the GAG content, DNA was quantified using a Quant-iT Picogreen dsDNA assay (Thermo Fisher Scientific) according to the manufacturer's instructions.

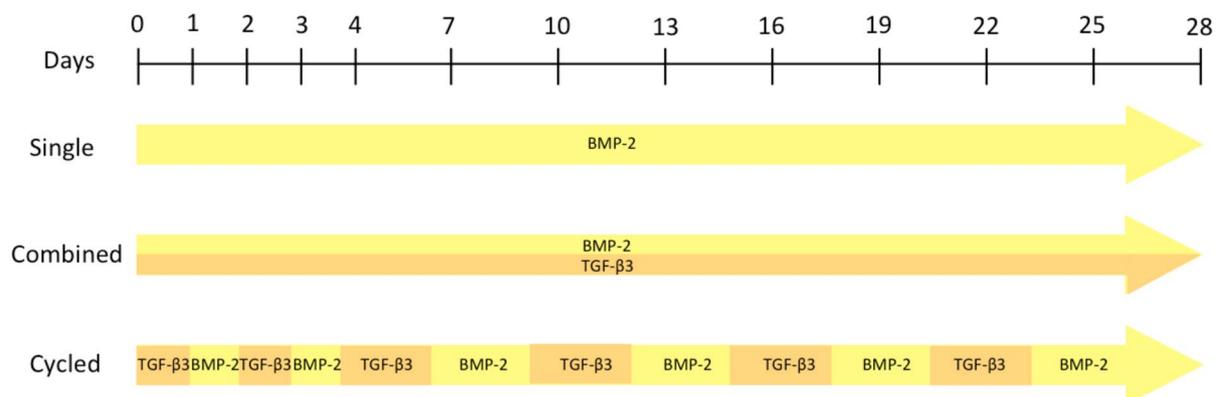


Fig. 1. Schematic representation of the three different patterns of medium change. BMP-2 is used as an example to explain the patterns. The same patterns were applied for BMP-2, BMP-6, BMP-7 and IGF-1, all in combination with TGF- β 3. After 28 d of culture, samples were harvested and processed for analysis.

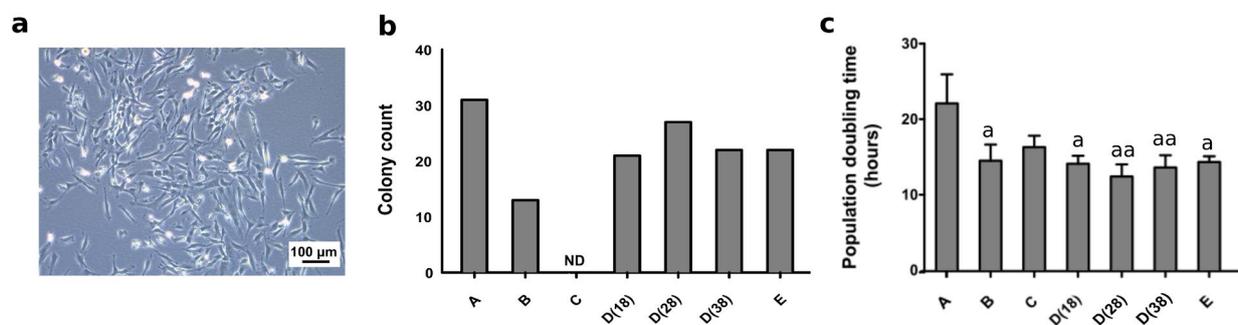


Fig. 2. DPSCs characterisation after isolation. (a) Representative images of DPSCs fibroblastic morphology and (b) number of colonies (mean of two individual countings of the same DPSC culture) formed by primary DPSCs. (c) Proliferation potential of all DPSC cultures expressed as PDT determined at passage 3. Statistical differences are shown as ^a $p \leq 0.01$, ^{aa} $p \leq 0.001$. ND: not determined because individual colonies could not be distinguished.

Hypertrophy induction

To assess whether chondrogenically stimulated DPSCs were capable of responding to hypertrophic induction, pellets were formed at passage 4, as described above [$n = 3$, donor A, D(28) and E]. Since none of the tested (pattern of) GFs outperformed the others in terms of GAG deposition, chondrogenic differentiation was carried out by supplementing the medium with TGF- β 3. After 28 d, the medium was changed to hypertrophic induction medium consisting of high-glucose DMEM supplemented with 1 % ITS + Premix, 1 nM dexamethasone, 0.2 mM L-ascorbic acid-2-phosphate, 1 % pen/strep, 10 mM

β -glycerophosphate and 1 nM triiodothyronine (Sigma-Aldrich) for 7 additional days. At day 35, pellets were fixed and processed for histology as described above. In addition to toluidine blue staining, collagen type X immunostaining and von Kossa staining for mineralisation were performed to assess hypertrophic matrix formation. von Kossa staining was done by incubating the sections for 1 h with 1 % silver nitrate (Sigma-Aldrich) directly under a light bulb (Philips Master TL5 HO 54W 830, 1 m distance). Subsequently, the samples were washed with 5 % sodium thiosulphate (Alta Aesar, Haverhill, MA, USA) and counterstained with haematoxylin.

