Formation of Cartilage and Synovial Tissue by Human Gingival Stem Cells

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Human gingival stem cells (HGSCs) can be easily isolated and manipulated in culture to investigate their multipotency. Osteogenic differentiation of bone-marrow-derived mesenchymal stem/stromal cells has been well documented. HGSCs derive from neural crests, however, and their differentiation capacity has not been fully established. The aim of the present report was to investigate whether HGSCs can be induced to differentiate to osteoblasts and chondrocytes. HGSCs were cultured either in a classical monolayer culture or in three-dimensional floating micromass pellet cultures in specific differentiation media. HGSC differentiation to osteogenic and chondrogenic lineages was determined by protein and gene expression analyses, and also by specific staining of cells and tissue pellets. HGSCs cultured in osteogenic differentiation medium showed induction of Runx2, alkaline phosphatase (ALPL), and osterix expression, and subsequently formed mineralized nodules consistent with osteogenic differentiation. Interestingly, HGSC micromass cultures maintained in chondrogenic differentiation medium showed SOX9-dependent differentiation to both chondrocyte and synovocyte lineages. Chondrocytes at different stages of differentiation were identified by gene expression profiles and by histochemical and immunohistochemical staining. In 3-week-old cultures, peripheral cells in the micromass cultures organized in layers of cuboidal cells with villous structures facing the medium. These cells were strongly positive for cadherin-11, a marker of synovocytes. In summary, the findings indicate that HGSCs have the capacity to differentiate to osteogenic, chondrogenic, and synovocyte lineages. Therefore, HGSCs could serve as an alternative source for stem cell therapies in regenerative medicine for patients with cartilage and joint destructions, such as observed in rheumatoid arthritis.

Introduction

RECONSTRUCTION AND REGENERATION of lost skeletal substance and joint structures that are composed of multiple differentiated tissues is a major challenge in orthopedic surgery and regenerative medicine. In this respect, stem cell therapy could offer a new approach for treatment. One promising approach of stem cell therapy is to induce in stem cells *in vitro* the developmental processes that generate different tissue components. These preconditioned cells or tissue constructs would then be used *in vivo* to generate appropriate functional tissues [1].

Skeleton anlagen constitute the primordia of the bone and cartilage through two successive processes, namely, con-

densation of undifferentiated mesenchymal cells and an early differentiation into chondrocytes allowing the formation of a cartilage template. These primordia can lead to two skeleton derivatives, specifically growth plate and joint structures.

In growth plates in axial and appendicular bones, cells mature into hypertrophic chondrocytes leading to cartilage mineralization by a process called endochondral ossification [2,3]. Other bone anlagen mature via intramembranous ossification process that takes place in the majority of the craniofacial skeleton. In this process, undifferentiated mesenchymal cells condense and directly differentiate into osteoblast cells, allowing bone matrix production and mineralization.

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Another fate of these primordia is the generation of joint structures. Synovial joints in limbs contain several distinct components, including the synovial cavity, the articular cartilage, and the synovial membrane [4]. Their morphogenesis occurs through segmentation of the cartilage template. During this process, chondrocytes change their phenotype and become elongated and start to express type I collagen [5,6]. Proliferation of these cells leads to an interzone formation within the cartilage primordium [5]. Then, the interzone cavitates and gives rise to the synovial space [5,6]. This cavity is surrounded by a synovial membrane that is composed of two types of synoviocytes. The first type is derived from macrophage-like cells, while the second type is called fibroblast-like synoviocytes [7]. These latter cells provide nutrients and lubrication for cartilage. Especially, they secrete a high amount of hyaluronic acid (HA). Little is known about commitment of these cells [4], but cadherin-11 is necessary for synovial membrane formation, and is a highly specific marker for fibroblast-like synoviocytes during adulthood [7].

Formation of the cartilaginous template critical for both endochondral ossification and articular cartilage development needs well-coordinated multiple cues, including cell–cell and cell–extracellular matrix (ECM) interactions and growth factors, that act in concert to induce the cells into a specific lineage. Replicating the early processes of articular cartilage development during *in vitro* engineering of cartilage for regenerative therapy would potentially help to optimize the fate of the construct when grafted. Moreover, recreating these developmentally regulated steps *in vitro* facilitates systematical studies about mechanisms of cell commitment and role of master genes during cartilage differentiation in a controlled setting.

