




# Temporomandibular Joint Fibrocartilage Contains CD105 Positive Mouse Mesenchymal Stem/Progenitor Cells with Increased Chondrogenic Potential

Janis R. Thamm<sup>1</sup> · Youssef Jounaidi<sup>3</sup> · Max-Laurin Mueller<sup>1</sup> · Vicki Rosen<sup>4</sup> · Maria J. Troulis<sup>1,2</sup> · Fernando Pozzi Semeghini Guastaldi<sup>1,5</sup> 

Received: 24 June 2021 / Accepted: 8 April 2022 / Published online: 9 May 2022  
© The Association of Oral and Maxillofacial Surgeons of India 2022

## Abstract

**Objective** A specific type of mesenchymal stem/progenitor cells (MSPCs), CD105<sup>+</sup> is reported to aid in cartilage regeneration through TGF- $\beta$ /Smad2-signalling. The purpose of this study was to identify and characterize CD105<sup>+</sup> MSPCs in temporomandibular joint (TMJ) cartilage.

**Materials and Methods** MSPCs were isolated from mouse TMJ condyle explants and evaluated for their clonogenicity and pluripotential abilities. MSPC were examined for CD105 antigen using immunohistochemistry and flow cytometry.

**Results** Immunohistochemistry revealed presence of CD105<sup>+</sup> MSPCs in the proliferative zone of condyle's cartilage. Only 0.2% of isolated MSPCs exhibited CD105, along with the stem cell surface markers CD44 and Sca-1. In CD105<sup>+</sup> MSPCs, intracellular immunostaining revealed

significantly higher ( $p < 0.05$ ) protein levels of collagen type 1, 2, proteoglycan 4. Ability for chondrogenic differentiation was found to be significantly higher ( $p < 0.05$ ) after 4 weeks compared to CD105<sup>-</sup> cells, using alcian blue staining. CD105<sup>+</sup> cells were found to resemble an early MSPC subgroup with significantly higher gene expression of biglycan, proteoglycan 4, collagen type 2, Gli2, Sox5 ( $p < 0.001$ ) and Sox9 ( $p < 0.05$ ). In contrast, significantly lower levels of Runx2 ( $p < 0.05$ ), Osterix, Trps1, Col10a1 ( $p < 0.01$ ), Ihh ( $p < 0.001$ ) related to chondrocyte senescence and commitment to osteogenic lineage, were observed compared to CD105<sup>-</sup> cells.

**Conclusion** The study showed the existence of a CD105<sup>+</sup> MSPC subgroup within TMJ fibrocartilage that may be activated to aid in fibrocartilage repair.

**Keywords** CD105 · Mesenchymal stem/progenitor cells · Cartilage regeneration · Temporomandibular joint

✉ Fernando Pozzi Semeghini Guastaldi  
fguastaldi@mgh.harvard.edu

<sup>1</sup> Department of Oral and Maxillofacial Surgery, Massachusetts General Hospital, Harvard School of Dental Medicine, Boston, MA, USA

<sup>2</sup> Walter C. Guralnick Professor of Oral and Maxillofacial Surgery, Department of Oral and Maxillofacial Surgery, Massachusetts General Hospital, Harvard School of Dental Medicine, Boston, MA, USA

<sup>3</sup> Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

<sup>4</sup> Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA, USA

<sup>5</sup> Skeletal Biology Research Center, Department of Oral and Maxillofacial Surgery, Massachusetts General Hospital, Harvard School of Dental Medicine, 50 Blossom St, Thier 513A, Boston, MA 02114, USA

## Introduction

Temporomandibular joint disorders reflect a broad cluster of degenerative conditions including TMJ osteoarthritis, which can lead to remodeling and/or degeneration and/or functional distortion of the physiological TMJ anatomy, and subsequent dysfunction [1, 2].

TMJ degeneration results in an imbalance between the synthesis and degradation of the extracellular matrix (ECM) of articular fibrocartilage and subchondral bone. This can have negative functional implications. Physiologically, chondrocytes precursors and MSPCs, residing in the cartilage proliferative zone, adapt the cartilage to ongoing changes in the application of friction and load onto the cartilage surface [3–6]. MSPCs of this zone express

CD44 as well as stem cell antigen 1 (Sca-1) on their surface [7]. These cells also show considerable expression of collagen type 2 (Col2a1, Indian hedgehog (Ihh) and parathyroid-hormone-related protein (PTHrP/PTHLH), signals that regulate chondrogenesis and endochondral ossification [4, 8, 9]. Suzuki and Iwata highlighted the importance of Ihh, which leads in its absence to loss of the articular disc, the lower articular cavity and severe TMJ deformities [10]. Cells of the growth plate proliferative zone show strong expression of SRY (Sex-Determining Region Y)-Box 9 (Sox9) protein, which is required for chondrocyte differentiation and therefore responsible for initiating the development of the mandibular condyle [10–12]. Sox9 is expressed by MSPCs until they become pre-hypertrophic [4]. Sox9 together with Sox5 and Sox6 are termed as the chondrogenic/Sox trio and are essential for early chondrogenesis [13]. The Sox trio provides sufficient intrinsic signaling adequate to form permanent cartilage and also activates specific enhancers in the Col2a1, Col9a1, Col11a2 and aggrecan (Agc1) genes to stimulate chondrogenic differentiation [14–18]. Senescent chondrocytes of the chondroblastic and hypertrophic cartilage zone lose their ability to synthesize collagen type 2 and show increased Col10a1 gene expression [4, 11, 19].

CD105 also known as Endoglin is an accessory part of the transforming growth factor (TGF $\beta$ ) receptor (TGF $\beta$ -R) complex [20, 21]. CD105 was first described by Quackebush et al. and found in human pre-B leukemia cells [22]. CD105 is expressed on human pre-erythroblasts, macrophages, leukemic cells, the syncytiotrophoblast of the placenta and vascular endothelium [22–25]. In most mesenchymal and epithelial cells the TGF $\beta$ -R complex consists of the type I and II receptor type [21].

Previous studies found increased ability for chondrogenic differentiation of CD105<sup>+</sup> MSPCs in synovia and adipose tissue, due to the maintenance of transforming growth factor-beta (TGF- $\beta$ ) and Smad2 signaling and the subsequent higher expression of the Col2a1, Sox9 and aggrecan genes [26–28]. Those are main components and transcriptional targets for chondrogenic differentiation.

In contrast, little is known about MSPCs of the TMJ, which are known to be critical for tissue regeneration [29]. We hypothesized that MSPCs would be present in the TMJ condyle's cartilage, and could be identified using CD105 antigen staining.

## Materials and Methods

### Mice

The use of mice followed the Institutional Animal Care and Use Committee (IACUC) guidelines approved and

conducted by Massachusetts General Hospital (Protocol #2017N000086). Two eight-week-old C57BL/6 female mice were obtained from Charles River Laboratories (Wilmington, MA).

