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Discoidin domain receptor 2 (DDR2) regulates proliferation of endochondral cells in mice

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ABSTRACT

Discoidin domain receptor 2 (DDR2) is a receptor tyrosine kinase that is activated by fibrillar collagens. DDR2 regulates cell proliferation, cell adhesion, migration, and extracellular matrix remodeling. The decrement of endogenous DDR2 represses osteoblastic marker gene expression and osteogenic differentiation in murine preosteoblastic cells, but the functions of DDR2 in chondrogenic cellular proliferation remain unclear. To better understand the role of DDR2 signaling in cellular proliferation in endochondral ossification, we inhibited *Ddr2* expression via the inhibitory effect of miRNA on *Ddr2* mRNA (mi*Ddr2*) and analyzed the cellular proliferation and differentiation in the prechondrocyte ATDC5 cell lines. To investigate DDR2's molecular role in endochondral cellular proliferation *in vivo*, we also produced transgenic mice in which the expression of truncated, kinase dead (KD) DDR2 protein is induced, and evaluated the DDR2 function in cellular proliferation in chondrocytes. Although the mi*Ddr2*-transfected ATDC5 cell lines retained normal differentiation ability, DDR2 reduction finally promoted cellular proliferation in proportion to the decreasing ratio of *Ddr2* expression, and it also promoted earlier differentiation to cartilage cells by insulin induction. The layer of hypertrophic chondrocytes in KD *Ddr2* transgenic mice was not significantly thicker than that of normal littermates, but the layer of proliferative chondrocytes in KD-*Ddr2* transgenic mice was significantly thicker than that of normal littermates. Taken together, our data demonstrated that DDR2 might play a local and essential role in the proliferation of chondrocytes.

1. Introduction

Discoidin domain receptor 2 (DDR2) is a receptor tyrosine kinase that is activated by fibrillar collagens, which act as its endogenous ligand [1–5]. DDR2 binds to and is activated by collagen I, II, III, V, and X, with the notable exception of basement membrane collagen IV [2,4,6–8]. DDR2 is expressed in connective tissues arising from embryonic mesoderm [9–12]. DDR2 regulates cell proliferation, cell adhesion, and migration as well as extracellular matrix remodeling [11,13–15].

We previously identified a recessive, loss-of-function allele for *Ddr2* (designated $Ddr2^{slie/slie}$) that causes dwarfism and infertility [12]. DDR2 null allele mice exhibit dwarfing with a shortening of the long bones and a reduction in body weight, which caused by a reduced proliferation rate of chondrocytes [11]. A decrease in endogenous DDR2 by shRNA represses osteoblastic marker gene expression and osteogenic differentiation in murine preosteoblastic cells [16].

The clonal cell line ATDC5 was isolated from the feeder-independent teratocarcinoma stem cell line AT805 on the basis of chondrogenic potentials in the presence of insulin [17,18]. ATDC5 cells can keep track of the multistep proliferation and differentiation process encompassing all endochondral ossification stages, from mesenchymal condensation to calcification *in vitro* [19–23]. To better understand the role of DDR2 signaling in cellular proliferation in endochondral ossification, we produced ATDC5 cell lines with stably lower *Ddr2* expression by using miRNA's inhibitory effect on *Ddr2* mRNA. We then analyzed cell proliferation and searched for differentiation in the stable mi*Ddr2*-expressed ATDC5 cell lines.

The formation of mammalian endochondral bone is controlled spatially and environmentally by many signaling molecules [24–26]. To investigate the molecular role of DDR2 in endochondral cellular proliferation *in vivo*, we also produced transgenic mice in which the expression of dominant-negative DDR2 protein is induced, and we evaluated the DDR2 function in cellular proliferation by analyzing the phenotypes. Dominant-negative DDR2 was made of kinase-dead DDR2 mutant (KD-DDR2), which is a truncated form lacking the kinase domain but retaining the extracellular and transmembrane domains [27,28].

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The purposes of this study are to investigate the molecular function of DDR2, especially in cellular proliferation in prechondrocytes, and to examine the relationship between the proliferation and differentiation of prechondrocytes through both *in vitro* and *in vivo* analysis.

