

COLLAGEN TYPE X IS ESSENTIAL FOR SUCCESSFUL MESENCHYMAL STEM CELL-MEDIATED CARTILAGE FORMATION AND SUBSEQUENT ENDOCHONDRAL OSSIFICATION

C.A. Knuth¹, E. Andres Sastre^{1,2}, N.B. Fahy^{1,2}, J. Witte-Bouma¹, Y. Ridwan^{3,4}, E.M. Strabbing¹, M.J. Koudstaal¹, J. van de Peppel⁵, E.B. Wolvius¹, R. Narcisi² and E. Farrell^{1,*}

¹Department of Oral and Maxillofacial Surgery, Special Dental Care and Orthodontics, Erasmus MC, University Medical Centre, 3015 CE Rotterdam, the Netherlands

²Department of Orthopaedics, Erasmus MC, 3015 CE Rotterdam, the Netherlands

³Department of Molecular Genetics, University Medical Centre Rotterdam, Erasmus MC, 3015 CE Rotterdam, the Netherlands

⁴Department of Radiology and Nuclear Medicine, Erasmus MC, University Medical Centre Rotterdam, 3015 CE Rotterdam, the Netherlands

⁵Department of Internal Medicine, Erasmus MC, 3015 CE Rotterdam, the Netherlands

Abstract

In tissue engineering, endochondral ossification (EO) is often replicated by chondrogenically differentiating mesenchymal stromal cells (MSCs) *in vitro* and achieving bone formation through *in vivo* implantation. The resulting marrow-containing bone constructs are promising as a treatment for bone defects. However, limited bone formation capacity has prevented them from reaching their full potential. This is further complicated since it is not fully understood how this bone formation is achieved. Acellular grafts derived from chondrogenically differentiated MSCs can initiate bone formation; however, which component within these decellularised matrices contribute to bone formation has yet to be determined. Collagen type X (COLX), a hypertrophy-associated collagen found within these constructs, is involved in matrix organisation, calcium binding and matrix vesicle compartmentalisation. However, the importance of COLX during tissue-engineered chondrogenesis and subsequent bone formation is unknown. The present study investigated the importance of COLX by shRNA-mediated gene silencing in primary MSCs. A significant knock-down of COLX disrupted the production of extracellular matrix key components and the secretion profile of chondrogenically differentiated MSCs. Following *in vivo* implantation, disrupted bone formation in knock-down constructs was observed. The importance of COLX was confirmed during both chondrogenic differentiation and subsequent EO in this tissue engineering setting.

Keywords: Collagen type X, endochondral ossification, regenerative medicine, bone tissue engineering, mesenchymal stem cells, chondrogenesis.

***Address for correspondence:** Dr Eric Farrell, Department of Oral and Maxillofacial Surgery, Special Dental Care and Orthodontics, Erasmus MC, 3000 DR Rotterdam, the Netherlands.

Telephone number: +31 107043655 Fax number: +31 107033098 Email: e.farrell@erasmusmc.nl

Copyright policy: This article is distributed in accordance with Creative Commons Attribution Licence (<http://creativecommons.org/licenses/by-sa/4.0/>).

Introduction

Endochondral ossification (EO) is the process by which long bones are formed. It relies on mesenchymal cells, which condense and undergo chondrogenic differentiation, resulting in the formation of a cartilage anlage (Ornitz, 2005). Subsequently, this anlage is remodelled by osteoclasts (Ortega *et al.*, 2004), invaded by nearby vasculature (Maes *et al.*, 2010) and, ultimately, serves as the template for bone formation by osteoblasts (Mackie, 2003). EO

can be mimicked in a tissue engineering setting by chondrogenically differentiating mesenchymal stem cells (MSCs) pre-implantation (Farrell *et al.*, 2011; Farrell *et al.*, 2008; Scotti *et al.*, 2013; Scotti *et al.*, 2010; van der Stok *et al.*, 2014). Although in the future such grafts could offer an attractive alternative to currently available treatment options, these constructs exhibit variable degrees of bone formation, with variable success in large-defect repair, depending on the approach used (Knuth *et al.*, 2017; van der Stok *et al.*, 2014). In recent years, the importance of donor

cells in experimental models has been identified, as they are capable of directly contributing to bone formation. There is evidence suggesting that, during development (Cervantes-Diaz *et al.*, 2017; Yang *et al.*, 2014; Zhou *et al.*, 2014), implanted cells can directly contribute to osteoblast-like cell populations (Bahney *et al.*, 2014; Scotti *et al.*, 2010). However, there is still much to learn about these grafts. Recently, it has been shown that devitalised grafts derived from chondrogenic MSCs form endochondral bone following *in vivo* implantation (Bourguine *et al.*, 2014; Cunniffe *et al.*, 2015), indicating that components within the chondrogenic matrix itself are capable of inducing bone formation independent of the presence of MSCs. Cunniffe *et al.* (2015) further showed that biomaterials containing decellularised matrices, specifically those originating from hypertrophic chondrogenically differentiated MSCs, are far better at inducing this bone formation than non-hypertrophic counterparts. However, what exactly within these hypertrophic matrices is responsible for this difference remains to be determined.

During chondrogenic differentiation, collagen type X (COLX), the major marker of hypertrophic chondrocytes, can be detected within the first few days (Knuth *et al.*, 2017; Pelttari *et al.*, 2006). Developmentally, following hypertrophic differentiation, the extracellular matrix (ECM) is remodelled to allow for invasion of the template by the nearby vasculature (Bahney *et al.*, 2015; Colnot and Helms, 2001). Next, mineralisation of the cartilage template is initiated, osteoclastic remodelling occurs throughout the process of matrix remodelling, allowing for easier cell migration and vascularisation to occur (Colnot and Helms, 2001; Engsig *et al.*, 2000; Touaitahuata *et al.*, 2014), and, ultimately, endochondral bone formation takes place (Colnot and Helms, 2001; Deckers *et al.*, 2002). COLX is produced by hypertrophic chondrocytes (HCs) and comprises more than 45 % of the total collagens produced (Luvalle *et al.*, 1992). It plays several important roles during EO, even contributing to the structural support of the pericellular network (Schmid and Linsenmayer, 1985; Shen, 2005), support that is essential during matrix remodelling (Shen, 2005). Additionally, COLX may play a role in initiating biomineralisation by binding annexin V (Kirsch and Pfäffle, 1992; Wu *et al.*, 1991). Annexin V facilitates the influx of Ca²⁺ into matrix vesicles, initiating mineralisation (Anderson *et al.*, 2005). Others have hypothesised that COLX is also essential for the proper distribution of collagens and proteoglycans throughout the ECM (Chan and Jacenko, 1998; Jacenko *et al.*, 2001), further supporting a role of COLX in proper matrix organisation. However, these hypotheses have been hard to verify since previous studies utilising transgenic (Tg) mouse models in which COLX is removed, truncated or disrupted using different methods produce conflicting data (Campbell *et al.*, 2004; Jacenko *et al.*, 2001; Kim *et al.*, 2003; Kwan *et al.*,

1997; Rosati *et al.*, 1994). Some groups have shown early developmental irregularities attributed to a lack of COLX, including perinatal death, dwarfism and various skeletal abnormalities (Campbell *et al.*, 2004; Jacenko *et al.*, 2001). Rosati *et al.* (1994) reported a complete absence of phenotypic changes. Studies have also reported that a lack of COLX results in a compromised immune system, with an inability to elicit and control a proper immune reaction (Jacenko *et al.*, 2002; Sweeney *et al.*, 2008; Sweeney *et al.*, 2010), while others find no such deficiency (Rosati *et al.*, 1994). These different findings could come down to the genetic variation among different strains of mice used, variation in the methodology used to achieve knock-down/interference of COLX or differences in sample processing, analysis or staining techniques. Nevertheless, this makes understanding the role of COLX increasingly challenging. Furthermore, COLX has only been studied from a developmental stand point. How COLX contributes to both chondrogenic differentiation of MSCs and subsequent endochondral bone formation has not been studied. The goal of the present study was to determine if COLX is essential for MSC-mediated chondrogenic differentiation and subsequent endochondral bone formation. *COL10A1* expression was knocked-down using a lentivirus-delivered short hairpin RNA (shRNA) directed against *COL10A1* mRNA sequence. In this way, it was possible to specifically knock-down the production of COLX in MSCs and determine its contribution to tissue engineered chondrogenesis and endochondral bone formation. *COL10A1* knock-down resulted in disrupted chondrogenic differentiation. When the knock-down was highly efficient, the production of key ECM components was severely decreased. Several secreted factors linked to the recruitment of key cell types involved in EO were also down-regulated. Following *in vivo* implantation, EO was likewise affected. However, this was only observed when knock-down efficiency was more than 80 %, indicating that a minimal production of *COL10A1* was sufficient to prevent these adverse effects. The study showed the importance of COLX in MSC-mediated chondrogenesis and EO, furthering the understanding of tissue-engineered endochondral bone formation.

Materials and Methods

MSC culture

MSCs were isolated from leftover material obtained from patients undergoing alveolar bone graft surgery (Medical Ethical Testing Commission approval code 2014-106; donor age range: 8-12 years old) following parental consent in accordance with relevant guidelines and regulations, as previously described (Knuth *et al.*, 2018). Cells were plated at approximately 2,300 cells/cm² at 37 °C and 5 % CO₂ in a humidified atmosphere in complete expansion medium [alpha modification minimum

essential medium (α MEM; Gibco) containing 10 % foetal bovine serum (41Q204K; Gibco), 50 μ g/mL gentamycin (Thermo Fisher Scientific), 1.5 μ g/mL fungizone (Thermo Fisher Scientific), 25 μ g/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich) and 1 ng/mL fibroblast growth factor-2 (Instruchemie, Delfzijl, the Netherlands)]. Medium was replenished every 3-4 d until cells reached approximately 90 % confluency. Then, they were passaged using 0.05 % trypsin. Donor 1 and 2 were transdifferentiated following passage 6 and donor 3 was used at passage 7. At each passage, cells performed approximately 3 population doublings, with no difference among donors (data not shown). Population doublings were calculated by determining the \log_2 of the total cell number following growth for one passage, divided by the initial population count.

Lentiviral generation

MISSION[®] TRC1.5 shRNA targeting *COL10A1* mRNA (TRCN0000082798; Sigma-Aldrich) and control plasmids [SHC001 (empty vector) and SHC002 (non-target control); Sigma-Aldrich] were transformed into DH5- α *Escherichia coli* (Invitrogen). shRNA targeting *COL10A1* mRNA was analysed using the basic local alignment search tool on the NCBI website and no sequence homology with any other gene was identified. Likewise, no human homology was found for the non-target plasmid. Plasmids were isolated using an EndoFree Plasmid Maxi Kit (QIAGEN) as per manufacturer's instructions. HEK293FT cells were expanded in complete HEK medium [high-glucose Dulbecco's modified Eagle medium (DMEM; Gibco), 10 % foetal bovine serum, 50 μ g/mL gentamycin, 1.5 μ g/mL fungizone] supplemented with 50 μ g/mL geneticin (Thermo Fisher Scientific) and maintained at 37 °C and 5 % CO₂ in a humidified atmosphere. 24 h prior to transfection, 3×10^5 cells/cm² were plated in tissue culture Petri dishes (Corning) in high-glucose DMEM containing 10 % foetal bovine serum. Cells were co-transfected with plasmids by calcium phosphate precipitation [3 μ g pLP1 plasmid, 3 μ g pLP2 plasmid, 3 μ g pLP/VSVG plasmid (ViraPower Lentiviral Packaging Mix, Thermo Fisher Scientific) and 7.5 μ g shRNA plasmid]. Medium was refreshed 24 h post transfection with HEK medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Viral supernatant was harvested and filtered (0.45 μ m filter) 48 h following transfection and stored at -80 °C until use. p24 concentration was determined using INNOTEST[®] HIV Antigen mAb enzyme immunoassay (Fujirebio, Gent, Belgium) performed as per manufacture's instruction.

