



# Inhibitory effect of retinoic acid receptor agonists on in vitro chondrogenic differentiation

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## Abstract

Retinoic acid (RA), an active metabolite of vitamin A, plays pivotal roles in a wide variety of biological processes, such as body patterning, organ development, and cell differentiation and proliferation. RA signaling is mediated by nuclear retinoic acid receptors,  $\alpha$ ,  $\beta$ , and  $\gamma$  (RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$ ). RA is a well-known regulator of cartilage and skeleton formation and RARs are also essential for skeletal growth and hypertrophic chondrocyte-specific gene expression. These important roles of RA and RARs in chondrogenesis have been widely investigated using in vivo mouse models. However, few reports are available on the function of each subtype of RARs on in vitro chondrocyte differentiation. Here, we examined the effect of specific agonists of RARs on chondrogenic differentiation of ATDC5 and C3H10T1/2 cells. Subtype-specific RAR agonists as well as RA decreased the expressions of chondrogenic differentiation marker genes and inhibited chondrogenic differentiation, which was accompanied with morphological change to spindle-shaped cells. Among RAR agonists, RAR $\alpha$  and RAR $\gamma$  agonists revealed a strong inhibitory effect on chondrogenic differentiation. RAR $\alpha$  and RAR $\gamma$  agonists also hampered viability of ATDC5 cells. These observations suggested that RAR $\alpha$  and RAR $\gamma$  are dominant receptors of RA signaling that negatively regulate chondrogenic differentiation.

**Keywords** Retinoic acid · Retinoic acid receptor · Chondrocyte · Chondrogenesis · Differentiation

## Introduction

Retinoic acid (RA), the main active metabolite of vitamin A (retinol) is a pivotal morphogenic factor for early embryonic patterning, organogenesis, and homeostasis in several tissues and organs. RA is a diffusible, lipophilic molecule that easily penetrates into cell cytoplasm. RA signaling transduces via nuclear retinoic acid receptors (RARs) and retinoid X

receptors (RXRs), which constitute heterodimers. The heterodimerized RAR–RXR complex recognizes the retinoic acid response elements (RAREs) in the promoter region of various genes and regulates their transcription (van Neerven and Mey 2007). RARs consist of three subtypes encoded by different genes: RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$ . They are thought to have distinct functions and reveal different expression patterns depending on the tissue and cell type. Therefore, the specificity and range of RA signaling are fine-tuned by the temporospatial expression patterns of RARs and the gradient intracellular levels of RA (Williams et al. 2010).

RA is one of the important factors for skeletal development and regulates cartilage formation and growth. RA has been reported to be a potent inhibitor of chondrogenesis (Pacifci et al. 1980). Subsequent reports have shown that endogenous RA signaling is suppressed during chondrogenesis, which is essential for ordinary chondrogenic differentiation (Weston et al. 2000, 2002). We also investigated the effect of the synthetic agonists selective for RAR $\alpha$  and RAR $\gamma$  on the ectopic ossification in the animal model of Fibrodysplasia Ossifications Progressiva (FOP), which is

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a genetic disorder characterized with progressive ectopic ossification (Shimono et al. 2010, 2011; Chakkalakal et al. 2016). We found that the agonists for RAR $\gamma$  successfully inhibited chondrogenesis and ectopic ossification (Chakkalakal et al. 2016). Conversely, pharmacological inhibition of RAR $\gamma$  enhanced endochondral ossification by stimulating BMP signaling (Uchibe et al. 2017).

Although the importance of RAR subtypes on chondrogenesis has been well investigated by in vivo studies using animals with/without genetical modification, the precise effect of each RAR subtype on in vitro chondrogenic differentiation is not yet fully understood. The present study examines the effect of agonists specific for each subtype of RAR on chondrogenic differentiation.

