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Human Primary Articular Chondrocytes, Chondroblasts-Like Cells, and Dedifferentiated Chondrocytes: Differences in Gene, MicroRNA, and Protein Expression and Phenotype

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In this study we have isolated human primary uncultured articular chondrocytes. When these cells are allowed to proliferate within their own extracellular matrix (ECM), they begin to produce hyaline ECM molecules similar to embryological chondroblasts. These cells are called chondroblast-like cells. Upon continued culture these cells spread onto the plastic surface and dedifferentiate. We have characterized these three stages of chondral cells by gene expression and expression of microRNAs (miRNAs) and proteins. Gene expression was quantified by real-time reverse transcriptase (RT) polymerase chain reaction, miRNA expression by miRNA arrays, and protein synthesis by extra- and intracellular flow cytometry. Many of the genes, miRNAs, and proteins were differentially expressed in the different stages of chondral cells. In the context of cellular therapy, expression of some genes is a cause for concern. The best source of cells for treatment of lesions of hyaline cartilage has not yet been identified. Adult chondroblast-like cells may be strong candidates. Profound understanding of how expression of genes and synthesis of proteins are regulated in these cells, for instance, by miRNAs, may reveal new strategies for improving their synthesis of hyaline ECM. This insight is important to be able to use these cells in the clinic.

Introduction

Autologous chondrocyte implantation (ACI) is now standard therapy in many centers for patients with focal lesions of hyaline articular cartilage.[1,2] ACI requires the removal of a small piece of articular cartilage, followed by *ex vivo* expansion of its content of articular chondrocytes (AC) to obtain large number of cells for implantation. *Ex vivo* expansion of AC in monolayer cultures invariably leads to dedifferentiation of the chondrocytes.[3,4] This means that the AC revert to a less specialized cell type, where synthesis of the hyaline cartilage-associated proteins type II collagen (COL2) and aggrecan (ACAN) is replaced by synthesis of COL1 and versican (VCAN), respectively.[5] These extracellular matrix (ECM) components are typical of fibrocartilage.[6,7] Transplantation of dedifferentiated chondrocytes frequently leads to the formation of fibrocartilage, or a mixture of fibrocartilage and hyaline cartilage within the lesion.[8,9] It is believed that this repair tissue has inferior functionality and durability, and that an end result may be arthrosis in that joint.[10] Most likely, a cell population more similar to the AC found *in vivo* is needed for implantation to improve the outcome of ACI. Recently, we established a new culture system where AC were cultured in their own ECM.[11] At day 7–10 of culture the cells maintained a rounded shape and expressed genes for several hyaline ECM molecules while still proliferating. As these cells have many of the features of embryological chondroblasts, they are called chondroblast-like cells (CBLC).

Although the use of CBLC in clinical transplantation might well improve on the clinical results obtained by using dedifferentiated chondrocytes, this has not yet been demonstrated clinically. We are presently conducting clinical trials using these cells. However, to further improve cell therapy strategies it is important to fully understand the biology of the cell populations used. This includes quantification of the genes expressed, the proteins they encode, and understanding the mechanisms that regulate gene expression. MicroRNAs (miRNAs) are a recently discovered class of molecules that affect both mRNA expression and protein synthesis. miRNAs are small single-stranded RNA molecules (~21–22 nt). miRNAs are derived from long primary transcripts (pri-miRNA, 2 kb in length) that are mainly transcribed by the RNA polymerase II. In the nucleus the pri-miRNAs are processed by the RNase Drosha to create short stem-loop molecules called pre-miRNAs (~70 nt) that are exported to the cytoplasm. In the cytoplasm the pre-miRNAs are further processed by the cytoplasmic RNase Dicer into a 21–22 nt miRNA duplex. The miRNA duplex is then assembled into the RNA-induced silencing complex where one of the strands (mature miRNA) base-pair with complementary mRNA transcripts.[12,13] It is thought that perfect complementary leads to degradation of the mRNA,

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whereas imperfect complementary results in translational inhibition.[14,15] It is generally accepted that the main function of miRNA is to knock down gene activity, although it has been shown to activate transcription also.[16] One-third of all human genes have been estimated to be regulated by miRNA.[17] As expression of individual miRNA relatively easily may be either increased or decreased by introducing miRNA precursor molecules or antagonists,[18] insight into expression of miRNA in the different types of chondral cells may introduce a new strategy for enhancing expression of genes expressing ECM molecules or decreasing expression of genes and proteins associated with dedifferentiation.

The objective of this study was to investigate the three cell populations: primary uncultured chondrocytes, CBLC, and dedifferentiated chondrocytes with respect to their expression of a large number of genes, proteins, and miRNA related to chondrogenesis. The results add to our understanding of the biology of *in vitro* chondrogenesis, and may also introduce new strategies for manipulation of chondrocytes used in clinical protocols.