Lentiviral transduction

MSCs were transduced at 30 % confluency. Cells were incubated for 48 h with 40 or 60 ng/mL p24 particles (depending on the batch used and the optimal dose determined by titration experiments) and 20 μ g/mL protamine sulphate (Sigma-Aldrich) in complete

expansion medium (Fig. 1-3). Following incubation, cells were washed, expanded and selected after reaching 80 % confluency. Selection was achieved in 72 h using 5 μ g/mL puromycin in complete expansion medium. After selection, cells were washed and expanded until 90 % confluency, when they were either harvested for RNA [homogenised in 350 μ L Trizol (Thermo Fisher Scientific) and stored at -80 °C until isolated] or used for differentiation studies. p24 particle, protamine sulphate and puromycin concentrations were determined prior to experimental start through titration experiments (Fig. 1-3). Knock-down percentage was calculated by subtracting the average *COL10A1* expression in *COL10A1* knock-down pellets from the average *COLX* expression in non-treated pellets. This was divided by the average of the non-treated *COL10A1* expression and then multiplied by 100 to obtain a percentage.

Chondrogenic differentiation

MSCs were chondrogenically differentiated in complete chondrogenic medium [high-glucose DMEM supplemented with 50 μ g/mL gentamycin, 1.5 μ g/mL fungizone, 1 mM sodium pyruvate (Gibco), 40 μ g/mL proline (Sigma-Aldrich), 1 : 100 v/v insulin-transferrin-selenium (ITS+; BD Biosciences), 10 ng/mL transforming growth factor β 3 (Peprotech), 25 μ g/mL L-ascorbic acid 2-phosphate and 100 nM dexamethasone (Sigma-Aldrich)] using 2×10^5 MSCs resuspended in 15 mL polypropylene tubes and centrifuged at 300 \times g for 8 min. Cultures were maintained in a humidified atmosphere at 37 °C and 5 % CO₂ and medium was replenished every 3-4 d for 21 d. Samples were harvested following 7 and 21 d in culture for RNA isolation (as previously described), glycosaminoglycan (GAG)/DNA analysis (stored at -80 °C), Western blot (snap-frozen), histology (4 % formalin fixed for 2 h), enzyme-linked immunosorbent assay (ELISA) (supernatant) or migration (conditioned medium; described below).

Osteogenic differentiation

MSCs were plated at a density of 3×10^3 cells/cm² in expansion medium. 24 h after plating, the medium was changed to osteogenic induction medium (high-glucose DMEM supplemented with 10 % v/v foetal bovine serum, 50 μ g/mL gentamycin, 1.5 μ g/mL fungizone, 10 mM glycerol-3-phosphate, 0.1 μ M dexamethasone and 0.1 mM L-ascorbic acid 2-phosphate). Cultures were maintained at 37 °C and 5 % CO₂ in a humidified atmosphere. Medium was refreshed every 3-4 d for 13-21 d. Once cell sheets began to detach from the well outer periphery, culture was ended and samples were fixed in 4 % (w/v) formalin. von Kossa staining was performed as previously described (Knuth *et al.*, 2018).

Adipogenic differentiation

MSCs were plated at a density of 2.1×10^4 cells/cm² in adipogenic induction medium [high-glucose

Table 1. Primer sequences used for real time PCR. *B2M*: beta-2-microglobulin; *VEGFa*: vascular endothelial growth factor a; *COL1A1*: collagen type I; *COL2A1*: collagen type II; *COL10A1*: collagen type X; *BMP2*: bone morphogenetic protein 2; *MMP13*: matrix metalloproteinase 13; *AGCN*: aggrecan.

Gene names	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>B2M</i>	TGCTCGCGCTACTCTCTCTTT	TCTGCTGGATGACGTGAGTAAAC
<i>VEGFa</i>	CTTGCCTTGCTGCTCTACC	CACACAGGATGGCTTGAAG
<i>COL1A1</i>	CAGCCGCTTCACCTACAGC	TTTTGTATTCAATCACTGTCTTGCC
<i>COL2A1</i>	CCCCATCTGCCCAACTGA	CTCCTTTCIGTCCCTTTGGT
<i>COL10A1</i>	ACTTCTCTTACCACATACACG	CCAGGTAGCCCTTGATGATGTACT
<i>BMP2</i>	AACACTGTGCGCAGCTTCC	CTCCGGGTTGTTTTCCAC
<i>MMP13</i>	AAGGAGCATGGCGACTTCT	TGGCCAGGAGGAAAAGC
<i>AGCN</i>	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA

DMEM supplemented with 10 % foetal bovine serum, 50 µg/mL gentamycin, 1.5 µg/mL fungizone, 0.2 mM indomethacin (Sigma-Aldrich), 0.01 mg/mL insulin (Sigma-Aldrich), 0.5 mM 3 iso-butyl-1-methyl-xanthine (Sigma-Aldrich) and 1 µM dexamethasone]. Cultures were maintained at 37 °C and 5 % CO₂ in a humidified atmosphere. Medium was refreshed as every 3-4 d for 19-21 d. Then, samples were prepared for histology [4 % (w/v) formalin-fixed]. Oil red O staining was performed as previously described (Knuth *et al.*, 2018).

Cell viability testing

MSCs were plated at a density of 2.1×10^4 cells/cm² and treated for 48 h at 37 °C, 5 % CO₂ in a humidified atmosphere with either protamine sulphate (10, 25, 50, 75 or 100 µg/mL) or puromycin (1.25, 2.5, 5, 7.5 or 10 µg/mL) in MSC expansion medium. Then, cells were washed with phosphate-buffered saline (PBS) and incubated in the dark for 3 h at 37 °C and 5 % CO₂ in a humidified atmosphere in 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich)-supplemented MSC expansion medium. Next, medium was aspirated and cells were washed with PBS. MTT-tetrazolium crystals formed by healthy cells were dissolved in 100 % ethanol. Absorbance was measured at 570 nm and 670 nm; 570 : 670 ratio was compared to non-treated control MSCs.

Western blot

Pellets were snap-frozen in liquid nitrogen and homogenised in 100 µL M-PER (Mammalian Protein Extraction Reagent, Thermo Fisher Scientific) with 1× Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific) followed by water bath sonication (10 cycles of 30 s sonication and 30 s rest). Total protein was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). 10 µg total protein was incubated at 95 °C and separated on a gradient gel (Bolt 4-12 % Bis-Tris Plus, Thermo Fisher Scientific) under non-reducing conditions. Proteins were transferred to a polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membrane and blocked with 5 % w/v fat-free milk

in 0.1 % v/v tris-buffered saline (TBS)-Tween 20. The membrane was cut at 70 kDa and each piece was incubated overnight with its respective antibody: 1 : 1,000 mouse monoclonal anti-COLX, clone X53 (Quartett, Berlin, Germany) and 1 : 2,000 rabbit monoclonal anti α -tubulin (Cell Signaling). Next, incubation for 1 h at room temperature with the respective secondary antibody was performed: 1 : 2,000 horseradish peroxidase (HRP)-conjugated goat anti-mouse and 1 : 2,000 HRP-conjugated goat anti-rabbit (both Thermo Fisher Scientific). Protein bands were visualised using the Super Signal West detection kit (Thermo Fisher Scientific) on the digital Δ camera Alliance 2.7 (Uvitec, Cambridge, UK) with an exposure time of 15 (COLX) or 17 min (μ -tubulin).

RNA isolation and gene expression

Samples were homogenised in 350 µL Trizol to which 70 µL 100 % chloroform was added and thoroughly mixed. Following a 10 min incubation at room temperature and phase separation at 10,000 \times g for 15 min, the aqueous phase was mixed with an equal volume of 70 % ethanol and transferred onto an RNeasy® kit column. RNA was isolation and purified using the RNeasy® Micro Kit (QIAGEN) following manufacturer's instructions. cDNA was reverse-transcribed as per manufacturer's instruction using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and PCR was performed as previously described (Table 1) (Knuth *et al.*, 2015. Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming, towards scale-up. Proceedings of the 24th NBTE Annual Meeting, Lunteren, The Netherlands. pp: 65). For gene expression of monolayer, day 7 and day 14 of chondrogenic differentiation, 3 different MSC donors were analysed. For gene expression at day 21, only one MSC donor was analysed (3 pellets/donor/condition). Gene expression is shown as $\Delta\Delta$ CT.

Biochemical assay

Chondrogenically differentiated samples stored at - 80°C were digested overnight at 60 °C in buffer containing papain [0.2 M NaH₂PO₄, 0.01 M ethylenediaminetetraacetic acid (EDTA),

0.01 M cysteine HCl, 250 µg/mL papain (Sigma-Aldrich), pH 6]. Then, GAGs were quantified by dimethylmethylene blue assay (pH 3) with chondroitin sulphate C (Sigma-Aldrich) as a standard, utilising an absorbance 530 : 590 ratio. Ethidium bromide was used to determine sample DNA content. Calf thymus DNA (Sigma-Aldrich) was used as a standard.

Cytokine quantification

Culture medium was refreshed 24 h before harvesting the pellets. Supernatant was applied to a human cytokine angiogenic multiplex chemiluminescent ELISA (150251HU; Quansys Biosciences, Logan, UT, USA) as per manufacturer's instructions. This angiogenic multiplex included the following targets: angiotensin II (Ang-2), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), interleukin-8 (IL-8), platelet derived growth factor-BB (PDGF-BB), tissue inhibitor of metalloproteinase 1 and 2 (TIMP-1 and TIMP-2), tumour necrosis factor (TNF). The ELISA plate was imaged using the Amersham Imager 600 (GE Healthcare) and images were quantified using the Q-view software[®] provided by the ELISA manufacturer (Quansys Bioscience).

Histology

In vitro samples were fixed for 2 h in 4 % formalin prior to paraffin-wax embedding. *In vivo* samples were formalin-fixed for 24 h and decalcified for 10 d in 10 % EDTA w/v in H₂O refreshed every other day. Following paraffin-wax embedding, 6 µm-thick sections were cut from all samples. Sections were deparaffinised and stained with haematoxylin-eosin (H&E), safranin O, tartrate-resistant acid phosphatase (TRAP) and antibodies against COLII or COLX, as previously described (Knuth *et al.*, 2015. Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming, towards scale-up. Proceedings of the 24th NBTE Annual Meeting, Lunteren, The Netherlands. pp: 65).

