Identification of novel long noncoding RNA associated with chondrocyte hypertrophy

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Directed by Professor Jin Woo Lee

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2018년부터 시작된 저의 실험실 생활이 어느덧 막바지에 이르러 졸업을 마주하게 되었습니다. 크고 높게만 보였던 석사 학위를 얻을 수 있게 도와주신 많은 분들께 감사의 말을 전하고자 합니다.

먼저, 석사 학위 시작의 기회를 주시고 연구자로서의 자세를 가르쳐 주신 이진우 교수님께 감사드립니다. 교수님께서 회식 때마다 해주신 삶의 이정표 같은 교훈들과 좋은 말씀들 항상 마음에 새기며 나아가겠습니다.

바쁘신 중에도 저의 부족한 학위 연구가 올바르게 나아갈 수 있도록 함께 봐주시고 많은 조언을 해주신 박상욱 교수님, small RNA라는 주제로 연구할 수 있게 해주시고 항상 따뜻한 조언으로 방황하고 있던 저의 연구를 바로 잡아주신 김성환 교수님께도 감사드립니다. 실험실 생활 중 가장 가까이에서 조언과 도움을 주신 박광환 교수님께도 감사드립니다.

저의 학위 주제의 시작과 끝을 함께 해주시고 많은 도움을 주신 윤동석 박사님 감사합니다. 학부 졸업 후 ABMRC 201호라는 곳에서 가족보다 긴 시간 함께한 연구실 멤버들, LJWOS 라는 곳을 처음 소개해주시고 적응할 수 있도록 많은 도움 주신 이경미 박사님, 처음 실험을 알려주신 고은애, 김지현 선생님, 대학원 동기이자 함께 해줘서 존재만으로도 고마웠던 수진이, 궂은일도 마다치 않고 웃는 얼굴로 항상 도와준 세희와 유지에게도 고맙습니다. 지금은 졸업하고 취업해서 멋진 백 박사님으로 지내고 있는 다운 언니에게도 항상 응원해주고 격려해줘서 정말 고맙다고 전하고 싶습니다.

학위 과정 동안 아무 걱정 없이 공부에만 집중할 수 있는 환경을 만들

어준 엄마, 아빠, 언니 그리고 주말마다 이모랑 놀아주느라 바빴던 동윤이와 주말에 갈 때마다 맛있는 음식들을 소개해준 형부에게도 감사합니다.

힘들 때마다 묵묵히 들어주고 응원해주고 믿어준 많은 친구들에게도 고맙다고 전하고 싶습니다. 훗날 아무리 바쁘고 힘들어도 많은 사랑과 기도를 받으며 보냈던 이 시간을 기억하며 주변을 돌보고 항상 감사할 줄 아는 사람이 되도록 노력하겠습니다. 그리고 이곳에서 배운 배움과 깨달음들을 사회에 나가서 많은 사람에게 공유하고 세상의 변화에 이바지할 수 있는 연구원이 되겠습니다.

감사합니다.

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ABSTRACT

Identification of novel long noncoding RNA associated with chondrocyte hypertrophy

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Background: Osteoarthritis (OA) is characterized by chondrocyte hypertrophy and its progression causes the focal calcification of joint cartilage. The molecular mechanisms regulating the progression of OA and hypertrophic chondrocytes are poorly understood. Recently, lncRNA has proven to be critical to various developmental and epigenetic regulatory processes of treatment for OA [1,2]. The aim of this study was to investigate lncRNA expression patterns in hypertrophic chondrocytes and determine the characteristics of gene regulation processes associated with hypertrophic differentiation.

Methods: TC28a2 human-immortalized chondrocytes were used to study hypertrophic chondrocytes by differentiation induction. The expression of the hypertrophic markers COL10A1, RUNX2, and osteocalcin was investigated by qRT-PCR and immunoblotting during hypertrophic differentiation in TC28a2 cell line. To examine the activity of the osteocalcin promoter, a human osteocalcin reporter assay was performed.

Results: Immunoblotting results showed that hypertrophic marker expression increased during hypertrophic differentiation but COL2A1 expression decreased. Using RNA-seq, transcriptomic differences were examined and lncRNAs were observed to be expressed more hypertrophic chondrocytes than normal chondrocytes. The top five up-regulated differentially expressed lncRNAs are LINC02593, H19, OBSCN-AS1, FOXD2-AS1, and LOC101927811. Further experiments showed that silencing those lncRNAs inhibit the activity of the transcriptional factor RUNX2. FOXD2-AS1 and LOC101927811 were shown to inhibit the expression of COL10A1 and rescue the chondrogenesis marker COL2A1.

Conclusion: This study identified that lncRNA expression was upregulated in hypertrophic chondrocytes and FOXD2-AS1 and LOC101927811 regulate chondrocyte hypertrophy and could serve as targets for osteoarthritis therapies.

Key words: long noncoding RNA, bone development, RNA-sequencing, cell-based therapy, chondrocytes, hypertrophic differentiation, FOXD2-AS1, LOC101927811

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I. INTRODUCTION

Osteoarthritis (OA) is a chronic and degenerative joint disease that seriously affects the quality of life of sufferers. Patients with OA experience joint pain, deformities, and chronic disability. OA causes changing the structure of articular cartilage to change and degrade, subchondral bone sclerosis, and synovial inflammation that is caused by inflammatory cytokines, such as tumor necrosis factor-α, Interleukin-1 β (IL-1β), growth factor and matrix metalloproteinases (MMP). MMP13, disintegrin and metalloproteinase with thrombospondin motif 5 (ADAMTS5) play critical roles in

cartilage degradation[3]. However, cartilage is not supplied with blood, and has only a limited ability to repair itself [4].

During growth plate development, chondrocytes are altered by their environment. Under normal conditions, chondrocytes maintain their surrounding extracellular matrix (ECM) comprising collagens, proteoglycans and noncollagenous matrix proteins[5]. Healthy mature chondrocytes remain in a quiescent state and resist proliferation and, hypertrophy through mineralization that comprises the bone matrix. However, in diseased conditions, chondrocytes recapitulate the differentiation processes that promote the development of hypertrophic chondrocytes[6]. In early stage of hypertrophic differentiation, RUNX2 has been reported to directly regulate the expression of type X collagen by binding its promoter[7]. It makes hypertrophic differentiated chondrocytes express type X collagen and low expression of cartilaginous specific markers, such as collagen type II. Also, the runx regulatory element was found in the promoter region of osteocalcin gene promoter that led to development of an osteoblast phenotype[8]. Hypertrophic chondrocytes are characterized by an enlarged size leads to apoptotic death, mineralization of the diseased cartilage, and replacement by calcification[9]. Chondrocytes degenerate in similar ways. Uncovering the mechanism of the induction of chondrocyte hypertrophy can lead to the development of OA treatments which inhibit these pathological changes. It was difficult to efficiently obtain sufficient numbers of primary chondrocytes that were isolated from articular cartilage. It was also difficult to

maintain chondrocyte with a cartilage-specific phenotype. Thus, the immortalized, human-origin TC28a2 chondrocyte cell line was selected to induction of hypertrophic differentiation. The TC28a2 cell line was primary cultured in chondrocyte transfected with simian virus 40 (SV40) large T antigen and neomycin resistance genes for five days. It expressed mRNAs encoding cartilage matrix marker proteins such as collagen, proteoglycans and sulfated glycosaminoglycans (Figure 1)[10]. The expression patterns of these key hypertrophic factors is summarized in Figure 1.