Material and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Isolation and culture of the cells

Articular cartilage was obtained from the femoral surface of three knees obtained from legs amputated for reasons other than cartilage disease. The donors were 84, 79, and 76 years of age, and all provided written informed consent. The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway, Section A. The cells was isolated as previously described.[11] Briefly, all the articular cartilage with normal and healthy appearance was harvested and cut into small pieces. Only for one donor (donor 2) there were minor areas of the cartilage with abnormal appearance. This patient had no previous history of osteoarthritis in the knee. The cartilage pieces were digested with Collagenase type XI (1.2 mg/mL) at 37°C for 90–120 min. The ensuing structures, called chondrocytes in autologous ECM, were washed several times and resuspended in culture medium consisting of Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum, 50 μg/mL ascorbic acid, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. The culture medium was changed every 3–4 days. After the first passage (10–12 days) amphotericin B was removed. At 60% confluence, cells were detached with trypsin-ethylenediaminetetraacetic acid and seeded into new culture flasks.

Gene expression analysis using real-time reverse transcriptase polymerase chain reaction

Total RNA for gene expression analysis was isolated using the RiboPure kit following protocols from the manfacturer (Ambion, Austin, TX). After DNase treatment (Ambion), RNA was quantified by spectrophotometry (Nanodrop, Wilmington, DE). For each reaction 200 ng total RNA was reverse transcribed into cDNA (total volume of 20 μL) by using the High Capacity cDNA Reverse Transcription kit following protocols from the manufacturer (Applied Biosystems, Abingdon, United Kingdom). Relative quantification was performed using the 7300 Real-Time reverse transcriptase (RT) PCR system (Applied Biosystems) and TaqMan® Gene Expression assays following protocols from the manufacturer (Applied Biosystems). TaqMan assay numbers are listed in Table 1. All samples were run in technical triplicates containing 1.0 μL cDNA in a total volume of 25 μL. The thermocycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Validation of 32 different endogenous controls was performed using the TaqMan Human Endogenous Control Plate following protocols from the manufacturer (Applied Biosystems). Subsequently, all samples were scaled relative to expression level of *CDKN1A*, which was found to be one of the most suitable endogenous controls in our experimental settings.

miRNA array and real-time RT polymerase chain reaction validation

Total RNA enriched with small RNA molecules (including miRNA) was extracted from the cells using the Trizol (Invitrogen, Carlsbad, CA) procedure with the following modifications: 1 mL of isopropanol was included in the precipitation step and the samples were kept at −80°C for minimum 30 min. After centrifugation the RNA pellet was dissolved in Trizol and column-purified using reagents and following protocols from the miRNeasy kit (Qiagen, Hilden, Germany).

After DNase treatment (Ambion), the total RNA was quantified by spectrophotometry (Nanodrop). miRNA profiling was obtained through the miRNA microarray service, including in depth analysis, from LC Sciences (Houston, TX, www.lcsciences.com). Analysis of variance ($p < 0.01$) was used for clustering to make a heat map. The arrays were based on the Sanger miRBase Release 13.0 and contained 875 unique probes. For real-time reverse transcriptase (RT) polymerase chain reaction (PCR) validation, the miRNAs were reverse transcribed to cDNA using primers included in the TaqMan miRNA assays (Table 1) and reagents from the TaqMan miRNA RT kit following protocols from the manufacturer (Applied Biosystems). Relative quantification was performed using the same parameters as described above for the real-time RT PCR analysis. *CDKN1A* was used as endogenous control. The prediction tools PicTar (http://pictar.mdc-berlin.de/), TargetScan (www.targetscan.org/), miRDB (http://mirdb.org/), Diana Micro-T (http://diana.cslab.ece.ntua.gr/), and miRNA viewer (http://cbio.mskcc.org/mirnaviewer/) were used to search for possible miRNA targets.

Flow cytometric analysis

For flow cytometric analysis uncultured cells were digested with collagenase for 3–4 h and passed first through 100 μm and then 40 μm pore size filters. The following directly conjugated monoclonal antibodies (Mab) were used for cell surface marker staining: CD49b (integrin α2)/PE, CD49f (α6)/PE, CD90/PE, CD104 (β4)/FITC, CD106/FITC, CD146/FITC (AbD Serotec, Kidlington, United Kingdom), CD34/FITC, 45/FITC, CD49a (α1)/FITC, CD49c (α3)/PE, CD49e (α5)/PE, CD29 (β1)/APC, CD44/PE, CD73/PE, CD117/PE (BD Biosciences, San Diego, CA), CD51 (αV)/PE (Chemicon Internationals, Temecula, CA), CD19/APC, CD105/APC, HLA-DR/APC (Diatec, Oslo, Norway), CD144/PE, Integrin α5/FITC (eBioscience, San Diego, CA),