## Materials and methods

### Materials

Dulbecco's modified Eagle's minimal essential medium (D-MEM)/Ham's F-12 was purchased from Life Technologies (Paisley, UK). Plastic dishes were obtained from Thermo Fisher Scientific (Rochester, NY, USA) and fetal bovine serum (FBS) from Sigma-Aldrich (St. Louis, MO, USA). RA was purchased from Sigma-Aldrich (St. Louis, MO, USA), and AM580, CD2314, CD1530, and BMS493 were obtained from Tocris Bioscience (Bristol, UK). ITS Premix Universal Culture Supplement (Insulin, Transferrin, and Selenious Acid) was purchased from Corning (Corning, NY, USA). Other materials used were of the highest grade commercially available.

### Cell culture and differentiation

ATDC5 cells were cultured in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 comprising 5% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air. To induce the differentiation of ATDC5 cells to chondrocytes, ITS and ascorbate were added to the culture medium when cells reached 90% confluency. C3H10T1/2 cells were used for high-density micromass culture, as previously described (Denker et al. 1999). Chondrogenic differentiation was induced by adding recombinant human BMP-2 (BioLegend, San Diego, CA, USA) into the culture medium. For further experiments, the cultures were treated with 100 nM RA, AM580 (RAR $\alpha$  agonist), CD2314 (RAR $\beta$  agonist), CD1530 (RAR $\gamma$  agonist), or BMS493 (pan-RAR inverse agonist). Bright field images were acquired using BZ-X700 microscope equipped with a BZ analyzer (Keyence, Osaka, Japan).

### Alcian blue staining

To determine cartilage nodule formation, we cultured C3H10T1/2 cells in the chondrogenic differentiation medium in a 24-well plate for 15 days in the presence of various types of RAR agonists or an inverse agonist of RAR. The cells were fixed with 4% PFA for 10 min and stained with 0.1% alcian blue dissolved in 0.1 M hydrochloric acid for 16 h at ambient temperature. The cells were then rinsed three times with distilled water.

### Real-time PCR analysis

Total RNA was extracted by the Trizol method (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Real-time PCR of each gene was performed in triplicate, for at least three independent experiments, with a CFX96 Real-time System (Bio-Rad, Hercules, CA, USA) using Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA). The sequences of primers used were as follows: mouse *ribosomal protein S29* (NM\_009093.2): 5'-GGAGTCACCCACGGAAGTTCCGG-3' (Forward), 5'-GGAAGCACTGGCGGCACATG-3' (Reverse); mouse *Sox9* (NM\_011448.4): 5'-TCAGATGCA GTGAGGAGCAC-3' (Forward), 5'-CCAGCCACAGCA GTGAGTAA-3' (Reverse); mouse *Col2a1* (NM\_031163.3): 5'-GGGCAACAGCAGGTTACATACAC-3' (Forward), 5'-TCAATAATGGGAAGGCGGGAGGTC-3' (Reverse); and mouse *Mmp13* (NM\_008607.2): 5'-CCAGACTAT GGACAAAGATT-3' (Forward), 5'-ATGCGATTACTC CAGATACT-3' (Reverse); mouse *Rara1* (NM\_009024.2): 5'-CCATGCCCCGAGGAAGA-3' (Forward), 5'-CAC AGTCAGAAGAGCAGG-3' (Reverse); mouse *Rara2* (NM\_001177302.1): 5'-CGAGATGTACGAGAGTGT GGAAGT-3' (Forward), 5'-TCTGGTTATAAAAGTCCA CCACTAGGA-3' (Reverse); mouse *Rarb2* (NM\_011243.2): 5'-AAGCAGGAATGCACAGAGAGCTAT-3' (Forward), 5'-TGGGCTTTCGGATCTTCT-3' (Reverse); mouse *Rarg1* (NM\_011244.4): 5'-GCTGCAGAGTCCAAG GGATTC-3' (Forward), 5'-GCGCAAAGAGTCTCTCCT TATTG-3' (Reverse); mouse *Rarg2* (NM\_001042727.2): 5'-TCTAGCACCCAGTTTCTCTCCAA-3' (Forward), 5'-CCGGGACAAACGATTCCA-3' (Reverse).

