

SERPINA9 and SERPINB2: Novel Cartilage Lineage Differentiation Markers of Human Mesenchymal Stem Cells with Kartogenin

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Abstract

Objective. Human mesenchymal stem cells (hMSCs) are a promising source for regenerative medicine, especially mesodermal lineages. Clinical applications require an understanding of the mechanisms for transcriptional control to maintain the desired cell type. The aim of this study was to identify novel markers for differentiation of hMSCs into bone or cartilage with the use of Kartogenin, by RNA analysis using microarray technology, and explore the role of RhoA-Rho associated protein kinase (ROCK) inhibition in these. **Methods.** Commercial human bone marrow derived primary mesenchymal stem cells were purchased from ATCC. Cells were differentiated *in vitro* in 2-dimensional cultures using Kartogenin as the main cartilage inducer and bone morphogenetic protein 2 for bone differentiation; cells were cultured with and without ROCK inhibitor Y-27632. After 21 days of culture, whole RNA was extracted and analyzed via Affimetrix microarrays. The most significant hits were validated by quantitative polymerase chain reaction. **Results.** We found a total of 1,757 genes that were either up- or downregulated on differentiation, when compared to P1 hMSC (control) at day 0 of differentiation. Two members of the Serpin superfamily, *SERPINA9* and *SERPINB2*, were significantly upregulated in the cartilage groups, whereas they were unchanged in the bone groups with and without ROCK inhibition. **Conclusions.** *SERPINA9* and *SERPINB2* are novel differentiation markers, and molecular regulator candidates for hMSC lineage commitment toward bone and cartilage, providing a new tool for regenerative medicine. Our study highlights the roles of these 2 genes, with significant upregulation of both in cell cultures stimulated with Kartogenin.

Keywords

mesenchymal stem cells, *SERPINA9*, *SERPINB2*, chondrocytes, osteoblasts

Introduction

Human mesenchymal stem cells (hMSCs), multipotent stem cells able to differentiate into 4 main lineages including osteoblasts and chondrocytes, are a very promising cell source for regenerative medicine.¹ Clinical applications require an understanding of the mechanisms for transcriptional control to maintain the desired cell type. Cartilage regeneration techniques rely primarily in cell therapy, with the first technique describing autologous chondrocyte implantation.² The best results have been accomplished with the use of chondrocytes for cartilage restoration, since the ultimate goal is hyaline cartilage.² Despite progress been made, new cell sources are being researched, namely, bone marrow-derived hMSCs. These must express the following cell surface receptors in at least 95%: CD105, CD73, and CD90; and be negative for CD45, CD14, CD34, CD19, and Class II HLA.³ Use of these cells has given mixed results over the years, the

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main concern being that quality of regenerated cartilage is not as good as the hyaline variety found in articular cartilage.⁴ Also, several studies have seen deterioration of this neocartilage after a median of 18 months.^{2,5}

In the past years another approach to regenerate hyaline cartilage for articular purposes has focused on the use of stem cells from several origins, and commitment for having a developmental recapitulation of cartilage *in vitro*. In order to do so, developmental gene regulation must be taken into account. The master regulator of cartilage development is the gene *SOX9*.⁶ In early studies, it was demonstrated that both cartilage and bone lineages share a common osteochondro progenitor and that the *RUNX2* gene, which is master regulator for bone formation, is downstream of *SOX9*, at least in the case of endochondral ossification, whereas for intramembranous bone formation mesenchymal stem cells can differentiate directly into bone cells.⁶

Differentiation of mesenchymal stem cells into cartilage has previously been achieved using members of the transforming growth factor (TGF) β family, especially TGF- β 3.⁴ Differentiation to bone has been extensively described in the past, also using other members of the superfamily named bone morphogenetic proteins (BMP), with the best one characterized being BMP2.⁷ The new strategy to induce certain genetic expression patterns is to bypass cell surface receptors using small chemical molecules that are able to target the cytosolic or nuclear proteins that are transcription factors.⁸ Kartogenin has recently been reported to induce chondrogenesis directly by dissociating CBF β from filamin A, to later translocate to the nucleus and activate *RUNX1*.⁹ On the other hand, inhibition of BMP type I receptor kinase has recently demonstrated to commit hMSCs to articular cartilage.¹⁰

The RhoA-Rho associated protein kinase (ROCK) branch of the small Rho GTPases has been the most researched pathway in lineage commitment, due to easy manipulation with chemical ROCK inhibitors, such as Fasudil and Y-27632.¹¹ This pathway has been implicated in lineage commitment of mesenchymal stem cells to many cell types.¹¹ Interestingly, ROCK inhibition can promote or inhibit chondrogenesis and/or osteogenesis, depending on the developmental stage of the cell type used for the *in vitro* assays.¹¹

The aim of this study was to identify novel markers for differentiation of hMSCs into bone and cartilage. In order to do this, a commercial bone marrow-derived hMSC line previously validated for differentiation to adipocytes, chondrocytes, and osteocytes was used, differentiating them *in vitro* into cartilage and bone. Cells were cultured with and without ROCK inhibitor Y-27632. RNA was extracted and analyzed via Affimetrix microarrays, with the most significant hits validated by quantitative polymerase chain reaction (qPCR).