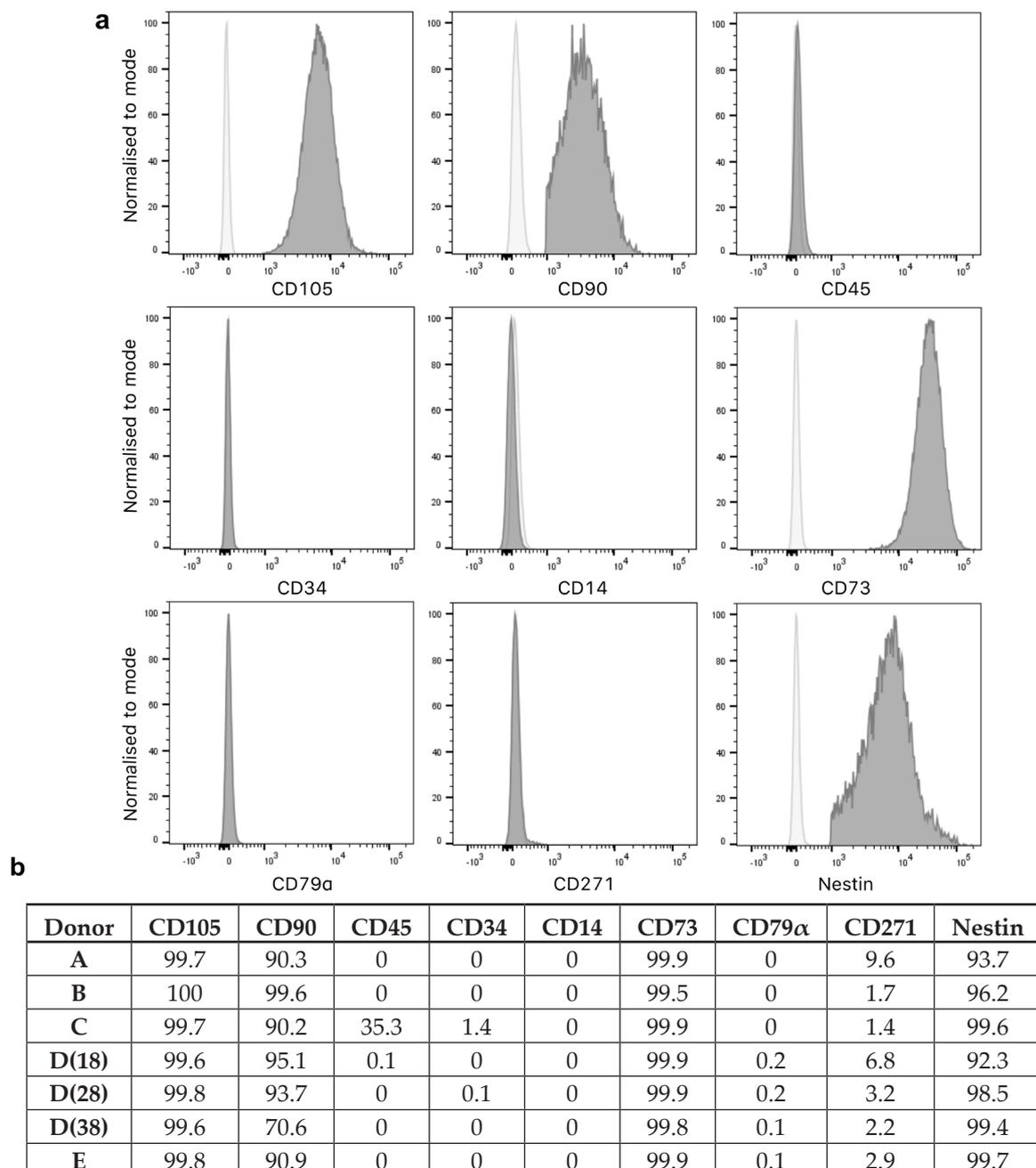


Fig. 3. Expression of stem cell surface markers per DPSC culture. (a) An example of the graphs obtained from donor E (dark grey) and the relative isotype controls (light grey). (b) Percentage of positive cells for mesenchymal, haematopoietic and neural crest cell markers are depicted for DPSCs derived from the different donors.

Immunohistochemistry

To define the collagen types present in the matrix, immunohistochemical staining with primary antibodies directed against aggrecan (Novus Biologicals, Centennial, CO, USA), collagen type I (Abcam), collagen type II (DSHB, University of Iowa, Iowa City, IA, USA) and collagen type X (Quartett, Berlin, Germany) was performed. All sections were deparaffinised and rehydrated as described above. For aggrecan and collagen type I stainings, antigens were retrieved by boiling the samples for 15 min at 95 °C in 10 nM citrate buffer. For collagen type II detection, antigens were retrieved by treating the sections at 37 °C with 1 mg/mL pronase (Sigma-Aldrich) and 10 mg/mL hyaluronidase (Sigma-Aldrich) for 30 min each. Finally, for collagen type X staining, antigens were retrieved by sequential incubation for 2 h with 1 mg/mL pepsin (Sigma-Aldrich) at pH 2.0 and for 30 min with 10 mg/mL hyaluronidase, both at 37 °C. The endogenous peroxidase activity was blocked by treating the sections for 10 min with 0.3 % H₂O₂ solution. Furthermore, to prevent aspecific binding, the samples were incubated with 5 % BSA (Roche) in PBS for 30 min. Primary antibodies were diluted in 5 % BSA/PBS (1 : 150 aggrecan, 3 µg/mL collagen type I, 0.6 µg/mL collagen type II, 10 µg/mL collagen type X) and incubated overnight at 4 °C. Mouse isotypes (Dako) were used as negative controls at concentrations or dilutions matched with those of the primary antibodies. Samples were incubated for 30 min at room temperature with EnVision+ System-HRP labelled secondary antibody (Dako). Antigen detection was performed by incubation with 3,3'-diaminobenzidine tetrahydrochloride hydrate for no longer than 10 min. Sections were

counterstained with 1 : 1 Mayer's haematoxylin in deionised water (Merck), dehydrated and mounted with permanent mounting medium (Depex; Merck).

Statistical analysis

GAG/DNA-ratios are presented as means of at least three biological replicates and their standard deviations. A randomised block design with Bonferroni *post-hoc* analysis was applied to the GAG/DNA ratios, absolute DNA values and absolute GAG content, in order to evaluate statistical differences between groups. An unpaired *t*-test with Welch's correction was performed in order to evaluate the differences in gene expression between DPSCs and MSCs. SPSS (version 24, IBM) was used for all statistical analyses. Statistical significance was defined as $p < 0.05$.

