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Retinoic Receptor Signaling Regulates Hypertrophic Chondrocyte-specific Gene Expression

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Abstract. *Background/Aim: Retinoid signaling is important for the maturation of growth-plate chondrocytes. The effect of retinoid receptor gamma (RARγ) signaling on the expression of genes in hypertrophic chondrocytes is unclear. This study investigated the role of RARγ signaling in regulation of hypertrophic chondrocyte-specific genes. Materials and Methods: The gene expression in mouse E17.5 tibial cartilage was examined by in situ hybridization analysis. Real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting were used for analysis of mRNA and phosphorylated mitogen-activated protein kinase (MAPK). Results: mRNA expression of Rarg and connective tissue growth factor (Ccn2) was detected in maturing chondrocytes throughout the cartilaginous skeletal elements. In chondrogenic ATDC5 cells, an RARγ agonist induced the gene expression of type-X collagen (Col10A1), transglutaminase-2 (Tg2), matrix metalloproteinase-13 (Mmp13), and Ccn2 mRNA, whereas a retinoic acid pan-agonist suppressed RARγ agonist-stimulated gene expression. Phosphorylated extracellular signal regulated-kinases (pERK1/2), p-p38, and phosphorylated c-Jun N-terminal kinase (pJNK) MAPK were time-dependently increased by RARγ agonist treatment. Experimental p38 inhibition led to a severe drop in the RARγ agonist-stimulated expressions of Col10A1, Tg2, Mmp13, and Ccn2 mRNA. Conclusion: RARγ signaling is required for the differentiation of hypertrophic chondrocytes, with differential cooperation with p38 MAPK.*

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Skeletal growth is a tightly controlled process of growth plates that involves the proliferation and differentiation of chondrocytes (1) through the process of chondrocyte proliferation and the production of a matrix consisting mainly of type-II collagen and a large proteoglycan, aggrecan. When proliferative chondrocytes differentiate into hypertrophic chondrocytes, the extracellular matrix is replaced by type-X collagen (COL10A1).

Numerous studies have contributed to the characterization of the precise interplay of diverse factors that regulate hypertrophic chondrocyte differentiation, extracellular matrix (ECM) degradation, and bone formation and remodeling (2-7). Among these factors, cellular communication network factor 2 (CCN2), also known as connective tissue growth factor, is specifically expressed in hypertrophic chondrocytes, and is important for matrix remodeling, osteoclastogenesis, and angiogenesis in endochondral ossification (2, 3). *Ccn2*-knockout mice exhibited skeletal defects that included changes in chondrocyte proliferation and matrix gene expression (2). CCN2 seems to be a significant effector molecule in skeletogenesis, and appears

to play particularly important roles in the terminal phases of chondrocyte maturation and endochondral ossification, phases that are also particularly sensitive to retinoid signaling (8). Several matrix metalloproteinases (MMPs), notably MMP13, are also crucial for endochondral ossification, catalyzing the degradation of collagen and aggrecan (5). Mice lacking MMP13 had severely impaired endochondral bone characterized by diminished ECM remodeling, prolonged chondrocyte survival, delayed vascular recruitment, and defective trabecular bone formation (6).

Retinoids are important regulators of the differentiation and cell proliferation in skeletal development, and they have been detected at higher concentrations in hypertrophic chondrocytes (9). Retinoids exert their effects by the modulation of gene expression by two distinct classes of nuclear receptors, the retinoic acid receptors (RARα, -β and-γ) and the retinoid X receptors (RXRα, -β and-γ), and an analysis of the effects of single and double RAR gene ablations in a murine model revealed compelling evidence that RARs are required for skeletal growth, matrix homeostasis, and growth plate function in postnatal mouse (10). RARγ has been demonstrated to be expressed specifically in the growth plate from the proliferative zone to the hypertrophic zone of chondrocytes (11). These and other studies have provided strong evidence that retinoids are involved in, and are required for, chondrocyte maturation. However, RARγ downstream effectors and the mechanisms of the signaling action in those processes remain largely unclear. The data that we obtained in the present study provide novel insights regarding the role of RARγ signaling in regulation of hypertrophic chondrocyte-specific genes.