Table 1. TaqMan Gene and MicroRNA Expression Assays

Gene symbol	*Gene name*	*TaqMan assay no.*
ACAN	Aggrecan	Hs00202971_m1
ALPL	Alkaline phosphatase	Hs00758162_m1
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	Hs00192708_m1
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	Hs00199841_m1
BGLAP	Bone gamma-carboxyglutamate protein (Osteocalcin)	Hs01587813_g1
CDKN1A	Cyclin-dependent kinase inhibitor 1A	Hs00355782_m1
COL1A1	Type I collagen	Hs00164004_m1
COL2A1	Type II collagen	Hs00264051_m1
COL9A1	Type IX collagen	Hs00156680_m1
COL10A1	Type X collagen	Hs00166657_m1
COL11A1	Type XI collagen	Hs00266273_m1
COMP	Cartilage oligomeric matrix protein	Hs00164359_m1
DCN	Decorin	Hs00754870_s1
HAS2	Hyaluronan synthase 2	Hs00193435_m1
ITGA2	Integrin, alpha 2	Hs00158148_m1
ITGA6	Integrin, alpha 6	Hs00173952_m1
ITGA10	Integrin, alpha 10	Hs00174623_m1
MMP1	Matrix metallopeptidase 1	Hs00899658_m1
MMP3	Matrix metallopeptidase 3	Hs00869308_m1
MMP13	Matrix metallopeptidase 13	Hs00233992_m1
OMD	Osteomodulin	Hs00192325_m1
RUNX2	Runt-related transcription factor 2	Hs00231692_m1
SOX5	SRY (sex determining region Y)-box 5	Hs00374709_m1
SOX6	SRY (sex determining region Y)-box 6	Hs00264525_m1
SOX9	SRY (sex determining region Y)-box 9	Hs00165814_m1
VCAN	Versican	Hs01007941_m1
MicroRNA		
miR-140-3p	microRNA 140	002234
miR-145	microRNA 145	002278
miR-222	microRNA 222	002276
miR-451	microRNA 451	001141

CD49d (α4)/FITC (Immunotec, Quebec, Canada), CD133/FITC (Miltenyi Biotec, Gladbach, Germany), and CD14/FITC (Sigma-Aldrich). The cells were coated with directly conjugated Mab at room temperature for 20 min, washed with phosphate-buffered saline, and fixed in 1% paraformaldehyde. Intracellular staining was performed following the protocols from the Cytofix/Cytoperm™ Fixation/Permeabilization kit (BD Biosciences). The following unconjugated antibodies were used: Aggrecan (Biosource, Nivelles, Belgium), Type I Collagen, Type II Collagen (MP biomedicals, Aurora, OH), Versican (R&D Systems, Abingdon, United Kingdom), Integrin α7a, Integrin α7b (gift from Dr. Tarone), Integrin α10 (gift from Hansa Medical AB), and Integrin α11 (gift from Dr. Donald Gullberg). Cells were first incubated with unconjugated primary antibodies against the molecule of interest for 20 min, washed, and then incubated with a fluorochrome-conjugated species and isotype-specific secondary antibody. Irrelevant Mabs were used as controls for all fluorochromes. Cells were analyzed using a FACSCalibur flow cytometer (Becton, Dickinson, San Jose, CA; www.bd.com). Median fluorescence index was calculated as follows: median fluorescence intensity of target molecule − median fluorescence intensity of isotype control.

Statistical analysis

The in-depth analysis of miRNA from LC Sciences (www.lcsciences.com) included analysis of variance ($p < 0.01$), which was used for clustering to make a heat map.

Results

During the first 7–10 days of culture, most of the AC were proliferating inside the chondrocytes in autologous ECM (three-dimensional culture) as previously described.[11] These cells have previously been shown to produce hyaline matrix molecules,[11] and are here called CBLC.[19] After 10–13 days increasing numbers of cells adhered to the surface of the culture flasks, and after 17–19 days all the cells were proliferating in adherent monolayer cultures. AC from donor 2 spread onto the plastic surface 2–3 days earlier than cells from donors 1 and 3. At day 28 cells from all donors had been in monolayer with fibroblastic morphology for at least 10 days and were termed dedifferentiated chondrocytes.

Endogenous control for real-time RT PCR

As recommended,[20] several endogenous controls were analyzed before the gene and miRNA expression experiments were performed. Of the 32 possible endogenous controls on the TaqMan Human Endogenous Control Plate *CDKN1A* had one of the most stable gene expression in our experimental settings. Several of the standard housekeeping genes such as *GAPDH*, β-*actin*, and *18s RNA* were expressed at much lower levels in primary uncultured chondrocytes than in proliferating cells (Supplementary Fig. 1; Supplementary Data are available online at www.liebertonline.com/ten).