To recreate a functional cartilage, the cell source is critical. In this context, bone-marrow-derived mesenchymal stem/stromal cells (BMMSCs) have been the most studied cells. They are able to differentiate into both chondroblasts and osteoblasts, and to form a mineralized matrix through endochondral differentiation *in vitro* and *in vivo* [1,8–10]. However, chondrocytes differentiated from BMMSCs are unstable and tend to follow an endochondral process characterized by type X collagen synthesis and ALPL activity *in vitro* and *in vivo* [11,12]. This indicates that the cells acquired a hypertrophic phenotype and that BMMSCs are more predisposed for osteogenic commitment rather than cartilage formation [13], which may limit their utility for joint therapy.

An alternative source of stem cells with bone/cartilage differentiation potential is the neural crest stem cells. Neural crest cells (NCCs) arise from the border of the neural plate and the non-neural ectoderm [14]. Cranial neural crest cells (CNCCs) are multipotent and can generate both neural and mesenchymal derivatives [15], whereas trunk NCCs do not form skeleton derivatives *in vivo* [16,17]. CNCCs generate facial and oral mesenchymal tissue, such as bone, cartilage, dental pulp, dentin, cementum, and periodontal ligament, as well as certain mesenchymal cells associated with distinct tissue compartments, including myofibroblasts and adipocytes. Facial bone is mainly produced by an intramembranous process although some endochondral ossification may occur in specific areas. These latter include the condylar and coronoid processes. Further, cartilages of the face, such as nasal septum and mandibular condyle, also derive from the neural crests [14]. We hypothesized that the high plasticity during morphogenesis of the CNCCs could be partially recreated *in*

vitro by using postmigratory adult CNCCs. To better understand neural crest cartilage differentiation and to assess utility of CNCCs in cartilage engineering, we used adult stem cells from gingival tissue of oral mucosa. Several studies have shown that neural-crest-derived stem cells persist during adulthood and are multipotent [18]. In particular, recent studies have demonstrated the presence of stem cells in gingival connective tissue and in gingival fibroblast (GF) cultures. These cells express neural crest markers; have self-renewal, multipotency, and immunomodulation properties; and have been used with success in several animal models of cell therapy [19–26].

Materials and Methods

Cell isolation and culture

GF cultures were obtained from human gingival biopsies from three healthy patients with no gingival inflammation. All patients gave their informed consent according to the Helsinki Declaration (1975) and denied having recently taken drugs that could affect connective tissue metabolism. For culture of cells, each biopsy was divided into two identical pieces that were then used to establish cell cultures using the explant or enzymatic digestion with collagenase II (2 mg/mL; Sigma-Aldrich, Oakville, ON, Canada) methods, respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with 50 µg/mL of ascorbic acid, nonessential amino acids, and penicillin/streptomycin (Gibco, Life Technologies, Inc. Burlington, ON, Canada). For colony-forming unit fibroblasts (CFU-F)-enriched cultures, single-cell suspensions obtained by incubation of the primary plastic-adherent monolayer cultures with trypsin/EDTA were seeded in 10-cm, tissue-culture-treated plates at low density (<50 cells/cm²), and cultured as earlier until colonies that contained at least 50 cells were formed. The growing colonies in each plate were collected by trypsinization and pooled. These pooled CFU-F populations were considered to represent human gingival stem cells (HGSCs). Three HGSC strains from three cell lines isolated by the digestion method from three different donors, and five HGSC strains from explant cultures from five different biopsies were used to assess the osteogenic and adipogenic differentiation capabilities. In addition, three palatal gingival cell lines were used for micromass cultures (two from the enzymatic digestion method and one obtained by the explant method). Two of the cell lines from palatal gingiva were used for SOX9 siRNA experiments.

Osteogenic differentiation assay

Confluent cell cultures were treated with osteogenic induction medium that consisted of DMEM supplemented with 10% FBS, 50 µg/mL of ascorbic acid, 100 nM dexamethasone, 100 nM vitamin D3, 10 mM β-glycerophosphate (Sigma-Aldrich), and antibiotic mixture (Gibco). After 21–35 days of culture, mineralization was analyzed by Alizarin Red S staining.