Results

DPSC isolation and characterisation

7 cultures were successfully established from the dental pulp tissue derived from 5 healthy human donors (Table 1). Cells adhering to the tissue-culture plastic were visible within a maximum of 9 d after plating the tissue digests and grew out into distinct colonies. The numbers of colonies formed and the proliferation rates varied between DPSCs isolated from different donors (Fig. 2b,c). After the first passage, all DPSCs displayed an elongated, fibroblastic morphology (Fig. 2a). Most DPSC cultures expressed cell surface markers (> 90 % positive) associated with mesenchymal stem cells, including CD105, CD90 and CD73 and no expression

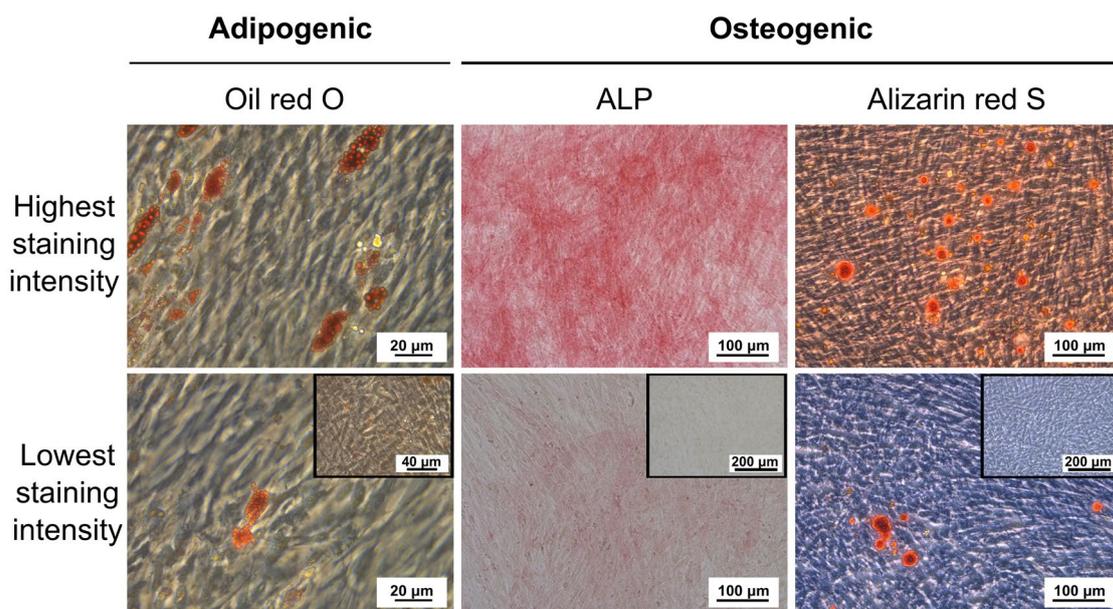


Fig. 4. Microscopical evaluation of the differentiation potential of DPSCs towards the adipogenic and osteogenic lineages. Variations in lipid vacuole formation (orange/red vesicles), ALP activity (red staining) and mineralised nodule (bright red nodules) formation were observed between the DPSCs derived from different donors and within cultures derived from the same donor. Inserts represent the negative undifferentiated controls.