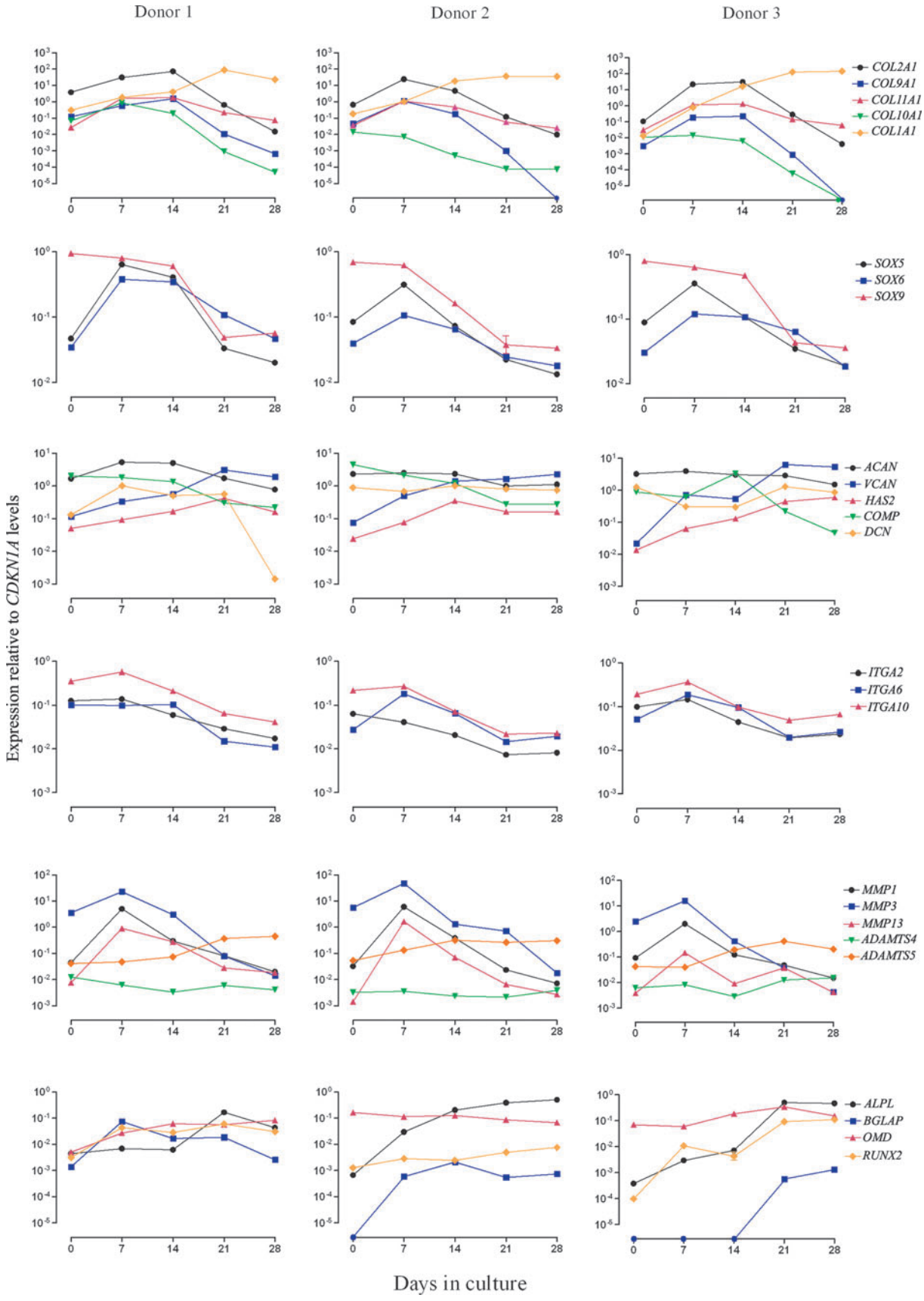

FIG. 1. Relative quantification of gene expression by real-time RT PCR. Real-time reverse transcriptase (RT) PCR analysis of relevant genes expressed during the three stages of *in vitro* culture. Stage 1: uncultured primary AC (day 0). Stage 2: adult chondroblast-like cells (day 7 and 14). Stage 3: dedifferentiated chondrocytes (day 28). Gene expression levels were scaled relative to *CDKN1A* expression levels. Data are shown as mean ± standard deviation for technical triplicates in each of three donors. Note log scale for the different *Y* axes. PCR, polymerase chain reaction.

Changes in gene expression during in vitro *culture*

Real-time RT PCR was used to determine changes in gene expression of a large number of genes (Fig. 1). The AC went through three stages during the 28 days of *in vitro* culture: (1) primary, uncultured nonproliferating AC (day 0); (2) the chondroblast-like stage (day 7–14); and (3) the dedifferentiation stage at the end of the culture. These stages were characterized by differential expression of genes encoding many of the collagens and relevant transcription factors (TFs) (Fig. 1). *COL2A1*, *COL9A1*, and *COL11A1* levels were high or moderate in uncultured cells. Expression increased 10–300-fold during stage 2, and then fell to low levels in stage 3, particularly for *COL9A1* and *COL2A1*. *COL10A1* levels were moderate in uncultured cells, were slightly downregulated during stage 2, and fell to undetectable or very low levels in stage 3. Moderate levels of *COL1A1* were detected in uncultured AC, and expression increased gradually to very high levels in dedifferentiated cells.