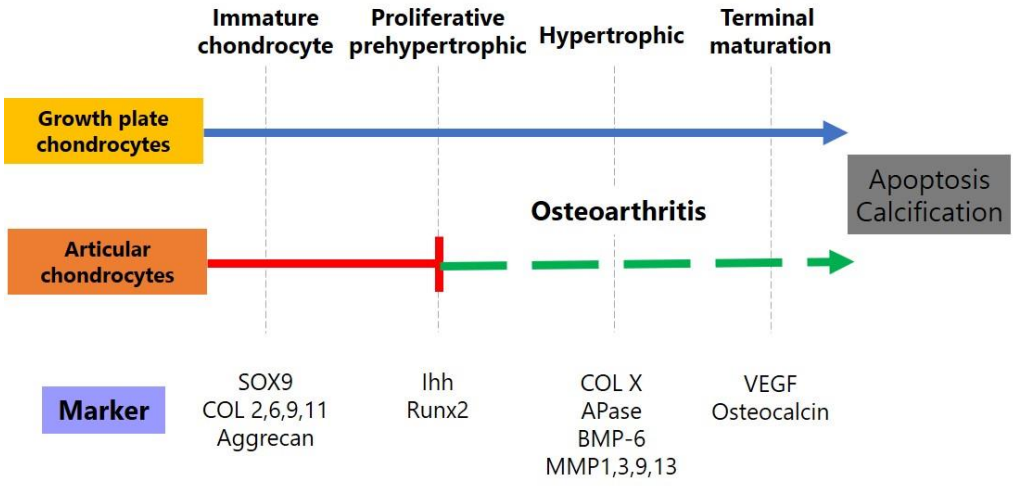


Figure 1. The expression patterns of key hypertrophic marker genes during the progression of hypertrophic differentiation.

An ncRNA is a functional RNA molecule that is not translated into proteins. Generally, the central process in molecular biology is DNA being transcribed into messenger RNA (mRNA) which are then translated into proteins. Also, protein-coding genes in the genome are reported that they are comprised less than 2% of the human genome[11]. Their relatively small population suggests that most transcription factors are noncoding RNAs (ncRNA) in genome. ncRNA that have more than 200 nucleotides are called long noncoding RNAs (LncRNA). They are structurally similar to protein-coding mRNA but have little or no protein-coding ability. LncRNAs are derived from both intergenic and overlapping protein-coding regions of genes and are transcribed by Polymerase II [12]. LncRNAs are classified as one of the following according to their orientation and position in the genome: antisense, long intergenic noncoding RNA (lincRNA), sense-overlapping, sense intronic, and processed transcript[13]. lncRNAs can regulate the genes that govern in skeletal muscle myogenesis and contribute to skeletal muscle malfunction by binding to other DNA, RNA, or protein[14]. lncRNAs have been reported to play important epigenetic regulatory roles in molecular processes[1]. lncRNAs can regulate OA, but the mechanism by which it does is still unclear[15]. In this study, RNA-sequencing (RNA-seq) was performed to identify novel lncRNAs which can be used to inhibit chondrocyte hypertrophy. This result suggests that regulating the expression of lncRNAs might inhibit the occurrence of pathological changes in OA cartilage.

II. MATERIALS AND METHODS

1. Cell lines and cell culture

TC28a2 cell line was purchased from the ATCC. TC28a2 cell line was established by transfecting primary cultures (day 5) of costal cartilage from a 15-year-old female with a retroviral vector expressing simian virus SV40 large T antigen[10]. TC28a2 cell line is widely used as a model cell line for studying normal and pathological cartilage repair mechanisms related to chondrocyte biology and physiology. The cells were cultured with Dulbecco's modified Eagle's medium with high Glucose (DMEM-HG; Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; RDT) and 1% (v/v) antibiotic antimycotic solution (Gibco). The cells were harvested at 80% confluences using 0.05% trypsin-EDTA (Gibco). Cells were washed, centrifuged, resuspended and seeded in new plates. The medium was replaced every 2-3 days with fresh medium.

2. Hypertrophic differentiation of TC28a2 cell line

For hypertrophic differentiation, cells were cultured in DMEM supplemented with 1% FBS, 1% insulin Transferrin Selunium-A (ITS, Invitrogen), 100 ng/ml BMP-2 (NKMAX), 10 ng/ml GDF-5 (PeproTech), 1 μM 3,3,5-Triiodo-L-thyronine (T3) (Sigma), 50 μg/ml L(+)-Ascorbic acid, 10 nM dexamethasone, 10 mM β-

glycerophosphate and 1% (v/v) penicillin–streptomycin. The hypertrophy medium was replaced every 2-3 days.

3. Quantitative real-time-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells using an AccuPrep Universal RNA Exraction Kit (K-3141, Bioneer, Daejeon, South Korea). RNA (2 μg) was then reverse-transcribed using an Omniscript kit (205113, Qiagen). Quantitative real-time-polymerase chain reaction (qRT-PCR) was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and 2×qPCRBIO SyGreen Mix (PB20.12-05, PCR Biosystems, London, UK) according to the manufacturers' guidelines. Primer sequences are shown in Table 1**.**

Table 1. Primer sequences for qRT-PCR

Gene names		Sequence (5' → 3')
18S rRNA	Forward	ACACGGACAGGATTGACAGA
	Reverse	GCCAGAGTCTCGTTCGTTAT
COL10A1	Forward	P261909 (Bioneer)
	Reverse	
COL2A1	Forward	P298511 (Bioneer)
	Reverse	
RUNX2	Forward	P229954 (Bioneer)
	Reverse	
OCN	Forward	P128146 (Bioneer)
	Reverse	
GAPDH	Forward	P267613 (Bioneer)
	Reverse	

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, 18S rRNA: 18S ribosomal RNA, COL10A1: Collagen Type X Alpha 1 Chain, COL2A1: Collagen type II Alpha 1, RUNX2: Runt-related transcription factor 2.

4. Immunoblotting

Total proteins were prepared from the cells using Pro-prep protein extraction solution (Intron). 30μg of each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with tris-buffered saline with tween 20 (TBST) containing 5% skim milk, blots were incubated for overnight at 4°C with primary antibodies against RUNX2 (1:1,000, sc390351, Santa Cruz Biotechnology, CA, USA), COL10A1 (1:1,000, ab182563 Abcam, Cambridge, United Kingdom), COL2A1 (1:1,000, sc-518017, Santa Cruz Biotechnology), β-actin (1:1,000, sc-47778, Santa Cruz Biotechnology). All membranes were washed three times with TBST for 10 minutes and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000, SA001-500 or SA002-500, GenDEPOT, Barker, TX, USA) for 1 hour at room temperature. Finally, all membranes were washed three times with TBST for 10 minutes and western blot images were detected by LAS 4000 (Fujifilm, Tokyo, Japan).

5. Immunocytochemistry

For hypertrophic differentiation, cells were dotted on the center of each well on the 24-well plates. Single drop of 1X Phosphate-Buffered Saline (PBS, Thermo scientific, Logan, Utah, USA) was placed in between each well to suppress over-drying of the

cells. Cells were then placed on 37°C incubator maintained with 5% CO_2 for 2 hours to stimulate adherence of the cells to the plate and then, maintained for 7 days in hypertrophy medium. The hypertrophy medium was replenished every 3 days. For, immunocytochemistry, the cells were washed with PBS and fixed in 4% paraformaldehyde for 10 minutes and washed twice with PBS. To permeabilization the cells were incubated with 0.25% PBST (PBS + 0.25% Triton-X 100) for 10 minutes and washed twice with PBS. To reduce nonspecific background staining, the cells were incubated in 3% BSA (in PBST) for 1 hour. The cells were incubated with RUNX2 (1:1,000, sc390351, Santa Cruz Biotechnology, CA, USA), COL10A1 (1:1,000, ab182563 Abcam, Cambridge, United Kingdom), COL2A1 (1:1,000, sc-518017, Santa Cruz Biotechnology) and Osteocalcin (1:1,000, sc-30045, Santa Cruz Biotechnology) overnight at 4°C and then washed with PBS. Detection of the signal was performed with AEC substrate kit (Abcam).

6. Human osteocalcin (OCN) pGreenZeo differentiation reporter assay

To establish TC28a2 cell line that stably express GFP where the *OCN* promoter/enhancer is active, Lentiviral plasmid for human *OCN* pGreenZeo differentiation reporter was purchased (System Biosciences, LLC; SBI SR1003PA-1). To obtain lentiviral particle with human *OCN* pGreenZeo differentiation reporter, HEK293T cells were seeded in T75 flask at a density of 5 $x10^6$ cells per dish. On the

next day, the cells were transfected with lentiviral plasmid for human *OCN* pGreenZeo differentiation reporter with the psPAX2 (http://www.addgene.org/12260/) and pMD2.G (http://www.addgene.org/12259/) using Lipofectamine 2000 (Invitrogen). After 6 hours of transfection, the medium was replaced. The transfected cells were maintained for 2 days, and then the supernatants were collected and stored at − 70 °C. To monitor GFP activity in TC28a2 cell lines infected with lentivirus of *OCN* pGreenZeo differentiation reporter, the cells were seeded in 6-well plates at a density of 1 x 10^5 cells under hypertrophic stimuli. After 7 days of hypertrophic differentiation, GFP signal was observed under fluorescence microscopy.