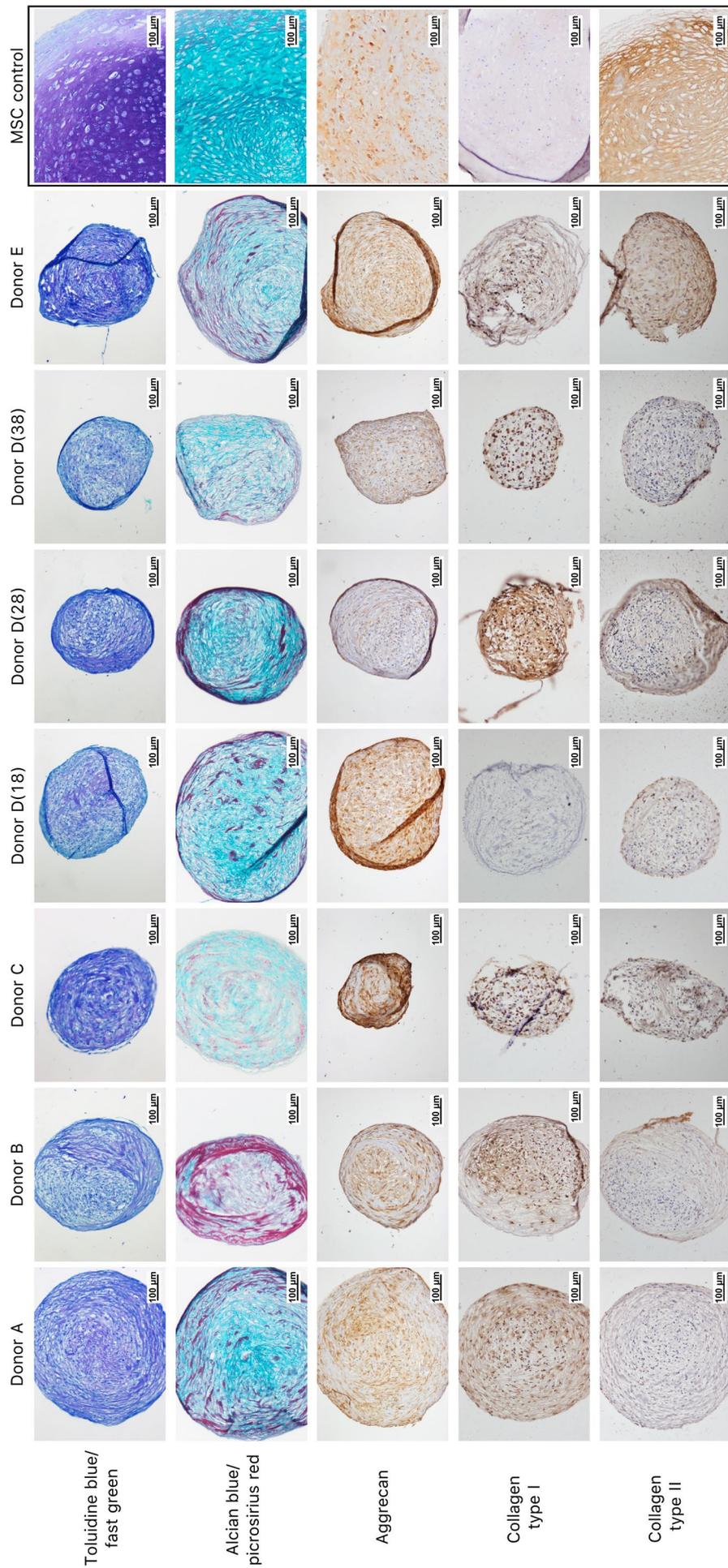


Fig. 5. Overview of the chondrogenic differentiation potential of DPSCs from different molars and donors. DPSCs from all donors displayed chondrogenic differentiation, highlighted by GAG (toluidine blue and alcian blue staining) and aggrecan deposition. The presence of collagen was determined by picrosirius red staining and specific immunohistochemistry for collagen types I and II. Bone-marrow-derived MSCs cultured with the same differentiation protocol were included as a positive control for chondrogenic differentiation.

(< 1.0 % positive) of the haematopoietic stem cell markers CD34 and CD45, monocyte marker CD14 and B cell marker CD79 α (Fig. 3). Additionally, most of the DPSCs were positive for the neural crest marker nestin. Finally, CD271 was only expressed in a small subpopulation of each DPSC culture (1.4-9.6 %). After characterisation, DPSCs from donor C were excluded from further experiments as a relatively large cell population was positive for CD45 (35.3 %), suggesting the presence of a haematopoietic subpopulation.

In contrast to the limited donor variation observed with respect to proliferation potential (Fig. 2), the

DPSCs exhibited heterogeneous differentiation capacity (Fig. 4,5). Overall, all osteogenically stimulated DPSC cultures displayed ALP activity, an early osteogenesis marker, to various extents and mineralisation nodules, a late osteogenesis marker. More variation was observed when DPSCs were differentiated towards the adipogenic lineage as the presence of intracellular lipid vesicles was observed in 5 out of 7 DPSC cultures. GAGs and aggrecan were present in all DPSC pellets after chondrogenic induction, although inter- and intra-donor variation was observed (Fig. 5). Furthermore, for most DPSC

