Gene expression profile in human induced pluripotent stem cells: Chondrogenic differentiation *in vitro*, part A

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Abstract. Human induced pluripotent stem cells (hiPSCs) offer promise in regenerative medicine, however more data are required to improve understanding of key aspects of the cell differentiation process, including how specific chondrogenic processes affect the gene expression profile of chondrocyte-like cells and the relative value of cell differentiation markers. The main aims of the present study were as follows: To determine the gene expression profile of chondrogenic-like cells derived from hiPSCs cultured in mediums conditioned with HC-402-05a cells or supplemented with transforming growth factor β3 (TGF-β3), and to assess the relative utility of the most commonly used chondrogenic markers as indicators of cell differentiation. These issues are relevant with regard to the use of human fibroblasts in the reprogramming process to obtain hiPSCs. Human fibroblasts are derived from the mesoderm and thus share a wide range of properties with chondrocytes, which also originate from the mesenchyme. Thus, the exclusion of dedifferentiation instead of chondrogenic differentiation is crucial. The hiPSCs were obtained from human primary dermal fibroblasts during a reprogramming process. Two methods, both involving embryoid bodies (EB), were used to obtain chondrocytes from the hiPSCs: EBs formed in a chondrogenic medium supplemented with TGF-β3 (10 ng/ml) and EBs formed in a medium conditioned with growth factors from HC-402-05a cells. Based on immunofluorescence and reverse transcription-quantiative polymerase chain reaction analysis, the results indicated that hiPSCs have the capacity for effective chondrogenic differentiation, in particular cells differentiated in the HC-402-05a-conditioned medium, which present morphological features and markers that are characteristic of mature human chondrocytes. By contrast, cells differentiated in the presence of TGF-β3 may demonstrate hypertrophic characteristics. Several genes [paired box 9, sex determining region Y-box (*SOX*) *5*, *SOX6*, *SOX9* and cartilage oligomeric matrix protein] were demonstrated to be good markers of early hiPSC chondrogenic differentiation: Insulin-like growth factor 1, Tenascin-C, and *β-catenin* were less valuable. These observations provide valuable data on the use of hiPSCs in cartilage tissue regeneration.

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Abbreviations: AGC1, aggrecan; Akt, protein kinase B; BMP-2, bone morphogenetic protein-2; COL2A1, type II collagen; COMP, cartilage oligomeric matrix protein; CTNNB1, β-catenin; EBs-embryoid bodies; ECM, extracellular matrix; EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; FGFR3, fibroblast growth factor receptor 3; HC-402-05a, human primary chondrocyte cell line; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; IGF-1, insulin-like growth factor 1; IHH, indian hedgehog; MMP, matrix metalloproteinase; NCAM, neural cell adhesion molecule; NKX3.2, NK-related homeodomain protein; PAX9, paired box 9; P13K, phosphatidylinositol 3-kinase; PHDFs, primary human dermal fibroblasts; RUNX2, runt-related transcription factor 2; SCs, stem cells; SOX2, 5, 6, 9, sex determining region Y-box 2, 5, 6, 9; Tcf-Lef, T-cell factor/lymphoid enhancer factor; TCFP2L1, transcription factor CP2-like 1; TGF-β3, transforming growth factor β3

Key words: human induced pluripotent stem cells, differentiation, chondrocytes, chondrogenic markers, early chondrogenesis, transforming growth factor-β3, conditioned medium

Introduction

Mature articular cartilage is unable to heal spontaneously and, consequently, lesions eventually progress to osteoarthritis. This lack of capacity for self-repair has prompted intensive research into methods of articular cartilage regeneration, including cell-based cartilage tissue engineering (1,2). The use of stem cells (SCs) may help to overcome the drawbacks of autologous chondrocytes, which include the limited number of chondrocytes available for cell culture, preservation of the cells' chondrogenic potential, and re-differentiation of cells during tissue formation following implantation. Human mesenchymal stem cells and

human induced pluripotent stem cells (hiPSCs) may be useful for cartilage regeneration (3-5). It is possible to use defined transcription factors to transorm terminally-differentiated cells, including fibroblasts, into hiPSCs, which share characteristics with human embryonic SCs (hESCs) (6). However, this strategy is not without risk, given that hESCs and hiPSCs are potentially tumorigenic and must therefore be monitored carefully if they are to be applied safely (7). Patient-derived hiPSCs differentiate into derivatives of three germ layers, ecto-, meso- and endoderm, and may be ideal autologous cells for chondrocyte generation because they are not subject to immune rejection and are easily expanded prior to chondrocyte generation (8).

Numerous techniques are available for chondrogenic differentiation of SCs, although the most common and efficient method of obtaining chondrocyte-like cells from hiPSCs is the formation of embryoid bodies (EB). Depending on the specific approach, chondrogenic differentiation may require the use of selected growth factors, scaffolds, or other biomaterials, as well as specific culture dishes (2- or 3-dimensional culture). Although it is possible to use a variety of mediums for chondrogenic differentiation, the optimal medium remains unclear (9-12). Similarly, during the chondrogenic differentiation process, a wide range of markers are available to assess cell differentiation, but the relative utility of these markers is not well-understood, in particular in the context of hiPSC differentiation, which is a novel approach in regenerative medicine (13,14).

Given this context, the primary aims of the present study were as follows: To determine the gene expression profile of chondrogenic-like cells derived from hiPSCs cultured in mediums conditioned with HC-402-05a cells or supplemented with transforming growth factor β3 (TGF-β3), and to assess the relative utility of the most commonly used chondrogenic markers as indicators of cell differentiation.

The cells were differentiated in chondrogenic mediums supplemented with either TGF-β3, the member of the TGF-β superfamily with the most chondrogenic potential (15) or conditioned with growth factors from the human primary chondrocyte cell line HC-402-05a. The gene expression profile of the chondrogenic-like cells derived from the hiPSCs cultured in the TGF-β-supplemented medium (TGF-β medium) were them compared with the cells cultured in the HC-402-05a-conditioned medium (condtioned medium). Notably, the type of medium had a notable impact on gene expression profiles. A total of 20 markers of chondrogenic differentiation were also evaluated, and paired box 9 *(PAX9)*, sex determining region Y-box 5 *(SOX5)*, sex determining region Y-box 6 *(SOX6)*, sex determining region Y-box 9 *(SOX9)* and cartilage oligomeric matrix protein *(COMP)* were demonstrated to be good markers of hiPSC differentiation, whereas insulin-like growth factor 1 *(IGF-1)*, Tenascin-C *(TNC)*, and *β-catenin* were less valuable indicators of cell differentiation. Furthermore, the origin (mesoderm) of fibroblasts and chondrocytes should be taken into consideration, due to the fact that several genes are common for stem cell-derived chondrocytes and human fibroblasts (e.g., *SMAD3* and *BMP-2*), decreasing their utility in the evaluation of chondrogenic process *in vitro*.

The findings of the present study demonstrated that cells differentiated in the conditioned medium present features that are characteristic of mature chondrocytes, whereas the features of cells cultured in the presence of TGF-β3 are characteristic of hypertrophic chondrocytes, thus underscoring the potential of the HC-402-05a-conditioned medium for *in vitro* chondrogenesis. The present study contributes to an improved understanding of the changes in gene expression that occur during the *in vitro* chondrogenic process and short-term culture of stem-derived chondrocytes, in addition to helping to clarify the relative value of a wide range of chondrogenic differentiation markers.

The present study is a two-part study. Part A, presented here, describes the markers that are characteristic for pluripotency state and early-stage chondrogenesis (Table I). The second part of the study (16) focused on markers that are characteristic of late stage chondrogenesis, hypertrophy and ossification.

Materials and methods

Culturing human induced pluripotent stem cells. The hiPSCs obtained during the reprogramming process as previously described (17) were seeded on 10 cm Petri dishes in Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) that had previously been coated with inactivated murine embryonic fibroblasts as a feeder layer ($1x10^6$). Following 24 h preparation of the feeder layer, hiPSCs were seeded at $2x10^6$ in hiPSC growth medium: Dulbecco's modified Eagle's medium (DMEM) F12 with L-glutamine (Merck Millipore, Darmstadt, Germany), 20% knockout serum replacement (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% non-essential amino acids (Merck Millipore), 0.1 mM β-mercaptoethanol (Merck Millipore), 1% penicillin/streptomycin (P/S; Merck Millipore). Prior to use, the medium was supplemented with fibroblast growth factor 2 (FGF-2; 10 ng/ml; Merck Millipore). The complete hiPSC growth medium was supplemented with ciprofloxacin (0.5 μg/ml; Sigma Aldrich; Merck Millipore) to avoid *Mycoplasma spp*. contamination for the first 7 days of culture. The culture medium was changed daily. Experiments using hiPSCs do not need approval from a local ethics committee.

Embryoid body formation. At 80% confluency, hiPSC colonies were passaged and dissociated into clumps with 0.1% collagenase IV solution (Thermo Fisher Scientific, Inc.). The cells were centrifuged (300 x *g*, 5 min, room temperature) in order to remove the collagenase and transferred into non-adherent 96-well plates (1,000 cells per well; Brand GmbH, Wertheim, Germany) in EB growth medium, which is a hiPSC growth medium without FGF-2. Embryoid bodies (EBs) formed within 24 h and were observed as free-floating aggregates. The culture medium was changed every 48 h. On day 7 the EBs were used for chondrogenic differentiation.

Chondrogenesis in vitro. A standard chondrogenic medium was used: DMEM F12 with L-glutamine (Merck Millipore), 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 50 μM L-proline (Sigma Aldrich; Merck Millipore), 50 μM ascorbic acid (Sigma Aldrich; Merck Millipore), 1 mM sodium pyruvate (Biowest), 1% ITS + Premix (Corning Life Sciences, Big Flats, NY, USA), 1% P/S (Merck Millipore) and 10^{-7} M dexamethasone (Sigma Aldrich; Merck Millipore).