7. RNA-Seq analysis

Isolated RNAs submitted to the Macrogen Inc. (Korea) for total RNA-seq. The overall quality of the total RNA was validated using spectrophotometry. To remove low quality and adapter sequence, the raw was read by the sequencer before analysis and align the processed reads to the Homo sapiens using HISAT2 v2.1.0[16]. The reference genome sequence of Homo sapiens (hg19) and annotation data were downloaded from the NCBI. And then, transcript assembly of known transcripts was processed by StringTie v 2.1.[17,18]. Base on the result of that, expression abundance of transcript and gene were calculated as read count or FPKM value (Fragments Per Kilobase of exon per Million fragments mapped) per sample. The expression profiles are used to do additional

analysis such as DEG (Differentially Expressed Genes). The relative abundances of gene were measured in read count using StringTie. Genes with one more than zeroed read count values in the samples were excluded. Filtered data were log2-transformed and subjected to RLE Normalization. Statistical significance of the differential expression data was determined using DESeq2 nbinomWaldTest[19] and fold change in which the null hypothesis was that no difference exists among groups. False discovery rate (FDR) was controlled by adjusting p value using Benjamini-Hochberg algorithm. For DEG set, hierarchical clustering analysis was performed using complete linkage and Euclidean distance as a measure of similarity. Enrichment of gene ontology analysis was performed for DEGs using g:Profiler[20] and KEGG pathway analysis was tested based on KEGG pathway (https://www.genome.jp/kegg/) database. We used multidimensional scaling (MDS) method to visualize the similarities among samples. MDS is one of the methods that convert the structure in similarity matrix to a simple geometrical picture as scatter plots. The larger the dissimilarity between 2 samples, the further apart the points representing the experiments in the picture should be. We applied to the Euclidean distance as the measure of the dissimilarity. Hierarchical clustering analysis also was performed using complete linkage and Euclidean distance as a measure of similarity to display the expression patterns of differentially expressed transcripts which are satisfied with $|\text{fold change}| \geq 2$ and raw $p < 0.05$.

All data analysis and visualization of differentially expressed genes was conducted

using R 3.6.1(www.r-project.org).

8. Validation of gene expression in mRNA level

For cDNA synthesis, 2μg RNA was reverse transcribed using the Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany). The list of primer sets used for specific specific lncRNA amplification was obtained from Bioneer. GAPDH was used to normalize for relative expression intensity of all genes.

Table 2. Sequences of primers used for lncRNA PCR analysis

Gene names		Sequence (5' → 3')
FOXD2	Forward	CCTCCTACCAATTACACGCC
	Reverse	GTACACACAGGTCGCTATGG
LINC02593	Forward	CCGAGCTTCAGAGGAGAGG
	Reverse	CTCCCAGATCGTGAGCTTTC
H19	Forward	AAGAAATGGTGCTACCCAGC
	Reverse	GTGCAGTGGTTGTAAAGTGC
OBSCN	Forward	CTCCTCGATGCCGTACTTG
	Reverse	CCACGTTCCTATGTGAGGTG
LOC101927811	Forward	TTAAACGAGCCCTGGATCTG
	Reverse	CCCGAAACAGACTTCTGAAC

FOXD2-AS1: FOXD2 adjacent opposite strand RNA 1, LINC02593: long intergenic non-protein coding RNA 2593, H19: H19 imprinted maternally expressed transcript, OBSCN-AS1: OBSCN antisense RNA 1, LOC101927811: uncharacterized LOC 101927811.

9. Knockdown of lncRNAs

TC28a2 cell line was seeded at 2 x 10^5 cells in 6-well plates. After 24 hours of culture, cells were transfected with 200nM siRNAs (Bioneer) using calfectin (Signagen laboratories, Rockville, USA) according to manufacturer's instructions. After 24 hours of transfection, the medium was replaced with hypertrophy medium and then incubated

7 days. The hypertrophy medium was replaced every 2-3 days. SiRNAs sequence are shown in Table 3.

Table 3. siRNA siRNA duplex sequences

Gene Name	Sequence
FOXD2	TCGTCTCTCCCTCTCTTCCTTTT
LINC02593	CTGATTACAGAGTCATTCATTCT
H19	GAGCAGCCTTCAAGCATTCCATT
OBSCN-AS1	CTGAGGAAAGCAAATGTCACTGT
OBSCN-AS1	TTCTCTGACTGTGTGTCTCTACC
LOC101927811	ATCATCTTTAGGGTTTTCTTAAA

10. Statistical analysis

In all applicable settings, appropriate statistical analysis had been carried out to derive at statistically significant result. In all figures, graphs and tables, * indicates p-value < 0.05, while ** indicates p-values < 0.01.

III. RESULTS

1. Hypertrophic differentiation of cultured TC28a2 occurs after 7 days

Hypertrophic differentiation was induced for 7 days and it was evaluated by expression of hypertrophic marker genes at 0, 1, 4 and 7 days to determine the timing of hypertrophy in TC28a2 cell line. From previous studies, the expression pattern of certain key marker genes such as COL10A1 and RUNX2 during hypertrophic differentiation has been elucidated. Immunoblotting analysis showed that the expression of COL10A1 and RUNX2 expression increased. However, the expression of COL2A1 was markedly decreased at day 7 (Figure 2).

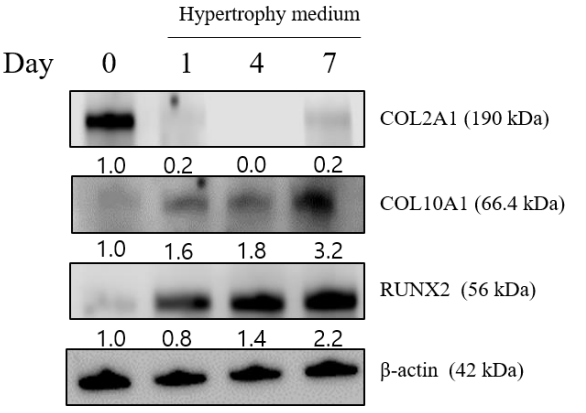


Figure 2. Changes in the expression of marker proteins expression during hypertrophic differentiation. β-actin was used for normalization. TC28a2 cell line was seeded in 6 well plates at a density of 2 x 10^5 cells/well. TC28a2 cell line was maintained for 1,4, and 7 days in hypertrophy medium, respectively. Then, the cell was harvested at designated time points. Day 0 means undifferentiated TC28a2 cell line.

Next, qRT-PCR analysis was performed to establish whether the expression pattern of the key marker genes from day 0, 1, 4, and 7 days during hypertrophic differentiation. By qRT-PCR, the results was investigated that COL10A1, and osteocalcin was increased during hypertrophic differentiation. However, the expression patterns of COL2A1 was also increased during hypertrophic differentiation. This result was not correlated with expression patterns of immunoblotting in Figure 1. Also, the expression levels of RUNX2 expected increase during hypertrophic differentiation remained relatively unchanged.

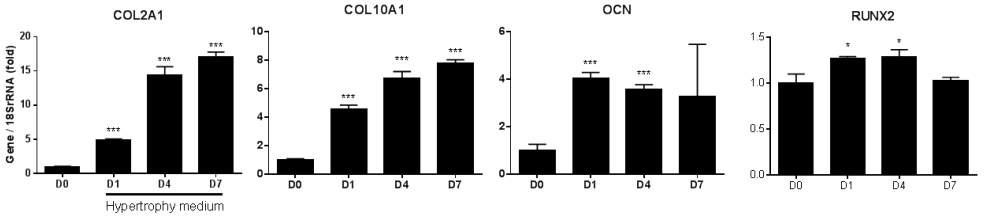


Figure 3. Relative mRNA expression levels in hypertrophic differentiation of TC28a2 cell line. Relative mRNA expression of hypertrophic marker (RUNX2, OCN, COL10A1) measured by qRT-PCR in hypertrophic differentiation of TC28a2. 18S rRNA was used for normalization. TC28a2 cell line was seeded in 6 well plates at a density of 2 x 10^5 cells/well. TC28a2 cell line was maintained for 1, 4 and 7 days in hypertrophy medium, respectively. The cells were harvested at designated time points. Day 0 means undifferentiated TC28a2 cell line.

