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Retinoic acid disrupts osteogenesis in pre-osteoblasts by down-regulating WNT signaling

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ABSTRACT

The skull bones are formed by osteoblasts by intramembranous ossification. WNT signaling is a regulator of bone formation. Retinoic Acid (RA) act as a teratogen affecting craniofacial development. We evaluated the effects of RA on the differentiation and mineralization of MC-3T3 cells, and on the expression of WNT components. MC-3T3 were cultured with or without 0.5 μM RA in osteogenic medium and mineralization was assessed by alizarin red staining. The expression of osteogenic marker genes and WNT genes was evaluated at several time points up to 28 days. RA significantly inhibited MC-3T3 mineralization ($p < 0.01$), without affecting ALP activity or *Alp* gene expression. Both parameters gradually increased in time. During culture, RA stimulated *Runx2* expression at 14 and 28 days compared to the respective controls ($p < 0.05$). Also, RA significantly reduced *Sp7* expression at days 14 and 21 ($p < 0.05$). Simultaneously, RA significantly reduced the expression of the WNT genes *cMyc, Lef1, Lrp5, Lrp6* and *Wnt11* compared to the controls ($p < 0.05$). In contrast, RA increased the expression of the WNT inhibitors *Dkk1* at day 21 and *Dkk2* at days 14 and 21 ($p < 0.01$). Our data indicate that RA disrupts osteogenic differentiation and mineralization by inhibiting WNT signaling.

1. Introduction

The skull bones are formed by osteoblasts in a process known as intramembranous ossification. This process starts with the condensation of mesenchymal progenitor cells and the activation of "osteogenic master transcriptional regulators" such as the Runt-related transcription factor 2 (*Runx2*) and Osterix (*Sp7*), the main drivers of early osteoblast differentiation (Rutkovskiy et al., 2016; Liu and Lee, 2013). Subsequently, these cells, now defined as pre-osteoblasts, enter a three-stage process that includes proliferation, deposition of extracellular matrix proteins (ECM) and mineralization. This leads to the differentiation of pre-osteoblasts to mature osteoblasts (James, 2013). In the first stage, pre-osteoblasts proliferate and deposit extracellular matrix proteins such as fibronectin, collagen and osteopontin (*Spp1*). During the second stage, pre-osteoblasts leave the cell cycle and start to express alkaline phosphatase (*Alp*). The final stage, matrix mineralization, is promoted by osteocalcin (*Bglap*) and bone sialoprotein (*Bsp*) (Rutkovskiy et al., 2016; Barbe et al., 2009). Many other transcription factors and signaling molecules are involved in osteogenesis and are controlled by signaling pathways including bone morphogenetic protein (BMP) and wingless-INT signaling (WNT) (Beederman et al., 2013; Long, 2011).

WNT signaling is a highly conserved pathway essential for the growth and development of many tissues and organs. WNT signaling regulates cellular processes such as cell fate, differentiation, migration and proliferation (Brugmann et al., 2007). The canonical WNT pathway stabilizes cytoplasmic β-catenin and facilitates its translocation into the nucleus, where it binds to lymphoid enhancer-binding factor 1/T cell-specific transcription factor (*Lef1/Tcf*). This complex induces target gene expression. In the absence of a WNT stimulus, β-Catenin is

Abbreviations: RA, retinoic acid; *Runx2*, Runt-related transcription factor 2; *Sp7*, Osterix; ECM, extracellular matrix proteins; *Spp1*, osteoponttin; *Alp*, alkaline phosphatase; *Bglap*, osteocalcin; *Bsp*, bone sialoprotein; BMP, bone morphogenetic protein; WNT, wingless-INT signaling; *Lef1/Tcf*, lymphoid enhancer-binding factor 1/T cell-specific transcription factor; CRABPs, cellular retinoic acid-binding proteins; RARs/RXRs, acid receptors and retinoid X receptors; *Rare*, retinoic acid response elements; *Cyp26b1*, cytochrome P450 family 26 subfamily B member 1; *Crabp2*, cellular retinoic acid binding protein 2; *Rarβ*, retinoic acid receptor beta; -, *Lrp5Lrp5Lrp6Lrp6*low-density lipoprotein receptor-related protein 5 - 6; *Lef1*, lymphoid enhancer binding factor 1; *Dkk1- Dkk2*, dickkopf WNT signaling pathway inhibitor 1 - 2

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phosphorylated and degraded in the cytoplasm (Gordon and Nusse, 2006; Moon, 2005c). Canonical WNT signaling stimulates the expression of *Runx2* as well as other osteogenic genes as demonstrated in *in vitro* studies and animal models (Gaur et al., 2005; Krishnan et al., 2006). However, other studies show that canonical WNT signaling can also suppress osteogenesis by down-regulating the transcription of osteoblast-marker genes (Kahler and Westendorf, 2003; Kahler et al., 2006).