Subcutaneous implantation

Experiments were conducted with approval by the Animal Experiments Committee at the Erasmus Medical Centre, Rotterdam (EU license number 15-114-02) adhering to all relevant guidelines and regulations. 8 week-old athymic nude mice (Balb/c nudes, Charles River) were housed under standard light-dark cycles with food and water *ad libitum* (12 mice in total; housed in groups of 2). Pain medication (buprenorphine 0.05 mg/kg body weight) was administered pre-operatively under anaesthesia (1-3 % isoflurane). Dorsal incisions were created in which a subcutaneous pocket was made. 4 subcutaneous pockets were created in the back of each mouse through two incisions, one in the upper and one in lower back. At each incision, 2 pockets (left and right) were created by blunt dissection. 3 control or virally transduced pellets per pocket

were implanted subcutaneously (conditions were randomised among mice and physical positions). 3 implantations were performed per experimental condition (3 pellets or 600,000 cells/implantation). Incisions were closed using staples. Animals were euthanised 8 or 10 weeks post implantation by CO₂ asphyxiation.

X-ray micro-computed tomography (µCT) longitudinal imaging

µCT scans were performed at the Applied Molecular Imaging Erasmus MC facility using the Quantum FX (Perkin-Elmer). While animals were anaesthetised (1-2.5 % isoflurane), scans were acquired using a field of view of 60 mm (90 kV/160 mA, 4.5 min) bi-weekly. Using the Analyze 11.0 software (AnalyzeDirect, Nieuwe Niedorp, the Netherlands), scans were quantified by converting original linear attenuation coefficient measurements by linear transformation to Hounsfield units (HU). Median spatial filters were applied with a 3 × 3 × 3 kernel size. Values corresponding to a density of over 0.133 g/cm³ (determined by phantom scans) were segmented out and quantified as calcified tissue.

Figures and statistical analysis

Graph figures were created using GraphPad Software (La Jolla, CA, USA). Graphs depict ΔCT values (Livak and Schmittgen, 2001), setting non-treated value to 1. Linear mixed model with Bonferroni *post-hoc* comparison was performed using SPSS (Ver 21. SPSS Inc) on expansion data in Fig. 1.

Results

Lentivirally transduced MSCs maintain proliferative and differentiation capacity following puromycin selection

Concentrations of protamine sulphate (20 ng/mL) and puromycin (5 µg/mL) were optimised by titration experiments (data not shown). Protamine sulphate was used to aid trafficking of the lentivirus particles into the cell by neutralising the charge between the MSC membrane and viral envelope. Puromycin was used to select only transduced cells due to the presence of a puromycin resistance cassette in the lentivirus. Cells not containing the cassette, meaning non-transduced cells, were killed in the presence of puromycin. Minimal doses of both drugs were chosen to avoid unnecessary cell stress during transduction. For each viral batch, the optimal concentration (as determined by p24 viral antigen concentration) was determined based on minimum dose at which cells maintained proliferative capacity (compared to non-transduced controls from the same donor) following selection. Following transduction with optimal lentiviral and protamine sulphate concentrations, MSCs maintained a healthy spindle-like morphology, which was retained after puromycin selection (data not shown). MSCs recovered quickly from selection

stress and all grew at a similar rate, undergoing comparable population doublings as non-treated controls (Fig. 1a,b). Furthermore, transduced cells maintained their differentiation potential upon osteogenic and adipogenic induction (Fig. 1c). Chondrogenesis as well as hypertrophy was observed by day 7 as evidenced by a clear upregulation in *COL2A1*, *COL10A1* and *COL1A1* expression (Fig. 1d). The expression of *COL2A1* and *COL10A1* was strongly affected by *COL10A1* knock-down (Fig. 1d).

COL10A1 could be efficiently knocked-down by lentivirally delivered shRNA

MSCs from each condition were chondrogenically differentiated for 21 d by pellet culture. Following differentiation, *COL10A1* expression was effectively knocked-down with an efficiency ranging between 80 and 99 %, depending on the MSC donor (Fig. 2a; donor 1: > 99 %; donor 2: 99 %; donor 3: 80 %). Gene and protein expressions of COLX were significantly downregulated as compared to non-

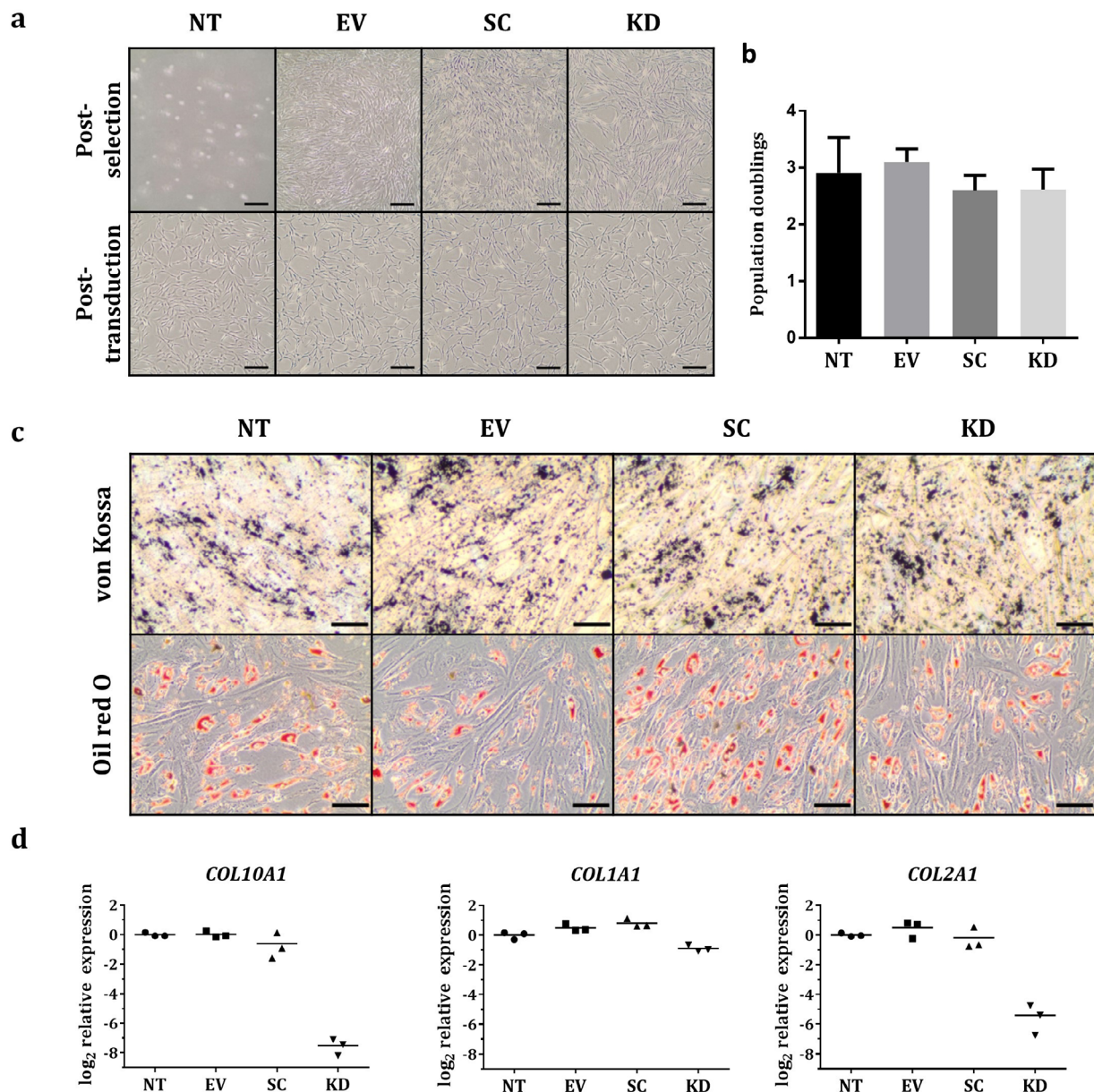


Fig. 1. Lentiviral infection did not hinder MSC expansion or differentiation capabilities. (a) MSCs were transduced with lentivirus containing an empty vector, non-target shRNA or *COL10A1* shRNA. Then, MSCs were selected based on puromycin resistance. Following transduction, cells maintained MSC-like morphology. Post-selection, positively infected cells maintained both morphology and proliferative capacity; scale bar: 200 μ m. (b) Transduced MSCs post-selection expanded at a similar rate to non-treated MSCs, undergoing a similar number of population doublings ($n = 4$; linear mixed model with Bonferroni *post-hoc* comparison: non-significant). (c) Transduced MSCs maintained the capacity to differentiate osteogenically and adipogenically. Scale bar: 50 μ m. (d) mRNA expression of *COL10A1*, *COL1A1* and *COL2A1* following 7 d of chondrogenic differentiation in donor 1. NT: non-treated; EV: empty vector; SC: non-target control; KD: knock-down.

treated, empty vector and non-target controls, which exhibited normal expression levels for all donors (Fig. 2a-c). COLX downregulation was confirmed by immunohistochemistry and Western blot for all donors and, therefore, both virus batches used (Fig. 2b,c). *COL10A1* knock-down pellets from donor 1 were too small to be processed for immunohistochemistry (Fig. 2c; donor 1). A complete absence of positive staining in *COL10A1* knock-down pellets from donor 2 and a slight positivity in the pellets from donor 3 were observed; however, the

quantity was far less than that observed in controls (Fig. 2c; donor 2, donor 3). Overall, the validity of the silencing approach was demonstrated at both transcript and protein level.