Medium conditioning. Standard chondrogenic medium was used: DMEM F12 with L-Glutamine (Merck Millipore),

Table I. Assessment of selected markers for early hiPSC chondrogenic differentiation *in vitro*.

Marker	Function of marker (stage of presentation)	Influence on chondrogenesis: positive (+) or negative (-)	Utility of the marker to assess chondrogenic progression (+, ++, +++)
NANOG	Maintenance of pluripotency (SCs)	-	+++
OCT-4	Maintenance of pluripotency (SCs)	-	+++
SOX2	Maintenance of pluripotency (SCs)	-	+++
E-CADHERIN	Maintenance of pluripotency (SCs)	-	+++
BRACHYURY	Cells from mesodermal stage	-/+	+++
CXCR4	Cells from mesodermal and endodermal stage	-	+++
TENASCIN-C	ECM of articular cartilage/condensation stage	+	+
PAX9	Induction of chondrogenesis (chondroprogenitors)	+	+++
NCAM	ECM/osteoblasts (condensation stage)	-/+	++
NKX3.2	Chondroprogenitors	+	++
The SOX trio: SOX5, 6 and 9	Chondrogenesis	+	+++
IGF-1	Pluripotency/chondrocytes/hypertrophic chondrocytes/osteoblasts	+	+
CD44	Cell-surface glycoprotein	+	++
COMP	Cartilage ECM	+	+++
AGGRECAN	Cartilage ECM	+	++
β-CATENIN	Pluripotency/mesoderm/chondrocytes/ hypertrophic chondrocytes/osteoblasts	+	+
EGF	SCs/cell proliferation/chondrogenesis	+/-	+
FGFR3	SCs/cell proliferation/chondrogenesis	+/-	+

The number of plus symbols (+, ++, +++) indicate the utility of the marker as follows: Average (+), good (++), very good (+++). OCT-4, octamer-binding transcription factor 3/4; SOX2, SRY (sex determining region Y)-box 2; CXCR4, C-X-C motif chemokine receptor 4; PAX9, paired box 9; NCAM, neural cell adhesion molecule; NKX3.2, NK-related homeodomain protein; SOX5, -6, -9, SRY (sex determining region Y)-box 5,-6,-9; IGF-1, insulin-like growth factor 1; COMP, cartilage oligomeric matrix protein; EGF, epidermal growth factor; FGFR3, fibroblast growth factor receptor 3.

10% FBS (Biowest), 50 μM L-proline (Sigma Aldrich; Merck Millipore), 50 μM ascorbic acid (Sigma Aldrich; Merck Millipore), 1 mM sodium pyruvate (Biowest), 1% ITS + Premix (Corning Life Sciences), 1% P/S (Merck Millipore) and 10^{-7} M dexamethasone (Sigma Aldrich; Merck Millipore), which was conditioned on the HC-402-05a cell line (up to 3 passages). Medium was collected following 24 h conditioning and administered to the differentiated EBs.

Chondrogenesis using embryoid bodies. The mature EBs were transferred onto 6-well plates (10 EBs per well) previously coated with 0.1% gelatin (Merck Millipore) and allowed to adhere for the next 24 h, following which the medium was replaced with a chondrogenic medium. This was either supplemented with TGF-β3 (10 ng/ml; ImmunoTools GmbH, Friesoythe, Germany), as a growth factor with the most chondrogenic potential, or conditioned on the HC-402-05a cell line as above. The positive influence of standard chondrogenic medium with the addition of exogenous TGF-β3 (10 ng/ml) on pluripotent SCs was previously tested and confirmed (18). The chondrogenic medium was changed every 48 h. The culture period lasted 21 days. In order to confirm that chondrocyte-like cells had been obtained, immunofluorescence analysis was performed on passage 0 (p0). Next, to evaluate the expression profile of chondrogenic markers (p3), reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was performed (Fig. 1). In all analyses, the stable adult human articular chondrocyte cell line (HC-402-05a) served as a positive control, as the European Collection of Authenticated Cell Cultures recommended it for the evaluation of the differentiation process in *in vitro* model systems.

Culture of differentiated cells. The derived stem cells were cultured in 0.1% gelatin (Merck Millipore) in DMEM F12 with L-glutamine (Merck Millipore), 10% FBS (Biowest), and 1% P/S (Merck Millipore) up to 3 passages.

Immunofluorescence analysis. The cells (p0; $0.5x10^5$) were transferred into a gelatin-coated (1:50) 48-well plate for 48 h. The cells were washed with PBS (Sigma Aldrich; Merck Millipore) and fixed for 20 min in 100% methanol (intercellular antigens; CHEMPUR, Piekary Śląskie, Poland) or 4% formaldehyde (extracellular antigens; CHEMPUR; 400 μl methanol/formaldehyde per well). Then, the cells were

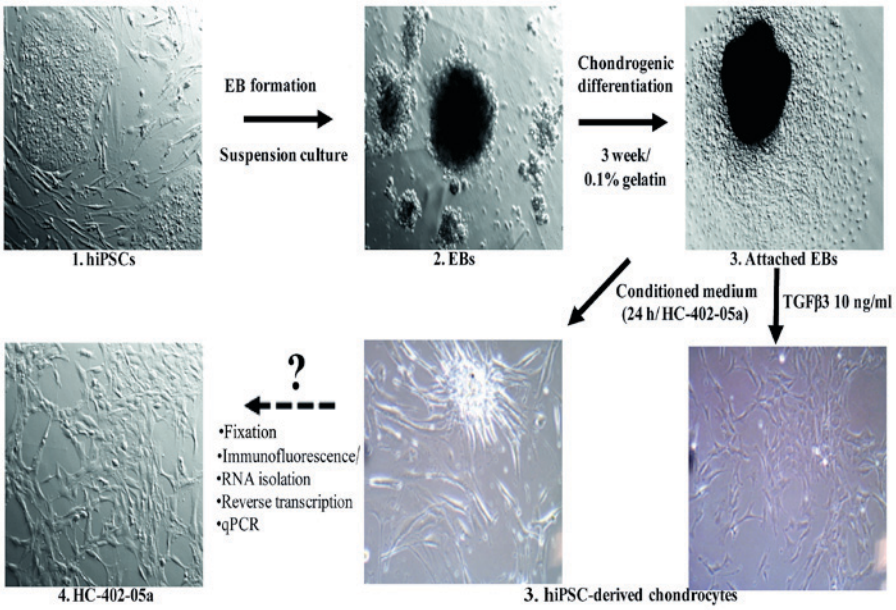

Figure 1. Schematic overview of the experiment. hiPSCs, human induced pluripotent stem cells; EB, embryoid bodies; TGF-β3, transforming growth factor β3; qPCR, quantitative polymerase chain reaction.

rinsed with PBS containing 1% FBS (Sigma Aldrich; Merck Millipore) and incubated for 30 min in PBS containing 1% FBS and 0.2% Triton X-100 (Sigma Aldrich; Merck Millipore) at room temperature. The cells were subsequently washed with PBS containing 1% FBS. The cells were incubated overnight at 4˚C with the following primary antibodies: COMP (1:100; cat. no. ab128893), type II collagen (COL2A1; 1:100; cat. no. ab34712), type IX collagen (COL9A1; 1:100; cat. no. ab134568), agreccan (AGC1; 1:85; cat. no. ab3778), SOX6 (1:50; cat. no. ab30455), SOX9 (1:50; cat. no. ab59252); all from Abcam, Cambridge, UK), Nanog (1:50; cat. no. MABD24) and octamer-binding transcription factor 3/4 (OCT3/4; 1:50; cat. no. MABD76); from BD Biosciences). The primary antibodies were diluted in PBS containing 1% FBS and 0.2% Triton X-100. Following conjugation with the primary antibodies, the cells were rinsed three times with PBS containing 1% FBS. The following Alexa Fluor 488 conjugated secondary antibodies were diluted with 1% FBS in PBS and were incubated in the dark for 1 h at 37˚C: Mouse monoclonal anti-immunoglobulin G (cat. no. 715-545-150), mouse monoclonal anti-immunoglobulin M (cat. no. 715-545-140) and rabbit polyclonal antibody (cat. no. 711-546-152; 1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Following washing three times with 1% FBS in PBS, the cells were stained for 5 min with diamidino-2-phenylindole dye (Sigma Aldrich; Merck Millipore) solution in water (1:10,000) followed by washing with PBS and fluorescent microscopic analysis. The intensity of the signals was evaluated using the bioinformatics programme ImageJ, version 1.49j (developed by National Institutes of Health, Bethesda, MD, USA).

RT-qPCR. Total RNA was extracted from cells (p3; $2x10^6$ cells) with TRIzol (Sigma Aldrich; Merck Millipore). Total RNA (1 μg per 20 μl reaction volume) free of genomic DNA contamination was reverse-transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol (25˚C for 5 min, 42˚C for 30 min, 85˚C for 5 min). cDNA was prepared three times for each repetition. qPCR reactions were performed using the LightCycler 480 Probes Master mix and appropriate probes labeled with fluorescein for each primer (Roche Diagnostics, Basel, Switzerland). The reaction conditions for all amplicons were as follows: Initially 95˚C for 10 min, followed by 45 cycles at 94˚C for 10 sec, 60˚C for 15 sec and 72˚C for 1 sec. All reactions were performed in the presence of 3.2 mM $MgCl_2$. cDNA samples (2.5 μl for a total volume of 10 μl) were analyzed for genes of interest and for the reference gene glyceraldehyde 3-phosphate dehydrogenase, which were selected based on the latest literature data concerning chondrogenic differentiation of hiPSCs (19). The level of expression of each target gene was calculated as $-2^{\Delta\Delta Cq}$ (20). The reaction was performed in triplicate for the genes of interest. Primer information is available upon request.

Statistical analysis. All experiments were performed a minimum of three times. The results are reported as the mean ± standard deviation. Comparisons between the study groups and controls were performed using one-way analysis of variance. Where the analysis of variance results were significant, post hoc analysis was performed via Tukey's multiple comparison test with a single pooled variance. Statistical tests were performed with GraphPad Prism (version 5.0a; GraphPad Software, Inc., San Diego, CA, USA). $^*P<0.05$ was considered to indicate a statistically significant difference.