### Immunofluorescence

ATDC5 cells were fixed in 4% neutralized formalin, permeabilized with acetone/ethanol mixture (50:50, v/v) at -20 °C for 1 min, and incubated with primary rabbit anti-Aggregan

polyclonal antibodies (Millipore, AB1031, 1:150) overnight at 4 °C followed by incubation with goat anti-Rabbit Alexa Fluor 488 IgGs (Invitrogen, A32731, 1:1000) for 30 min. Cultures were also stained with 0.1 µg/ml DAPI (Sigma-Aldrich, St. Louis, MO, USA) for nuclear staining and with Alexa Fluor 546-phalloidin (Invitrogen, A22283, 1:500) for cytoplasmic F-actin staining. Samples were mounted and viewed with BZ-X700 microscope (Keyence, Osaka, Japan).

### Cell viability measurement by CCK-8 assay

Viability of ATDC5 cells was measured by the CCK-8 quantitative colorimetric assay for cell proliferation/cytotoxicity (Dojindo, Kumamoto, Japan). The assay detects the signals, which correlate with the levels of mitochondrial activity. Subconfluent cells were grown in 96-well culture plates and treated with RAR agonists or an inverse agonist for 5 days. The absorbance of the medium was measured at 450 nm by microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The experiments were performed three times with three independent samples.

### Statistical analysis

All data are expressed as means  $\pm$  SD and a minimum of three independent experiments were performed for each assay. Analysis of variance (ANOVA) was used for performing statistical analysis (Prism 6; GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was indicated with “\*” ( $P$  value  $<$  0.05).

## Results

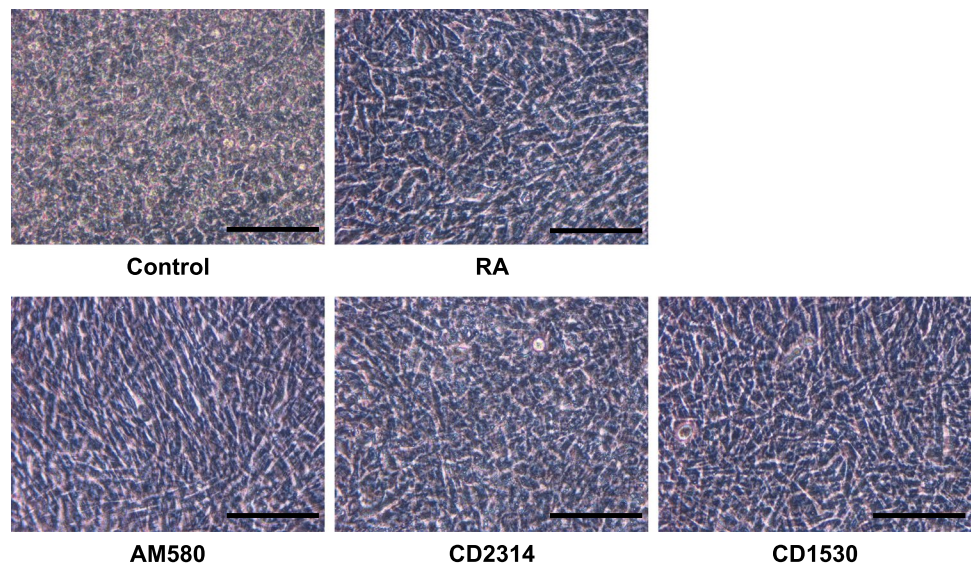
### RAR agonists altered the morphology of ATDC5 cells

ATDC5 cells were grown in 6-well plates in chondrogenic differentiation medium for 5 days. Figure 1 shows the bright field images of the cells treated with RA or RAR agonists (AM580, CD2314, or CD1530). In the vehicle-treated control group, the cells underwent cellular condensation and chondrogenic differentiation, which is characterized by extracellular matrix deposition and round- or hexagon-shaped cells morphology. In contrast, the cells treated with RA or subtype-specific agonists showed spindle-shaped morphology and less extracellular matrix deposition, which are also characterized by altered arrangement of actin filaments as shown in Fig. 1. This morphological change was prominent in cells treated with RA, AM580, and CD1530, compared to CD2314-treated cells, which showed relatively mild morphological change.

