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LRP4 induces extracellular matrix productions and facilitates chondrocyte differentiation

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ABSTRACT

Endochondral ossification is an essential step for skeletal development, which requires chondrocyte differentiation in growth cartilage. The low-density lipoprotein receptor-related protein 4 (LRP4), a member of LDLR family, is an inhibitor for Wnt signaling, but its roles in chondrocyte differentiation remain to be investigated. Here we found by laser capture microdissection that LRP4 expression was induced during chondrocyte differentiation in growth plate. In order to address the roles, we overexpressed recombinant human LRP4 or knocked down endogenous LRP4 by lentivirus in mouse ATDC5 chondrocyte cells. We found that LRP4 induced gene expressions of extracellular matrix proteins of type II collagen (*Col2a1*), aggrecan (*Acan*), and type X collagen (*Col10a1*), as well as production of total proteoglycans in ATDC5 cells, whereas LRP4 knockdown had opposite effects. Interestingly, LRP4-knockdown reduced mRNA expression of *Sox9*, a master regulator for chondrogenesis, as well as *Dkk1*, an extracellular Wnt inhibitor. Analysis of Wnt signaling revealed that LRP4 blocked the Wnt/β-catenin signaling activity in ATDC5 cells. Finally, the reduction of these extracellular matrix productions by LRP4-knockdown was rescued by a β-catenin/TCF inhibitor, suggesting that LRP4 is an important regulator for extracellular matrix productions and chondrocyte differentiation by suppressing Wnt/β-catenin signaling.

1. Introduction

Endochondral ossification is an essential step during skeletal development. Unlike intramembranous ossification, chondrocytes play an essential role in endochondral ossification. Five distinct zones can be histologically recognized at the light-microscope level: (1) zones of resting; (2) proliferation/cell columns; (3) maturation/hypertrophy for chondrocytes; (4) zones of calcification; and (5) ossification of bone-forming cells (osteoblasts). During the first three steps, the cartilage mold grows in length by continuous cell divisions as well as by extracellular matrix secreted from chondrocytes. The control of the processes plays pivotal roles for further induction of calcification and ossification. Dysregulation of either step leads to severe skeletal dysplasia in both mice and humans. In years, sex-determining region Y-type high mobility group box protein, Sox9, is known to promote chondrocyte differentiation as a master transcriptional factor [1]. Accompanied with the differentiation, chondrocytes start to produce cartilage-specific matrix proteins such as type II collagen (Col2a1) and aggrecan (Acan) and then undergo hypertrophy and terminal differentiation, which are characterized by the secretion of type X collagen (Col10a1).

In the past 10 years, the Wnt signaling pathway has emerged as a critical regulator of cartilage development. Some of components in the Wnt signaling pathway are shown to be involved in chondrocyte differentiation *in vitro* and in cartilage development *in vivo* [2]. With secreted Wnt ligands and its receptors, frizzled (Frz) family and LRP5/6, three distinct intracellular signaling cascades are known to be activated thus far: the Wnt/β-catenin pathway, the Wnt/JNK pathway and the Wnt/Ca^{2+} pathway. Sox9 and Wnt/β-catenin thus control chondrocyte differentiation in a mutually antagonistic manner [3]. Responsible membrane proteins in the Wnt signaling pathway, however, are not fully elucidated in chondrocyte differentiation.

The low-density lipoprotein receptor (LDLR)-related protein 4 (LRP4) is a transmembrane protein and a member of the LDLR family [4]. In osteoblasts, LRP4 is a receptor for Wnt signaling inhibitors of Dkk1, Sost, and Sostdc1 (Wise), and reduces Wnt/β-catenin signaling during bone development [5,6]. Indeed, homozygous *Lrp4*-deficient mice have skeletal dysplasia including growth retardation with fully penetrant polysyndactyly in their limbs and

