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In Vitro and in Silico Analysis of ADAMTS5 Transcription in Human Chondrocytes

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Abstract

Osteoarthritis is a major cause of disability in the adult population. Exercise is commonly prescribed, but the mechanisms underlying mechanotransduction of joint tissues are not well understood. Since Lrp5 is an important mechano-sensitive receptor in Wnt signaling, we examined its role in the mRNA expression of A Disintegrin and Metalloproteinase with Thrombospondin Motifs 5 (ADAMTS5), a major proteolytic aggrecanase that degrades extracellular matrix in articular cartilage. Using genome-wide expression data for C28/I2 chondrocytes with and without Lrp5-specific siRNA, we employed a systems biology approach and built a regulatory network model. Experimental data revealed that silencing Lrp5 significantly altered Wnt signaling gene expression and elevated the mRNA level of ADAMTS5 and several cytokines. A series of experiments using RNA interference showed that the expression of ADAMTS5 was at least in part stimulated by p38 MAPK and IL1β, while Lrp5 acted as a suppressor of their upregulation. Regulatory network analysis using an algorithm predicted the potential involvement of Wnt3a, Myc and CCAAT/Enhancer-Binding Protein β (CEBPB). Collectively, the systems biology approach helped develop an Lrp5-mediated network model in regulation of ADAMTS5, and the model predicted that a secretary factor such as Wnt3a might be involved in Lrp5-mediated homeostasis of ADAMTS5.

Keywords: ADAMTS5; Lrp5; Wnt signaling; RNA interference; Regulatory network

Introduction

Wnt signaling is involved in various signal transductions for cellular development and differentiation and cell-cell communications, and it plays a critical role in the mechanotransduction of bone [1,2]. Specifically, low-density-Lipoprotein Receptor-Related Protein 5 (Lrp5) is a co-receptor in the canonical Wnt signaling pathway and global deletion of Lrp5 in mice as well as conditional deletion in osteocytes results in a deficiency in load-driven bone formation and presents a phenotype of low bone mass [3-5]. Although the Wnt signaling pathway is essential for the maintenance of skeletal tissues, the role of Lrp5in cartilage is not well understood. It is reported that expression of Lrp5 is up regulated in osteoarthritic cartilage [6]. A hallmark of osteoarthritis is activation of aggrecanases, which cleave cartilage-specific proteoglycan core protein, i.e., aggrecan [7]. Downregulation of aggrecanases, in particular, A Disintegrin and Metalloproteinase with Thrombospondin Motifs 5 (ADAMTS5), is critically important for the protection of cartilage [8]. Mice deficient in ADAMTS5, for instance, are protected from cartilage erosion in mouse models of osteoarthritis [9,10]. In this study, we employed a systems biology approach and examined the role of Lrp5 in chondrocytes ontranscription of ADAMTS5, a major aggrecanase highly expressed in osteoarthritic cartilage.

Herein, we addressed a pair of questions: Does silencing Lrp5 in chondrocytes using RNA interference alterthe mRNA expression level of ADAMTS5? What signaling pathway is linked to Lrp5-mediated regulation of ADAMTS5? The role of canonical Wnt signaling in cartilage is controversial. It is reported that activation of β-catenin signaling leads to premature chondrocyte differentiation and the development of osteoarthritis-like phenotype [11]. Since β-cateninactivates the transcription of Wnt-responsive genes in conjunction with T-cell factor transcription factors [12], this report indicates that Wnt signaling is a stimulator of arthritic symptoms. It is also reported, however, that loss-of-function of Lrp5 increases cartilage degradation associated with low bone mass density in subchondralbone [13].Collectively, existing data do not allow us to determine the role of Lrp5 specifically in chondrocytes and the regulation of ADAMTS5.

In this study, we employed C28/I2 human chondrocyte cells and transfected them with two independent siRNAsspecific to Lrp5. The expression of ADAMTS5 is reported to be regulatedin part by p38 mitogen-activated protein kinase (p38 MAPK) [14], but it has not been shown whether there is any linkage between Lrp5 mediated signaling and activation of p38 MAPK.In order to identify the Lrp5-mediated regulatory mechanism of transcription of ADAMTS5, we conducted a genome-wide mRNA expression analysis using the Lrp5 siRNA treated chondrocytes. Quantitative real-time PCR was performed to verify microarray data, and pathway analysis was carried outto evaluate the effects of Lrp5 silencing on signal transduction. To identify other regulatory elements, we conducted a series of RNA silencing experiments using siRNAs specific to Lrp5, IL1β and/or p38 MAPK.