2. Materials and methods

2.1. Plasmid construction of miR-RNAi

Expression plasmids of miR-RNAi for *Ddr2* mRNA (mi*Ddr2*) were designed using the Invitrogen web site, and cloning them into the pcDNA6.2-GW/EmGFP-miR vector (BLOCK-iT Pol II miR RNAi expression vector, Invitrogen, La Jolla, CA, USA). The oligonucleotides used were as follows: mi*Ddr2*, 5′-TGCTGTAAGCATGGGATCATAGGTGGGTTTTGGCCACTGACTGACCCACCTATTCCCATGCTTA-3′ (sense) and 5′-CCTGTAAGCATGGGAATAGGTGGGTCAGTCAGTGGCCAAAACCCACCTATGATCCCATGCTTAC-3′ (antisense).

2.2. Cell culture and transfection

ATDC5 cells were maintained in a medium consisting of a 1:1 mixture of DMEM and nutrient F-12 ham (Sigma, St. Louis, MO, USA) containing 5% fetal bovine serum (FBS; Bio West, Nuaillé, France), 10 μg/ml human transferrin (Sigma), and 3×10^{-8} M sodium selenite (Sigma). For the induction of chondrogenesis, the cells were cultured in maintenance medium supplemented with 10 μg/ml bovine insulin (Sigma) at 37 °C in a humidified atmosphere of 5% CO2 in air as previously described [17,18]. The medium was replaced every 3 days. To determine the growth curve, ATDC5 cells were placed in 12-well microplates (Iwaki, Tokyo, Japan) and cultured in maintenance medium. The cells were then rinsed with PBS, treated with trypsin, and counted with a hemocytometer for 4 days. pcDNA6.2-GW/EmGFP-miR (BLOCK-it) plasmids were transfected using Fugene 6 Transfection Reagent (Roche, Basel, Switzerland). Stable lines were obtained by blasticidin selection.

2.3. Alcian blue staining

At day 15 of culture, ATDC5 cells were rinsed with PBS and fixed with 95% methanol for 20 min. They were stained with 0.1% Alcian blue 8GX (Sigma) in 0.1 M HCl overnight [18].

2.4. Construction of the transgene for mice

The fusion gene for microinjection was constructed by inserting a 1.6 kb *Not*I restriction fragment bearing KD-*Ddr2*, which was designed based on [28], into a unique *Not*I site of the α2 (XI) collagen gene-based expression vector, 742*lacZ*Int. This vector contains the *Col11a2* promoter, a SV40 RNA, the β-galactosidase reporter gene, the SV40 polyadenylation signal, and the first intron sequence of *Col11a2* as an enhancer [29]. An insulator sequence was inserted into the upstream from the *Col11a2* promoter region to integrate transcriptional efficiency [30].

2.5. Production of transgenic mice

The plasmid pInsulator-*Col11a2*-KD-*Ddr2* was digested with *Eco*RI and *Sca*I to release the insert. Transgenic mice were produced by microinjecting several hundred molecules of the DNA fragment into the pronuclei of fertilized eggs from F1 hybrid mice (C57BL/6 × DBA) as describe elsewhere [31]. The transgenic mice and their progenies were screened for the presence of transgene by PCR assays of genomic DNA extracted from the tail. Genomic DNA was amplified by transgene-specific PCR using primers derived from the SV40 intron region (5′-GCCACCATGATCCCCATTCCCAGAATGC-3′) and from the mouse *Ddr2* cDNA (5′-TCACAGTTTCCTGGGGAACTCTTC-3′) (arrowheads).

2.6. Quantitative RT-PCR

For quantitative RT-PCR, total RNAs were isolated from ATDC5 cell lines of plasmids of miR-*Ddr2* transfect or nontransfect using the SV Total RNA Isolation system (Promega, Madison, WI, USA) and isolated from cartilage of KD-*Ddr2* transgenic and wild-type mice using TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA with a Superscript III first-strand synthesis kit for RT-PCR (Invitrogen). Table 1 lists the primers used to detect *Ddr2*, *Runx2*, KD-*Ddr2,* and β-2 microglobulin. Quantitative PCR was performed using a Light Cycler rapid thermal cycler system (Roche) in a 20 μl volume with 3.4 μM $MgCl_2$, 1 μM each of the forward and reverse primers, and a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA). The amplification protocol consisted of 95 °C for 10 s followed by 45 cycles at 95 °C for 10 s and 65 °C for 5 s.