Methods

Cell Culture

Multipotent mesenchymal stem cells with the ability to differentiate to both cartilage and bone lineages PCS-500-012 from ATCC were used.⁹ This cell line has been validated for differentiation to adipocytes, chondrocytes, and osteocytes, being positive for CD29, CD44, CD73, CD90, CD105, and CD166 and negative for CD14, CD19, CD34, and CD45, which are markers considered as International Society for Cellular Therapy (ISCT) criteria for characterizing MCSs.¹² Cells were cultured with DMEM:F12 (Dulbecco's modified Eagle medium), 10% FCS (fetal calf serum), Pen/Strep 1:100, and Glutamine 1:100 (Gibco-Invitrogen, Carlsbad, CA).

In Vitro Chondrogenic Differentiation

To set up the differentiation assay, 3 technical replicates were cultured in parallel in 24-well plates; 5×10^4 cells per 1 cm^2 were seeded. Basal media was supplemented with Kartogenin (KGN) 100 nM (Sigma) and ascorbic acid 50 μM (Sigma).⁹

In Vitro Osteogenic Differentiation

To set up the differentiation assay, 3 technical replicates were cultured in parallel in 24-well plates; 5×10^4 cells per 1 cm^2 were seeded. Basal media was supplemented with BMP-2 at 50 ng/mL (Peprotech), ascorbic acid 50 μM (Sigma), dexamethasone at 1×10^{-6} , and β -glycerol phosphate at a final concentration of 10 mM.

ROCK Inhibition

In order to inhibit RhoA downstream effector ROCK, the chemical inhibitor Y-27632 (Sigma) was used at a final concentration of 10 $\mu\text{M}/\text{mL}$ in both groups differentiated to chondrocyte and osteoblast lineage.

RNA Isolation

After 21 days of culture, differentiation medium was removed and the cell monolayer was washed twice with phosphate-buffered saline. QIAGEN RNEasy mini kit was used as recommended by the manufacturer. Briefly, monolayers were removed from the tissue culture plates with a 10-minute incubation time at 37°C with Accutase (Sigma); cells were dislodged mechanically with a P1000 tip and transferred to a clean Eppendorf tube. Cells were pelleted and the supernatant removed. They were then lysed with the RLT buffer and processed until the end of the protocol, eluting with 50 μL of RNase-free water. Integrity and purity of RNA concentration was assessed using ND-1000

Spectrophotometer (Nano-Drop Technologies, Wilmington, DE). The quality of each RNA sample was validated on an Agilent BioAnalyzer 2100 (Agilent Technologies, Germany), and 150 ng of the best-quality samples (RIN values between 8.0 and 9.7) was processed using Affymetrix Whole Transcript Sense Target Labelling Kit (Affymetrix, Santa Clara, CA).

DNA Microarray Global Gene Expression Profiling

For microarray experiments, target cDNA was prepared according to the Whole-Transcript PLUS (WT) Sense Target Labelling Protocol (Affymetrix, Inc., Santa Clara, CA). Briefly, pooled RNA (200 ng) from 3 biological replicates was converted to first-strand cDNA using Superscript II reverse transcriptase primed by a poly(T) oligomer. Second strand cDNA synthesis was followed by an *in vitro* transcription to generate cRNA. The cRNA products were used as templates for a second cycle cDNA synthesis where dUTPs are incorporated to the new strand. The cDNA was fragmented using uracil-DNA glycosylase and apurinic apyrimidinic endonuclease. The fragments (40-70 mers) were then labelled by means of a biotin-labelled deoxynucleotide terminal addition reaction. Fragmented and labelled cDNA was hybridized using Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Santa Clara, CA). The arrays were washed, stained for biotinylated cDNA, and scanned according to the manufacturer's recommendations. The array represents 47,000 total RefSeq transcripts covered with a median of 22 probes per gene, which assures an accurate detection for genome-wide transcript expression changes (<http://www.affymetrix.com>).

Gene Expression Profiling

Samples were classified into 5 groups with 3 replicates each: (1) Control hMSCs; (2) samples from chondrogenic media (Chondro); (3) samples from chondrogenic media ROCK inhibited with Y-27632 (Chondro Y); (4) osteogenic samples (Osteo); and (5) samples from osteogenic media ROCK inhibited with Y-27632 (Osteo Y). Hierarchical cluster analysis was performed as described previously.¹³

Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis

In order to validate microarray findings, master transcription factors for each type of tissue (*SOX9* for cartilage and *RUNX2* for bone) along with those genes with highest differential expression and biological significance (*SERPINB2* and *SERPINA9*) were measured in total RNA from the *in*

vitro differentiation assays. qPCR assays for each gene were carried out in triplicate and normalized to the most stably expressed reference gene in hMSC: peptidyl-prolyl isomerase A (*PPIA*).¹⁴

cDNA Synthesis for qPCR Validation

Complementary DNA (cDNA) synthesis was carried out with QuantiTect Rev. Transcription Kit (QIAGEN) starting from 900 ng of total RNA of each sample. We checked for genomic DNA (gDNA) contamination before the cDNA synthesis by agarose electrophoresis and by real-time PCR after the DNA Wipeout step of the cDNA synthesis procedure. All samples were free of gDNA contamination.

The PCR reaction was performed with RT2 qPCR Primer Assays (QIAGEN) using the following conditions: 1 μ L of cDNA, 1 μ L of the primer assay, 12.5 μ L of RT2 SYBR Green ROX Mastermix (QIAGEN), and 10.5 μ L of nuclease-free water. Samples were amplified using the following steps: 1 hold of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds, and finally a melting curve ramping from 50°C to 99°C.

