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Role of the Low-Density Lipoprotein Receptor-Related Protein-1 in Regulation of Chondrocyte Differentiation

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The low-density lipoprotein receptor-related protein 1 (LRP1) is known as an endocytic and signal transmission receptor. We formerly reported the gene expression and the localization of LRP1 in cartilage tissue and chondrocytes, but its roles in the differentiation of chondrocytes remained to be investigated. Here, in order to address this issue, we employed RNAi strategy to knockdown *lrp1* in chondrocytic cells and obtained findings indicating a critical role therein. As a result of *lrp1* knockdown, *aggrecan* and *col2a1* mRNA levels were decreased. However, that of *col10a1* or *mmp13* mRNA was rather increased. Under this condition, we performed a promoter assay for Axin2, which is known to be induced by activation of the WNT/β-catenin (βcat) signaling pathway. Thereby, we found that Axin2 promoter activity was enhanced in the *lrp1* knockdown cells. Furthermore, when the WNT/β–catenin pathway was activated in chondrocytic cells by WNT3a or SB216763, which inhibits the phosphorylation of GSK3β, the mRNA levels of *aggrecan* and *col2a1* were decreased, whereas that of *mmp13* was increased. Additionally, the level of phosphorylated protein kinase C (PKC) ζ was also decreased in the *lrp1* knockdown cells. When the phosphorylation of PKCζ was selectively inhibited, *aggrecan* and *col2a1* mRNA levels decreased, whereas the *mmp13* mRNA level increased. These data demonstrate that LRP1 exerts remarkable effects to retain the mature phenotype of chondrocytes as a critical mediator of cell signaling. Our findings also indicate that the onset of hypertrophy during endochondral ossification appears to be particularly dependent on the WNT and PKC signaling initiated by LRP1.

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The low-density lipoprotein receptor (LDLR)-related protein-1 (LRP1), is a 600-kDa type I membrane protein and a member of the LDLR family (Herz and Strickland, 2001). By interacting with over 40 distinct ligands, LRP1 is thought to regulate lipid homeostasis, extracellular proteolysis, growth factor/cytokine activity, composition of the extracellular matrix (ECM), and even immune responses (Herz and Strickland, 2001). A significant part of these LRP1 functions is thought to be related to endocytosis (van Kerkhof et al., 2005) and cellular signal transduction pathways (Herz, 2001). Consistent with its functional diversity, LRP1 is essential for embryonic development. It was reported that conventional *lrp1*-deficient animals failed to develop normally and died during early to mid-gestation (Herz et al., 1992). Of note, the involvement of LRP1 in the prevention of atherosclerosis was also indicated by utilizing conditional gene targeting technology (Boucher et al., 2003). Also, the expression and function of LRP1 in the central nervous system (May and Herz, 2003), vascular smooth muscle cells (Boucher et al., 2003), and macrophages (Gardai et al., 2003) have been relatively well characterized.

Recently, LRP1 was shown to interact with human Frizzled-1 (HFz1) to down-regulate the canonical WNT/β-catenin (βcat) signaling pathway (Zilberberg et al., 2004). WNT signaling is divided into canonical and non-canonical pathways. Canonical WNT signaling regulates the protein level of βcat (Peifer et al., 1994; Peifer and McEwen, 2002). When WNT/βcat signaling is

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inactivated, βcat is phosphorylated by glycogen synthase kinase 3β (GSK3β), which leads to ubiquitination of βcat and its degradation by proteasomes (Giles et al., 2003). When WNT/βcat signaling is activated, GSK3β is inhibited. This inhibition in turn stabilizes βcat, thereby increasing the amount of βcat, which accumulates in the nucleus and regulates gene expression in conjunction with the TCF/Lef family of transcription factors. Being other members of the LDLR family, LRP5/6 interact with Frizzled (Fz) receptors to function as WNT co-receptors. Non-canonical WNT signaling pathways include the WNT/calcium pathway. This WNT/calcium pathway depends on an intracellular release of Ca^{2+} to activate calcium-sensitive enzymes, such as protein kinase C (PKC; Kuhl, 2004). In addition, the mitogen-activated protein kinase (MAPK) pathway activates PKC as well. According to a previous study, LRP1 also activates PKC (Hayashi et al., 2007).

A hierarchy of WNT signaling regulates diverse developmental processes. WNT signaling has a vital role during chondrogenesis and chondrocyte differentiation. Vertebrate cartilage is of two distinct types, permanent cartilage represented by articular cartilage, and temporary cartilage represented by the growth plate cartilage where endochondral ossification occurs. During endochondral ossification, chondrocytes first proliferate, and then become mature cells, which produce ECM components such as aggrecan and type II collagen. Thereafter, the cells eventually differentiate into hypertrophic chondrocytes, which produce type X collagen and matrix metalloproteinase (MMP) 13. At the terminal stage of endochondral ossification, the cartilage matrix becomes mineralized and is invaded by blood vessels; and hypertrophic chondrocytes are then thought to undergo apoptosis. Through this process, cartilage is replaced by bone (Nakanishi et al., 1997; Takigawa et al., 2003).

The outcomes of WNT signaling in chondrocytes and chondroprogenitor cells are complex, occasionally resulting in either stimulation and inhibition of differentiation (Hartmann and Tabin, 2000, 2001; Chimal-Monroy et al., 2002; Church et al., 2002). For example, WNT3A-inducible transcription factor Twist1 inhibits the expression of *aggrecan* and *col2a1* (Reinhold et al., 2006), and WNT5a inhibits *col2a1* expression (Yang et al., 2003).

In contrast, PKC is a classical signal transducer in chondrocytes. Inhibition or down-regulation of PKC blocks chondrogenesis (Choi et al., 1995), whereas activation of this kinase causes dedifferentiation of rabbit costal and articular chondrocytes (Takigawa et al., 1983; Bouakka et al., 1988). It should be noted that PKC mediates chondrocyte differentiation promoted by CCN2/connective tissue growth factor (CTGF; Yosimichi et al., 2006).

In our series of studies, we have uncovered critical roles of CCN2/CTGF that are played throughout endochondral ossification (Nakanishi et al., 1997, 2000; Nishida et al., 2002; Ivkovic et al., 2003; Takigawa et al., 2003; Perbal and Takigawa, 2005) and articular cartilage regeneration (Nishida et al., 2004). Currently, a number of cell-surface molecules have been nominated as candidates for specific receptors for CCN2/CTGF. However, specific receptors for CCN2/CTGF have not yet been defined in chondrocytes. Since LRP1 is one such candidate that directly binds to CCN2/CTGF (Segarini et al., 2001; Gao and Brigstock, 2003; Perbal, 2004; Yang et al., 2004), we earlier investigated LRP1 in cartilage and demonstrated its presence in all chondrocytes except for hypertrophic chondrocytes (Kawata et al., 2006). However, the roles of LRP1 in the differentiation of chondrocytes are not currently understood. In this study, we evaluated the cell biological significance of LRP1 in chondrocytes and precisely characterized its function in those cells, in which involvement of WNT/βcat and PKC signaling pathways were indicated.

