




# Progenitor Cells in Healthy and Osteoarthritic Human Cartilage Have Extensive Culture Expansion Capacity while Retaining Chondrogenic Properties

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## Abstract

**Objective.** Articular cartilage-derived progenitor cells (ACPCs) are a potential new cell source for cartilage repair. This study aims to characterize endogenous ACPCs from healthy and osteoarthritic (OA) cartilage, evaluate their potential for cartilage regeneration, and compare this to cartilage formation by chondrocytes. **Design.** ACPCs were isolated from full-thickness healthy and OA human cartilage and separated from the total cell population by clonal growth after differential adhesion to fibronectin. ACPCs were characterized by growth kinetics, multilineage differentiation, and surface marker expression. Chondrogenic redifferentiation of ACPCs was compared with chondrocytes in pellet cultures. Pellets were assessed for cartilage-like matrix production by (immuno)histochemistry, quantitative analyses for glycosaminoglycans and DNA content, and expression of chondrogenic and hypertrophic genes. **Results.** Healthy and OA ACPCs were successfully differentiated toward the adipogenic and chondrogenic lineage, but failed to produce calcified matrix when exposed to osteogenic induction media. Both ACPC populations met the criteria for cell surface marker expression of mesenchymal stromal cells (MSCs). Healthy ACPCs cultured in pellets deposited extracellular matrix containing proteoglycans and type II collagen, devoid of type I collagen. Gene expression of hypertrophic marker type X collagen was lower in healthy ACPC pellets compared with OA pellets. **Conclusions.** This study provides further insight into the ACPC population in healthy and OA human articular cartilage. ACPCs show similarities to MSCs, yet do not produce calcified matrix under well-established osteogenic culture conditions. Due to extensive proliferative potential and chondrogenic capacity, ACPCs show potential for cartilage regeneration and possibly for clinical application, as a promising alternative to MSCs or chondrocytes.

## Keywords

articular cartilage, progenitor cell, endogenous, osteoarthritis, cartilage repair

## Introduction

Cartilage defects larger than 2 cm<sup>2</sup> are currently treated by transplantation of autologous culture-expanded chondrocytes.<sup>1,2</sup> As chondrocytes are the resident cell type in cartilage, this cell type is the prime candidate for cartilage repair. Although long-term results are promising with good patient reported outcomes and radiographic signs of cartilage formation,<sup>1,3</sup> drawbacks of the treatment remain. Extensive culture of chondrocytes for autologous administration induces dedifferentiation and loss of phenotype.<sup>4</sup> In addition, graft hypertrophy can lead to continued ailments and may necessitate further revision surgery.<sup>5</sup> These drawbacks can potentially be resolved by other cell sources. Mesenchymal stromal cells (MSCs) are frequently used<sup>6</sup> due to the relatively non-invasive methods for isolation, extensive culture-expansion potential,<sup>7,8</sup> and efficient *in vitro* differentiation

into chondrocytes producing cartilaginous tissue.<sup>9,10</sup> However, the associated risk of MSCs differentiating into hypertrophic chondrocytes and subsequent endochondral ossification poses a challenge.<sup>11</sup> While chondrocytes and

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MSCs are impacting the way cartilage defects are treated, different cell sources overcoming potential limitations may further advance the quality of repair tissue, and hence possibly clinical outcomes, leaving a gap for improvement in cell-based cartilage tissue engineering.

Initially, a small portion of isolated articular chondrocytes was described to grow clonally and differentiate into several lineages.<sup>12</sup> Next, a distinct progenitor cell with stem cell-like characteristics was identified in the superficial zone, first in bovine<sup>13</sup> and later also in human cartilage.<sup>14</sup> This endogenous progenitor population is referred to as cartilage stem cells, mesenchymal or chondrogenic progenitor cells, or articular cartilage-derived progenitor cells (ACPCs).

Besides extensive culture expansion,<sup>15</sup> ACPCs are successful at producing neo-cartilage *in vitro*<sup>16,17</sup> and *in vivo*.<sup>14,18</sup> Several reports indicate that ACPCs have no tendency to differentiate into hypertrophic chondrocytes, unlike MSCs.<sup>19,20</sup> These combined features give ACPCs a preference over chondrocytes and MSCs for cartilage regeneration. Furthermore, similar to chondrocytes,<sup>21</sup> pathological origin could influence ACPC performance. Indeed, OA-derived ACPCs were shown to possess chondrogenic properties, like healthy cartilage-derived ACPCs.<sup>22</sup> However, direct comparisons of chondrogenic potential of ACPCs from healthy and OA cartilage are limited.<sup>22,23</sup> Direct comparison can provide insight in the pathophysiology of OA and the potential role of ACPCs in health and disease.

The current study aims to characterize and compare fibronectin-selected ACPCs from healthy and OA human cartilage. By direct comparison of ACPC populations to full-depth cartilage cell populations derived from the same donors, their potential for cartilage regeneration is investigated.

## Methods

### Tissue Collection

Macroscopically healthy cartilage ( $n = 6$ , age = 46-49) was isolated post-mortem from full-weight-bearing and non-weight-bearing locations of the knee (Department of Pathology, University Medical Center Utrecht). Osteoarthritic (OA) cartilage was obtained from redundant material from patients ( $n = 6$ , age = 55-79) undergoing total knee arthroplasty. Anonymous collection of this tissue was performed according to medical ethics regulations of the University Medical Center Utrecht and the guideline "good use of redundant tissue for research" of the Dutch Federation of Medical Research Societies.<sup>24</sup> Human MSCs ( $n = 6$ , age = 30-66) were derived from bone marrow from the iliac crest of patients receiving spondylodesis or total hip arthroplasty surgery after their informed consent and

according to a protocol approved by the local medical ethical committee.

### Cell Isolation and Expansion

Cartilage from all parts of the joint (weight-bearing and non-weight-bearing) was pooled for each donor. Cartilage pieces were digested in 0.2% (w/v) pronase (Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 1% (v/v) penicillin/streptomycin (pen/strep; 100 U/mL, 100 µg/mL; Gibco) for 2 hours at 37°C, followed by overnight digestion in 0.075% (w/v) type II collagenase (Worthington, Lakewood, NJ) in DMEM with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Biowest) and 1% pen/strep under agitation. The population from here on referred to as "chondrocytes" is the total cell population isolated from cartilage, without any purification or selection.<sup>14</sup> Chondrocytes were expanded using chondrocyte expansion medium (DMEM, 10% FBS, 1% pen/strep). To isolate ACPCs, the digest was seeded at 500 cells/cm<sup>2</sup> at 37°C on culture plastic pre-coated for 1 hour with fibronectin (1 µg/mL in PBS containing MgCl<sub>2</sub> and CaCl<sub>2</sub>; Sigma-Aldrich) using serum-free medium (DMEM with 1% pen/strep). After 20 minutes, non-adhered cells were washed away and ACPC expansion medium was added (DMEM, 10% FBS, 1% pen/strep, 200 µM l-ascorbic acid 2-phosphate [ASAP; Sigma-Aldrich], and 5 ng/mL basic fibroblast growth factor [bFGF; PeproTech]). On day 6, colonies (>32 cells) were isolated using sterile glass cloning cylinders (Sigma-Aldrich). Collected colonies were pooled and ACPCs were further expanded on conventional tissue culture plastic with ACPC expansion medium.

The mononuclear fraction of bone marrow was separated by centrifugation using a Ficoll-Paque density gradient (GE Healthcare, The Netherlands) and plated using MSC expansion medium (Minimum Essential Media [ $\alpha$ MEM; Gibco, The Netherlands], 10% FBS, 1% pen/strep, 200 µM ASAP, and 1 ng/mL bFGF). MSCs were expanded up to passage 4 before use in differentiation assays.

### Flow Cytometry

Cells were washed in buffer (0.5% w/v bovine serum albumin [BSA], 2mM EDTA in PBS) and incubated with antibodies against CD49e, CD146, CD166 (Miltenyi Biotec), CD105 (Abcam), CD90, CD73, or a cocktail of markers (CD45, CD34, CD11b, CD79A, HLA-DR; all R&D Systems) according to the manufacturers' instructions. Labeled cells were analyzed using a BD FACSCanto II or BD LSRFortessa (BD Biosciences, USA). Dead cells were excluded using 100 ng/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) or 1 µg/mL 7-Aminoactinomycin D (7AAD; Molecular Probes). Results were analyzed using

FlowJo V10 data analysis software package (TreeStar, USA).

### Colony Forming Unit Fibroblast Assay

Chondrocytes directly after isolation (passage 0) and ACPCs and chondrocytes at passages 2 and 4 were seeded at 50 and 100 cells per 6-well plate well in duplicates. Cells were cultured with corresponding expansion medium. After 7 days, colonies were fixed with 10% formalin and stained for 45 minutes using 0.05% (v/v) Crystal Violet (Sigma-Aldrich) in Milli-Q water and counted.