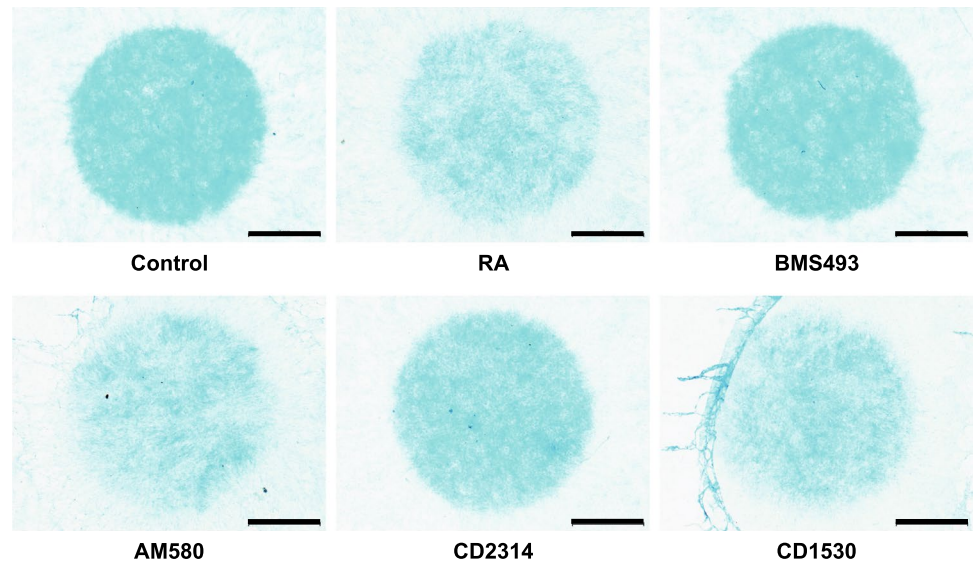
### RAR agonists impaired cartilage matrix formation

To examine the role of RARs in chondrogenic differentiation and subsequent formation of cartilage matrix in vitro, we stained the micromass cultures of C3H10T1/2 cells with alcian blue solution at day 15 after treatment with RAR agonists (AM580, CD2314, or CD1530) or inverse agonist, BMS493 (Fig. 2). This micromass culture model is suitable to analyze cartilage matrix formation compared to general monolayer culture methods, which take long time to produce appreciable matrix nodules. Untreated cells showed

**Fig. 1** Alteration in morphology of ATDC5 cells induced by RAR agonists. Bright field photomicrographs of ATDC5 cells treated with/without RA, RAR agonists (AM580, CD2314, or CD1530) for 5 days. Bars represent 100 µm



**Fig. 2** RAR agonist treatment inhibits cartilage matrix formation. C3H10T1/2 micromass cultures were maintained in chondrogenic differentiation medium with/without RA, RAR agonists, or RAR inverse agonist for 15 days. The cartilage matrix formation was determined by alcian blue staining. Cartilage nodule formation was observed in blue-stained tissues. Bar represents 2 mm



production of cartilage matrix and formation of cartilage nodules, indicated by intense staining with alcian blue. The cartilage nodules were dramatically decreased in the cells treated with RA or RAR agonists (AM580, CD2314, or CD1530), whereas RAR inverse agonist, BMS493, had little effect on cartilage matrix formation compared with the control. Among all the subtypes of RARs, RAR $\alpha$  and RAR $\gamma$  agonists (AM580 and CD1530, respectively) exhibited stronger inhibitory effect on cartilage matrix formation compared to RAR $\beta$  agonist (CD2314).