Results

Immunofluorescence analysis confirmed that chondrocyte-like cells were obtained. To confirm the presence of markers characteristic of chondrocytes, the cells (p0) following chondrogenic differentiation in the TGF-β3 and conditioned media were analyzed by immunofluorescent staining. These

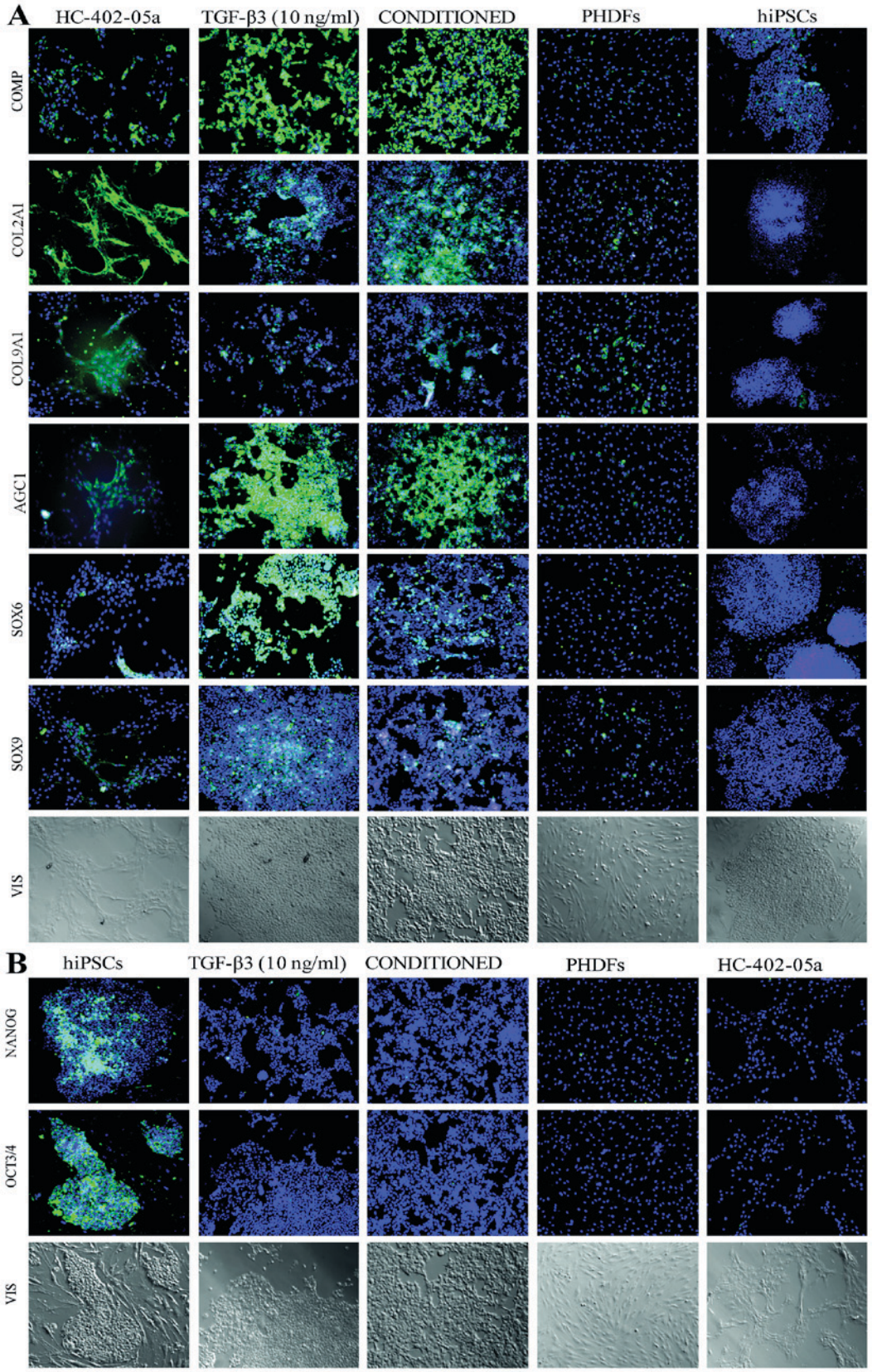

Figure 2. Immunofluoresence analysis of the chondrocyte-like cells instantly following the differentiation process, identified the presence of (A) markers characteristic of mature chondrocytes and (B) the simultaneous lack of pluripotency markers. hiPSCs, human induced pluripotent stem cells; TGF-β3, transforming growth factor β3; PHDFs, primary human dermal fibroblasts; COMP, cartilage oligomeric matrix protein; COL2A1, type II collagen; COL9A1, type IX collagen; AGC1, aggrecan; SOX, sex determining region Y-box; OCT3/4, octamer-binding transcription factor 3/4.

cells indicated the occurence COMP, COL2A1, COL9A1, AGC1, SOX6 and SOX9 at levels similar to those established in the HC-402-05a chondrocyte cell line (Figs. 2 and 3). Furthermore, the chondrocyte-like cells did not demonstrate the presence of

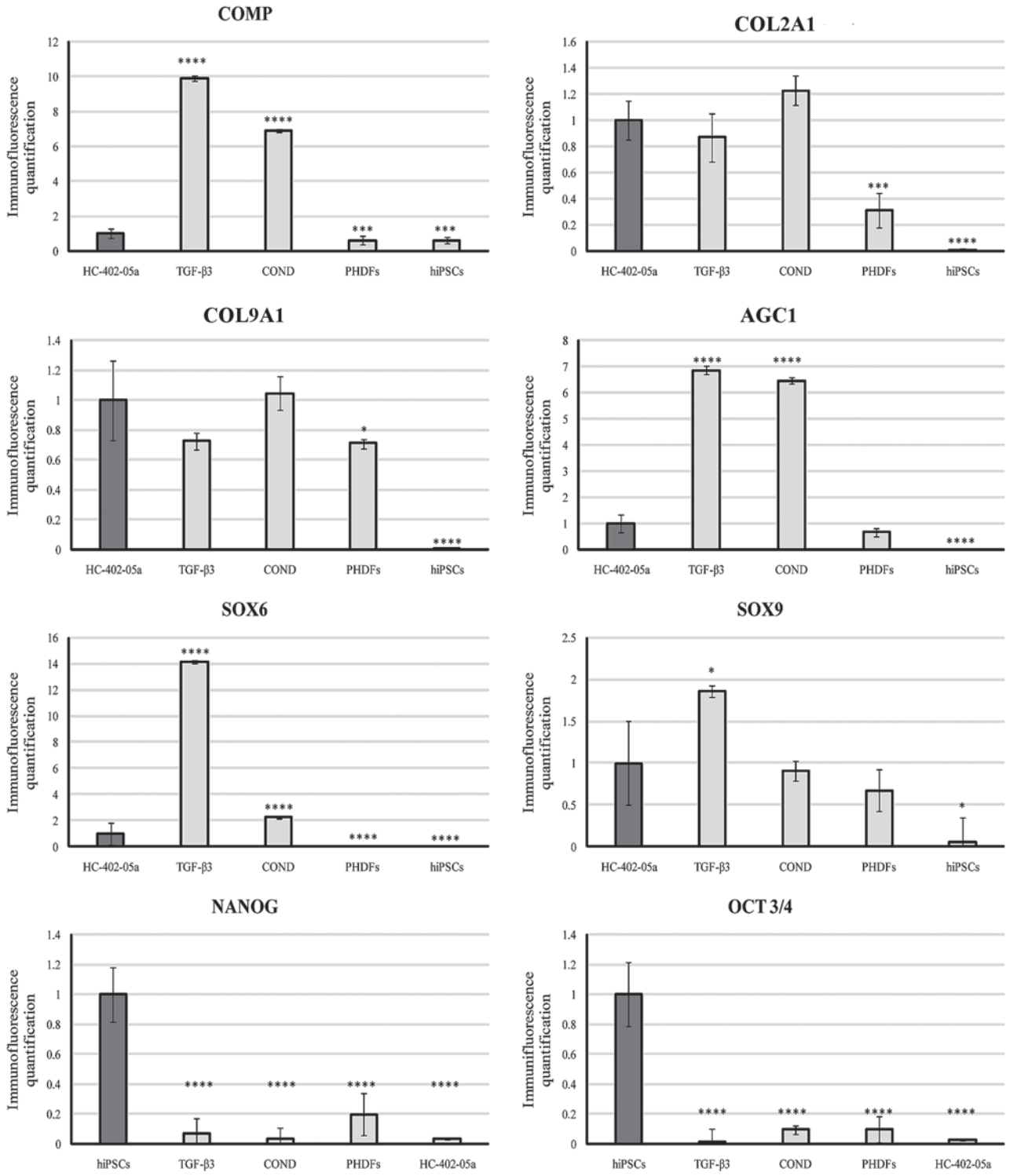

Figure 3. Quantified immunofluorescence analysis of chondrogenic markers and pluripotency markers. ****P<0.0001 vs. HC0402-05a. COMP, cartilage oligomeric matrix protein; COL2A1, type II collagen; COL9A1, type IX collagen; AGC1, aggrecan; SOX, sex determining region Y-box; OCT3/4, octamer-binding transcription factor 3/4; TGF-β3, transforming growth factor β3; COND, conditioned medium; PHDFs, primary human dermal fibroblasts; hiPSCs, human induced pluripotent stem cells.

pluripotency markers: Nanog and OCT3/4/OCT4 (Figs. 2 and 3). These results confirm that the obtained chondrocyte-like cells were fully differentiated from hiPSCs. Furthermore, they express the chondrocyte specific markers.