The best known regulators of expression of *COL2A1* are the TFs SOX9, 5, and 6.[21] High levels of *SOX9* where detected in uncultured AC at stage 1. Surprisingly, *SOX9* levels did not increase in parallel with *COL2A1* levels, but instead weakly decreased during stage 2 and dramatically decreased during stage 3. In contrast, *SOX5* and *SOX6* levels were low/moderate in uncultured cells and then changed in parallel with *COL2A1* during stage 2 and 3. Gene expression of other molecules of importance for hyaline matrix was also investigated. We saw gradual increases in *VCAN* and *HAS2* and gradual decrease in *COMP* in the course of cell culture, with minor changes observed for *ACAN* and *DCN*. *ITGA2* decreased from day 7, whereas *ITGA6* and *ITGA10* increased at the beginning of stage 2 (day 7) and then decreased. Figure 1 also shows gene expression of several matrix degrading enzymes. Interestingly, *MMP3* was detected at high levels in uncultured cells. *MMP1*, *MMP3*, and *MMP13* were greatly increased at day 7, followed by a decrease in expression to that observed at day 0 or lower. The gene encoding the aggrecan degrading enzyme ADAMTS4 was not drastically changed, whereas *ADAMTS5* slightly increased in dedifferentiated AC. Some bone-related genes were also examined. *ALPL* and *RUNX2* levels increased after stage 1. *BGLAP* (Osteocalcin) and *OMD* expression was not consistent between the donors.

miRNA profiling

miRNAs are a class of recently discovered small RNA molecules that may regulate important cellular functions during *in vitro* culture of AC. On the basis of our gene expression results, we chose day 0 (stage 1, uncultured cells), day 7 (stage 2), and day 28 (stage 3) for global miRNA analysis. Hierarchical clustering of differentially ($p < 0.01$) expressed miRNA revealed four different clusters (Fig. 2). One cluster consisted of miR-451, which was only upregulated in stage 1. Four miRNA, including miR-140-3p, were upregulated in *COL2A1* producing cells (stage 1 and 2). Another cluster consisted of five miRNA, including miR-221 and miR-222, that were upregulated in proliferating cells (stage 2 and 3). The last cluster consisted of 11 miRNA, including miR-143 and miR-145, upregulated only in dedifferentiated cells (stage 3). One representative miRNA from each cluster was validated using real-time RT PCR. Expression pattern of miR-451, miR-140-3p, miR-222, and miR-145 was very similar to that obtained from miRNA arrays (Fig. 3A–D).

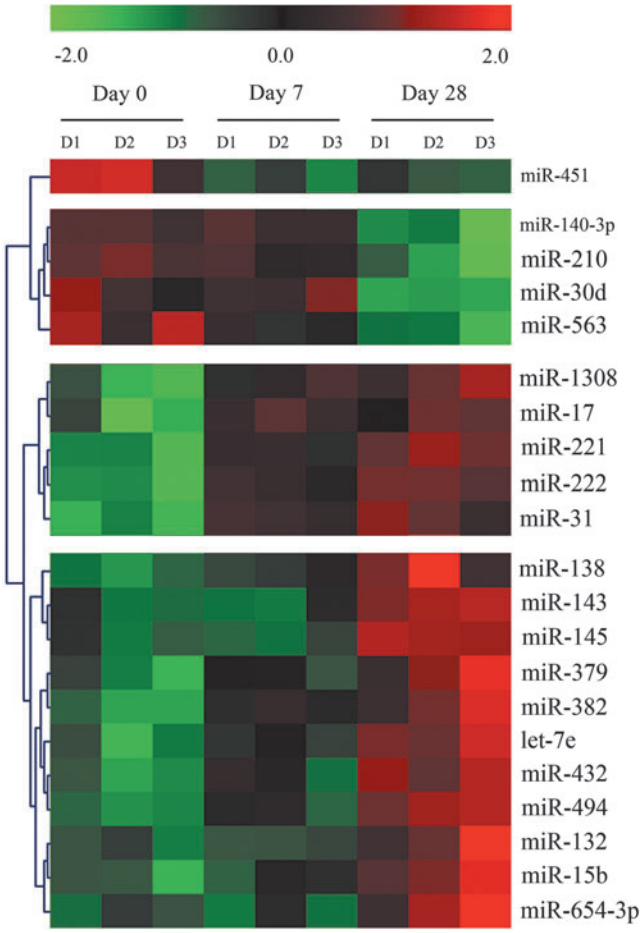

FIG. 2. Heat map of miRNA expressed in the three different stages of culture. Clustered presentation of miRNA expressed differentially ($p < 0.01$, analysis of vasriance) between the three stages of *in vitro* culture. Red and green color indicates high and low expression, respectively. D1 = Donor 1, D2 = Donor 2, and D3 = Donor 3. miRNA, microRNA.