2.7. Histology

Bones were collected, decalcified, and placed into Bouin's fixative overnight. The tissues were dehydrated, embedded in paraffin, cut into 5 μm sections, and processed for hematoxylin and eosin staining. Areas of proliferative cartilage and the length of hypertrophic chondrocytes were counted on serial sections and measured using NIH image software (National Institutes of Health, Bethesda, MD, USA).

2.8. Staining of the skeleton

Mice were dissected and fixed in 95% ethanol overnight, and then stained with Alcian blue followed by Alizarin Red S solution (Sigma) according to standard protocols.

2.9. Western blotting

Total protein was isolated from cartilage cells of wild-type and KD-*Ddr2* transgenic mice. The protein samples were separated by electrophoresis using a 10% sodium dodecyl sulfate polyacrylamide gel. Proteins were transferred to a polyvinylidene fluoride membrane (AE-6660; Atto, Tokyo, Japan). After the membrane was blocked with 3% skim milk for 20 min, it was incubated with anti-DDR2 polyclonal antibody (AF2538; R&D Systems, Minneapolis, MN, USA) or tubulin α monoclonal antibody (T9026; Sigma) and then processed with 1:5000 diluted anti-goat IgG antibody conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories, West Grove, PA, USA) or with 1:5000 diluted anti-mouse IgG antibody conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories).

2.10. Statistical analysis

To detect significant statistical differences, nonparametric Mann–Whitney or parametric Student's *t*-tests were used to compare experimental groups.

3. Results

3.1. Ddr2 expression in the miDdr2-transfected ATDC5 cell lines

To understand the biological functions of *Ddr2*, we then transfected precartilage cell line ATDC5 with miRNA for the *Ddr2*

Table 1
Oligonucleotide primer sequences used for RT-PCR or qPCR.

	Forward primer	Reverse primer
Ddr2	CACCACCTATGATCCCATGC	TGACTGTCATTTCATCATCCAG
Runx2	CAGCGGACGAGGCAAGAGTTTCAC	CTGCCTGGGATCTGTAATCTGACT
KD-*Ddr2*	TGTCCTGGGAAAGCATCTTG	CCTTGGTCTCGGAAGAACTCTC
β-2 Microglobulin	CCTGGTCTTTCTGGTGCTTG	TGCTGAAGGACATATCTGACATC

Ddr2 discoidin domain receptor family, member 2; KD-*Ddr2* knockdown discoidin domain receptor family, member 2.

expression plasmid (mi*Ddr2*) and produced stable cell lines in order to examine the effect of downregulation of *Ddr2* expression (Figs. S1 and 1A). After Blasticidin selection, we established three stable cell lines: mi*Ddr2* line 1, 2, and 3.

Ddr2 mRNAs were assessed by quantitative real-time PCR (qPCR) using triplicate samples from different ATDC5 cell lines after 24 h from transfection in transfected cells. The results showed that the relative ratio of each cell line to the negative control-transfected cells decreased *Ddr2* expression drastically; especially, endogenous *Ddr2* expression in mi*Ddr2* line 3 was around one-third that with in nontransfected cells (Fig. 1B).

3.2. Cell proliferation and differentiation in the miDdr2-transfected ATDC5 cell lines

To determine the effect of the reduction of endogenous *Ddr2* expression in ATDC5 cells, we first compared the rates of cellular proliferation in negative control-transfected cells and transfected cell lines. We compared the time course of cell proliferation determined by the growth curve for 4 d in transfected ATDC5 cells, and found significantly greater numbers of mi*Ddr2*-transfected cells than mock cells (Fig. 2A).

We processed the differentiation induction of ATDC5 cell lines to cartilage cells with bovine insulin and found many more Alcian blue-positive cells in mi*Ddr2* line 3 compared with mock cells after 15 d culture (Fig. 2B).

3.3. Alteration of Ddr2-related Runx2 in the miDdr2-transfected ATDC5 cell lines

To understand how mi*Ddr2* stable cell lines caused the DDR2 decrement, as well as the proliferation and differentiation of the precartilage cells, we analyzed the expression of *Runx2,* a gene that DDR2 regulates, by qPCR. The results showed that the relative ratio of mi*Ddr2* cell lines to mock cells decreased in *Runx2* as the ratio of *Ddr2* suppression decreased (Fig. 2C).