### Multilineage Differentiation

For chondrogenic and hypertrophic differentiation,  $2.5 \times 10^5$  ACPCs (passage 4) or chondrocytes (passage 2) were pelleted in 15 mL Falcon tubes by centrifugation at 320 g for 5 minutes. Pellets were cultured in chondrogenic medium (DMEM with 1% pen/strep, 2% (v/v) human serum albumin [HSA; Albuman, Sanquin Blood Supply Foundation], 1% insulin-transferrin-selenium-ethanolamine [ITS-X; Gibco], 0.1  $\mu$ M dexamethasone, 0.2 mM ASAP, and 10 ng/mL transforming growth factor [TGF]- $\beta$ 1 [PeproTech]). After 21 days of chondrogenic differentiation, half of the pellets was fixed using 10% buffered formalin. The other half of the pellets was exposed to hypertrophic medium (DMEM with 1% pen/strep, 1% ITS-X, 1 nM dexamethasone, 200  $\mu$ M ASAP, 10 mM  $\beta$ -glycerophosphate [BGP; Sigma-Aldrich], and 1 nM 3,3',5-Triiodo-L-thyronine [Sigma-Aldrich]) for 7 additional days. After a total of 28 days, the hypertrophic-treated pellets were fixed with 10% formalin and processed for histology.

For osteogenic and adipogenic differentiation, ACPCs (passage 4) and chondrocytes (passage 2) were seeded in 24-well plate wells using corresponding expansion media. Upon subconfluency, monolayers were treated with osteogenic medium ( $\alpha$ MEM with 10% FBS, 1% pen/strep, 200  $\mu$ M ASAP, 10 mM BGP, and 10 nM dexamethasone [Sigma-Aldrich]) or adipogenic medium ( $\alpha$ MEM with 10% FBS, 1% pen/strep, 1  $\mu$ M dexamethasone, 0.5 mM 3-Isobutyl-1-methylxanthine [IBMX; Sigma-Aldrich], 0.2 mM indomethacin [Sigma-Aldrich], and 1.72  $\mu$ M insulin [Sigma-Aldrich]) for 21 days. After 21 days, monolayers were fixed with 10% formalin and stained for calcium deposits by 40 mM alizarin red S in demineralized water (pH 4.1; Sigma-Aldrich) or intracellular lipid vacuoles by 7.3 mM oil red O (Sigma-Aldrich) in 60% isopropanol. For all assays, MSCs were ran in parallel as positive controls.

Additional osteogenic differentiation was performed by expanding and differentiating ACPCs using several batches of FBS (Biowest and Gibco) and platelet lysate<sup>25</sup> (Sanquin Blood Supply Foundation). Also, monolayers were treated

with osteogenic medium with 100 ng/mL recombinant human Bone Morphogenetic Protein-2<sup>26</sup> (InductOS) and cell pellets<sup>14</sup> were stimulated with osteogenic medium, both for 21 days. Results are shown in Supplementary Figure S1.

### Pellet Redifferentiation Culture

For the redifferentiation cultures,  $2.5 \times 10^5$  cells were pelleted in ultra-low attachment 96-well plate wells by centrifugation at 320 g for 5 minutes. Pellets were cultured for 28 days in redifferentiation medium (DMEM with 1% pen/strep, 2% HSA, 2% ITS-X, and 1% ASAP). Half of the pellets was supplemented with 10 ng/mL TGF- $\beta$ 1. Used medium was stored at  $-20^\circ\text{C}$  for further analysis. Pellets were processed for biochemical analyses, gene expression, and (immuno)histochemistry.

### Biochemical Analysis of Pellets

Pellets were digested overnight with papain (250  $\mu$ g/mL papain [Sigma-Aldrich], 0.2 M  $\text{NaH}_2\text{PO}_4$ , 0.1M EDTA, 0.01M cysteine, pH 6) at  $60^\circ\text{C}$ . Deposition of sulphated glycosaminoglycans (GAGs) and release into the medium was measured by a dimethylmethylene blue assay (pH 3). The 525 / 595 nm absorbance ratio was measured using chondroitin-6-sulfate (Sigma-Aldrich) as a reference. DNA was quantified using a Quant-iT PicoGreen assay (Invitrogen) according the manufacturer's instructions.

### Histological Analysis of Pellets

Pellets were processed for histology by dehydration through graded ethanol steps, clearing in xylene, and embedding in paraffin. Five- $\mu$ m sections were stained for proteoglycan production with 0.125% Safranin-O (Merck) counterstained with 0.4% fast green (Sigma-Aldrich) and Weigert's hematoxylin (Clin-Tech). Collagen deposition was visualized by immunohistochemistry. Sections were blocked in 0.3% (v/v) hydrogen peroxide. Antigen retrieval for type I and II collagen was performed with 1 mg/mL pronase (Sigma-Aldrich) and 10 mg/mL hyaluronidase (Sigma-Aldrich) and for type X collagen with 1 mg/mL pepsin (Sigma-Aldrich) and 10 mg/mL hyaluronidase for 30 minutes at  $37^\circ\text{C}$ . Sections were blocked with 5% (w/v) BSA in PBS for 1 hour at room temperature and incubated with primary antibodies for type I collagen (EPR7785 [BioConnect], 1:400 in 5% PBS/BSA), type II collagen (II-II6B3 [DHSB], 1:100 in 5% PBS/BSA), and type X collagen (X53 [Quartett], 1:20 in 5% PBS/BSA) overnight at  $4^\circ\text{C}$ . Appropriate IgGs were used as isotype controls. Next, type I collagen sections were incubated with BrightVision Poly-HRP-Anti Rabbit (ImmunoLogic) and type II collagen sections were incubated with goat-anti-mouse IgG HRP-conjugated (DAKO,

P0447; 1:100 in 5% PBS/BSA), both for 1 hour at room temperature. Type X collagen sections were incubated with biotinylated sheep-anti-mouse IgG (RPN1001V [GE Healthcare]) for 1 hour at room temperature, then with streptavidin-HRP for 1 hour at room temperature (DAKO, P0397; 1:1000 in 5% PBS/BSA). Next, all stainings were developed using 3,3'-diaminobenzidine (DAB, Sigma-Aldrich). Cell nuclei were counterstained with Mayer's hematoxylin (Klinipath).

### Gene Expression

Gene expression analysis was performed by real-time polymerase chain reaction (PCR). RNA was isolated from cells and pellets using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA (200-500 ng) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCRs were performed using iTaq Universal SYBR Green Supermix (Bio-Rad) in the LightCycler 96 (Roche Diagnostics) according to the manufacturer's instructions. Primers (Invitrogen) are listed in **Table S1**. Relative gene expression was calculated using 18S as a housekeeping gene. Amplified PCR fragments extended over at least one exon border (except for 18S). The primer for detection of 2 splice variants of COL2A1 extended across exon 2 of the gene and results in amplification of splice variants IIa and IIb. PCR products were separated on 2% (w/v) agarose gel electrophoresis and stained with SYBR Safe (Invitrogen).

### Statistical Analysis

Experiments for flow cytometry, colony-forming efficiency, and multilineage differentiation were performed with cells from 6 healthy and 6 OA cartilage donors, unless stated otherwise. Pellet redifferentiation culture was performed with cells from 3 donors and 3 technical replicates per donor. All data are expressed as mean  $\pm$  standard deviation (SD). Data were analyzed using the GraphPad Prism 8 software package (GraphPad Software, United States). Normality was confirmed with a Shapiro-Wilk test ( $p > 0.05$ ). Groups were compared using an unpaired  $t$  test, 1- or 2-way analysis of variance (ANOVA) with Bonferroni *post hoc* test. Gene expression data were not normally distributed and therefore analyzed with a Mann-Whitney or Kruskal-Wallis test with Dunn's *post hoc* test. A value of  $P < 0.05$  was considered statistically significant.

## Results

### Cells Isolated from Human Cartilage Have a Chondrogenic Profile

All samples consistently contained cells highly expressing the cartilage-specific marker cartilage link protein (HAPLN1),