The absence of *COL10A1* during chondrogenic differentiation of MSCs affected the ECM

After confirming the efficiency of *COL10A1* knock-down, how chondrogenic differentiation of MSCs was affected in its absence was investigated. For all donors, non-treated, empty vector and non-target

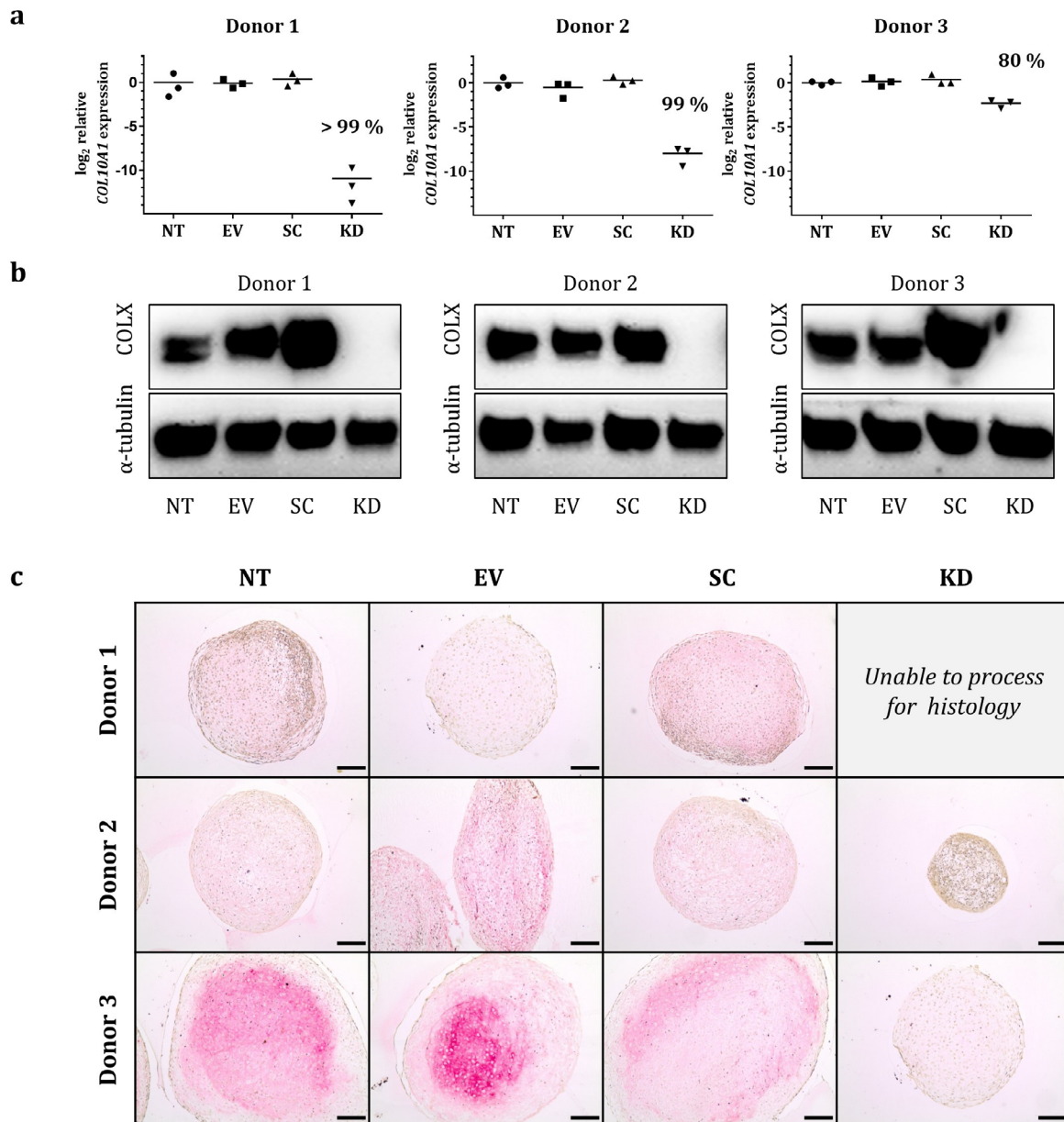


Fig. 2. *COL10A1* can be efficiently knocked-down using lentivirally delivered shRNA. MSCs were transduced with lentivirus containing an empty vector, non-target shRNA or *COL10A1* shRNA. Then, MSCs were selected based on puromycin resistance, expanded and chondrogenically differentiated for 21 d, when they were harvested for analysis. (a) Compared to control conditions, MSCs transduced with shRNA directed against *COL10A1* showed significant down regulation of gene expression, with the level of knock-down varying between donors (% NT vs. COLX KD; pooled data: linear mixed model with Bonferonni correction: ^a $p < 0.05$). (b) This down regulation was also observed at the protein level by Western blot and (c) COLX immunohistochemistry. Scale bar: 200 μm. NT: non-treated; EV: empty vector; SC: non-target control; KD: knock-down.

controls maintained their chondrogenic potential, indicating that lentiviral transduction and RNA-induced silencing complex activation did not negatively affect chondrogenic differentiation of MSCs (Fig. 3). *COL10A1* knock-down pellets were significantly smaller than all controls for all donors, prompting the investigation of whether the absence of COLX affected other matrix components. When COLX production was mostly absent, as in donors 1 and 2, a significant decrease in GAGs (Fig. 3b) and *ACAN* expression (Fig. 3c) was observed. However, when a less effective knock-down of *COL10A1* was achieved, as in donor 3, matrix production

was less affected (Fig. 3). A similar trend was observed with regard to COLII at both protein (Fig. 4a) and gene expression (Fig. 4b) level. A similar result was observed for *COL1A1* (Fig. 4c). Overall, *COL10A1* knock-down affected the production of crucial chondrogenic ECM components following differentiation.

A complete cartilage template was essential for tissue-engineered MSC-mediated EO

After observing that *COL10A1* knock-down in MSCs affected tissue-engineered cartilage formation and confirming that control conditions for each

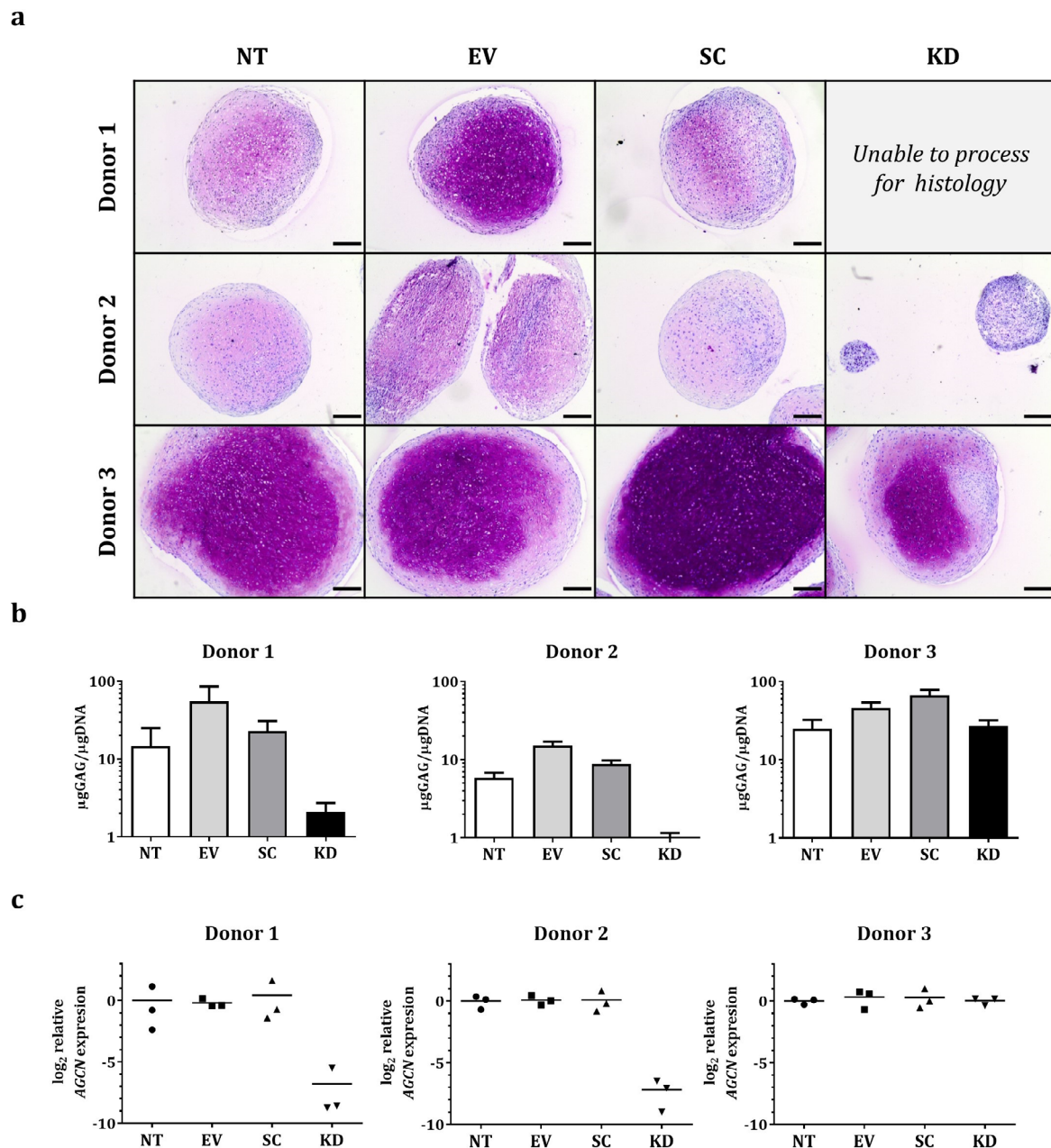


Fig. 3. A small quantity of *COL10A1* was required for the production of GAGs during chondrogenic differentiation of MSCs. (a) Thionine staining and GAG quantification showed that, upon over 99% *COL10A1* knock-down, GAG production was decreased (donors 1 and 2). **(b)** Production of 20% of the original *COL10A1* quantity was enough to trigger GAG production (donor 3). **(c)** A similar pattern was also observed for *ACAN* expression. Staining and expression of genes did not vary greatly among non-treated, empty and non-target conditions. Scale bar: 200 μ m. NT: non-treated; EV: empty vector; SC: non-target control; KD: knock-down.

donor were unaffected, how a lack of *COL10A1* affected endochondral bone formation following subcutaneous implantation in nude mice, a model shown to result in endochondral bone formation (Farrell *et al.*, 2011; Knuth *et al.*, 2017), was determined. Chondrogenically differentiated MSC pellets were cultured for 21 d *in vitro* and implanted subcutaneously in nude mice. All control conditions from these donors underwent mineralisation (Fig. 5). *COL10A1* knock-down pellets from donors 1 and 2 did not show *in vivo* mineralisation in μ CT scans (Fig. 5b). No pellets containing *COL10A1* shRNA were retrieved from donor 1 and only 1 of the 9 implanted pellets was retrieved from donor 2. This pellet

showed calcification around the outer periphery but no marrow formation and was substantially smaller than control pellets (Fig 5a, donor 2). All pellets from donor 3 containing shRNA against *COL10A1* not only provided evidence of calcification in μ CT scans (Fig. 5b; donor 3), albeit less than that observed in other conditions, but also after 10 weeks *in vivo* showed the presence of mature bone, bone marrow and calcified cartilage.

An almost complete down-regulation of *COL10A1* in MSCs pre-implantation hindered *in vivo* bone formation; however, when down-regulation was not as efficient, bone formation proceeded normally.