### Harvesting of TMJ Condyle, MSPC Isolation and Colony-Forming Assay

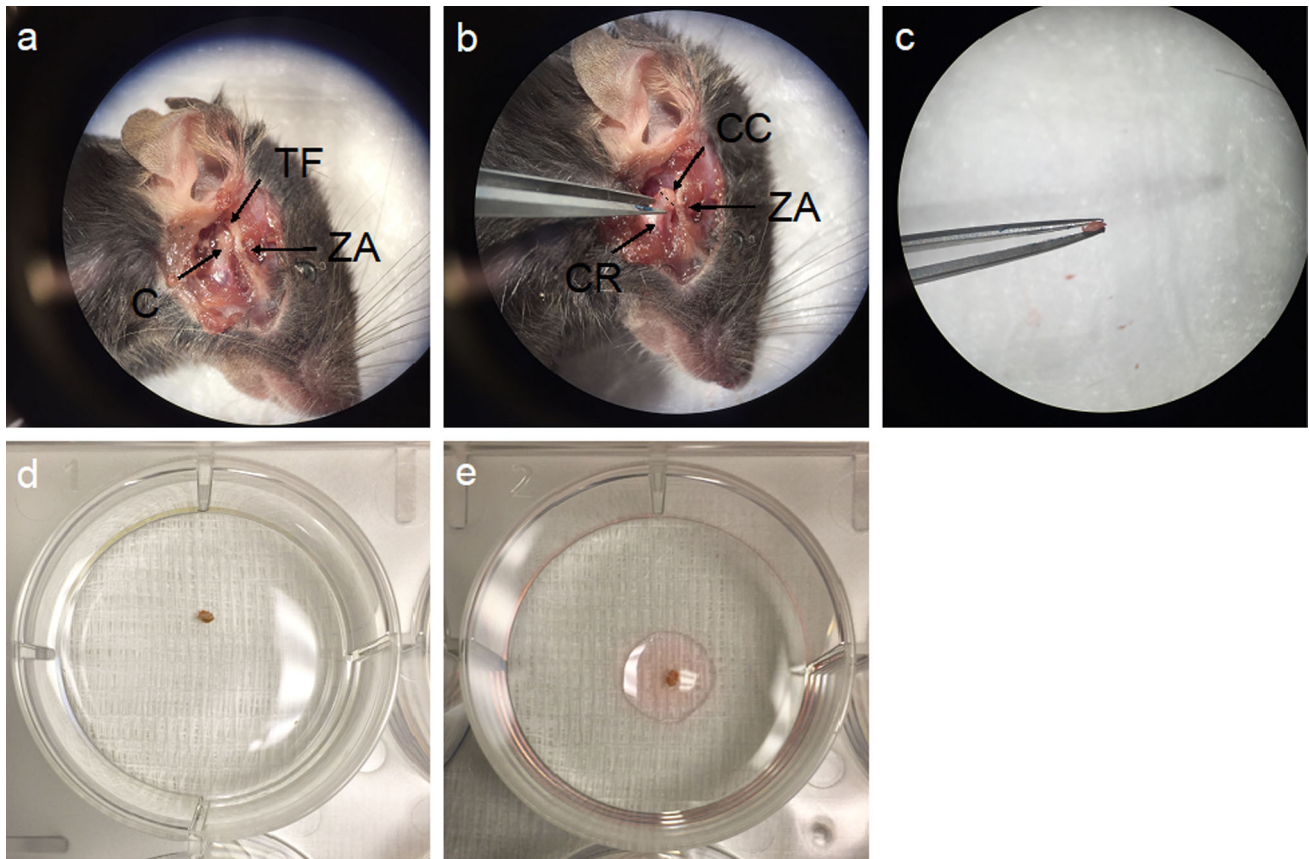
TMJs were removed in a sterile manner and condyles were explanted ( $n = 4$ ). The fibrocartilage cap was carefully removed from the TMJ condyles, cleaned from surrounding soft tissue and washed using sterile phosphate-buffered saline (PBS). 50  $\mu$ L of growth media, consisting of 900 mL filtered Dulbecco's Modified Eagle medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 10 mL of 10% fetal bovine serum (FBS) (GenDEPOT, Katy, Tx), 10 mL antibiotic, antimycotic solution (10,000 units/mL penicillin, 10 mg/mL streptomycin, 25  $\mu$ g/mL amphotericin B) (Sigma-Aldrich, St. Louis, MO) and 10 mL non-essential amino acids (NEAA) (Millipore, Burlington, MA) were applied, to allow the tissue to adhere to the plate. After 2 h (h) of incubating at 37 °C, 5% CO<sub>2</sub>, 42% humidity, 1.5 mL of DMEM was carefully added to each plate. After a period of 3 days (d) an additional volume of 1.5 mL DMEM was added. When the specimen detached from the plate after 48 h, it was removed under sterile conditions. To remove all detritus of the specimen, the plate was washed gently, using PBS. Then, another 3 mL of fresh DMEM growth medium were added. At a cell confluency of 60–80% cells were transferred onto a 25 cm<sup>2</sup> CO<sub>2</sub>-permeable flask and passaged for further use (Fig. 1a–e).

Flasks of 25 cm<sup>2</sup> size were seeded with 1000–2000 cells per flask and incubated for 7 d. Nuclei were stained using 0.5% crystal violet staining solution (Sigma-Aldrich, St. Louis, MO). Cells gathering at a number of at least 50 cells, were considered to be a colony (Fig. 1c).

### Histology and Immunostaining

Explanted TMJs ( $n = 4$ ) were stored in 10% neutral buffered formalin (EMD Millipore, Burlington, MA), decalcified and finally embedded in paraffin. Hematoxylin and Eosin (HE) staining and Safranin-O staining were performed from 5  $\mu$ m thick deparaffinized histological slides.

For immunostaining, deparaffinized sections were initially blocked for endogenous peroxidase activity and unspecific secondary antibody binding applying Bloxall Endogenous Peroxidase blocking solution (VectorLabs, Burlingame, CA) and subsequently 2.5% goat/horse serum for 20 min each. Sections were incubated in primary antibody solution over night at 4 °C, followed by secondary antibody binding for 1 h at room temperature. Immunohistochemical detection was performed using



**Fig. 1** Harvesting of TMJ condyle cartilage. **a** After removing the muscle layer, TMJ anatomy was exposed, showing the condyle (C) and the temporomandibular fossa (TF), as well as the zygomatic arch (ZA). **b** TMJ was opened and the condyle was liberated by

dissecting adherent muscle and connective tissue. Condyle cartilage (CC) was cut from the condyle ramus (CR). **c** harvested condyle was rinsed **d** and left for attachment in **e** 50  $\mu$ l regular DMEM

Vectastain Elite ABC HRP Kit and ImmPACT DAB kit (both VectorLabs, Burlingame, CA) according to the manufacturer's instructions. Nuclear counterstaining was performed using Hematoxylin QS solution. Primary antibodies used were anti-CD44 (1:100), anti-CD105 (1:100), anti-aggrecan (1:200), anti-collagen-type-I (1:100), anti-Ihh (1:200), anti-PTH1LH (1:50) (all Abcam, Cambridge, UK), anti-Sox9 (1:600, Cell Signaling Technology Inc., Danvers, MA) and anti-collagen-type-II (1:200, SouthernBiotech, Birmingham, AL). Secondary antibodies used for immunofluorescence were goat anti-rat (Texas Red, ThermoFisher Scientific, Waltham, MA) and donkey anti-rabbit (Alexa Fluor 488, Abcam, Cambridge, UK).

### Flow Cytometry (FACS) Analysis

MSPCs (P1) were seeded and expanded after reaching approximately 70% confluency on a 25 cm<sup>2</sup> Flask. MSPCs were harvested at a cell viability rate over 90%. Cells were resuspended in ice-cold staining buffer (BioLegend, San Diego, CA) at a concentration of  $10 \times 10^6$  cells/mL per tube. Primary antibodies used to stain for cluster of

differentiation (CD) MSPC antigens were: Anti-CD44 (FITC, 1:200), anti-Sca-1 (APC/Cy7, 1:80), anti-CD73 (PE/Cy7, 1:160) and anti-CD105 (Pacific Blue, 1:50). Anti-CD34 (APC, 1:20), displaying hematopoietic stem cells, was chosen for negative control. Stained cells were stored on ice for 20 min in dark, followed by washing the cells 3 times at  $350 \times g$  for 5 min each. 7-AAD viability staining solution was added at 5  $\mu$ L per tube to exclude non-viable cells from further evaluation. Cells were analyzed on a FACSaria IIU machine (BD Biosciences, San Jose, CA) and sorted into two groups, CD34-/CD44 + /Sca-1 + /CD105 + and CD34-/CD44 + /Sca-1 + /CD105-. Groups differed in whether expressing CD105 or not. Surface antigen antibodies were obtained from BioLegend, San Diego, CA. Data analysis was performed using FlowJo software program (BD Biosciences, San Jose, CA).

### RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) Analysis

RNA isolation from first passage (P1) MSPCs was performed using TRIzol RNA isolation reagent (ThermoFisher

Scientific, Waltham, MA). RNA concentration and quality were determined using spectrophotometry and gel electrophoresis. Synthesis of cDNA was performed, using SMARTScribe Reverse Transcriptase Kit (Takara Bio USA Inc., Mountain View, CA). qRT-PCR was performed using Platinum SYBR Green qPCR Super Mix (Invitrogen, Carlsbad, CA) on a CFX96 Real-Time PCR System (Bio-Rad Laboratories Inc., Hercules, CA) and primers as outlined in Table 1. Measured values were normalized to

cyclophilin B and calculated using the  $2^{-\Delta\Delta Ct}$  method. Gene expression was evaluated in an unsorted sample of harvested MSPCs, serving as a control, and also in preselected CD105<sup>+</sup> and CD105<sup>-</sup> subgroups.