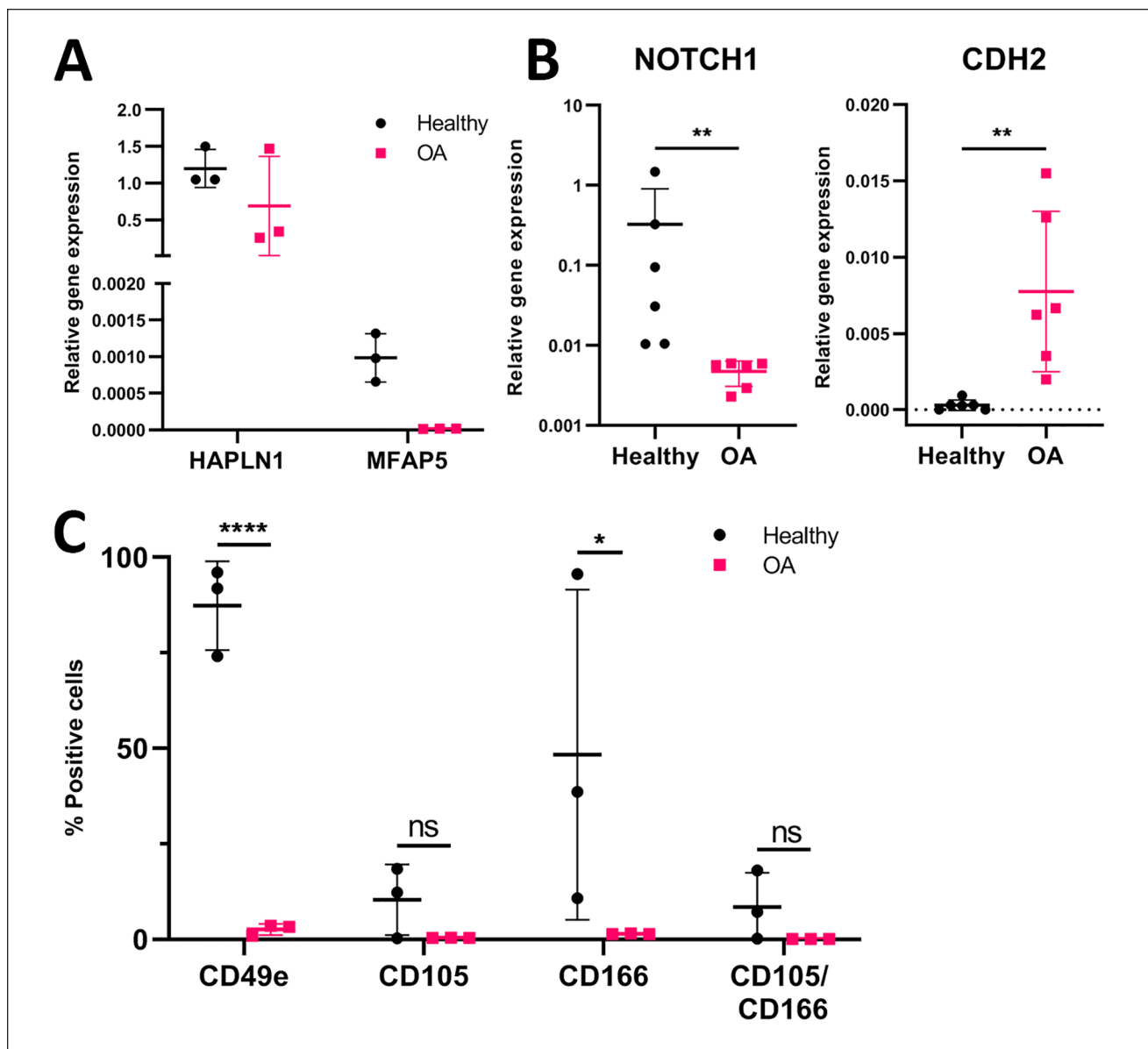
while having low expression of the synovial-specific marker microfibril-associated glycoprotein-2<sup>7</sup> (MFAP5; **Fig. 1A**). Expression of HAPLN1 was comparable between cells directly after digestion of healthy versus OA cartilage ( $1.199 \pm 0.259$  vs.  $0.687 \pm 0.678$ ). Likewise, expression of MFAP5 was low in cells from both disease states ( $0.0010 \pm 0.0003$  vs.  $0.000016 \pm 0.000001$ ), confirming successful isolation of cartilage cells without contamination of synoviocytes from the tissues.

### Expression of Progenitor-Specific Markers in Freshly Isolated Chondrocytes

Gene expression of freshly isolated chondrocytes revealed a significant difference in Notch receptor 1 (NOTCH1) between healthy and OA-derived cells ( $0.3274 \pm 0.5821$  vs.  $0.0047 \pm 0.0016$ ;  $p = 0.0022$ ; **Fig. 1B**). Notably, expression of n-cadherin (CDH2) was significantly higher in cells derived from OA cartilage compared with healthy ( $0.0003 \pm 0.0003$  vs.  $0.0077 \pm 0.0053$ ;  $p = 0.0061$ ; **Fig. 1B**). Cells positive for cell surface marker CD49e (integrin- $\alpha 5$ ) were significantly decreased in OA-derived cells compared with healthy ( $87.3\% \pm 11.6\%$  vs.  $2.6\% \pm 1.4\%$ ;  $P < 0.0001$ ; **Fig. 1C**). Likewise, expression of CD166 was decreased in OA-derived cells ( $48.4\% \pm 43.2\%$  vs.  $1.4\% \pm 0.1\%$ ;  $p = 0.0122$ ). Marker expression of CD105 as well as co-expression of CD105 and CD166 did not differ between the groups.

### Selection by Adhesion to Fibronectin Results in a Population with High Clonogenicity and Proliferative Capacity

Colony-forming efficiency of ACPCs at passage 2 and 4 was higher than of the full population ( $P < 0.0001$  for all groups; **Fig. 2A**). Morphology of representative Crystal Violet-stained colonies can be seen in Supplementary **Figure S2**. Healthy and OA ACPCs underwent  $18.1 \pm 1.5$  and  $13.0 \pm 1.0$  population doublings, respectively, until reaching the fourth passage after  $29.3 \pm 1.0$  and  $28.0 \pm 3.3$  days, healthy and OA chondrocytes had  $6.8 \pm 0.9$  and  $6.4 \pm 1.0$  population doublings, respectively, until reaching passage 4 after  $40.7 \pm 2.1$  and  $41.0 \pm 3.3$  days (**Fig. 2B**). Expansion of chondrocytes and ACPCs in ACPC expansion medium resulted in similar population doublings per passage and similar passage length (Supplementary **Fig. S3A and B**), while expansion in chondrocyte expansion medium resulted in a less population doublings by chondrocytes while their culture time per passage decreased (Supplementary **Fig. S3C and D**). OA ACPCs and chondrocytes lost expression of type II collagen (COL2A1) during expansion (**Fig. 2C**). Expression of type II collagen splice variants IIa and IIb did not reveal any distinct differences



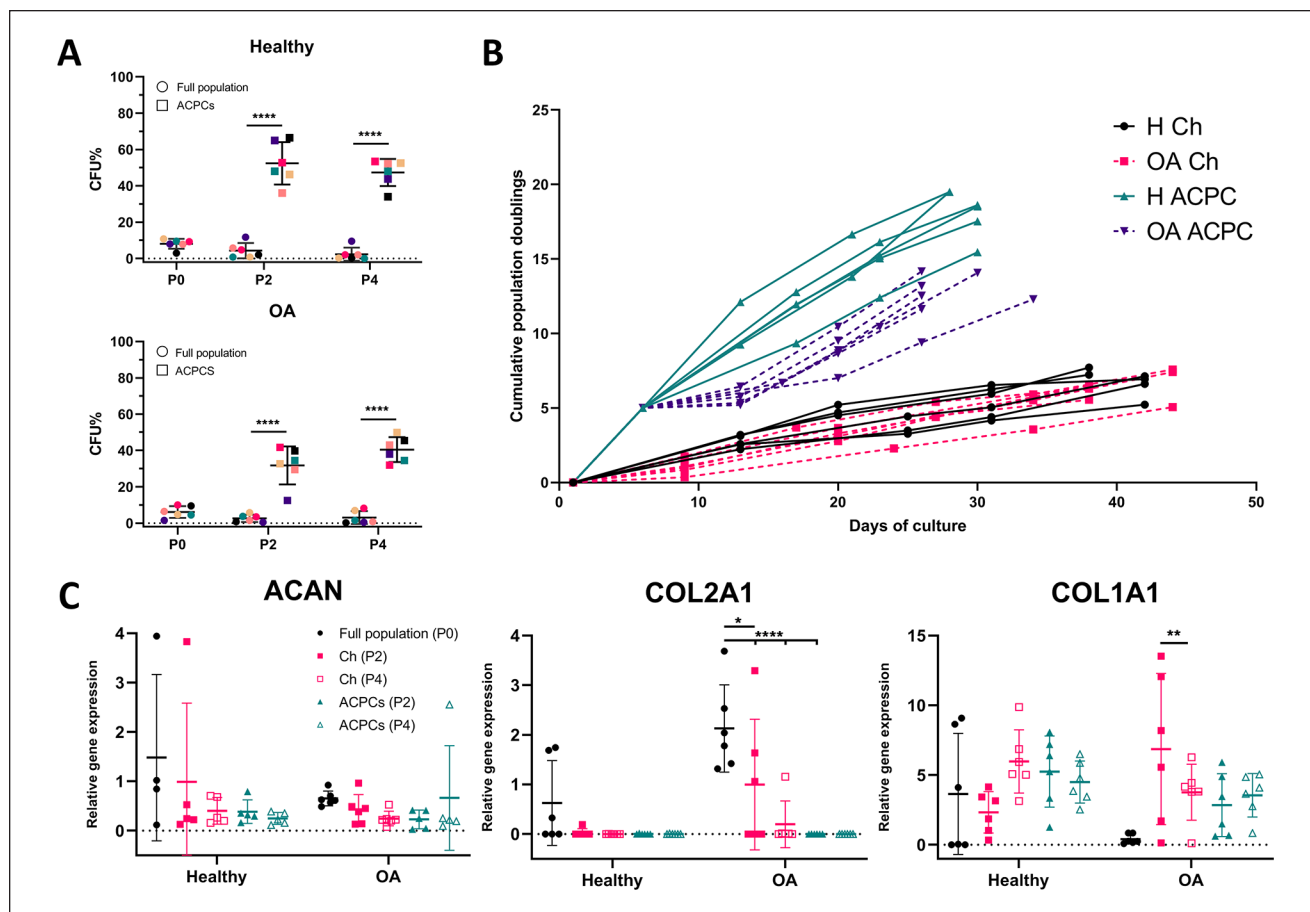
**Figure 1.** Characterization of full-depth cartilage cell populations. **(A)** Expression of cartilage- and synovial-specific genes in freshly isolated chondrocytes ( $n = 3$  for both). Gene expression of cartilage-specific gene hyaluronan and proteoglycan link protein I (HALPN1) is consistently high in chondrocytes isolated from healthy and osteoarthritic (OA) cartilage, while synovial-specific gene microfibril-associated protein 5 (MFAP5) is low in cells from both tissues. **(B)** Gene expression of Notch receptor I (NOTCH1) was higher in healthy compared with OA cartilage. Expression of n-cadherin (CDH2) was significantly increased in OA cartilage-derived cells ( $n = 6$  for both). **(C)** Surface marker expression of CD49e and CD166 were decreased in OA-derived cells compared with healthy cartilage-derived cells ( $n = 3$  for all). Three technical replicates per donor, each data point represents data of one donor. \* $P < 0.05$ . \*\* $P < 0.01$ . \*\*\* $P < 0.0001$ .

between chondrocytes and ACPCs or between passage numbers (Supplementary Fig. S4).