Osteogenesis is also influenced by environmental factors such as vitamins (Green et al., 2016; Aghajanian et al., 2015). Retinoic acid (RA), the main vitamin A derivate, is an essential signaling molecule for embryonic development and bone formation. RA is involved in cell proliferation and apoptosis, and is crucial in cell differentiation specifically in the limbs and the craniofacial skeleton (Laue et al., 2011). Human epidemiologic studies have demonstrated that both a shortage and an excess of RA can cause congenital diseases including facial anomalies such as cleft lip and/or palate, midfacial hypoplasia, and craniosynostosis (Chenevixtrench et al., 1992; Baroni et al., 2006). RA regulates gene expression by binding to cellular retinoic acid-binding proteins (CRABPs) that transport RA to the nucleus, where it binds to the retinoic acid receptors and retinoid X receptors (RARs/RXRs). This complex then binds to retinoic acid response elements (*Rare*) in the DNA thus activating target gene expression (Rhinn and Dolle, 2012).

Several studies show contradictory effects of RA on osteogenic differentiation, which may depend on the cell source and RA concentration. Varying concentrations of RA have been used to study its effects during osteogenic induction. The effects range from inhibition of differentiation and down-regulation of osteogenic marker genes (*Alp, Runx2, Sp7*) at low RA levels from 0.001 to 1 μM, to changes in cell morphology, decreased cell number and variable effects on mineralization at high RA levels (> 5 μM) (Lind et al., 2013; Bi et al., 2013; Mattinzoli et al., 2012; Masuda et al., 2015; Nagasawa et al., 2005). Concentrations commonly used are in the range of 0.01 to 1 μM (Lind et al., 2013; Bi et al., 2013; Masuda et al., 2015; Nagasawa et al., 2005). Additionally, studies on chondrocytes, embryonic stem cells and neural cells show a RA–WNT cross-regulation (Osei-Sarfo and Gudas, 2014; Yasuhara et al., 2010). We hypothesized that RA affects bone formation and mineralization through interaction with WNT signaling. To test our hypothesis, we induced osteogenesis in the broadly used pre-osteoblast cell line MC-3T3 in the presence or absence of RA in an average concentration of 0.5 μM based on previous studies (Lind et al., 2013; Bi et al., 2013; Masuda et al., 2015; Nagasawa et al., 2005). We found that RA strongly disrupts the differentiation and mineralization of these cells as shown by the down-regulation of osteogenic genes and alizarin red staining. This was accompanied by a down-regulation of WNT signaling.

2. Materials and methods

2.1. Cell culture

MC-3T3-E1 murine pre-osteoblasts were seeded (95,000 cells/cm^2) in proliferation medium composed of minimum essential medium α (MEMα, Gibco, Invitrogen; A1049001) with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (1% P/S, Sigma) and cultured at 37 °C in a humidified atmosphere of 5% CO2 in air. Once cultures were 90% confluent, the medium was switched to osteogenic differentiation medium composed of minimum essential medium α (MEMα, Gibco, Invitrogen; 22571), 10% FBS, 1% P/S, 10 mM β-glycerophosphate, 10 nM dexamethasone and 50 μg/ml vitamin C in the absence or presence of 0.5 μM RA (Sigma-Aldrich). Medium was changed every other day for 28 days. Each experiment was performed in triplicate and cells were harvested after 7, 14, 21 and 28 days.

2.2. Alkaline phosphatase activity assay

To assess early osteoblast differentiation, ALP activity levels were measured with a colorimetric assay to quantify the hydrolysis of p-nitrophenol. Cells were washed two times with PBS, then lysed with three freeze-thaw cycles in 1 ml of ddH2O, and stored at −20 °C. 80 μl of each sample or standard (0–25 nM serial dilutions of 4-dinitrophenol) were mixed with 20 μl buffer solution (0.5 M 2-amino-2-methyl-1-propanol) and 100 μl substrate solution (p-nitrophenyl phosphate) in a 96 well plate, and incubated at 37 °C for 60 min. The reaction was stopped by adding 20 μl of 0.3 M NaOH and absorbance was measured in a plate reader at 405 nm. The total DNA content was determined using the QuantifluorTM dsDNA kit (Promega) according to the manufacturer's protocol. The ALP activity values were expressed as nmol 4-NP/hour/ng DNA.