### RAR agonists decreased chondrogenic gene expression

To examine the effect of RARs on the expressions of chondrocyte differentiation marker genes, we treated ATDC5 cells with RA, RAR agonists, or RAR inverse agonist for 14 days, and extracted total RNA from the cells. After reverse transcription of the total RNA to cDNA, we performed qPCR using specific primer pairs of *Col2a1* and *Sox9*, which are early differentiation markers of chondrocytes, and *Mmp13*, a marker gene of matured chondrocytes. RAR $\alpha$  and RAR $\gamma$  agonists (AM580 and CD1530) as well as RA significantly decreased the expression levels of *Col2a1* and *Sox9* in ATDC5 cells, whereas RAR $\beta$  agonist (CD2314) showed weak inhibitory effect (Fig. 3a, b). In contrast, RAR inverse agonist, BMS493, increased the expressions of both *Col2a1* and *Sox9* compared to the control group. *Mmp13* expression was enhanced by RA, RAR $\alpha$ , and RAR $\gamma$  agonists (AM580 and CD1530) (Fig. 3c). Although RAR $\beta$  agonist (CD2314) also increased *Mmp13* expression, the effect was less prominent than that of other specific agonists (Fig. 3c). We also examined the expression

levels of RAR subtypes to determine endogenous levels of each receptors in our experimental model at day 1 (Fig. 3d). *Rara* and *Rarg* were highly expressed subtypes compared to *Rarb2* in ATDC5 cells.

Expression of Aggrecan protein was investigated by immunofluorescence using the same culture model at day 3. Aggrecan-positive cells were observed in control and BMS493-treated groups; however, we could not see clear signal in cultures treated with RAR agonists (Fig. 4).

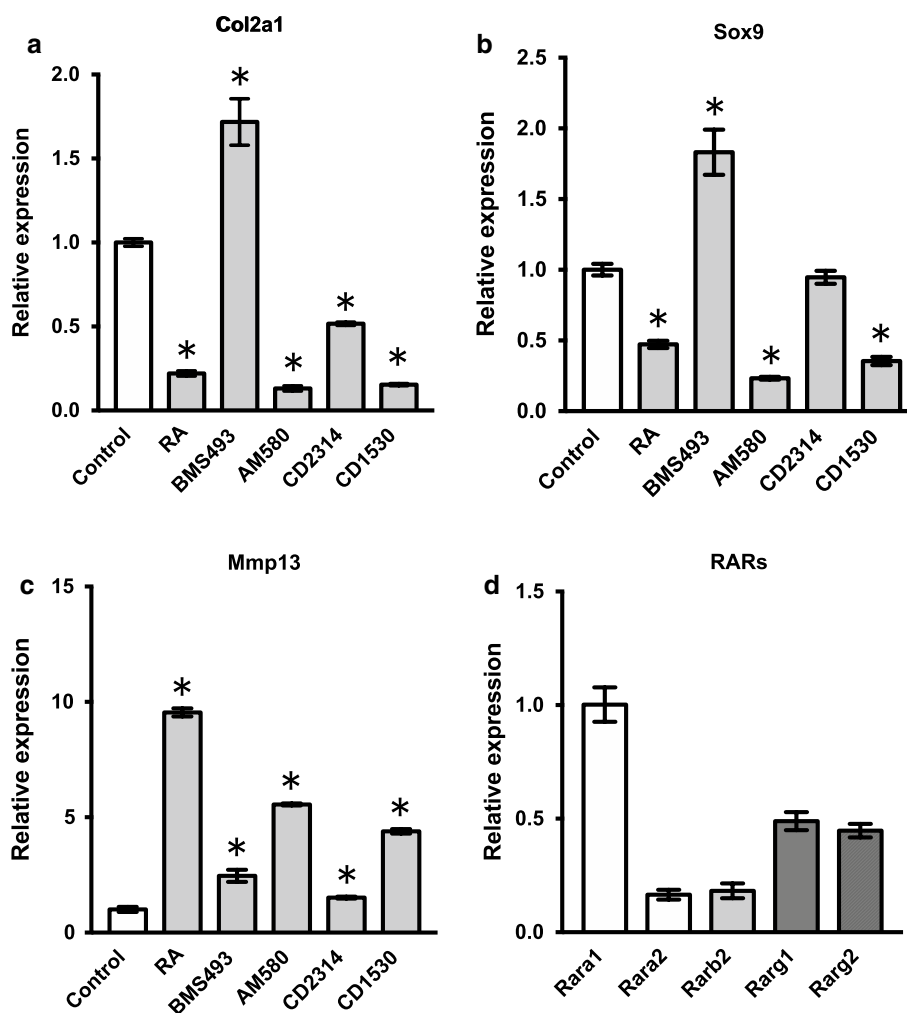
### RAR agonists decreased viability of ATDC5 cells