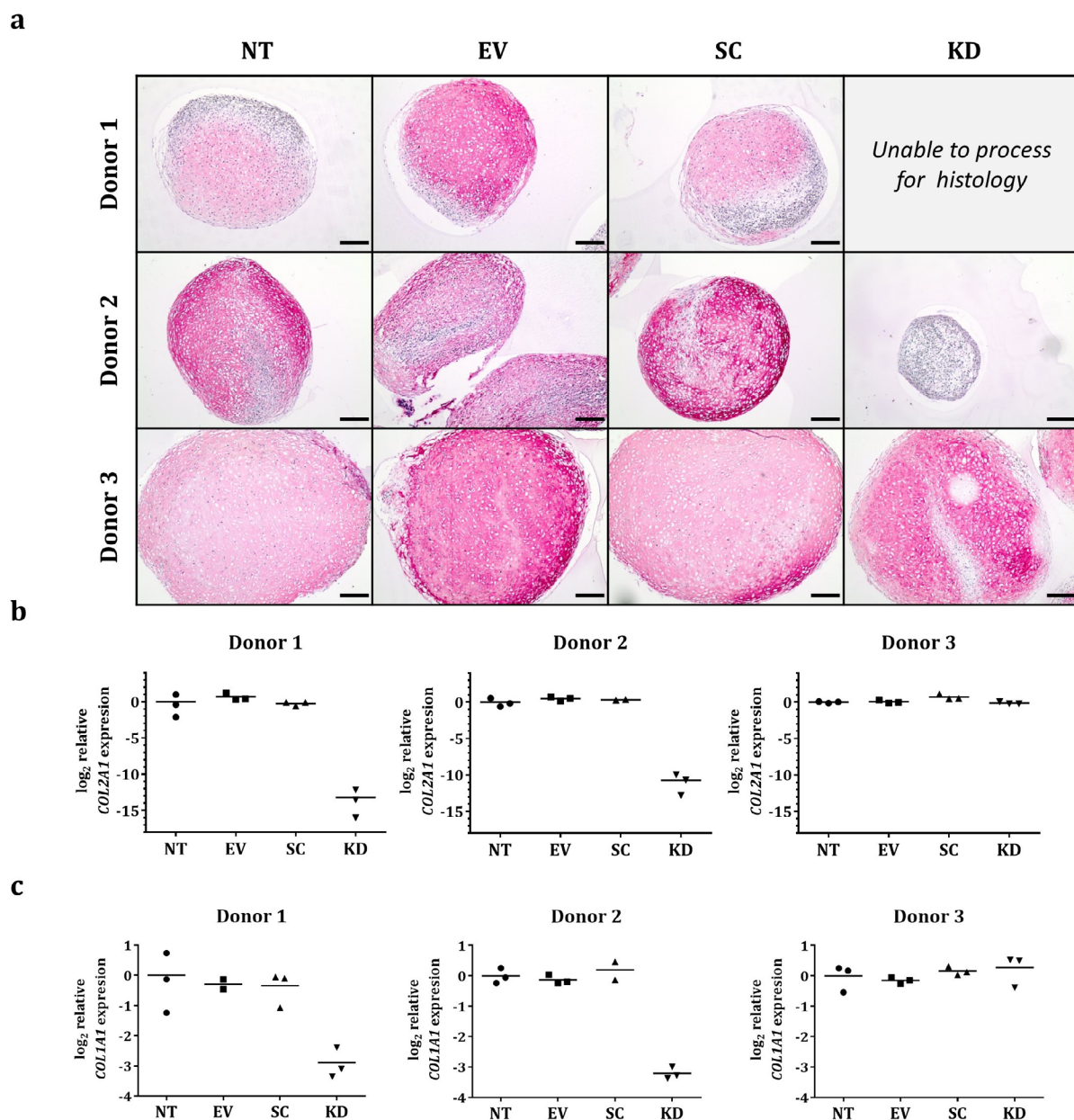


Fig. 4. A small quantity of *COL10A1* was required for proper *COL2A1* production during chondrogenic differentiation of MSCs. (a) *COL2A1* immunohistochemistry and (b) gene expression showed that, when *COL10A1* was knocked-down over 99 %, *COL2A1* production was affected (donors 1 and 2). When the knock-down was less efficient (donor 3), *COL2A1* production was not affected. (c) The same trend was observed for *COL1A1*. Staining and expression of genes did not vary greatly between non-treated, empty and non-target conditions. Scale bar: 200 μ m. NT: non-treated; EV: empty vector; SC: non-target control; KD: knock-down.

Chondrogenic matrices with decreased COL10A1 exhibited decreased osteoclastic remodelling

As *in vivo* endochondral bone formation was differentially affected depending on the efficiency of COL10A1 knock-down, next step was to determine if the presence of osteoclasts – important for matrix remodelling, a central process to successful endochondral bone formation (Ortega *et al.*, 2004) – was also affected in a similar manner. TRAP was performed and a different staining pattern was observed in donor 2 COL10A1-shRNA-containing pellet *versus* controls (Fig. 6). Non-treated, empty vector and scrambled pellets showed positive staining in the central marrow cavity whereas COL10A1-shRNA-containing pellets only showed staining around the outer periphery. This was starkly different from COL10A1-shRNA-containing pellets from donor 3 which exhibited a similar staining pattern to what

was observed in all different control condition pellets (Fig. 6). When COL10A1 expression was significantly knocked-down, less osteoclasts were observed *in vivo*; however, this could be an indirect rather than direct effect of COL10A1 knock-down. Further research will be required to determine this.

Down-regulation of COL10A1 affected the secretion profile of chondrogenic MSCs

Following implantation, some calcification and altered cell recruitment were observed in COL10A1 knock-down pellet from donor 2. Less extensive differences were observed in COL10A1 knock-down pellets from donor 3 (Fig. 7). In order to further understand why these differences occurred, the secreted profile of factors produced by chondrogenic MSCs and influencing EO was investigated. PDGF-BB and FGF-2, two factors involved in endothelial migration and

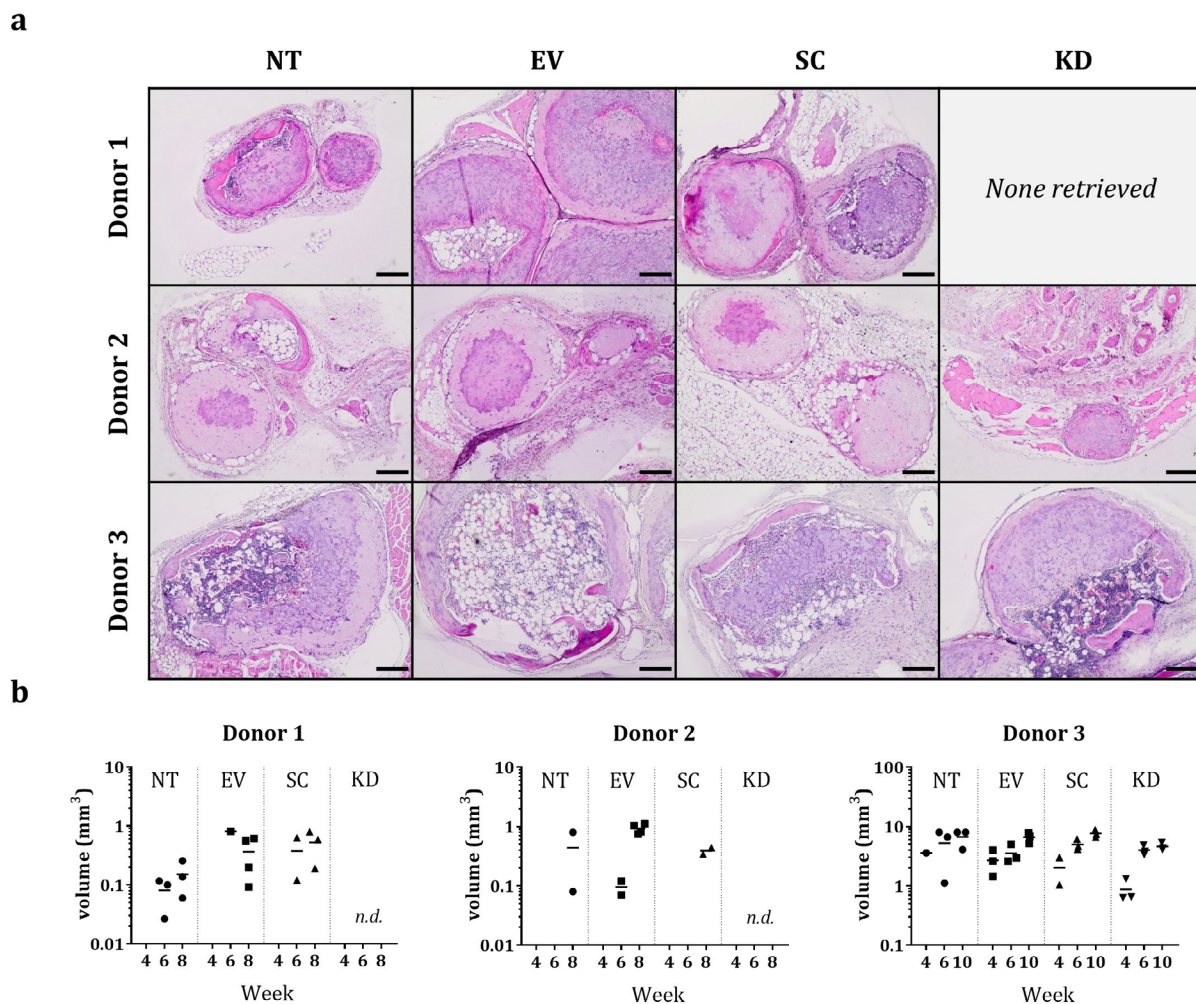


Fig. 5. When COL10A1 production was absent in chondrogenically differentiated MSCs, bone formation was hindered. However, a less significant knock-down resulted in normal bone formation. (a,b) Following 8 (donors 1 and 2) or 10 weeks (donor 3) of *in vivo* implantation, control conditions showed the production of bone, bone marrow and calcified cartilage. Donor 1 COL10A1 knock-down pellets could not be retrieved following implantation; however, one pellet from donor 2 showed signs of mineralisation. Pellets from donor 3 appeared to be similar to control conditions; however, mineralisation was initially delayed, as observed in longitudinal μ CT. Given that the samples were retrieved at 8 weeks for donors 1 and 2 and at 10 weeks for donor 3, it was not possible to pool the donors for statistical analysis. Scale bar: 200 μ m. NT: non-treated; EV: empty vector; SC: non-target control; KD: knock-down; n.d.: non-detectable.

osteoblastic migration/maturation (Kim *et al.*, 2007; Ornitz, 2005; Xie *et al.*, 2014), were lowly expressed (Fig. 7a,b). These factors were both detected at a higher level in donors 2 and 3. A similar trend was observed for TNF α , a factor linked to inflammation and osteoclastic maturation (Gerstenfeld *et al.*, 2003; Kimble *et al.*, 1997) (Fig. 7c). This showed that the secreted profile of chondrogenically differentiated MSCs, at least in part, was influenced by *COL10A1* production.