We performed the gene expression assays by triplicate in a Rotor-Gene Q instrument (QIAGEN) obtaining a global mean variation coefficient of 1.37%. The information of the primers used in the expression assays is shown in Supplemental Table 1. The manufacturer (QIAGEN) experimentally verified all primers to amplify a single amplicon of the correct size with uniform PCR efficiency.

Cell Culture Protocol with TGF- β 1

In order to further strengthen our results, a standard 21-day differentiation protocol using TGF- β 1 was performed in the same primary cells. Cells were cultured with DMEM:F12, 10% FCS, Pen/Strep 1:100, and glutamine 1:100 (Gibco-Invitrogen, Carlsbad, CA). To set up the differentiation assay, 3 technical replicates were cultured in parallel in 24-well plates; 5×10^4 cells per 1 cm² were seeded. Basal media was supplemented with Transforming Growth Factor β 1 (TGF- β 1) 10 ng/mL (Peprotech) and ascorbic acid 50 μ M (Sigma).¹⁵ RNA isolation and qPCR analyses were performed from 3 independent experiments ($n = 3$) in the same manner as previously described.

Statistical Analysis

For microarray analyses, all possible pairwise comparisons between the 5 groups generated some contrasts of interest. Raw data were background-corrected using Robust Multiarray Average (RMA)¹⁶ and normalized using Quantile Normalization.¹⁷ Differential expression was determined

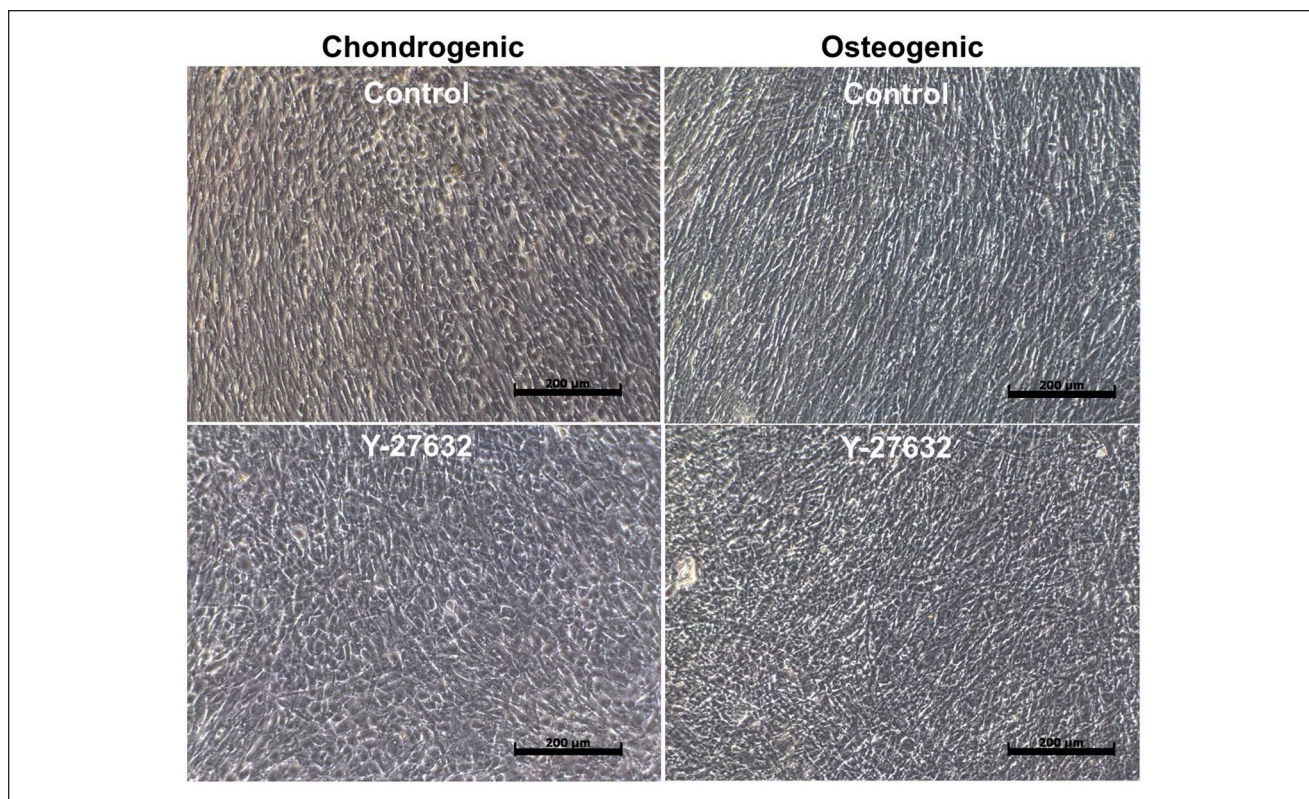


Figure 1. High-density monolayer culture in chondrogenic and osteogenic conditions. Phase contrast images of 7-day high-density monolayer cultures in chondrogenic (first column) and osteogenic (second column) conditions, with and without ROCK inhibitor Y-27632. Scale bar: 200 μm .

using statistical linear models with arbitrary coefficients; contrasts of interest were analyzed using the bioconductor library “limma.”^{18,19} Correction for multiple hypotheses was applied using false discovery rate, and genes were selected as differentially expressed based on a fold-change (FC) < -2 or > 2 and considered significant when P value was below 0.05.