To examine the effect of RAR agonists on cell viability, we treated ATDC5 cells with/without RA, RAR agonists, or RAR inverse agonist for 5 days, and measured the cell viability using CCK-8 assay. Treatment of the cells with RA and RAR $\alpha$ / $\gamma$  agonists significantly decreased cell viability (Fig. 5). However, viability of RAR $\beta$  agonist-treated cells was comparable to that of the vehicle-treated control. Treatment with RAR inverse agonist significantly increased the viability of ATDC5 cells.

### Discussion

In this study, we investigated the effect of subtype-specific RAR agonists on chondrogenic differentiation. The agonists for RAR $\alpha$ , RAR $\beta$ , or RAR $\gamma$  suppressed chondrogenic differentiation with downregulation of chondrocyte-specific gene expression. Among them, RAR $\alpha$  and RAR $\gamma$  agonists exhibited higher inhibitory effect on the chondrogenic differentiation and cell viability compared to RAR $\beta$  agonist.

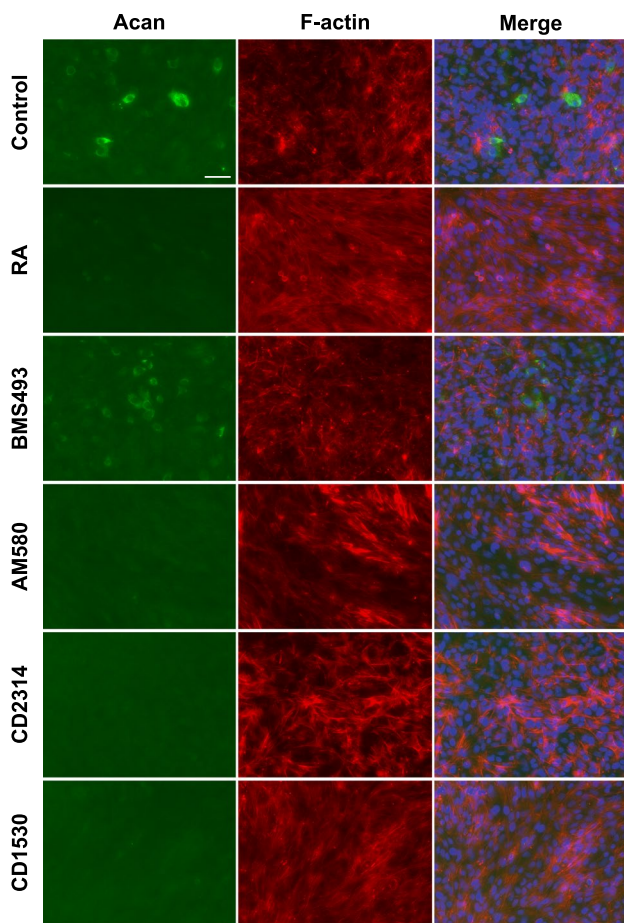
**Fig. 3** Alteration in chondrogenic gene expression by RAR agonists. ATDC5 cells were cultured in chondrogenic differentiation medium with/without RA, RAR agonists, or RAR inverse agonist. The quantitative expression levels of **a** *Col2a1*, **b** *Sox9*, and **c** *Mmp13* were determined by qPCR at day 14. Expression levels of RAR subtypes were determined at day 1 (**d**). *Rps29* was used as internal control. Data are represented by mean  $\pm$  SD and the experiments were independently conducted with three replicates ( $n=3$ ). \* $P < 0.05$  (one-way ANOVA with Dunnett's post hoc multiple comparison test)