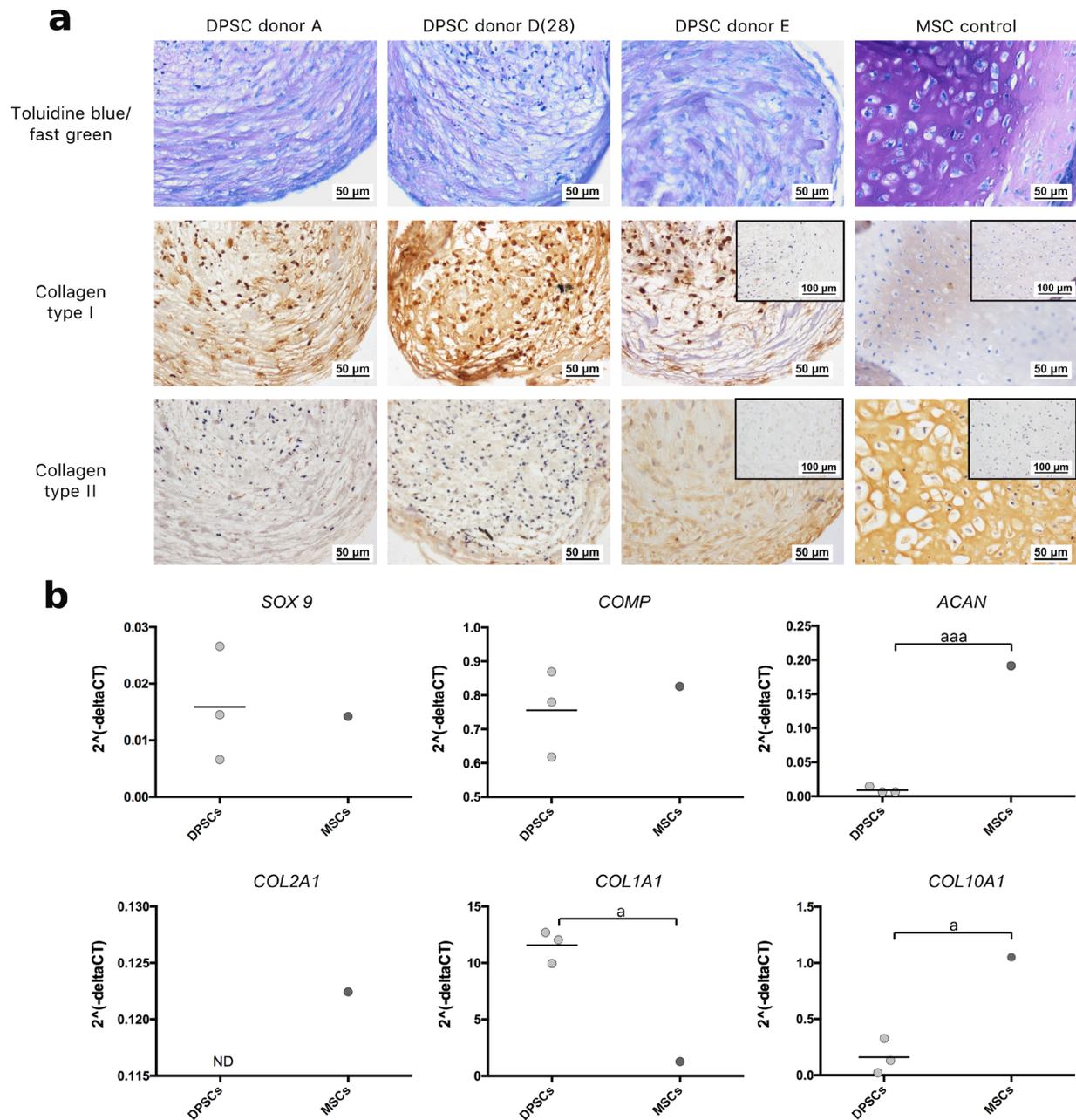


Fig. 6. Evaluation of cell morphology and expression of chondrogenic markers for 3 DPSC donors. (a) A higher magnification of the toluidine-blue-stained sections highlighted the absence of the typical chondrocyte lacunae. Furthermore, the presence of collagen type I and II was highlighted when compared to the isotypes control depicted in the inserts. (b) Gene expression analysis confirmed the presence of chondrogenic markers after 21 d. MSC pellets cultured with the same differentiation protocol were included as a positive chondrogenic control. Dots represent the expression of the individual donors. ND: not detectable. Statistical differences are shown as ^a $p \leq 0.05$, ^{aaa} $p \leq 0.001$.

pellets, collagen type I was most distinctly present in the ECM when compared to collagen type II (Fig. 5). Gene expression analysis of the DPSCs confirmed the presence of chondrogenic markers including *SOX9*, *COMP* and *ACAN* in the pellet cultures derived from 3 donors. Consistent with the immunohistochemistry results, *COL1A1* expression was upregulated in all cases. In contrast, *COL2A1* expression was undetectable after 21 d of differentiation (Fig. 6). In addition, the DPSCs adopted a flattened morphology, indicative of fibrocartilage formation, rather than a round morphology in lacunae (Fig. 6), a characteristic of hyaline cartilage tissue.

Assessing DPSC potential for hyaline cartilage differentiation

To evaluate whether DPSCs also possessed the capacity to differentiate into the hyaline chondrogenic lineage *in vitro*, additional formulations and various patterns of exposure to chondro-inductive media were evaluated.

Overall, the toluidine blue staining highlighted the GAG deposition for the different GFs and patterns of exposure (Fig. 7a). No statistically significant differences were found in the GAG/DNA-ratios of the pellets that were cultured with the evaluated factors applied singularly or in an alternating pattern with TGF- β 3 (Fig. 7b,c). Interestingly, the GAG/DNA-ratio of pellets exposed only to CDM was comparable to those receiving GFs in a singular or cycled fashion

(Fig. 7b,c). Furthermore, pellets that received a BMP isotype combined with TGF- β 3 (Fig. 7d) displayed the lowest GAG/DNA-ratio. Immunohistochemical evaluation highlighted that none of the conditions promoted extensive collagen type II deposition, while collagen type I remained an abundant component of the ECM in all conditions (Fig. 8). In addition, cells remained flat and were not enclosed in lacunae.

DPSCs did not differentiate into the hypertrophic chondrogenic phenotype

Although differentiation into the hyaline cartilage lineage appeared limited, whether DPSCs were at all capable of continuing differentiation along this lineage into the hypertrophic chondrogenic phenotype was explored. Toluidine blue staining confirmed GAG production. However, no mineral deposition or collagen type X was present in the ECM of the pellets that were either exposed to chondrogenic medium only or to hypertrophic medium (Fig. 9).

Discussion

Stem cells derived from the cranial neural crest, which during development are involved in the formation of Meckel's cartilage and TMJ disc, can still be found after birth in the dental pulp (Chai *et al.*, 2000). Thus, the use of DPSCs was explored in the context