Materials and Methods

In situ hybridization. Serial paraffin tissue sections of C57Bl/6 mouse E17.5 tibial cartilage were pre-treated with 10 μg/ml proteinase K (Sigma) for 10 min at room temperature, post-fixed in 4% paraformaldehyde, washed with phosphate-buffered saline (PBS) containing 2 mg/ml glycine, and treated with 0.25% acetic anhydride in triethanolamine buffer (12). Sections were hybridized with antisense or sense ^{35}S-labeled probes (approximately 1×10^6 disintegrations/min (dpm)/section) at 50˚C for 16 h. Mouse cDNA clones included: *Ccn2* (nt. 357-1180 and full length; NM_001901); matrix metalloproteinase-13 (*Mmp13*, nt. 11-744; NM_008607); collagen x (*Col10A1*) (nt. 1302-1816; NM009925); *Rarg* (nt. 1673-2628; NM_011244) and collagen II (*Col2A1*) (nt. 1095-1344; X57982). After hybridization, slides were washed with 2× saline-sodium citrate (SSC) containing 50% formamide at 50˚C, treated with 20 μg/ml RNase A for 30 min at 37˚C, and washed three times with 0.1× SSC at 50˚C for 10 min/wash. Sections were dehydrated with 70, 90, and 100% ethanol for 5 min/step, coated with Kodak NTB-3 emulsion (Carestream Health, Inc., Rochester, NY, USA) diluted 0.8:1 with water, and exposed for 10 to 14 days. Slides were developed with Kodak D-19 at 20˚C and stained with hematoxylin. Dark and bright field images for radiolabeled riboprobes of each cDNA clone were captured using a digital camera.

Cell culture. A murine chondrogenic cell line, ATDC5, was purchased from the RIKEN Cell Bank (Tsukuba Science City, Japan). ATDC5 cells were cultured at a density of 1×10^4 cells/cm^2 in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (GIBCO/BRL, Gaithersburg, MD, USA) containing 5% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), followed by replacement with DMEM/F12 containing 5% FBS, 10 μg/ml human recombinant insulin (Wako Pure Chemical, Osaka, Japan), 10 μg/ml transferrin (Roche Diagnostics, Mannheim, Germany) and 3×10^{-8} M sodium selenite (Sigma) for the promotion of cell differentiation, and subsequent culture at 37˚C for different periods up to 12 days under 5% CO_2. RNA was extracted from cultured cells when the ATDC5 cells became confluent (4 days after plating) and was then extracted every 2 days after confluence.

Day-10 cultures of ATDC5 cells were treated with 100 nM all-*trans*-retinoic acid (ATRA; Sigma St Louis, MO, USA); 100 nM RARγ-selective agonist AGN204647 (10), 100 nM RARα-selective agonist AGN195183 (13), the 100 nM RAR inverse agonist AGN194310 (10), 20 μM of selective inhibitor of extracellular signal regulated-kinases (ERK1/2) kinase PD98059 (Calbiochem, La Jolla, CA, USA), 20 μM of selective inhibitor of p38 kinase SB203580 (Calbiochem), 20 μM of selective inhibitor of c-Jun *N*-terminal kinase (JNK) SP60012 (Calbiochem), or combinations of these agents for 24 h.

Immunoblot analysis. An immunoblot analysis for mitogen-activated protein kinase (MAPK) and activated MAPK by using cell lysates from the experimental cultures. The cells were lysed in an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 1% NP-40, 10 mM NaF, 100 mM leupeptin, 2 mg/ml aprotinin, and 1 mM phenylmethyl sulfonyl fluoride). The lysates were centrifuged at 16,000 × *g* for 20 min at 4˚C, and the protein concentrations in the supernatant were determined by a BCA assay. A 50-μg sample of each lysate was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nylon membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membrane was incubated with primary and secondary antibodies according to the ECL chemiluminescence protocol (RPN2109; Amersham Biosciences, Buckinghamshire, UK) to detect secondary antibody binding. Antibodies to ERK1/2, phosphorylated (p)ERK1/2, p38, p-p38, JNK, p-JNK, activating transcription factor 2 (ATF2), and p-ATF2 were purchased from Cell Signaling Technology (Danvers, MA, USA) and used at 1:200 dilution. Horseradish peroxidase-conjugated anti-mouse, rabbit, or goat immunoglobulins (IgGs) were used as the secondary antibodies at 1:1,000 dilution.