For qPCR, relative expression units were calculated with REST software (QIAGEN) using the reference gene *PPIA* and normalizing with the control group. We compared the expression levels among the 5 groups of each gene using the Kruskal-Wallis test adjusted by multiple comparisons. Statistical analysis and the graphs were performed in the IBM SPSS Statistics 21 statistical package. P values below 0.05 were considered significant.

Results

Molecular Phenotype of Chondrocytes and Osteoblasts with and without ROCK Inhibition

Global gene expression profiling was conducted on hMSCs treated with or without Y-27632 (10 $\mu\text{M}/\text{mL}$) in the presence of osteogenic or chondrogenic media

(**Fig. 1**). Hierarchical clustering based on differentially expressed genes revealed clear separation of control cells treated with the chondrogenic media or osteogenic media regardless of ROCK inhibition. We identified 1,757 genes differentially expressed within 2.0 FC, $P < 0.05$, 635 were upregulated with KGN treatment and 768 genes upregulated in the BMP2 treatment group. The most representative 100 genes, 51 upregulated with KGN treatment and 49 upregulated in the BMP2 treatment group, are shown in **Figure 2** and **Table 1**. The complete list of genes and their differential expression between groups are shown in Supplemental Tables S2 to S5.

Master Transcription Factors for Cartilage and Bone Expression

SOX9 and *RUNX2* did not show statistically significant differential expression in microarray analysis; nevertheless, these results were further validated by qPCR since they are considered the master transcription factors for each type of tissue. Results of qPCR analysis are shown in **Table 2** and **Figure 3**. *SOX9* expression was significantly upregulated in the chondrogenic group without ROCK inhibition and downregulated in both osteogenic

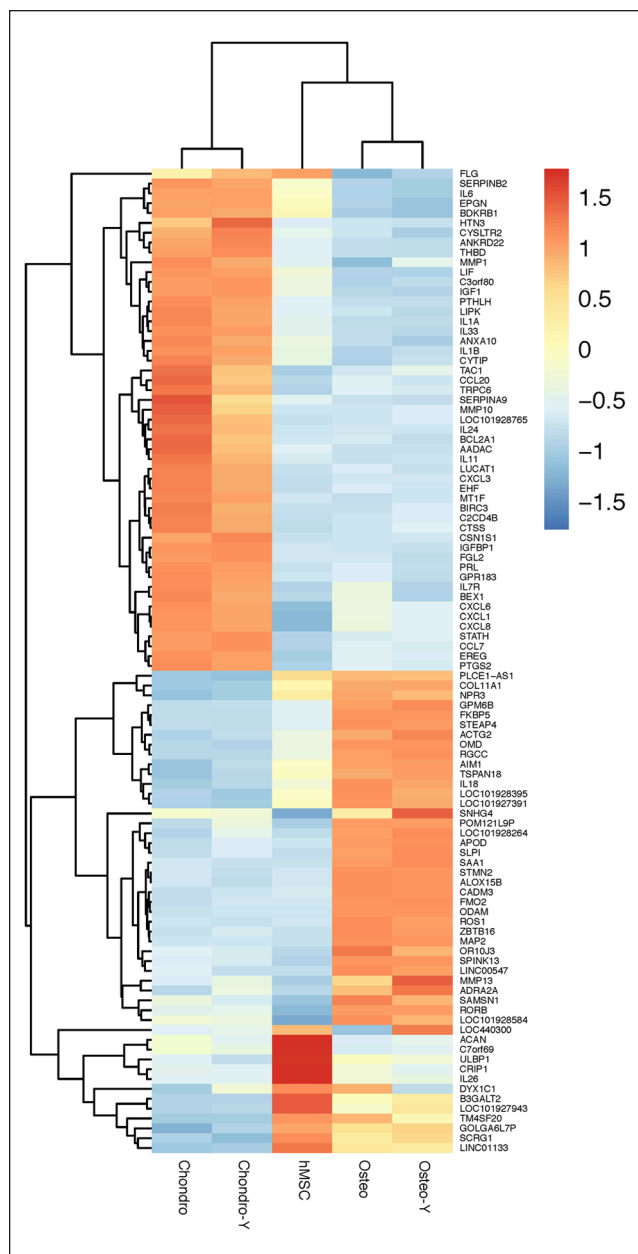


Figure 2. Hierarchical cluster analysis of genes involved in chondrocyte and bone differentiation. Chondro = chondrocytes; Chondro-Y = chondrocytes with ROCK inhibitor Y27632; hMSC = human bone marrow mesenchymal stem cells; Osteo = osteoblasts; Osteo-Y = osteoblasts with ROCK inhibitor Y27632; ROCK = Rho-associated coiled-coil containing protein kinase.

differentiation groups ($P < 0.001$; **Fig. 3A**). Relative expression was maintained at basal level in the ROCK inhibited chondrogenic group. *RUNX2* expression was upregulated in the osteogenic group without ROCK inhibition when compared with both chondrogenic groups ($P < 0.001$; **Fig. 3B**).

Identification of *SERPINB2* and *SERPINA9* as Cartilage Differentiation Genes

Among genes highly regulated in the cartilage differentiation group, and without change in the osteogenic differentiation group, were 2 members of the Serpin peptidase inhibitor superfamily: *SERPINB2* and *SERPINA9*. *SERPINB2* was 10-fold overexpressed between control and cartilage groups ($P = 0.003$) and highly significant when comparing both bone groups against cartilage without ROCK inhibition ($P < 0.001$; **Fig. 3C**). *SERPINA9* was more than 60-fold overexpressed between control and cartilage groups ($P = 0.003$) and also highly significant when comparing both groups without ROCK inhibition ($P < 0.001$; **Fig. 3D**).