To further evaluate the effects of Lrp5, IL1β, and p38 MAPK on the transcriptional regulation of ADAMTS5 and predict quantitative interplay among them, two computational approaches were employed. First, Transcription-Factor Binding Motifs (TFBMs) involved in regulation of ADAMTS5 were predicted using ant algorithm. Ant

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algorithm is a heuristic search procedure for evaluating a potential set of TFBMs from genome-wide expression data [15]. Second, a mathematical pathway model was built using data from single and double deletions with siRNAs as well as the predicted TFBMs. The model included a set of parameters which provided a predictive weight for stimulatory and inhibitory effects of Lrp5, IL1β, and p38 MAPK on transcription of ADAMTS5.

Materials and Methods

Cell culture

C28/I2 human chondrocytes were cultured in DMEM containing 10% fetal bovine serum and antibiotics (50 units/ml penicillin, and 50 μg/ml streptomycin; Life Technologies, Grand Island, NY, USA). Cells were maintained at 37˚C and 5% CO_2 in a humidified incubator. One ng/ml IL1β (R&D Systems, Minneapolis, MN, USA) stimulation was performed after 10 h serum-free conditions.

Knockdown of Lrp5, p38 MAPK and IL1β by siRNA

Cells were treated with siRNA specific to Lrp5, IL1β (Life Technologies) and p38 MAPK (Cell Signaling, Danvers, MA, USA). The selected target sequences for knockdown of Lrp5a, Lrp5b, p38 MAPK, and IL1β were: Lrp5a, 5'- GUACAGGCCCUACAUCAUU-3'; Lrp5b, 5'-CGUUCGGUCUGACGCAGUA-3';p38 MAPK, 5'- GCCCAUAAGGCCAGAAACU -3'; and IL1β, 5'-CGAUGCACCUGUACGAUCA-3'.Note that Lrp5a siRNA and Lrp5b siRNA are two independent siRNAs specific to Lrp5.As a nonspecific control, negative siRNA (Silencer Select #1, Life Technologies and SignalSilence Control siRNA, Cell Signaling) were used. Cells were transiently transfected with siRNA in Opti-MEM I medium with Lipofectamine RNAiMAX (Life Technologies). Six hours later, the medium was replaced by regular culture medium. The efficiency of silencing was assessed with immunoblotting and quantitative real-time PCR at 48 h after transfection.

Quantitative real-time PCR

Total RNA was extracted using an RNeasy Plusmini kit (Qiagen, Germantown, MD, USA). Reverse transcription was conducted with high capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, CA, USA), and quantitative real-time PCR was performed using ABI 7500 with Power SYBR green PCR master mix kits (Applied Biosystems). We evaluated mRNA levels of ADAMTS5, IL1β, IL6, IL8, IL12A, IL15, IL16, and IL18 with the PCR primers listed in Table 1. GAPDH was used for internal control, and the relative mRNA abundance for the selected genes was obtained with respect to the level of GAPDH mRNA.

Western Immunoblotting

Cells were lysed in a Radioimmunoprecipitation Assay (RIPA) buffer containing protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphatase inhibitors (Calbiochem, Billerica, MA, USA). Isolated proteins were fractionated using 10% SDS gels and electro-transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membrane was incubated for 1 h with primary antibodies followed by 45 min incubation with goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Cell Signaling). We used antibodies against p38 MAPK, p-p38 MAPK, Lrp5 (Cell Signaling) and β-actin (Sigma). Protein levels were assayed using SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific), and signal intensities were quantified with a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan).

Microarray and pathway analysis

Microarray experiments were conducted using Agilent Whole Human Genome arrays (Human 8 X 60K array, Agilent). Eight RNA samples isolated from 4 pairs of C28/I2 chondrocyte cells (transfected with control and Lrp5 as iRNAs) were labeled with the Agilent low RNA input fluorescent linear amplification kit. They were hybridized to 8 one-color arrays using the *in situ* hybridization kit (Agilent). Microarray data were filtered to remove background noise and a modified t-test was performed to identify a group of genes that were altered >2-fold or <0.5-fold with statistical significance at $p< 0.01$. The list of genes was imported into Pathway-Express which identified gene signaling pathways through calculation of an impact factor [16].

Computational Analysis

Using an ant algorithm-based prediction method, potential TFBMs were found [15]. In brief, the ant algorithm is a meta-heuristic optimization technique based on the biological behavior of ant colonies. Ants initially wander randomly until they find a food source. When they do, they return to the colony, depositing pheromones along the way. Other ants find and follow these pheromones so that shorter routes to better food sources will be reinforced. In our application, paths were sets of 20 6-basepair TFBMs [17-19]. Pheromone levels were found by finding the error between the actual gene expression levels and the predicted expression levels from the contributions of the chosen motifs [20]. After each iteration, the pheromone levels were updated and the next TFBMs were chosen based on the current pheromone levels. At the end of the analysis, the TFBMs with the highest pheromone levels were further analyzed using the TRANSFAC 7.0 Public 2005 database [21] by finding promoter sites containing the predicted motif sequences.