3.4. Generation of cartilage-specific expressed KD-Ddr2 transgenic mouse lines

To elucidate the DDR2 function of endochondral proliferation and ossification *in vivo*, we ligated the *Ddr2* partial cDNA that encodes the receptor region to *Col11a2* promoter sequences and the insulator sequence to construct the transgene *Col11a2*-KD-*Ddr2* (Fig. 3A). The KD-*Ddr2* transgenic lines appeared normal. Five transgenic founders derived from microinjections were confirmed by the genomic PCR primers shown in Fig. 3A (arrowheads), and transgenic offspring with mild phenotypes generated from three transgenic founders (lines 1, 2, and 4). qPCR using total RNAs extracted from costal cartilage showed that the number of KD-*Ddr2* mRNAs of transgenic line 4 was 17-fold that of the littermates, but those of line 1 and 2 were less than that of the littermates (Fig. 3B). Immunoblotting demonstrated the expression of a KD-*Ddr2* protein in line 4 of the cartilage of KD-*Ddr2* transgenic mice (Fig. 3C, arrowhead). Immunohistochemistry using anti-DDR2 antibody showed another signal for original DDR2 proteins in the cartilage of KD-*Ddr2* transgenic mice (Fig. 3C).

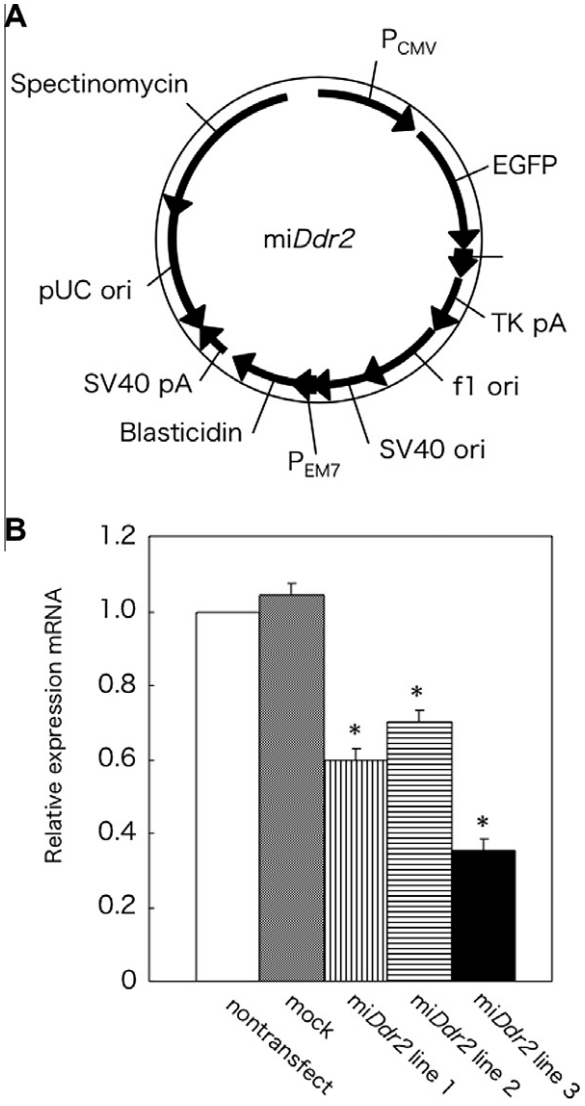

Fig. 1. Construction and expression of endogenous *Ddr2* in mi*Ddr2*-transfected ATDC5 cell lines. (A) Plasmid structure of miRNA in the *Ddr2* expression system for the production of stable cell lines to eliminate *Ddr2* expression. (B) Relative expression levels of endogenous *Ddr2* in mi*Ddr2*-transfected ATDC5 cell lines (mi*Ddr2* cell lines) as determined by qPCR. $n = 3$, $^{*}p < 0.05$.

3.5. Phenotypes in cartilage-specific expressed KD-Ddr2 transgenic mice

The transgenic mice (KD-*Ddr2* line 4) tended to weigh less than their littermates, but the differences were not significant from 2 to 6 weeks of age (Fig. S2). Because the transgene was highly expressed in cartilage, we examined the skeleton at 6 weeks of age.