#### ACPCs Fail to Produce Mineralized Matrix upon Osteogenic and Hypertrophic Induction

All healthy and OA ACPCs differentiated into the chondrogenic and adipogenic lineage, indicated by Safranin-O and

oil red O stainings (Fig. 3A and B). Chondrocyte pellets stained less for proteoglycans than ACPC pellets [Fig. 3A, left panels]. Osteogenic differentiation was evident in chondrocytes [Fig. 3C, left panels], while ACPCs stained negative for mineralized matrix by alizarin red [Fig. 3C, right panels]. Osteogenic differentiation was also unsuccessful when ACPCs were expanded with different batches of FBS and platelet lysate, and when the osteogenic differentiation



**Figure 2.** Culture expansion of articular cartilage-derived progenitor cells (ACPCs) versus non-selected chondrocytes. **(A)** Colony-forming units (CFU) at increasing cell passages (P0, P2, P4). ACPCs form significantly more colonies at passage 2 and 4, compared with chondrocytes. Colors within the graphs represent different donors ( $n = 6$  for all). **(B)** Cumulative population doublings of ACPCs and chondrocytes. Healthy and osteoarthritic (OA) ACPCs underwent  $18.1 \pm 1.5$  and  $13.0 \pm 1.0$  population doublings, respectively, until reaching the fourth passage; healthy and OA chondrocytes underwent  $6.8 \pm 0.9$  and  $6.4 \pm 1.0$  population doublings, respectively, until reaching passage 4 ( $n = 6$  for all). **(C)** Expression of type II collagen (collagen type I alpha I chain; COL2A1) decreased in OA chondrocytes and ACPCs during expansion. Expression of type I collagen (collagen type II alpha I chain; COL1A1) was increased in passage 2 chondrocytes ( $n = 6$  for all). Three technical replicates per donor, each data point represents data of one donor. ACAN = aggrecan. \* $P < 0.05$ . \*\* $P < 0.01$ . \*\*\* $P < 0.0001$ .

medium was supplemented with 100 ng/mL BMP-2 or when ACPC pellets were stimulated with osteogenic medium (Supplementary Fig. S1). All chondrocytes and ACPCs were unable to differentiate into hypertrophic chondrocytes, indicated by negative staining for type X collagen (Fig. 3D).

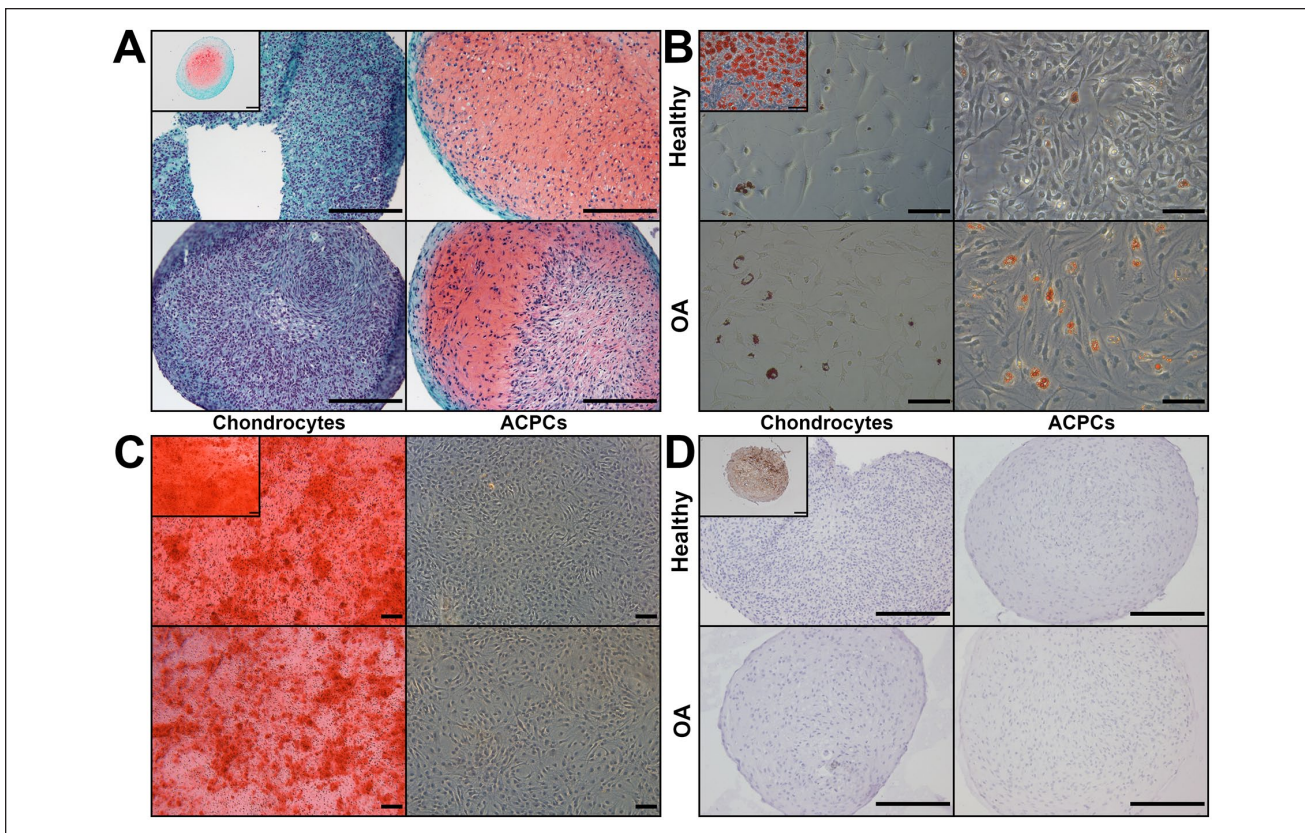
### Expression of Bone Marrow-Derived MSC Surface Markers in Culture-Expanded ACPCs

Expression of cell surface markers defined for bone marrow-derived MSCs in monolayer culture,<sup>28</sup> CD90, CD105, and CD73, was >95% in all populations. CD166-expression was >99% in all ACPC donors, but lower in OA chondrocytes ( $87.8\% \pm 13.6\%$ ) compared with healthy ACPCs

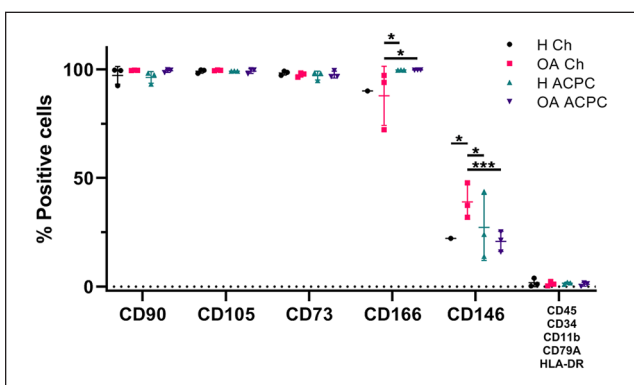
( $99.7\% \pm 0.1\%$ ;  $p = 0.0322$ ) and OA ACPCs ( $99.8\% \pm 0.1\%$ ;  $p = 0.0315$ ). Expression of CD146 was higher in OA chondrocytes ( $39.0\% \pm 8.1\%$ ) compared with healthy chondrocytes ( $22.2\% \pm 0.0\%$ ;  $p = 0.0322$ ), healthy ACPCs ( $27.3\% \pm 15.2\%$ ;  $p = 0.0352$ ), and OA ACPCs ( $20.9\% \pm 4.6\%$ ;  $p = 0.0003$ ). Expression of several markers was tested using a cocktail containing antibodies against CD45, CD34, CD11b, CD79A, and HLA-DR. All cell types were <2% positive for this cocktail of markers (Fig. 4).