p38 MAPK assay. Cell extracts were incubated overnight with immobilized p38 MAPK (Thr180/Tyr182) monoclonal antibody (Cell Signaling Technology). A kinase reaction was performed in the presence of 100 μM of cold ATP (Cell Signaling Technology) and 2 μg of ATF2 fusion protein (Cell Signaling Technology). The phosphorylation of ATF2 at Thr71 was measured by western blotting using phospho-ATF2 (Thr71) antibody (Cell Signaling Technology).

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from ATDC5 cells by using TRIZOL reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's recommendations, and the transcripts were analyzed

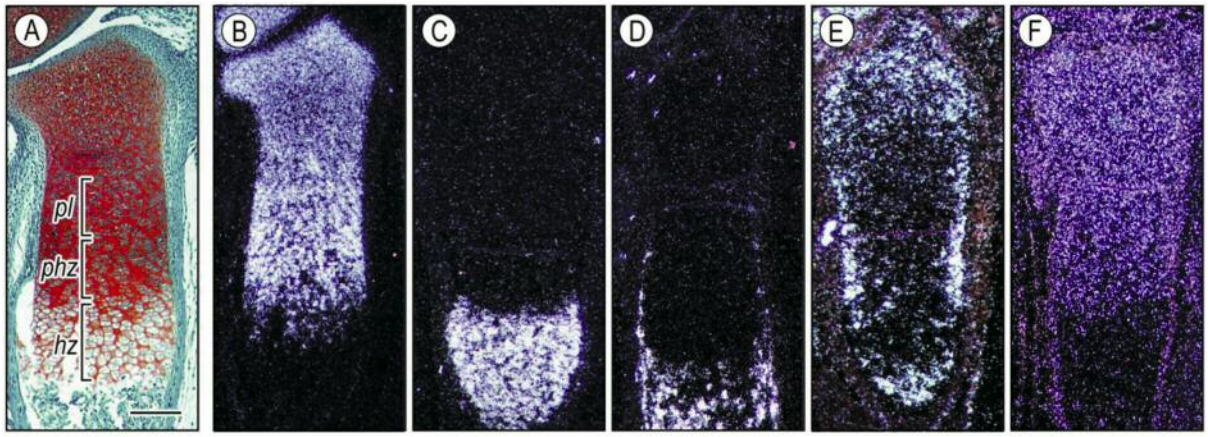

Figure 1. *In situ hybridization analysis of gene expression in mouse E17.5 tibial cartilage. Longitudinal serial sections were processed for histochemical proteoglycan staining with safranin O (A), in situ hybridization with 35S-UTP riboprobes encoding type-II collagen (B), type-X collagen (C), matrix metalloproteinase-13 (D), connective tissue growth factor (Ccn2) (E) and retinoid receptor gamma (Rarg) (F). Note the strong expression of Rarg and Ccn2 in maturing chondrocytes and up-regulation of Ccn2 transcripts in hypertrophic chondrocytes in tibial growth plates. pl: Proliferating chondrocyte zone, phz: pre-hypertrophic chondrocyte zone, and hz: hypertrophic chondrocyte zone. Scale bar in A for A-F: 75 μm.*