Chondrogenic differentiation assay

Cells were released from monolayer cultures by trypsin treatment, 500,000 cells were centrifuged (500 g/5 min), and

then they were cultured as micromass cultures in 15-mL polypropylene tubes (Falcon, BD Biosciences, Mississauga, ON, Canada). Two media were used successively as previously described [1]. The first serum-free medium contained high-glucose DMEM, 100 nM dexamethasone, 1% ITS + 1, L-proline (40 µg/mL), and ascorbic acid (50 µg/mL) (Sigma-Aldrich); sodium pyruvate (1 mM; Gibco); and 10 ng/mL transforming growth factor-β3 (TGF-β3) (BioVision, Edmonton, Alberta, Canada), and was used during the first 3 weeks of culture to induce chondrogenic differentiation. To induce hypoxic conditions, this medium was then replaced for the following 2 weeks with a medium composed of high-glucose DMEM, 1 nM dexamethasone, 1% ITS + 1, L-proline (40 µg/mL), ascorbic acid (50 µg/mL), L-thyroxine (50 nM), and β-glycerophosphate (20 mM) (Sigma-Aldrich) and sodium pyruvate (50 mM; Gibco), and cells were cultured in a hypoxic condition that was achieved by closing the cap of the culture tube tightly to prevent gas exchange.

Histology and immunostaining

For histological assessment, micromass cultures were fixed in cold (4°C) ethanol solution (50%), dehydrated in ethanol, and embedded in paraffin. Paraffin sections (7-µm thick) were cut using a microtome. Histological staining was performed using alcian blue (pH 1), alizarin red S, toluidine blue, hematoxylin-eosin, and picosirius red stains. The following primary antibodies were used for immunostaining: anti-type II collagen (CIIC1; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti-type X collagen (X-AC9; Developmental Studies Hybridoma Bank), anti-cadherin-11 (MAB1790; R&D Systems, Minneapolis, MN), anti-tubulin, anti-α-SMA (ab5694; ABCAM, Paris, France), and anti-Ki67 (M0722; Dako, Les Ulis, France). For HA staining, biotinylated hyaluronic acid binding protein (HABP) was used (Calbiochem, Millipore, Toronto, ON, Canada). Apoptosis was detected using ApopTag kit (Millipore, Molsheim, France). Prior to immunostaining, nonspecific binding of antibodies was blocked by incubating the samples with phosphate-buffered saline containing 3% bovine serum albumin and 0.1% glycine for 30 min. Incubation with primary antibodies was carried out at 4°C overnight, and immunolabeling was detected using Vectastain ABC kit with VIP or DAB substrates (Vector Laboratories, Burlington, ON, Canada). For every immunostaining, a negative control was added without primary antibody. The sections were observed using an Axioplan light microscope (Zeiss, North York, ON, Canada).

For immunofluorescence stainings, goat anti-rabbit Alexafluor 594- and goat anti-mouse Alexafluor 488-conjugated secondary antibodies were used (Life Technologies Corporation). Cell nuclei were stained using DAPI (Life Technologies Corporation). Samples were observed and digital images were recorded on the Axioplan fluorescent light microscope (Zeiss).

Quantitation of Ki67-positive and apoptotic cells in tissue sections (three sections per condition) was performed counting cells in 5–10 acquisition fields per section per culture condition using the ImageJ software (NIH).

Electron microscopy

Paraffin-embedded sections from micromass cultures were rehydrated, rinsed in PIPES, and fixed in 2.5% glutaraldehyde

for 1 h. After rinsing, sections were postfixed with 0.5% osmium tetroxide for 20 min. Portions of the glass slides were trimmed to contain only the sections, and then mounted on to aluminum stubs using conductive adhesive and colloidal silver (Canemco-Marivac, St. Laurent, QC, Canada), followed by sputter coating with 5-nm gold/palladium (Leica, EM MED020, Leica Microsystem, Inc., Concord, ON, Canada). The samples were examined using Helios NanoLab 650 Dual Beam Scanning Electron Microscope (FEI, Hillsboro, OR).

Gene expression analysis

Total RNA was extracted from cultured cells using Aurum™ Total & RNA Mini Kit (Bio-Rad Laboratories, Mississauga, ON, Canada), and treated with DNase (Fermentas, Burlington, ON, Canada) according to the manufacturer's protocol. Total RNA concentration and purity was measured by nanodrop (Agilent, Mississauga, ON, Canada) and only samples with OD260/280 ratio of 1.8 to 2.1 were used for the study. cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's instructions: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. All primers (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd) were designed on the boundaries of exons whenever possible, and analyzed by BLASTn for their specificity and by RNAfold software for their secondary structures. Efficiency of target amplification was optimized for each primer set using a 10-fold dilution series of cDNA while standard curves were made. For the reaction, equal amount of retro-transcriptase (RT) product from each sample was diluted to a concentration such that the Ct values were well within the range of their standard curves (in general equivalent to 5 ng/mL RNA), and 3 µL of diluted RT products was mixed with 7.5 µL of 2 X iQ SYBR Green I Supermix (Bio-Rad Laboratories), and 5 pmols of primers, for a final volume of 15 µL. Real-time PCR amplification was performed on the CFX96 System (Bio-Rad Laboratories) using the following program: one cycle at 94°C for 3 min, 35 cycles at 95°C for 5 s, gene-specific annealing for 10–20 s, and reaction completion with reading plate and a melt curve analysis from 65°C to 95°C, 5 s for each 0.5°C. For some genes (collagen type II and aggrecan), a touchdown PCR cycle was implemented. In brief, a cycling program where the annealing temperature was gradually reduced (2°C/every cycle) was followed by the standard quantitative polymerase chain reaction (QPCR) protocol. In preliminary experiments, reaction products were run on agarose gels (1.2%) to confirm the correct amplicon sizes. For a given experiment, a reference gene was chosen based on its variance calculation using CFX manager software (Bio-Rad Laboratories). Nontranscribed RNA samples (nonreverse transcribed sample) and a water control were used as negative controls. The PCRs were performed in triplicate for each sample. The data were analyzed based on 2ΔΔCt method (CFX Manager Software Version 2.1; Bio-Rad Laboratories).