Materials and Methods

Antibodies and reagents

For immunoblotting, mAb 5A6 (Progen, Heidelberg, Germany) recognizing the 85-kDa LRP1 light chain, anti-GAPDH (Chemicon, Temecula, CA), anti-βcatenin C-18, anti-PKC α C-20, anti-PKC ε C-15, anti-PKC ζ C-20 (Santa Cruz Biotech, Santa Cruz, CA), anti-PKC alpha (phospho T638), anti-PKC epsilon (phospho S729) (Abcam, Tokyo, Japan), anti-PKC zeta (pT560) Phospho Rabbit Monoclonal (Epitomics, Inc., Burlingame, CA), and anti-HA (Bethyl Laboratories, Inc., Montgomery, TX) antibodies were employed. The secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG antibodies were purchased from American Qualex (San Clemente, CA), Chemicon, and Santa Cruz Biotech, respectively. Recombinant Mouse Wnt3a was purchased from R&D Systems, Inc. (Minneapolis, MN). GSK3β inhibitor (SB216763) and PKC inhibitor (calphostin C) were purchased from Sigma–Aldrich (St. Louis, MO). Myristoylated PKCζ pseudosubstrate peptide inhibitor (PKCζ inhibitor) was purchased from Calbiochem (San Diego, CA).

Cells

Cells of the human chondrocytic cell line HCS-2/8 (Takigawa et al., 1989; Takigawa et al., 1991) were cultured in Dulbecco's modification of minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS). Primary chicken chondrocytes were isolated from the caudal one-third portion (LS) of the sterna, the peripheral regions (USP) and central core regions (USC) of the cephalic portion of the sterna of day 17 chick embryo using the method described (Iwamoto et al., 1995) and were maintained in high-glucose DMEM containing 10% FBS. The cells were cultured at 37°C in humidified air with 5% CO_2. For inhibitor assays, 2.5–20 μM SB216763, 100–200 nM calphostin C, or 7.5–15 μM PKCζ inhibitor was added to the culture. Unless otherwise specified, recombinant mouse WNT3a was added to a final concentration of 75 ng/ml to DMEM cultures containing 0.5% FBS.

LRP1 siRNA transfection

To knockdown LRP1 protein production, we used RNA interference technology. Two designed pairs of oligoduplexes targeted against human *lrp1* (gene accession No. NM_002332) were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). The target nucleotide sequences of those oligoduplexes were 5′-UGG ACU AUA UUG AAG UGG UGG ACU AAG-3′ and 5′-CCU GUA CCA UGA ACA GCA AAA UGA UAG-3′. The former was termed LRP-1163; and the latter, LRP-13157. A nonspecific oligoduplex (nonsilencing control, targeting 5′-UUA GGG GAU AAG UAC GGU UGA AUC UAG-3′) was used as a negative control at the same final concentrations as the human LRP1-targeting RNA duplexes. A pair of oligoduplexes targeted against chicken *lrp1* (gene accession No. NM_205242) was designed and purchased from Invitrogen Corporation (Carlsbad, CA). The target nucleotide sequence of that oligoduplexes was 5′-GGU GUU CUU CAC UGA CUA CTT-3′. Non-targeting siRNA (Ambion, Inc., Austin, TX) was used as a negative control at the same final concentrations as the chicken LRP1-targeting RNA duplex. Prior to transfection, the cells were transferred to each well in 6-well plates (density: 4×10^5 cells/well). The transient transfections with a 30 nM concentration of each human siRNA and a 70 nM concentration of each chicken siRNA were performed by using siPORT NeoFX™ Transfection Agent (Applied Biosystem, Foster City, CA) according to the manufacturer's protocol. At 24 h after the transfection, the medium was exchanged for fresh medium, and the cells were cultured for another 48–72 h. The mRNA of the cells was harvested after 48 h, whereas the protein was harvested after 48 and 72 h.

RNA extraction and cDNA synthesis

Cells were collected, and total RNA was extracted, following the manufacturer's instructions (RNeasy kit, Qiagen, Hilden, Germany). Total RNA (500 ng) was reverse-transcribed by AMV Reverse Transcriptase (TaKaRa, Ohtsu, Japan) at 42°C for 30 min.

Real-time PCR

Real-time PCR was performed by using TOYOBO SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan) in a LightCycler™ system (Roche, Basel, Switzerland). Reactions were performed in 10 μl containing 1 μl of cDNA, 0.4 μl of each primer (5 μM), and 5 μl of 1× SYBR Green master mix. Primer sets and optimized conditions for the PCR of each target are listed in Tables 1 and 2. Absence of non-specific PCR products was checked by melting curve and electrophoresis analyses. Relative copy numbers were computed based on data obtained with a serial dilution of a representative sample for each target gene. The experiments were repeated at least twice, and similar results were obtained.

Immunoblot analysis

HCS-2/8 cells were lysed in lysis buffer (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na [VO_4], 5% glycerol, 40 mM ammonium molybdate, and 1 mM phenylmethanesulfonyl fluoride). The lysate diluted in 1× SDS sample buffer (50 mM Tris–HCl [pH6.8], 2% SDS, 5% glycerol, 2% bromphenol blue) with or without 2-mercaptoethanol was boiled for 3 min, and was then subjected to SDS–PAGE in 9% or 12% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes with a blotting apparatus. The membranes were then incubated for 1 h in a blocking buffer [3% non-fat milk in phosphate-buffered saline (PBS) or bovine serum albumin (BSA) in Tris-buffered saline (TBS)] and subsequently incubated overnight with anti-LRP1 (5A6, 1:100), anti-GAPDH (1:100), anti-βcatenin (C-18, 1:400), anti-PKC α (C-20, 1:200), anti-PKC ε (C-15, 1:100), anti-PKC ζ (C-20, 1:200), anti-PKC alpha (phospho T638) (1:5,000), anti-PKC epsilon (phospho S729) (1:1,000) or anti-PKC zeta (pT560) Phospho Rabbit Monoclonal (1:2,000) antibody in the blocking buffer. The membrane was washed 5 times in PBS or TBS and incubated for 2 h with HRP-conjugated anti-mouse IgG (1:2,000), anti-rabbit IgG (1:5,000), or anti-goat IgG (1:5,000) in the blocking buffer. After extensive washes with PBS or TBS, immunoreactive proteins were detected by using an ECL Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ). The signal intensities were quantified with Image J (http://rsb.info.nih.bov/ij/). The experiments were repeated at least twice, and similar results were obtained.

Construction of plasmids

The constructs of Axn2-Luc and mLRP4 were kindly provided by Dr. Frank Constantini (Jho et al., 2002) and Dr. Guojun Bu (Obermoeller-McCormick et al., 2001) respectively. A retroviral vector that expresses the short hairpin RNA (shRNA) targeting LRP1 (shLRP1) or scrambled shRNA was constructed by inserting the double-stranded DNA fragments comprising the following sense-loop-antisense DNA sequences: shLRP1, 5′-T GGA CTA TAT TGA AGT GGT GGA CTA GTG TGC TGT CCT AGT CCA CCA CTT CAA TAT AGT CCA-3′; scrambled shRNA, 5′-T TAG GGG ATA AGT ACG GTT GAA TCT GTG TGC TGT CCA GAT TCA ACC GTA CTT ATC CCC TAA-3′, with pSINsi-hU6 (Takara) used as the parental vector

DNA transfection

DNA transfection of HCS-2/8 cells was performed at 48 h after the siRNA transfection, or at 80% confluence in 12-well dishes. For transfection, 0.5 μg plasmid DNA was mixed with serum-free DMEM in a total volume of 25 μl, which mixture was then added to 25 μl of serum-free DMEM containing 2 μl FuGene 6 Transfection Reagent (Roche Applied Science, Glendale, CA); and this resultant mixture was incubated for 15 min at room temperature and then added directly to the cells.