by real-time RT-PCR. The primers used were the following: 5'-TGAGAGAGGCGAATGGAACG-3' (forward) and 5'-TTCTGCCCGAGGGTTCTAGC-3' (reverse) for aggrecan (*Agr*); 5'-AAGAACAGCATCGCCTACCT-3' (forward) and 5'-CTTACCAGTGTGTTTCGTGC-3' (reverse) for *Col2*; 5'-TCTGTACAATAGGCAGCAGC-3' (forward) and 5'-TAGGCGTGCCGTTCTTATAC-3' (reverse) for *Col10A1*; 5'-TTCCGAGTATGACT-3' (forward) and 5'-GCCAATATCAGTCGGGAACA-3' (reverse) for TG-2; 5'-CCAGACTATGGACAAAGATT-3' (forward) and 5'-ATGCGATTACTCCAGATACT-3' (reverse) for *Mmp13*; 5'-CCAATGACAATACCTTCTGC-3' (forward) and 5'-GAAAGCTCAAACTTGACAGG-3' (reverse) for *Ccn2*; and 5'-TGAACGGGAAGCTCACTGG-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse) for glyceraldehyde 3-phosphate dehydrogenase Gapdh. The real-time RT-PCR was performed with a Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany) in Light Cycler capillaries with a commercially available master mix containing Taq DNA polymerase and SYBR-Green I deoxyribonucleoside triphosphate (Light Cycler DNA Master SYBR-Green I; Roche Molecular Biochemicals). After the addition of primers (final concentration: 10 μM), $MgCl_2$ (3 mM), and template DNA to the master mix, 65 cycles of denaturation (95˚C for 15 s) and extension (60˚C for 45 s) were performed. After the completion of PCR amplification, a melting curve analysis was conducted.

Statistical analysis. The data were analyzed using the unpaired Student's *t*-test for the comparisons of two groups and a one-way analysis of variance (ANOVA) for the analysis of repeated multiple group comparisons. Results are expressed as the mean±standard deviation (SD). *p*-Values of less than 0.05 were considered significant.

Results

Expression of Rarg and Ccn2 in developing tibial cartilage. In a first set of experiments, we determined the expression patterns of *Rarg* and *Ccn2* in mouse developing cartilage. Longitudinal serial sections of tibial cartilage from E17.5 mice were processed for *in situ* hybridization using 35S-labeled antisense riboprobes. Tibial growth plates exhibited distinctive morphological characteristics and chondrocyte organization. They displayed prospective articular chondrocytes at their epiphyseal ends and long growth plates with well-define proliferative, prehypertrophic and hypertrophic zones occupying the metaphysis and diaphysis (Figure 1A). In addition, the diaphysis underwent the process of endochondral ossification and was surrounded by intramembranous bony collar. *In situ* hybridization on serial sections of tibial cartilage revealed that while Col2 was strongly expressed throughout most chondrocytes except for hypertrophic chondrocytes, *Col10A1* was markedly up-regulated in the hypertrophic chondrocytes (Figure 1B and C, respectively). *Mmp13* transcripts were confined to the post-hypertrophic chondrocytes, and cells residing in the primary spongiosa and intramembranous bony collar (Figure 1D). Expression of *Ccn2* and *Rarg* was detected in maturing chondrocytes throughout the cartilaginous skeletal elements, indicating a significant similarity between topographical distribution between the two molecules (Figure 1E and F, respectively). Importantly, *Ccn2* expression became abundant in the hypertrophic zone of growth plate. These data clearly indicate that expression of *Rarg* and *Ccn2* detected in maturing chondrocytes precedes chondrocyte hypertrophy characterized by *Col10A1* and *Mmp13* expression.

RARγ agonist-dependent modulation of Col10A1, Tg2, Mmp13 and Ccn2 gene expression. Confluent ATDC5 cells were switched to low-serum-containing medium and treated