Discussion

The role of COLX in EO is often debated. Where some developmental studies have found significant perinatal death, delayed bone formation and a compromised immune system, others have reported no major difference between knock-out and wild-type controls (Jacenko *et al.*, 2001; Jacenko *et al.*, 2002; Kwan *et al.*, 1997; Rosati *et al.*, 1994). In these studies, matrix production was significantly altered, resulting in the distribution of matrix components around hypertrophic cells and throughout the growth plate (Jacenko *et al.*, 2001; Jacenko *et al.*, 2002). COLX also plays a role in the movement and accumulation of proteoglycans and associates with other collagens within the matrix (Chen *et al.*, 1990; Chen *et al.*, 1992; Poole and Pidoux, 1989). However, how COLX

contributes to tissue-engineered cartilage formation and subsequent endochondral bone formation has yet to be determined. The present study investigated the role of COLX from a tissue engineering perspective using culture-expanded MSCs. Using chondrogenically differentiated MSCs, which are prone to hypertrophic differentiation (Mueller and Tuan, 2008; Narcisi *et al.*, 2015; Pelttari *et al.*, 2006), GAG quantity was found to be significantly decreased and other major ECM components, including COLII and aggrecan, significantly downregulated or almost absent when COLX knock-down was more than 80 % (gene expression). As chondrogenically differentiated MSCs express COLX early during differentiation and have previously been shown to form bone following *in vivo* implantation (Farrell *et al.*, 2011; Farrell *et al.*, 2008; van der Stok *et al.*, 2014), this system was a suitable model for the present study. Several lentiviral controls were included to ensure that transfection of primary MSCs with a lentivirus [empty vector control (SHC001)] and the activation of RNA-induced silencing complex (RISC) [by a non-target control shRNA (SHC002)] was not having detrimental effects on cell behaviour. To ensure that the proper concentration of protamine sulphate, puromycin and lentiviral supernatant were used, extensive testing was completed prior to experimental work start. This allowed to minimise unnecessary stress to MSCs throughout the study. In some instances,

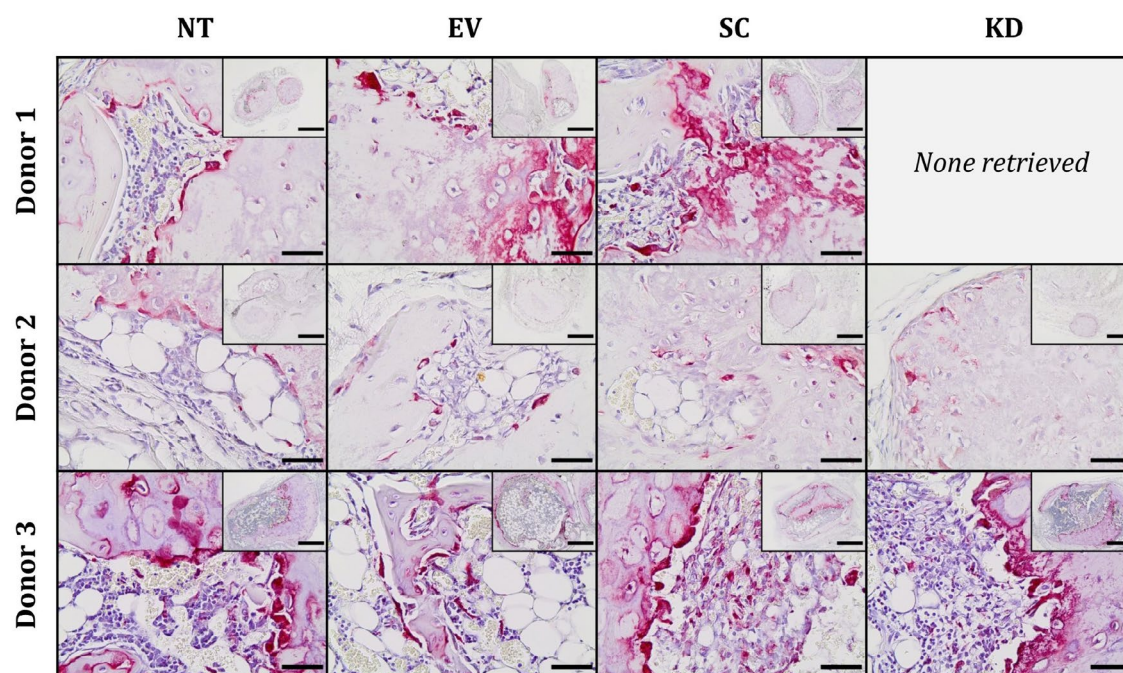


Fig. 6. Osteoclast activity in response to *COL10A1* knock-down in chondrogenically differentiated MSCs following *in vivo* implantation was affected in relation to the efficiency of the knock-down. TRAP staining of implanted pellets following 8 (donors 1 and 2) or 10 (donor 3) weeks of *in vivo* implantation. Pellets containing *COL10A1* shRNA could not be recovered following implantation from donor 1. 1 of the 9 *COL10A1*-shRNA-containing pellets from donor 2 was recovered but only exhibited TRAP positive staining on the outer periphery. All implanted pellets from donor 3 were recovered and displayed TRAP positivity throughout the construct. Staining did not vary greatly among non-treated, empty and non-target conditions. Scale bar: 200 μ m (bigger squares) and 50 μ m (smaller squares). NT: non-treated; EV: empty vector; SC: non-target control; KD: knock-down.

an increased chondrogenic potential was observed in lentivirally transduced cells (empty vector and non-target vector) as compared to non-treated MSCs. The addition of protamine sulphate could have contributed to this increase, as it has been shown to increase proliferation and differentiation potential when used in transduction protocol (Lin *et al.*, 2012). Protamine sulphate was not included in non-treated control in order to assess the chondrogenic and bone-forming potential of truly non-treated cells which had undergone the same number of passages. Given the extensive passaging required to accumulate the number of MSCs required for these experiments and the inherent donor variability observed in MSCs, each donor was shown independently in order to give a clear impression of the results.

Knuth *et al.* (2017) observed a close relation between COLII and COLX, which are expressed almost simultaneously during early chondrogenic differentiation. It is possible that during chondrogenic differentiation of MSCs, COLX expression is essential for COLII and in the absence of COLX, not only is COLII affected, but also other ECM components which are essential for proper chondrogenic differentiation.

Rosati *et al.* (1994), in a *COL10A1* knock-down and Tg mouse model study, showed that adjacent non-hypertrophic chondrocytes, which are unaffected by the knock-down, are still able to produce normal cartilage matrix components. As these non-affected cells were not present in the present model, this could explain why matrix production was more severely affected. Knuth *et al.* (2017) and Peltari *et al.* (2006) have previously reported that *COL10A1* expression occurs early in chondrogenic differentiation of MSCs, being observed within the first few days. In tissue engineering, it appears as if a certain threshold of *COL10A1* production is crucial for proper cartilage matrix formation during chondrogenic differentiation. When *COL10A1* was efficiently down-regulated, proper matrix formation was hindered. Not only were crucial ECM components not produced, but the secreted profile of the constructs was affected. A small quantity of *COL10A1* (roughly 20 % of the original; donor 3) was sufficient to allow MSC construct to undergo ossification and marrow formation *in vivo*, albeit less than in non-knock-down conditions. This might indicate that a minimum threshold of COLX production allowed for these

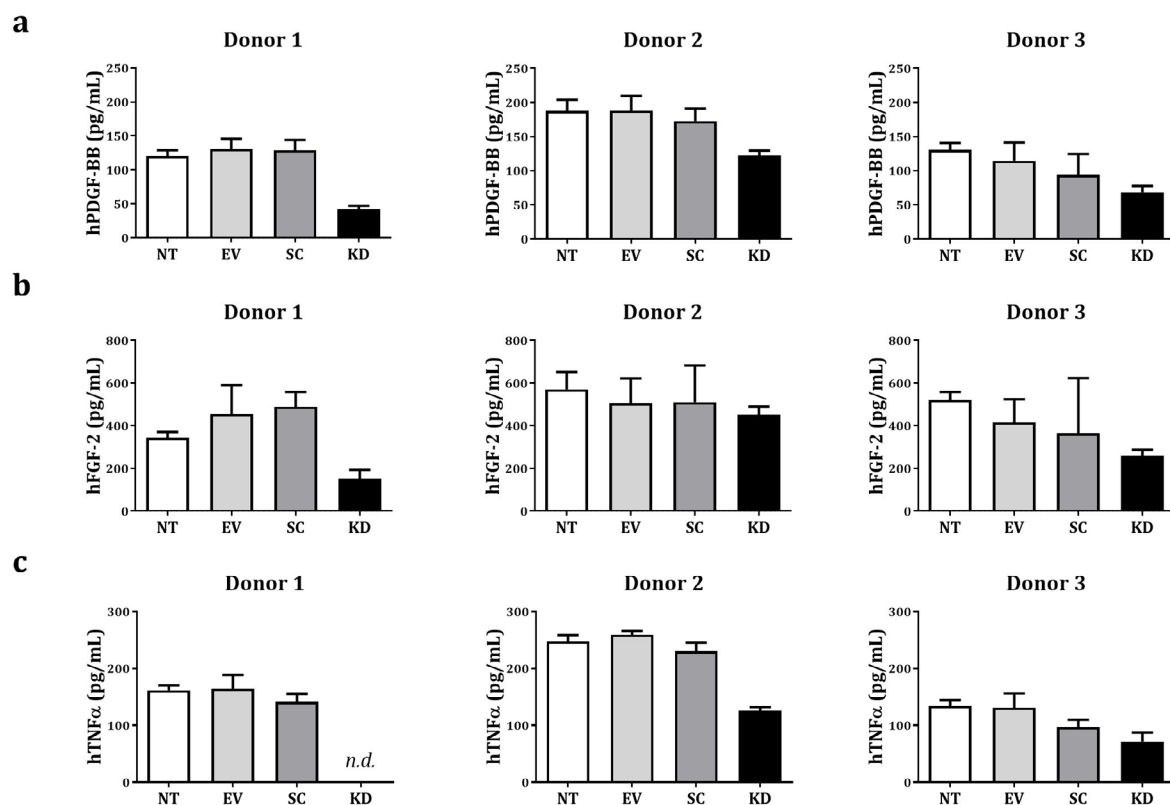


Fig. 7. When *COL10A1* production was absent, the production of factors related to osteoclastic and osteoblastic migration and maturation was decreased but a small quantity rescued this effect. (a,b) A significant knock-down of *COL10A1* was required for other factors related to osteoblastic migration and maturation, hPDGF-BB and hFGF-2, to be down-regulated (donor 1). A small quantity of *COL10A1* remaining in knock-down conditions showed no inhibition of hPDGF-BB and hFGF-2 (donors 2 and 3). (c) Production of hTNFα was down regulated when *COL10A1* was downregulated in chondrogenic MSCs. Production did not vary greatly between non-treated, empty and non-target conditions. As donor were not pooled, no statistical analysis was performed. NT: non-treated; EV: empty vector; SC: non-target control; KD: knock-down. n.d.: not determined.

processes to still occur. A larger number of donors would be helpful to determine exactly what this threshold is.