We first examined the effect of RA on chondrogenic differentiation in ATDC5 cells. RA changed the cell morphology to fibroblast-shape and suppressed the expression of chondrogenic marker genes. It is already known that physiological gradient distribution of RA is naturally found in the developing limbs, implying that different concentrations of RA elicit diverse effects during the formation of growth plate cartilage (Iwamoto et al. 1993). Therefore, response of chondrocytes to RA is thought to change according to differentiation status of cells and the availability of RA. Consequently, RA was reported to play a variety of functions including differentiation or dedifferentiation of chondrocytes, morphological alterations, regulation of cell proliferation, and different phenotype of collagen (Hein et al. 1984; Horton & Hassell 1986; Takishita et al. 1990). It was also reported that high concentration of RA ( $2 \times 10^{-7} - 1 \times 10^{-6}$  M) inhibited chondrocyte differentiation (Takigawa et al. 1980), whereas lower concentration of RA ( $1 \times 10^{-8}$  M) did not show inhibitory effects on chondrocyte differentiation and its shape (Enomoto et al. 1990). A recent study by Li et al. showed that RA ( $5 \times 10^{-7}$  M) treatment

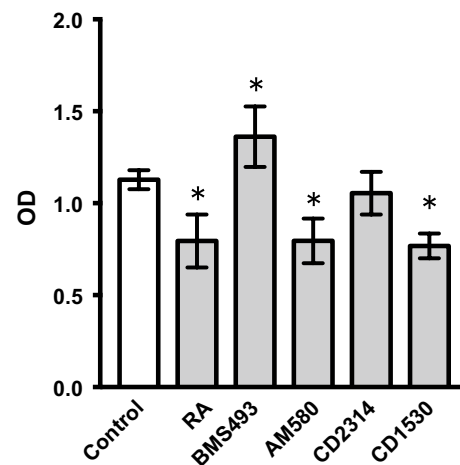
in human palatal chondrocytes inhibited proliferation and ossification of chondrocytes by upregulating Wnt/ $\beta$ -catenin signaling, which is an important signaling pathway for palatal development and chondrogenesis (Li et al. 2014). They also described that RA stimulates chondrogenic maturation and terminal differentiation by decreasing the expressions of related marker genes and increasing the expressions of osteogenic marker genes (Li et al. 2014). In other reports, the treatment of RA inhibited chondrocyte proliferation by decreasing cartilage proteoglycan and changed their typical polygonal morphology to a fibroblastic-spindle shape (Hein et al. 1984; Horton W and Hassell JR 1986). As described above, the role of RA signaling in chondrogenesis is considerably complicated and controversial. These diverse responses to RA are probably because RA treatment can broadly activate all subtypes of RARs. Therefore, here, we attempted to investigate the role of each RAR subtype using highly specific agonists.

In this study, all agonists inhibited chondrocyte differentiation, suggesting that all RARs are involved in the process of chondrocyte differentiation. RA seems to distribute



**Fig. 4** Immunofluorescence and actin staining of RAR agonists treated ATDC5 cells. ATDC5 cells were cultured in chondrogenic differentiation medium with/without RA, RAR agonists, or RAR inverse agonist. Cells were stained with anti-Aggregan antibody followed by phalloidin (F-actin) staining at day 3 post-treatment. Bar represents 50  $\mu\text{m}$