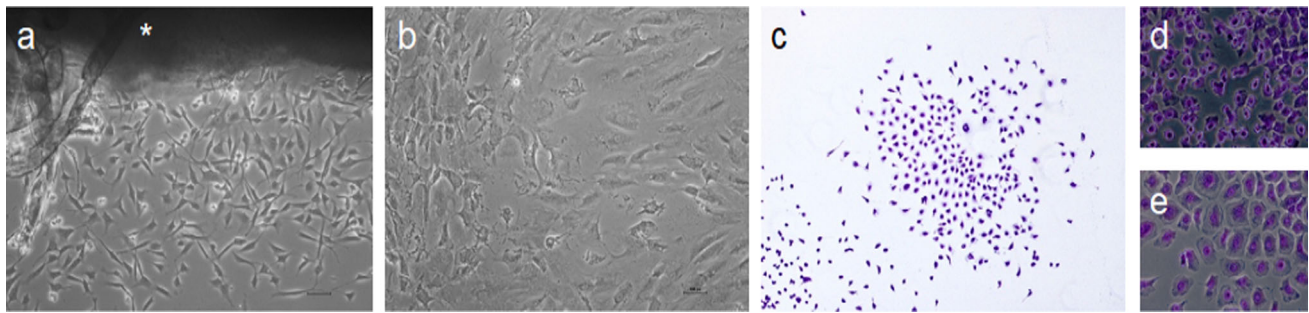
### Intracellular Immunostaining

CD105<sup>±</sup> and unsorted MSPCs (all P1) were seeded at a number of 1000 cells/mL on chamber slides and were left

**Table 1** Listing of all chosen primer pairs (5'-3' direction) for measuring gene expression in mus musculus, using qRT-PCR

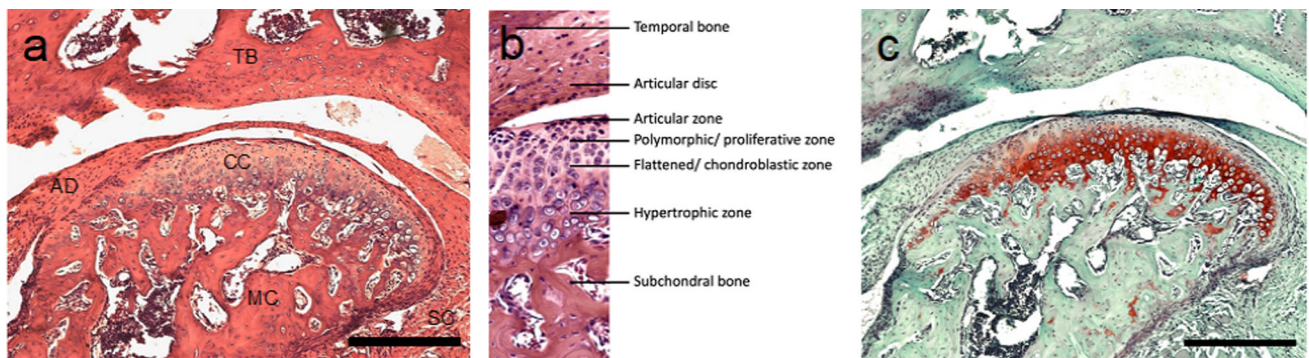
Gene	Primer pair (5'-3' direction)
Cyclophilin B	TTCTTCATAACCACAGTCAAGACC ACCTTCCGTACCACATCCAT
Biglycan (Bgn)	GACAACCGTATCCGCAAAGT GTGGTCCAGGTGAAGTTCGT
Insulin-like growth factor (Igf1)	GACCGAGGGGCTTTACTTC CATCCACAATGCCTGTCTGA
Aggrecan (Acr1)	CCTGCTACTTCATCGACCCC AGATGCTGTTGACTCGAACCT
Collagen Type I (Col1a1)	TGGTTTGGAGAGAGCATGACCGA TTGGTCGATGTAGGCTACGCTGTT
Collagen Type II (Col2a1)	ACCAAATTCCTGTTC ACTGGTAAGTGGGGCAAGAC
Collagen Type X (Col10a1)	CTTTGTGTGCCTTTCAATCG GTGAGGTACAGCCTACCAGTTTT
Decorin (Dcn)	GAGGGAACCTCACTTGGACA TTGTTGTTGTGAAGGTAGACGAC
Lysyl Oxidase (Lox)	ATGCGTTTCGCTGGGCTGTGC CTAATACGGTGAAATTGTGCAGCC
SRY-box 5 (Sox5)	ATGGAAGTCGATGGCAATAAAGT CCACCACATCCGCTAAGCTG
SRY-box 6 (Sox6)	AATGCACAACAAACCTCACTCT AGGTAGACGTATTTCCGGAAGGA
SRY-box 9 (Sox9)	CAGCAAGACTCTGGGCAAG TCCACGAAGGGTCTCTTCTC
Osterix (Osx)	AGCGACCACTTGAGCAAACAT GCGGCTGATTGGCTTCTTCT
Proteoglycan 4 (Prg4)	ACTTCAGCTAAAGAGACACGGAGT GTTTCAGGTGGTTTCCTTGGTTGTAGTAA
Runt related transcription factor 2 (Runx2)	CCACAAGGACAGAGTCAGATTACA TGGCTCAGATAGGAGGGGTA
Transcriptional repressor GATA binding 1 (Trps1)	CAAATCTCAGGCCTGAGTGA GTGAAGAGCTGATATCCTGCAG
Indian hedgehog signaling molecule (Ihh)	GACTCATTGCCTCCCAGAAGT CCAGGTAGTAGGGTCACATTGC
GLI family zinc finger 2 (Gli2)	TGCTGTGGACTAGGAATAGG AACCTTCCGCTCAACCACAA





**Fig. 2** Culturing of explanted condyle, colony forming assay. **a** Cell outgrowth from explanted condyle (\*) at third day. **b** Dense cell layer after 14 d of culturing. Bright-field microscopy (Mag = 10x).

**c** Representative cell colony, consisting of > 50 cells after 7 d of incubation (Mag = 10x). Isolated cells differed in small **d** and large **e** phenotype (Mag = 20x). Scale bar = 50  $\mu$ m



**Fig. 3** Histology of mouse TMJ and distinct zonal organization of the cartilage layer. **a** H&E Staining (Mag = 10x) of the temporal bone (TB), mandibular condyle (MC), articular disc (AD), cartilage of condyle (CC), synovial capsule (SC). **b** Histological organization in

distinct layers of the articular cartilage (Mag = 20x). **c** Safranin O/fast green staining of the condyle (Mag = 10x). Proteoglycans and chondrocytes are stained red. Scale bar = 100  $\mu$ m

for cell attachment overnight. Cell permeabilization and intracellular immunostaining were finally performed according to the protocol reported by Khanna-Jain et al. [30]. Primary collagen-type-1, type-2, aggrecan, Sox9 and Ihh antibodies were used at the same concentrations as previously reported. Proteoglycan 4 primary antibody (Abcam, Cambridge, UK) was used at a concentration of 1:250. Secondary antibodies used were donkey anti-rabbit (Alexa Fluor 488, 1:200) and donkey anti-mouse (Alexa Fluor 568, 1:200) (Abcam, Cambridge, UK). Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI) solution. Primary antibody was omitted in negative controls.