Dual-Glo luciferase assay

The dual luciferase assay was carried out by using the Dual-Glo luciferase assay system (Promega, Madison, WI) with a modified protocol. Luminescence was measured in a Fluoroskan Ascent FL (Labsystems, Helsinki, Finland). To determine firefly luciferase activity, we mixed 50 μl of cell lysate and 50 μl of Dual-Glo luciferase reagent in each well of a 96-well plate and incubated the plate for 10 min. The luminescence was then measured 4 times. For determination of *Renilla* luciferase activity, 50 μl of Dual-Glo Stop and Glo reagent were subsequently added to the reactions. After mixing and incubation for 10 min, the luminescence was measured 10 times. The average and standard deviation for each measure were computed with these values. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity. The experiments were repeated at least twice, and similar results were obtained.

Preparation of LRP1 shRNA expressing pseudo retrovirion

Retroviral vectors expressing shRNA targeting human LRP1 and scramble shRNA were produced by using a Retrovirus Packaging Kit Ampho (TaKaRa) according to the manufacturer's protocol. Briefly, 3×10^6 293T cells were seeded into one 6-cm collagen-coated dish. After 24 h, the culture medium was changed

TABLE 1. Primers and experimental conditions for real-time PCR with HCS-2/8 cells

Target gene (human)	Primer direction	Sequence (5′ → 3′)	Length of PCR product	Denature time (s)	Annealing temperature (°C)
gapdh	S	gccaaaagggtcatcatctc	215	5	65
	AS	gtcttctgggtggcagtgat			
lrp1	S	acatatagcctccatcctaatc	152	0	65
	AS	ttccaatctccacgttcat			
lrp6	S	caagcaccaaaggcactta	148	0	60
	AS	cggtagctatatggcctgta			
aggrecan	S	ttcgggcagaagaaggac	182	5	60
	AS	cgtgagctccgcttctgt			
col2a1	S	gagggcaatagcaggttcacgta	133	5	65
	AS	tgggtgcaatgtcaatgatgg			
col10a1	S	gagtatgtccactcctctt	148	5	60
	AS	cattcttttcagcctacctc			
mmp13	S	tggtggtgatgaagatgatttgtct	375	5	60
	AS	agttacatcggaccaaactttgaag			
sox9	S	caaccagaattccctttgga	199	5	60
	AS	tgctccatttagccaaggtt			

S, sense; AS, anti-sense.

TABLE 2. Primers and experimental conditions for real-time PCR with chicken cells

Target gene (chicken)	Primer direction	Sequence (5′ → 3′)	Length of PCR product	Denature time (s)	Annealing temperature (°C)
gapdh	S	aggctgtggggaaagtca	202	5	60
	AS	gacaacctggtcctctgtgtat			
lrp1	S	ggctgagccatccctttca	114	5	62
	AS	ggttggtgacggatttgt			
sox9	S	aggaagctggctgaccagta	192	5	60
	AS	cgttcttcaccgacttcctc			
aggrecan	S	cctgcctgacctctttgc	277	5	61
	AS	tggggaggagggcaacat			
col2a1	S	agaaaggaatccagcccaat	236	5	61
	AS	acacctgccagattgattcc			
col10a1	S	acatgcatttacaaatatcgttac	160	5	55
	AS	aaaatagtagacgttaccttgactc			

S, sense; AS, anti-sense.

to DMEM containing 10% FBS and 25 μM chloroquine. The mixture of a recombinant retrovirus vector, pGP, and pE-ampho was added to Transfection Buffer, and was dropped onto the cell dish evenly. After the cells had been Incubated for 7–11 h in a 5% CO_2 incubator at 37°C, the medium was removed from the dish and then replaced with fresh DMEM containing 10% FBS. At 24 h after transfection, the culture medium was refreshed again. Finally, at 48 h post-transfection, the culture supernatant was collected and filtered through a 0.45-μm sterilized filter. The viral suspension was dispensed in small aliquots and stored at −80°C until used.

Infection with LRP1 shRNA expressing pseudo retrovirion

HCS-2/8 cells were seeded at 2×10^5/well in each well of 6-well plates. After 24 h, the culture medium was changed to 900 μl of DMEM containing 10% FBS with 18 μg/ml of polybrene. The virus suspension was diluted 10-fold with DMEM containing 10% FBS, and 100 μl of diluted virus suspension was then added to each well. After the cells had been incubated for 4–6 h in 5% CO_2 at 37°C, 1 ml of DMEM containing 10% FBS was added to each well. At 24 h post-infection, the medium was replaced with fresh DMEM containing 10% FBS.

Statistics

Data were presented as means ± standard deviations, and the statistical significance of differences in mean values was assessed by Student's unpaired *t*-tests. Differences among the mean values were considered significant at a P of <0.05.

Results

Effect of *lrp1* knockdown on chondrocytic phenotype

For transient *lrp1* knockdown experiments, siRNAs were designed to target the *lrp1* sequence at position 1163 or 13157. The former was termed LRP1-1163; and the latter, LRP1-13157 (Fig. 1A). The expression of *lrp1* was knocked down to approximately 10% of the control by LRP1-1163 and to 50% by LRP1-13157, whereas the expression of *lrp6* was not affected (Fig. 1B). These results suggest that the *lrp1* knockdown was specific. Moreover, the production of LRP1 protein was substantially knocked down to 30% by LRP1-1163 and to 70% by LRP1-13157 (Fig. 1C). Under the same conditions, the expression of *aggrecan*, a differentiation marker of chondrocytes, was decreased by approximately 50% with LRP1-1163 and by 70% with LRP1-13157 (Fig. 2A). Moreover the expression of *col2a1*, another differentiation marker of chondrocytes, was decreased by approximately 90% with LRP1-1163 and by 80% with LRP1-13157 (Fig. 2A). However, the expression of *col10a1*, a differentiation marker of hypertrophic chondrocytes, was increased by approximately 2.5-fold with LRP1-1163 and LRP1-13157 (Fig. 2A). These results suggest that LRP1 plays a significant role in the differentiation of chondrocytes.

Repressive function of LRP1 in the canonical WNT signaling pathway in chondrocytic cells

Since the involvement of WNT signaling in chondrocyte differentiation has been suggested (Reinhold et al., 2006), we evaluated the effect of *lrp1* knockdown on the canonical WNT signaling pathway. By *lrp1* knockdown, the accumulation of βcat was increased in the HCS-2/8 cells (Fig. 2B), indicating that the WNT/βcat signaling pathway was repressed by LRP1. To confirm this repression, we co-transfected LRP1-1163 or LRP1-13157-treated cells with the reporter construct designated Axin2-Luc (Jho et al., 2002); because the Axin2 promoter is known to be activated, when the WNT/βcat signaling pathway is activated. As a result, the Axin2 promoter activity was increased by approximately 1.3- or 1.6-fold, respectively, in the *lrp1*-knocked down HCS-2/8 cells compared with the control activity (Fig. 2C). These data suggest that LRP1 mediates an inhibitory signal in the WNT/βcat signaling pathway.

Long-term effect of *lrp1* knockdown in chondrocytes

In order to further investigate the long-term effect of *lrp1* knockdown in chondrocytes, we applied a retrovirus vector expressing LRP1-targeted shRNA to HCS-2/8 cells (Fig. 3). The cells were maintained for 14 days after the infection, and approximately 70% decreases in *aggrecan* and *col2a1* mRNA levels were seen in the HCS-2/8 cells infected with the LRP1 shRNA-expressing retrovirus vector (Fig. 3A). In contrast, the expression of *mmp13*, another differentiation marker of hypertrophic chondrocytes, was increased by approximately 1.5-fold in *lrp1*-knocked down HCS-2/8 cells (Fig. 3A). These findings indicate that long-term expression of the *lrp1* shRNA promoted late chondrocytic differentiation. Additionally, the accumulation of βcat was increased in HCS-2/8 cells, when they were infected with LRP1 shRNA expressing retrovirus vector (Fig. 3B), just as in the case of treatment with *lrp1* siRNA. Collectively, these results are comparable to those shown in Figure 2.