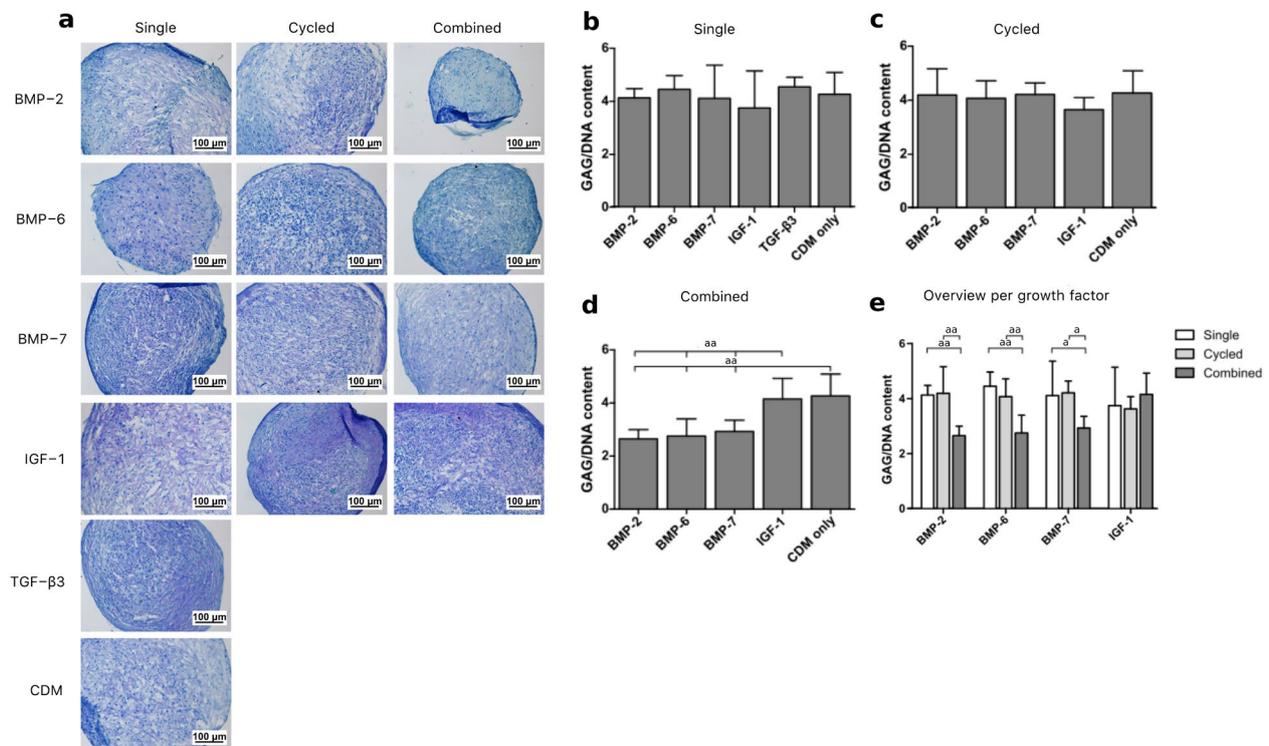


Fig. 7. Effect of the different GFs and their administration patterns on GAG deposition. (a) Toluidine blue staining highlighted GAG deposition in all conditions. GAG/DNA ratios when GFs were applied (b) singularly, (c) in an alternating cyclic pattern or (d) combined. (e) Combined overview. In the cycled group, GFs were alternated with TGF- β 3 whereas, in the combined group, they were supplemented together with TGF- β 3. No GFs were added in the CDM group. Statistical differences are shown as ^a $p \leq 0.01$, ^{aa} $p \leq 0.001$.

of cartilage TE for maxillofacial applications. In particular, the present study extensively analysed the DPSC capacity to form hyaline and fibrous cartilage *in vitro* by applying various differentiation regimes.

DPSC pellets derived from all 7 third molars extracted produced an ECM consisting of GAGs, aggrecan and collagen types I and II, which was consistent with other reports (Grottkau *et al.*, 2010; Iohara *et al.*, 2006; Koyama *et al.*, 2009; Zhang *et al.*, 2006). This, together with the expression of *SOX9*

and *COMP*, confirmed the competence of these cells to differentiate toward the chondrogenic lineage. However, histological analysis identified collagen type I as the predominant collagen type of the ECM in most of the cultures. Consistently, expression of *COL1A1* was also upregulated. Additionally, DPSCs displayed an elongated morphology typical of the fibroblast-like cells residing in the TMJ disc (Allen and Athanasiou, 2006). In contrast, GAG and collagen type II deposition was limited when compared to the

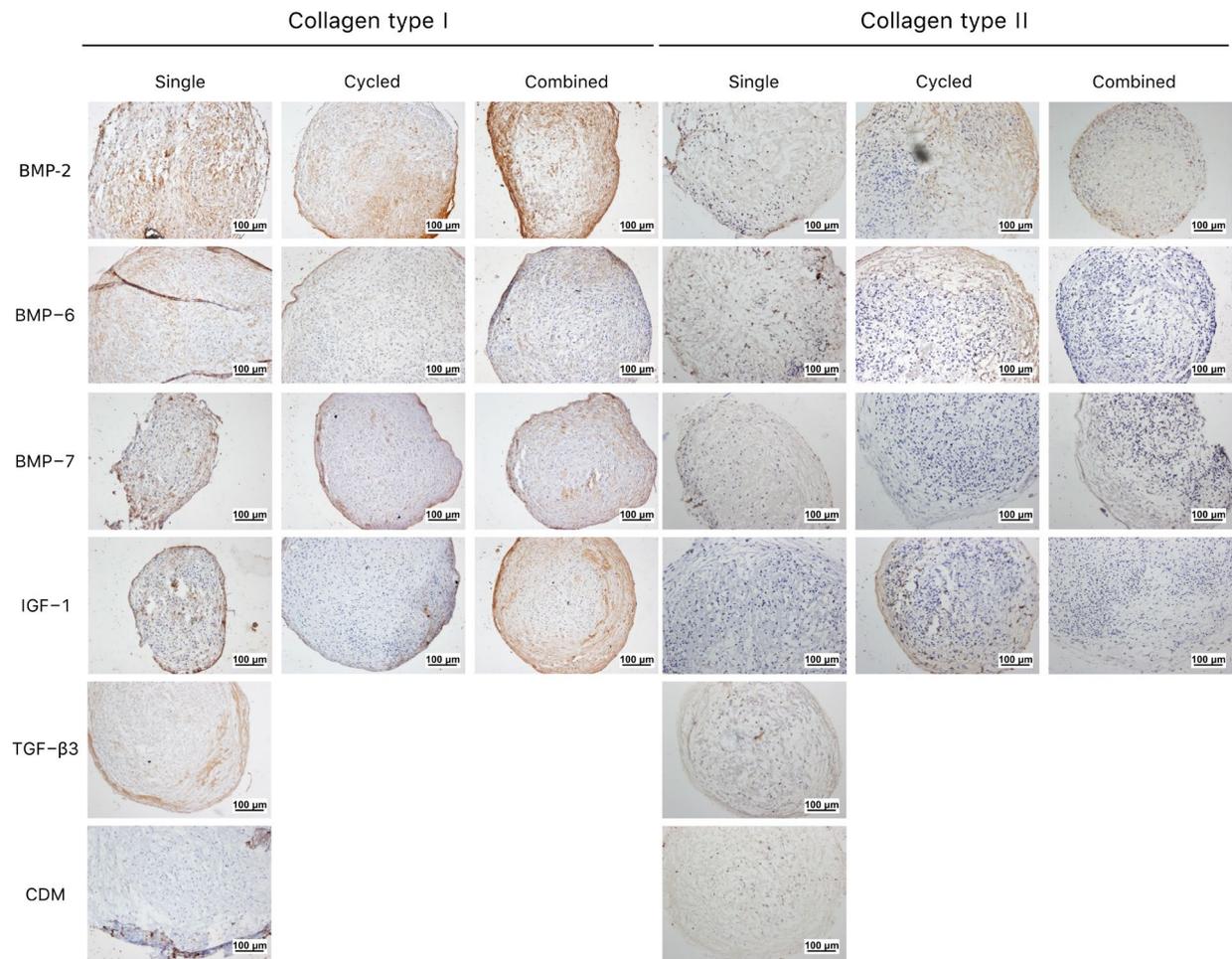


Fig. 8. Effect of the different GFs and their administration patterns on collagen deposition. Immunohistochemical analysis highlighted the presence (brown) of collagen type I (left) and collagen type II (right) after 4 weeks of culture.