### Multi-Differentiation Potential Assays

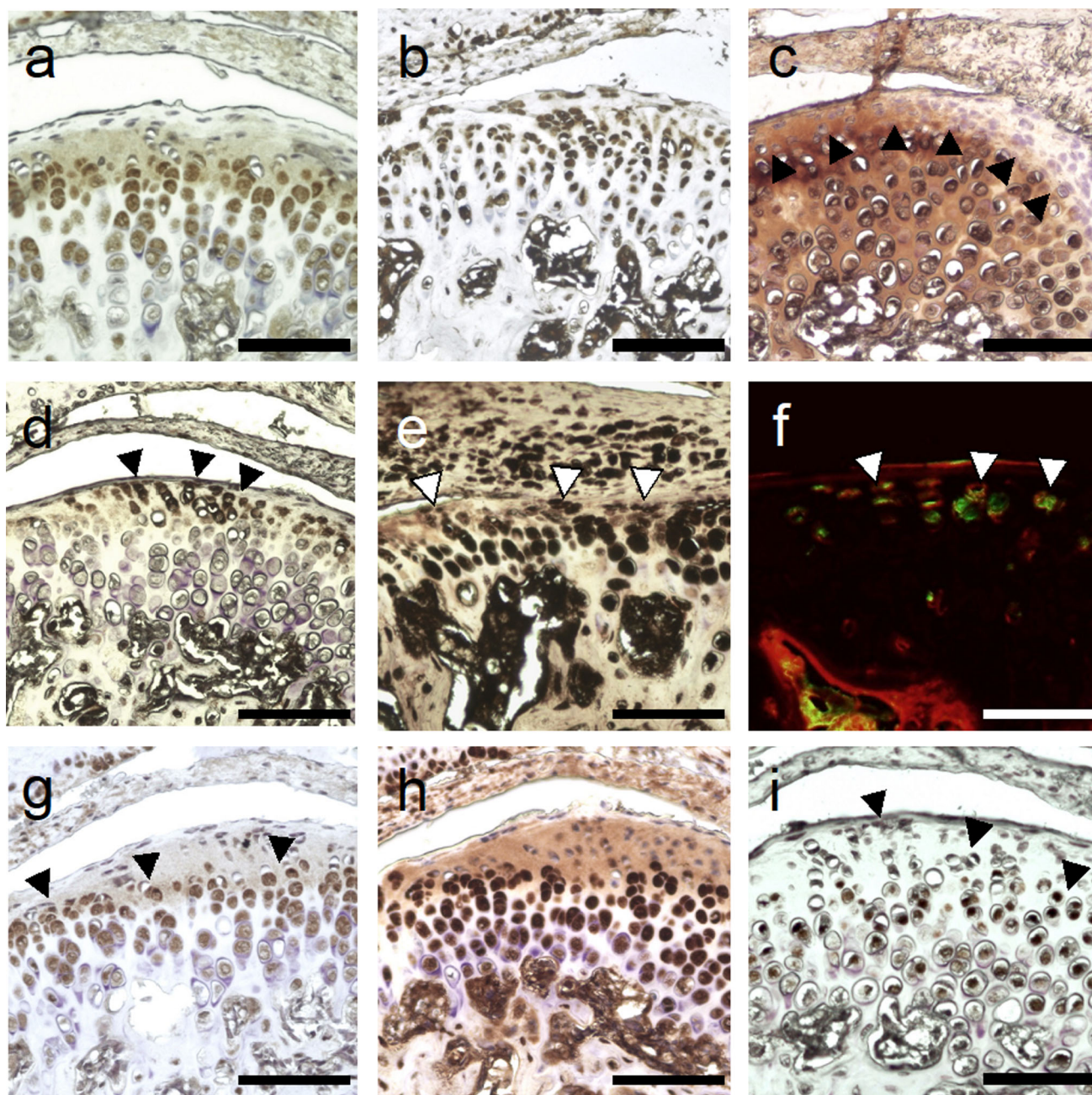
CD105<sup>±</sup> cells were used at 2000 cells per 25 cm<sup>2</sup> flask and cultured for 7 d. Osteogenic and adipogenic media were produced as described elsewhere [8]. Chondrogenic media was obtained by adding dexamethasone (100 nM), 50  $\mu$ g/mL of ascorbic acid, L-proline (40  $\mu$ g/mL) (Sigma-Aldrich, St. Louis, MO), sodium pyruvate (2 mM) (Gibco, Grand Island, NY), 1% Insulin-Transferrin-Selenium (ITS)

solution (Corning, Corning, NY) and TGF- $\beta$ 1 at 10 ng/mL (Invitrogen, Carlsbad, CA). After 7 d of cell growth, media was changed to either chondrogenic, osteogenic or adipogenic media and further incubated for 4 weeks. Media was changed every other day. Staining protocols were carried out as described elsewhere [7], using alcian blue, alizarin Red S and oil Red O (Thermo Fisher Scientific, Waltham, MA). Five flasks each, were evaluated for differentiation capacity of CD105<sup>±</sup> cells and each lineage was separated from unstained cells using ImageJ software setting the appropriate threshold level, leading to the proportionate area of differentiated cells.

### Statistical Analysis

Statistical analysis was performed, using SPSS software (IBM, Armonk, NY). T-Test or Mann-Whitney-U-Test for independent samples was carried out. For evaluation of intracellular immunostaining, results were conservatively estimated using Kruskal-Wallis-Test. Level of significance was set to  $p < 0.05$  for all statistical testing. Results are displayed as mean  $\pm$  standard deviation (SD). Level of significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .





**Fig. 4** Immunohistochemistry/Immunofluorescence of TMJ condyle's cartilage (Mag = 20x, scale bar = 100  $\mu$ m). Zonal distribution of aggrecan **a**, collagen type 1 **b**. Collagen type 2 **c** distribution defines border of upper localized proliferative zone. CD44 positive **d**,

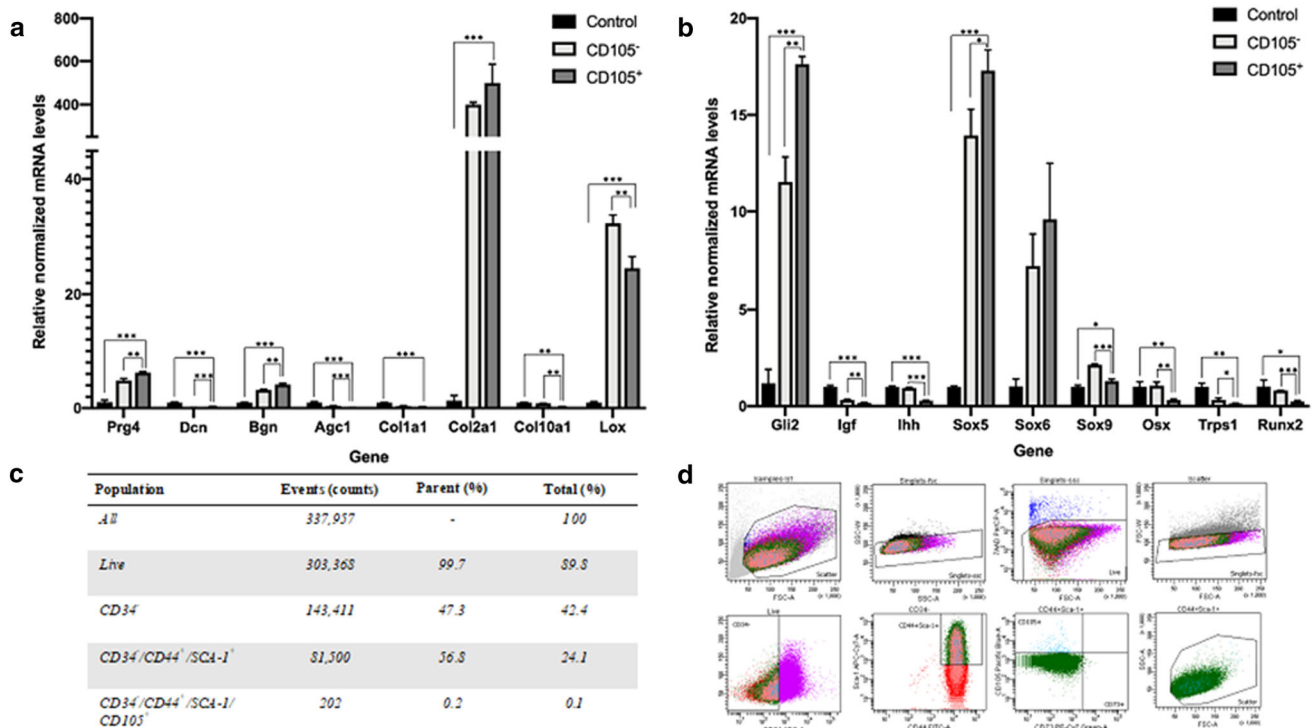
CD105 positive **e**, Ihh positive **g**, PTHLH positive **h**, and Sox9 positive **i** cells in the TMJ condyle of 8-weeks-old mice. CD44 (red)/CD105 (green) positive **f** cells within the proliferative cartilage zone

## Results

### Colony-Forming Assay

Cell escape from the explant ( $n = 4$ ) was observed after 3 d of culturing (Fig. 2a) showing the typical spindle-shape of MSCs. Violet nuclear staining showed the formation of MSCP colonies (> 50 cells/colony) over all the 25  $\text{cm}^2$

plate after 7 d of incubation. Colony forming ability of the harvested cells revealed clonogenicity of the isolated stem cells (Fig. 2b,c). Cells differed in phenotype and were observed at small (Fig. 2d) and large cell (Fig. 2e) size. Cultured CD105<sup>+</sup> and CD105<sup>-</sup> cells showed similar proliferation rates and displayed only homogenous small cell phenotype.