A linear model of the pathway involving Lrp5, p38, IL1β and ADAMTS5 was builtusing data from siRNA-knockout experiments. The model consists of three state variables, x_1(LRP5), x_2(IL1β), and x_3(p-p38), and one measurement variable, y (ADAMTS5 mRNA level). Parameters a,b and c linked the effects of Lrp5 to the three state

target	forward primer	backward primer
ADAMTS5	5'- CACTGTGGCTCACGAAATCG-3'	5'- CGCTTATCTTCTGTGGAACCAAA-3'
IL1β	5'- GCTGAGGAAGATGCTGGTTC -3'	5'- TCCATATCCTGTCCCTGGAG -3'
IL6	5'- TACCCCCAGGAGAAGATTCC -3'	5'- TTTTCTGCCAGTGCCTCTTT -3'
IL8	5'- GTGCAGTTTTGCCAAGGAGT -3'	5'- ACTTCTCCACAACCCTCTGC -3'
IL12A	5'- GATGGCCCTGTGCCTTAGTA -3'	5'- TCAAGGGAGGATTTTTGTGG -3'
IL15	5'- AGCTGGCATTCATGTCTTCA -3'	5'- TGGGGTGAACATCACTTCC -3'
IL16	5'- CACGGTGACACTGGAGAAGA -3'	5'- TGATGATGTTCCAGGCTTCA -3'
IL18	5'- GCATCAACTTTGTGGCAATG -3'	5'- ATAGAGGCCGATTTCCTTGG -3'
GAPDH	5'-GCACCGTCAAGGCTGAGAAC -3'	5'- ATGGTGGTGAAGACGCCAGT -3'

Table 1: Real-time PCR primers used in this study.

variables, while parameters d to g governed the other interactions in the pathway. With parameter h representing all interactions not affected by Lrp5, the measurement variablewas expressed:

$$y = y_0 + \Delta y$$

$$\Delta y = (-c - af - db - adg)x_1 + (f + dg)x_2 + dx_3 - ex_1x_2 + aex_1^2 + h$$

Where y_0 is the basal level of ADAMTS5 set to 1. The parameters were solved using the Moore-Penrose pseudoinverse matrix conversion.

Statistical analysis

Three or four independent experimental trials were conducted and data were expressed as mean ± S.D. For comparison among multiple samples, ANOVA followed by post *ad hoc* tests were conducted. Statistical significance was evaluated at $p< 0.05$. The single and double asterisks indicate $p< 0.05$ and $p< 0.01$, respectively. To determine intensities in immune blotting, band images were quantified using Image J.

Results

Elevation of ADAMTS5 mRNA level by Lrp5 siRNA

Both microarray and quantitative real-time PCR data revealed that the deletion of Lrp5 by RNA interference using two different sequences (Lrp5a and Lrp5b) elevated the level of ADAMTS5 mRNA (Figure 1a, b). Pathway analysis using the microarray-derived expression profiles confirmed that silencing Lrp5 significantly altered the mRNA profiles in canonical Wnt signaling, as well as non-canonical Wnt signaling such as planar cell polarity and Wnt/Ca^{2+} pathways (Figure 1c).

Response of interleukins by Lrp5 siRNA

In response to treatment with Lrp5 siRNA, mRNA levels of to 6 interleukin genes (IL6, IL1β, IL12A, IL15, IL18, IL16) were elevated while IL8 mRNA was suppressed (Figure 2a). To validate the potential linkage of the Lrp5 deletion to expression of these interleukins, quantitative real-time PCR was conducted after IL1β treatment. Compared with Lrp5 siRNA, IL1β treatment caused a similar elevation to mRNA abundance of IL6, IL1β and ADAMTS5. However, administration of IL1β did not elevate the mRNA levels of IL12A, IL15, IL18, IL8 and IL16 (Figure 2b).

Involvement of p38 MAPK in ADAMTS5 regulation from LRP5 silencing

Western blot analysis revealed that both sequences of LRP5 siRNA elevated the phosphorylation of p38 MAPK (Figure 3a, b). Applying