Characterization of *SERPINB2* and *SERPINA9* in TGF- β 1 Cultures

Both Serpins were evaluated in TGF- β 1 cultures in order to confirm previous results. *SERPINA9* was more than 20-fold upregulated in the TGF- β 1 group ($P < 0.001$; **Fig. 4A**); however, *SERPINB2* was found downregulated in the TGF- β 1 group ($P < 0.001$; **Fig. 4B**), as compared to controls (data shown are representative from 3 independent experiments, $n = 3$).

Discussion

Serpins (serine proteinase inhibitors) are a superfamily of proteins (350-500 amino acids in size) that fold into a conserved structure and employ a substrate-like inhibitory mechanism.²⁰ To our knowledge, this is the first study to demonstrate *SERPINB2* and *SERPINA9* are highly overexpressed in hMSCs when stimulated with KGN, and to describe a role of this protein family in chondrogenesis.

In recent years, it has been acknowledged that not only growth factors or chemical molecules drive differentiation of a certain progenitor to a desired lineage; external physical cues can also influence differentiation to the point of restricting, directing or favoring certain lineages.⁷ Members of the Rho family of small GTPases, specially the downstream effectors ROCKs, have been implicated in multiple functions such as differentiation, maturation, and remodeling of the extracellular matrix.¹¹ ROCK inhibition has been previously reported, via the small chemical inhibitor Y-27632, as promoting differentiation and maturation of osteoblasts in rat primary calvarial cell culture.²¹ In contrast, ROCK inhibition has been reported to interfere with osteogenesis in hMSCs with BMP2 via downstream effectors SMADs.¹¹ In the present work, we have shown how ROCK inhibition affected gene expression of cells differentiated to bone and cartilage in high-density monolayer cultures from hMSCs. High-density monolayer cultures are

Table 1. Hundred Most Representative Genes Revealed in Microarray Analysis.

Upregulation with Kartogenin		Upregulation with BMP2	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
AADAC	Arylacetamide deacetylase	ACAN	Aggrecan
ANKRD22	Ankyrin repeat domain 22	ACTG2	Actin, gamma 2, smooth muscle, enteric
ANXA10	Annexin A10	ADRA2A	Adrenoceptor alpha 2A
BCL2A1	B-cell CLL/lymphoma 2-related protein A1	AIM1	Absent in melanoma 1
BDKRB1	Bradykinin receptor B1	ALOX15B	Arachidonate 15-lipoxygenase, type B
BEX1	Brain expressed, X-linked 1	APOD	Apolipoprotein D
BIRC3	Baculoviral IAP repeat containing 3	B3GALT2	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2
C2CD4B	C2 calcium-dependent domain containing 4B	C7orf69	Chromosome 7 open reading frame 69
C3orf80	Chromosome 3 open reading frame 80	CADM3	Cell adhesion molecule 3
CCL20	Chemokine (C-C motif) ligand 20	COL11A1	Collagen, type XI, alpha 1
CCL7	Chemokine (C-C motif) ligand 7	CRP1	Cysteine-rich protein 1 (intestinal)
CSN1S1	Casein alpha s1	DYX1C1	Dyslexia susceptibility 1 candidate 1
CTSS	Cathepsin S	FKBP5	FK506 binding protein 5
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	FMO2	Flavin containing monooxygenase 2 (nonfunctional)
CXCL3	Chemokine (C-X-C motif) ligand 3	GOLGA6L7P	Golgin A6 family-like 6
CXCL6	Chemokine (C-X-C motif) ligand 6	GPM6B	Glycoprotein M6B
CXCL8	Chemokine (C-X-C motif) ligand 8	IL18	Interleukin 18
CYSLTR2	Cysteinyl leukotriene receptor 2	IL26	Interleukin 26
CYTIP	Cytohesin 1 interacting protein	LINC00547	Long intergenic non-protein coding RNA 547
EHF	Ets homologous factor	LINC01133	Long intergenic non-protein coding RNA 1133
EPGN	Epithelial mitogen	LOC101927391	Uncharacterized LOC101927391
EREG	Epiregulin	LOC101927943	Uncharacterized LOC101927943
FGL2	Fibrinogen-like 2	LOC101928264	Uncharacterized LOC101928264
FLG	Filaggrin	LOC101928395	Uncharacterized LOC101928395
GPR183	G protein-coupled receptor 183	LOC101928584	Uncharacterized LOC101928584
HTN3	Histatin 3	LOC440300	Chondroitin sulfate proteoglycan 4 pseudogene
IGF1	Insulin-like growth factor-like family member 1	MAP2	Microtubule-associated protein 2
IGFBP1	Insulin-like growth factor binding protein 1	MMP13	Matrix metalloproteinase 13
IL11	Interleukin 11	NPR3	Natriuretic peptide receptor 3
IL1A	Interleukin 1, alpha	ODAM	Odontogenic, ameloblast associated
IL1B	Interleukin 1, beta	OMD	Osteomodulin
IL24	Interleukin 24	OR10J3	Olfactory receptor, family 10, subfamily J, member 3
IL33	Interleukin 33	PLCE1-AS1	Phospholipase C, epsilon 1 antisense RNA 1
IL6	Interleukin 6	POM121L9P	POM121 transmembrane nucleoporin-like 9, pseudogene
IL7R	Interleukin 7 receptor	RGCC	Regulator of cell cycle
LIF	Leukemia inhibitory factor	RORB	RAR-related orphan receptor B
LIPK	Long intergenic non-protein coding RNA 1133	ROS1	ROS proto-oncogene 1, receptor tyrosine kinase
LOC101928765	Uncharacterized LOC101928765	SAA1	Serum amyloid A-like 1
LUCAT1	Lung cancer associated transcript 1 (non- protein coding)	SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1
MMP1	Matrix metalloproteinase 1	SCRG1	Stimulator of chondrogenesis 1
MMP10	Matrix metalloproteinase 10	SLPI	Secretory leukocyte peptidase inhibitor
MTIF	Metallothionein IF	SNHG4	Small nucleolar RNA host gene 4
PRL	Prolactin	SPINK13	Serine peptidase inhibitor, Kazal type 13 (putative)
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	STEAP4	STEAP family member 4