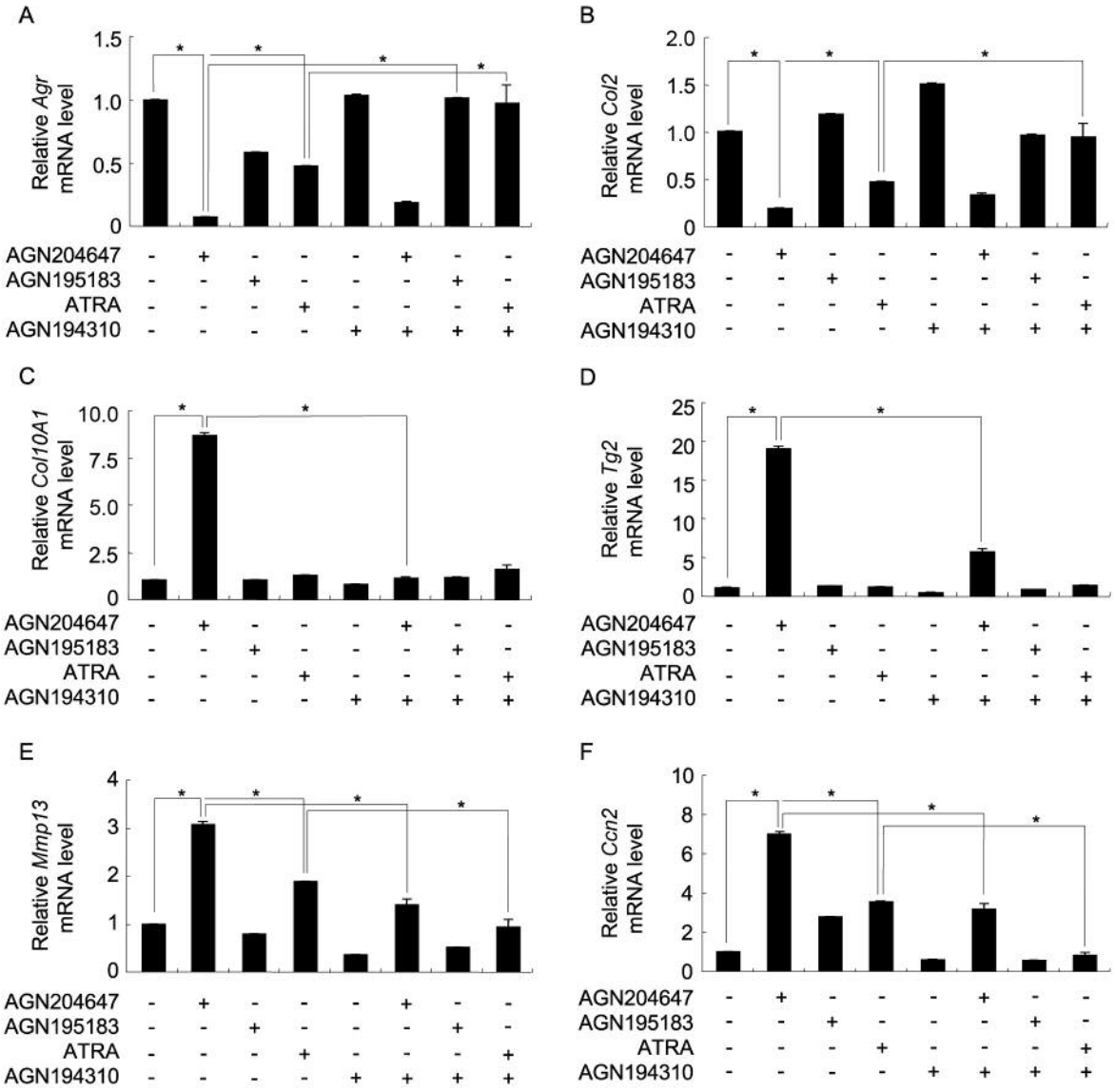

Figure 2. *Modulation of aggrecan (Agr) (A), type II collagen (Col2) (B), type X collagen (Col10A1) (C), transglutaminase-2 (Tg2) (D), matrix metalloproteinase-13 (Mmp13) (E), and connective tissue growth factor (Ccn2) (F) mRNA expression by retinoid receptor (RAR)-α (AGN195183) or -γ (AGN204647) agonists with/without retinoid pan-antagonist (AGN194310) in 10-day cultures of ATDC5 cells. ATDC5 cells were exposed to 100 nM AGN204647, AGN195183, or all-trans-retinoic acid (ATRA) with or without 100 nM AGN194310 for 24 h. Total RNA from these cells was used for real-time reverse transcription-polymerase chain reaction analysis. The values in the graph indicate the relative mRNA level. *Significantly different at $p<0.05$ between the indicated groups.*