siRNA transfection

Transfections with siRNA were performed using lipofectamine RNAiMax kit (Invitrogen, Life Technologies, Burlington, ON, Canada) in 60-mm culture dishes by the indirect method. The siRNA duplexes were designed using

Whitehead Institute's siRNA prediction tool following the N19 + dTdT rule. Among these duplexes, three were designed to inhibit SOX9 (iSOX9a, b, c), one was to inhibit a gene unrelated to SOX9 (vimentin: iVIM), and one was composed of a scrambled iSOX9a sequence (iScr) used as a control (Mock) (Supplementary Table S1). In brief, cells were treated with siRNA (2 μ L of 10 mM siRNA in 100 μ L Opti-MEM, final concentration at 100 nM) in RNAiMax (6 μ L in 100 μ L Opti-MEM) in serum-free Opti-MEM medium (Gibco) for 12 h, before replacing the medium with the normal growth medium. A set of cultures was also treated with a mixture of all three SOX9 siRNAs at the same time (100 nM each). To assess transfection efficiency by real-time PCR, cells were cultured for various time points and RNA was isolated as earlier. Optimal siRNA and RNAiMax concentrations were determined in preliminary experiments. For a set of experiments to visualize siRNA inside the cells by fluorescence microscopy, iSOX9b was coupled with Alexafluor 488 fluorochrome (Eurogentec, Liège, Belgium). To assess cadherin-11 function in synovocyte differentiation, an siRNA inhibiting cadherin-11 (iCDH11, see Supplementary Table S1) was used during micromass cultures in the same conditions than the iSOX9.

Statistical analysis

Real-time PCR results were analyzed using the non-parametric Friedman test with Graph Pad software (Graphpad Software, Inc., La Jolla, CA).

Results

Gingival cell characterization

Human gingival cell lines were derived from independent donors, and each biopsy was divided into two samples in order to assess two cell-harvesting strategies (enzyme digestion and explant culture). Because long-term in vitro culture impairs progenitor cell properties [27,28], all experiments were performed using cells up to the third passage. As previously described [20], GFs were able to form CFU-F when cultured at low density ($<50/\text{cm}^2$) as cell-culture-plastic-adherent cells. For all subsequent experiments, CFU-F-pooled cells, named HGSCs, were used. Analysis of mRNA expression of neural crest markers by PCR showed that the cells expressed TWIST1 and SNAIL1 ($n=5$ cell lines, three from palatal and two from buccal gingiva) (Supplementary Fig. S1). Cells were also positive for osteochondro-lineage markers Runt-related transcription factor 2 (RUNX2) and SOX9 ($n=5$) (Supplementary Fig. S1). Further, they displayed stable cell proliferation rate with a constant about 40-h population doubling time over 3 weeks (Fig. 1A), and with osteogenic and adipogenic differentiation potential in vitro ($n=3$) (Fig. 1B, C). Both enzymatically and explant-derived cell cultures from these three different patients showed similar osteogenic differentiation capacity (Fig. 1D). For the subsequent experiments, we used the three HGSC lines, which were all derived from palatal gingiva.