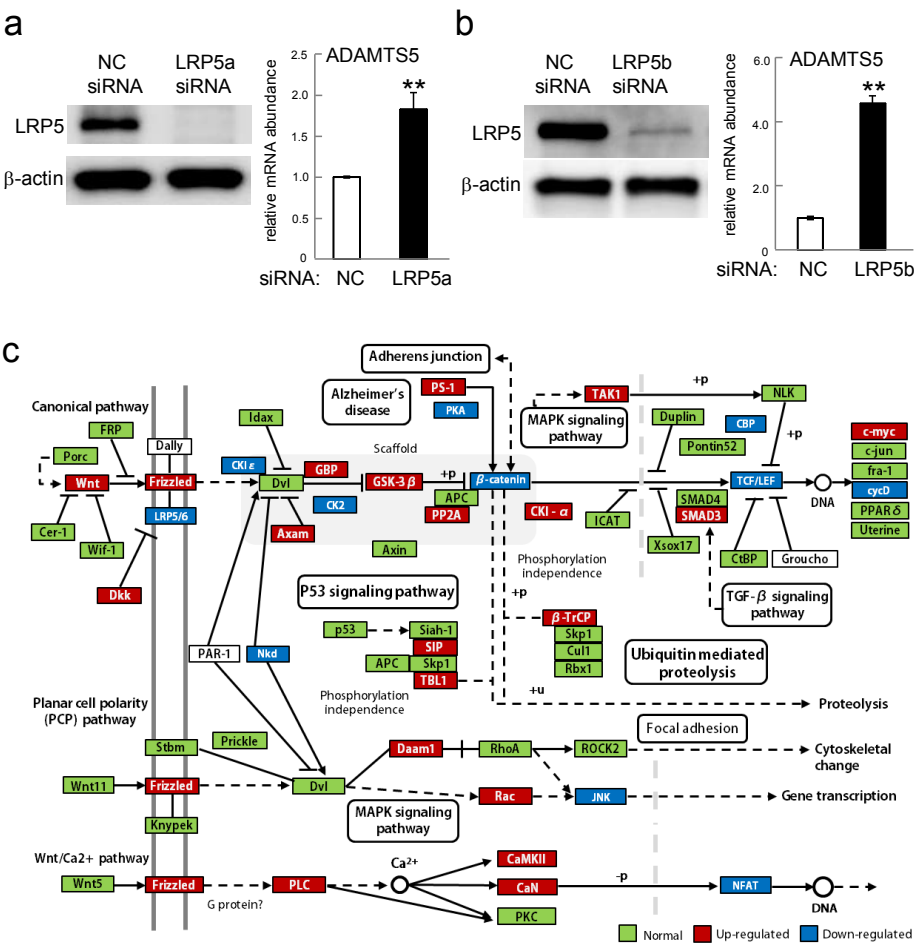

Figure 1: Effects of Lrp5 silencing. NC: non-specific siRNA
(A-B): RNA interference using two Lrp5 siRNAs and elevation of ADAMTS5 mRNA level
(C): Wnt pathway affected by Lrp5 silencing. The mRNA levels of the genes marked in green, red, and blue are unchanged (p>0.05), upregulated and downregulated, respectively.