### ACPC Pellets Produce Proteoglycans and Type II Collagen In Vitro

Chondrocytes and ACPC pellets stained positive for proteoglycans when stimulated with 10 ng/mL TGF- $\beta$ 1. When the



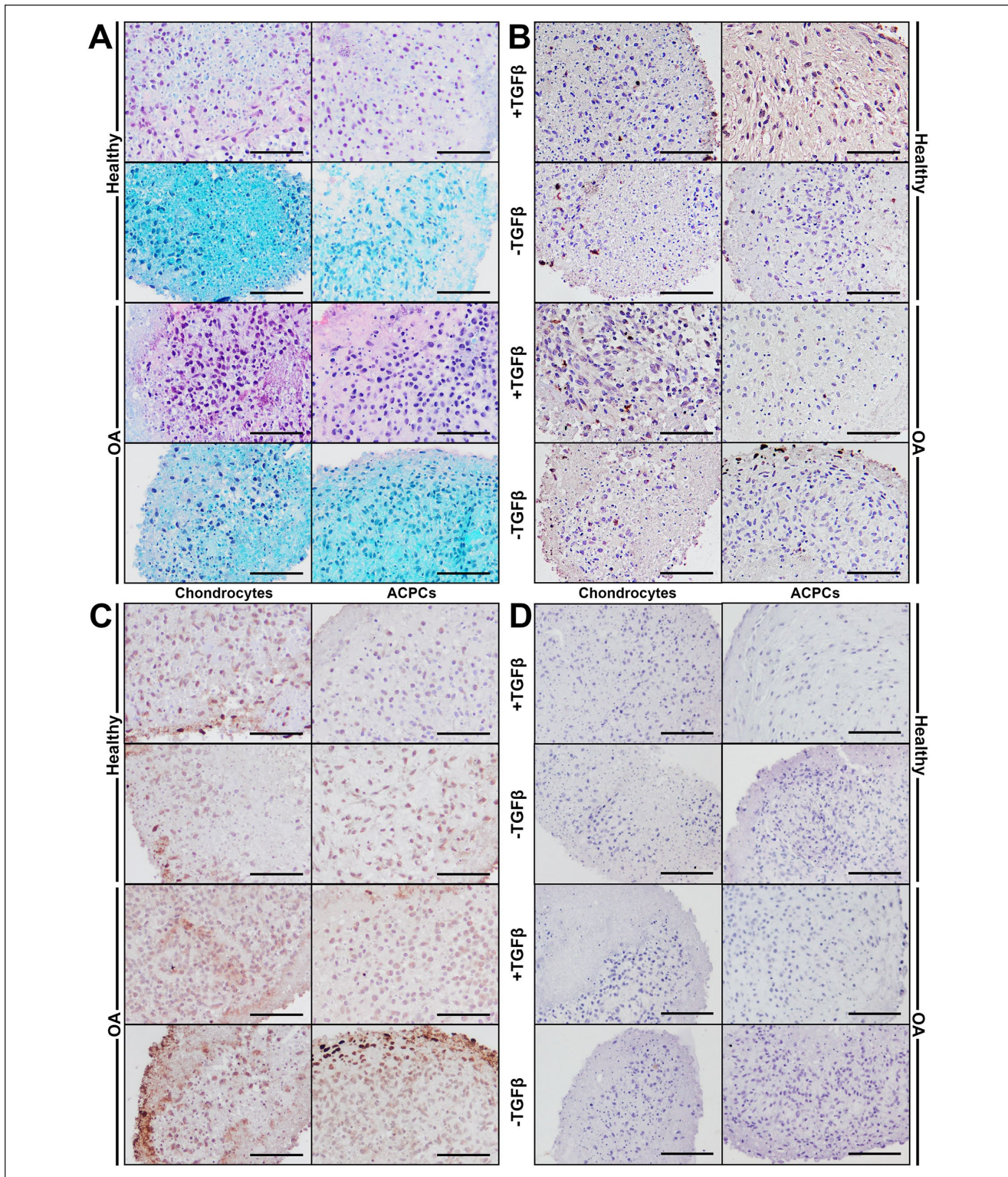
**Figure 3.** Differentiation of chondrocytes and articular cartilage-derived progenitor cells (ACPCs) into 4 lineages. **(A)** Chondrogenic differentiation was more effective in ACPCs than chondrocytes, indicated by staining of proteoglycans by Safranin-O. **(B)** Adipogenic differentiation was achieved in chondrocytes and ACPCs, indicated by staining of lipid droplets by oil red O. **(C)** Osteogenic differentiation was successful in chondrocytes, but not in ACPCs, shown by staining of the mineralized matrix by alizarin red. **(D)** Chondrocytes and ACPCs did not differentiate into hypertrophic chondrocytes indicated by absent staining for type X collagen. Inserts show bone marrow-derived mesenchymal stromal cells (MSCs) differentiated in parallel as positive controls. *N* = 6 for all cell types, a representative image per cell type was selected. All scale bars = 200  $\mu$ m. OA = osteoarthritic.



**Figure 4.** Cell surface marker expression by flow cytometry. Expression of CD90, CD105, and CD73 was >95% in all donors (*n* = 3). CD166 expression was lower in osteoarthritic (OA) chondrocytes compared with healthy and OA articular cartilage-derived progenitor cells (ACPCs). CD146 expression was higher in OA chondrocytes compared with the other cell types. All cell types were <2% positive for CD45, CD34, CD11b, CD79A, and HLA-DR. Three technical replicates per donor, each data point represents data of one donor. \**P* < 0.05. \*\*\**P* < 0.001.

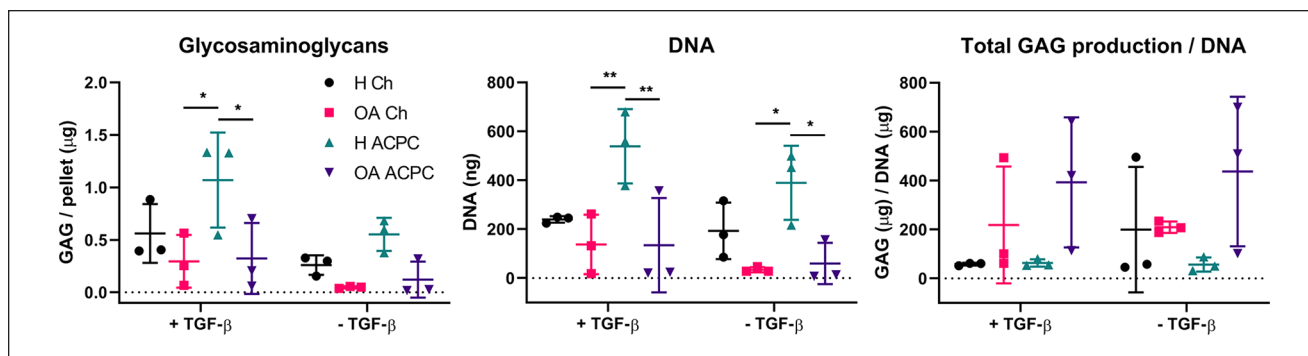
redifferentiation medium was not supplemented with TGF- $\beta$ 1, pellets stained negative for proteoglycans (**Fig. 5A**). Type II collagen production was only found in healthy ACPC pellets cultured in the presence of TGF- $\beta$ 1. All other conditions showed no type II collagen-positive matrix (**Fig. 5B**). In addition, healthy ACPC pellets stimulated with TGF- $\beta$ 1 were the only condition negative for type I collagen (**Fig. 5C**). All cultures were negative for X collagen (**Fig. 5D**).

Healthy ACPC pellets cultured with TGF- $\beta$ 1 contained more GAGs compared with OA chondrocyte and OA ACPC pellets ( $1.1 \pm 0.4 \mu\text{g}$  vs.  $0.3 \pm 0.3 \mu\text{g}$  and  $0.3 \pm 0.3 \mu\text{g}$ , *p* = 0.0115 and *p* = 0.0152, respectively; **Fig. 6**, left panel). Similarly, DNA content was higher in healthy ACPC pellets, independent of TGF- $\beta$ 1 (with TGF- $\beta$ 1:  $539.0 \pm 152.0$  ng vs.  $137.4 \pm 121.8$  ng and  $134.2 \pm 192.8$  ng, *p* = 0.0058 and *p* = 0.0054, respectively, without TGF- $\beta$ 1:  $389.4 \pm 151.5$  ng vs.  $34.0 \pm 10.7$  ng and  $59.7 \pm 84.5$  ng, *p* = 0.0153 and *p* = 0.0264, respectively [**Fig. 6**, middle panel]). Production of GAG corrected for the amount of DNA was not different between the groups (**Fig. 6**, right panel).



**Figure 5.** Histological staining of redifferentiated pellets. **(A)** Chondrocyte and articular cartilage-derived progenitor cells (ACPC) donor-matching pellets stained positive for proteoglycans by Safranin-O when stimulated with transforming growth factor (TGF)- $\beta$ 1 for 4 weeks. **(B)** TGF- $\beta$ 1-stimulated healthy ACPC pellets were positive for type II collagen and **(C)** negative for type I collagen. **(D)** None of the groups stained positive for type X collagen **(D)**.  $N = 3$  for all cell types, a representative image per cell type was selected. Scale bars = 100  $\mu$ m. OA = osteoarthritic.





**Figure 6.** Glycosaminoglycan (GAG) and DNA content in redifferentiated pellets after 4 weeks. Quantification of GAGs shows significantly more GAGs produced in healthy articular cartilage-derived progenitor cells (ACPC) pellets cultured in the presence of transforming growth factor (TGF)- $\beta$ 1 (left panel). DNA quantification shows significantly more DNA present in healthy ACPC pellets, suggesting bigger pellet sizes (middle panel). Total GAG production corrected for DNA content reveals no differences between the experimental groups (right panel).  $N = 3$  for all cell types. Three technical replicates per donor, each data point represents data of one donor. \* $P < 0.05$ . \*\* $P < 0.01$ .

### Reduced Expression of Hypertrophic Marker Type X Collagen

Gene expression analysis was only performed on TGF- $\beta$ 1-treated pellets, as insufficient amounts of RNA could be isolated from non-TGF- $\beta$ 1-treated pellets. No difference was found between expression of chondrogenic genes aggrecan (ACAN), type II collagen (COL2A1), and SRY-box transcription factor 9 (SOX9) between the experimental groups (Fig. 7A). Noteworthy, COL2A1 expression in ACPC pellets was close to zero. Expression of the hypertrophic marker type X collagen (COL10A1) was lower in healthy ACPC pellets compared with OA ACPC pellets ( $0.00074 \pm 0.00071$  vs.  $0.01315 \pm 0.00393$ ;  $p = 0.0028$ ) and compared with OA chondrocyte pellets ( $0.005664 \pm 0.002154$ ;  $p = 0.0296$ ). Expression of type I collagen (COL1A1) and matrix metalloproteinase 13 (MMP13) was not different between the groups (Fig. 7B).