Osteogenic differentiation of HGSC monolayer cultures

To further characterize the HGSCs, we tested their osteogenic differentiation capacity in more detail, and assessed

expression of osteogenic-differentiation-related genes over time in monolayer cultures. The cells were able to form mineralized nodules when stimulated by an osteoinductive medium for 3–5 weeks (Fig. 1D). This involved an early upregulation of ALPL (days 4 and 7), an early and extended upregulation of RUNX2 (days 4–21), and an about 20-fold late upregulation of SP7 (day 21). Expression of SOX9 remained moderately upregulated from day 4 to 21, while MSX2 showed an early (day 4) and late (day 21) peak, but these changes in gene expression were not statistically significant (Fig. 1E). Gene expression studies showed comparable mRNA expression kinetics over time among the different cell strains. The capability to form mineralized nodules decreased at late passage ($>P6$) (Fig. 1D), which is consistent with data from dental-pulp-derived stem cells [29].

Chondrogenic differentiation and maturation of HGSC micromass cultures

To assess the capability of the cells for chondrogenic differentiation along the endochondral pathway, HGSCs were cultured in chondrogenic induction medium as micromass cultures for 3 weeks, followed by 2 weeks of hypoxic condition to induce hypertrophy ($n=3$). When expression of key genes involved in chondrogenesis was followed over time, an upregulation of SOX9, ACAN, and COL2A1 mRNA expression appeared after 2 weeks of chondrogenic induction (Fig. 2G). At day 21, histological sections from the cultures showed strong inhomogeneous glycosaminoglycan staining (Fig. 2A). Organization of the collagen-rich ECM was then assessed by picrosirius red staining (Fig. 2B, C). In the most peripheral surface layers of the cultures, collagen was parallel to the surface, while in the more-central areas it was randomly oriented (Supplementary Fig. S2). The cultures were positive for type II collagen (Fig. 2D) especially in the more-central areas of the micromass that were relatively acellular reminiscent of natural cartilage. Immunostaining for type X collagen was mostly negative especially in the central areas of the cultures, but areas closest to the outer surface showed some weak staining (Fig. 2E, F). Examination with a higher magnification showed a heterogeneous composition of the micromass culture with a central cell-free area located next to typical chondrocyte-like cells embedded in a cartilaginous-like matrix (Fig. 2E). This differentiation is comparable to what is classically obtained with BMMSCs (Supplementary Fig. S3).

During cartilage development, hypoxic condition due to a lack of blood supply within the cartilage induces hypertrophy [30]. Hypertrophic cells are characterized by an increase of the cell volume and a rounded cell shape [31]. To assess the effect of hypoxia in our micromass culture model, cells grown for 21 days in the chondrogenic induction medium in normoxic conditions were switched to hypoxic condition for 14 days by adding L-thyroxine and closing the caps of the culture tubes. After 1 week, expression of genes implicated in the maturation of hypertrophic chondrocytes, that is, Indian Hedgehog (IHH), collagen type X alpha 1 (COL10A1), vascular endothelial growth factor α (VEGFA), matrix metalloproteinase 13 (MMP13), RUNX2, and collagen type I alpha 1 (COL1A1), was upregulated (Fig. 3G), while chondroblast-related genes SOX9, ACAN, and

FIG. 1. Characterization of gingival-connective-tissue-derived cells. Analysis of growth kinetics showed an increase in cell numbers with a constant doubling time over 3 weeks (A). When incubated in the appropriate differentiation medium, cells showed osteogenic-like differentiation with deposition of mineralized matrix (B, alizarin red S staining) and differentiation into adipocyte-like cells (C, oil red O staining). Osteogenic differentiation at low ($P \leq 3$) and high passages ($P \geq 6$) was further tested with three HGSC lines from three different donors and isolated by the explant or enzymatic digestion method. All low-passage cell lines showed similar osteogenic response (D), while cells at high passage showed a decreased differentiation potential. Gene expression analysis of key genes involved in osteodifferentiation was also followed over time in osteogenic medium. Results from real-time PCR showed that the master genes were upregulated during this process and followed classical kinetics over time (E). The results show mRNA expression relative to GAPDH as housekeeping gene according to the $2^{-\Delta\Delta Ct}$ method. Each QPCR was performed in triplicate. Results show mean $\pm$ standard deviation from four parallel cell lines (* $P < 0.05$; ** $P < 0.01$). NS, no statistically significant differences. HGSCs, human gingival stem cells; QPCR, quantitative polymerase chain reaction. Color images available online at www.liebertpub.com/scd