Effect of LRP1 overexpression on chondrocytic phenotype

In order to support the findings obtained by the loss of function experiments, we performed overexpression studies of mLRP4 (Obermoeller-McCormick et al., 2001) that was a functional equivalent of LRP1. After 48 h following transfection of an mLRP4 overexpression vector, the production of mLRP4 protein by HCS-2/8 cells was confirmed by immunoblotting using anti-HA and anti-LRP1 antibodies (Fig. 4A). Under the same conditions, the expression of *sox9*, another differentiation marker of chondrocytes, was increased by approximately twofold with mLRP4 (Fig. 4B). Moreover the expression of *aggrecan and col2a1* was increased by approximately 2- and 1.5-fold with mLRP4 (Fig. 4B). In contrast, the expression of

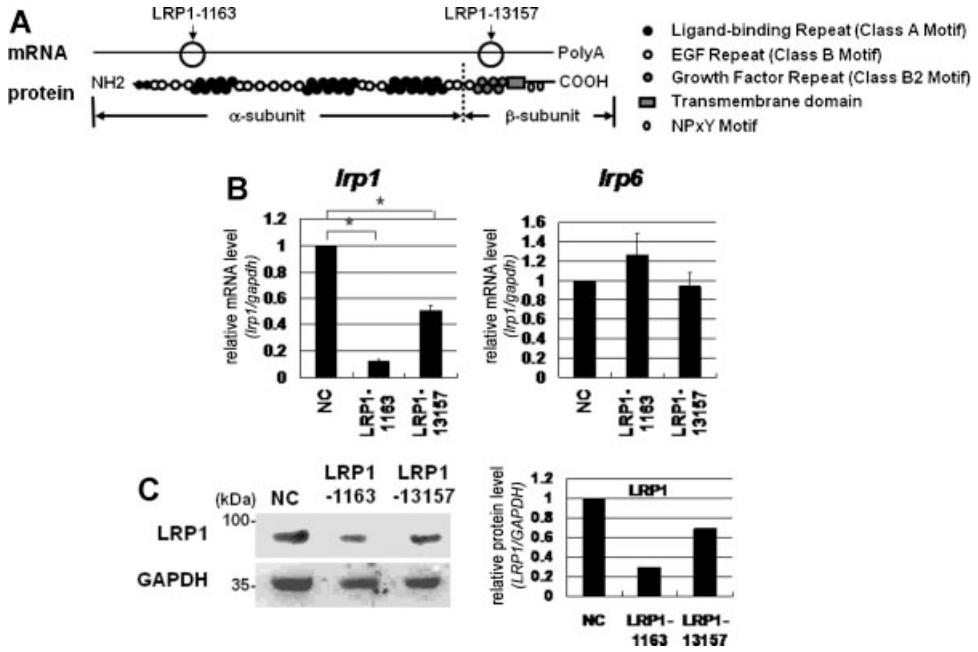

Fig. 1. Expression level of *lrp1* mRNA and production level of LRP1 protein following LRP1 siRNA (30 nM) transfection in HCS-2/8 cells. A: LRP1 siRNAs, LRP1-1163 and LRP1-13157, were designed to target the sequences at the positions of 1163 and 13157, respectively. A brief description of the symbols used to illustrate the LRP1 structure is included. B: Expression level of 2 LDLR family members 48 h after siRNA transfection. The expression of *lrp1* was efficiently knocked down; whereas the expression of *lrp6* was not decreased, thus indicating the efficacy and specificity in the reduction in *lrp1* mRNA. The values represent the means ± SD. *$P < 0.05$. C: Representative immunoblot analysis of LRP1 72 h after siRNA transfection. Positions of molecular weight markers (100, 35 kDa) are shown at the left of the images. The intensity of each signal was quantified with Image J (http://rsb.info.nih.bov/ij/) and was standardized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Substantial and specific reductions in protein levels are seen. NC, non-silencing scrambled siRNA as a negative control.

mmp13 was decreased by approximately 70% with mLRP4 (Fig. 4B). The expression of *lrp6* was not affected (Fig. 4B), suggesting that the observed effects were LRP1 specific. Moreover, the accumulation of βcat was decreased (Fig. 4C). These data firmly support the finding indicated in Figs. 2 and 3.

Activation of the canonical WNT signaling pathway promotes late chondrocytic differentiation

To ascertain the contribution of the canonical WNT signaling pathway to chondrocytic differentiation directly, we activated WNT/βcat signaling in HCS-2/8 cells by using SB216763, an inhibitor of GSK3β. Our data confirmed that WNT/βcat signaling was actually activated by SB216763, as represented by Axin2 promoter activity and the accumulation of βcat in whole cells (Fig. 5A,B). Under this condition, the mRNA levels of *aggrecan* and *col2a1* were decreased, whereas the level of *mmp13* was increased by SB216763 (Fig. 5C). We observed no significant effect of SB216763 on the expression of *lrp1*. Comparable changes in chondrocytes differentiation marker mRNA expression was obtained by using recombinant WNT3a (data not shown). These results indicate that activation of the WNT/βcat signaling pathway promotes the terminal differentiation of chondrocytes.

LRP1-dependent activation of PKCζ in chondrocytic cells

The effects of activation of the WNT/βcat signaling pathway by SB216763 on chondrocytic differentiation were weaker than those by *lrp1* knockdown (Fig. 5C). Therefore, suspecting the involvement of another signaling pathway, we performed immunoblot analysis to evaluate the phosphorylation of PKC in HCS-2/8 cells with or without the *lrp1* siRNA. The results showed that phosphorylation of the conventional type of PKC, PKCα, and that of a novel type PKC, PKCε, were unchanged, but that the phosphorylation of the atypical type PKC, PKCζ, was decreased by *lrp1* knockdown in chondrocytes (Fig. 6A). Moreover, we evaluated the phosphorylation of PKC in HCS-2/8 cells with or without LRP1 overexpression. The results showed that phosphorylation of PKCα and PKCε were unchanged, whereas that of PKCζ was increased by LRP1 overexpression in chondrocytes (Fig. 6B). These data suggest that LRP1 mediates specifically the phosphorylation of PKCζ as well.