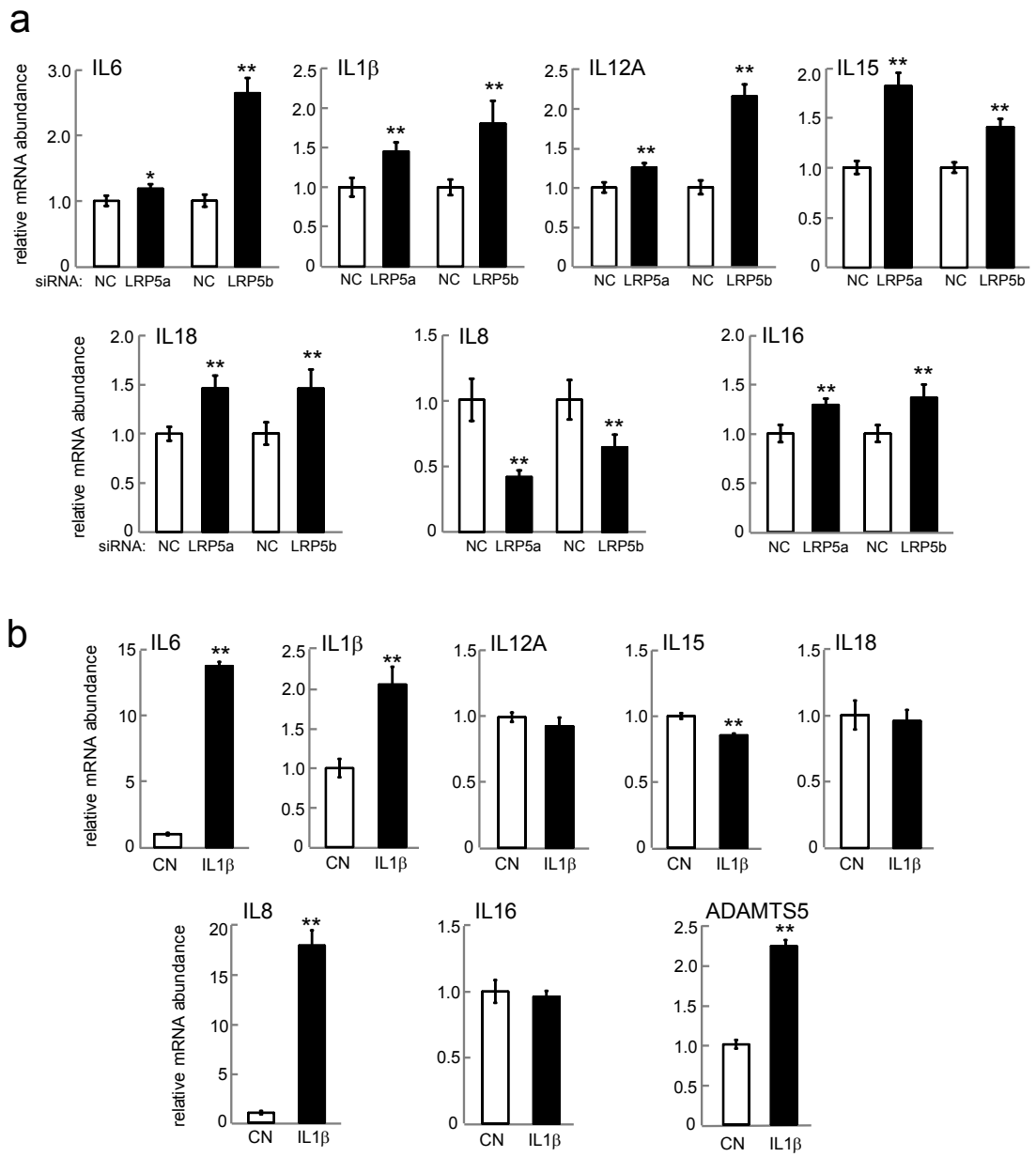

Figure 2: Quantitative real-time PCR for evaluating the effects of LRP5 siRNA and administration of IL1β on the mRNA levels of IL6, IL1β, IL12A, IL16, IL18, IL8, and IL16.
Note that the single and double asterisks denote $p<0.05$ and $p<0.01$, respectively
(A) Relative mRNA abundance in response to RNA interference with two LRP5 siRNAs. NC: non-specific siRNA. (B) Relative mRNA abundance in response to administration of IL1β. CN: Control.

p38 siRNA decreased total and phosphorylated p38 MAPK, while a double knockdown with LRP5 siRNA showed a slight recovery in the phosphorylated p38 MAPK level (Figure 3c). ADAMTS5 mRNA levels decreased after p38 siRNA (Figure 3d). Double knockdown of both p38 siRNA and LRP5 siRNA slightly decreases the elevation of ADAMTS5 mRNA caused by a single knockdown of just LRP5 siRNA.

Effects of LRP5 and IL1β silencing on mRNA levels of ADAMTS5 and IL6

IL1β is significantly silenced from a single knockdown with IL1β siRNA (Figure 4a), while a double knockdown with both IL1β siRNA and LRP5 siRNA mitigated the IL1β mRNA elevation induced by a single knockdown with LRP5 siRNA (Figure 4b). Knockdown with only IL1β siRNA did not affect ADAMTS5 mRNA, but a double knockdown with IL1β siRNA and LRP5 siRNA decreased the ADAMTS5 mRNA increase observed in a single knockdown with LRP5 siRNA (Figure 4c). However, knockdown with IL1β siRNA does not significantly affect IL6 mRNA expression (Figure 4d).

In silico analysis of LRP5-mediated regulation

The ant algorithm software predicted several potential TFBMs. The top four TFBMs predicted were AAAGCC, CGTAGC, CTTTTA