2.3. Alizarin red staining and quantification

Cells were washed with PBS, fixed for 15 min in 10% formalin and rinsed with water. One ml of 40 mM Alizarin red (pH 4.1–4.2) was added to each well at room temperature and the plates were shaken for 20 min. Unincorporated dye was discarded and cells were washed with water several times. To quantify the mineral deposition, incorporated Alizarin red was solubilized by the addition of 800 μl of 10% (v/v) acetic acid at room temperature for 30 min. The monolayer of cells was scraped off and transferred with the supernatant to a clean tube. Samples were heated at 85 °C for 10 min, transferred to ice for 5 min and then centrifuged at 20,000 *g*for 15 min. The supernatant was pipetted in a new tube and 75 μl of 10% ammonium hydroxide was added to neutralize the acid. Aliquots of 150 μl were read at 405 nm in a plate reader. The results were compared with a standard curve generated with increasing Alizarin red concentrations. Data were expressed as mM of Alizarin red.

2.4. Real-time quantitative PCR

Total RNA was isolated from the cells using the RNeasy MiniKit (Quiagen, Germany) according to the manufacturer's protocol. Equal amounts of RNA from each sample (1 μg) was reverse transcribed using the iScriptTM Reverse Transcriptase system (Bio-Rad, USA). Quantitative real-time PCR reactions were carried out in 25 μl containing 5 μl cDNA (12.5 ng), 4.5 μl RNA-free water, 2.5 μM forward and reverse primers and 12.5 μl SYBR Green Supermix (Bio-Rad). The amplifications were performed in a CX96 Real Time System (Bio-Rad) using the following conditions: initial denaturalization at 95 °C for 3 min, followed by 39 cycles performed at 95 °C for 15 s and 60 °C for 30 s. All data were normalized to the expression of three reference genes (*Gapdh, β-actin* and *18 s rRNA*). Relative expression was calculated according to the $2^{-\Delta Ct}$ method. Primers were obtained from Biolegio (Nijmegen, the Netherlands) and their sequences are summarized in Table 1.

2.5. Statistical analysis

Each culture experiment was performed in triplicate, and the results are presented as mean ± standard deviation. Differences between the groups were evaluated by the analysis of variance (ANOVA). Post hoc comparisons were made using the Bonferroni test. Differences were considered significant if $p < 0.05$. All statistical tests were performed with Graphpad Prism software (Graphpad software, La Jolla, CA, USA).

3. Results

3.1. Decreased mineralization by retinoic acid

MC-3T3 cells were cultured with or without (controls) 0.5 μM of RA

Table 1
Primer sequences.

Gene category	symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')
Housekeeping	*Gapdh4*	GGCAAATTCAACGGCACAGT	CCTCACCCCATTTGATGTTAGTG
	Bactin	CGGTTCCGATGCCCTGAGGCTCTT	CGTCACACTTCATGATGGAATTGA
	18 s rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Osteogenic marker	*RunX2*	CGGACGAGGCAAGAGTTTCA	GGATGAGGAATGCGCCCTAA
	Sp7	CCTACCCAGGAAGAAGCTCAC	TGGTTTTGGGGGCTGAAAGG
	Alp	CCAGCAAGAAGAAGCCTTTG	AACCCAGACACAAGCATTCC
	Bglap	CCGGGAGCAGTGTGAGCTTA	CCATACTGGTCTGATAGCTC
	Bsp	GTTTGAAGTCTCCTCTTCCTCC	TTTATCCTCCTCTGAAACGGT
RA responsive	*Rarβ*	GAAAACGACGACCCAGCAAG	TTACACGTTCGGCACCTTTC
	Cyp26b1	GATCCTACTGGGCGAACACC	GGAGAAGACCTTGCGCTTGT
	Crabp2	TGATCTCGACTGCTGGCTTG	TCCCATCGGGTTCCCATAAAG
Wnt components	*Wnt10b*	ACCACGACATGGACTTCGGAG	CCGCTTCAGGTTTTCCGTTACC
	Wnt11	CTCAAGACCCGCTACCTGTC	ACCACTCTGTCCGTGTAGGG
	Wnt5a	ATGCAGTACATTGGAGAAGGTG	CGTCTCTCGGCTGCCTATTT
	Lrp5	ACGTCCCGTAAGGTTCTCTTC	GCCAGTAAATGTCGGAGTCTAC
	Lrp6	TGGGGCAAGCACACTGATAAAAA	TGGGGAGAAGTGCCAAAGATAGAAC
	cMyc	GGTTTGCCTCTTCTCCACAG	TCCTGTACCTCGTCCGATTC
	Lef1	TATGAACAGCGACCCGTACA	TCGTCGCTGTAGGTGATGAG
	cJun	CGCAACCAGTCAAGTTCTCA	GCCAGCCTCCACATGGCGTC
	Dkk1	CGGGGGATGGATATCCCAGAA	ACGGAGCCTTCTTGTCCTTTG
	Dkk2	CCTGCATGCTCTGTAGGAGG	GATTCCATTATTGCAGCGGGT