to cartilage tissue and expresses its function via binding to RARs inside the cells. Therefore, temporal and spatial expression of RAR subtypes in the tissues is also considered to be important for chondrocyte differentiation. RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$  show various expression patterns during the initial stage of limb skeletogenesis. Although RAR $\alpha$  and RAR $\gamma$  transcripts are similarly distributed in the limb bud at embryonic day 10, RAR $\gamma$  transcripts were dominant in chondrocyte lineage at later stage and specifically distributed in all cartilages (Dollé et al. 1989; Ruberte et al. 1990; Mollard et al. 2000). Williams et al. demonstrated that RAR $\gamma$  transcripts were strongly expressed in resting, proliferative, and pre-hypertrophic zones in vivo (Williams et al. 2009). Our previous study showed that RAR $\gamma$  is a crucial factor for the suppression of chondrogenesis mediated by retinoid and the stimulation of RAR $\gamma$  effectively impaired ectopic ossification (Chakkalakal et al. 2016). RAR $\alpha$  has also been reported to act as a therapeutic target for experimental mouse models



**Fig. 5** Negative effect of RAR agonists on cell viability. ATDC5 cells were grown in 96-well plates and cultured with/without RA, RAR agonists, or RAR inverse agonist for 5 days. The cell viability was determined using CCK-8 assay and compared with the control. Results are indicated as OD (means  $\pm$  SD) (n=6). Significant differences compared to control are indicated by an asterisk, \* $P < 0.05$  (one-way ANOVA with Dunnett's post hoc multiple comparison test)

of heterotopic ossification (Shimono et al. 2010). However, these observations from in vivo studies could have been resulted from systemic effect of RAR agonists. Also, it is difficult to reveal RA distribution at local cartilage tissues in animal studies. Therefore, we investigated the effect of RAR agonists in vitro to focus directly on chondrocyte differentiation in this study. Consistent with previous in vivo studies, RAR $\alpha$  and RAR $\gamma$  agonists showed higher inhibitory effect on chondrogenic differentiation of ATDC5 cells compared with RAR $\beta$  agonist. This difference in strength of inhibitory effect between RAR subtypes is potentially due to the difference in the expression levels of each RAR during chondrocyte differentiation. Taken together, RAR $\alpha$  and RAR $\gamma$  are likely dominant receptors also in in vitro chondrogenesis. Interestingly, RAR agonists increased the expression level of *Mmp13*, one of the maturation marker genes of chondrocyte. This upregulation suggests that RA signaling stimulates maturation of chondrocytes, whereas it inhibits differentiation of immature cells into chondrogenic lineage. Enhanced expression of *Mmp13* could also contribute to the diminished matrix production, which is shown as attenuated staining intensity with alcian blue.

We also demonstrated that RAR $\alpha$  and RAR $\gamma$  agonists decreased viability of ATDC5 chondroprogenitor cells. Moreover, interference with RAR signaling by inverse agonist showed opposite effect in the same cell line. The previous study by Minegishi et al. has shown that excess RA signaling resulted in decreased proliferation rate of growth plate chondrocytes (Minegishi et al. 2014). These observations suggested that RAR signaling, especially RAR $\alpha$  and

RAR $\gamma$ , inhibits not only chondrogenic differentiation, but also proliferation of chondrogenic cells. Thus, the reduction in cartilage matrix formation could be due to the dual and independent effects of RARs.

In conclusion, the present study showed that RA signaling negatively regulates in vitro chondrogenic differentiation. Although treatment with agonists for each RAR subtype used in this study decreased chondrogenic differentiation, RAR $\alpha$  and RAR $\gamma$  agonists showed critical inhibitory effect on the differentiation. Furthermore, we demonstrated that RAR $\alpha$  and RAR $\gamma$  agonists also decreased viability of ATDC5 chondroprogenitor cells. These distinct effects of RARs on chondrogenesis finally caused the reduction of cartilage matrix formation, which is beneficial for the treatment of heterotopic ossification.

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**Authors' contribution** KU designed the experiments. YS, KU, KY, YW, JG, HY, MI, HK, and HO conducted the experiments, evaluated the results, and prepared the manuscript. All authors have read and approved the final submitted manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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