with RARγ agonist AGN204647, RARα agonist AGN195183, or ATRA for 24 h in the absence or presence of retinoid antagonist AGN194310 and were then subjected to real-time RT-PCR analysis. RARγ agonist significantly down-regulated aggrecan and *Col2* mRNA, whereas retinoid antagonist reversed down-regulation of aggrecan and *Col2* mRNA induced by RARγ agonist (Figure 2A and B). RARγ agonist significantly boosted the mRNA expressions of *Mmp13* and *Ccn2* compared with the control, whereas retinoid antagonist reversed RARγ agonist action as well as *Col10A1* and *Tg2* mRNA expression (Figure 2C-F).

Involvement of MAPK. To verify that RARγ agonist treatment modulated the activation of MAPK, cultures were treated with AGN204647 for different lengths of time and the cultures were processed for an immunoblot analysis of

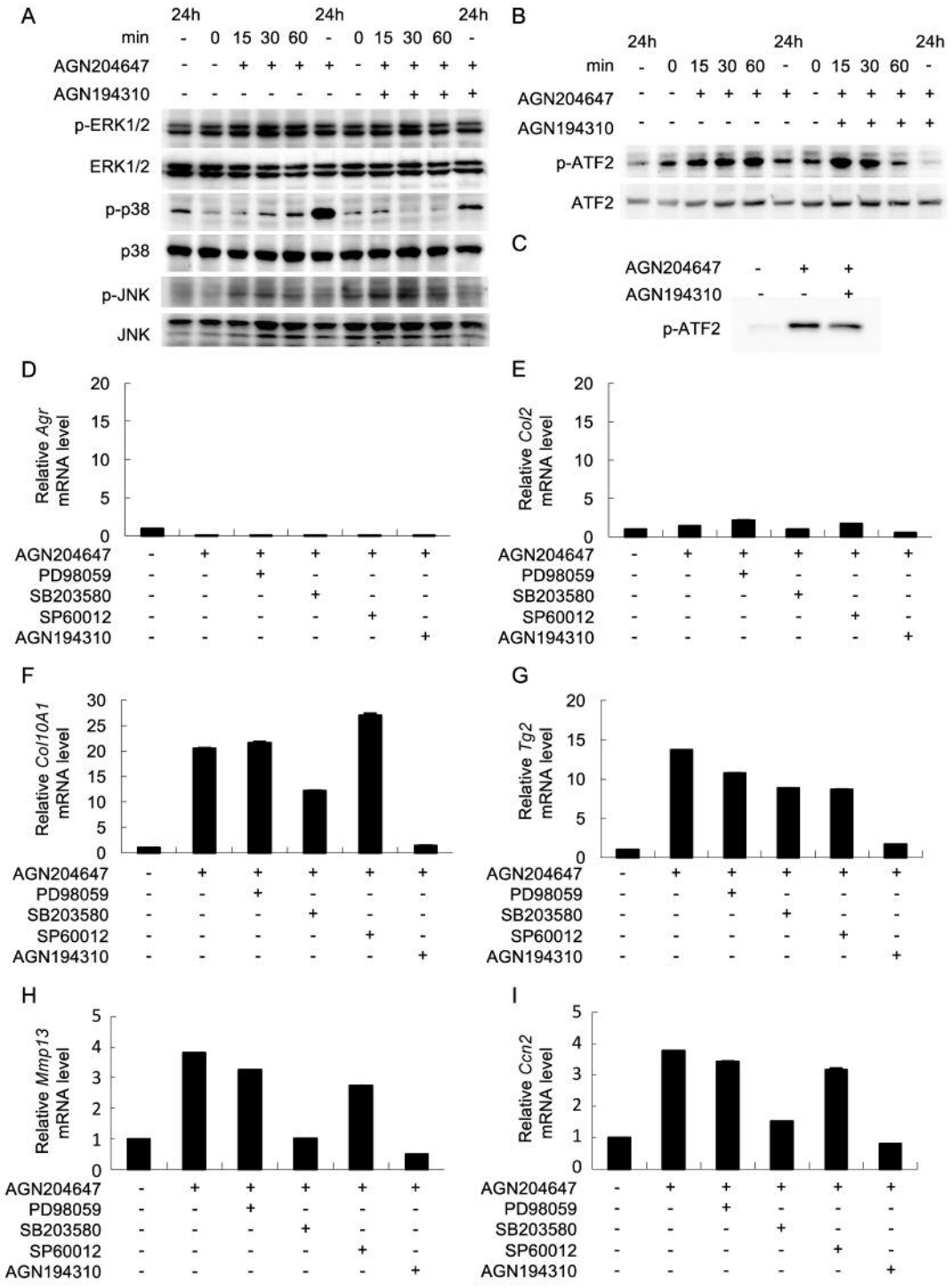

Figure 3. *Analysis of the action of mitogen-activated protein kinase (MAPK) on aggrecan (Agr), type II collagen (Col2), type X collagen (Col10A1), transglutaminase-2 (Tg2), matrix metalloproteinase-13 (Mmp13), and connective tissue growth factor (Ccn2) mRNA induction by retinoid receptor gamma (RARγ) agonist AGN204647. ATDC5 cells were exposed to 100 nM AGN204647 with/without 100 nM retinoid antagonist AGN194310 for the indicated times, and the cell lysates were processed for immunoblot determination of phosphorylated extracellular signal regulated-kinases (pERK1/2), p-p38 and phosphorylated c-Jun N-terminal kinase (pJNK) mitogen-activated protein kinase (MAPK) (A), and phosphorylated activating transcription factor 2 (p-ATF2) (B). C: The level of activated p38 MAPK activity was determined by a kinase activity assay. ATDC5 cells were treated with 20 μM ERK1/2 kinase inhibitor PD98059, p38 MAPK SB203580 or JNK inhibitor SP60012 and then stimulated with 100 nM AGN204647 for 24 h. Total RNA from these cells was