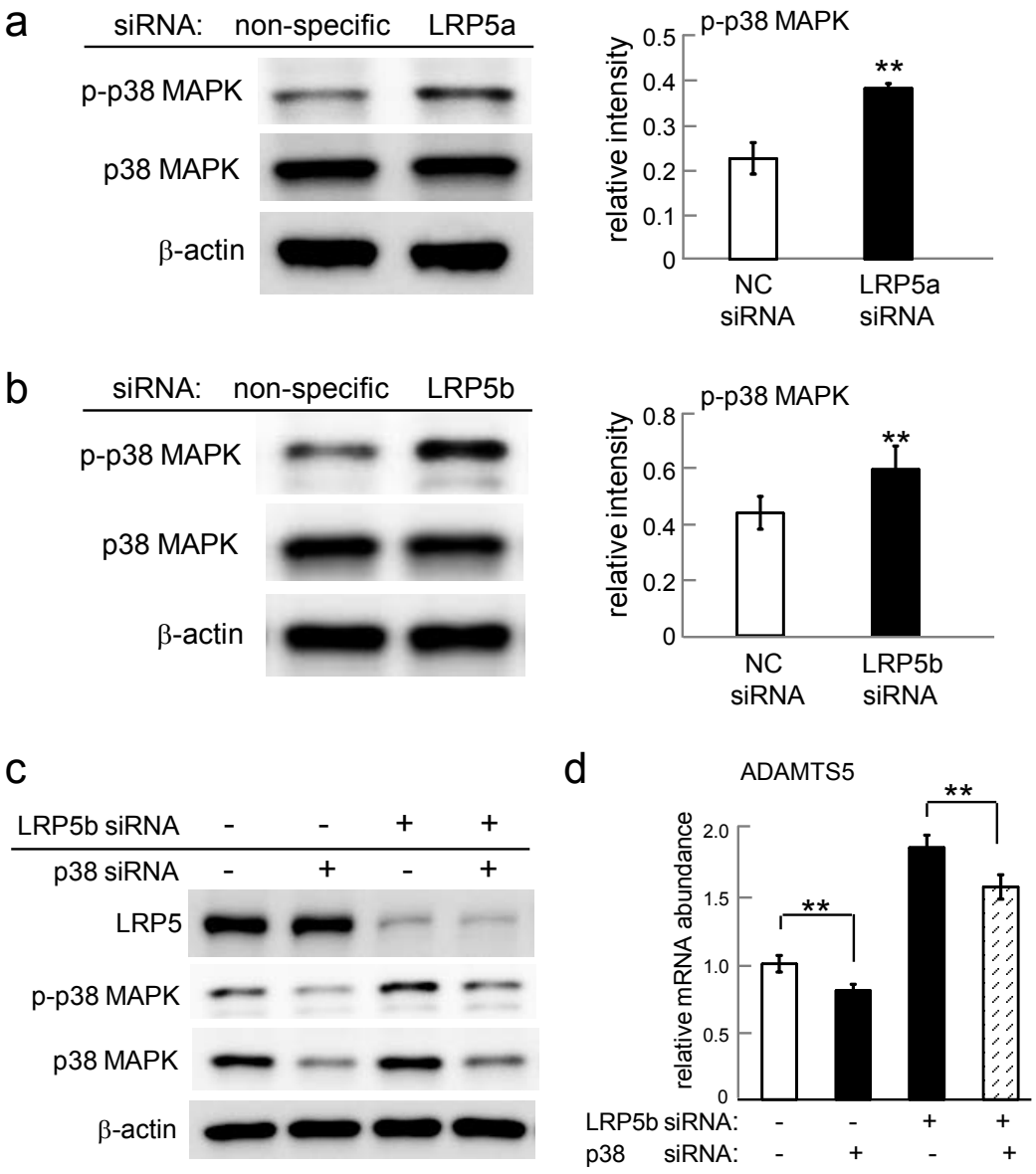

Figure 3: Involvement of p38 MAPK in regulation of ADAMTS5 mRNA level in LRP5 silencing. Note that the double asterisk denotes p<0.01
(A-B): Elevated phosphorylation level of p38 MAPK to LRP5 silencing using two LRP5 siRNAs
(B): Regulation of ADAMTS5 mRNA level in response to p38 siRNA and/or LRP5b siRNA.

and TAACCA (Figure 5a).The genes ATF2, MYC, FURIN, SERPINA1, and CEBPB were found to be related to Wnt signaling, p38 MAPK signaling, IL1β, or ADAMTS5 through a literature search (Figure 5b). From the filtered ($p< 0.05$) microarray data, LRP5 and WNT3A were found to be downregulated, while MYC, CEBPB, IL1β, and ADAMTS5 were upregulated (Figure 5c).

The results from the experiments led to a linear model of the pathway of LRP5, p38, and IL1β regulation of ADAMTS5 (Figure 5d). Using experimental data (Table 2), the values of several parameters were estimated (Table 3). The parameters (d,e and h) were explicitly determined, while the others (a, b, c, f, and g) were predicted as a hyperplane in a parameter space. The allowable parameter values relating a, b and c are depicted as a plane in three-dimensional space (Figure 5e).

Discussion

osteoarthritis-like phenotype [11] and expression of Lrp5 is reported to be elevated in human osteoarthritic chondrocytes [10]. However, knockout mice lacking Lrp5 presented an increase in cartilage degradation in surgically induced osteoarthritis [13]. The current study demonstrated that transfection of Lrp5 siRNAin chondrocytes elevated the mRNA levels of many interleukins, and increased the phosphorylation of p38 MAPK. Silencing p38 and IL1β each attenuated the Lrp5 siRNA-induced increase in ADAMTS5. The results herein using a systems biology approach indicate that Lrp5-mediated Wnt signaling in response to a secretory ligand such as Wnt3ainteracts with p38 MAPK signaling and interleukins to regulate transcription of ADAMTS5 in chondrocytes.