PKCζ as a promoter of late chondrocyte differentiation

For the examination of the role of PKCζ in chondrocyte differentiation, a specific inhibitor of PKCζ phosphorylation was employed. First of all, we confirmed that only the phosphorylation of PKCζ was actually inhibited by the PKCζ inhibitor, while the level of p-PKCα was unchanged (Fig. 7A). In the presence of the PKCζ inhibitor, the mRNA levels of *aggrecan* and *col2a1* were decreased, whereas the level of *mmp13* was significantly increased (Fig. 7B), as exactly observed upon *lrp1* knockdown. Next, the phosphorylation of all PKC subtypes except the atypical type was inhibited by calphostin C in HCS-2/8 cells. We confirmed that the phosphorylation of the conventional PKC, PKCα, was actually inhibited by calphostin C, whereas the level of p-PKCζ was unchanged (Fig. 7C). When the phosphorylation of all PKC subtypes except the atypical type was inhibited by calphostin C, the mRNA levels of *aggrecan* and *col2a1* were decreased; and, interestingly, the level of *mmp13* was also decreased (Fig. 7D). These results suggest that

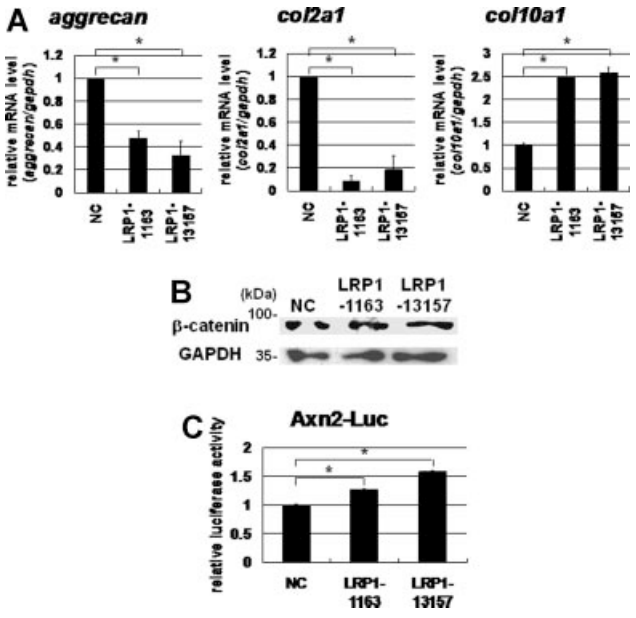

Fig. 2. A: *Aggrecan*, *col2a1*, and *col10a1* mRNA levels in *lrp1* knocked down chondrocytic HCS-2/8. Decreases of approximately 50–90% in *aggrecan* and *col2a1* mRNA levels are seen with LRP1 siRNA. In contrast, the expression of *col10a1* was increased by approximately 2.5-fold in the *lrp1* knocked down chondrocytes. The values represent the means ± SD. *$P < 0.05$. B: Slight increase in βcat level by *lrp1* knockdown in chondrocytes. Positions of molecular weight markers (100, 35 kDa) are shown at the left of the images. C: Induction of *Axin2/* luciferase activity by *lrp1* knockdown. Axin2-Luc and pRL-TK (*Renilla* luciferase under the thymidine kinase promoter) were co-transfected into HCS-2/8 cells together with the indicated plasmids, and the ratio of Axin2-driven firefly luciferase to TK-driven constitutively expressed *Renilla* luciferase activity was measured and computed. *Lrp1* knockdown increased the relative ratio, indicating the induction of the promoter activity. The values represent the means ± SD. *$P < 0.05$. NC, non-silencing scrambled siRNA as a negative control.

the phosphorylation of a particular subtype, PKCζ, promotes the terminal differentiation of chondrocytes.

Effect of *lrp1* knockdown in chondrocytes at different differentiation stages on chondrocytic phenotype

Finally, to evaluate the effects of LRP1 knockdown in normal chondrocytes at different differentiation stages, we performed RNAi-mediated gene silencing experiments with chicken primary chondrocytes. The chondrocytes were isolated from the caudal one-third portion (LS), the peripheral regions (USP) and central core regions (USC) of the cephalic portion from the sterna of day 17 chick embryo using the method described (Iwamoto et al., 1995). LS, USP and USC cells represent resting chondrocytes, proliferating chondrocytes, and hypertrophic chondrocytes, respectively. The expression of *lrp1* was knocked down to approximately 40–50% of the control by LRP1-siRNA in chondrocytes at all differentiation stages (Fig. 8). Under the same conditions, the expression of *sox9* and *aggrecan* was decreased by approximately 60–70%, and 30–50%, respectively, with LRP1-siRNA in chondrocytes of all differentiation stages. In contrast, decrease of approximately 50% in *col2a1* mRNA level was seen only in the *lrp1* knocked down USC cells, whereas in the *lrp1* knocked down LS and USP cells, it showed modest increase. Interestingly, the expression of *col10a1* was increased by approximately 1.3-fold only in the *lrp1* knocked down USC cells, whereas in the *lrp1* knocked down LS, it showed a decrease of approximately 90%, and in the *lrp1* knocked down USP cells, it was unchanged. These results suggest that LRP1 downregulation in hypertrophic chondrocytes specifically promotes their terminal differentiation.

Discussion

In the present study, we showed that LRP1 was critically involved in chondrocyte differentiation. In fact, LRP1 knockdown in cultured chondrocytes inhibited the expression of marker genes associated with the mature phenotype but boosted the characteristics associated with hypertrophy (Figs. 2A and 3A) especially in chondrocytes at a hypertrophic stage (Fig. 8). Conversely, LRP1 overexpression in cultured

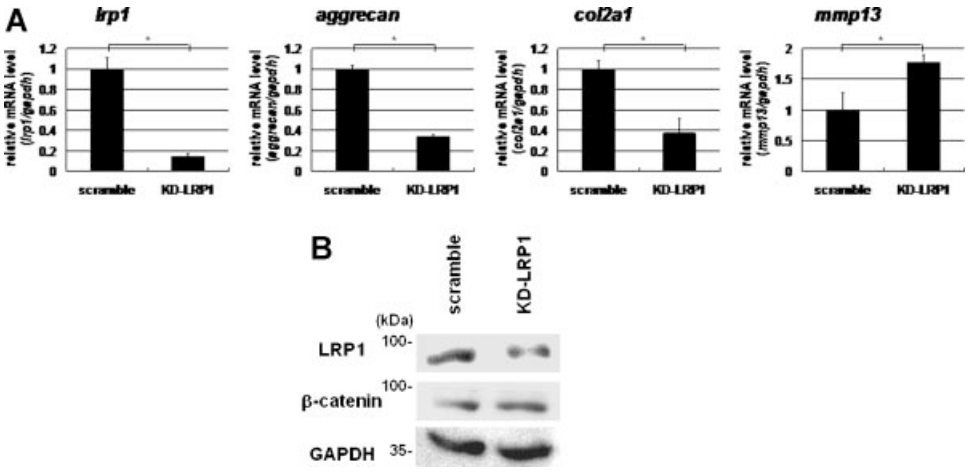

Fig. 3. A: *Aggrecan, col2a1*, and *mmp13* mRNA levels in chondrocytic HCS-2/8 after *lrp1* knockdown by a retrovirus vector. Decreases of approximately 70% in *aggrecan* and *col2a1* mRNA levels are seen in HCS-2/8 cells treated with LRP1 shRNA-expressing retroviral vector. By contrast, the expression of *mmp13* was increased by approximately 1.5-fold in the LRP1 knocked down chondrocytic cells. The values represent the means ± SD. *$P < 0.05$. B: Immunoblot analysis of the retrovirally transduced cells. Accumulation of βcat was observed in HCS-2/8 cells treated with the LRP1 shRNA-expressing retroviral vector. Positions of molecular weight markers (100, 35 kDa) are shown at the left of the images.