**Fig. 5** qRT-PCR analysis. **a** Gene expression of genes relevant for building up the extracellular matrix of fibrocartilage. **b** genes relevant for stimulation of skeletogenesis, and cell differentiation. All gene expression is normalized to cyclophilin B expression. All gene expression of CD105<sup>+</sup> and CD105<sup>-</sup> cells is displayed relative to gene

expression of unsorted cells (control). Error are means ± SD. **c** Flow cytometry analysis of MSCs. **d** Ratio and gating strategy of detected antigens labeled within the sample. %Total gives the ratio of antigens within the whole sample, while %Parent outlines the ratio of each antigen-subgroup

### Histology and Immunostaining

Organization of the condyle articular cartilage in 4 distinct layers was observed (Fig. 3a–c). The proliferative zone was identified through immunohistochemical analysis, revealing absence of collagen type 2, but positive Sox9 antigen staining (Fig. 4b, i). CD44 positive MSCs were localized in the proliferative zone (Fig. 4d). Collagen Type 2 and aggrecan were mainly distributed in the chondroblastic zone (Fig. 4a, c). CD44 positivity decreased dramatically in deeper cartilage layers towards subchondral bone. CD105 presence was observed over all cartilage layers (Fig. 4e). Double immunofluorescence staining certified presence of CD44 and CD105 positive cells in the proliferative zone (Fig. 4f). Proliferative zone and chondroblastic zone were also found to be rich in Ihh, and PTHLH, and Sox9 (Fig. 4g–i).

### FACS Analysis

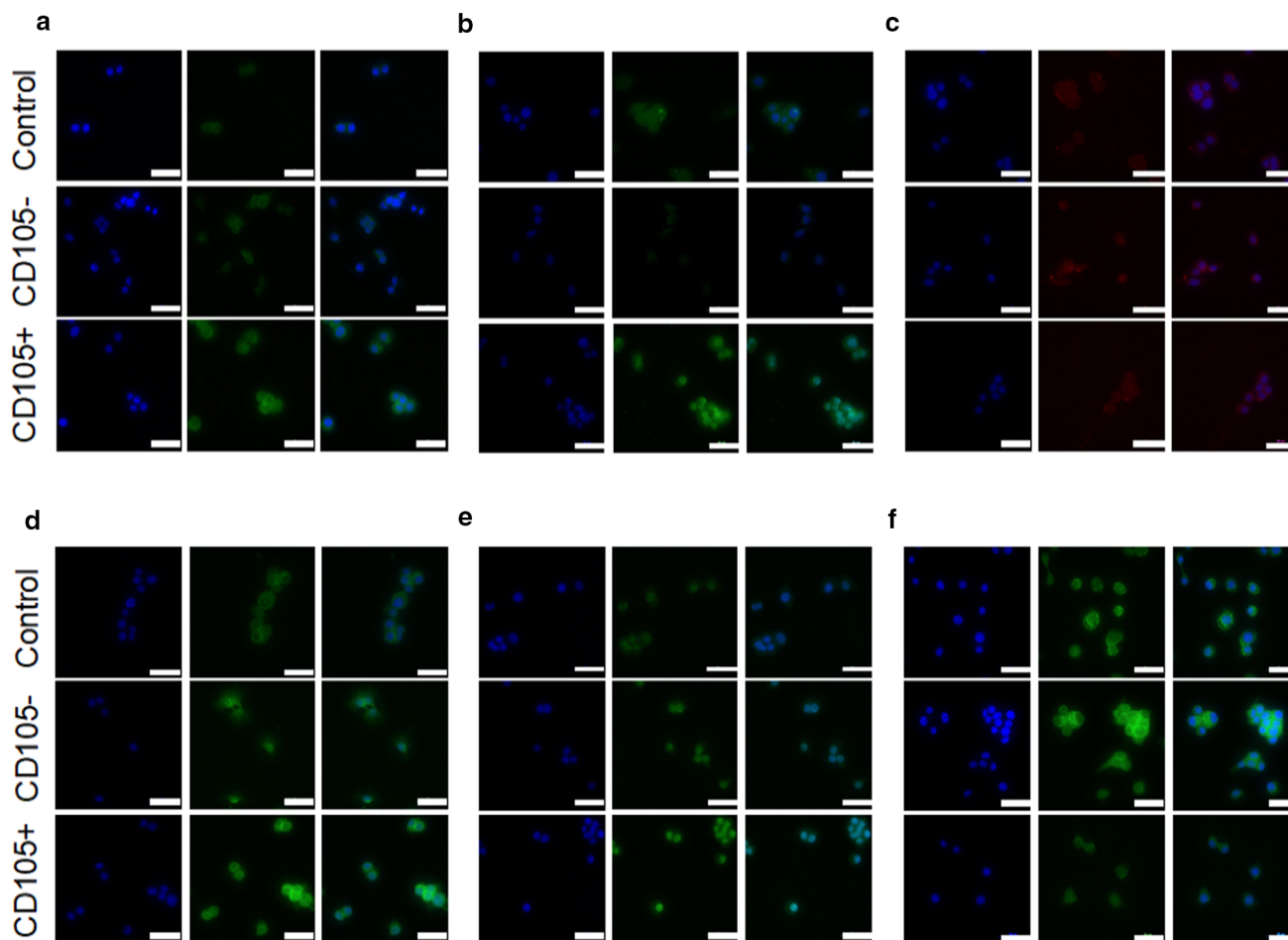
It was found that 42.4% of isolated MSCs (P1) were CD34 negative (–). CD34<sup>-</sup> cell population was further screened for expressing both CD44 and Sca-1, which was found in 56.8% of the sample. Only 0.2% of CD34<sup>-</sup>/CD44<sup>+</sup>/Sca-1<sup>+</sup> cells expressed CD105 antigen and 0.1% of

the non-preserved sample. CD73<sup>+</sup> expression was not observed within the isolated cell population. CD34<sup>-</sup>/CD44<sup>+</sup>/Sca-1<sup>+</sup>/CD105<sup>-</sup> and CD34<sup>-</sup>/CD44<sup>+</sup>/Sca-1<sup>+</sup>/CD105<sup>+</sup> cell populations were separately sorted and subsequently cultured for further qRT-PCR analysis (Fig. 5c).

### RNA Isolation and qRT-PCR Analysis

Comparing CD105<sup>+</sup> and CD105<sup>-</sup> MSC subgroups, gene expression of Bgn, Prg4 ( $p < 0.01$ ) and Dcn ( $p < 0.001$ ) were found significantly higher in CD105<sup>+</sup> cells (Fig. 5a). Agc1 expression was higher in the CD105<sup>-</sup> cells (Fig. 5a). Gene expression of Col2a1 was significantly higher in CD105<sup>+</sup> cells compared to the unsorted sample ( $p < 0.001$ ), Col1a1 did not differ significantly between CD105<sup>+</sup> and negative cells (Fig. 5a). Significant Col2a1 upregulation correlated with increased Sox9 levels of CD105<sup>+</sup> cells, compared to the unsorted control sample. Col10a1 gene was found to be significantly increased ( $p < 0.01$ ) in the unsorted and CD105<sup>-</sup> sample, compared to CD105<sup>+</sup> cells (Fig. 5a). Sox 5, 6, 9, Runx2, Transcriptional repressor GATA binding 1 (Trps1) and Gli2 were observed at significantly increased levels ( $p < 0.001$ ) in the CD105<sup>+</sup> sample (Fig. 5b). Sox5 and 9 were significantly expressed ( $p < 0.001$  and  $p < 0.05$ ) in preselected cells





**Fig. 6** Intracellular immunostaining (Mag = 40x, scale bar 50  $\mu$ m). Protein levels of collagen type 1 **a**, collagen type 2 **b**, aggrecan **c**, proteoglycan 4 **d**, Sox9 **e** and Ihh **f** were quantified by intracellular staining

compared to the unsorted control sample. *Osx* ( $p < 0.01$ ), *Runx2* ( $p < 0.05$ ), *Ihh* ( $p < 0.001$ ), *Trps1* ( $p < 0.01$ ) relevant for osteogenesis were found to be significantly downregulated in  $CD105^+$  cells (Fig. 5b).