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variable craniofacial defects [7,8]. Similarly, mutations in *LRP4* cause congenital syndactyly in cattle, as well as polysyndactyly in Cenani-Lenz syndrome in human [9–13]. *LRP4* mutations also cause sclerosteosis-2 in human by abrogating the sclerostin activity [14]. In addition, LRP4 serves as a receptor for an agrin at the neuromuscular junction, the synapse between motor neuron and skeletal muscle [15,16]. We recently reported *LRP4* mutations in congenital myasthenic syndrome that compromised the agrin-signaling pathway, but not the Wnt/β-catenin signaling pathway [17]. Although skeletal dysplasia in *Lrp4*-deficient mice may be partly accounted for by defects in endochondral ossification, little is known about LRP4 function in chondrocyte differentiation.

This study was carried out to determine the role of LRP4 in chondrocyte differentiation by modulating LRP4 expression. We first confirmed by laser capture microdissection that expression of LRP4 is increased with chondrocyte differentiation in the growth plate. Overexpression and knocking down of LRP4 revealed that LRP4 is an essential factor for proper chondrocyte maturation and extracellular matrix productions in chondrogenic cell line, ATDC5 cells. We also showed that LRP4 functions as an inhibitor for Wnt/β-catenin signaling in chondrocytes. We propose that LRP4 is an essential Wnt/β-catenin signaling inhibitor in endochondral ossification.

2. Materials and methods

2.1. Microdissection and mRNA purification with chondrocytes in mouse

All mouse studies were approved by the Animal Care and Use Committee of the Nagoya University Graduate School of Medicine. Nine-day-old ICR mice were purchased from Japan SLC, Inc. Mice were sacrificed under deep anesthesia with ether. The tibiae were harvested, embedded in OCT (Sakura Finetek), and immediately frozen in dry ice. Frozen sections (10 μm) were processed and stained using 0.02% toluidine blue. Each growth plate zone was visually identified and clipped out with laser microdissection (LMD7000, Leica). Total RNA was isolated from the tissues using RNeasy Micro Kit (Qiagen). RNA quality was assayed by the Agilent Model 2100 Bioanalyzer (Agilent Technologies). RNA was linearly amplified by Ovation Pico WTA System (NuGEN Technologies).

2.2. ATDC5 cell culture

ATDC5 cells were purchased from the Riken BioResource Center. ATDC5 cells were cultured in DMEM/F12 (a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium) (Sigma–Aldrich) supplemented with 5% fetal bovine serum (FBS, Thermo Scientific) and insulin–transferrin–selenite (ITS, Invitrogen) in a 37 °C humidified atmosphere for its differentiation. (The) Medium was changed every other day until harvesting cells. Full-length human LRP4 cDNA (Open Biosystems) was cloned into the EcoRI site of the pcDNA3.1 mammalian expression vector. For *Lrp4* overexpression, cells were transfected with pcDNA3.1-hLrp4 using FuGENE6 (Roche). The following double stranded oligonucleotides were synthesized and cloned into pLenti-CMV-GFPx2-DEST to make shRNA-expressing lentiviral vectors (Supplementary Fig. S1A):

shLrp4-1, 5′-GATCCCGGAAGTTTCCTGACATAAATTCAAGAGATTTATGTCAGGAAACTTCCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGGAAGTTTCCTGACATAAATCTCTTGAATTTATGTCAGGAAACTTCCGG-3′

shLrp4-2, 5′-GATCCCGGGCTTTATGATAATTTAATTCAAGAGATTAAATTATCATAAAGCCCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGGGCTTTATGATAATTTAATCTCTTGAATTAAATTATCATAAAGCCCGG-3′. pLenti-CMV-GFPx2-DEST was kindly provided by Dr. Eric Campeau at the University of Massachusetts Medical School. Lrp4 gene was silenced using lentivirally introduced constructs producing shRNA against Lrp4 mRNA. Quercetin (ChromaDex Inc.) was added into the culture medium at a concentration of 10 μM.