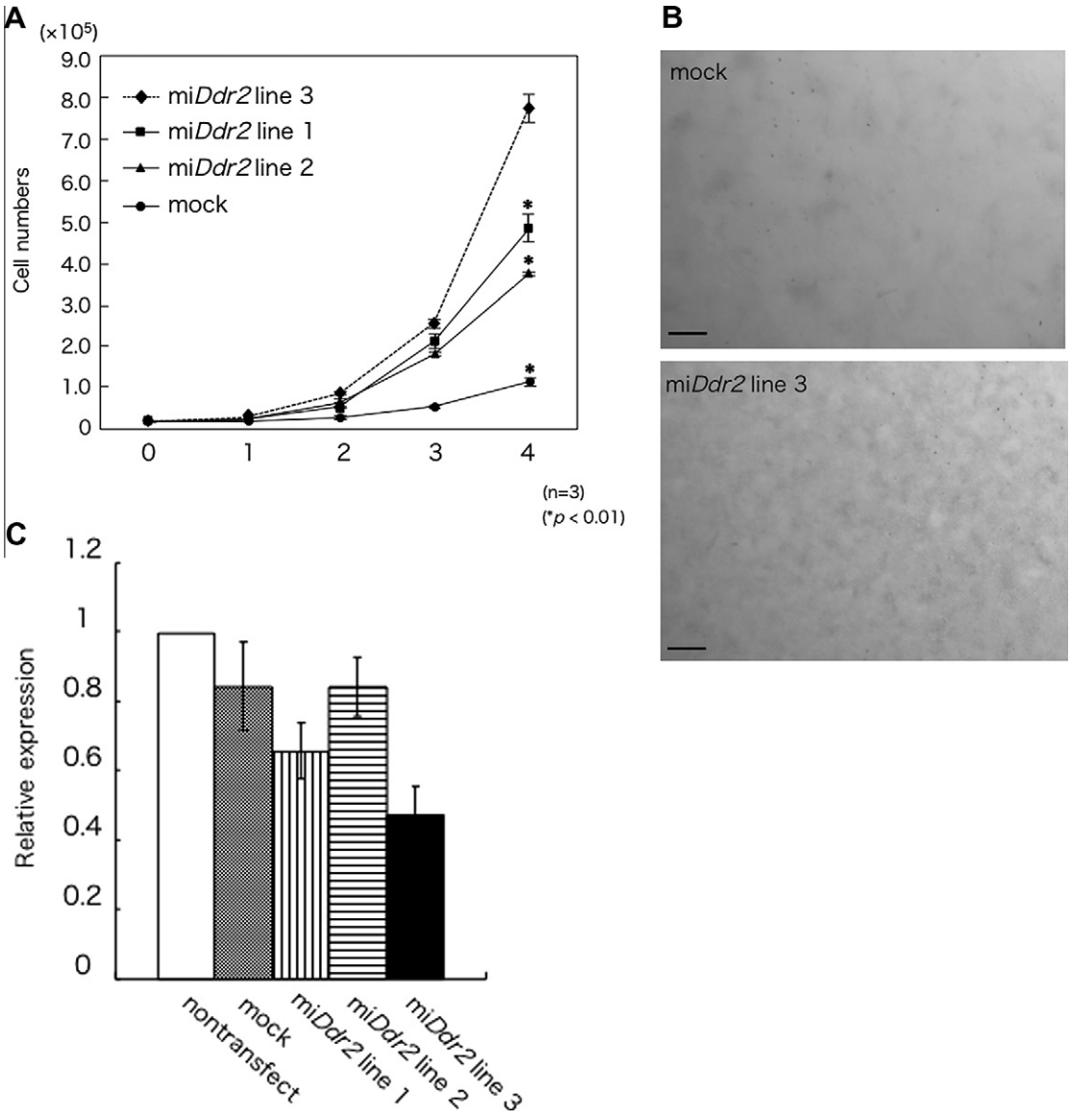

Fig. 2. Effects of cell proliferation and differentiation in the mi*Ddr2*-transfected ATDC5 cell lines. (A) Growth curves of mi*Ddr2* cell lines. Total cell numbers were determined for three wells at each time point. All mi*Ddr2*-transfected cells were significantly increased in number compared with mock cells $n = 3$, $^{*}p < 0.01$. (B) Induction of cartilage nodules in the presence of bovine insulin. mi*Ddr2* (line 3) cells were cultured for 15 days and stained with 0.1% Alcian blue. There are more Alcian blue-positive cells in mi*Ddr2* line 3 than mock cells. Scale bar: 1 mm. (C) Relative expression levels of *Runx2* of mi*Ddr2* cells. The relative ratio of mi*Ddr2* cells to mock cells decreased in *Runx2*.