### Intracellular Protein Expression

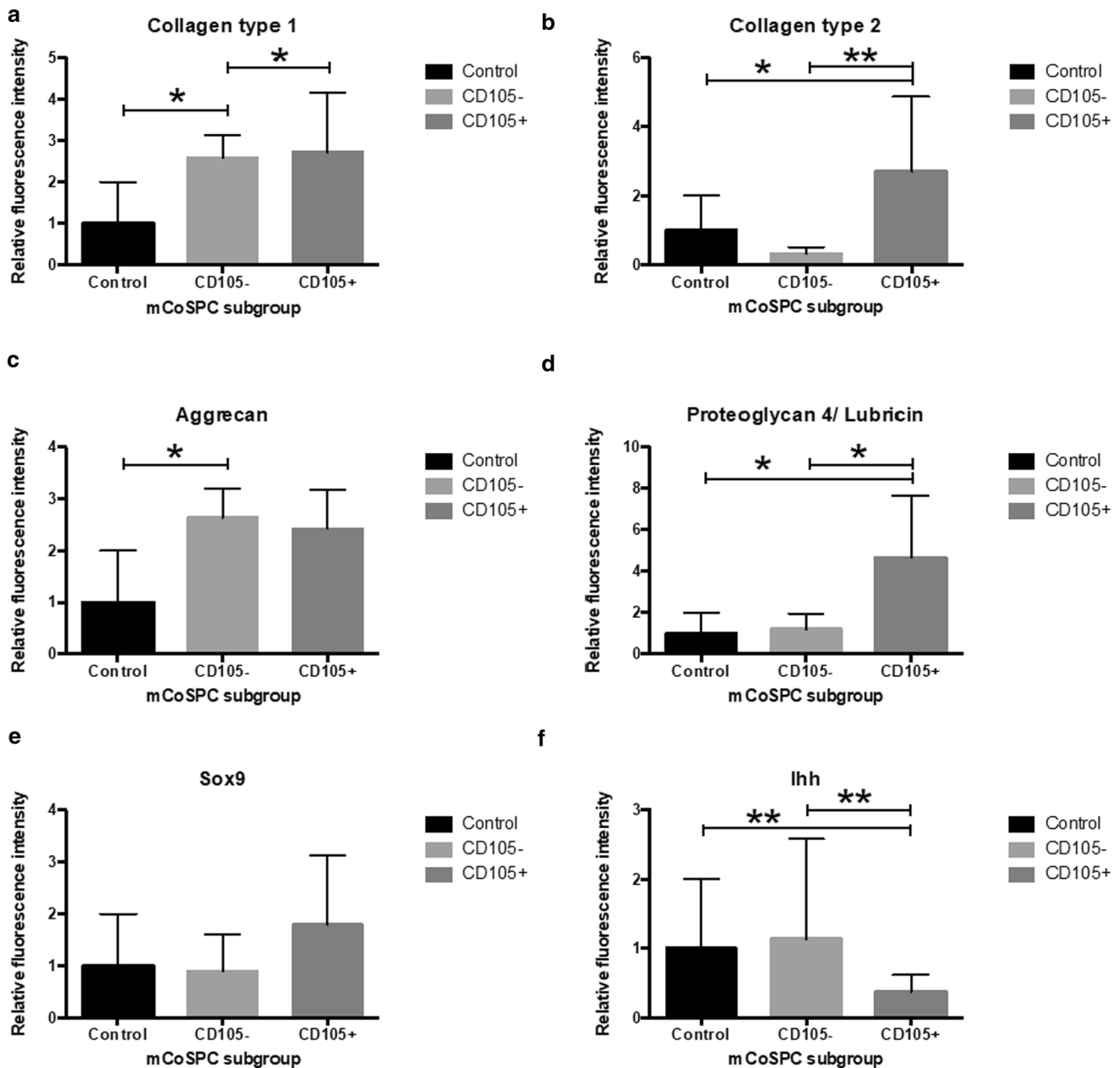
Significantly higher intracellular collagen type 1 was observed comparing  $CD105^+$  and  $CD105^-$  to the control sample ( $p < 0.05$ ) (Fig. 6a, 7a). Collagen type 2 protein expression aligned with qPCR findings of the *Col2a1* gene. Collagen type 2 protein levels of  $CD105^+$  cells were found at significantly increased levels compared to  $CD105^-$  cells ( $p < 0.001$ ) and the control ( $p < 0.05$ ) (Fig. 6b, 7b). Levels of aggrecan were found approximately two times higher in  $CD105^+$  cohorts compared to the control, but only levels of aggrecan were found significantly elevated for  $CD105^-$  group ( $p < 0.05$ ) (Fig. 6c, 7c). Between  $CD105^{\pm}$  groups no significant difference in aggrecan protein expression was found. In accordance to the qPCR

findings of proteoglycan 4, intracellular staining revealed a significant increase ( $p < 0.05$ ) in  $CD105^+$  cells (Fig. 6d, 7d). Intranuclear Sox9 levels did not differ significantly between groups (Fig. 6e, 7e), while *Ihh* protein expression was significantly increased in the control and  $CD105^-$  cohort compared to  $CD105^+$  cells, in line with the previously described gene expression (Fig. 6f, 7f).

### Multi-Differentiation Potential Assay

Alcian blue staining visualized extracellular glycosaminoglycan secretion of differentiated cells. Extracellular free calcium deposits within the colonies were visualized by deep red and bright orange ECM staining, after applying alizarin red staining solution. Adipogenic differentiation was observed, and appeared as red vacuoles and reddish colony staining as oil red O binds to triglycerides of fatty acids.  $CD105^+$  cells were found to show stronger chondrogenic differentiation than  $CD105^-$  cells ( $p < 0.05$ ) after 4 weeks (Fig. 8a). In contrast,  $CD105^-$  cells trended to





**Fig. 7** Bar graphs (mean ± SD) visualize relative protein expression of CD105<sup>±</sup> cohorts as measured fluorescence intensity signal to the control

show higher osteogenic and adipogenic differentiation without reaching a significant level (Fig. 8b,c).

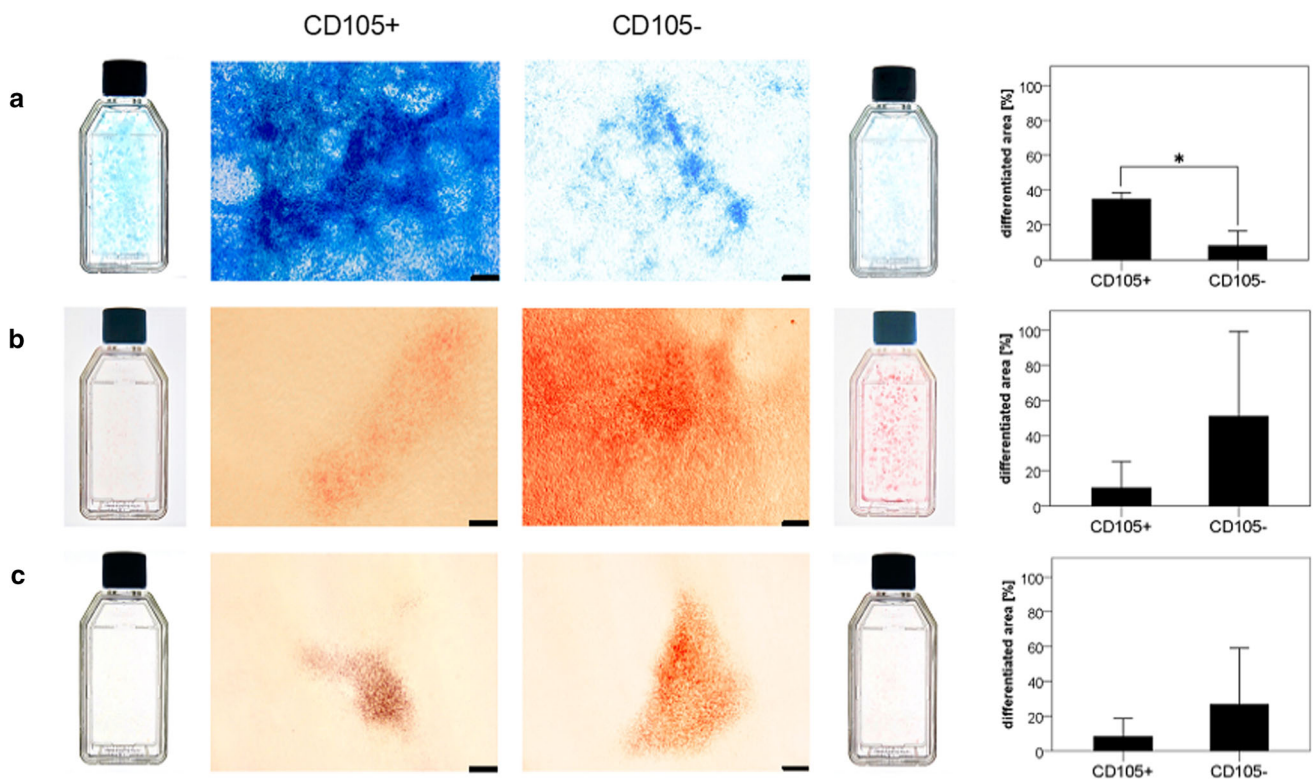
**Discussion**

Application of MSCs is widely studied for regeneration and/or repair of many tissues, including fibrocartilage. Numerous authors agree that autologous cells would be the best choice for restoring damaged tissue. However, it appears that in situ cells are also required to maintain organ hemostasis [31–35]. Underlining this fact, it was found that