used for a real-time reverse transcription-polymerase chain reaction analysis for Agr (D), Col2 (E), Col10A1 (F), Tg2 (G), Mmp13 (H), and Ccn2 (I). The values in the graph indicate the relative mRNA level. Two independent experiments were performed, and the average of the mRNA level is shown.*

the levels of phosphorylated *versus* total levels of the three kinases. As shown in Figure 3A, expression of ERK1/2, p38, and JNK-activated MAPK started to increase after the RARγ agonist treatment. ERK1/2 and JNK MAPK activation reached a plateau at 30 min, whereas p38 MAPK activation reached a maximum at 24 h. Retinoid antagonist at 100 nM completely inhibited the p38 MAPK activation for 24 h, and weakly suppressed pERK1/2 for 60 min. However, pJNK was not changed by the treatment with retinoid antagonist.

To clarify the phosphorylation of ATF2 through RARγ signaling, cells were treated with retinoid antagonist and RARγ agonist. The treatment of cells with retinoid antagonist blocked RARγ-induced ATF2 phosphorylation (Figure 3B). We further confirmed the activation of ATF2 by conducting a p38 MAPK activity assay. p38 MAPK activity was up-regulated by treatment with RARγ agonist and inhibited by retinoid antagonist (Figure 3C).

To determine whether MAPKs were involved in RARγ action on the mRNA expression of aggrecan, *Col2, Col10A1, Tg2, Mmp13* and *Ccn2*, ATDC5 cells were treated with RARγ agonist for 24 h with/without specific inhibitors of MAPK/ERK kinase (MEK), p38 MAPK, or p-JNK. The aggrecan and *Col2* mRNA levels were not significantly changed by the treatment with any of the inhibitors (Figure 3D and E). The treatment with p38 MAPK inhibitor reversed RARγ agonist-induced mRNA expressions of *Col2, Col10A1, Tg2, Mmp13* and *Ccn2*. However, MEK inhibitor did not affect the RARγ agonist-induced *Col10A1, Mmp13* and *Ccn2* expression (Figure 3F, H and I).