(continued)

Table 1. (continued)

Upregulation with Kartogenin		Upregulation with BMP2	
Gene Symbol	Gene Name	Gene Symbol	Gene Name
<i>PTH1H</i>	Parathyroid hormone-like hormone	<i>STMN2</i>	Stathmin 2
<i>SERPINA9</i>	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9	<i>TM4SF20</i>	Transmembrane 4 L six family member 20
<i>SERPINB2</i>	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	<i>TSPAN18</i>	Tetraspanin 18
<i>STATH</i>	Statherin	<i>ULBP1</i>	UL16 binding protein 1
<i>TAC1</i>	Tachykinin, precursor 1	<i>ZBTB16</i>	Zinc finger and BTB domain containing 16
<i>THBD</i>	Thrombomodulin		
<i>TRPC6</i>	Transient receptor potential cation channel, subfamily C, member 6		

BMP2 = bone morphogenetic protein 2. Genes shown in alphabetical order.

Table 2. qPCR Analysis of the Different Culture Conditions. Gene Expression Analyses of *SOX9*, *RUNX2*, *SERPINB2*, and *SERPINA9* in hMSCs, and After Cell Differentiation Into Chondrocytes and Osteoblasts, with and without ROCK Inhibition.

Gene	Relative Gene Expression Units				P Value
	Chondro	Chondro-Y	Osteo	Osteo-Y	
<i>SOX9</i>	2.14 (2.06)	1.11 (0.15)	0.22 (0.20)	0.39 (0.16)	<0.001
<i>RUNX2</i>	0 (0.0)	1.69 (2.50)	3.74 (0.75)	1.23 (1.23)	<0.001
<i>SERPINB2</i>	10.82 (6.62)	4.10 (0.99)	0.02 (0.01)	0.03 (0.01)	<0.001
<i>SERPINA9</i>	64.32 (21.88)	19.79 (2.94)	0.23 (0.07)	0.38 (0.08)	<0.001

Data shown as median and interquartile range, normalized by the control group (hMSCs). hMSCs = human bone marrow mesenchymal stem cells; Chondro = chondrocytes; Chondro-Y = chondrocytes with ROCK inhibitor Y27632; Osteo = osteoblasts; Osteo-Y = osteoblasts with ROCK inhibitor Y27632; ROCK = Rho-associated coiled-coil containing protein kinase.

considered an appropriate model to explore cartilage differentiation, when at the end of 21 days of culture the so-called monolayer is composed of several layers of cells and in reality is a 3-dimensional model.²² It is also considered an ideal experimental model that prevents the formation of a local hypoxic microenvironment, since pellet cultures may present variations between conditions at the periphery and center of the cultures.²³

Microarray results gave no significant upregulation of either *SOX9* or *RUNX2*, which are the master regulators for cartilage and bone, respectively. When validation by qPCR was performed, we identified that Serpins, in particular *SERPINB2* and *SERPINA9*, are highly upregulated when hMSCs are differentiated to cartilage using KGN, suggesting their expression is downstream of the *RUNX1* transcription factor.⁸ In contrast, gene expression was not affected in cells differentiated toward osteoblasts, regardless of ROCK inhibition. These findings could indicate that bone cells might not require these 2 protease inhibitors while differentiating, or that they serve as angiogenic inhibitors, since articular cartilage is avascular.²⁴ In contrast, *SERPINF1* was previously reported as a positive regulator for osteoblasts, and mutations of the gene have been implicated in osteogenesis imperfecta and otosclerosis.²⁵

The *SERPINA9* gene has been previously described as implicated in B cell development and maturation,²⁶ confined to germinal centers in secondary lymphoid organs.²⁷ *SERPINB2* has been characterized as implicated in intracellular stress response.²⁸ Only recently have members of this superfamily been studied in lineage specification of 2 tissues typically arising from mesenchymal stem cells. *SERPINB2* was found as a novel negative regulator of osteogenesis and adipogenesis; the authors stimulated hMSCs via TGF- β 1 and found a 3-fold downregulation of the *SERPINB2* gene. On siRNA *SERPINB2* gene inhibition, osteogenic and adipogenic differentiation was stimulated, suggesting TGF- β 1 suppresses *SERPINB2*, promoting osteogenesis and adipogenesis.²⁹ Our findings also confirm downregulation of *SERPINB2* on TGF- β 1 cultures; however, our study demonstrates upregulation of *SERPINA9* in hMSCs when stimulated with either KGN and TGF- β 1, which had not previously been described. Furthermore, KGN showed upregulation of *SERPINB2*, which suggests these Serpins may be differentiation markers of lineage commitment toward cartilage, which has been recently published as the default pathway for these cells.³⁰