To investigate the transcriptional activation of osteocalcin during hypertrophic differentiation, the hOCN copGFP reporter assay was performed. Transient expression of hOCN can activate the expression of green fluorescent protein through the hOCN promoter. The GFP expression was detected during hypertrophic differentiation. These results showed that hOCN promoter was activated during hypertrophic differentiation in hOCN-transfected TC28a2 cell line.

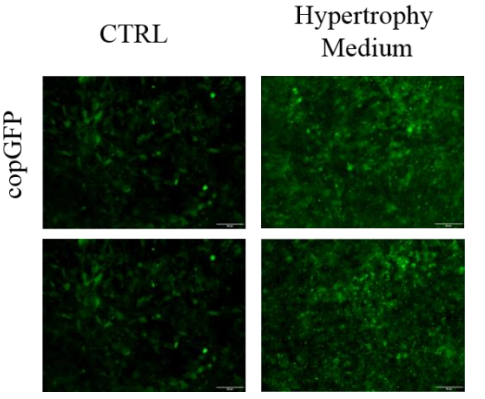


Figure 4. GFP expression in hOCN-transfected cells. Transfected TC28a2 cell line was maintained for 7 days in hypertrophy medium. Expression of OCN protein after differentiation measured by immunofluorescence.

To examine the expression of hypertrophy marker induced by hypertrophy medium, immunocytochemistry was performed. At day 7, the results showed the phenotypic increase of COL10A1, RUNX2 and decrease of COL2A1 by immunocytochemistry (Figure 5). These data suggested that TC28a2 tend to acquire a hypertrophic phenotype after 7 days of hypertrophy induction.

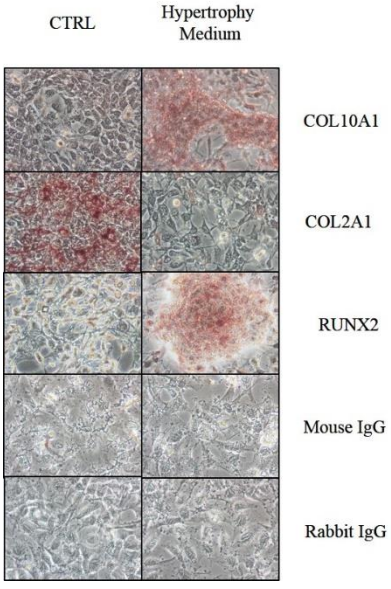


Figure 5. Detection of hypertrophic markers by immunocytochemistry. Immunocytochemistry was performed at day 7 after hypertrophic differentiation.

2. Overview of lncRNAs and mRNA expression profiles

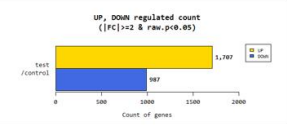
Next, total RNA-seq analysis has been performed to screen lncRNAs with differential expression patterns in hypertrophic-induced TC28a2 cell line. Total RNA was extracted using AccuPrep universal RNA Extraction Kit (Bioneer). We compared the differential expression genes in chondrocytes (CTRL) versus hypertrophy chondrocytes. The RNA integrity number (RIN) was assessed. All RIN value were > 7, and 260:280 ratios were > 1.5 (Table 4).

Table 4. Quality control results for RNA-seq samples

Sample Name	Conc. (ng/ul)	Final Volume (ul)	Total Amount (ug)	RIN	rRNA Ratio	Result
CTRL1	154.904	15	2.324	9.6	4.4	Pass
CTRL2	181.99	15	2.73	9.8	3.6	Pass
HYPERTROPHY1	62.545	16	1.001	9.3	2.2	Pass
HYPERTROPHY3	78.881	17	1.341	7.1	1.5	Pass
HYPERTROPHY2	124.129	17	2.11	9.5	2.9	Pass

A total of 1707 was upregulated (log2 [fold-change] > 2, FDR < 0.05) and 987 were downregulated (log2 [fold-change] < -2, FDR < 0.05) in response to induce hypertrophic differentiation. Furthermore, among 2181 protein-coding genes and 223 lncRNAs screened. The volcano plots display the variations in the expression of lncRNAs and mRNA between CTRL and Hypertrophic differentiation groups. Hierarchical clustering shows differential expression of lncRNAs and mRNA between CTRL and Hypertrophic differentiation groups.

A



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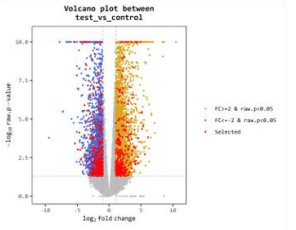

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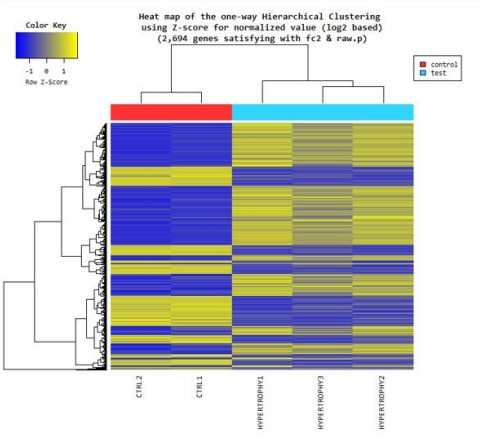

Figure 6. Differential expression of mRNAs and lncRNAs in hypertrophic differentiation. (A) Numbers of up-regulated (yellow) and down-regulated (blue). (B) Volcano plot of differentially expressed genes of hypertrophic differentiation versus normal chondrocyte. Red points indicate differentially expressed lncRNAs. X-axis represents fold change (log 2) and Y-axis represents P (−log 10). (C) Hierarchical clustering of RNA-seq data. Heatmaps showing hypertrophic differentiation-related lncRNAs and mRNAs. The relative expression of lncRNAs and mRNAs is depicted according the colour scale shown on the left. Yellow represents high relative expression and blue represents low relative expression, and -1, 0 and 1 are fold changes in the corresponding spectrum.

To further understand the regulatory functions of the differential expressed lncRNAs, all predicted target genes were annotated according to GO and KEGG pathway. Among the GO Enrichment terms the most abundance GO terms in the biological process (Figure 7) categories were, extracellular structure organization, extracellular matrix organization, urogenital system development, renal system development, axonogenesis, neuron projection guidance, kidney development, axon guidance, connective tissue development and cell-cell adhesion via plasmamembrane adhesion molecules. The most abundance GO terms in the cellular component categories (Figure 8) were, collagen-containing extracellular matrix, membrane raft, membrane microdomain, membrane region, endoplasmic reticulum lumen, cell-cell junction, cell-substrate junction, focal adhesion, collagen trimer and glutamatergic synapse. The most abundance GO terms in the molecular function categories (Figure 9) were, transmembrane receptor protein kinase activity, extracellular matrix structural constituent, glycosaminoglycan binding, transmembrane receptor protein tyrosine kinase activity, growth factor binding, extracellular matrix binding, growth factor activity, heparin binding, integrin binding and collagen binding.