2.3. Total RNA extraction and real-time RT-PCR analysis

Total RNA was isolated from ATDC5 cells using Trizol reagent (Invitrogen). The first strand cDNA was synthesized with ReverTra Ace reverse-transcriptase (Toyobo). We quantified mRNAs for *Lrp4*, *Col2a1*, *Acan*, *Col10a1*, *Sox9*, *Runx2*, *MMP13*, *Dkk1*, *SOST* and *Axin2* using LightCycler 480 Real-Time PCR (Roche) and SYBR Green (Takara). The mRNA levels were normalized for that of *Gapdh*. Primer sequences are shown in Table S1.

2.4. Alcian blue staining

ATDC5 cells were differentiated into chondrocytes for 15 days, and were fixed with methanol for 30 min at −20 °C, and stained overnight with 0.5% Alcian Blue 8 GX (Sigma) in 1 N HCl. For quantitative analysis, Alcian blue-stained cells were lysed in 200 μl of 6 M guanidine HCl for 6 h at room temperature. The optical density of the extracted dye was measured at 630 nm using PowerScan 4 (DS Pharma Biomedical).

2.5. Luciferase assays

ATDC5 cells were transfected with either pcDNA3.1-hLrp4 or siLrp4-1 against mouse Lrp4 (5′-GGAAGUUUCCUGACAUAAA-3′ and 5′-UUUAUGUCAGGAAACUUCC-3′) (Supplementary Fig. S1B) in addition to TOPFlash firefly luciferase reporter vector (M50 Super 8× TOPFlash plasmid, Addgene) and phRL-TK encoding Renilla luciferase (Promega) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocols. At 24 h after transfection, the cells were incubated for 24 additional hours with or without 5 ng/ml of mouse Wnt3A protein (R&D Systems). Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) and PowerScan4 (DS Pharma Biomedical). Firefly luciferase activity was normalized by Renilla luciferase activity and relative luciferase units (RLU) were indicated.

3. Results

3.1. LRP4 is highly expressed in the hypertrophic zone of the growth plate

As the first step in evaluating the potential role of LRP4 on chondrocyte differentiation for endochondral ossification, we analyzed mRNA expressions in the tibial proximal growth plates of mice. We clipped out cells from the resting zone, the proliferating zone, and the hypertrophic zone of the growth plate with laser-capture microdissection in 9-day-old ICR mice in triplicate. Expressions of *Col10a1*, a specific marker for the hypertrophic zone, and *Lrp4* were quantified by real-time RT-PCR. We first confirmed that *Col10a1* was highly expressed in the hypertrophic zone (Fig. 1B). We found that *Lrp4* expressions were similarly 3.5-times higher in the hypertrophic zone compared to the resting zone (Fig. 1A). Reanalysis of microarray data of previously reported laser capture microdissection of the mouse growth plate similarly revealed that the ratio of *Lrp4* expression in the hypertrophic zone compared to the resting zone was 7.0 ± 3.5 (mean and SD, $n = 2$) [18]. These results suggest that Lrp4 expression is tightly upregulated in chondrocyte differentiation for endochondral ossification.