Changes in cell surface marker and ECM protein expression during in vitro *culture*

Cells use adhesion molecules, such as integrins, to attach to their ECM ligands *in vivo* and to the plastic surface of the culture flasks during *in vitro* expansion. Therefore, we also analyzed expression of different integrin subunits and other cell surface markers using flow cytometry. All integrin subunits except for α6, α7a, α7b, and α11 were detected on uncultured cells. α2, α3, β3, and β4 were only detected in one donor (low levels). After 28 days of *in vitro* culture expression of most integrin subunits increased (Fig. 4A). CD44, CD73, CD90, CD105 (markers expressed by mesenchymal stem cells [MSC]), and CD106 were also expressed by the uncultured AC. Again expression increased at day 28. To ensure that there was no contamination of haematopoietic cells in our cultures, we also analyzed expression of CD14, CD19, CD34, and CD45. These markers were not found on any of the cells. Also CD117, CD133, CD144, and HLA-DR were not detected.

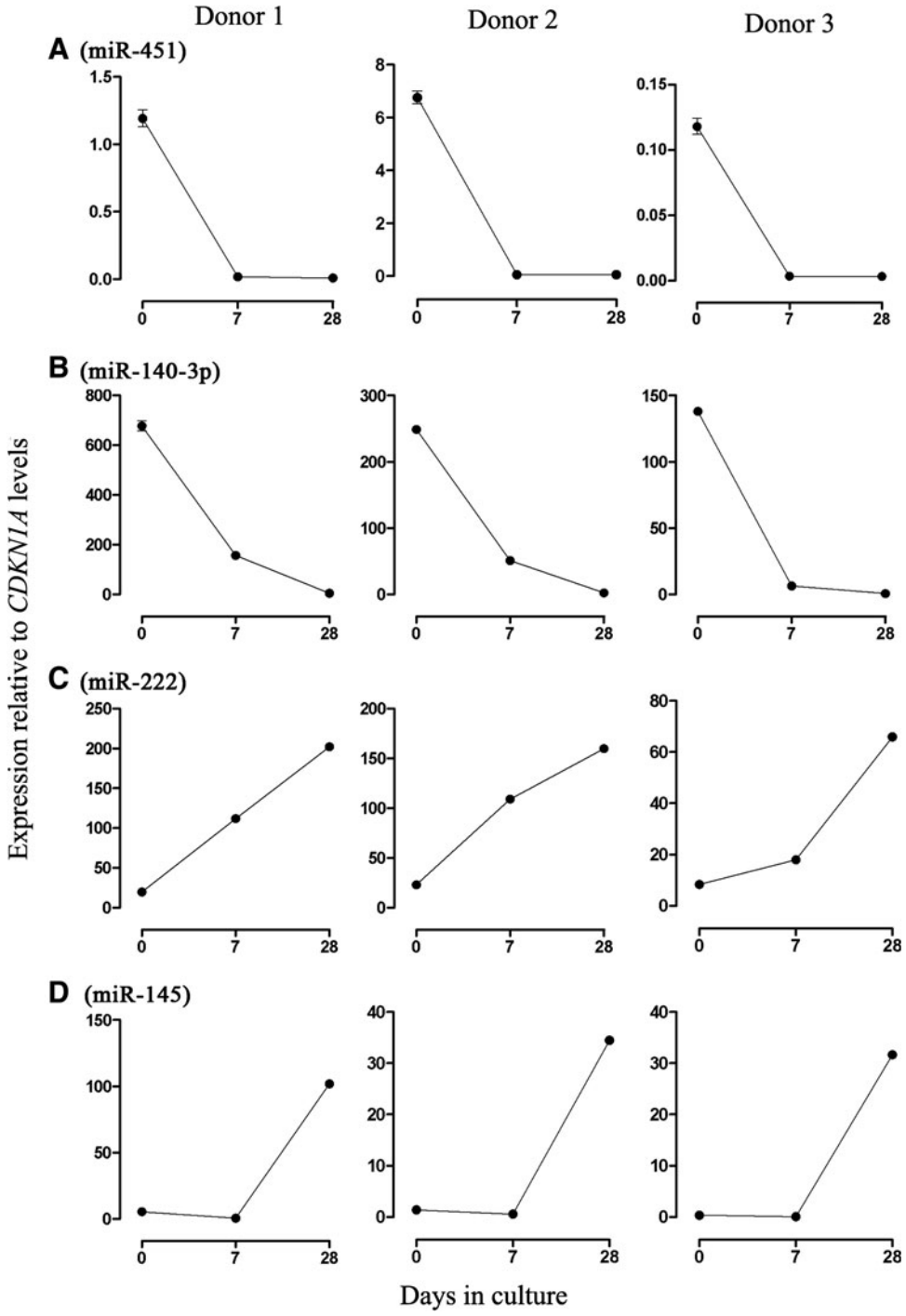

FIG. 3. Validation of miRNA expression by real-time RT PCR. One miRNA from each of the four clusters, i.e., miR-451 **(A)**, miR-140-3p **(B)**, miR-222 **(C)** and miR-145 **(D)**, was validated with real-time RT PCR. miRNA levels were scaled relative to *CDKN1A* expression levels. Data are shown as mean ± standard deviation for technical triplicates in each of three donors. Note linear scale for the different *Y* axes.