FIG. 2. Analysis of chondrogenic differentiation. After 21 days of micromass culture in chondrogenic induction medium, cultures generated cartilage-like tissue. (A) Alcian-blue-stained (pH=1) histological sections from the cultures that displayed a cartilage-like ECM particularly at the center of the micromass. Positive area is outlined by the arrow. (B) Picrosirius red staining showed collagen-rich (red) areas in both periphery and center of the culture. (C) Observation under polarized light of the periphery of the culture revealed collagen fibers that were aligned parallel to the micromass surface, and showed a more intense green color, indicating a difference in the diameter of the collagen fibers in the peripheral and central areas. Cultures showed also positive immunostaining for type II collagen (D), while the staining for type X collagen was mostly negative, with some areas closest to the outer surface showing a weak staining (E). (F) Negative control staining without primary antibody showed no positive immunoreaction. Representative images from three experiments performed with three parallel HGSC lines are shown. Magnification bar: (B, C) 200 μ m; (D, E) 100 μ m; and (F, G) 30 μ m. (G) In the micromass cultures, expression of type II collagen, aggrecan, and SOX9 mRNA was strongly upregulated after 14 days of culture in chondrogenic medium, and was then downregulated after 1 week under hypoxia (corresponding to day 28 after initiation of the experiment). Results show QPCR analysis of mRNA expression relative to GAPDH ($2^{-\Delta\Delta C_t}$ method). ECM, extracellular matrix. Color images available online at www.liebertpub.com/scd

COL2A1 were downregulated (Fig. 2G). These differences did not reach statistical significance due to variation in the magnitude of gene expression changes in the examined three different HGSC cultures. At day 35, a fibrillar collagen network was reorganized compared with day 21 (Fig. 3A), and was characterized by a lower fiber density and absence

of peripheral aligned fibers. A glycosaminoglycan-rich ECM (Fig. 3B) was observed in two out of three micromass cultures. In all the cultures, cells acquired a hypertrophic phenotype characterized by a round shape and an increased cell volume (Fig. 3C, D). In addition, cells were surrounded by a collagen network composed of cartilage-specific type II

FIG. 3. Analysis of chondrocyte maturation. After 3 weeks of chondrogenic differentiation, followed by 2 weeks of hypoxic conditions, chondrogenic cells acquired a hypertrophic phenotype. Histological sections stained with picosirius red showed that cells inside the micromass cultures were surrounded by a mature cartilage-like fibrillar collagen network (**A**) and (**D**) associated with a toluidine-blue-positive ECM (**B**). (**C–F**) Examination with higher magnification revealed a typical organization of mature cartilage. Many cells displayed morphology reminiscent of hypertrophic chondrocytes when examined by SEM analysis (**C**), and were surrounded by a collagen network composed of type II (**E**) and type X collagens (**F**). However, no mineralization of the matrix was noted (data not shown). Representative images from three experiments performed with three parallel HGSC lines are shown. Magnification bar: (**B**) 200 μ m; (**C**) 100 μ m; and (**E–G**) 20 μ m. (**G**) QPCR analysis showed an upregulation of key genes associated or involved in chondrocyte hypertrophy after 1 week of hypoxia (corresponding to day 28 after initiation of the experiment). Results show mRNA expression relative to GAPDH ($2\Delta\Delta C_t$ method). IHH, Indian Hedgehog; COL10A1, collagen type X α 1; VEGFA, vascular endothelial growth factor α ; MMP13, matrix metalloproteinase 13; RUNX2, Runt-related transcription factor 2; COL1A1, collagen type I α 1. Results show mean of relative mRNA expression from two parallel HGSC lines. SEM, scanning electron microscopy. Color images available online at www.liebertpub.com/scd

collagen (Fig. 3E) and hypertrophy-associated type X collagen (Fig. 3F). However, cells were unable to complete endochondral ossification, and to form mineralized matrix, even when treated with β -glycerophosphate. Moreover, alkaline phosphatase activity was negative (data not shown). Thus, HGSCs were able to give rise to hypertrophic chondrocytes, but did not induce ossification in vitro.

Peripheral cell lining in the HGSC micromass cultures differentiates into fibroblast-like synoviocytes

At day 21, a peripheral surface cell lining had formed and was oriented perpendicularly to the underlying layers of the micromass cultures (Fig. 4A–F). The cell lining was mainly composed of only one layer of polarized cells (Fig. 4E), but