Pluripotency and mesodermal markers were not observed in the gene expression profiles of stem cell-derived chondrocytes. All cells were collected and analyzed following the third passage. Neither the cells differentiated in the TGF-β3 medium nor those differentiated in the conditioned medium expressed any of the following protein-coding genes assigned to pluripotency state: *Nanog*, *OCT4* and *SOX2* (Fig. 4). Furthermore, *E-cadherin*, a glycoprotein that is involved in embryogenesis by mediating cell-cell contact in hESCs, was

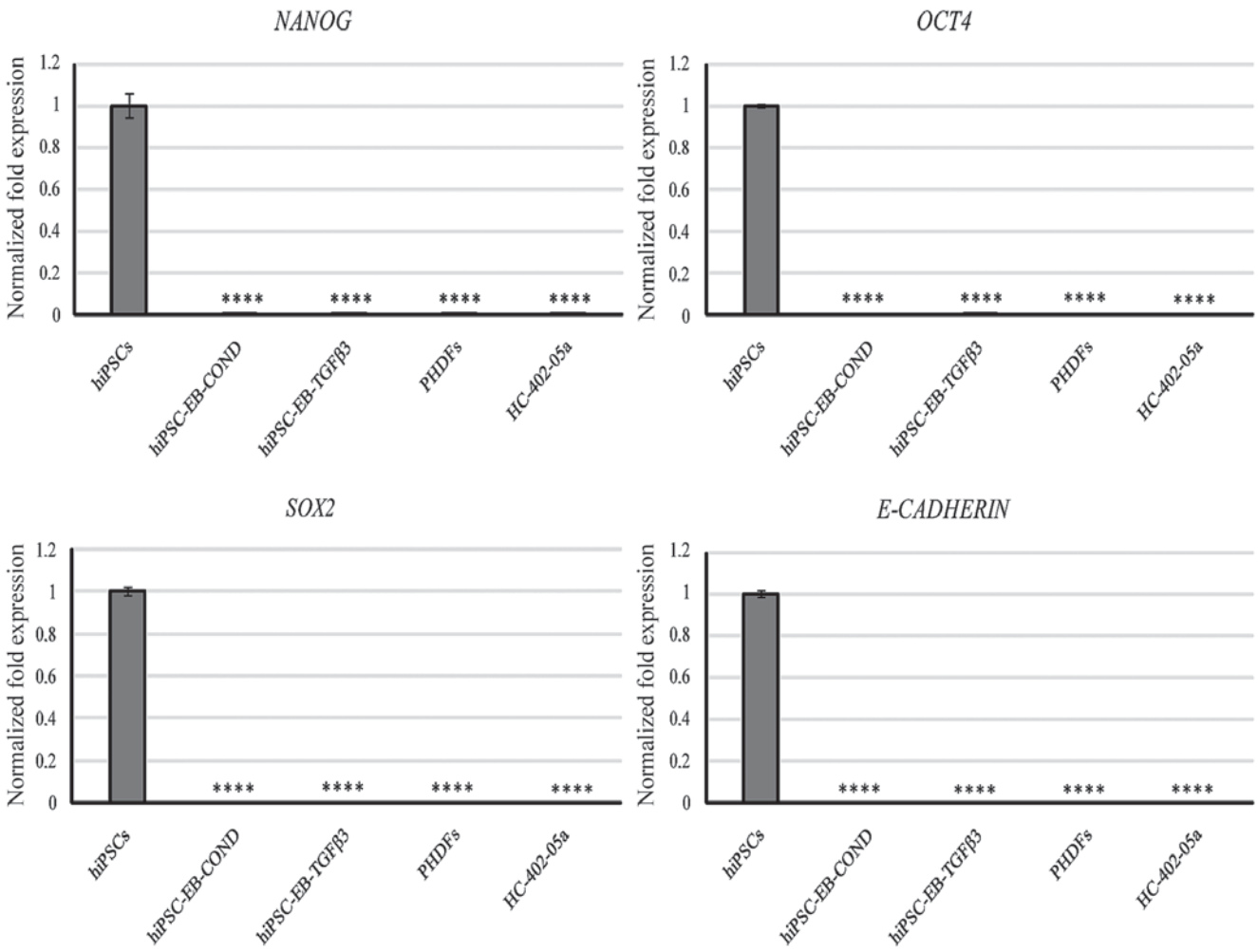

Figure 4. Based on quantitative polymerase chain reaction analysis, the hiPSC-derived chondrocytes differentiated in chondrogenic medium with TGF-β3 (10 ng/ml) or following conditioning on HC-402-05a cells lost markers characteristic for pluripotency. hiPSCs were used as a positive control. PHDFs and HC-402-05a cells were used as negative controls. ****P<0.0001 vs. hiPSCs. hiPSCs, human induced pluripotent stem cells; TGF-β3, transforming growth factor β3; EB, embryoid bodies; COND, conditioned medium; PHDFs, primary human dermal fibroblasts; OCT4, octamer-binding transcription factor 4; SOX2, sex determining region Y-box 2.

not expressed either (Fig. 4). In contrast, the positive control hiPSCs expressed these markers at a high level (Fig. 4). This finding indicates that these cells lost their pluripotent state. These markers are specific and may be useful to evaluate the loss of pluripotency state.

None of the investigated cells expressed the *Bra* gene (coding brachyury; data not shown), which is present in cells from the primitive streak or nascent mesoderm. This may indicate that the differentiated cells did not stop differentiating in the early stage of chondrogenesis. It is possible to assume that they had lost their pluripotent nature and were no longer mesodermal precursors. The forced chondrogenesis *in vitro* may have given rise to chondrocyte-like cells lacking mesodermal features. Furthermore, brachyury is a particularly specific marker because none of the controls [HC-402-05a, primary human dermal fibroblasts (PHDFs), and hiPSCs] expressed the *Bra* gene.

Likewise, expression of C-X-C motif chemokine receptor 4 (*CXCR4*) was not observed, which is active in the primitive streak, the endoderm and in later stages of embryogenesis, including intermediated and lateral plate mesoderm. *CXCR4* expression was present in HC-402-05a and hiPSCs (Fig. 5). This finding confirmed that the obtained cells did not present features characteristic of the mesoderm.

Assessment of markers engaged in induction of chondrogenesis. The presence of several markers necessary to induce chondrogenesis was also assessed: *PAX9*, neural cell adhesion molecule (*NCAM*) and NK-related homeodomain protein (*NKX3.2*). *PAX9* was observed only in HC-402-05a cells and in cells differentiated in the conditioned medium (Fig. 6). *NCAM* was expressed by all the studied cells, but at varying levels, with the most prominent expression observed in PHDFs and cells differentiated in the TGF-β3 medium (Fig. 6). *NKX3.2* was also present at a more stable level in all the study cells, in contrast to the more variable *NCAM*. *NKX3.2* expression was highest in HC-402-05a cells (Fig. 6).

Assessment of SOX gene expression. Next, the expression of a trio of transcription factors (*SOX5*, also called *L-SOX5* or *SOX5L*; *SOX6* and *SOX9*) belonging to the SRY family (encoded by the sex-determining region on the Y chromosome) was assessed. *SOX5* was expressed at low levels by all cells except for HC-402-05a (Fig. 7). Similar results were observed