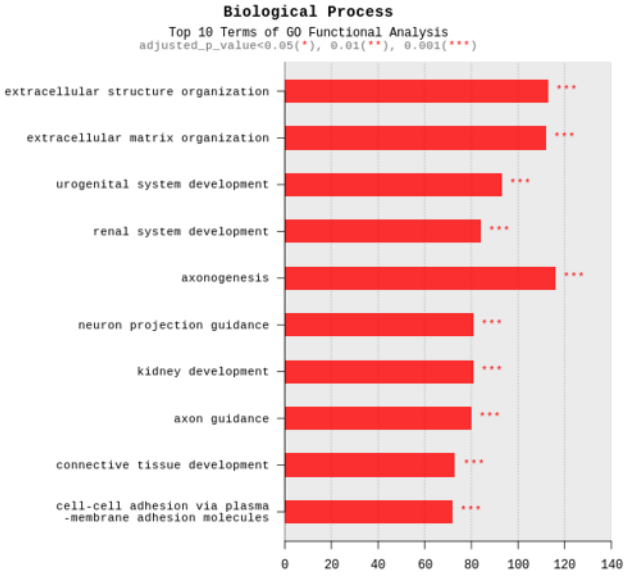

Figure 7. Top 10 potential biological functions of altered mRNAs and lncRNAs associated with hypertrophic differentiation according to $p < 0.05$

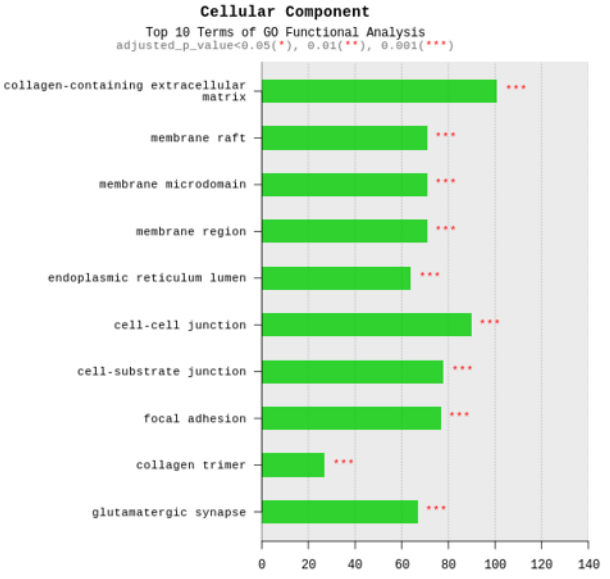

Figure 8. Top 10 potential cellular functions of altered mRNAs and lncRNAs associated with ten lncRNAs hypertrophic differentiation according to $p < 0.05$.

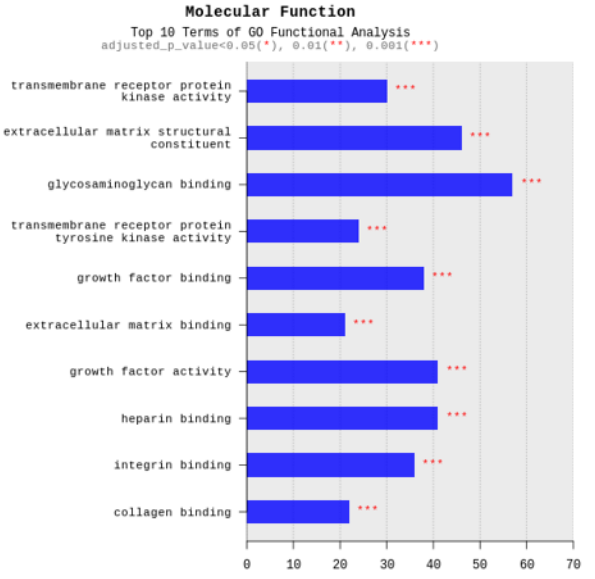

Figure 9. Top 10 potential molecular functions of altered mRNA and lncRNAs associated with ten lncRNAs hypertrophic differentiation according to $p < 0.05$.

Among KEGG pathway analysis (Figure 10), the most abundant were, pathways in cancer, MAPK signaling pathway, metabolic pathways, cytokine-cytokine receptor interaction, PI3K-AKT signaling pathway, proteoglycans in cancer, ras signaling pathway, AGE-RAGE signaling pathway in diabetic complications, focal adhesion, hippo signaling pathway, amoebiasis, fox0 signaling pathway, chagas disease (American trypanosomiases), rap1 signaling pathway, jak-STAT signaling pathway, TGF-beta signaling pathway, breast cancer, EGFR tyrosine kinase inhibitor resistance, hypertrophic cardiomyopathy (HCM) and cellular senescence.

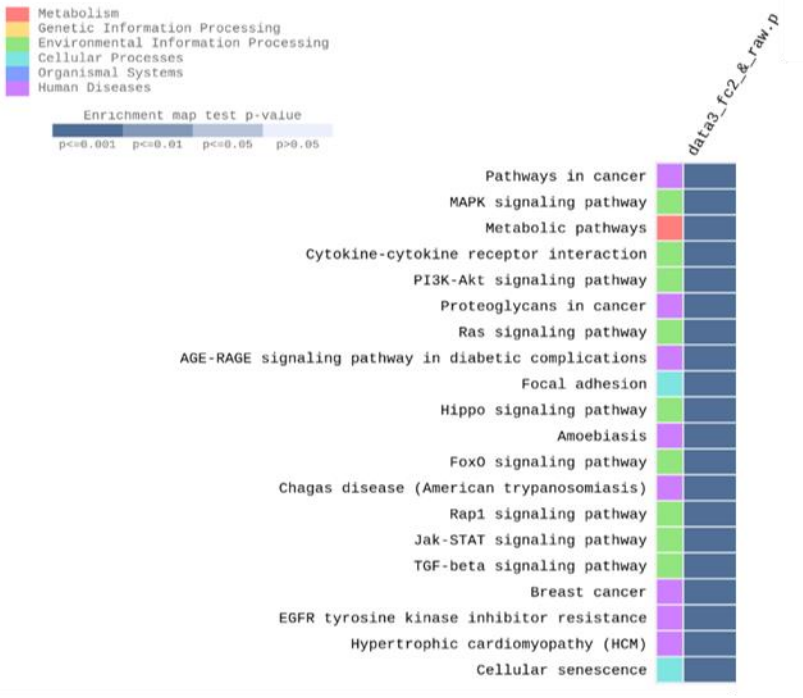

Figure 10. Kegg pathway annotation categories for target gene functions.

3. Differentially expressed lncRNAs in TC28a2 cell lines

A heatmap was revealed the differential expressed 21 lncRNAs that was more than 4 fold upregulated during hypertrophic differentiation (Figure 11). For further study, the top five upregulated differential expressed lncRNAs (DEL) were selected. LINC02593, H19, OBSCN-AS1, FOCD2-AS1 and LOC101927811 which were at least 6 fold difference in expression between groups during hypertrophic differentiation. The information of each lncRNAs on heatmap was described on Table 5.

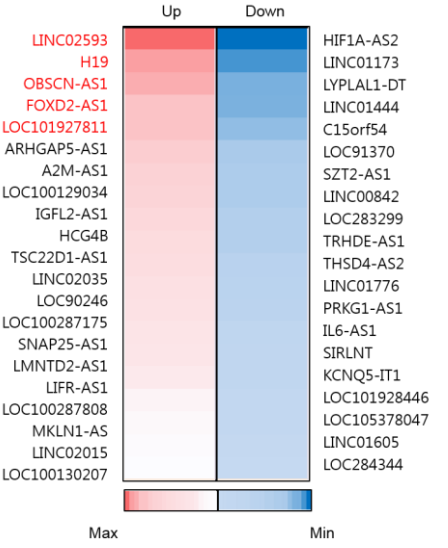

Figure 11. Heatmap of differential expressed lncRNAs in hypertrophic differentiation of TC28a2 cell line.

Table 5. Top 21 lncRNA which have shown distinctively increased expression pattern (> fold change 4) in hypertrophic differentiation induced group

Up-regulated lncRNAs day 7 versus day 0			
Gene symbol	Fold change	P-value	Gene ID
LINC02593	10.340648	0.000000	100130417
H19	8.905702	0.000275	283120
OBSCN-AS1	6.293102	0.000077	574407
FOXD2-AS1	6.191152	0.000000	84793
LOC101927811	6.157640	0.000031	101927811
ARHGAP5-AS1	5.913140	0.023821	84837
A2M-AS1	5.567017	0.002291	144571
LOC100129034	5.430814	0.000000	100129034
IGFL2-AS1	5.223893	0.011123	645553
HCG4B	5.077622	0.012678	80868
TSC22D1-AS1	4.737170	0.004025	641467
LINC02035	4.619645	0.000000	100129550
LOC90246	4.599669	0.000000	90246
LOC100287175	4.492012	0.001786	100287175
SNAP25-AS1	4.480039	0.001536	100131208
LMNTD2-AS1	4.381653	0.006470	692247
LIFR-AS1	4.379457	0.000001	100506495
LOC100287808	4.376561	0.000001	100287808
MKLN1-AS	4.241651	0.000000	100506881
LINC02015	4.182272	0.015586	102724550
LOC100130207	4.130780	0.000000	100130207

To validate the candidate lncRNAs from RNA-seq data, qRT-PCR analysis was performed (Figure 12). First, each primer of lncRNAs was designed for qRT-PCR. In agreement with the RNA-seq data, five lncRNAs were increasing expression during hypertrophic differentiation. The overexpression of each lncRNAs was 2 fold, 3 fold, 1.5 fold, 2 fold and 1.5 fold in turns at 7 days of hypertrophic differentiation.