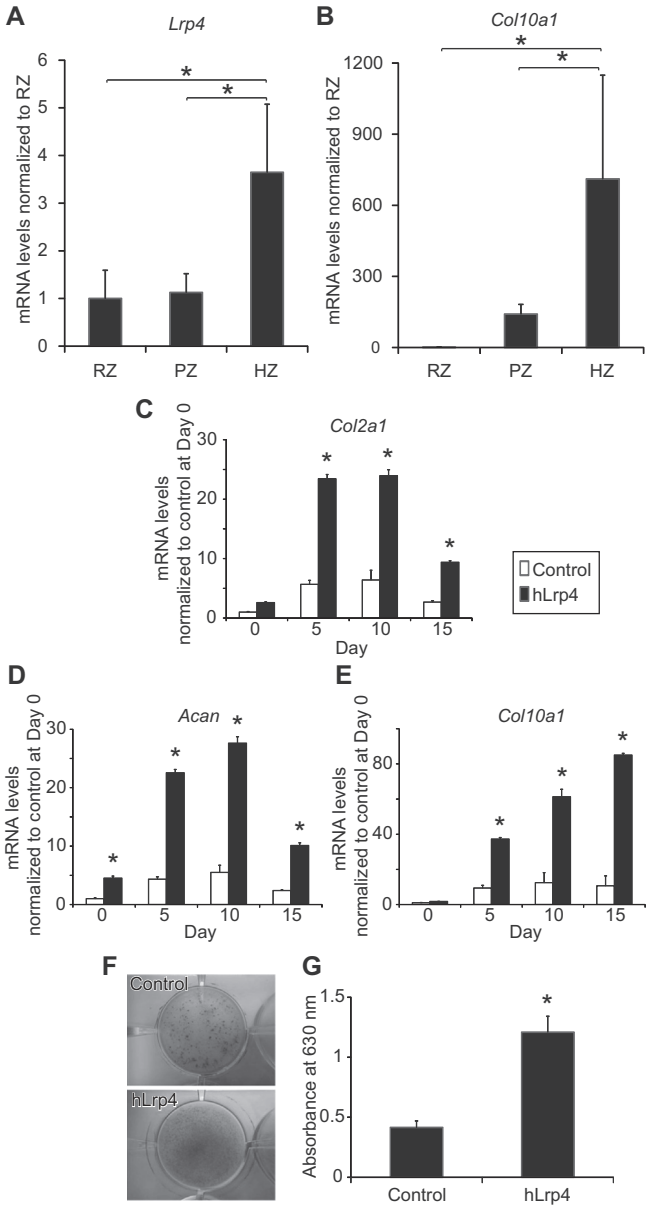

Fig. 1. Endogenous LRP4 is induced with endochondral ossification in mouse and overexpression of LRP4 induces expressions of extracellular matrix proteins in ATDC5 cells. (A and B) *Lrp4* and *Col10a1* expressions of the tibial proximal growth plate in mice are quantified by real-time RT-PCR. Values are normalized to that in the resting zone (RZ). RZ, the resting zone; PZ, proliferating zone; and HZ, hypertrophic zone. $^{*}P < 0.05$ by one-way ANOVA followed by Bonferroni post hoc test. (C–E) Gene expressions of *Col21a1*, *Acan* and *Col10a1* are enhanced by human LRP4 overexpression during chondrogenic differentiation of ATDC5 cells. ATDC5 cells are cultured with insulin–transferrin–selenite (ITS) for differentiation and harvested at the indicated days. The gene expression levels are quantified by real-time RT-PCR. Values are normalized to that at Day 0. $^{*}P < 0.05$ by two-way repeated measure ANOVA followed by Bonferroni post hoc test. (F) Alcian blue staining of ATDC5 cells that are differentiated to chondrocytes with ITS for 15 days. (G) Alcian blue-stained proteoglycans are quantified by measuring OD630 of the cell lysates. $^{*}P < 0.01$ by Student's *t*-test. (A, B, C, D, E, and G) Mean and SD ($n = 3$) are indicated.