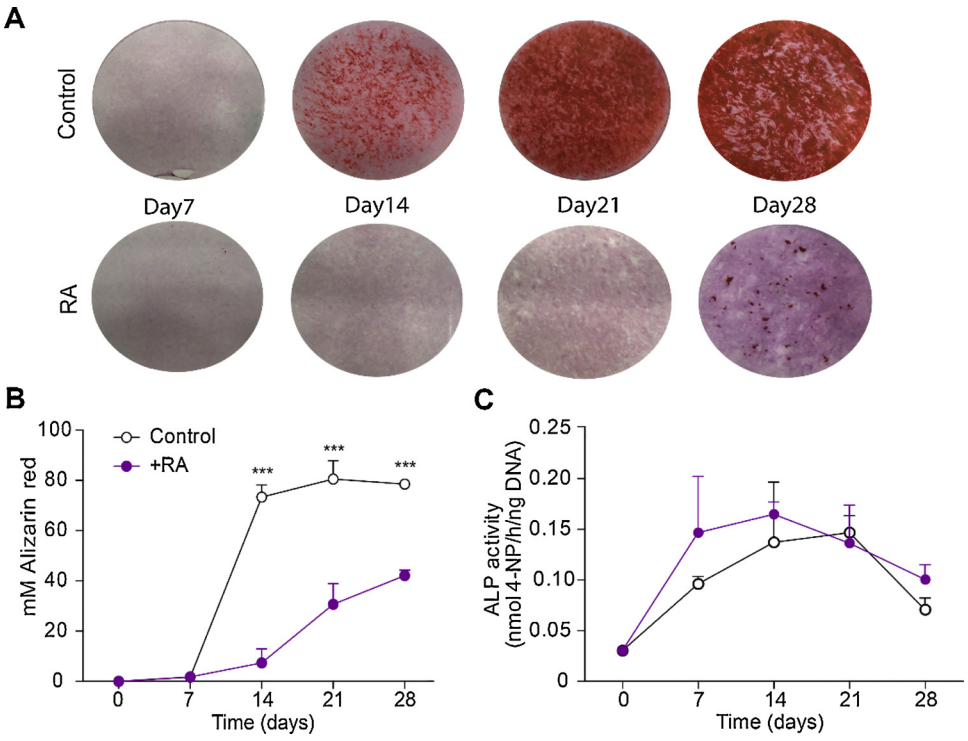

Fig. 1. RA inhibits mineralization of MC-3T3 cells without affecting ALP activity. MC-3T3 cells were cultured with and without (control) 0.5 μM RA for up to 28 days. (A) Alizarin Red staining was performed on controls and RA treated MC-3T3 cells at 7, 14, 21 and 28 days. The Figure shows representative pictures of the staining at each time point. (B) Semi-quantification of mineralization after Alizarin Red staining. RA treatment significantly decreased the calcium accumulation compared to the control group after 14, 21 and 28 days. *** $p < 0.001$. (C) Quantification of ALP activity. RA did not affect ALP activity. Data are represented as the average ± SD (N = 3).

in osteogenic induction medium for 28 days to investigate matrix mineralization using Alizarin red staining. The controls showed a clear increase in Alizarin red staining, starting from day 14 (Fig. 1A). Alizarin Red staining showed a significant reduction by RA at days 14, 21 and 28 as compared to the control group of about 90%, 62% and 46% respectively ($p < 0.001$, Fig. 1B).