Another member of the Serpin superfamily, *SERPINH1* or heat shock protein 47, had previously been described

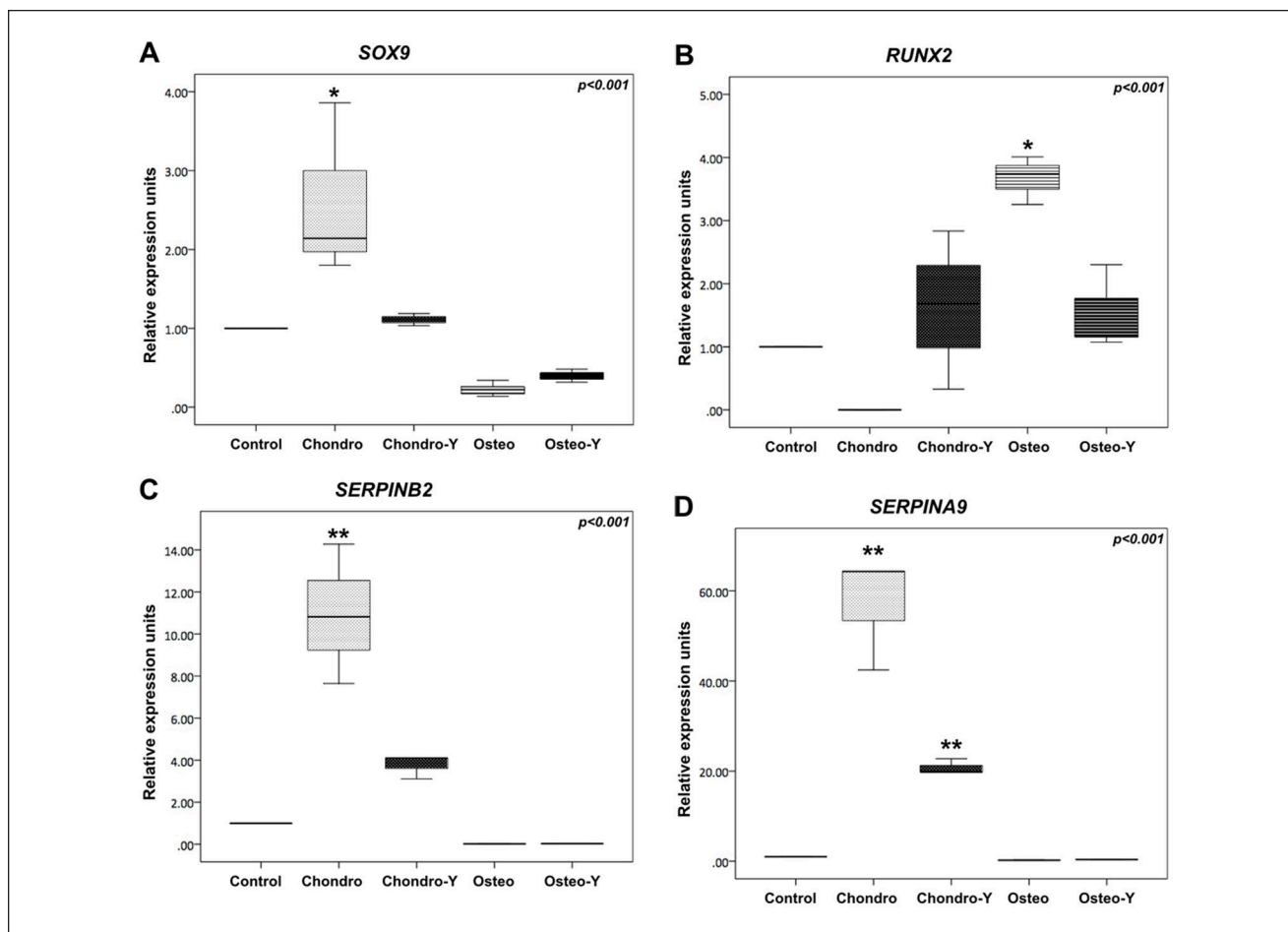


Figure 3. Real-time PCR analysis of the different culture conditions. Gene expression analyses of (A) *SOX9*, (B) *RUNX2*, (C) *SERPINB2*, and (D) *SERPINA9* in hMSCs, and after cell differentiation into chondrocytes, osteoblasts with and without ROCK inhibition. Box-plots normalized by the control group (hMSCs). Chondro = chondrocytes; Chondro-Y = chondrocytes with ROCK inhibitor Y27632; hMSCs = human bone marrow mesenchymal stem cells; Osteo = osteoblasts; Osteo-Y = osteoblasts with ROCK inhibitor Y27632; ROCK = Rho-associated coiled-coil containing protein kinase. * $P < 0.05$ as compared to Control; ** $P < 0.05$ as compared to all other study groups.

as an essential molecular chaperone for cartilage and endochondral bone formation, at collagen last stage assembly.³¹ Its role was characterized downstream of genes controlling chondrocyte and osteoblast commitment and differentiation. *SERPINE2* has been recently reported to be upregulated in cartilage on IL-1 α stimulation, inhibiting metalloproteinase activity at the cartilage level to decrease collagen breakdown.³² *SERPINA1* and *SERPINA3* were also previously explored as markers of chondrogenic differentiation and dedifferentiation; however, bone marrow aspirates were not characterized with ISCT criteria and lacked information for *SOX9* expression.³³ Finally, *SERPINI2* has also been described in MSCs but not in articular cartilage, in a previous study with bone marrow aspirates that were not also not fully characterized or evaluated during differentiation.³⁴ Taking

into account what has been previously reported, Serpin superfamily members have antagonist roles for cell differentiation from mesoderm precursors toward cartilage, bone, and adipose tissue.