Based on a genome-wide microarray of siRNA-based experiments,

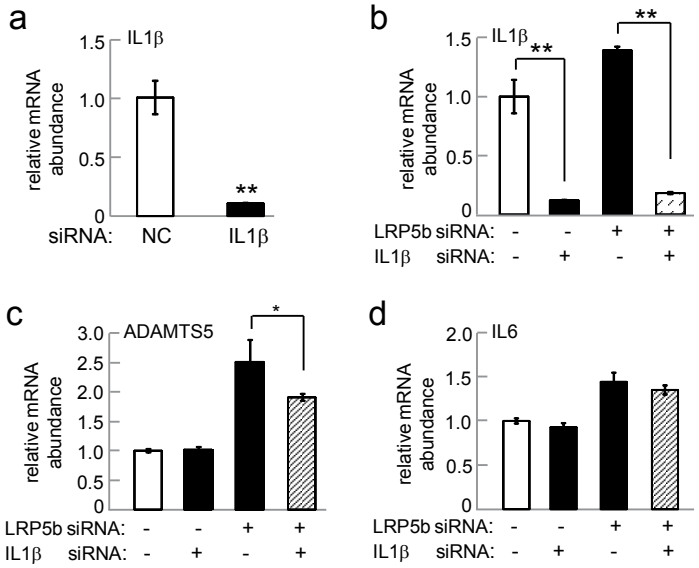

Figure 4: Effects of LRP5 and IL1β silencing on the mRNA levels of ADAMTS5 and IL6.
Note that the double asterisk denotes p<0.01, and "NC" denotes non-specific siRNA.
(A): IL1β silencing
(B-D): Levels of IL1β mRNA, ADAMTS5 mRNA and IL6 mRNA in response to LRP5 siRNA and/or IL1βsiRNA, respectively.

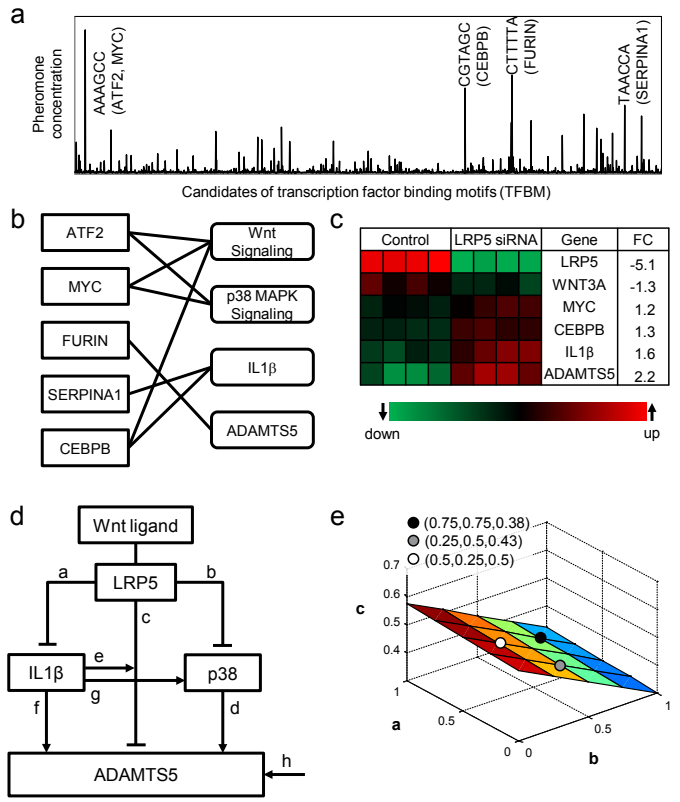

Figure 5: Computational analysis of Lrp5 siRNA
(A): Pheromone level of potential transcription factor binding motifs. Top 4 motifs are labeled with relevant genes. (B): Involvement of the targeted genes (ATF2, MYC, FURIN, SERPINA1 and CEBPB) in Wnt signaling, p38 MAPK signaling, IL1β, and ADAMTS5 from a literature search
(C): Heat map of relevant genes from Lrp5 siRNA microarray data
(D): Potential network model for LRP5, IL1β, and p38 regulation of ADAMTS5
(E): Plane on which parameter values a, b, and c reside. The three dots mark sample parameter values

x_1	x_2	x_3	y	remark
1	1	1	1	control
0	1	1	2.1	Lrp5 siRNA
1	0	1	1.05	IL1β siRNA
1	1	0	0.8	P38 siRNA
0	0	1	1.6	Lrp5 siRNA and IL1β siRNA
0	1	0	1.8	Lrp5 siRNA and p38 siRNA

Note: the values of (x_1, x_2, x, and y) are normalized by the values for controls.