3.2. Retinoic acid does not modulate ALP activity

Next, we evaluated the effects of RA treatment on ALP activity in cultured MC-3T3 cells to assess early osteoblast differentiation. In the controls, ALP activity increased from day 0 to 21 and thereafter decreased. RA did not affect ALP activity significantly ($p > 0.05$, Fig. 1C). The peak levels of ALP activity at day 14 were 0.16 ± 0.05 and 0.13 ± 0.012 nmol 4-NP/h/ng DNA, with and without RA, respectively.

3.3. Confirmation of retinoic acid activity

To confirm the functionality of RA, we evaluated the expression levels of Cytochrome P450 Family 26 Subfamily B Member 1 (*Cyp26b1*), Cellular Retinoic Acid Binding Protein 2 (*Crabp2*) and Retinoic Acid Receptor Beta (*Rarβ*) that are involve in the retinol-dependent signaling (Fig. 2). In controls, the expression of *Rarβ* was continuously low, while RA induced an up-regulation of *Rarβ* at 7 and 14 days compared to the controls ($p < 0.01$, Fig. 2A). In the controls, *Cyp26B1* expression increased at days 7 and 14 followed by a decrease until the end of culture (Fig. 2B). RA induced a significant up-regulation of *Cyp26b1* at days 21 and 28 ($p < 0.05$). In controls, a gradual down-regulation of *Crabp2* expression occurred (Fig. 2C). RA strongly up-

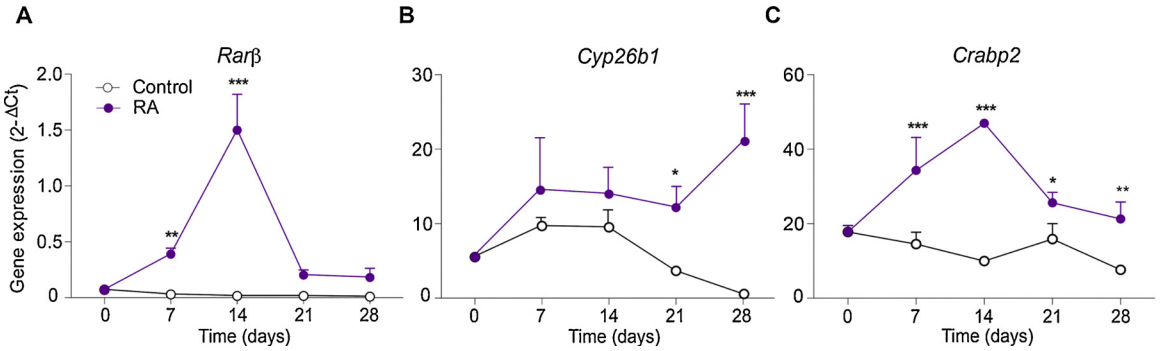

Fig. 2. The expression of RA responsive genes. RA significantly stimulated the expression of three metabolic genes known to respond to RA: *Rarβ, Cyp26b1,* and *Crabp2*. (A) RA significantly stimulated *Rarβ* expression at days 7 and 14. (B) *Cyp26b1* expression was significantly increased at days 21 and 28. (C) RA significantly stimulated *Crabp2* expression at all time points. Data are represented as the average ± SD (N = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the controls at each time point.

regulated *Crabp2* expression from day 7 until day 28 of treatment compared to controls ($p < 0.01$).

3.4. Retinoic acid affects osteogenic marker genes

To evaluate the influence of RA on osteoblast differentiation, we examined the expression of early (*Runx2, Sp7* and *Alp*) and late (*Bglap* and *Bsp*) osteogenic related genes (Fig. 3). In the controls, *Runx2* expression increased slightly from day 0 to day 28. Compared to the controls, *Runx2* was strongly up-regulated by RA at days 14, 21 and 28 ($p < 0.05$). For *Sp7*, gene expression slightly increased from day 0 to 7 in the controls, followed by a stronger up-regulation at days 14 and 21. Compared to the controls, RA inhibited *Sp7* expression from day 14 on ($p < 0.05$, Fig. 3A). *Alp* expression was not affected by RA treatment and showed a gradual increase with a peak at day 21 followed by a drop to day 28.