Intracellular levels of COL1 were barely detected in two of the donors in the uncultured cells. However, higher levels were found at days 14 and 28 in all three donors (Fig. 4B). COL2 was detected at all time points, with slightly higher levels at day 14. ACAN was expressed at moderate levels and was not differentially expressed during culture. VCAN was not expressed in the uncultured cells, whereas it was present in all three donors at days 14 and 28.

Discussion

Presumably, the best therapeutic strategy for lesions of articular cartilage will be based on the use of cells that are able to produce perfect hyaline ECM. The only cells known to make hyaline ECM are embryonic chondroblasts[19] and adult chondrocytes. Thus, our therapeutic cells should perhaps resemble embryonic chondroblasts.

Genes encoding several collagens changed in the course of cell culture according to three different patterns. The first pattern was followed by *COL2A1*, *COL9A1*, and *COL11A1* levels, which increased during stage 2 and then decreased during stage 3. In hyaline cartilage the collagen fibrils consist of bundles of COL2 with small amounts of COL11. COL9 binds in a regular pattern to the surface of the COL2/COL11 fibrils and is thought to mediate interaction with other col-

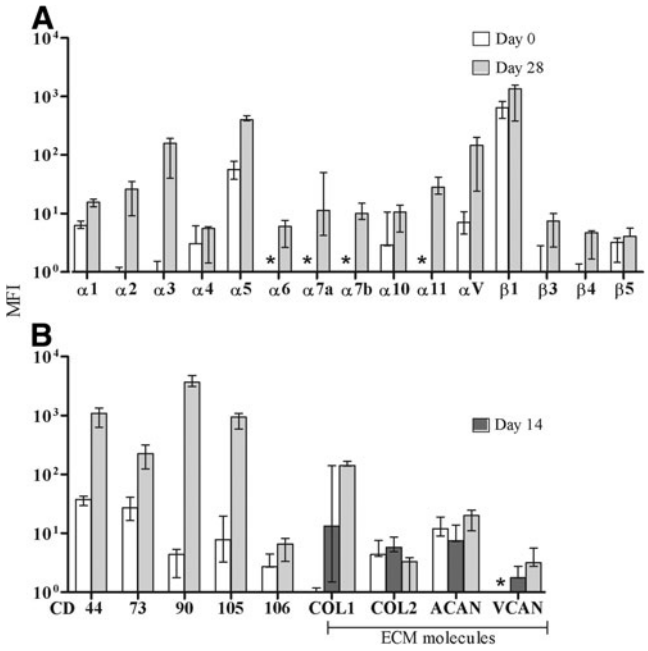

FIG. 4. Cell surface marker and intracellular ECM staining in uncultured and dedfferentiated AC. **(A)** Integrin expression on day 0 (uncultured chondrocytes) and day 28 (dedifferentiatied chondrocytes) of culture. **(B)** Cell surface marker and intracellular ECM molecule expression at days 0, 14, and 28. The bars represent MFI for the three donors, with error bars representing the range. *Not detected. ECM, extracellular matrix; MFI, median values for fluorescence intensity.

lagen fibrils and matrix proteins.[22] Given this relationship *in vivo*, and the fact that *COL2A1*, *COL9A1*, and *COL11A1* are activated by SOX9,[23–25] it is perhaps not surprising that they seem to be co-regulated. For COL2, we observed the same expression pattern at the protein level, though at low levels and with relatively small differences between the cell populations. The low level of COL2 in the uncultured cells may be explained by the fact that collagen in cartilage has a half-life of >100 years.[26] The requirement for new COL2 in adult cartilage would thus be expected to be low. We have previously shown that CBLC produce hyaline matrix containing COL2 fibrils.[11] The relatively small increase in intracellular COL2 levels between uncultured chondrocytes and CBLC may reflect rapid transport of COL2 molecules to the extracellular space.

A second pattern was followed by *COL10A*, which remained largely unchanged in the CBLC, and then dropped to very low or undetectable levels in dedifferentiated cells. Expression of *COL10A1* is a marker for hypertrophy during embryogenesis.[19,27] However, COL10 is found in both the superficial and near the tidemark zone in normal human articular cartilage.[28] Thus, *COL10A1* expression in these cells may possibly reflect physiological requirements for COL10, and not the ominous onset of hypertrophy.

Following a third pattern, *COL1A1* gradually increased from moderate levels to very high levels in dedifferentiated cells. *In vivo* COL1 is known to be a part of the ECM in the superficial zone, but is not normally synthesized by AC in other zones.[29] By intracellular flow cytometry we found very small amounts of COL1 in the uncultured cells. This may suggest a blockage of translation in these cells. For day 14 and 28 the intracellular COL1 matched the *COL1A1* levels. This may explain why dedifferentiated chondrocytes, when used for clinical transplantation, frequently produce fibrocartilage dominated by COL1.[9]

Expression of *SOX5* and *SOX6* increased in stage 2 cells, whereas *SOX9* was already highly expressed in the uncultured cells. During embryogenesis, *SOX9* is expressed in prechondrocytes, before *SOX5* and *SOX6*. Low levels of *COL2A1* are also expressed in these cells.[19] In embryological chondroblasts all these genes are expressed, and COL2 protein and hyaline cartilage is being synthesized. Thus, similar to embryological chondrogenesis, the upregulation of SOX5 and SOX6 on a background of SOX9 availability may be important effectors of chondroblast differentiation.[19]

ACAN and *VCAN* levels were mirrored by protein expression for both molecules. Although the results for *VCAN* were entirely as expected, *ACAN* levels are frequently found to be reduced in dedifferentiated chondrocytes.[30] One possible explanation for the persistently high *ACAN* levels in these cells could be that day 28 cells may not have entirely completed the dedifferentiation process.