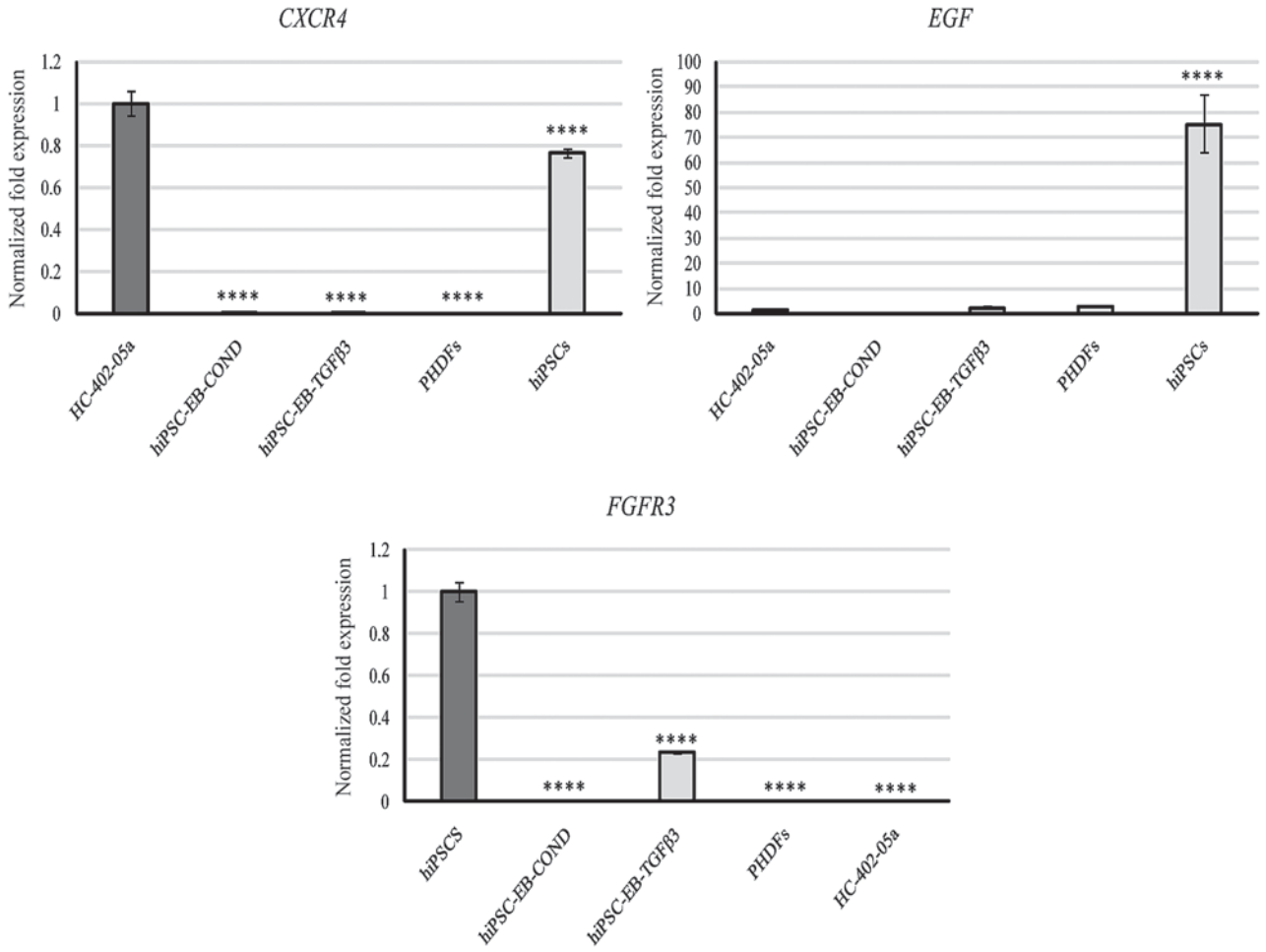

Figure 5. Based on quantitative polymerase chain reaction analysis, the hiPSC-derived chondrocytes differentiated in chondrogenic medium with TGF-β3 (10 ng/ml) or in medium conditioned with HC-402-05a cells did not remain in the first stage of chondrogenesis because of the lack of expression of *CXCR4*. Furthermore, *EGF* and *FGFR3* mRNA levels were low. The HC-402-05a cell line served as a positive control. PHDFs and hiPSCs were used as negative controls. ****P<0.0001 vs. HC-402-05a cells. hiPSCs, human induced pluripotent stem cells; TGF-β3, transforming growth factor β3; CXCR4, C-X-C motif chemokine receptor 4; EGF, epidermal growth factor; FGFR3, fibroblast growth factor receptor 3; PHDFs, primary human dermal fibroblasts; hiPSCs, human induced pluripotent stem cells; EB, embryoid bodies; COND, conditioned medium.

for *SOX6*, although cells differentiated in the conditioned medium expressed this marker at significantly higher levels than cells cultured in TGF-β3 (Fig. 7). The results obtained in the cells cultured in the conditioned medium were promising because expression of *SOX9*, one of the most important markers of chondrogenesis, was expressed highly in these cells compared with all other groups (Fig. 7).

Assessment of markers that are activated throughout the entirety of chondrogenesis. Next, the expression of markers involved in the entire chondrogenic process were assessed, including *IGF-1*, *CD44*, *β-catenin* and the components of the cartilage extracellular matrix (ECM; *TNC* and *COMP*). Cells differentiated in the conditioned medium expressed genes required for production of CD44, COMP, and β-catenin, while the cells cultured with TGF-β3 expressed β-catenin and, in particular, IGF-1 (Figs. 8 and 9). *TNC* was expressed by all cells, but expression was significantly higher only in PHDFs (Fig. 9).

Assessment of markers responsible for cell proliferation rate and the inhibition or enhancement of chondrogenesis. Finally, two markers used to evaluate the proliferation rate of cultured cells were examined: fibroblast growth factor receptor 3 (*FGFR3*) and epidermal growth factor (*EGF*). *FGFR3* expression was detected in cells differentiated in TGF-β3 medium and in hiPSCs (Fig. 5). The hiPSCs demonstrated significantly higher levels of *EGF* expression than all other groups (Fig. 5), suggesting a strong proliferative potential. *EGF* was also observed in the cells differentiated with TGF-β3 and in PHDFs, although *EGF* expression was significantly decreased in those cells compared with hiPSCs (Fig. 5).

Discussion

Current methods of differentiating hiPSCs into chondrocyte-like cells are not efficient and require further improvement. The present study evaluated and compared two different mediums used to differentiate hiPSCs into chondrocyte-like cells, and revealed that medium conditioned with human cartilage chondrocytes was a highly effective chondrogenic stimulator. Furthermore, the chondrogenic properties were demonstrated to change, even during short-term culture (passage 0 vs. 3). Immunofluorescence analysis confirmed that chondrocyte-like

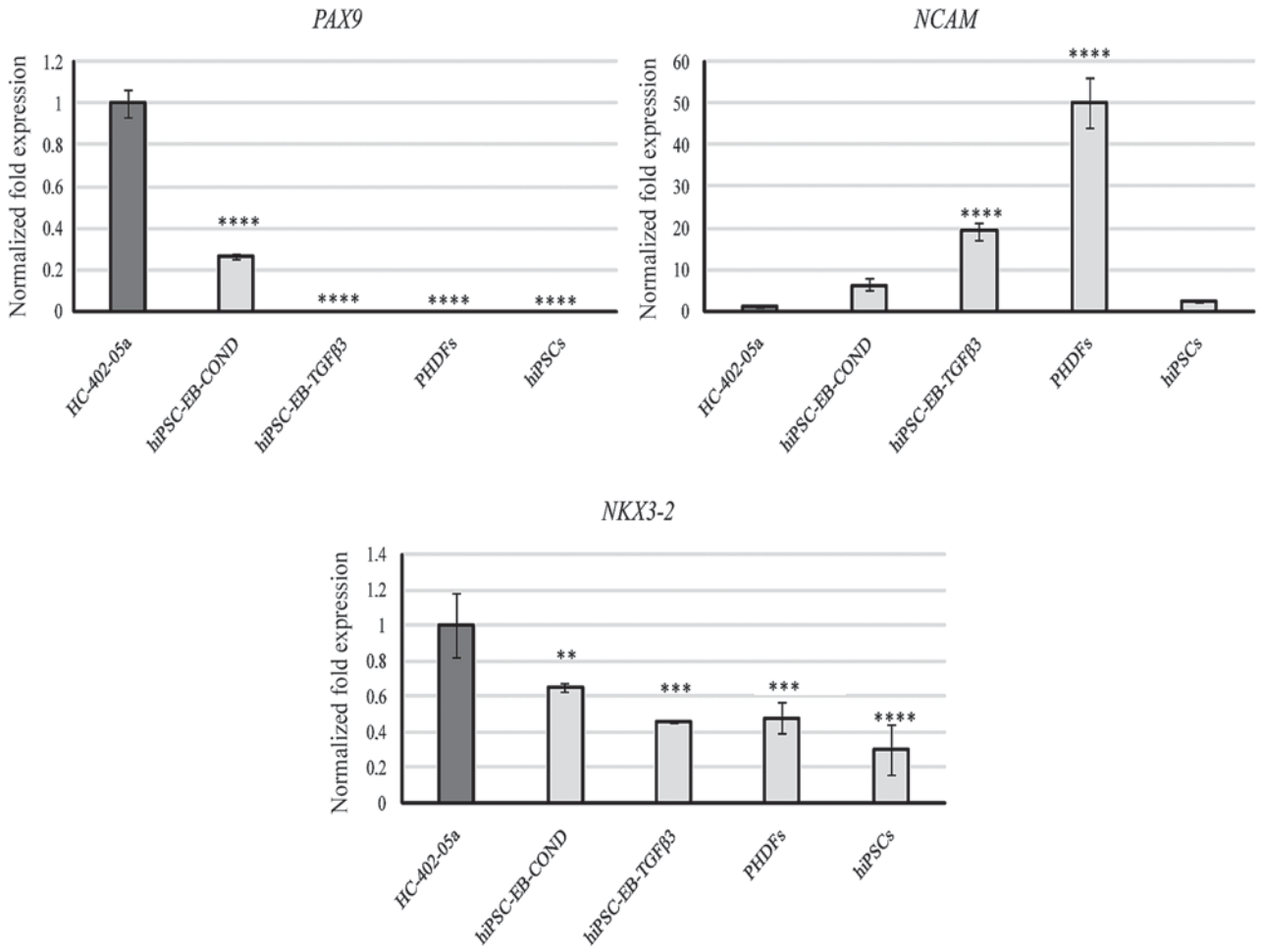

Figure 6. Based on quantitative polymerase chain reaction analysis, the hiPSC-derived chondrocytes differentiated in chondrogenic medium with TGF-β3 (10 ng/ml) or in medium conditioned with HC-402-05a cells revealed the expression of genes responsible for induction of chondrogenesis. The HC-402-05a cell line served as a positive control. PHDFs and hiPSCs were used as negative controls. **P<0.01, ***P<0.001, ****P<0.0001 vs. HC-402-05a cells. hiPSCs, human induced pluripotent stem cells; TGF-β3, transforming growth factor β3; PHDFs, primary human dermal fibroblasts; PAX9, paired box 9; NCAM, neural cell adhesion molecule; NKX3.2, NK-related homeodomain protein; EB, embryoid bodies; COND, conditioned medium.

cells were obtaine, and. qPCR analysis assessed the relative utility of the most commonly used chondrogenic markers as indicators of cell differentiation. The main aim of the present study was to evaluate the relative value of a wide range of chondrogenic markers to assess the progress of chondrogenic differentiation. Among the 20 different chondrogenic markers evaluated, it was possible to identify those that were the most useful as indicators of differentiation (Table I). This finding will help to improve and accelerate research involving hiPSC and chondrogenic differentiation.

The results of the present study confirmed that all the differentiated cells lost their pluripotent nature (Fig. 4). Furthermore, the results indicated that these cells did not preserve properties characteristic of early-phase differentiation involving the mesodermal stage (Fig. 5). Below, the markers of early chondrogenesis are discussed.

PAX1 and *PAX9* belong to the PAX family and are involved in the formation of the axial skeleton. They are characterized by the presence of a highly-conserved DNA binding domain, the paired box. *PAX1* and *PAX9* are the main mediators of Sonic hedgehog, which belongs to the Indian hedgehog (IHH) family, and are required to induce chondrogenesis. Once chondrogenesis has been initiated, expression of the *PAX* genes is downregulated (21,22). PAX9 is required to induce the chondrogenic process, and *PAX9* expression was demonstrated to be associated with *IHH* expression. The presence of *PAX9* mRNA was visible in cells differentiated in the conditioned medium, and these cells also exhibited a high level of *IHH* expression (16). In cells differentiated in the TGF-β3 medium, expression of *IHH* was lower (16) and *PAX9* expression was lower compared with cells cultured in the conditioned medium. This observation indicated that these cells originated from late stages of chondrogenesis, during which *PAX9* expression is downregulated (Fig. 6). These two signaling pathways are associated and were previously confirmed to be dependent on each other (21,22). *PAX* genes offer a promising strategy to evaluate the progression of *in vitro* chondrogenesis, because *PAX9* is not expressed by hiPSCs nor by PHDFs.