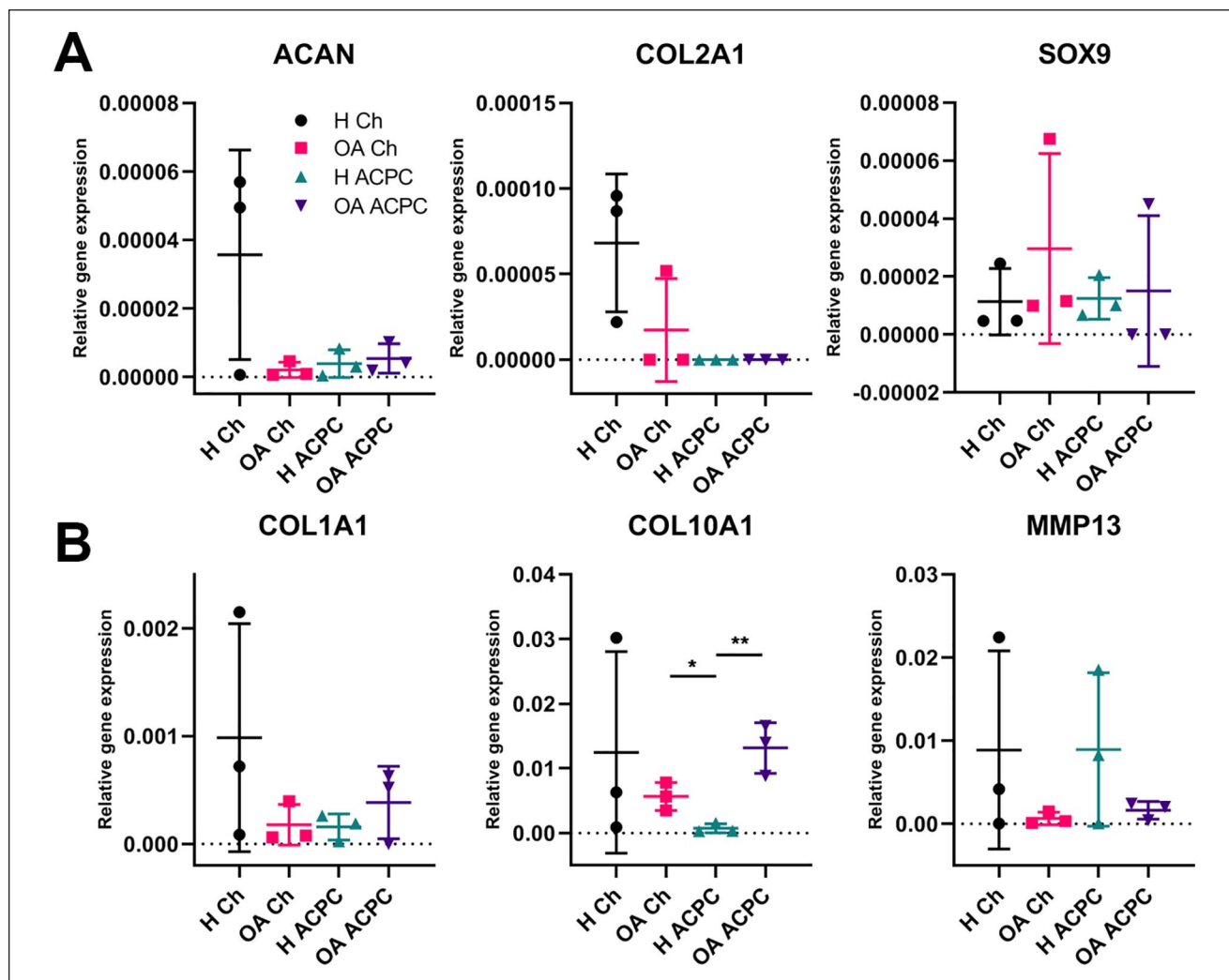
### Discussion

The current study aimed to characterize human ACPCs from healthy and OA cartilage and determine their potential for cartilage regeneration. While fibronectin-selected progenitor populations have been described in healthy<sup>14</sup> and OA human cartilage,<sup>22,29</sup> this study is the first to directly compare chondrogenic functionality of ACPCs from healthy and OA cartilage to chondrocytes derived from matching donors. The results confirm the presence of an ACPC population in human articular cartilage.<sup>14,22,23,29</sup> Differential adhesion to fibronectin resulted in a cell population that was capable of clonal growth, extensive culture expansion, multilineage differentiation, and had a limited tendency to produce mineralized matrix and terminally differentiate into hypertrophic chondrocytes. As chondrogenic

potential of chondrocytes can be dependent on the disease state of cartilage,<sup>21</sup> ACPCs derived from healthy and OA cartilage might provide as good candidates for cartilage repair. The current side-by-side comparison of healthy- with OA-derived ACPCs and donor-matched chondrocytes provides an overview of these cells' potential for cartilage regeneration.

We investigated full-depth healthy and OA cell populations attempting to find correlations between previously reported progenitor markers and ACPC quantity. Expression of NOTCH1 was found to be higher in healthy cells compared with OA, confirming previous findings.<sup>30</sup> At the same time, CDH2 was significantly upregulated in OA cells. The cell-cell adhesion molecule N-cadherin is related to cellular condensation in early chondrogenesis during development and absent in differentiated cartilage.<sup>31,32</sup> This might be a result of chondrocyte clustering in OA<sup>33,34</sup> and the cells potentially obtaining a more premature chondrogenic phenotype. CDH2 in culture-expanded ACPCs was previously found to be higher in non-OA ACPCs compared with OA ACPCs,<sup>35</sup> indicating that this difference is lost upon selection for progenitors and culture expansion. Therefore, NOTCH1 and CDH2 might be used as markers to distinguish between healthy- and OA-derived cells.

Expression of surface markers CD49e (integrin- $\alpha$ 5, part of the key fibronectin receptor) and CD166 was lost in the total population of OA cells compared with healthy cells, and no difference in CD105/CD166-double positive cells was found. These findings are in contrast to previous ones,<sup>23</sup> where a higher percentage of double positive cells was found in OA tissue. However, others found similar amounts<sup>36</sup> or more CD105/CD166-double positive cells in healthy cartilage versus OA.<sup>37</sup> The OA cartilage in the current study was obtained from end-stage OA patients and was not scored on OA severity. Severely degraded OA



**Figure 7.** Gene expression of redifferentiated pellets. **(A)** Expression of chondrogenic genes aggrecan (ACAN), type II collagen (collagen type I alpha I chain; COL2A1), and SRY-box transcription factor 9 (SOX9) was not different between the groups. **(B)** Type X collagen expression was lower in healthy articular cartilage-derived progenitor cells (ACPC) pellets compared with osteoarthritic (OA) ACPC pellets. Expression of type I collagen (collagen type II alpha I chain; COL1A1) and matrix metalloproteinase 13 (MMP13) was not different between the groups.  $N = 3$  for all cell types. Three technical replicates per donor, each data point represents data of one donor. \* $P < 0.05$ . \*\* $P < 0.01$ .

cartilage has lost most of its superficial layer and would subsequently also have lost superficial zone cells, which express CD49e<sup>13</sup> and CD166.<sup>36</sup> Separation based on CD49e-expression could lead to a population with improved chondrogenic potential, like healthy cartilage-derived ACPCs.

Our results show separation based on differential adhesion to fibronectin results in a population with enriched colony-forming efficiency and increased proliferative potential. Fibronectin-selected ACPCs were previously found to maintain telomerase activity and telomere length up to at least 22 population doublings,<sup>14,38</sup> which is more than the number of population doublings reached in our study. When using the same expansion media to expand

both cell types, population doublings in chondrocytes were limited compared with ACPCs with chondrocyte expansion medium, supporting the findings of higher cell yields of ACPCs. To add, culture time of chondrocytes decreased over passaging, indicating increasing cell size and possible dedifferentiation. On the contrary, OA-derived ACPCs lost mRNA expression of type II collagen upon culture expansion. Articular chondrocytes are known to dedifferentiate in monolayer expansion,<sup>4</sup> but regain their phenotype when exposed to appropriate culture conditions.<sup>12,39</sup> Similarly, our ACPCs regained their differentiation potential and especially healthy cartilage-derived ACPCs were successful in producing type II collagen- and proteoglycan-rich

matrix *in vitro*, while chondrocytes were less effective. Gene expression and protein deposition after the culture period of 4 weeks did not correspond, for which type II collagen is the most evident example. While H-ACPCs pellets cultured with TGF- $\beta$ 1 did not express the gene corresponding for type II collagen, immunohistochemistry revealed a slightly positive staining in these pellets. While discrepancies between protein and gene expression are regularly seen,<sup>40</sup> the chondrogenic response of ACPCs in this case might have been earlier than that of chondrocytes, resulting in differences in gene expression between the cell types at the 4-week evaluation point. Evaluation of gene expression throughout the culture period would give insight into the timing of the response. Furthermore, evaluation of individual clones of ACPCs would shed more light on cell performance and would allow for selection of populations with optimal characteristics, as was investigated before.<sup>14,38</sup> Yet, for the purpose of the current study it would considerably delay expansion time to obtain sufficient amounts of cells for tissue engineering and limit clinical application.