FIG. 4. Fibroblast-like synoviocyte differentiation. (A–D) SEM analysis of the histological sections from the micromass cultures at day 21 showed a distinct peripheral lining cell layer that was formed on the outer surface of the micromass culture. Hematoxylin staining (E) and tubulin immunostaining (F) showed that the cells in the peripheral layer were polarized and oriented perpendicularly to the underlying layer that was aligned more parallel to the outer surface of the micromass culture. Immunostaining showed that the peripheral cell lining was positive for cadherin-11 (CDH-11) (G, J) and hyaluronic acid (HA) (H, K). Inhibition of cadherin-11 by siRNA transfection showed an inhibition of the formation of the peripheral cell lining (I, L). Representative images from experiments with three parallel HGSC lines are shown. Magnification bar: (E) 20 μ m; (F) 50 μ m; (G) 100 μ m; and (H–J) 50 μ m. Color images available online at www.liebertpub.com/scd

in some locations it reached a thickness of up to five layers (not shown). Scanning electron microscopy showed that cells of the external layers had a cuboidal shape and presented an irregular villous outer surface (Fig. 4A–D). To find out whether these cells shared key properties of fibroblast-like synoviocytes found *in vivo*, we performed immunostaining for cadherin-11 [7]. Results showed that the peripheral cell lining was strongly positive for cadherin-11, and that the subperipheral layer also contained positive cells (Fig. 4G, J). The first cadherin-11-positive cells appeared in the peripheral surface layers at day 14 after induction of the micromass cultures (Supplementary Fig. S4). Moreover, similar to normal fibroblast-like synoviocytes [4] these cells produced HA as indicated by strong HABP reactivity (Fig. 4H, K).

SOX9 plays a critical role in gingival cell chondrogenic micromass culture self-organization

Role of SOX9, a key transcription factor expressed by chondrocyte precursors that regulates *COL2A1* and *ACAN* expression in chondrogenesis [32], was studied by using SOX9 siRNA transfection of the micromass cultures. Control cultures were transfected with siRNA against an unrelated vimentin gene (iVIM), and using scrambled (mock) SOX9 siRNA. Real-time PCR showed that different SOX9 siRNAs caused variable SOX9 downregulation (Fig. 5A). iSOX9a had a marked effect on SOX9 expression (52% downregulation of SOX9), while iSOX9b and iSOX9c caused a 74% and 64% downregulation, respectively (Fig. 5A). Combining all three SOX9 siRNAs caused the most potent SOX9 downregulation (87% downregulation) (Fig. 5A), but it also resulted to poor generation of micromass cultures likely due to toxicity (data not shown). Downregulation of SOX9 by the different duplexes of siRNA lasted at least for 8 days (Supplementary Fig. S5). Based on the aforementioned data, iSOX9b was chosen for the further experiments. To assess the role of SOX9 in the development of the micromass cultures, we compared them histologically with and without SOX9 siRNA treatment. Histological analysis showed that compartmentalization of the micromass culture was already underway in control samples at day 7. In particular, control cultures had formed a well-defined area containing fewer cells and increased apoptotic activity (Fig. 5B, C). Accordingly, in the peripheral area, around 3.5% of the cells were apoptotic, while 30.9% of the cells near the central acellular area were apoptotic in the control cultures. In contrast, this apoptotic compartment was almost missing from SOX9-siRNA-treated samples (<1% of apoptotic cells) (Fig. 5B, C). Proliferating cells were present in the peripheral area for both mock (Fig. 5D, left panel) and SOX9-siRNA-treated micromass cultures (Fig. 5D, right panel) with similar proportions ($11.44\% \pm 5.25\%$ for mock vs. $10.5\% \pm 5.69\%$ for siSOX9-treated cultures). However, while proliferating cells were present in the center of SOX9-siRNA-treated cultures, they were missing from the control cultures (Fig. 5D). Immunostaining of α -smooth muscle actin, a marker for myofibroblasts, showed that inhibition of SOX9 resulted to areas with high numbers of α -SMA-positive myofibroblasts (Fig. 5E) while these cells were missing from the control cultures (Fig. 5F). To further assess cell morphology and abundance of glycosaminogly-

cans, the cultures were stained with toluidine blue. In 14-day-old cultures, toluidine blue staining showed cells with well-defined polarized surface layer, a distinct elongated layer underneath the surface layers, and chondrocyte-like cells in the deeper areas of the cultures (Fig. 4I). In contrast, some areas of the SOX9-siRNA-treated cultures harbored predominantly cells with a prehypertrophic-like phenotype (round shape) and the surface layers were less well-defined (Fig. 5G, H).

Discussion

This study provides novel insight into the properties of postmigratory NCCs. First, while these cells were derived from adult human gingival soft tissue, they were able to differentiate into hypertrophic chondrocytes. Second, when cultured as micromasses, they self-organized to generate distinct areas that resembled native cartilage templates. These included a peripheral area composed of two distinct layers that mimic synovial cell lining and the perpendicularly oriented subintimal layer. This area also contained aligned fibrils of collagen. These characteristics are similar to chondrodifferentiation achieved using induced pluripotent stem cells [33]. In the micromass cultures in the present study, the central area also contained a type II-collagen-rich cartilaginous matrix where the cells further differentiated in hypertrophic chondrocytes. Thus, this *in vitro* chondrogenesis by HGSCs reproduced a set of physiological developmental processes resulting in different cell phenotypes, highlighting the plasticity of gingival cells.