Schmid type metaphyseal chondrodysplasia, a disease resulting in dwarfism, coxa vara and other skeletomuscular defects, is the result of mutated or deficient COLX production (Chan *et al.*, 1998; McIntosh *et al.*, 1994; Warman *et al.*, 1993). As a significant effect was observed when COLX production was decreased during chondrogenesis, how this affected bone formation following implantation was investigated. A severe decrease in COLX production had not only major consequences on MSC chondrogenic differentiation but also affected endochondral bone formation. When COLX was significantly down regulated, as in donor 1, bone formation was not observed. However, in donor 2, who also had a highly efficient knock-down, only one pellet from the implant pockets was retrieved, with calcification of the construct but no marrow formation. Overall, this donor appeared to be slightly delayed in bone formation, as controls had very little marrow formation following implantation. It is possible that had the *in vivo* time frame been increased, marrow formation would have occurred, but this would require verification by further investigation. In donor 3, where knock-down efficiency was under 90 %, matrix remodelling and bone formation appeared relatively unaffected; however, mineralisation was still less than in non-treated controls. Due to the inherent variability in these types of experiments, because of inter-donor as well as lentivirus batches variations, it was difficult to make a clear broad statement on the knock-down effect in all donors. However, the knock-down variability enabled the conclusion that some degree of COLX expression was sufficient for bone formation to take place, since bone formation was only inhibited in the samples where COLX was knocked-down completely. It is possible that the greater chondrogenic potential of this donor required a higher lentiviral load in order to completely prevent *COL10A1* expression due to the presence of more mRNA per cell. However, a higher virus concentration increases the risks of off-target effects. It would be of great interest to study if the overexpression of *COL10A1* in this model would improve chondrogenic differentiation or increase the secretion of factors which influence *in vivo* matrix remodelling and bone formation. Alternative knock-down approaches, for example a CRISPR-Cas system using hTERT-immortalised MSCs, would be beneficial to confirm those findings.

To further understand these differences, the secretion of relevant molecules was investigated. During EO, recruitment and invasion of the nearby vasculature, initiation of osteoclast migration, remodelling of the cartilaginous matrix and osteoblastic maturation and bone deposition are all essential. Those processes can be initiated by different secreted factors (Boeuf and Richter, 2010; Scotti *et al.*,

2013; Scotti *et al.*, 2010; Tonnarelli *et al.*, 2014), which were released or found within the matrix of the implanted chondrogenic pellets. TNF α production was downregulated when COLX expression was decreased. TNF α is produced at high levels by hypertrophic chondrocytes (Kon *et al.*, 2001). TNF α is not only a major inflammatory factor involved in bone fracture healing (Gerstenfeld *et al.*, 2003) but also an important factor in bone formation and metabolism. It is involved in osteoclast differentiation and mediates bone turnover (Bertolini *et al.*, 1986; Kimble *et al.*, 1997). In fracture repair studies, a decreased recruitment of osteoclasts as well as delayed endochondral bone formation are observed in mice with TNF α signalling deficiency (Gerstenfeld *et al.*, 2003). Other factors, including PDGF-BB and FGF-2, which are expressed consistently during all stages of fracture repair (Bolander, 1992), were also found to be affected by a lack of COLX. PDGF is important for osteoblastic differentiation (Li *et al.*, 2014; Yu *et al.*, 1997) and contributes to the stabilisation of blood vessels by orchestrating migration and assembly of pericytes, which contribute to proper vasculature formation (Caplan and Correa, 2011; Xie *et al.*, 2014). FGFs play a role in EO and are also important for the proliferation and differentiation of chondrocytes (Kronenberg, 2003; Ornitz and Marie, 2002). Additionally, FGFs play a role in osteoclast formation and maturation (Hurley *et al.*, 1998; Kawaguchi *et al.*, 2000; Nakagawa *et al.*, 1999). A significant decrease in TNF α , PDGF-BB and FGF-2 was found when *COL10A1* was knocked-down over 99 % as compared to controls which did not show a difference in the production of this or other reported factors (donor 1). In donor 1 *COL10A1* knock-down, constructs were never retrieved following implantation. These constructs were most likely resorbed or degraded by the host. However, donors which had a decreased COLX expression but still maintained TNF α , PDGF-BB and FGF-2 expression were retrieved and formed bone following implantation (donors 2 and 3). In retrieved pellets from these donors, bone and signs of osteoclastic remodelling were observed, as evident by TRAP positive staining. Possibly, the production of these secreted factors was high enough to initiate the migration of osteoclasts and osteoblasts required to initiate bone formation. A full secreted profile analysis would be helpful to further elucidate the effects of a *COL10A1* knock-down on the chondrogenic secretome of differentiated MSCs.

In the present study, significant effects of COLX during both cartilage and bone formation, were observed. However, Rosati *et al.* (1994) found no major defects or skeletal abnormalities when COLX production is inhibited. This could be explained by the species' differences, as neither mouse cells nor a fully immune competent environment were part of the present study. Additionally, it is possible that, in Rosati *et al.* (1994) study, other collagens and components in the ECM were able to compensate

for the absence of COLX by surrounding cells in the growth plate. As the present model was a single-cell culture followed by subcutaneous implantation in a normally non-bony environment, no such cells close to the construct were present to compensate for the lack of COLX. As is often the case with MSC studies, a certain amount of inter- and intra-donor variation was observed. Although inter-donor variation is well documented in MSCs, the intra-donor variation observed could be attributed to the random integration nature of lentiviral delivery. This could have been controlled partially for by single-cell expansion, which is extremely difficult to achieve when using a primary MSC cell source. This variation has also been observed by others utilising primary MSCs (Lin *et al.*, 2012). However, it is clear from a tissue engineering perspective that COLX is essential for proper cartilage and bone formation. Its presence is required for proper cartilage differentiation pre-implantation and necessary for subsequent endochondral bone formation. With this in mind, it could be possible to further improve graft performance with chondrogenically differentiated MSCs by over expressing or increasing hypertrophic differentiation pre-implantation, augmenting the production of important factors which are influenced by the presence of COLX. However, further studies are required to prove this. By improving the understanding of how these constructs achieve endochondral bone formation, graft performance and clinical relevance of such constructs will also improve.

Conclusion

The present study showed how the absence of COLX in MSCs not only hindered the ability of MSCs to undergo chondrogenesis but also affected subsequent endochondral ossification following *in vivo* implantation. From a tissue engineering perspective, the study improved the understanding of how MSC-mediated endochondral ossification is achieved. Understanding COLX plays a crucial role. The present method of bone formation could further improve graft construction in the future.

Acknowledgments

The work was supported using imaging equipment provided by the Applied Molecular Imaging Erasmus MC facility.

Authors declare no conflict of interest.

References

Anderson HC, Garimella R, Tague SE (2005) The role of matrix vesicles in growth plate development and biomineralization. *Front Biosci* **10**: 822-837.

Bahney CS, Hu DP, Taylor AJ, Ferro F, Britz HM, Hallgrimsson B, Johnstone B, Miclau T, Marcucio RS (2014) Stem cell-derived endochondral cartilage stimulates bone healing by tissue transformation. *J Bone Miner Res* **29**: 1269-1282.

Bahney CS, Hu DP, Theodore Miclau Iii RSM (2015) The multifaceted role of the vasculature in endochondral fracture repair. *Front Endocrinol* **6**: 4. DOI: 10.3389/fendo.2015.00004.

Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR (1986) Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumour necrosis factors. *Nature* **319**: 516-518.

Boeuf S, Richter W (2010) Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors. *Stem Cell Res Ther* **1**: 31. DOI: 10.1186/scrt31.

Bolander ME (1992) Regulation of fracture repair by growth factors. *Proc Soc Exp Biol Med* **200**: 165-170.

Bourguine PE, Scotti C, Pigeot S, Tchang LA, Todorov A, Martin I (2014) Osteoinductivity of engineered cartilaginous templates devitalized by inducible apoptosis. *Proc Natl Acad Sci U S A* **111**: 17426-17431.

Campbell MR, Gress CJ, Appleman EH, Jacenko O (2004) Chicken collagen X regulatory sequences restrict transgene expression to hypertrophic cartilage in mice. *Am J Pathol* **164**: 487-499.

Caplan AI, Correa D (2011) PDGF in bone formation and regeneration: new insights into a novel mechanism involving MSCs. *J Orthop Res* **29**: 1795-1803.

Cervantes-Diaz F, Contreras P, Marcellini S (2017) Evolutionary origin of endochondral ossification: the transdifferentiation hypothesis. *Dev Genes Evol* **227**: 121-127.

Chan D, Jacenko O (1998) Phenotypic and biochemical consequences of collagen X mutations in mice and humans. *Matrix Biology* **17**: 169-184.

Chan D, Weng YM, Graham HK, Sillence DO, Bateman JF (1998) A nonsense mutation in the carboxyl-terminal domain of type X collagen causes haploinsufficiency in schmid metaphyseal chondrodysplasia. *J Clin Invest* **101**: 1490-1499.

Chen Q, Gibney E, Fitch JM, Linsenmayer C, Schmid TM, Linsenmayer T (1990) Long-range movement and fibril association of type X collagen within embryonic cartilage matrix. *Proc Natl Acad Sci* **87**: 8046-8050.

Chen Q, Linsenmayer C, Gu H, Schmid TM, Linsenmayer TF (1992) Domains of type X collagen: alteration of cartilage matrix by fibril association and proteoglycan accumulation. *J Cell Biol* **117**: 687-694.

Colnot CI, Helms JA (2001) A molecular analysis of matrix remodeling and angiogenesis during long bone development. *Mech Dev* **100**: 245-250.

Cunniffe GM, Vinardell T, Murphy JM, Thompson EM, Matsiko A, O'Brien FJ, Kelly DJ (2015) Porous decellularized tissue engineered hypertrophic cartilage as a scaffold for large bone defect healing. *Acta Biomater* **23**: 82-90.