Since there is a need for identification of unique markers for selecting ACPCs, we specifically looked into gene expression of type II collagen splice variants in order to investigate whether this marker could be used for discerning ACPCs and chondrocytes. Splice variant IIa is an established marker for juvenile chondrocytes or mesenchymal cells, while variant IIb is expressed by mature chondrocytes.<sup>41,42</sup> Although ACPCs would be chondrogenic precursors and were expected to express the immature variant of type II collagen, no differences were found here between the cell types or passage numbers, a possible result of the cells being in the expansion phase rather than in redifferentiation and are not actively producing extracellular matrix.

ACPCs are generally referred to as MSC-like as they, besides holding multilineage differentiation potential, meet the surface marker criteria to identify MSCs.<sup>28</sup> In addition, there are some indications that ACPCs have similar anti-inflammatory properties as MSCs.<sup>43</sup> More than 95% of the ACPCs described here expressed MSC-markers CD90, CD105, CD73, and CD166, and expression of a panel of negative markers is <2%. Noteworthy, ACPCs were negative for HLA-DR, making these populations potentially interesting for allogeneic applications. Culture-expanded chondrocytes exhibit a similar pattern of surface marker expression. While the expression pattern of the ACPC populations investigated here are in line with previous reports,<sup>14,16,44</sup> caution should be taken when drawing conclusions. Evaluating expressed surface markers straight after ACPC-selection from the total pool of cells is the only way to directly compare cell populations and avoid the effect of culture expansion on the expression profile.

Both ACPC populations were unable to produce mineralized matrix upon stimulation with various osteogenic differentiation media and protocols. While osteogenesis is

generally confirmed in human ACPCs,<sup>14,22,29</sup> indications of reduced osteogenic potential exist. Interestingly, consistent results have been reported on decreased or absent expression of hypertrophic chondrocyte marker type X collagen<sup>16,19,20,45</sup> or early osteogenic marker alkaline phosphatase.<sup>17</sup> The differences in osteogenic differentiation potential between the populations investigated here and fibronectin-isolated ACPC populations described by others are remarkable. As ACPCs originate from the cartilage, the cells might be more primed toward the chondrogenic lineage rather than to differentiate into osteoblasts or continue toward terminal hypertrophic differentiation. Since others do report on osteogenic differentiation of ACPCs, minor differences in culture media composition might explain the discrepancies. Isolation and culture protocols should be conducted side by side to elucidate differences between ACPC populations. Bone marrow-derived MSCs are associated with the risk of hypertrophic cartilage formation, when cells either differentiate or deposited matrix is remodeled into bone.<sup>19,46</sup> Because hypertrophy in autologous chondrocyte implantation (ACI) continues to be a challenge,<sup>5</sup> the reduced osteogenic drift of ACPCs holds great promise for these cells.

Healthy cartilage-derived ACPCs produce cartilage ECM *in vitro* containing proteoglycans and type II collagen, and are devoid of type I collagen. In addition, these healthy ACPC pellets had low expression of type X collagen mRNA. Cartilage harvest site and tissue quality can be important for eventual cartilage production. To obtain healthy ACPCs, we combined all load-bearing and non-load-bearing cartilage from healthy knee joints, while chondrocytes isolated for ACI procedures are generally from non-load-bearing areas. However, chondrocytes from macroscopically healthy, full-weight bearing cartilage were shown to produce more proteoglycans and type II collagen *in vitro*.<sup>21</sup> Separating sub-groups of ACPC populations based on the degree of weight-bearing might provide further insights into the physiological role of progenitors in cartilage homeostasis. While we have not investigated it in the current study, several studies report on an increased number of ACPCs in OA cartilage<sup>22,23,47,48</sup> and numbers of ACPCs increasing after mechanical stimulation.<sup>49,50</sup>

ACPCs were used in a caprine model and had good lateral integration with the native cartilage,<sup>14</sup> showing potential for use in a 2-step cartilage repair procedure. Furthermore, a pilot study with 15 patients employing matrix-assisted autologous chondrocyte transplantation reported satisfactory histological and pain scoring 1 year after surgery.<sup>18</sup> ACPCs were expanded for a maximum of 3 weeks, substantially shorter than the expansion time needed for chondrocytes, which is generally 4 to 8 weeks,<sup>51-53</sup> depending on growth speed and defect size. In spite of these promising early clinical results, direct comparisons between chondrocytes and ACPCs are necessary to identify

advantages in length of culture expansion and quality of the repair tissue.

To conclude, ACPCs isolated here show potential for cartilage regeneration, possibly in an autologous approach replacing chondrocytes. The limited potential of these ACPC populations to produce mineralized matrix and absence of type X collagen protein and mRNA expression in healthy cartilage-derived ACPCs is promising. These observations combined with extensive *in vitro* expansion potential of ACPCs can have major implications for future cartilage repair treatments.

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### Declaration of Conflicting Interests


The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Ethical Approval

This study was approved by our institutional review board.

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### References

- Peterson L, Vasiliadis HS, Brittberg M, Lindahl A. Autologous chondrocyte implantation: a long term follow-up. *Am J Sports Med.* 2010;38(6):1117-24. doi:10.1177/0363546509357915.
- Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *New Engl J Med.* 1994;331(14):889-95. doi:10.1007/s40262-012-0001-1.
- Andriolo L, Reale D, Di Martino A, De Filippis R, Sessa A, Zaffagnini S, *et al.* Long-term results of arthroscopic matrix-assisted autologous chondrocyte transplantation: a prospective follow-up at 15 years. *Am J Sports Med.* 2020;48(12):2994-3001. doi:10.1177/0363546520949849.
- Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vécsei V, *et al.* Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. *Osteoarthritis Cartilage.* 2002;10(1):62-70. doi:10.1053/joca.2001.0482.
- Niethammer TR, Pietschmann MF, Horng A, Roßbach BP, Ficklscherer A, Jansson V, *et al.* Graft hypertrophy of matrix-based autologous chondrocyte implantation: a two-year follow-up study of NOVO CART 3D implantation in the knee. *Knee Surg Sports Traumatol Arthrosc.* 2014;22(6):1329-36. doi:10.1007/s00167-013-2454-7.
- Vonk LA, De Windt TS, Slaper-Cortenbach ICM, Saris DBF. Autologous, allogeneic, induced pluripotent stem cell or a combination stem cell therapy? where are we headed in cartilage repair and why: a concise review. *Stem Cell Res Ther.* 2015;6(1):94. doi:10.1186/s13287-015-0086-1.
- Salerno A, Brady K, Rijkers M, Li C, Caamaño-Gutierrez E, Falciani F, *et al.* MMP13 and TIMP1 are functional markers for two different potential modes of action by mesenchymal stem/stromal cells when treating osteoarthritis. *Stem Cells.* 2020;38:1438-53. doi:10.1002/stem.3255.
- Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells.* 2007;25:2739-49. doi:10.1634/stemcells.2007-0197.
- Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, *et al.* The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg Am.* 1998;80(12):1745-57. doi:10.2106/00004623-199812000-00004.
- Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng.* 1998;4(4):415-28. doi:10.1089/ten.1998.4.415.
- Gawlitza D, Farrell E, Malda J, Creemers LB, Alblas J, Dhert WJ. Modulating endochondral ossification of multipotent stromal cells for bone regeneration. *Tissue Eng Part B Rev.* 2010;16(4):385-95.
- Barbero A, Ploegert S, Heberer M, Martin I. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. *Arthritis Rheum.* 2003;48(5):1315-25. doi:10.1002/art.10950.
- Dowthwaite GP, Bishop J, Redman S, Khan I, Rooney P, Evans D, *et al.* The surface of articular cartilage contains a progenitor cell population. *J Cell Sci.* 2004;117(6):889-97. doi:10.1242/jcs.00912.
- Williams R, Khan IM, Richardson K, Nelson L, McCarthy HE, Analbelsi T, *et al.* Identification and clonal characterization of a progenitor cell sub-population in normal human articular cartilage. *PLoS One.* 2010;5(10):e13246. doi:10.1371/journal.pone.0013246.
- Archer C, Williams R, Nelson L, Khan I. Articular cartilage-derived stem cells: identification, characterisation and