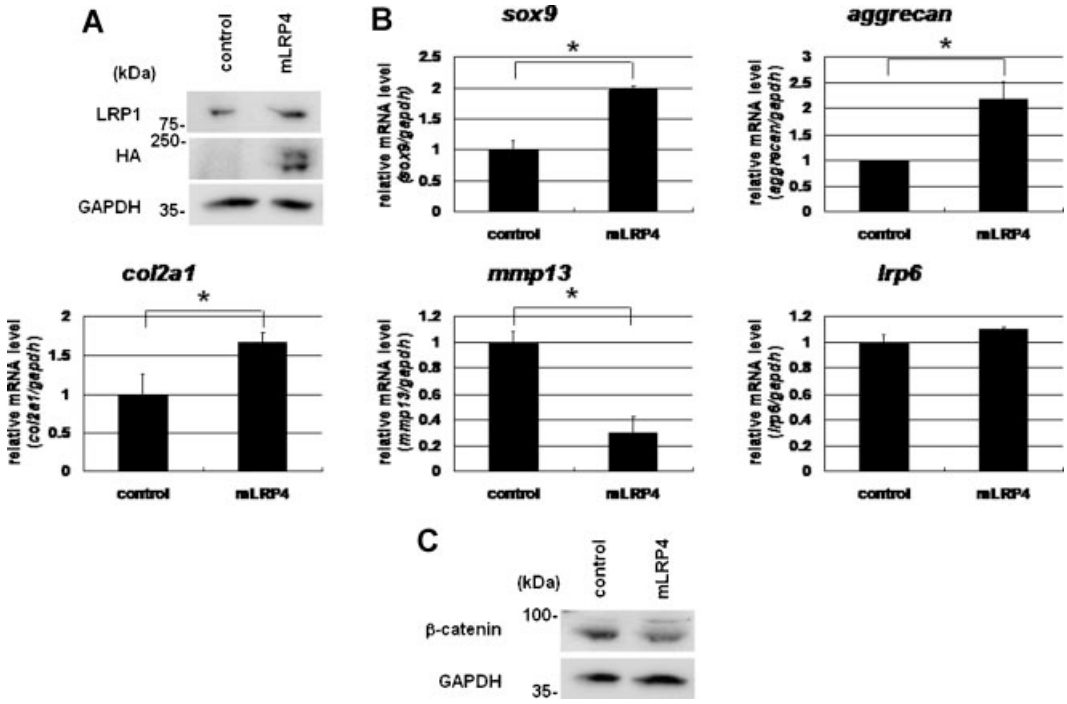

Fig. 4. ***SoX9*, *aggrecan*, *col2a1*, and *mmp13* mRNA levels in LRP1-overexpressed chondrocytic HCS-2/8. A: After 48 h following the transfection of an mLRP4 overexpression vector, the production of mLRP4 protein was confirmed by immunoblotting using anti-HA and anti-LRP1 antibodies. Positions of molecular weight markers (75, 250, 35 kDa) are shown at the left of the images. B: Under the same conditions, the expression of *sox9* was increased by approximately 2 fold with mLRP4. The expression of *aggrecan and col2a1* was also increased by approximately 2- and 1.5-fold with mLRP4, respectively. In contrast, the expression of *mmp13* was decreased by approximately 70% with mLRP4. The expression of *lrp6* was not affected. The values represent the means ± SD. $^{*}P < 0.05$. C: Accumulation of βcat was decreased by mLRP4 overexpression. Positions of molecular weight markers (100, 35 kDa) are shown at the left of the images.**

chondrocytes boosted the expression of marker genes associated with the mature phenotype but inhibited the characteristics associated with hypertrophy (Fig. 4B). These findings are consistent with our previous report that LRP1 is expressed in the growth plate except for the hypertrophic zone (Kawata et al., 2006). Our study provides clear evidence that LRP1 is a crucial and direct regulator of chondrocyte phenotype and may strongly impact skeletogenesis.

We showed that LRP1 was related to the WNT/βcat signaling pathway (Figs. 2B,C, 3B, and 4C). In this context, there also is a previous study indicating that LRP1 negatively regulates this pathway (Zilberberg et al., 2004). WNT/βcat signaling plays a crucial role in various developmental processes (Moon et al., 1997; Wodarz and Nusse, 1998; Polakis, 2000). These data support LRP1's possible roles in WNT/βcat signaling in chondrocytes, which finely balance the signaling as a part of multiple positive and negative regulatory mechanisms. WNT signaling is transduced via βcat and TCF/Lef proteins (Willert and Nusse, 1998; Eastman and Grosschedl, 1999; Vleminckx et al., 1999). Although most immunologically detectable βcat is cytoplasmic in the upper zones of the growth plate and in proliferating chondrocytes in culture, it is mostly nuclear in hypertrophic mineralizing chondrocytes (Enomoto-Iwamoto et al., 2002). This change in βcat distribution is consistent with the sharp difference in LRP1 distribution between the proliferating and hypertrophic zones. It is thus intriguing to consider that LRP1 and presumably other LDLR family members may be implicated in the negative regulation of various Wnt/Fz/LRP5/6-induced signaling events during development. The versatility of the effects of certain LDLR members on WNT signaling is reminiscent of several other factors, such as Dickkopf family members (Krupnik et al., 1999) and the Frizzled-related proteins (Melkonyan et al., 1997), which occasionally antagonize or synergize with WNT ligands and thus serve to fine-tune the WNT signaling pathway, whose tightly controlled regulation is essential for proper coordination of various developmental processes.

Moreover, we showed that LRP1 was related to the PKC signaling pathway (Fig. 6). A previous study revealed that LRP1 activates PKC (Hayashi et al., 2007), which is also a classical signal transducer in chondrocytes. Indeed, inhibition or down-regulation of PKC blocks chondrogenesis (Choi et al., 1995), whereas activation of this kinase causes dedifferentiation of rabbit costal and articular chondrocytes (Takigawa et al., 1983; Bouakka et al., 1988). Our present data indicate LRP1's positive role in the phosphorylation of PKC, particularly, PKCζ (atypical type PKC) in chondrocytes. There are previous studies showing that inhibition of PKCζ regulates nitric oxide-induced apoptosis and dedifferentiation of articular chondrocytes (Kim et al., 2002) and that PKCζ is up-regulated in osteoarthritic cartilage by the induction of ECM degradation (LaVallie et al., 2006). More recently, fibroblast growth factor (FGF)-2-mediated activation of atypical PKC has been reported to inhibit chondrocyte proliferation (Krejci et al., 2007). Thus, it is also intriguing to consider that LRP1 may be implicated in regulating various PKCζ signaling events during cartilage development.

In our data, the mRNA level of *mmp13* was increased approximately 1.5-fold when WNT/βcat signaling was activated (Fig. 5C), while it was increased approximately 3.5-fold when phosphorylation of PKCζ was specifically inhibited in HCS-2/8 cells (Fig. 7A). These results implicate both WNT/βcat and PKCζ signaling pathways in the regulation of chondrocyte