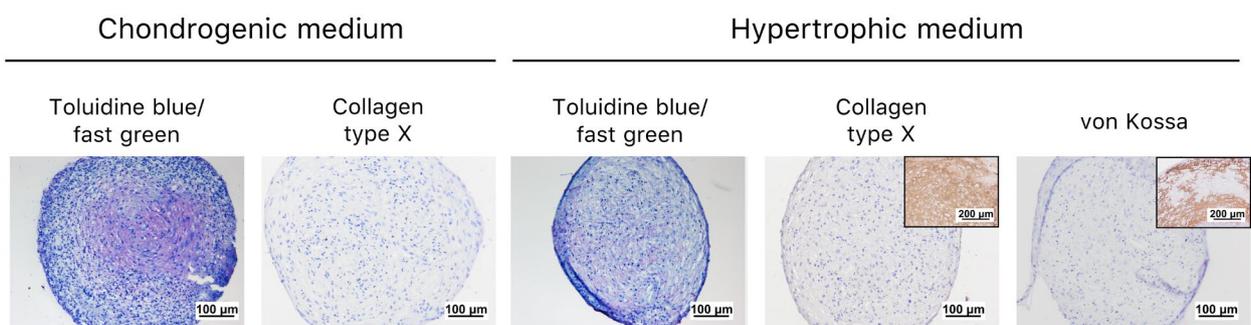


Fig. 9. Hypertrophic marker presence in DPSCs after chondrogenic and hypertrophic induction for 35 d. Positive controls of differentiated BMSCs are depicted in the inserts.

MSC cultures used as control condition. Interestingly, no *COL2A1* expression was detected. This apparent discrepancy between PCR quantification and histological results could be due to several factors, including post-transcriptional regulatory mechanism and difference in speed between the transcription and translation process and in half-life between mRNA and protein (2.6-7 h for mRNA *versus* 46 h for proteins) (Liu *et al.*, 2016; Vogel and Marcotte, 2012). Similarly, other studies have reported collagen type I expression (Spath *et al.*, 2010; Sternberg *et al.*, 2012) in DPSC pellets and inferior (hyaline) chondrogenic potential when compared to other adult stem cell sources (Alge *et al.*, 2010; Isobe *et al.*, 2016; Koyama *et al.*, 2009; Sternberg *et al.*, 2012). As far as it can be ascertained, only one study explored the potential of using DPSCs embedded in a chitosan/alginate scaffold for the regeneration of the TMJ disc (Bousnaki *et al.*, 2018). Consistent with the present study results, Bousnaki *et al.* (2018) observed an upregulation of collagen type I gene expression, together with expression of SOX9, aggrecan and COMP during chondrogenic differentiation. These evidences indicated that, although it has been proposed that DPSCs could be applied for hyaline cartilage regeneration (Mata *et al.*, 2017; Nuti *et al.*, 2016), the *in vitro* deposited ECM had robust characteristics of fibrous cartilage tissue.

Several strategies have been reported in the literature to elicit the limited hyaline chondrogenic differentiation capacity of DPSCs, including transduction with an adeno-associated viral vector to induce TGF- β 3 expression (Rizk and Rabie, 2013), co-culture with chondrocytes (Dai *et al.*, 2012) and use of nanopatterned hydrogels as a support material (Nemeth *et al.*, 2014). These strategies result in enhanced GAG (Dai *et al.*, 2012; Rizk and Rabie, 2013), collagen type II (Dai *et al.*, 2012; Nemeth *et al.*, 2014; Rizk and Rabie, 2013) and aggrecan (Rizk and Rabie, 2013) deposition. In the present study, the possibility of enhancing DPSC chondrogenesis was evaluated by tailoring the composition of the differentiation medium. This is particularly appealing from a clinical translational perspective, as changing the supplied GFs represents a simpler and more straightforward option to implement. Thus, the present study evaluated if GAGs and collagen type II deposition could be enhanced by supplementing BMP-2, BMP-6, BMP-7 or IGF-1, alone or in combination with TGF- β 3. These (combinations of) GFs were selected because they are classically used in MSCs chondrogenic differentiation protocols (Vater *et al.*, 2011). Furthermore, BMP-2 and BMP-7 are particularly interesting candidates, not only because of their common use to stimulate MSC chondrogenesis, but also because they are expressed in early developing Meckel's cartilage (Wang *et al.*, 2013) and TMJ condyle (Stocum and Roberts, 2018). Finally, since the administration of different GFs in an alternating, cyclic pattern can effectively enhance MSC chondrogenesis (Indrawattana *et al.*, 2004), its effect on DPSC differentiation was assessed. Results

showed that culturing DPSC pellets with CDM alone already resulted in limited GAG deposition, which was not enhanced by any of the GFs or patterns evaluated. Remarkably, the combination of BMP-2, -6 or -7 with TGF- β 3 resulted in significantly lower GAG/DNA-ratios than when the DPSCs were cultured with either of these factors alone. This suggested that the continuous combination of one of the BMP isoforms with TGF- β 3 did not have a synergistic effect on DPSC chondrogenesis as observed for example for MSCs and adipose-derived stem cells (Shen *et al.*, 2009; Shen *et al.*, 2010). Furthermore, collagen type I remained an abundant component in the ECM in all conditions and the cells maintained an elongated morphology after differentiation, a phenomenon that is typical for fibrochondrocytes (Allen and Athanasiou, 2006). These results further confirmed the DPSC predisposition to produce a fibrocartilaginous matrix under a large variety of chondro-inductive culture conditions.