Condensation, the first stage of chondrogenesis, depends on expression of the cell-cell adhesion proteins N-cadherin and NCAM. Expression of these molecules is rapidly reduced when cells shift into the differentiation phase, resulting in the release of cells from strong interactions with each other (23). In healthy cartilage, there are no cell-cell contacts, however there are functional cell-matrix contacts that are primarily integrin-mediated (24). In addition, as *NCAM* is expressed in

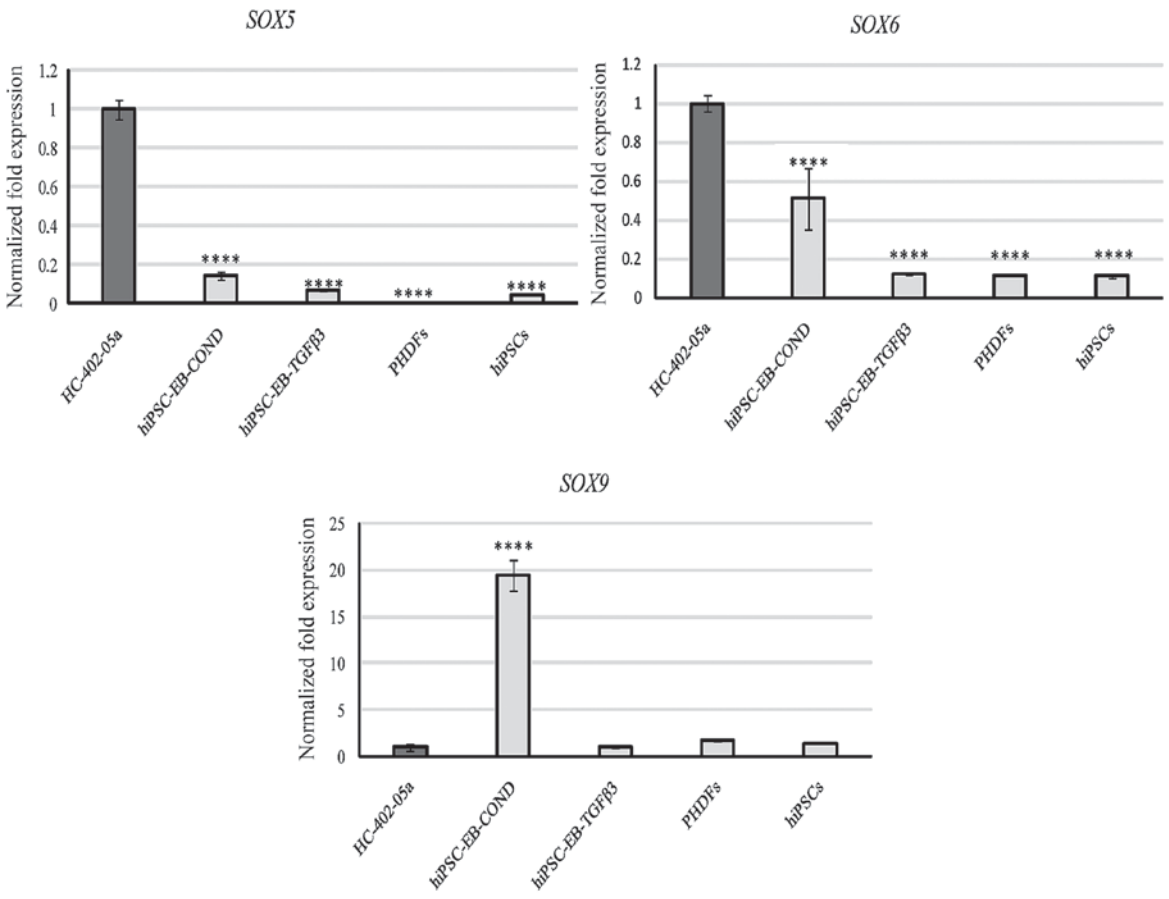

Figure 7. Based on quantitative polymerase chain reaction analysis, the markers of early chondrogenesis were highly expressed by the cells differentiated in HC-402-05a-conditioned medium. The HC-402-05a cell line served as a positive control. PHDFs and hiPSCs were used as negative controls. ****P<0.0001 vs. HC-402-05a cells. PHDFs, primary human dermal fibroblasts; hiPSCs, human induced pluripotent stem cells; SOX, sex determining region Y-box; EB, embryoid bodies; COND, conditioned medium.

osteoprogenitor cells and osteoblasts but not in chondrocytes, chondroprogenitor cells, or chondroblasts, NCAM is involved in the induction of secondary chondrogenesis (25). NCAM mediates not only cell-to-cell binding, but also the interaction between cells and components of the ECM, including heparin sulfate proteoglycans and collagens. It is relevant to the regenerative process, where fibroblasts serve an important function (26).

Francavilla *et al* (27) demonstrated that NCAM has the ability to repress several FGF-induced processes, including signal transduction and cell proliferation. The negative effect of NCAM depends on its capacity to compete with FGF to bind to the FGF receptor. However, the data from the present contradict the results from the study by Francavilla *et al* (27): Cells differentiated in the presence of TGF-β3 demonstrated high levels of *FGFR3* and *NCAM* in the present study. Due to the presence of other hypertrophic and osteogenic markers, the high level of NCAM observed in cells cultured in TGF-β3 is likely to be associated with secondary chondrogenesis rather than the condensation stage. The cells differentiated in the conditioned medium additionally presented with a relatively high level of *NCAM* expression (Fig. 6). Nevertheless, due to the fact that other hypertrophic and osteogenic markers were observed at very low levels, it is possible to assume that these cells scarcely shifted to advanced chondrogenesis or the hypertrophic stage. NCAM is characteristic of fibroblasts, thus it is not surprising that these cells presented with high levels of these markers, thereby reducing the usefulness of NCAM as a marker of chondrogenesis.

The pro-chondrogenic NK-related homeodomain protein NKX3.2 is required to activate the master chondrogenic transcriptional regulator SOX9. The presence of this protein results in the expression of chondrocyte phenotypic genes including *AGC1*, *COL2A1,* and components of cartilaginous ECM. A feedback loop exists between NKX3.2 and runt-related transcription factor 2 (RUNX2), leading to repression of RUNX2. In osteoprogenitor cells, the ability of NKX3.2 to repress RUNX2 is abrogated, suggesting the existence of a switching mechanism from chondrogenesis to osteoblast formation (28,29). The initial induction of NKX3.2 in chondrocyte precursor cells during early-stage chondrocyte formation and its downregulation in the terminal-stage of chondrogenesis is controlled by the IHH pathway, a key regulator of chondrocyte hypertrophy (30).

NKX3.2 was expressed by all the investigated cells. The presence of *NKX3.2* would appear to suggest that differentiated cells underwent chondrogenesis. Unfortunately, because *NKX3.2* was also observed at low levels in hiPSCs and PHDF, its use in the evaluation of chondrogenesis may be limited (Fig. 6).

The SOX trio of transcription factors (SOX5, SOX6, and SOX9) belong to the SRY family (encoded by the