Discussion

The importance of RAR in chondrocyte maturation and function was demonstrated in *in vitro* studies (11, 14) and has been re-affirmed by a recent analysis of mice deficient in RARα/RARγ or RARβ/RARγ, that exhibited significant skeletal growth retardation (10). Our results here show that *Rarg* was specifically expressed in proliferative and pre-hypertrophic zones of mouse tibial long bone growth plates, with *Col10A1, Mmp13* and *Ccn2* being the most abundantly expressed members and their expressions prominently characterizing hypertrophic chondroyctes.

It was recently reported that a general deletion of *Rarg* but not *Rara* in mice caused bone marrow defects characterized by hematopoietic stem cell alteration and even a marked reduction in trabecular bone during aging (15, 16). It is, thus, clear that there are intimate cross-talk interactions between the hypertrophic growth plate and bone marrow that are essential for the physiological progression of skeletal growth. Our present data indicate that RARγ signaling may bring about appropriate expression levels and the actions of these important hypertrophic chondrocyte-specific downstream effectors, contributing to a seamless transition from hypertrophic cartilage to trabecular bone. The *Rarg* gene expression is consistent with the gene expressions of *Mmp13* and *Ccn2*, which have important roles in the resorption of hypertrophic cartilage from growth plates and the remodeling of newly deposited trabecular bone during long bone development (2, 4-6).

Our evidence obtained from ATDC5 cell cultures shows that an exogenous RARγ agonist stimulated *Mmp13* and *Ccn2* expression, and a retinoid antagonist reversed this action. Based on these and other observations, it is clear that retinoid signaling is an important regulator of the expression of *Mmp13* and *Ccn2* in chondrocytes, and that *Mmp13* and *Ccn2* may be important downstream effectors. The three major MAPK signaling pathways are involved in the program of chondrogenic differentiation from embryonic through postnatal stages of development (17). The murine ATDC5 cell line has been used as monolayer culture to identify the MAPK transducers downstream of the chondro-stimulatory growth factors (18). Insulin also induced the phosphorylation of all three MAPK pathways in ADTC5 cells (19). Indeed, the p38 MAPK pathway regulates the expression of *CoI2* and aggrecan and proteoglycan synthesis in response to growth factors (18, 20, 21).

In our analyses using ATDC5 cells, it was difficult to evaluate the effect of the MAPK inhibitor on the Col II and aggrecan mRNA expressions. Notably, p38 MAPK signaling has been shown to positively regulate hypertrophic chondrocyte differentiation with an up-regulation of *Col10A1*, and *Mmp13* (22). In a related study, Zhang *et al.* (23) found that the expression in transgenic mouse chondrocytes of a constitutively active mutant of *Mmk6* (a MAPK kinase that specifically activates p38) had a dwarf phenotype characterized by reduced chondrocyte proliferation, the inhibition of hypertrophic differentiation, and a delay in the formation and secondary ossification centers. These studies reached the conclusion that activated p38 MAPK has a positive role in chondrocyte maturation, hypertrophy, and the remodeling of newly-deposited trabecular bone during long bone development.

Retinoids inhibit cell proliferation by suppressing the phosphorylation of ERK1/2 (24). In contrast, p-p38 stimulated genes *Rarg* and *Col10A1*, whereas p-ERK stimulated the expression of genes including *Ccn2* and osteopontin in primary cultured chick hypertrophic chondrocytes by ATRA (8). In contrast to our finding, p38 MAPK reduced RARγ signal-induced *Ccn2* expression in ATDC5 cells. We observed that treatment with the JNK inhibitor SP60012 had no impact on the chondrocyte differentiation.

All these studies point to an involvement of p38 pathway signaling in the regulation of chondrocyte differentiation hypertrophy. However, further studies are needed to clarify the specific roles of RARγ signaling on the p38 cascade in endochondral bone formation.

Conflicts of Interest

None of the Authors have any conflict of interest in regard to this study.

Acknowledgements

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