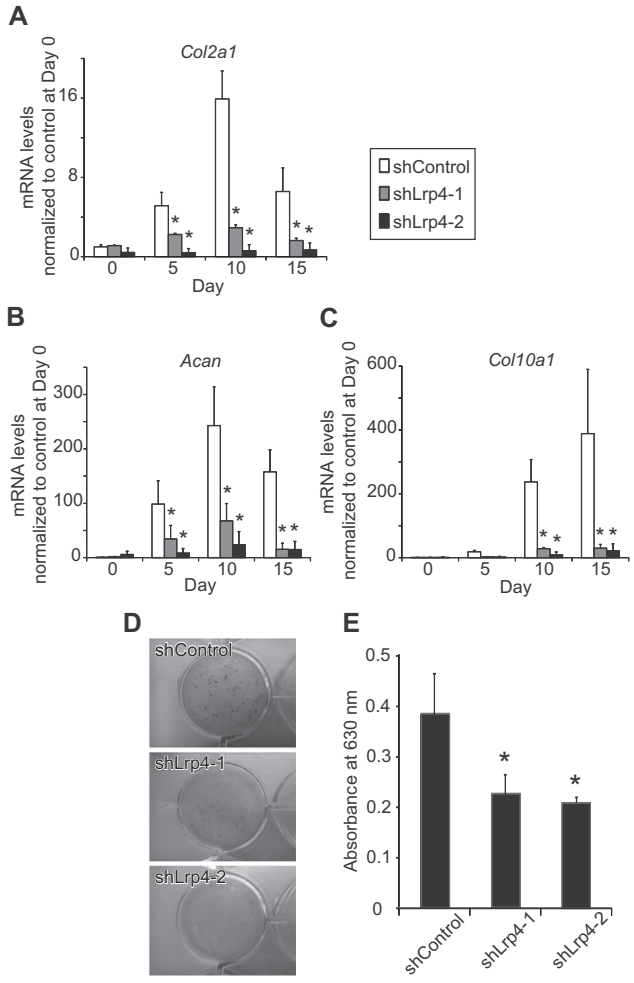

Fig. 2. *Lrp4* knockdown reduces expressions of extracellular matrix proteins. (A–C) Gene expressions of *Col2a1*, *Acan*, and *Col10a1* are reduced by lentiviral infections of two different shLrp4s (shLrp4-1 and shLrp4-2) during chondrogenic ATDC5 differentiation, as shown in Fig. 1. Values are normalized to that at Day 0. $^{*}P < 0.05$ by two-way repeated measure ANOVA followed by Bonferroni post hoc test between shControl and shLrp4. (D) Alcian blue staining of ATDC5 cells that are differentiated to chondrocytes with ITS for 15 days. (E) Alcian blue-stained proteoglycans are quantified by measuring OD630 of the cell lysates. $^{*}P < 0.05$ by Student's *t*-test. (A, B, C, and E) Mean and SD ($n = 3$) are indicated.

3.2. LRP4 promote gene expressions of extracellular matrix proteins and glycosaminoglycan productions

To investigate distinct roles of LRP4 in chondrocyte differentiation, we used the mouse chondrogenic cell line ATDC5, which is commonly used to study multistep processes of chondrocyte differentiation [19]. Expressions of extracellular matrix genes of *Col2a1*, *Acan*, and *Col10a1* were induced in the course of ATDC5 differentiation for 15 days (Fig. 1C–E). Overexpression of Lrp4 in ATDC5 cells further induced expressions of *Col2a1*, *Acan*, *Col10a1* in the course of differentiation. In addition, *Lrp4* overexpression upregulated Alcian blue staining, presenting the amount of glycosaminoglycans in extracellular matrix of ATDC5 cells (Fig. 1F and G). Conversely, lentivirus-mediated knockdown of *Lrp4* suppressed expression levels of extracellular matrix genes in ATDC5 cells (Fig. 2A–C). Knockdown of *Lrp4* also resulted in the decrease of Alcian blue staining (Fig. 2D and E). These results suggest that LRP4 is required for extracellular matrix productions in chondrocytes.

3.3. LRP4 knockdown reduces Sox9 and Dkk1 mRNAs, but has no effect on Sost mRNA

Downregulation of the Wnt/β-catenin signaling by Sox9 is essential for chondrocyte differentiation [3]. Quantitative RT-PCR showed that *Lrp4* knockdown reduced *Sox9* expression (Fig. 3A). These results suggest that Lrp4 is an inducer for chondrocyte differentiation partly via *Sox9* expression.