The size and shape of each skeleton component stained with Alcian blue and Alizarin red of transgenic mice were the same as those of their normal littermates (Fig. S3). We also examined endochondral bone formation by histological analysis. At 3 weeks of age, the layer of proliferative chondrocytes in KD-*Ddr2* transgenic mice was significantly thicker than that of normal littermates (Fig. 4A and B).

4. Discussion

Endochondral proliferation and ossification are very important but complex processes for bone and skeletal formation, which is controlled by the spatial and temporal environments. To date, little is known about the exact role of extracellular matrix molecules, specifically collagen and its receptors, in endochondral ossification. DDR2 null allele dwarf mice have chondrocyte abnormalities [11], along with four different mutations in the DDR2 gene in the patients affected with the rare autosomal-recessive spondylo-meta-epiphyseal dysplasia, short limb-abnormal calcification-type syndrome (SMED-SL) [32,33]. However, the details of DDR2 regulation about ligands, molecules, receptors, and intracellular signaling pathways of the extracellular matrix involved in endochondral proliferation and ossification are not well known.

Here we used the ATDC5 cell line as a model of endochondral proliferation and ossification. The mi*Ddr2*-transfected ATDC5 cell lines are useful models of precursor endochondral cells because we found that they retained normal differentiation ability. DDR2 reduction induced by miRNA designed for *Ddr2* finally promoted cellular proliferation in proportion to the decrease in the ratio of *Ddr2* expression, indicating that DDR2 might play a negative role in the proliferation of chondrocytes. We also identified that mi*Ddr2*-transfected ATDC5 cell lines showed earlier differentiation to cartilage cells by insulin induction compared with mock cells after 15 d culture. This study suggested that DDR2 might play an inhibitory regulatory role on chondrocyte differentiation. Taken together, our results indicate that the decrease in DDR2 leads to cellular proliferation due to the direct signal reduction of DDR2,