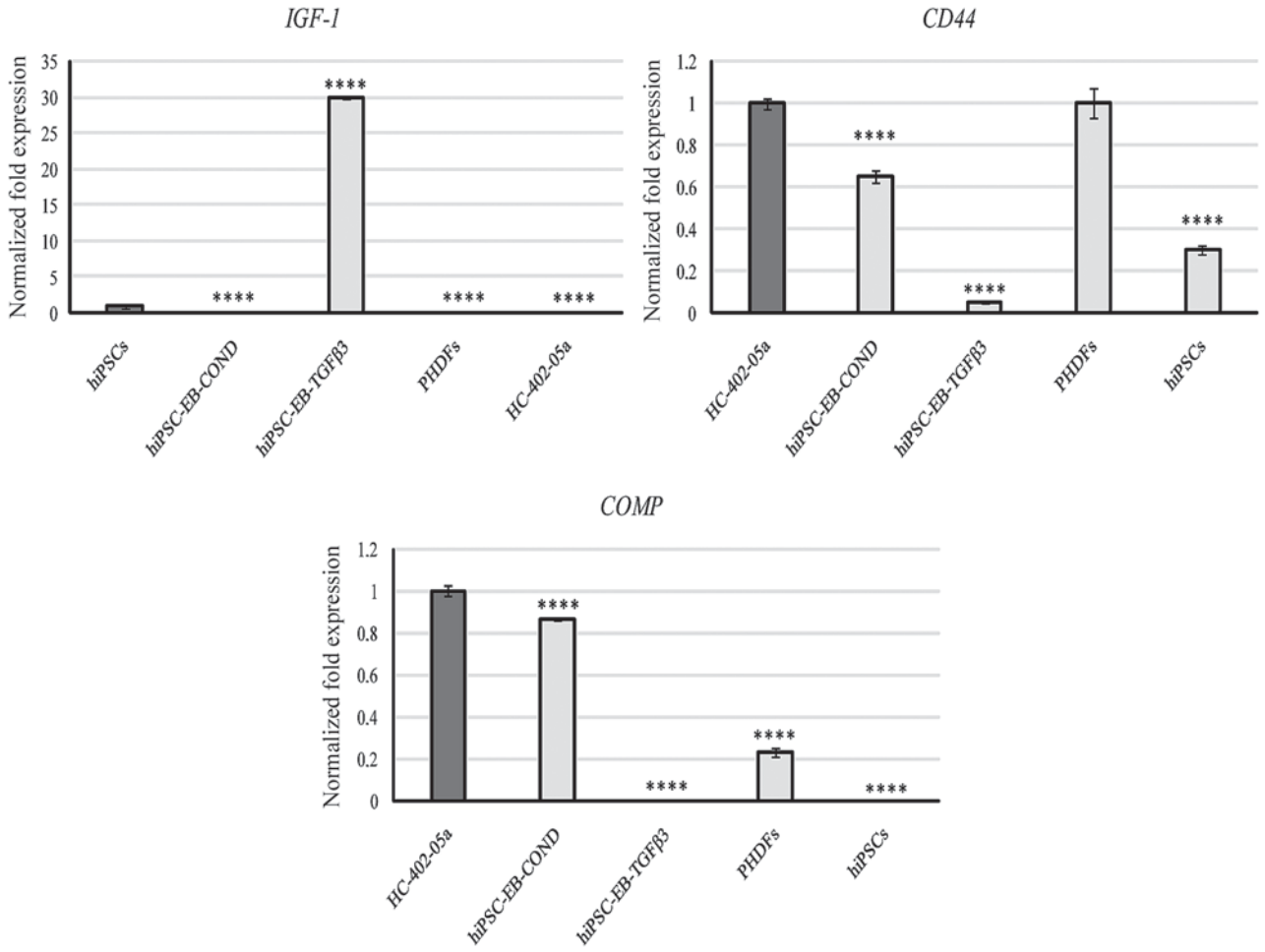

Figure 8. Based on quantitative polymerase chain reaction analysis, the hiPSC-derived chondrocytes differentiated in chondrogenic medium with TGF-β3 (10 ng/ml) or in medium conditioned with HC-402-05a cells demonstrated diverse expression of genes assigned to chondroprogenitors and chondrocytes. The HC-402-05a cell line served as a positive control. PHDFs and hiPSCs were used as negative controls. ****P<0.0001 vs. HC-402-05a cells. hiPSCs, human induced pluripotent stem cells; TGF-β3, transforming growth factor β3; PHDFs, primary human dermal fibroblasts; IGF-1, insulin-like growth factor 1; COMP, cartilage oligomeric matrix protein; EB, embryoid bodies; COND, conditioned medium.

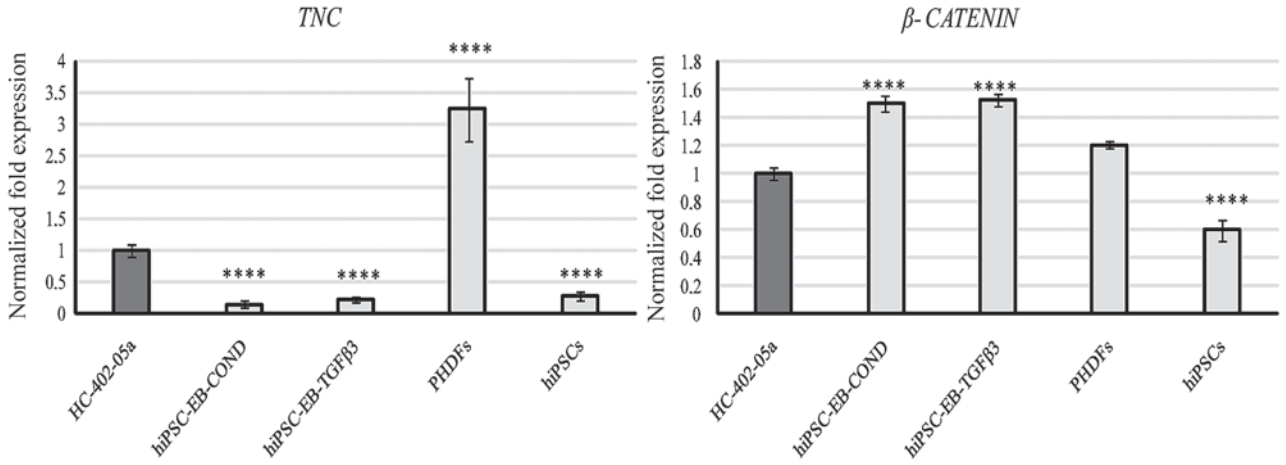

Figure 9. Based on quantitative polymerase chain reaction analysis, the hiPSC-derived chondrocytes differentiated in chondrogenic medium with TGF-β3 (10 ng/ml) or in medium conditioned with HC-402-05a cells expressed *TNC* and *β-catenin* at low and high levels, respectively. The HC-402-05a cell line served as a positive control. PHDFs and hiPSCs were used as negative controls. ****P<0.0001 vs. HC-402-05a cells. hiPSCs, human induced pluripotent stem cells; TGF-β3, transforming growth factor β3; TNC, Tenascin-C; PHDFs, primary human dermal fibroblasts; EB, embryoid bodies; COND, conditioned medium.

sex-determining region on the Y chromosome). They are expressed in proliferating and prehypertrophic chondrocytes. However, in hypertrophic chondrocytes, expression of *SOX* genes is turned off. In contrast to *SOX5* and *SOX6, SOX9* is

required for chondrogenesis. Nevertheless, the lack of *SOX5* and *SOX6* results in defective skeletogenesis. *SOX9* is also upregulated via FGFR3 signaling, and there is a positive regulatory loop between these (31). *SOX9* is required to activate other cartilage genes including *COL2A1* and *AGC1*. *SOX5* and *SOX6* increase the binding efficiency of SOX9 to other cartilage-specific enhancers. In the absence of *SOX5* and *SOX6*, the expression of *COL2A1*, *AGC1* and other chondrocyte markers is either very low or undetectable. Without the presence of SOX5 and SOX6, SOX9 has a limited capacity to bind to the other cartilage enhancers (32). Yamamizu *et al* (33) examined the involvement of SOX9 in the repression of SOX2, and reported that SOX9 has a significant influence on the cyclin dependent kinase inhibitor 1A (CDKN1A)-SOX2 pathway. SOX9 activity induces the formation of p21, which subsequently binds to the SRR2 enhancer of SOX2, inhibiting its expression and facilitating differentiation. Furthermore, SOX9 has the ability to compete with T-cell factor/lymphoid enhancer factor (Tcf-Lef) to bind to β-catenin, resulting in its degradation. This suggests that the chondrogenic process is regulated by the interaction between SOX9 and the WNT/β-catenin signaling pathway. SOX9 also inhibits the activity of the cyclin D1 promoter, which has a high affinity for the β-catenin/Tcf-Lef complex. The WNT/β-catenin signaling pathway inhibits the differentiation of chondrocyte precursors and initiates the progression of mature chondrocytes towards hypertrophy (34). SOX9 prevents osteogenic bone morphogenetic protein-2 (BMP-2) and RUNX2-induced osteogenic differentiation and endochondral ossification, respectively. BMP-2 has a high capacity to induce chondrogenic differentiation but also undesirable hypertrophic differentiation. The forced overexpression of SOX9 in BMP-2-mediated chondrogenic differentiation seems to be a promising strategy for cartilage tissue engineering (35).

Based on the results of the present study, it appears that cells cultured in the conditioned medium may originate from early or advanced chondrogenesis, in which the expression of *SOX9* is most prominent (Fig. 7). In addition, these cells did not express hypertrophic markers, which include *RUNX2* (16). In contrast, the cells differentiated in the TGF-β3 medium expressed low levels of *SOX9* and high levels of *RUNX2*, an observation that suggests these cells underwent hypertrophy. The low levels of *SOX5* and *SOX6* mRNA explain the lack of expression of the *COL2A1* and *AGC1* genes in all differentiated cells. This result is supported by the fact that the mRNA level of *COL2A1* and *AGC1* in cultured chondrocytes abruptly decreased following the first passage while, by contrast, the expression of genes that code for type I and X collagen increased or remained unchanged, respectively (36). This may also explain the lack of expression of *COL2A1* and *AGC1* genes in the cell samples obtained following the third passage. Growth factors including TGF-β and bone morphogenetic protein-7, in addition to growth and differentiation factor 5, promote AGC1 synthesis while simultaneously preventing its degradation (37). Indeed, it is this collagen/AGC1 network that gives cartilage its viscoelastic nature with stiff elastic polymer properties, making it resistant to sudden impact loading with slow inelastic deformation under sustained load (38).

The results of the present study indicate that the expression of the *SOX trio* is likely to be a good prognostic marker for cells undergoing chondrogenic differentiation. By contrast, interpretation of *COL2A1* and *AGC1* as markers of chondrogenic differentiation is more complex because, as other authors have previously demonstrated (36), the expression of these genes rapidly decreases as the number of passages and the culture period increases, and these are highly dependent on the expression of other markers, including the *SOX* trio genes. Based on the immunofluoresence and qPCR analyses, earlier literature data indicating that the production of COL2A1 and AGC1 decreases with passage number and duration of culture was confirmed (passage 0 vs. 3).

IGF-1 is active during the entire chondrogenic process. It promotes the synthesis of COL2A1 and proteoglycans and stabilizes the chondrocyte phenotype in pathological conditions. IGF-1 and BMP-2 are predominantly present in the proliferative and hypertrophic layers, however additionally, rarely, in the calcified chondrocyte zone, in contrast with TGF-β1 (39). Activation of IGF-1 correlates with the presence of the type 1 IGF receptor, which becomes elevated in human osteoarthritic chondrocytes as a function of disease severity. IGF binding proteins regulate the density of IGF-1 receptors on the cell surface and the levels of activated IGF-1 (40). The IGF-1 signaling pathway is involved in the regulation of growth plate development and cell size during chondrogenesis. During phosphatidylinositol 3-kinase (P13K)-protein kinase B (Akt) signaling, IGF-2 and RUNX2 are dependent on each other to coordinate osteoblast and chondrocyte differentiation and migration. IGF-1 is suggested to be a major ligand for the activation of P13K-Akt signaling and RUNX2 (41,42). Although IGF-1 is associated with chondrogenesis, a previous study suggested that it may be involved in the maintenance of SC pluripotency (42). In mouse spermatogonial SCs, IGF-1 is secreted from Leydig cells as a key factor in sustaining a pluripotent state. Blockage of endocrine factor IGF-1 receptor phosphorylation and its downstream P13K/Akt signaling pathway reduces the activity and expression of pluripotency genes including *Nanog, OCT4* and PR domain zinc finger protein 1 (43). However, data concerning human pluripotent SCs are lacking.