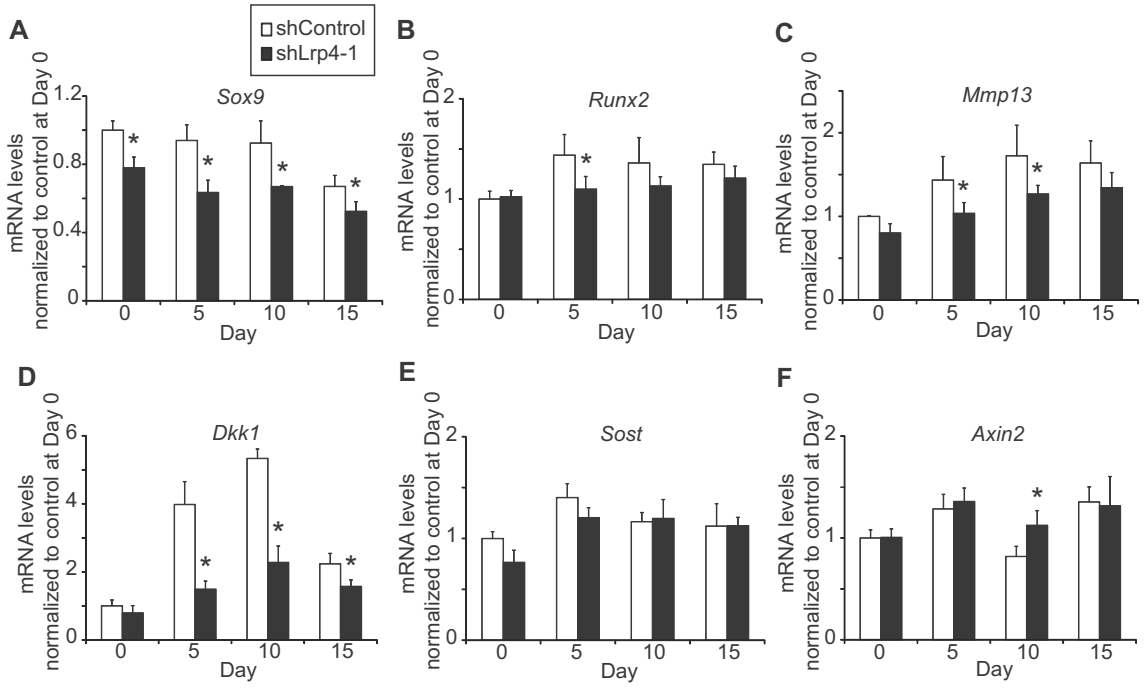

Fig. 3. *Lrp4* knockdown regulates gene expressions of *Sox9*, *Dkk1* and *Axin2*. (A–F) Expressions of chondrogenic markers (*Sox9*, *Runx2* and *MMP13*), extracellular Wnt inhibitors (*Dkk1*, *Sost*) and a target gene for Wnt/β-catenin signaling (*Axin2*) are quantified by real-time RT-PCR. ATDC5 cells are cultured in differentiation media as in Figs. 1 and 2. Values are normalized to that at Day 0. $^{*}P < 0.05$ by two-way repeated measure ANOVA followed by Bonferroni post hoc test. Mean and SD ($n = 3$) are indicated.

In osteoblasts, LRP4 binds to Wnt signaling inhibitors of Dkk1 and Sost [5], but no report addressed the relation between LRP4 and Dkk1/Sost in chondrocytes. We found in differentiation-induced ATDC5 cells that *Lrp4* knockdown suppressed expression levels of *Dkk1*, but not of *Sost* (Fig. 3D and E). LRP4 is thus likely to suppress Wnt/β-catenin signaling in chondrocyte differentiation along with Dkk1.

3.4. LRP4 reduces the Wnt/β-catenin signaling activity in ATDC5 cells

To confirm that LRP4 has an effect on Wnt signaling pathway in ATDC5 cells, we examined Wnt signaling activities with the TCF-responsive reporter, TOPFlash. The TOPFlash activity was increased by addition of Wnt3A ligand in ATDC5 cells (Fig. 4A and B). The reporter activities were decreased by overexpression of *Lrp4* and increased by knockdown of *Lrp4* in both native and Wnt3A-added medium. These results suggested that LRP4 is a negative regulator for Wnt/β-catenin in ATDC5 cells.