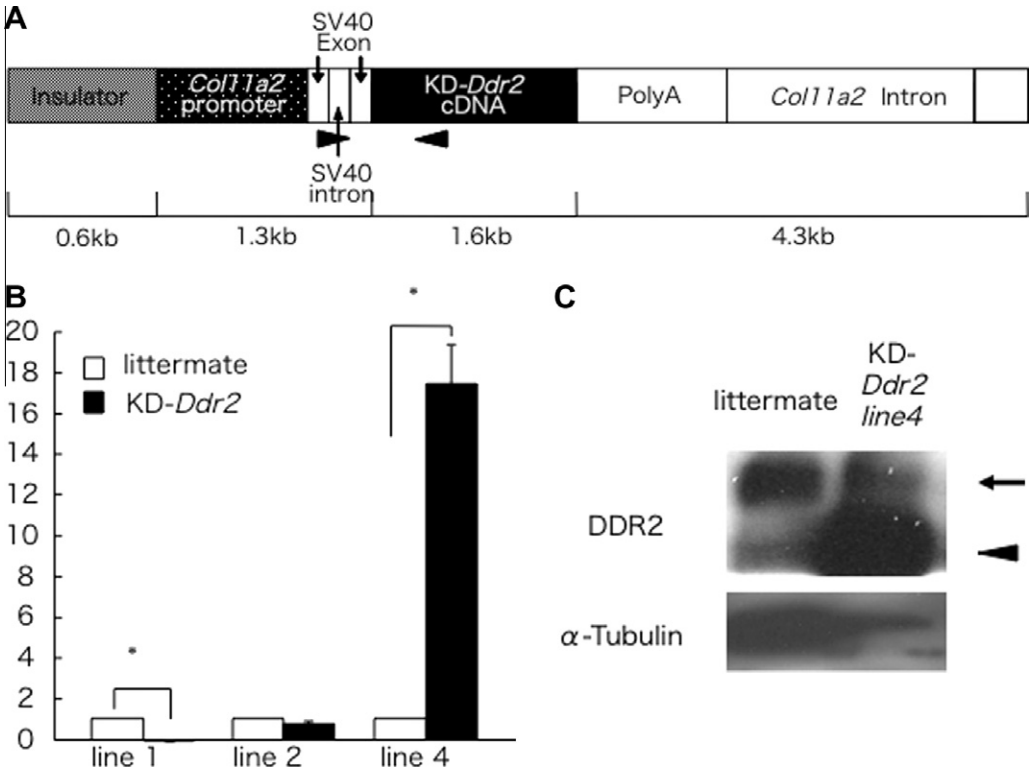

Fig. 3. Production of kinase-dead (KD) *Ddr2* transgenic mouse lines. (A) Structure of *Col11a2* promoter/KD-*Ddr2* fusion gene used for the production of transgenic mice. Mouse KD-*Ddr2* cDNA was introduced into a eukaryotic expression plasmid between the *Col11a2* promoter sequence and the polyadenylation site. The segment was microinjected into fertilized ova. The relative positions of the primers used (see Section 2) are indicated. (B) Relative expression levels of KD-*Ddr2* transgenic mouse lines at 6 weeks of age. The expression of KD-*Ddr2* mRNAs of transgenic line 4 was 17-fold that of littermates. (C) Protein expression of KD-*Ddr2* transgene. Immunohistochemistry using anti-DDR2 antibody showed a signal for endogenous DDR2 protein (arrow) and kinase-dead short DDR2 protein (arrowhead) in cartilage of KD-*Ddr2* transgenic mice. Immunoblotting for α-tubulin was performed as a positive control. Positive control consisted of lysates from testis of wild-type mice.

not to the loss of function of endochondral cells in mi*Ddr2*-transfected ATDC5 cells.

DDR2 activates *Runx2* through p38 MAPK to promote osteoblast differentiation [34]. We demonstrated that reduced expression of DDR2 may lead to the subsequent downregulation of *Runx2* in mi*Ddr2*-transfected ATDC5 cells in proportion to the expression reduction level of *Ddr2*. This result indicated that *Runx2* could be a downstream molecule of the *Ddr2* signal pathway in endochondral cells. DDR2 acts as a proliferative regulator to fibroblast and hepatic stellate cells through the extracellular matrix [11,13,27]. These results and our results may contradict each other, but there are two possibilities for explaining the discrepancy. First is the decrement of *Runx2* expression in mi*Ddr2*-transfected ATDC5 cells. *Runx2* is essential for chondrocyte maturation. It regulates limb growth by organizing chondrocyte maturation and proliferation and by repressing adipogenic differentiation and maintaining the cellular character of chondrocytes *in vitro* [35–37]. In mi*Ddr2*-transfected ATDC5 cells, the reduction of *Runx2* expression according to the DDR2 decrement resulted in consecutively proliferative cellular induction by maintaining the undifferentiated status of chondrocytes. Secondly, the reduction of DDR2 expression is systemic in *Ddr2* knockout mice and spontaneous mutant mice, and dwarfism also can be provoked by reasons other than only the decrease in the ability of chondrocyte cellular proliferation. In fact, DDR2 deletion yielded many phenotypes, including pituitary and reproductive functions [12]. Further research for DDR2 should be conducted, as our results did not reflect the temporal or partial ossification situation because osteoblasts and osteoclastic cells are dimensionally involved in endochondral ossification [24,25].

Next, we tried to specifically inhibit DDR2 function in endochondral proliferation and ossification *in vivo*. We used competitive inhibition with KD-*Ddr2* overexpression specifically in cartilage cells, because it is generally difficult to regulate genes by miRNA *in vivo*. We constructed a transgenic vector using an insulator sequence to stabilize the activity of the transgene expression [30]. Body size and skeleton length of KD-*Ddr2* overexpressed mice were not significantly different compared with the littermates. On the other hand, the layer of hypertrophic chondrocytes in KD-*Ddr2* transgenic mice was not significantly thicker than that of normal littermates, but the layer of proliferative chondrocytes in KD-*Ddr2* transgenic mice was significantly thicker than that of normal littermates. This cellular proliferation is similar to the phenotype of mi*Ddr2*-transfected ATDC5 cells *in vitro*, which indicated that DDR2 might have various molecular functions to regulate the proliferation of chondrocytes rather than the differentiation of hypertrophic chondrocytes.