Table 2: Set of experimental data points.

Parameter	Value
(a,b,c)	$-0.025a + 0.025b + c \cong 0.55$
d	~ 0.25
e	~ 0.5
(f,g)	$f + 0.3g \cong 0.0475$
h	~ 0.35

Table 3: Parameter values.

the ant algorithm predicted many potential TFBMs, including ATF2, MYC, FURIN, SERPINA1 and CEBPB. These molecules are all reported to be linked to activation of p38 MAPK, Wnt signaling, ADAMTS5, and/or inflammatory and degenerative responses. In response to Wnt3a, for instance, ATF2 is activated via p38 MAPK in mouse and human embryonic cell lines [22]. Also, activation of MYC is mediated by p38 MAPK [22] and it is inhibited by Wnt signaling [23]. FURIN codes a protein that converts ADAMTS5 to its active form [24].SERPINA1 is up-regulated in chondrocyte cells in response to IL1β stimulation [22] and CEBPB was reported to mediate IL1β-induced expression of collagenases such as MMP1 and MMP13 [25].

Following the hints provided by the computational analysis, silencing experiments were performed that show that Lrp5-mediated signaling is involved in reducing p38 MAPK signaling and interleukin responses. Silencing Lrp5 elevates p-p38 MAPK, and a double deletion with p38 siRNA and Lrp5 siRNA suppresses the upregulation of ADAMTS5 induced by Lrp5 siRNA. This suggests an intermediary role for p38 in Lrp5-mediated regulation of ADAMTS5. Similarly, a double deletion of IL1β and Lrp5 attenuates the increase in the level of ADAMTS5 mRNA. Unlike in p38 MAPK, however, a single deletion of IL1β caused no significant change to ADAMTS5 mRNA level indicating that p38 MAPK and IL1β act differently.

The results of siRNA-based experiments were quantitatively interpreted by the network model consistent with genome-wide expression data and the prediction of TFBMs with ant algorithm. In the network model, the parameters representing interactions among Lrp5-mediated Wnt signaling, IL1β, p38 MAPK, and ADAMTS5 were estimated. These parameters can be understood as the relative weights of each interaction. The results indicated that the components which are not directly linked to Lrp5-mediated Wnt, IL1β, or p38 MAPK, represented by h, also provide a contribution to ADAMTS5 transcription.The combinational effect of IL1β directly on ADAMTS5 and through p38 (f+dg) sums to 0.475, suggesting its efficacy is greater than those other componand ADAMTS5 (a,b and c respectively) suggest a larger range of possible values of a and b than for.For likely values of a and b, c will be close to f and d in value. Lrp5-mediated Wnt signaling ents represented by h. The planar relation between the parameters representing Lrp5's effects on IL1β, p38 MAPK, however, applies its most significant effect on ADAMTS5 through IL1β and p38 MAPK. More experiments will be required to fully solve the parameters and to reveal other possible pathway features.

The mechanism of the up regulation of Lrp5 in the cartilage of patients with osteoarthritis is not known. In phosphate metabolism, FGF23 stimulates excretion of phosphate but its expression is markedly elevated in chronic kidney diseases in which phosphate excretion is diminished [23,24]. The elevation of Lrp5 in arthritis patients might have a mechanism similar to the regulation of FGF23 in chronic kidney diseases. Namely, Lrp5 may act as an inhibitor of ADAMTS5 in arthritic cartilage, and its expression is up regulated in arthritis patients to cope with their pathogenic condition. However, the elevation of both Lrp5 and ADAMTS5 may result from other stimulating factors such as inflammatory cytokines. Alternatively, other tissues such as synoviocytes and subchondral bone may add complexity to the Lrp5-mediated regulation of ADAMTS5.

In this study we focused on transcriptional regulation of ADAMTS5, but other regulatory levels such as translation and post-translational modification are also important for regulation of cartilage ECM. Furthermore, this study is limited to the linkage of Lrp5 and ADAMTS5 in C28/I2 cell line but the role of other metalloproteinases including ADAMTS4 and MMP13 might be affected differently in osteoarthritic cells. Further analysis using primary cells from normal and patients with osteoarthritis may provide more insights on the Lrp5-mediated regulatory mechanism in pathophysiology of degenerative joint diseases.

In summary, the systems biology approach including experimentations and computational simulation in this study presented a novel Lrp5-mediated network model for transcription of ADAMTS5. The model contributes to pointing out an important role of Lrp5-mediated Wnt signaling in suppressing the mRNA expression of ADAMTS5 in chondrocytes andgeneratingtestable hypotheses forinteractions among Wnt ligands such as Wnt3a, Lrp5, IL1β and p38 MAPK.

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