3.5. Chemical inhibitor for Wnt/β-catenin signaling, quercetin, rescues the effects of LRP4 knockdown

Finally, to confirm that LRP4 functions in chondrogenesis, we performed rescue experiments on extracellular matrix production. Quercetin inhibits a transcriptional complex comprised of β-catenin and TCF, and specifically suppresses Wnt/β-catenin signaling. We found that reduced expression levels of *Col2a1*, *Acan*, and *Col10a1* by *Lrp4* knockdown were cancelled with the quercetin treatment (Fig. 4F and G). Similarly, the decrease of Alcian blue staining by Lrp4 knockdown was rescued by quercetin (Fig. 4D and E). These results demonstrated that Lrp4 regulates the chondrogenic gene expressions through the Wnt/β-catenin signaling pathway.

4. Discussion

4.1. LRP4 is instrumental for endochondral ossification

Lrp4 is expressed in several tissues including tooth primordia, ureteric buds, limb buds, neuromuscular junctions and osteoblast; each of these tissues developed abnormally in *Lrp4*-deficient mice [5,7,8,20]. Expression of Lrp4 in chondrocytes and its functions remain to be addressed. Here we demonstrated that *Lrp4* is highly expressed in hypertrophic chondrocytes during endochondoral ossification. We also showed that LRP4 is an important regulator for chondrocyte maturation and production of chondrogenic extracellular matrix. Consistent with our data, *Lrp4*-deficient mice have abnormal Alcian blue staining in cartilages [7,8] and impaired length of femur bone [5], but their underlying mechanisms have not been dissected in detail. In human, mutations in *LRP4* cause Cenani-Lenz syndrome [9–11], sclerosteosis-2 [14], and congenital myasthenic syndrome [17]. In contrast to *Lrp4*-deficient mice, none of these diseases present primary cartilage abnormality or dwarfism. In human diseases, the Wnt/β-catenin-suppressive activity of LRP4 may be sufficiently retained even in the presence of *LRP4* mutations. Alternatively, in these patients, other LRP family proteins may substitute for the defective LRP4 and rescues defective endochondral ossification.

4.2. LRP4 functions in the Wnt/β-catenin signaling pathway for chondrocyte differentiation

Wnt ligands, Wnt-5a and Wnt-5b, are expressed in prehypertrophic and hypertrophic chondrocytes and have roles in chondrocyte differentiation [2,21]. Frizzled proteins, Fzd-1 and Fzd-7, delay chondrocyte maturation [2] and LRP5/6 promote chondrocyte hypertrophy [22]. Here we confirmed that another transmembrane protein, Lrp4, induces chondrocyte maturation and extracellular matrix production by inhibition of Wnt/β-catenin signaling. An LRP4-binding partner, Dkk1, increases glycosaminoglycan synthe-