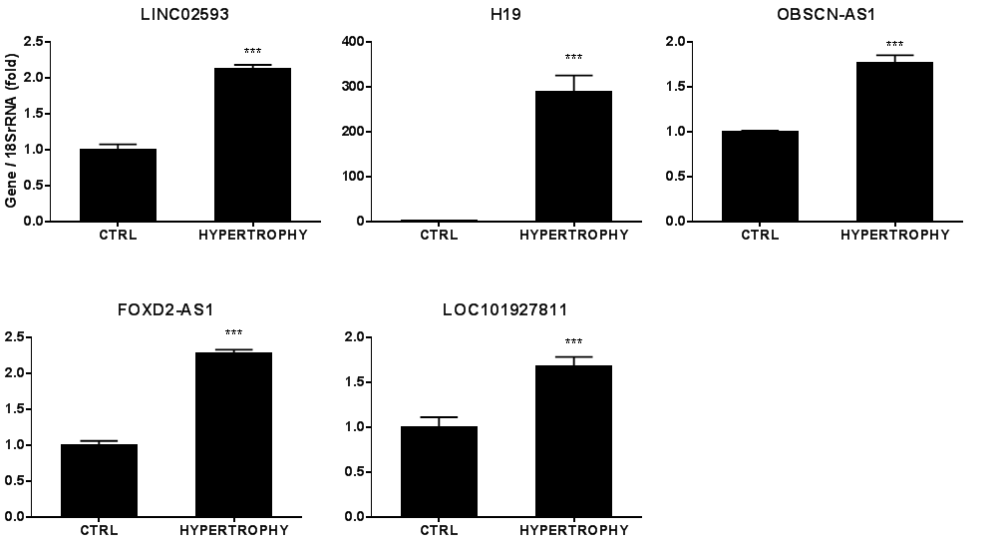

Figure 12. Validation of lncRNAs expression in mRNA level. The expression of lncRNAs in qPCR of lncRNAs (LINC02593, H19, OBSCN-AS1, FOXD2-AS1, LOC101927811) was performed. Total RNAs were isolated from hypertrophic differentiation induced chondrocytes for 7 days. 18S rRNA was used for normalization.

4. Knockdown efficiency of lncRNAs by siRNA in TC28a2

To investigate the function of five lncRNAs in hypertrophic differentiation, siRNA sequences was designed for silencing each candidates and confirmed the knockdown efficacy of lncRNAs by qRT-PCR. The OBSCN-AS1 has two isoforms but specific primer both of them are unavailable. So, two siRNA sequences was used for silencing OBSCN-AS1. Approximately knockdown efficiency was shown in TC28a2. The percentage of knockdown efficiency was 61%, 46%, 73%, 65%, 55%, 55% and 19% in turn (Figure 13). These results demonstrated that siRNA-mediated reduction of lncRNAs in TC28a2.

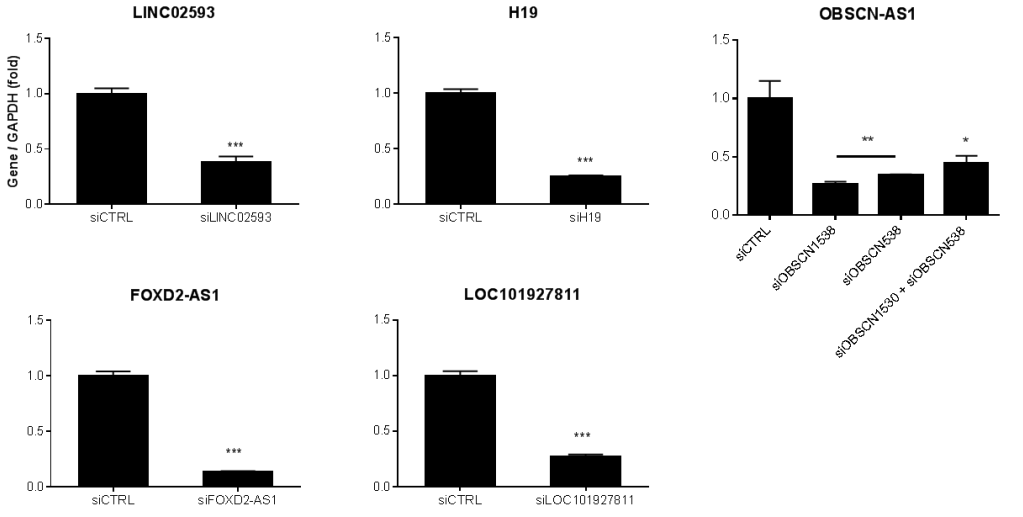

Figure 13. Knockdown efficiency of lncRNAs by siRNA in TC28a2 cell line. The knockdown efficiency for a siRNA targeting each lncRNAs were examined in TC28a2 cell lines by qRT-PCR.

5. Knockdown of lncRNAs by siRNA inhibits hypertrophic differentiation in TC28a2 cell line

To investigate the role of the 5 lncRNAs in hypertrophy induction, siRNA targeting each lncRNAs was used to knockdown lncRNAs in TC28a2. Hypertrophic differentiation was induced for 7 days after silencing the lncRNAs in TC28a2 cell line. The mRNA levels of hypertrophic markers were downregulated in silencing of lncRNAs significantly compared to hypertrophy group except for H19. The chondrogenic marker, COL2A1, was downregulated compared to CTRL group. The mRNA of RUNX2 seemed no difference except siOBSCN538 that was downregulated compared to hypertrophy chondrocytes (Figure 14A). Next, immunoblotting was performed to investigate the protein level during hypertrophy after silencing the lncRNAs. The protein level of COL2A1 was decreased when inducing hypertrophic differentiation. However, siOBSCN538, siFOXD2 group was rescued the level of COL2A1 in 3 fold and 2 fold respectively. The COL10A1 was only decreased in siFOXD2 and siLOC101927811. The protein level of master transcription marker of hypertrophic differentiation, RUNX2, was decreased in all silencing lncRNAs compared to CTRL (Figure 14B). To confirm the deactivating effect of osteocalcin by siRNA-mediated silencing lncRNAs, TC28a2 cell lines infected with lentivirus of *OCN* pGreenZeo differentiation reporter and induced hypertrophic differentiation after silencing lncRNAs with siRNAs. After 7 days of differentiation, the GFP

fluorescence was measured in decrease compared to hypertrophy. These results suggested that osteocalcin activity was suppressed by silencing lncRNAs. Next, immunocytochemistry was performed at day 7 of hypertrophic differentiation after silencing each lncRNAs. These results showed that silencing the lncRNAs suppressed the COL2A1 compared CTRL. But, suppressed protein level of COL2A1 was rescued by siFOXD2 and siLOC101927811.