tissue resident stem cells possess increased organ hemostasis ability [29].

Gamer et al. found that fibrocartilage of the mouse knee harbors different subpopulations of MSCs [7]. Moreover, CD105<sup>+</sup> MSCs, derived from the synovium or adipose tissue, are reported to have increased chondrogenic differentiation potential, due to TGF-β/ Smad2 signaling, as CD105 is an integral part of the TGF-β receptor complex [26–28].

We hypothesized that the triad of CD44, Sca-1 and CD105 and the lack of CD34 antigen identify MSCs of the TMJ’s fibrocartilage, similar to fibrocartilage of the



**Fig. 8** Multi-differentiation potential assay (Mag = 4x, scale bar = 200  $\mu$ m). Bar graphs (mean  $\pm$  SD) visualize area [%] found to be differentiated after 4 weeks of cell differentiation, comparing

CD105<sup>+</sup> and CD105<sup>-</sup> cell subgroups. **a** Chondrogenic differentiation, **b** osteogenic differentiation and **c** adipogenic differentiation

mouse knee joint [7]. Immunostaining of the TMJ revealed CD44<sup>+</sup> and CD105<sup>+</sup> MSCs in the proliferative zone. After harvesting the TMJ fibrocartilage cap, clonogenicity, tissue outgrowth and multi-differentiation potential were investigated to show that MSCs were isolated as a first step and, subsequently, verified sorting for CD44, Sca-1, CD73 and CD105, using FACS as reported by Gamer et al. [7].

As no single MSC marker exists, the triad of CD105, CD73 and CD90 and the lack of CD34 are proposed to identify mesenchymal stem cells in humans as shown by Dominici et al. [36]. Mouse TMJ MSCs lacked specific surface antigen markers that human species demonstrate. Stem cells harvested from the mouse condyle were found not to express CD73 (0%) in CD34<sup>-</sup> subgroup; and, almost no CD105 (0.2%), similar to the findings reported by Gamer et al., who identified and characterized adult mouse meniscus stem/progenitor cells [7]. Low expression rates of CD73 is consistent with the idea that surface antigen markers differ between species; CD73 expression is also negative in mouse MSCs [37, 38].

Gene expression of CD105<sup>-</sup> preselected cells was defined through increased Col10a1, Col1a1, Sox9 and Ihh levels, together with elevated levels of Osx, Runx2, Ihh, Trps1 genes, which are related to chondrocyte maturation,

subsequently leading to osteoblastic differentiation and endochondral ossification as shown elsewhere [12, 39–44]. Elevated gene expression of Runx2 correlated with findings of Runx2 upregulation in chondrocytes populating the OA affected joint [45]. Hence, Runx2 upregulation is accepted as an important player in cartilage maturation and in the pathogenesis of OA [45]. Those findings may validate the assumption, that CD105<sup>-</sup> cells were identified as matured MSCs of the chondroblastic or hypertrophic zone, expressing osteogenesis driving genes at a higher level while in the process of endochondral ossification.

In contrast, CD105<sup>+</sup> antigen is associated with an earlier MSC state, due to significant downregulation of Osx, Runx2, Ihh, Trps1 and Col10 gene expression and considerable high expression of Col2a1 and Prg4, compared to the control (unsorted MSCs) [4].

That CD105<sup>+</sup> subpopulation is able to deliver crucial proteins of the ECM and for physiological joint function at increased levels was shown by significant higher intracellular immunostaining of collagen type 1, type 2 and proteoglycan 4, as well as, due to increased intensity of alcian blue staining, probably correlating with higher secretion of glycosaminoglycans related to increased proteoglycan 4 secretion and higher expression of Bgn and Dcn gene. Considerable higher levels of the chondrogenic trio, Sox5,

6 and 9, on gene level and increased chondrogenic turnover potential deliver promising features for investigating further improvements in restoring fibrocartilage. From a preliminary in vivo study conducted by Guastaldi et al. we have promising findings that MSPCs aid in the restoration process of damaged TMJ cartilage [46]. Furthermore administered MSPCs to the damaged TMJ did not lead to increased inflammation vs. a sham group in which only a defect to the TMJ was created, without using any cell therapy [46]. This finding is in accordance to reports that MSCs have immunosuppressive capacities, like inhibition of T-Cell proliferation and are reported to decrease initiate acute immune reaction, when being transplanted [47–49]. Those abilities make the application of MSPCs favorable in tissue engineering and regenerative medicine.

In conclusion, fibrocartilage of the mouse TMJ is populated by a CD105<sup>+</sup> MSPC subgroup, a finding that is consistent with previous investigations of mesenchymal tissue [7, 26–28]. The characteristics of TMJ-resident CD105<sup>+</sup> MSPCs suggest that this subpopulation might be a favorable source of tissue resident stem cells for future improvements in stem cell-based therapies. To fully understand the role of CD105<sup>+</sup> MSPCs and their behavior in maintaining and/or recovering hemostasis of the TMJ fibrocartilage, further studies will need to investigate if this minor population can still be found in mice older than 8 weeks that have undergone a TMJ stress/ alteration process. If isolated CD105<sup>+</sup> MSPCs are also found to possess homing ability, using fluorescent protein-transfected cultures, a CD105<sup>+</sup> stem-cell-based injection therapy could be a possible next step for delivering CD105<sup>+</sup> MSPCs to the OA affected joint.

**Acknowledgements** This study was funded in part from grants: MGH-Department of Oral and Maxillofacial Surgery Education Research Fund (Boston, MA), Jean Foundation (NH), Fondation Bertarelli (Gstaad, Switzerland) and MGH-Walter C. Guralnick Fund (Haseotes-Bentas Foundation, Boston, MA).

**Authors' Contributions** JRT contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data. YJ contributed to the acquisition of data, analysis and interpretation of data. MLM contributed to the acquisition of data, analysis and interpretation of data. VR contributed to analysis and interpretation of data. MJT contributed to the conception and design of the study. FPSG contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data. JRT and MLM drafted the manuscript. YJ, VR, MJT and FPSG revised the manuscript. All authors contributed to final approval of the version to be submitted.