Our study has various strengths. Our study model was a cell line previously validated for differentiation to adipocytes, chondrocytes, and osteocytes, with complete criteria for characterizing MSCs, which we found is a limitation for most studies previously reported. We decided on the use of this model based on the study publishing the discovery of KGN, which also used a commercial cell line.⁹ Initially, our findings performed by microarray analysis with regard to Serpins were unexpected; however, we decided to further explore and validate by qPCR with novel constitutive genes specific for hMSC and validated commercial primers (QIAGEN).

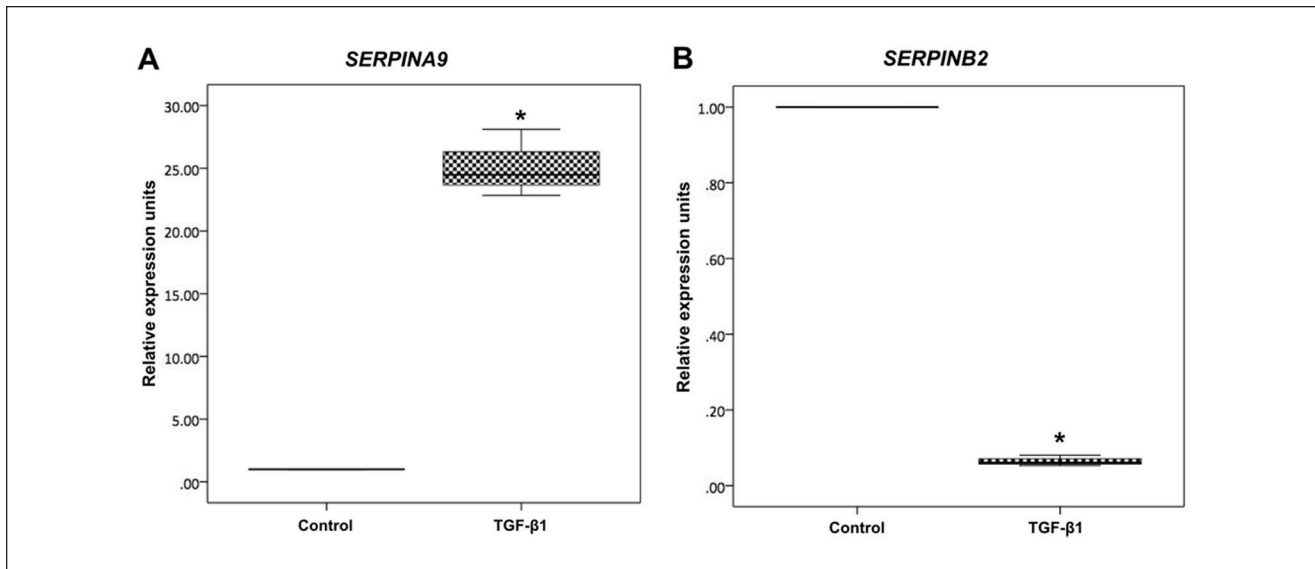


Figure 4. Real-time PCR analysis of culture with TGF- β 1. Gene expression analyses of (A) *SERPINA9* and (B) *SERPINB2* in hMSCs and after cell culture with transforming growth factor beta 1 (TGF- β 1). Box-plots normalized by the control group (hMSCs). * $P < 0.001$ as compared to Control.

Upregulation of these genes was very significant, with very stringent methodology. Results were very marked, particularly with *SERPINA9* upregulation showing a 60-fold increase with KGN, and a 20-fold increase with TGF- β 1, which identifies it as a novel differentiation marker for lineage commitment and potential regulator. We acknowledge that Serpins may provide a new tool for regenerative medicine. However, regenerative efforts must take into account that a single target (i.e., gene, protein) is not sufficient to obtain cell differentiation, and that the extracellular environment is as important.² Especially when cells are cultured *in vitro ex vivo*, this hurdle might be tackled using chemical compounds that mimic physical forces, by altering signaling pathways such as the Rho GTPases that translate external stimuli.¹¹ We also acknowledge that functional studies were not performed, that data from this study will require external validation, and that future research is required to check for basal expression of Serpins in native articular cartilage, as well as hypertrophic chondrocytes and subchondral bone.

Conclusions

In conclusion, we describe *SERPINA9* and *SERPINB2* of the Serpin superfamily of proteins as novel differentiation markers, and molecular regulator candidates for hMSC lineage commitment toward bone and cartilage, providing a new tool for regenerative medicine. Our study highlights the roles of these 2 genes, with significant upregulation of both in cell cultures stimulated with Kartogenin.

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Declaration of Conflicting Interests

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

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Supplemental material

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