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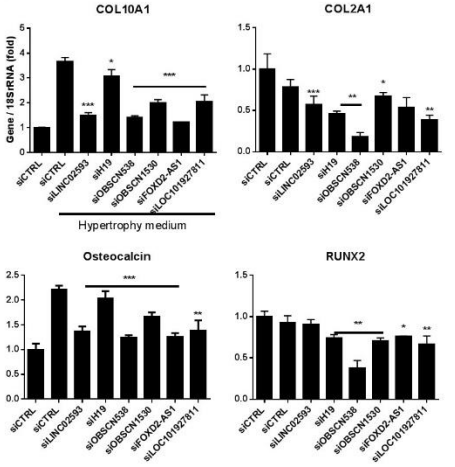

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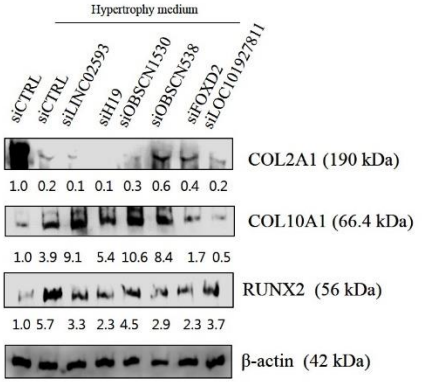

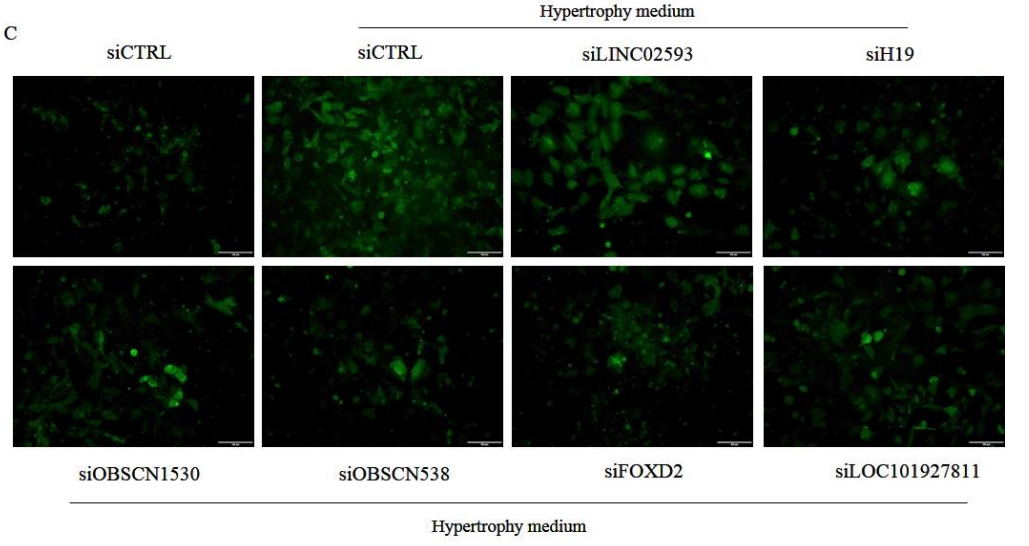

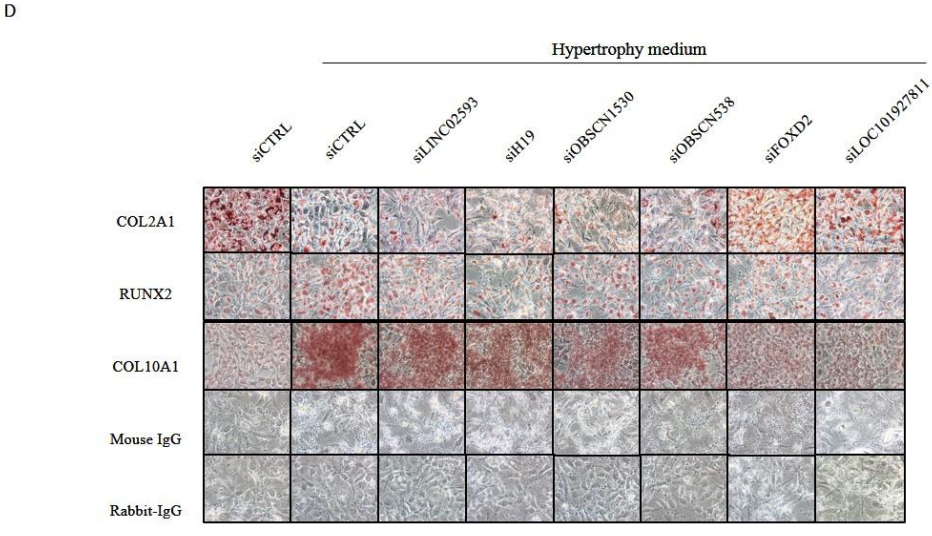

Figure 14. Knockdown of lncRNAs by siRNAs inhibits hypertrophic differentiation in TC28a2 cell line. (A) Relative expression level of Hypertrophic markers in 7 days induction of hypertrophic differentiation after siRNAs-mediated knockdown of

lncRNAs. P-values were calculated compared to hypertrophy group. *p<0.05, **p<0.01, ***<0.001 (B) Western analysis of expression of COL2A1, COL10A1 and RUNX2 in 7 days induction of hypertrophic differentiation after siRNAs-mediated knockdown of lncRNAs. (C) The inhibitory effect of silencing lncRNAs on hypertrophic differentiation was analyzed by GFP expression at day 7. (D) The inhibitory effect of silencing lncRNAs on hypertrophic differentiation was analyzed by immunocytochemistry at day 7.

IV. DISCUSSION

The purpose of cartilage regeneration is to create good quality of hyaline cartilage. However, generation of cartilage from progenital cells suppressed by hypertrophic differentiation. Recent studies reported lncRNAs affect the transcriptional and post-transcriptional regulation of extracellular matrix genes and cytoskeletal[21]. Microarray analysis was limited to detecting transcripts that according to existing genomic sequencing data. But RNA-seq investigated both unknown and known transcripts. Using RNA-seq, transcriptomic differences and unknown lncRNAs was investigated associated with hypertrophic differentiation. Before RNA-seq analysis, we prepared the samples after validating the protein and mRNA expression levels by hypertrophic marker genes. In the results, the protein levels of COL10A1 and RUNX2 were increased in western blot results. But, the protein level of COL2A1 was decreased during hypertrophic differentiation. Previous study reported that hypertrophy chondrocyte was contributed to the decrease of COL2A1[22]. The expression of corresponding marker gene, such as type X collagen, was predominantly confined to the lower cartilage zones, furthest removed from the articular surface[23]. The COL10A1 was specifically expressed in hypertrophic chondrocytes which was surrounded by a calcified extracellular matrix. Moreover, the RUNX2 was master transcriptional gene of hypertrophic chondrocyte. The RUNX2 regulated expression of COL10A1 during endochondral bone formation[24]. Next, qRT-PCR result showed that the mRNA level of

COL2A1 was increased and COL10A1, OCN was increasing during hypertrophic differentiation. The mRNA expression of RUNX2 was no difference during hypertrophic differentiation. Runx2 gene expression is known for regulating mRNA and protein levels on multiple levels, such as transcription, translation and post-transcriptional modification. So it suggested that RUNX2 was regulated by post-transcriptional during hypertrophic differentiation[25]. In previous study, osteocalcin started calcifying the cells and being accumulated in the extracellular matrix[26]. So, osteocalcin promoter activity was investigated during hypertrophic differentiation. For this study, transcriptional reporter vector, human OCN pGreenZeo was constructed. The results showed that expression level of GFP increased at 7 days of inducing hypertrophic differentiation. The control showed less GFP expression. These data indicated that GFP expression was specifically expressed by OCN promoter. Next, to visualize the localization of a specific protein or antigen in cells, the immunocytochemistry was performed. The results were correlated with immunoblotting analysis. Collectively, these data suggested that TC28a2 tend to acquire a hypertrophic phenotype after hypertrophy induction in culture for 7 days. Afterwards, samples were prepared for total RNA-seq analysis in triplicates. RNA-seq has been performed to screen lncRNAs with differentially expression pattern in hypertrophic-induced TC28a2 cell lines at day 7. RNA-seq data showed that total of 1707 was upregulated and 987 were downregulated in response to hypertrophic

differentiation. Furthermore, among 2181 protein-coding genes and 223 lncRNAs screened. The volcano plots display the variations in the expression of lncRNAs (53 upregulated genes and 72 downregulated genes) and mRNAs (736 upregulated genes and 427 downregulated genes) between CTRL and Hypertrophic differentiation groups with P-value<0.05 and |FC|>=2 as the cutoff. To further understand the molecular pathways, enrichment analysis was performed. GO and KEGG pathway analysis was used to uncover the potential mechanism in hypertrophy chondrocyte. In GO biological Process analysis, extracellular structure organization, extracellular matrix organization, and axonogenesis were top 3 GO terms related activities of gene product during hypertrophic differentiation. The extracellular matrix is comprised with COL2A1 and its essential role is support to disperse pressure and maintain the cartilage homeostasis[27]. Also, the structure of extracellular matrix provides the biomechanical propertied for synthesis of anabolic or catabolic genes[28]. The GO term definition browsers annotated the axonogenesis (GO: 0040770) that modulates the frequency, rate or extent of axonogenesis, the generation of an axon, the long process of a neuron. In cellular component analysis, collagen-containing extracellular matrix, cell-cell junction, and cell-substrate junction were top 3 GO terms which suggested where a gene product act in hypertrophy chondrocyte. In molecular function analysis, glycosaminoglycan binding, extracellular matrix structural constituent and growth factor activity was the most commonly recognized series of events during hypertrophic differentiation.