In stage 2, expression of *MMP1*, *3*, and *13* dramatically increased at day 7 before returning to baseline expression or lower. In embryological chondrogenesis MMPs are upregulated to soften the ECM,[31] thus, providing space for the cells to migrate and establish cell–cell contact important for condensation, the first step in chondrogenesis. Interestingly, several MMPs, including MMP3 and MMP13, were found to be expressed during chondrogenic differentiation of MSC.[32] MMP13 is also known to be a marker for hypertrophy during embryological chondrogenesis.[19,33] Further investigations are required to determine the significance of these *MMPs* in our culture system. The increase of *ALPL* and *RUNX2* is a potential concern as they are markers of osteogenesis, and also of chondroblast hypertrophy during embryogenesis.[19] These genes were expressed at low levels in adult CBLC. Importantly, no evidence of bone formation or unexpected cell death has so far been observed with the clinical use of these cells. Still, expression of these genes requires attention in further studies.

We found a number of changes in expression of integrins and other cell surface markers when AC were established in culture. These results correspond almost exactly to observations made by Woods *et al*.[34] At day 28 of culture expression of most subunits had increased. This suggests that ACs use different set of integrins to adhere to ECM and the culture flasks.

We also show here that many miRNAs were differently expressed in cells of the different stages. MiR-451 was the only miRNA that was highly expressed only in the primary uncultured AC. This miRNA has been shown to reduce proliferation in cancer cells[35,36] and may perhaps have a role as inhibitor of proliferation also in AC *in vivo*. Of the miRNA highly expressed in both stages 1 and 2, miR-140-3p is of particular interest as it was the miRNA with the highest expression in our dataset (the complementary sequence miR-140-5p showed the same expression pattern and was significant with $p < 0.05$; data not shown). miR-140 has been shown to be cartilage specific in mouse embryos where it targets histone deacetylace 4 (*HDAC4*).[37] Recently, miR-140

was also shown to regulate homeostasis and development of cartilage.[38] This group of miRNA was downregulated in dedifferentiated cells only, suggesting that they may inhibit molecules participating in the dedifferentiation process. Four of the five miRNAs that were upregulated in proliferating cells (stages 2 and 3) (miR-17, 31, 221, and 222) have been shown to induce proliferation in several other cell types,[39–43] again suggesting miRNA participation in the regulation of cell cycle in these cells. Of the 11 miRNAs that were upregulated in dedifferentiated AC only, miR-145, 132, 138, and let-7e are interesting. Several miRNA prediction tools predict miR-145 to target *SOX9* and *COL2A1. SOX5* is predicted to be a target of miR-132, *SOX6* and *COL11A2* are possible targets of miR-138, and let-7e may have P4HA2 (prolyl 4-hydroxylase, a key enzyme in collagen synthesis) as a target. This may, potentially, contribute to the dedifferentiation of the cells. In contrast to our observations, Dunn *et al.* found decreased miR-145, 221, and 222 levels in monolayer cells compared to native cartilage.[44] As our observations for miR-145 and 222 were made in global arrays and confirmed by real-time quantitative PCR, we believe that our results are correct for our experimental system. The discrepancy may be explained by the fact that Dunn *et al.* compared bovine AC derived from distinct zones and from anatomically distinct regions in the femur, which introduces both species and possibly anatomical reasons for the differences in miRNA expression. MiR-27[45,46] and miR-675[47] have also been shown to be involved in cartilage biology. However, these miRNAs were not differently expressed in the three different cell populations studied here.

In conclusion, we demonstrate that CBLC express genes and synthesize proteins typical of hyaline ECM. We describe differences in gene and miRNA expression between CBLC and uncultured chondrocytes as well as dedifferentiated chondrocytes. We also describe the differential expression of miRNAs in the three different cell types. As miRNAs regulate gene as well as protein expression, this insight may improve our understanding of chondrocyte biology and also introduce new therapeutic targets for cell-based treatment strategies for lesions of hyaline cartilage. Whether CBLC will produce better hyaline cartilage than dedifferentiated chondrocytes or MSCs need to be tested in formal assays for *in vitro* chondrogenesis[48] and, eventually, by clinical trials.

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Disclosure Statement

This work has not received financial support or other benefits from commercial sources, and none of the authors have financial interests that could create a conflict of interest with regard to this work.

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