To our knowledge, this is the first report that describes fibroblast-like synoviocyte differentiation during micromass chondrodifferentiation *in vitro* with cells other than those derived from the synovium. Distinct mechanical and other cell-adhesion-related stimuli due to lack of adhesion to the culture plate and abundant cell–cell contacts in the floating micromass cultures may act synergistically with cytokine signaling to polarize the most external cell lining to form this fibroblast-like synoviocyte layer. This is supported by the finding that no synoviocyte differentiation was noted in cultures where the micromass was attached to the culture dish (data not shown). Whether the fibroblast-like synoviocyte differentiation is a distinct property of neural-crest-derived gingival cells or a more general characteristic of connective tissue cells from other tissues in this culture environment remains to be shown.

SOX9 is considered a master gene for differentiation of chondrocytes [34]. Moreover, SOX9 drives chondrodifferentiation of stem cells derived from diverse sources [34]. Our results confirm the upregulation of this gene during chondrogenesis of HGSCs. In addition, SOX9 was also expressed by these cells at basal level without chondrogenic stimulation. This basal expression may explain the ability of HGSCs for efficient chondrogenesis.

In SOX9-knockout mice, cells are unable to generate normal cartilage after mesenchymal condensation but acquired a hypertrophic phenotype [34]. This is similar to the SOX9-siRNA-treated micromass cultures in the present study. However, in the knockout mice, inhibition of SOX9 was associated with apoptosis [34], which was not noted in our study. These opposite effects might be linked either to

FIG. 5. SOX9 regulates morphogenesis of cartilage-like micromass cultures. (A) Real-time PCR of SOX9 mRNA expression in SOX9 siRNA (siSOX9)- and control siRNA (mock)-transfected cells 1 day after siRNA transfection showed a downregulation of SOX9 expression. Results show mean and standard deviation from two experiments performed with two parallel HGSC lines. iSOX9a, first duplex against SOX9; iSOX9b, second duplex; iSOX9c, third duplex; N, cells treated without siRNA; Scr, transfection with a scrambled iSOX9a siRNA; iSOX9mix, cells were transfected with all three SOX9 siRNAs (iSOX9a, iSOX9b, and iSOX9c) at the same time. (B) Hematoxylin-eosin staining shows the presence of an early compartmentalization of micromass cultures at day 7 with the presence of a cell-free area at the center. (C) Staining of apoptotic cells by TUNEL assay at day 7. Control cultures showed areas that contained abundantly apoptotic cells especially at the center. However, apoptosis was not evident in SOX9-siRNA samples (B, C). (D) Analysis of cell proliferation by Ki67 immunostaining at day 7 showed that proliferating cells were present in the peripheral area for both mock and siSOX9 micromass cultures. They were also present in the center of SOX9-siRNA cultures, but missing from the control cultures. (E, F) Immunostaining of α -SMA (red fluorescence) at day 14 showed immunoreactivity only in SOX9-siRNA-treated samples. Blue color indicates nuclear staining with DAPI. (G, H) Toluidine blue staining shows the presence of round cells in SOX9-siRNA micromass cultures, but not in mock conditions. Toluidine blue metachromasia (purple staining) was mainly observed in SOX9-siRNA conditions. Magnification bar: (B, D, E, F) 100 μ m; (C, G, H) 50 μ m. The siRNA experiment was performed twice on two different cell strains. Color images available online at www.liebertpub.com/scd

To our knowledge, this is the first study to provide evidence that, in addition to chondrogenic cells, HGSCs are also able to differentiate into type X-collagen-secreting hypertrophic chondrocytes and fibroblast-like synoviocytes. However, unlike BMMSCs cultured in identical conditions [1,8], HGSCs showed no capability to form mineral deposits. It is possible that contrary to the mesodermal bone marrow MSCs, neural-crest-originating HGSCs need other cells and/or a distinct ECM niche to promote appropriate terminal differentiation and produce mineralized matrix. Interestingly, gingival cells expressed high levels of VEGFA, a potent proangiogenic factor, under the hypoxic condition. This characteristic may be of advantage to promote revascularization critical for regenerative therapies in the joint. The gingival cell population that had cartilage and synoviocyte-like differentiation potential was derived from pooled CFU population without further selection or fractionation. Although such cell population has been shown to

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