- Deckers MML, Van Bezooijen RL, Van Der Horst G, Hoogendam J, van der Bent C, Papapoulos SE, Löwik CWGM (2002) Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. *Endocrinology* **143**: 1545-1553.
- Engsig MT, Chen Q-J, Vu TH, Pedersen A-C, Therkildsen B, Lund LR, Henriksen K, Lenhard T, Foged NT, Werb Z (2000) Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. *J Cell Biol* **151**: 879-890.
- Farrell E, Both SK, Odorfer KI, Koevoet W, Kops N, O'Brien FJ, Baatenburg de Jong RJ, Verhaar JA, Cuijpers V, Jansen J, Erben RG, van Osch GJ (2011) *In-vivo* generation of bone *via* endochondral ossification by *in-vitro* chondrogenic priming of adult human and rat mesenchymal stem cells. *BMC Musculoskelet Disord* **12**: 31. DOI: 10.1186/1471-2474-12-31.
- Farrell E, van der Jagt OP, Koevoet W, Kops N, Van Manen CJ, Hellingman CA, Jahr H, O'Brien FJ, Verhaar JAN, Weinans H (2008) Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? *Tissue Eng Part C Methods* **15**: 285-295.
- Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Tsay A, Fitch J, Barnes GL, Graves DT, Einhorn TA (2003) Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. *J Bone Miner Res* **18**: 1584-1592.
- Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA (2003) Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem* **88**: 873-884.
- Hurley M, Lee S, Raisz L, Bernecker P, Lorenzo J (1998) Basic fibroblast growth factor induces osteoclast formation in murine bone marrow cultures. *Bone* **22**: 309-316.
- Jacenko O, Chan D, Franklin A, Ito S, Underhill CB, Bateman JF, Campbell MR (2001) A dominant interference collagen X mutation disrupts hypertrophic chondrocyte pericellular matrix and glycosaminoglycan and proteoglycan distribution in transgenic mice. *Am J Pathol* **159**: 2257-2269.
- Jacenko O, Roberts DW, Campbell MR, McManus PM, Gress CJ, Tao Z (2002) Linking hematopoiesis to endochondral skeletogenesis through analysis of mice transgenic for collagen X. *Am J Pathol* **160**: 2019-2034.
- Kawaguchi H, Chikazu D, Nakamura K, Kumegawa M, Hakeda Y (2000) Direct and indirect actions of fibroblast growth factor 2 on osteoclastic bone resorption in cultures. *J Bone Miner Res* **15**: 466-473.
- Kim SJ, Kim SY, Kwon CH, Kim YK (2007) Differential effect of FGF and PDGF on cell proliferation and migration in osteoblastic cells. *Growth Factors* **25**: 77-86.
- Kim TK, Sharma B, Williams CG, Ruffner MA, Malik A, McFarland EG, Elisseff JH (2003) Experimental model for cartilage tissue engineering to regenerate the zonal organization of articular cartilage. *Osteoarthritis Cartilage* **11**: 653-664.
- Kimble RB, Bain S, Pacifici R (1997) The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice. *J Bone Miner Res* **12**: 935-941.
- Kirsch T, Pfäffle M (1992) Selective binding of anchorin CII (annexin V) to type II and X collagen and to chondrocalcin (C-propeptide of type II collagen). Implications for anchoring function between matrix vesicles and matrix proteins. *FEBS Lett* **310**: 143-147.
- Knuth CA, Kiernan CH, Palomares Cabeza V, Lehmann J, Witte-Buoma J, ten Berge D, Brama PA, Wolvius EB, Strabbing EM, Koudstaal M (2018) Isolating paediatric mesenchymal stem cells with enhanced expansion and differentiation capabilities. *Tissue Eng Part C Methods* **24**: 313-321.
- Knuth CA, Witte-Bouma J, Ridwan Y, Wolvius EB, Farrell E (2017) Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming. *Eur Cell Mater* **34**: 142-161.
- Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, Gerstenfeld LC, Einhorn TA (2001) expression of osteoprotegerin, receptor activator of NF- κ B ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. *J Bone Miner Res* **16**: 1004-1014.
- Kronenberg HM (2003) Developmental regulation of the growth plate. *Nature* **423**: 332-336.
- Kwan KM, Pang MK, Zhou S, Cowan SK, Kong RY, Pfordte T, Olsen BR, Sillence DO, Tam PP, Cheah KS (1997) Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: implications for function. *J Cell Biol* **136**: 459-471.
- Li A, Xia X, Yeh J, Kua H, Liu H, Mishina Y, Hao A, Li B (2014) PDGF-AA promotes osteogenic differentiation and migration of mesenchymal stem cell by down-regulating PDGFR α and derepressing BMP-Smad1/5/8 signaling. *PloS One* **9**: e113785. DOI: 10.1371/journal.pone.0113785.
- Lin P, Lin Y, Lennon DP, Correa D, Schluchter M, Caplan AI (2012) Efficient lentiviral transduction of human mesenchymal stem cells that preserves proliferation and differentiation capabilities. *Stem Cells Transl Med* **1**: 886-897.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* **25**: 402-408.
- Luvalle P, Daniels K, Hay ED, Olsen BR (1992) Type X collagen is transcriptionally activated and specifically localized during sternal cartilage maturation. *Matrix* **12**: 404-413.
- Mackie E (2003) Osteoblasts: novel roles in orchestration of skeletal architecture. *Int J Biochem Cell Biol* **35**: 1301-1305.
- Maes C, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, Carmeliet G, Kronenberg HM (2010) Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Dev Cell* **19**: 329-344.

- McIntosh I, Abbott MH, Warman ML, Olsen BR, Francomano CA (1994) Additional mutations of type X collagen confirm COL10A1 as the Schmid metaphyseal chondrodysplasia locus. *Hum Mol Genet* **3**: 303-307.
- Mueller MB, Tuan RS (2008) Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. *Arthritis Rheum* **58**: 1377-1388.
- Nakagawa N, Yasuda H, Yano K, Mochizuki S-i, Kobayashi N, Fujimoto H, Shima N, Morinaga T, Chikazu D, Kawaguchi H (1999) Basic fibroblast growth factor induces osteoclast formation by reciprocally regulating the production of osteoclast differentiation factor and osteoclastogenesis inhibitory factor in mouse osteoblastic cells. *Biochem Biophys Res Commun* **265**: 158-163.
- Narcisi R, Cleary MA, Brama PA, Hoogduijn MJ, Tüysüz N, ten Berge D, van Osch GJ (2015) Long-term expansion, enhanced chondrogenic potential, and suppression of endochondral ossification of adult human MSCs *via* WNT signaling modulation. *Stem Cell Reports* **4**: 459-472.
- Ornitz DM (2005) FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev* **16**: 205-213.
- Ornitz DM, Marie PJ (2002) FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev* **16**: 1446-1465.
- Ortega N, Behonick DJ, Werb Z (2004) Matrix remodeling during endochondral ossification. *Trends Cell Biol* **14**: 86-93.
- Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, Aigner T, Richter W (2006) Premature induction of hypertrophy during *in vitro* chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum* **54**: 3254-3266.
- Poole AR, Pidoux I (1989) Immunoelectron microscopic studies of type X collagen in endochondral ossification. *J Cell Biol* **109**: 2547-2554.
- Rosati R, Horan GS, Pinero GJ, Garofalo S, Keene DR, Horton WA, Vuorio E, de Crombrughe B, Behringer RR (1994) Normal long bone growth and development in type X collagen-null mice. *Nat Genet* **8**: 129-135.
- Schmid TM, Linsenmayer TF (1985) Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. *J Cell Biol* **100**: 598-605.
- Scotti C, Piccinini E, Takizawa H, Todorov A, Bourguine P, Papadimitropoulos A, Barbero A, Manz MG, Martin I (2013) Engineering of a functional bone organ through endochondral ossification. *Proc Natl Acad Sci U S A* **110**: 3997-4002.
- Scotti C, Tonnarelli B, Papadimitropoulos A, Scherberich A, Schaeren S, Schauerte A, Lopez-Rios J, Zeller R, Barbero A, Martin I (2010) Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. *Proc Natl Acad Sci U S A* **107**: 7251-7256.
- Shen G (2005) The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. *Orthod Craniofac Res* **8**: 11-17.
- Sweeney E, Campbell M, Watkins K, Hunter CA, Jacenko O (2008) Altered endochondral ossification in collagen X mouse models leads to impaired immune responses. *Dev Dyn* **237**: 2693-2704.
- Sweeney E, Roberts D, Corbo T, Jacenko O (2010) Congenic mice confirm that collagen X is required for proper hematopoietic development. *PLoS One* **5**: e9518. DOI: 10.1371/journal.pone.0009518.
- Toben D, Schroeder I, El Khassawna T, Mehta M, Hoffmann JE, Frisch JT, Schell H, Lienau J, Serra A, Radbruch A, Duda GN (2011) Fracture healing is accelerated in the absence of the adaptive immune system. *J Bone Miner Res* **26**: 113-124.
- Tonnarelli B, Centola M, Barbero A, Zeller R, Martin I (2014) Re-engineering development to instruct tissue regeneration. *Curr Top Dev Biol* **108**: 319-338.
- Touaitahuata H, Cres G, de Rossi S, Vives V, Blangy A (2014) The mineral dissolution function of osteoclasts is dispensable for hypertrophic cartilage degradation during long bone development and growth. *Dev Biol* **393**: 57-70.
- van der Stok J, Koolen MKE, Jahr H, Kops N, Waarsing JH, Weinans H, van der Jagt OP (2014) Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. *Eur Cell Mater* **27**: 137-148.
- Warman ML, Abbott M, Apte SS, Hefferon T, McIntosh I, Cohn DH, Hecht JT, Olsen BR, Francomano CA (1993) A type X collagen mutation causes Schmid metaphyseal chondrodysplasia. *Nat Genet* **5**: 79-82.
- Wu LN, Genge BR, Lloyd GC, Wuthier RE (1991) Collagen-binding proteins in collagenase-released matrix vesicles from cartilage. Interaction between matrix vesicle proteins and different types of collagen. *J Biol Chem* **266**: 1195-1203.
- Xie H, Cui Z, Wang L, Xia Z, Hu Y, Xian L, Li C, Xie L, Crane J, Wan M (2014) PDGF-BB secreted by preosteoclasts induces angiogenesis during coupling with osteogenesis. *Nat Med* **20**: 1270-1278.
- Yang L, Tsang KY, Tang HC, Chan D, Cheah KS (2014) Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. *Proc Natl Acad Sci U S A* **111**: 12097-12102.
- Yu X, Hsieh SC, Bao W, Graves DT (1997) Temporal expression of PDGF receptors and PDGF regulatory effects on osteoblastic cells in mineralizing cultures. *Am J Physiol* **272**: C1709-C1716.
- Zhou X, von der Mark K, Henry S, Norton W, Adams H, de Crombrughe B (2014) Chondrocytes transdifferentiate into osteoblasts in endochondral

bone during development, postnatal growth and fracture healing in mice. *PLoS Genet* **10**: e1004820. DOI: 10.1371/journal.pgen.1004820.

Discussion with Reviewers

Daniel Kelly: COLX appeared to be important not only for endochondral ossification, but also for initiation of MSC chondrogenesis. What are the implications of this finding for the cartilage tissue engineering field and the widespread belief that COLX expression is always a ‘bad thing’ as it suggests hypertrophy? Should we also be viewing COLX as a key early marker of MSC chondrogenesis?

Authors: With regard to MSC-based chondrogenesis, we believe that COLX expression closely mirrors the expression of COLII and, therefore, is expressed quite early in chondrogenesis (Fig. 1d day 7 and Knuth *et al.*, 2017). However, it is still not desirable in the context of cartilage tissue engineering as it seems to always precede bone formation. In healthy chondrocytes, COLX expression is not observed and is usually also seen as a marker of negative processes in the joint related to osteoarthritis onset and progression. We believe, it is valid to say that it could be a key early marker of MSC chondrogenesis in the currently available protocols for differentiation *in vitro*, but with the caveat that this chondrogenesis will most probably lead to endochondral ossification.

Daniel Kelly: For the donors that failed to undergo endochondral ossification *in vivo*, it is difficult to link this directly to the absence of COLX, as, in general, chondrogenesis and ECM accumulation were also suppressed in these groups. In the future, might it be possible to silence *COL10A1* later in chondrogenesis to better elucidate its role in hypertrophy and endochondral ossification?

Authors: This would involve generating a lentivirus with an extra inducible cassette that enables overexpression of the shRNA for *COL10A1* when a specific antibiotic is added to the culture medium. Theoretically, this would enable the knock-down of the gene from a specific time point or for a desired duration. However, there would be some technical issues to overcome. For example, these systems can be very leaky, leading to some expression of the shRNA even without the addition of the specific inducer. Also, penetration of the antibiotic through the dense matrix of the chondrogenic pellet would have to be assessed and verified. In general, it would be very interesting to assess if there is a stage specific necessity for *COL10A1* expression.

Editor’s note: The Scientific Editor responsible for this paper was Martin Stoddart.