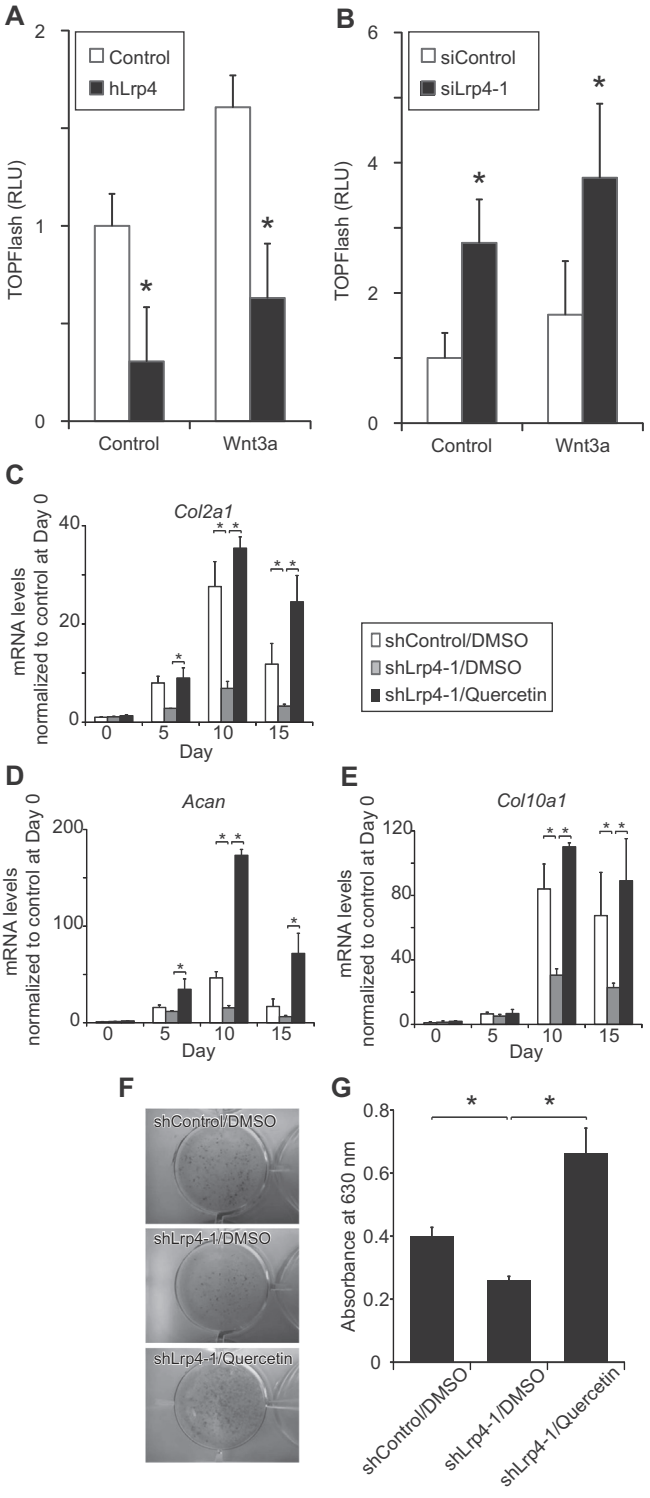

Fig. 4. LRP4 inhibits Wnt signaling pathway in ATDC5 cells. (A and B) ATDC5 cells are transfected with either human LRP4 or siLrp4-1 for 24 h. TCF-dependent TOPFlash reporter activities are normalized by the TK promoter-driven Renilla luciferase activity and expressed as relative luciferase units (RLU). $^{*}P < 0.05$ by Student's *t*-test. (C–E) ATDC5 are infected by lentivirus carrying either shControl or shLrp4-1 and cultured in differentiation medium with or without quercetin. Gene expressions of *Col2a1*, *Acan*, and *Col10a1* are quantified by real-time RT-PCR. Values are normalized to that at Day 0. $^{*}P < 0.05$ by two-way repeated measure ANOVA followed by Bonferroni post hoc test. (F) Alcian blue staining of ATDC5 cells that are differentiated to chondrocytes with ITS for 15 days. (G) Alcian blue-stained proteoglycans are quantified by measuring OD630 of the cell lysates. $^{*}P < 0.01$ by Student's *t*-test. (A, B, C, D, E, and G) Mean and SD ($n = 3$) are indicated.

sis and expressions of *Sox9* and *Col2a1* in early chondrogenesis of human mesenchymal stem cells [23]. In addition, Dkk1 is weakly and highly expressed in mature chondrocytes and osteoblasts, respectively, during endochondral ossification [24]. Another LRP4-binding partner, SOSTDC1, is broadly expressed in mouse limbs during endochondral ossification [25]. We propose that the functions of Dkk1 and SOSTDC1 in endochondral ossification are dependent on their binding partner, LRP4. These molecules orchestrate to downregulate Wnt/β-catenin signaling in endochondral ossification.

Competing interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.125.

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