- their role in spontaneous repair. *Rheumatol Curr Res*. 2012; S3:005. doi:10.4172/2161-1149.s3-005.
16. Carluccio S, Martinelli D, Palamà MEF, Pereira RC, Benelli R, Guijarro A, *et al.* Progenitor cells activated by platelet lysate in human articular cartilage as a tool for future cartilage engineering and reparative strategies. *Cells*. 2020;9(4):1052. doi:10.3390/cells9041052.
  17. Schmidt S, Abinzano F, Mensinga A, Teßmar J, Groll J, Malda J, *et al.* Differential production of cartilage ECM in 3D agarose constructs by equine articular cartilage progenitor cells and mesenchymal stromal cells. *Int J Mol Sci*. 2020;21(19):7071. doi:10.3390/ijms21197071.
  18. Jiang Y, Cai Y, Zhang W, Yin Z, Hu C, Tong T, *et al.* Human cartilage-derived progenitor cells from committed chondrocytes for efficient cartilage repair and regeneration. *Stem Cells Transl Med*. 2016;5(6):733-44. doi:10.5966/sctm.2015-0192.
  19. McCarthy HE, Bara JJ, Brakspear K, Singhrao SK, Archer CW. The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse. *Vet J*. 2012;192(3):345-51. doi:10.1016/j.tvjl.2011.08.036.
  20. Levato R, Webb WR, Otto IA, Mensinga A, Zhang Y, van Rijen M, *et al.* The bio in the ink: cartilage regeneration with bioprintable hydrogels and articular cartilage-derived progenitor cells. *Acta Biomater*. 2017;61:41-53. doi:10.1016/j.actbio.2017.08.005.
  21. Bekkers JEJ, Saris DBF, Tsuchida AI, van Rijen MH, Dhert WJ, Creemers LB. Chondrogenic potential of articular chondrocytes depends on their original location. *Tissue Eng Part A*. 2014;20(3-4):663-71. doi:10.1089/ten.TEA.2012.0673.
  22. Fellows CR, Williams R, Davies IR, Gohil K, Baird DM, Fairclough J, *et al.* Characterisation of a divergent progenitor cell sub-populations in human osteoarthritic cartilage: the role of telomere erosion and replicative senescence. *Sci Rep*. 2017;7(1):41421. doi:10.1038/srep41421.
  23. Alsalameh S, Amin R, Gemba T, Lotz M. Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. *Arthritis Rheum*. 2004;50(5):1522-32. doi:10.1002/art.20269.
  24. COREON. Human tissue and medical research: code of conduct for responsible use (2011). Available from: <https://www.coreon.org/wp-content/uploads/2020/04/coreon-code-of-conduct-english.pdf>. Published 2011. Accessed November 15, 2021.
  25. Prins H-J, Rozemuller H, Vonk-Griffioen S, Verweij VGM, Dhert WJA, Slaper-Cortenbach ICM, *et al.* Bone-forming capacity of mesenchymal stromal cells when cultured in the presence of human platelet lysate as substitute for fetal bovine serum. *Tissue Eng Part A*. 2009;15(12):3741-51. doi:10.1089/ten.TEA.2008.0666.
  26. Hanada K, Dennis JE, Caplan AI. Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. *J Bone Miner Res*. 1997;12(10):1606-14. doi:10.1359/jbmr.1997.12.10.1606.
  27. Rapko S, Zhang M, Richards B, Hutto E, Dethlefsen S, Duguay S. Identification of the chondrocyte lineage using microfibril-associated glycoprotein-2, a novel marker that distinguishes chondrocytes from synovial cells. *Tissue Eng Part C Methods*. 2010;16(6):1367-75. doi:10.1089/ten.TEC.2009.0772.
  28. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. *Cytotherapy*. 2006;8(4):315-7. doi:10.1080/14653240600855905.
  29. Nelson L, McCarthy HE, Fairclough J, Williams R, Archer CW. Evidence of a viable pool of stem cells within human osteoarthritic cartilage. *Cartilage*. 2014;5(4):203-14. doi:10.1177/1947603514544953.
  30. Grogan SP, Miyaki S, Asahara H, D'Lima DD, Lotz MK. Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis. *Arthritis Res Ther*. 2009;11(3):R85. doi:10.1186/ar2719.
  31. Oberlender SA, Tuan RS. Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. *Development*. 1994;120(1):177-87.
  32. Tuli R, Tuli S, Nandi S, Huang X, Manner PA, Hozack WJ, *et al.* Transforming growth factor- $\beta$ -mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and wnt signaling cross-talk. *J Biol Chem*. 2003;278(42):41227-36. doi:10.1074/jbc.M305312200.
  33. Hoshiyama Y, Otsuki S, Oda S, Kurokawa Y, Nakajima M, Jotoku T, *et al.* Chondrocyte clusters adjacent to sites of cartilage degeneration have characteristics of progenitor cells. *J Orthop Res*. 2015;33(4):548-55. doi:10.1002/jor.22782.
  34. Hoshiyama Y, Otsuki S, Oda S, Kurokawa Y, Nakajima M, Jotoku T, *et al.* Expression pattern and role of chondrocyte clusters in osteoarthritic human knee cartilage. *J Orthop Res*. 2015;33(4):548-55. doi:10.1002/jor.22782.
  35. Zhang S, An Q, Hu P, Wu X, Pan X, Peng W, *et al.* Core regulatory RNA molecules identified in articular cartilage stem/progenitor cells during osteoarthritis progression. *Epigenomics*. 2019;11(6):669-84. doi:10.2217/epi-2018-0212.
  36. Pretzel D, Linss S, Rochler S, Endres M, Kaps C, Alsalameh S, *et al.* Relative percentage and zonal distribution of mesenchymal progenitor cells in human osteoarthritic and normal cartilage. *Arthritis Res Ther*. 2011;13(2):R64. doi:10.1186/ar3320.
  37. Xia Z, Ma P, Wu N, Su X, Chen J, Jiang C, *et al.* Altered function in cartilage derived mesenchymal stem cell leads to OA-related cartilage erosion. *Am J Transl Res*. 2016;8(2):433-46. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27158337>.
  38. Khan IM, Bishop JC, Gilbert S, Archer CW. Clonal chondroprogenitors maintain telomerase activity and Sox9 expression during extended monolayer culture and retain chondrogenic potential. *Osteoarthritis Cartilage*. 2009;17(4):518-28. doi:10.1016/j.joca.2008.08.002.
  39. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell*. 1982;30(1):215-24. doi:10.1016/0092-8674(82)90027-7.
  40. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet*. 2012;13(4):227-32. doi:10.1038/nrg3185.
  41. Sandell LJ, Morris N, Robbins JR, Goldring MB. Alternatively spliced type II procollagen mRNAs define distinct populations

- of cells during vertebral development: differential expression of the amino-propeptide. *J Cell Biol.* 1991;114(6):1307-19. doi:10.1083/jcb.114.6.1307.
42. Zuscik MMJ, Hilton MJM, Zhang X, Chen D, O'Keefe RJ. Regulation of chondrogenesis and chondrocyte differentiation by stress. *J Clin Invest.* 2008;118(2):429-38. doi:10.1172/JCI34174.
  43. De Luca P, Kouroupis D, Viganò M, Perucca-Orfei C, Kaplan L, Zagra L, *et al.* Human diseased articular cartilage contains a mesenchymal stem cell-like population of chondroprogenitors with strong immunomodulatory responses. *J Clin Med.* 2019;8(4):423. doi:10.3390/jcm8040423.
  44. Bernstein P, Sperling I, Corbeil D, Hempel U, Fickert S. Progenitor cells from cartilage—no osteoarthritis-grade-specific differences in stem cell marker expression. *Biotechnol Prog.* 2013;29(1):206-12. doi:10.1002/btpr.1668.
  45. Anderson DE, Markway BD, Weekes KJ, McCarthy HE, Johnstone B. Physioxia promotes the articular chondrocyte-like phenotype in human chondroprogenitor-derived self-organized tissue. *Tissue Eng Part A.* 2018;24(3-4):264-74. doi:10.1089/ten.TEA.2016.0510.
  46. Mueller MB, Tuan RS. Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. *Arthritis Rheum.* 2008;58(5):1377-88. doi:10.1002/art.23370.
  47. Wang Y-X, Zhao Z-D, Wang Q, Li Z-L, Huang Y, Zhao S, *et al.* Biological potential alterations of migratory chondrogenic progenitor cells during knee osteoarthritic progression. *Arthritis Res Ther.* 2020;22(1):62. doi:10.1186/s13075-020-2144-z.
  48. Su X, Zuo W, Wu Z, Chen J, Wu N, Ma P, *et al.* CD146 as a new marker for an increased chondroprogenitor cell sub-population in the later stages of osteoarthritis. *J Orthop Res.* 2015;33(1):84-91. doi:10.1002/jor.22731.
  49. Seol D, McCabe DJ, Choe H, Zheng H, Yu Y, Jang K, *et al.* Chondrogenic progenitor cells respond to cartilage injury. *Arthritis Rheum.* 2012;64(11):3626-37. doi:10.1002/art.34613.
  50. Jang KW, Ding L, Seol D, Lim TH, Buckwalter JA, Martin JA. Low-Intensity pulsed ultrasound promotes chondrogenic progenitor cell migration via focal adhesion kinase pathway. *Ultrasound Med Biol.* 2014;40(6):1177-86. doi:10.1016/j.ultrasmedbio.2013.12.007.
  51. Saris D, Price A, Widuchowski W, Bertrand-Marchand M, Caron J, Drogset JO, *et al.* Matrix-applied characterized autologous cultured chondrocytes versus microfracture: two-year follow-up of a prospective randomized trial. *Am J Sports Med.* 2014;42(6):1384-94. doi:10.1177/0363546514528093.
  52. Ebert JR, Robertson WB, Woodhouse J, Fallon M, Zheng MH, Ackland T, *et al.* Clinical and magnetic resonance imaging-based outcomes to 5 years after matrix-induced autologous chondrocyte implantation to address articular cartilage defects in the knee. *Am J Sports Med.* 2011;39(4):753-63. doi:10.1177/0363546510390476.
  53. Niemeyer P, Laute V, John T, Becher C, Diehl P, Kolombe T, *et al.* The effect of cell dose on the early magnetic resonance morphological outcomes of autologous cell implantation for articular cartilage defects in the knee. *Am J Sports Med.* 2016;44(8):2005-14. doi:10.1177/0363546516646092.