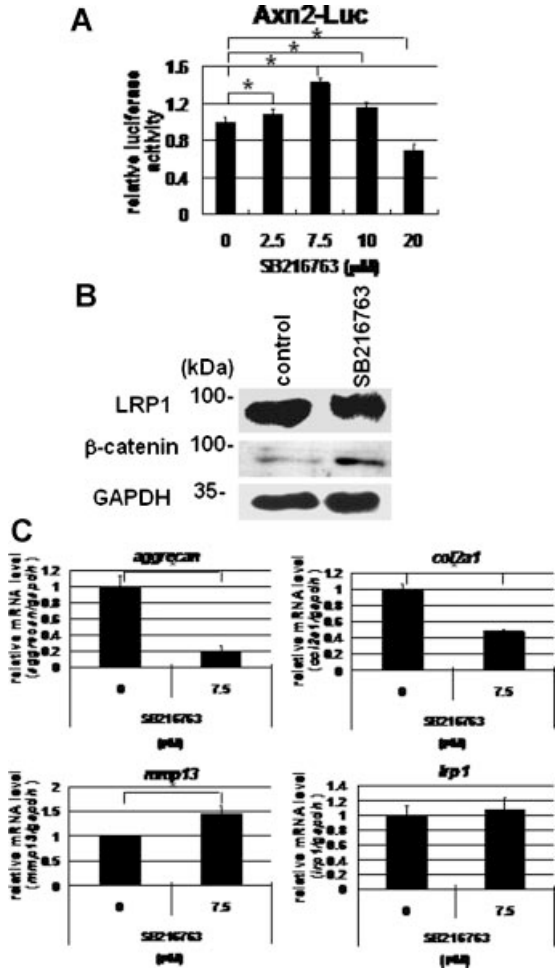

Fig. 5. Effects of the activation of WNT/βcat signaling in HCS-2/8 cells by SB216763, a GSK3β inhibitor. A,B: Activation of WNT/βcat signaling was confirmed by performing an Axin2 promoter assay (A) and by immunoblot analysis of βcat accumulated in whole cells (B). The values represent the means ± SD. *$P < 0.05$. Positions of molecular weight markers (100, 35 kDa) are shown at the left of the images. C: In the presence of SB216763, the mRNA levels of *aggrecan* and *col2a1* were decreased, whereas the level of *mmp13* was slightly increased. The values represent the means ± SD. *$P < 0.05$.

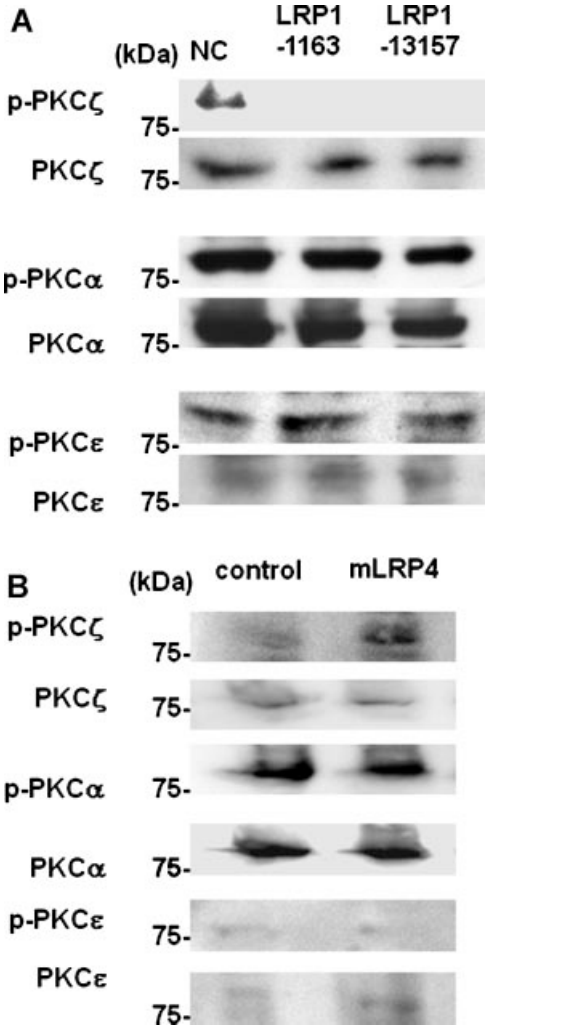

Fig. 6. A: Immunoblot analysis of the phosphorylation of PKC in HCS-2/8 cells in the presence or absence of LRP1 siRNA. By *lrp1* knockdown, phosphorylation of conventional-type PKC, PKCα, and that of the novel-type PKC, PKCε were unchanged; whereas phosphorylation of the atypical type PKC, PKCζ was decreased in chondrocytes. The position of the molecular weight marker used (75 kDa) is shown at the left of the images. NC, non-silencing scrambled siRNA as a negative control. B: Immunoblot analysis of the phosphorylation of PKC in HCS-2/8 cells with or without LRP1 overexpression. After 48 h following transfection of mLRP4 overexpression vector, the phosphorylation of PKCα and PKCε showed no change but the phosphorylation of PKCζ was increased by LRP1 overexpression in chondrocytes. The position of the molecular weight marker used (75 kDa) is shown at the left of the images.

hypertrophic differentiation, albeit the effect of the PKCζ signaling pathway may be dominant in this case (Fig. 9).

The change in chondrocytes marker gene expression appeared similar between *lrp1* knocked down HCS-2/8 cells (Figs. 2A and 3A) and *lrp1* knocked down USC cells representing hypertrophic chondrocytes (Fig. 8). These results suggest that LRP1 downregulation in hypertrophic chondrocytes specifically promotes terminal differentiation of chondrocytes.

What are the ligands that are involved in such signaling among chondrocytes? Various factors are known to play roles in endochondral ossification. For example, FGFs and their receptors negatively regulate longitudinal bone growth (De Luca and Baron, 1999). Epidermal growth factor (EGF) regulates skeletal development via an autocrine mechanism (Tajima et al., 1994). EGF stimulates chondrocyte proliferation, while inhibiting collagen synthesis and alkaline phosphatase activity (Vivien et al., 1990). Insulin-like growth factor I (IGF1) is reputed to augment longitudinal bone growth by stimulating growth plate chondrocyte proliferation and augmenting chondrocyte hypertrophy (Wang et al., 1999). However, surface plasmon resonance experiments demonstrated that basic FGF, EGF, or IGF1 did not bind to purified LRP immobilized on a sensor chip (Loukinova et al., 2002) but that platelet-derived growth factor (PDGF) did. PDGF stimulates the activation of PKCζ (Xu et al., 1996) and proliferation and differentiation of cultured chondrocytes from rat rib growth plate (Wroblewski and Edwall, 1992). Moreover, transforming growth factor β (TGFβ) also can directly bind to LRP1 (Harris-White et al., 2004; Tseng et al., 2004; Harris-White and Frautschy, 2005). During endochondral bone formation, TGFβ acts as a potent inhibitor of the terminal differentiation of epiphyseal growth plate chondrocytes. TGFβ