Finally, the capability of chondrogenically differentiated DPSCs to acquire a hypertrophic phenotype was investigated. Inferior expression of collagen type X as compared to the MSC control was already observed upon chondrogenic induction. In addition, the absence of mineralisation and collagen type X protein deposition after stimulation with both chondrogenic and hypertrophic media indicated that, unlike bone-marrow-derived MSCs (Pelttari *et al.*, 2006), DPSCs did not seem to have the potential to undergo hypertrophy under the differentiation conditions used in the study.

In conclusion, even if DPSCs are defined as MSC-like cells, they respond differently from the classical chondrogenic stimulation protocol for MSCs. The reasons for this behaviour could be multiple. Interestingly, already without any GF stimulation, the expression of chondro-related genes is dissimilar between MSCs and DPSCs. These differences dramatically increase upon chondrogenic stimulation in a micromass model system (Sternberg *et al.*, 2012). Comparably, other studies have previously reported a different extent of GAG and collagen type II production by MSCs isolated from different tissues (Isobe *et al.*, 2016) or within different regions of the same tissue (Sivasubramanian *et al.*, 2019) upon stimulation with the same chondrogenic medium. Even though the reason behind this heterogeneity in chondrogenic differentiation response is not clear, it can be hypothesised that it correlates with the presence of different GFs in the natural microenvironment (Sivasubramanian *et al.*, 2019), a different epigenetic profile or a different cell origin.

Conclusions

The results indicated that, despite existing indications that DPSCs could be used for engineering hyaline cartilage (Mata *et al.*, 2017) and hypertrophic chondrogenic tissues (Kruijt Spanjer *et al.*, 2017),

these cells would be more suitable for engineering of fibrocartilaginous tissues, including the disc and condylar cartilage, for TMJ regeneration.

Acknowledgements

This study was part of project number AOCMF-17-17G, supported by the AO Foundation, Davos, Switzerland. Part of the study was also supported by the BOOA research grant of the NVMKA. The antibody against collagen type II (II-II6B3), developed by T.F. Linsenmayer, was obtained from the DSHB developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242, USA.

The authors declare no conflict of interest.

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Discussion with Reviewers

Piefrancesco Pagella: How do the authors explain the poor tendency to form hyaline-like cartilage of DPSCs in their *in vitro* system, compared to previous reports?

Authors: One of the major differences between the present study and previous work where chondrogenic differentiation is claimed, is that the present study used a 3D pellet model rather than a 2D culture system (Alge *et al.*, 2010; Alvarez *et al.*, 2015; Pereira *et al.*, 2012). When 3D pellets are used, other authors have also reported inferior chondrogenic potential and lower presence of hyaline markers when compared to other cell types (Sternberg *et al.*, 2012; Sivasubramaniyan *et al.*, 2019; Pierdomenico *et al.*, 2005). This poor tendency to differentiate into hyaline-like cartilage could be attributed to the fact that even if DPSCs are defined as MSC-like cells, they upregulate different chondro-related genes when compared to the bone marrow-derived stem cells after chondrogenic induction (Sternberg *et al.*, 2012). Thus, it is possible that they require different stimuli to produce a more hyaline-like cartilaginous matrix.

Giovanna Orsini: Can the authors point out the future clinical implications of their work?

Authors: DPSCs have been mainly investigated for their application potential in bone regeneration and dentistry (Anitua *et al.*, 2018; Morad *et al.*, 2013). However, contrasting evidence exists regarding their suitability for cartilage TE. In addition, most of the efforts have been focused on the regeneration of hyaline cartilage (Sternberg *et al.*, 2012; Zhang *et al.*, 2006). The present study proposed a new and rather

unexplored application of DPSCs. The capability of DPSCs to secrete collagen type I and retain a fibrochondrocytes morphology under different stimuli makes them a viable option for fibrocartilage TE. This predisposition towards secreting ECM components typical of fibrocartilage, together with the simplicity of the DPSC isolation procedure as well as their high proliferative potential, makes them particularly attractive for clinical application when compared to other cell types. Finally, their use could be envisioned for the regeneration of various types of fibrocartilaginous tissues, including the mandibular condylar cartilage, menisci and intervertebral disc.

Anne Poliard: It is possible that collagen type II mRNA expression was already downregulated at day 21. Could this be shown with an earlier time point real-time PCR? Could it be that the antibody used recognised type IIA and IIB isoforms while primers would be specific of type IIB.

Authors: Although we understand that this might provide valuable information about the temporal pattern of *COL2A1* expression in DPSCs, we truly believe that this is beyond the scope of the study. The goal of the work was to determine the chondrogenic potential of DPSCs with regard to their application for engineering hyaline cartilage or fibrous cartilage. The data presented were sufficient to justify the conclusions as, next to the limited production of collagen type II protein, the presence of abundant collagen type I at the protein level, the upregulation of *COL1A1* expression as well as expression of other cartilage matrix genes and the cell morphology were shown. These data combined supported the conclusion that DPSCs are more suitable for fibrous cartilage engineering. In addition, throughout the manuscript, we never claimed an extensive collagen type II deposition, even at the protein level. In contrast, we highlighted that it was rather limited, making DPSCs unsuitable for hyaline cartilage engineering when following the various methods. Thus, even if we would provide additional data on *COL2A1* expression at earlier time points, this would not change the conclusions presented.

Editor's note: The Scientific Editor responsible for this paper was Thimios Mitsiadis.