The phenotypes of KD-*Ddr2* transgenic mice were less severe than expected from the results of mi*Ddr2*-transfected ATDC5 cells, which indicated that DDR2 might play important roles in endochondral ossification but neither critical nor essential roles in determining total body and skeleton size.

The greater thickness of the proliferative chondrocyte layer in KD-*Ddr2* transgenic mice could be an anomaly in the reduction of chondrocyte proliferation in DDR2-deficient mice [11]. This difference could come from regional and functional differences in DDR2 shortage; these differences indicate that DDR2 could have various roles in different conditions, either systemically or locally.

To summarize, we investigated the effects of both the decrement of DDR2 in mi*Ddr2*-transfected ATDC5 cells *in vitro* and in DDR2 dominant-negative overexpressed transgenic mice, and the results suggested that DDR2 might regulate proliferative chondrocytes.

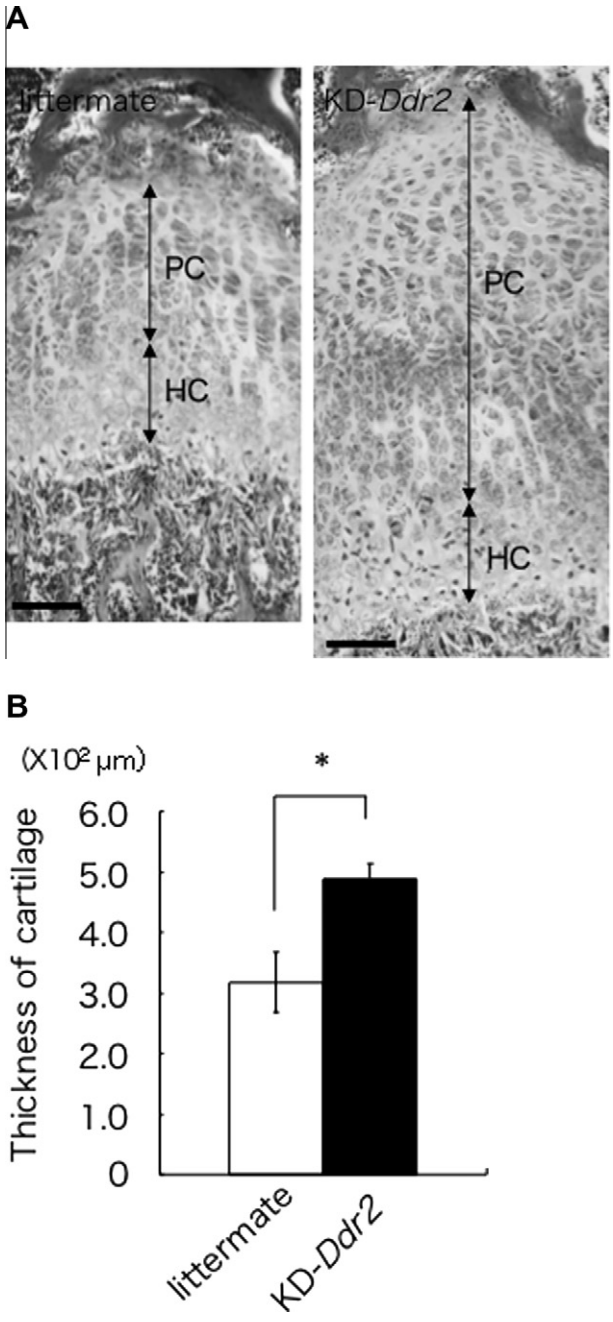

Fig. 4. Phenotypes of endochondral bone formation in cartilage-specific expressed KD-*Ddr2* transgenic mice. (A) Serial sections of hypertrophic chondrocytes (HC) and proliferative chondrocytes (PC) were stained with hematoxylin and eosin in KD-*Ddr2* transgenic mice (line 4) and littermates. (B) The heights of the zone of hypertrophic chondrocytes were measured and compared between littermates and transgenic mice at 3 weeks of age. The layer of proliferative chondrocytes in KD-*Ddr2* transgenic mice was significantly thicker than that of normal littermates. Scale bar: 100 μm.

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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.2012.09.106.

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