Several KEGG pathways participated in hypertrophic differentiation. The results of KEGG analysis revealed several key signaling pathways, such as pathways in cancer, MAPK signaling, Metabolic pathways, Cytokine-cytokine receptor interaction and PI3K-Akt signaling pathway. These above KEGG pathways suggested that participate in the regulation of hypertrophic chondrocyte. For further study, 5 lncRNAs were selected that were upregulated than 4 fold during hypertrophic differentiation by RNA-seq analysis. So, if silencing targeted lncRNAs, it may inhibit hypertrophic differentiation. To validate the RNA-seq analysis data, 5 lncRNAs called LINC02593, H19, OBSCN-AS1, FOXD2-AS1, and LOC101927811. Then, selected five lncRNAs were upregulated during hypertrophic differentiation was shown by qRT-PCR. These data indicated that RNA-seq results and in vitro experiments are in correlation. Additionally, each siRNA sequence was designed for silencing lncRNAs to investigate the function of lncRNAs in hypertrophic differentiation. Then, successfully knockdown efficiency of LINC02593, H19, FOXD2, LOC10192781, and OBSCN-AS1 was confirmed at 200nM concentration of siRNA transfection by qRT-PCR. For investigating the inhibitory effect of silencing lncRNAs, hypertrophic differentiation was induced after transfecting TC28a2. In qRT-PCR analysis results, mRNA expression level of COL10A1 and OCN was downregulated significantly compared to hypertrophy. The expression level of COL2A1 was not significantly recued in mRNA levels. Next, in immunoblotting analysis, the protein level of RUNX2 was decreased compared to

hypertrophy in silencing lncRNA groups. The COL10A1 was only decreased in siFOXD2 and siLOC101927811. The expression level of COL2A1 was rescued in siOBSCN538 and siFOXD2. The COL2A1 is important component of extracellular matrix molecules and it is important to extracellular signaling molecule that regulates chondrocyte proliferation, differentiation, metabolism[29-31]. Also, the initiation of matrix degradation is the process of hypertrophic differentiation. These results suggested that siFOXD2 seems to inhibit hypertrophic differentiation. The expression level of osteocalcin promoter activity was downregulated compared to hypertrophy. These data demonstrated the inhibitory effect for pre-osteoblast by silencing lncRNAs in TC28a2[32]. To investigate the phenotype of transfected cell, the ICC was perfomed at day 7 of hypertrophic differentiation after silencing lncRNAs and the results were correlated with immunoblotting results. The ICC analysis showed that RUNX2 nuclear staining had decreased in silencing lncRNAs groups compared to hypertrophy. These results suggested that mater transcriptional regulator factor, RUNX2, was suppressed by silencing the lncRNAs. L cao et al. reported that FOXD2-AS1 was upregulated in OA and it suppressed CCND1 by sponging miR-206 which was binding with CCND1[33]. In previous study, X liu et al. reported that silencing FOXD2-AS1 regulated TERT expression in post-transcriptional level in thyroid cancer[34]. A study by J liao et al. indicated that dysregulation of H19 led to impair normal osteogenesis through notch signaling[35]. Then, G dai et al. reported that H19 enhanced RUNX2 phosphorylation

during hypertrophic differentiation[36]. LINC02593 (long intergenic non-protein coding RNA 2593) was uncharacterized noncoding RNA. Recently, LOC101927811 was updated the official full name called ITGB8 antisense RNA1 by HGNC. But, there is no reports existed. Collectively, lncRNAs may regulate the gene in posttranscriptional levels and they may function with interacting target protein, RNA or DNA. Herein, we found that FOXD-AS1 has inhibiting effect of the protein expression of COL10A1 and RUNX2. Also, it rescued the protein level of COL2A1. Other 4 lncRNAs except for FOXD2-AS1 could inhibit the protein level of RUNX2 but not COL10A1. We suggested that 5 lncRNAs may function to interact with RUNX2. However, 3 lncRNAs (except FOXD2-AS1 and LOC101927811) have limitation for inhibiting hypertrophic differentiation alone. Based on our study, FOXD2-AS1 and LOC101927811 were identified as novel lncRNA that expected target for inhibiting chondrocyte hypertrophy, followed by noticeable regression for proper progression to cartilage regeneration.

V. CONCLUSION

In this study, we found top five up-regulated differentially expressed lncRNAs (DELs), LINC02593, H19, OBSCN-AS1, FOXD2-AS1 and LOC101927811 by RNA-seq. Then, we performed knockdown study of lncRNA by siRNA. From immunoblotting, reporter assay and immunocytochemistry, knockdown of FOXD2-AS1 rescued the COL2A1 expression and suppressed the expression level of COL10A1 and RUNX2. Other 4 lncRNAs also inhibited the expression level of RUNX2, but cannot rescued the COL2A1. The results of this study may provide 2 novel therapeutic lncRNAs : FOXD2-AS1 and LOC101927811 for inhibiting of hypertrophic differentiation during chondrogenesis. Disease-associated lncRNAs are promising and solving the biological functions and underlying mechanism of these lncRNAs are interested to gene therapy for inhibiting hypertrophic chondrocytes.

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ABSTRACT (IN KOREAN)

연골세포 비대화와 관련된 긴 비암호화 RNA 탐색

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골관절염은 연골세포 비대화와 관절 연골의 석회화를 유발한다. 관절염 및 비대화 연골 세포의 진행을 조절하는 분자 메커니즘은 잘 알려지지 않았다. 최근에 긴 비암호화 RNA가 다양한 발달 과정과 후성 유전학적 조절에서 중요한 역할을 하는 것으로 입증되었다. 이 연구의 목적은 비대화 연골세포에서 긴 비암호화 RNA 발현을 조사하고 비대화 분화와 관련된 유전자 조절의 특성을 탐색하는 것이다. 따라서 TC28a2 세포주에서 비대화 분화 동안 증가하는 비대화 관련

유전자의 발현을 실시간 중합효소 연쇄반응과 웨스턴 블랏을 시행하였으며, osteocalcin reporter assay를 통해 프로모터 활성을 확인하였다. 추가로 세포에 있는 펩타이드나 단백질 항원의 특정 항원결정기에 붙는 항체를 확인하는 방법인 면역세포화학 법을 시행하였다. 비대화 분화 동안 비대마커인 RUNX2, COL10A1, Osteocalcin은 증가하였으나 연골 형성 마커인 COL2A1은 감소하였다. RNA-시퀀싱을 통해 전사체 차이를 조사하였고 정상 연골 세포보다 비대화 연골세포에서 증가하는 긴비암호와 RNA 발견하였다. 이 연구에서는 정상연골세포보다 비대화 연골 세포에서 과발현하는 상위 5 개 긴 비암호화 RNA 인 LINC02593, H19, OBSCN-AS1, FOXD2-AS1 및 LOC101927811를 선택하였다. 위 긴 비암호화 RNA 들을 짧은 간섭 RNA를 이용하여 특정 긴 비암호화 RNA를 억제한 뒤 비대화 분화를 시키자 비대화 관련 마커 중 전사 조절 단백질 발현이 억제되는 것을 발견하였다. 또한, 비대화 마커인 COL10A1의 억제와 COL2A1의 구제하는데 기여하는 FOXD-AS1 과 LOC101927811을 발견하였다. 이러한 발견은 긴 비암호화 RNA가 연골 세포 비대화 억제 및 골관절염 치료에 도움을 줄 것이다.

핵심되는 말 : 연골세포, 연골 비대화, 긴 비암호화 RNA, 세포-유전자 치료, 골 발생, FOXD2-AS1, LOC101927811