#### Declarations

**Conflict of interest** The authors report no declarations of interest.

## References

1. U.S. Department of Health and Human Services NIOH, National Institute of Mental Health. TMJ Disorders. Bethesda, MD: U.S. Government Printing Office.; 2017. Report No.: NIH Publication No. 17-3487
2. McNeill C, Mohl ND, Rugh JD, Tanaka TT (1990) Temporomandibular disorders: diagnosis, management, education, and research. *J Am Dent Assoc* 120(3):253
3. Robinson J, O'Brien A, Chen J, Wadhwa S (2015) Progenitor cells of the mandibular condylar cartilage. *Curr Mol Biol Rep* 1(3):110–114
4. Dy P, Wang W, Bhattaram P, Wang Q, Wang L, Ballock RT et al (2012) Sox9 directs hypertrophic maturation and blocks osteoblast differentiation of growth plate chondrocytes. *Dev Cell* 22(3):597–609
5. Tanaka E, Detamore MS, Mercuri LG (2008) Degenerative disorders of the temporomandibular joint: etiology, diagnosis, and treatment. *J Dent Res* 87(4):296–307
6. Wadhwa S, Kapila S (2008) TMJ disorders: future innovations in diagnostics and therapeutics. *J Dent Educ* 72(8):930–947
7. Gamer LW, Shi RR, Gendelman A, Mathewson D, Gamer J, Rosen V (2017) Identification and characterization of adult mouse meniscus stem/progenitor cells. *Connect Tissue Res* 58(3–4):238–245
8. Zhang W, Chen J, Zhang S, Ouyang HW (2012) Inhibitory function of parathyroid hormone-related protein on chondrocyte hypertrophy: the implication for articular cartilage repair. *Arthritis Res Ther* 14(4):221
9. Yang J, Andre P, Ye L, Yang YZ (2015) The Hedgehog signalling pathway in bone formation. *Int J Oral Sci* 7(2):73–79
10. Suzuki A, Iwata J (2016) Mouse genetic models for temporomandibular joint development and disorders. *Oral Dis* 22(1):33–38
11. Shibukawa Y, Young B, Wu C, Yamada S, Long F, Pacifici M et al (2007) Temporomandibular joint formation and condyle growth require Indian hedgehog signaling. *Dev Dyn* 236(2):426–434
12. Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M et al (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* 372(6506):525–530
13. Ikeda T, Kamekura S, Mabuchi A, Kou I, Seki S, Takato T et al (2004) The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. *Arthritis Rheum* 50(11):3561–3573
14. Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW et al (1997) SOX9 directly regulates the type-II collagen gene. *Nat Genet* 16(2):174–178
15. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrughe B (1997) SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. *Mol Cell Biol* 17(4):2336–2346
16. Liu Y, Li H, Tanaka K, Tsumaki N, Yamada Y (2000) Identification of an enhancer sequence within the first intron required for cartilage-specific transcription of the alpha2(XI) collagen gene. *J Biol Chem* 275(17):12712–12718
17. Sekiya I, Tsuji K, Koopman P, Watanabe H, Yamada Y, Shinomiya K et al (2000) SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6. *J Biol Chem* 275(15):10738–10744
18. Zhang P, Jimenez SA, Stokes DG (2003) Regulation of human COL9A1 gene expression. Activation of the proximal promoter region by SOX9. *J Biol Chem* 278(1):117–123



19. Yahara Y, Takemori H, Okada M, Kosai A, Yamashita A, Kobayashi T et al (2016) Pterostatin B prevents chondrocyte hypertrophy and osteoarthritis in mice by inhibiting *Sik3*. *Nat Commun* 7:10959
20. Barbara NP, Wrana JL, Letarte M (1999) Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-beta superfamily. *J Biol Chem* 274(2):584–594
21. Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J et al (1992) Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. *J Biol Chem* 267(27):19027–19030
22. Quackenbush EJ, Letarte M (1985) Identification of several cell surface proteins of non-T, non-B acute lymphoblastic leukemia by using monoclonal antibodies. *J Immunol* 134(2):1276–1285
23. Gougos A, Letarte M (1990) Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. *J Biol Chem* 265(15):8361–8364
24. Lastres P, Bellon T, Cabanas C, Sanchez-Madrid F, Acevedo A, Gougos A et al (1992) Regulated expression on human macrophages of endoglin, an Arg-Gly-Asp-containing surface antigen. *Eur J Immunol* 22(2):393–397
25. Pierelli L, Bonanno G, Rutella S, Marone M, Scambia G, Leone G (2001) CD105 (endoglin) expression on hematopoietic stem/progenitor cells. *Leuk Lymphoma* 42(6):1195–1206
26. Fan W, Li J, Wang Y, Pan J, Li S, Zhu L et al (2016) CD105 promotes chondrogenesis of synovium-derived mesenchymal stem cells through *Smad2* signaling. *Biochem Biophys Res Commun* 474(2):338–344
27. Chang CB, Han SA, Kim EM, Lee S, Seong SC, Lee MC (2013) Chondrogenic potentials of human synovium-derived cells sorted by specific surface markers. *Osteoarthr Cartil* 21(1):190–199
28. Jiang T, Liu W, Lv X, Sun H, Zhang L, Liu Y et al (2010) Potent in vitro chondrogenesis of CD105 enriched human adipose-derived stem cells. *Biomaterials* 31(13):3564–3571
29. Goichberg P (2016) Current understanding of the pathways involved in adult stem and progenitor cell migration for tissue homeostasis and repair. *Stem Cell Rev* 12(4):421–437
30. Khanna-Jain R, Mannerstrom B, Vuorinen A, Sandor GK, Suuronen R, Miettinen S (2012) Osteogenic differentiation of human dental pulp stem cells on beta-tricalcium phosphate/poly(l-lactic acid/caprolactone) three-dimensional scaffolds. *J Tissue Eng* 3(1):2041731412467998
31. Qu D, Zhu JP, Childs HR, Lu HH (2019) Nanofiber-based transforming growth factor-beta3 release induces fibrochondrogenic differentiation of stem cells. *Acta Biomater* 93:111–122
32. Fang D, Jin P, Huang Q, Yang Y, Zhao J, Zheng L (2019) Platelet-rich plasma promotes the regeneration of cartilage engineered by mesenchymal stem cells and collagen hydrogel via the TGF-beta/SMAD signaling pathway. *J Cell Physiol* 234(9):15627–15637
33. Gupte MJ, Swanson WB, Hu J, Jin X, Ma H, Zhang Z et al (2018) Pore size directs bone marrow stromal cell fate and tissue regeneration in nanofibrous macroporous scaffolds by mediating vascularization. *Acta Biomater* 82:1–11
34. Jacer S, Shafaei H, Soleimani RJ (2018) An investigation on the regenerative effects of intra articular injection of co-cultured adipose derived stem cells with chondron for treatment of induced osteoarthritis. *Adv Pharm Bull* 8(2):297–306
35. de Souza TR, Takamori ER, Menezes K, Carias RBV, Dutra CLM, de Freitas AM et al (2018) Temporomandibular joint regeneration: proposal of a novel treatment for condylar resorption after orthognathic surgery using transplantation of autologous nasal septum chondrocytes, and the first human case report. *Stem Cell Res Ther* 9(1):94
36. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 8(4):315–317
37. Anderson P, Carrillo-Galvez AB, Garcia-Perez A, Cobo M, Martin F (2013) CD105 (endoglin)-negative murine mesenchymal stromal cells define a new multipotent subpopulation with distinct differentiation and immunomodulatory capacities. *PLoS ONE* 8(10):e76979
38. Boxall SA, Jones E (2012) Markers for characterization of bone marrow multipotential stromal cells. *Stem Cells Int*
39. Fu H, Doll B, McNelis T, Hollinger JO (2007) Osteoblast differentiation in vitro and in vivo promoted by *Osterix*. *J Biomed Mater Res A* 83(3):770–778
40. Tu Q, Valverde P, Chen J (2006) *Osterix* enhances proliferation and osteogenic potential of bone marrow stromal cells. *Biochem Biophys Res Commun* 341(4):1257–1265
41. Komori T (2017) Roles of *Runx2* in Skeletal Development. *Adv Exp Med Biol* 962:83–93
42. Kronenberg HM (2003) Developmental regulation of the growth plate. *Nature* 423(6937):332–336
43. Suemoto H, Muragaki Y, Nishioka K, Sato M, Ooshima A, Itoh S et al (2007) *Trps1* regulates proliferation and apoptosis of chondrocytes through *Stat3* signaling. *Dev Biol* 312(2):572–581
44. Chung UI, Schipani E, McMahon AP, Kronenberg HM (2001) Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. *J Clin Invest* 107(3):295–304
45. Komori T (2018) *Runx2*, an inducer of osteoblast and chondrocyte differentiation. *Histochem Cell Biol* 149(4):313–323
46. Guastaldi FPS, Hakim MA (2021) Are stem cells useful in the regeneration and repair of cartilage defects in the TMJ condyle? An *In Vivo* Study. *J Dent Oral Disord* 7(2):1159
47. Kim N, Cho SG (2016) Overcoming immunoregulatory plasticity of mesenchymal stem cells for accelerated clinical applications. *Int J Hematol* 103(2):129–137
48. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P et al (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or non-specific mitogenic stimuli. *Blood* 99(10):3838–3843
49. Le Blanc K, Ringden O (2007) Immunomodulation by mesenchymal stem cells and clinical experience. *J Intern Med* 262(5):509–525

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.