IGF-1 expression in cells differentiated in the presence of TGF-β3 was high compared with *IGF-1* expression in human articular chondrocytes (Fig. 8). This phenomenon is associated with the transition from chondrogenic-like cells into hypertrophic chondrogenic-like cells, rather than cells from early chondrogenesis. This observation is confirmed by the high expression of *RUNX2* (16), which is associated with IGF-1 via the P13K/Akt signaling pathway.

Hyaluronan (HA) is a linear polymer-glycosaminoglycan that is distributed throughout the extracellular space of connective tissues, including articular cartilage. HA forms the backbone of proteoglycan aggregates, which primarily consist of AGC1 interacting with COL2A1. HA, together with proteoglycan aggregates, ensures the load-bearing capacity of the tissue (44). Takahashi *et al* (45) demonstrated that fragmentation of HA receptor CD44 is a common phenomenon in dedifferentiated and osteoarthritic chondrocytes, caused by the secretion and activity of matrix metalloproteinases (MMP), leading to cleavage of CD44. The disruption of CD44 may cause matrix turnover and enhanced catabolism, which are hallmarks of early osteoarthritis. *CD44* is highly expressed in human parental fibroblasts and is gradually lost

during the reprogramming process. It influences the adhesion and motility of fibroblasts throughout TGF-β activation, and is critical in lesions because, as fibroblasts migrate to the site of injury, CD44 controls inflammation and initiates the repair process (46,47).

The cells differentiated in the conditioned medium presented *CD44* levels similar to those observed in human articular chondrocytes while, by contrast, cells obtained from the TGF-β3 medium did not express *CD44* (Fig. 8). This lack of *CD44* expression may be due to the activity of MMPs, which were highly expressed during the present study and may have presented undesirable features of dedifferentiated and/or osteoarthritic chondrocytes (16). It is necessary to be cautious when considering CD44 as a marker for the chondrogenic process *in vitro* due to the fact that, as the published data indicate (44,45), CD44 is present in fibroblasts and at the vestigial level in human pluripotent SCs.

COMP is an important component of the cartilage ECM. It has the ability to interact with COL2A1 and AGC1 as well as other ECM components. COMP has a large impact on cartilage phenotype development, and on the matrix organization and load support function of cartilage. A COMP deficiency in the joints is correlated with arthritis. This protein holds promise as a diagnostic and prognostic factor as a marker of disease progression and the effect of treatment (48,49).

In the present study, the conditioned medium stimulated expression of *COMP*. Furthermore, the level of expression was similar to that observed in human articular chondrocytes, suggesting that cells cultured in the conditioned medium have chondrogenic properties. In contrast, *COMP* was not observed in cells cultured in the presence of TGF-β. PHDFs and hiPSCs expressed this marker at low levels, thus it is possible to use this marker to assess the gene profile expression of differentiating cells (Fig. 8).

TNC is an oligomeric glycoprotein of ECM expressed during various processes, including neural development, tissue remodeling, wound healing, angiogenesis and tumorigenesis. This marker was suggested to be tissue-specific due to its high concentration in articular cartilage. However, compared with human articular chondrocytes, malignant cells produce TNC in higher quantities (50). TNC has proliferative and anti-adhesive properties and is considered, therefore, to have metastatic potential (50). Data indicate that TNC is highly active during early chondrogenic differentiation, for example during mesenchymal condensation, and is turned off in cartilage with progressive chondrocyte maturation. Although the fibrinogen-like domain of TNC is indispensable, it is not sufficient by itself for the induction of chondrogenesis (51). TNC is involved in fibroblast migration and infiltration into the provisional matrix in response to injury. This suggests that *TNC* expression and degradation is tightly controlled to ensure efficient tissue rebuilding (52).

TNC was expressed by all the cells evaluated in the present study. The highest levels of expression were detected in PHDFs vs. the positive controls (human articular chondrocytes) (Fig. 9). Although *TNC* is expressed in the articular cartilage, it is also involved in multiple cellular processes, thus reducing its value as a marker of chondrogenesis.

Control of the WNT/β-catenin signaling pathway helps to make the reprogramming process more efficient. Augmented reprogramming is observed as a result of interaction between WNT/β-catenin and reprogramming factors (*OCT4*, *SOX2* and Kruppel-like factor 4) and other endogenous core pluripotency genes, although it does not affect v-myc myelocytomatosis viral oncogene homolog expression. This signaling pathway is critical for the reprogramming process and its influence is most apparent during the initial stage where interaction with the T-cell factor is important. Nevertheless, WNT/β-catenin is not required to maintain cell pluripotency (53). Qiu *et al* (54) demonstrated that the self-renewal-promoting WNT/β-catenin effect is predominantly triggered by two of its downstream targets, *KLF2* and transcription factor CP2-like 1 (*TCFP2L1).* The downregulation of these two genes impairs mouse embryonic stem cell self-renewal mediated by WNT/β-catenin, and conversely the overexpression of *KLF2* and *TCFP2L1* recapitulates the self-renewal-promoting effect (54). Furthermore, Nanog and β-catenin (coded by *CTNNB1*) cooperate in establishing pluripotency during the reprogramming process. Nanog inhibits Dickkopf-related protein 1, which leads to β-catenin activation and accumulation, which, in turn, is essential for Nanog-dependent conversion of pre-miPSCs into miPSCs. Thus, the crosstalk between Nanog and the WNT/β-catenin signaling pathway is relevant for ESC physiology, as it results in a synergistic effect (55). In human PSCs, the contribution of the WNT/β-catenin signaling pathway in promoting self-renewal of hESCs is unclear. Certain data suggest that this signaling pathway is involved in hESC proliferation and self-renewal, but following multiple passages of hESCs the effect disappears. Davidson *et al* (56) demonstrated that the WNT/β-catenin signaling pathway is assigned to differentiation towards mesodermal lineages rather than self-renewal. They also demonstrated that OCT3/4, a key pluripotency factor, represses endogenous WNT/β-catenin signaling in hESCs.

The WNT/β-catenin signaling pathway is also involved in chondrogenesis. N-cadherin, required for temporal mitogen-activated protein kinase (MAPK) 1/2, p38 MAPK and BMP-2-mediated regulation of chondrogenic genes including *SOX9*, *AGC1* and *COL2A1*, modulates the potential WNT-induced nuclear activity of β-catenin. N-cadherin-mediated redistribution of β-catenin appears to be a mechanism by which WNT-mediated chondrogenesis is kept under control (57). The WNT/β-catenin pathway is involved in the fracture repair process and bone healing through early cartilage callus formation, endochondral ossification, induction of vascularization, late stage remodeling and recovery of mechanical strength. Inhibition of this pathway results in decreased expression of the following chondrogenic and osteogenic genes: Type I, II and X collagen, *MMP-13*, alkaline phosphatase, osteocalcin, *SOX9*, *RUNX2* and vascular endothelial growth factor (58). WNT/β-catenin signaling is activated by TGF-β-mediated SMAD family member 3 (SMAD3), which increases β-catenin signaling and its nuclear translocation. The cooperation between TGF-β members and β-catenin results in increased expression of cyclin D_1 in the chondrocytes (59).

The results of the present study confirm that the β-catenin signaling pathway may be involved in the self-renewal of pluripotent SCs and in the chondrogenic process. Expression of β-catenin was evident in cells differentiated in the presence of

TGF-β3, those differentiated in the HC-402-05a-conditioned medium and also in all control cells: Human articular chondrocytes, human primary fibroblasts and hiPSCs (Fig. 9). This level of expression in differentiated cells may be associated with N-cadherin-mediated redistribution of β-catenin during chondrogenesis. In turn, the high level of *β-catenin* mRNA in hiPSCs may be associated with interactions between WNT/β-catenin and pluripotency factors including Nanog. The presence of the activated β-catenin signaling pathway may explain the high level of *SMAD3* expression (16), as a result of SMAD3-mediated activation of β-catenin. Because the WNT/β-catenin signaling pathway is engaged in multiple processes, from self-renewal to differentiation, it is difficult to use as a marker for iPSC differentiation.

In the present study, two protocols to obtain chondrocyte-like cells from hiPSCs via embryoid bodies have been described, either with the addition of TGF-β3 to the chondrogenic medium, or using a chondrogenic medium conditioned with HC-402-05a cells. The chondrocyte-like cells obtained in the present study expressed genes that are present during early chondrogenesis. Furthermore, the value of several of these genes as markers of chondrogenic progression was demonstrated: *PAX9*, *SOX5*, *SOX6*, *SOX9* and *COMP* were all good markers of hiPSC differentiation. In contrast, other markers including *IGF-1*, *TNC* and *β-catenin* were less valuable. Notably, because certain markers are also expressed by PHDFs, these must be used with caution, taking into account the dedifferentiation process or transcriptional memory of their parental somatic cells. Thus, the origin of hiPSCs has an impact on their further differentiation toward chondrocyte-like cells deriving from the same germ layer as the parental cells of hiPSCs; reprogrammed fibroblasts.

The present study contributes to an improved understanding of the chondrogenic process. In addition, the obtained hiPSC-derived chondrocytes were demonstrated to be quite unstable and the chondrogenic features varied among the number of passages and duration of culture. Therefore, current protocols based on hiPSC differentiation require further improvements, particularly with regard to future scaled-up culture of differentiated hiPSCs and their subsequent application in clinical practice. The present study provides a method to more efficiently assess forced differentiation towards chondrocytes. Nevertheless, given the preliminary nature of the present study, more research is required to reach definitive conclusions.

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