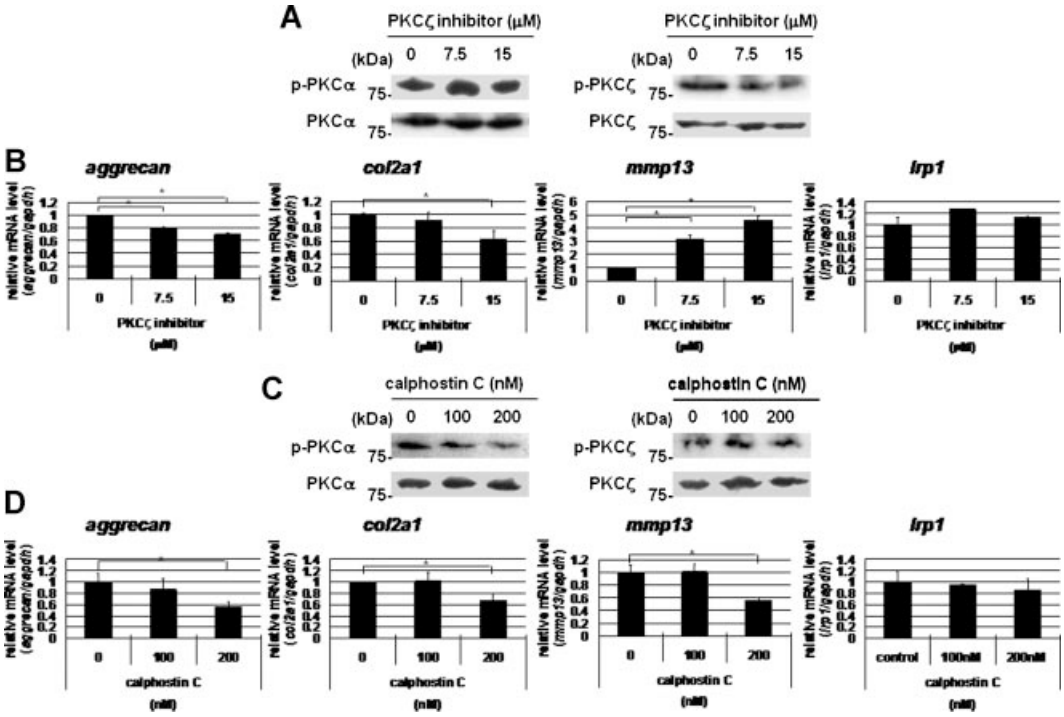

Fig. 7. Effects of PKC inhibitors on chondrocytic differentiation. A: Specific inhibition of the phosphorylation of PKCζ in HCS-2/8 cells by a PKCζ inhibitor. The phosphorylation of PKCζ was actually inhibited by the PKCζ inhibitor, whereas that of PKCα was not affected. The position of the molecular weight marker used (75 kDa) is shown at the left of the images. B: Effects on chondrocyte marker gene expression. When phosphorylation of PKCζ was selectively inhibited, the mRNA levels of *aggrecan* and *col2a1* were decreased, whereas the level of *mmp13* was significantly increased. The values represent the means ± SD. *$P < 0.05$. C: Effects of calphostin C, which inhibits the phosphorylation of all PKC subtypes except for the atypical type. The phosphorylation of PKCα was efficiently inhibited by calphostin C, whereas the p-PKCζ level was unchanged. The position of the molecular weight marker used (75 kDa) is shown at the left of the images. D: Effects of calphostin C on chondrocyte marker gene expression. When the phosphorylation of all PKC subtypes except the atypical type was inhibited, the mRNA level of *mmp13* was decreased, as well as that of *aggrecan* and *col2a1*. The values represent the means ± SD. *$P < 0.05$.

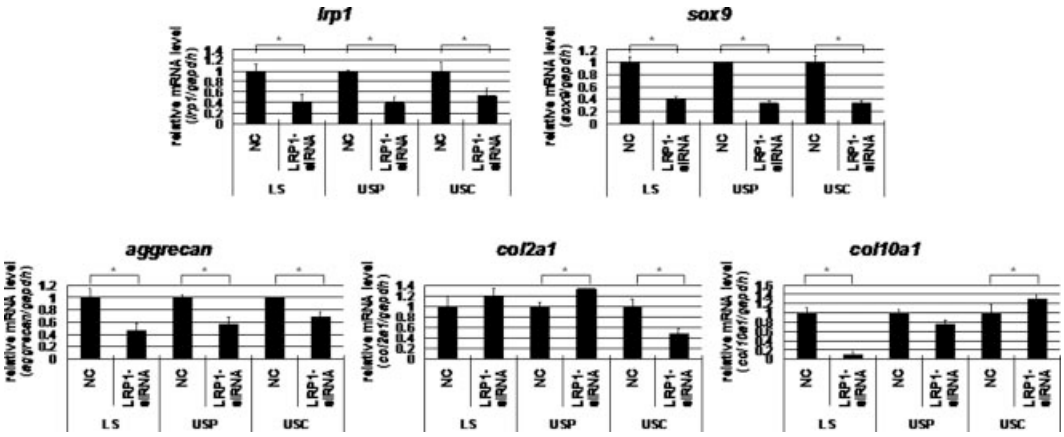

Fig. 8. *Sox9*, *aggrecan*, *col2a1*, and *col10a1* mRNA levels in *lrp1* knocked down chicken primary chondrocytes. The chondrocytes were isolated from chick sternum. LS, USP and USC cells represent resting chondrocytes, proliferating chondrocytes, and hypertrophic chondrocytes, respectively. All values represent relative ratios against NC at each differentiation stages. The expression of *lrp1* was knocked down to approximately 40–50% of the control by LRP1-siRNA in chondrocytes at all differentiation stages. Under the same conditions, the expression of *sox9* and *aggrecan* was decreased with LRP1-siRNA in chondrocytes at all differentiation stages. In contrast, decreases in *col2a1* mRNA level was seen only in the *lrp1* knocked down UCS cells. Interestingly, the expression of *col10a1* was increased only in the *lrp1* knocked down USC cells, whereas in the *lrp1* knocked down LS, it was drastically decreased, and in the *lrp1* knocked down USP cells, it remained unchanged. The values represent the means ± SD. *$P < 0.05$. NC, non-targeting siRNA as a negative control.

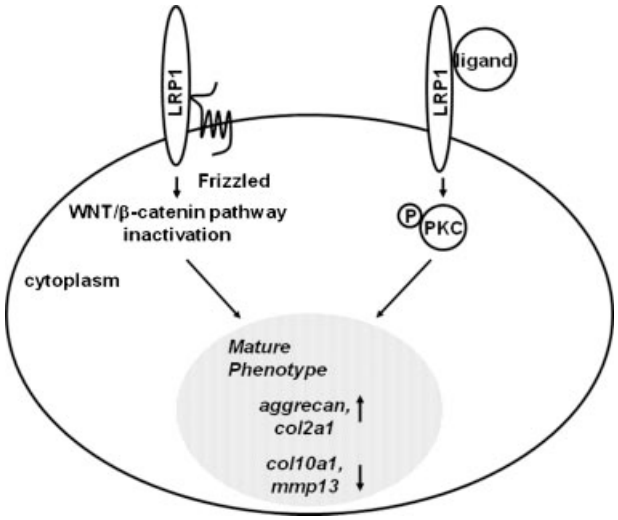

Fig. 9. Schematic representation of the role of LRP1 in maintaining mature chondrocytic phenotype. In chondrocytes, inhibition of WNT/β-catenin pathway and/or activation of PKC by LRP1 contribute to the maintenance of mature chondrocytic phenotype, which result in the enhancement of *aggrecan* and *col2a1* mRNA expression and inhibition of *col10a1* and *mmp13* mRNA expression.

also activates protein kinases, including PKC, and modulates gene expression via its delicate interaction with other signaling pathways (Li et al., 2005). Importantly, CCN2/CTGF binds directly to LRP1 (Segarini et al., 2001; Gao and Brigstock, 2003; Perbal, 2004; Yang et al., 2004). CCN2/CTGF activates PKCζ (Yosimichi et al., 2006), and plays critical roles throughout endochondral ossification (Nakanishi et al., 1997, 2000; Nishida et al., 2002; Ivkovic et al., 2003; Takigawa et al., 2003; Perbal and Takigawa, 2005) and articular cartilage regeneration (Nishida et al., 2004). Therefore, LRP1 would supposedly regulate endochondral ossification by its interaction with such ligands. Besides, based on its multiple functionality and molecular structure, we hypothesize that LRP1 may be cooperating also with other factors serving as negative modulators of chondrocyte hypertrophy.

Thus, it appears quite likely that LRP1 is a major cell-surface molecule that transduces the signals to repress the hypertrophic onset during chondrocyte differentiation. Therefore, by counterbalancing the signals that promote hypertrophy, LRP1 may play an important role in executing the endochondral ossification in a proper manner.

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