The expression of the late osteogenic marker gene *Bglap* in the controls slowly increased from day 0 to 14 followed by a strong upregulation at days 21 and 28. RA induced an up-regulation of *Bglap* at days 7 and 14, followed by a down-regulation at days 21 and 28 compared to the controls ($p < 0.05$). In the controls, *Bsp* expression progressively increased over the 28 days. Similarly, *Bsp* expression in RA-treated cells showed an increase in time with a peak at day 21 ($p < 0.05$, Fig. 3B).

3.5. RA inhibits WNT signaling during osteogenic differentiation

To evaluate the effects of RA on WNT signaling during osteogenic differentiation, the expression of several osteogenic marker genes and WNT genes was determined by qPCR (Fig. 4). Four different sets of WNT-related genes were evaluated: ligands (Canonical: *Wnt10b*. Non-canonical: *Wnt11* and *Wnt5a*), co-receptors (*Lrp5* and *Lrp6*), target genes (*cMyc*, *Lef1* and *cJun*) and WNT inhibitors (*Dkk1* and *Dkk2*). The controls showed a constant expression of the canonical WNT ligand *Wnt10b* (Fig. 4A). Similar *Wnt10b* expression was observed in RA-treated cells with a down-regulation at day 28 ($p < 0.01$). *Wnt11* expression was highly increased at days 14 and 21 in the controls, with a reduction at day 28 (Fig. 4B). Compared to the controls, RA inhibited *Wnt11* expression over the 28 days of treatment ($p < 0.001$, at days 14 and 21). *Wnt5a* expression in the controls showed a dip in expression

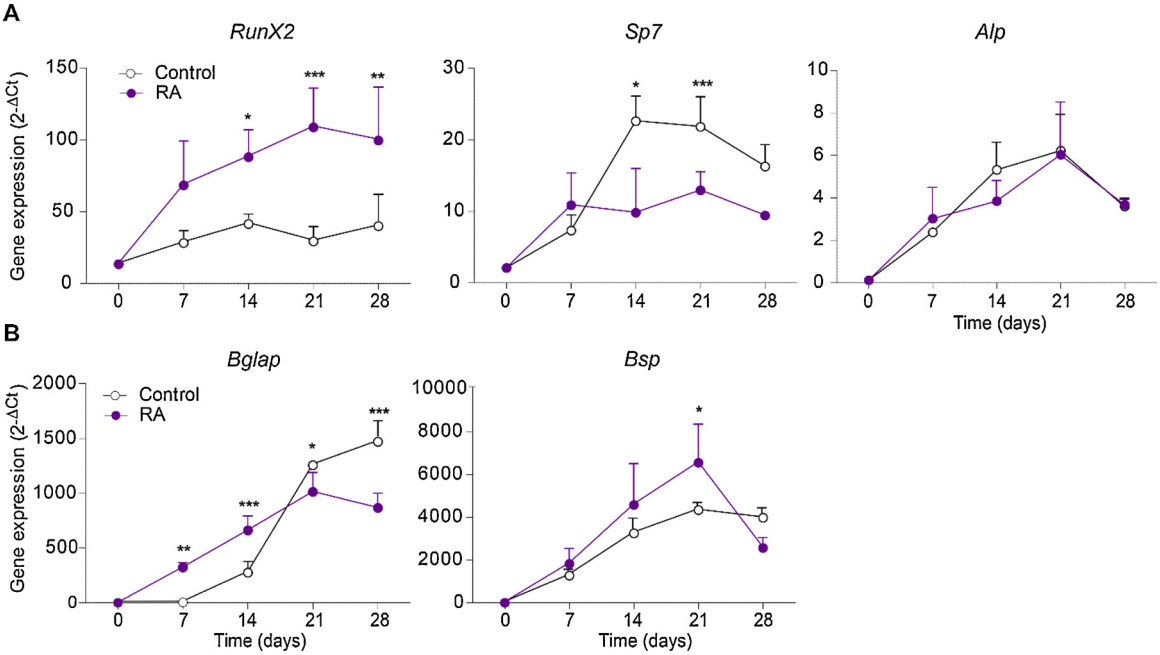

Fig. 3. RA affects the expression of osteogenic genes during MC-3T3 differentiation and mineralization. MC-3T3 cells were cultured with or without (control) 0.5 μM RA for up to 28 days. (A) The expression of early osteogenic genes: *Runx2, Sp7, Alp*; and (B) the expression of late osteogenic genes: *Bglap*, *Bsp*. Data are represented as the average ± SD (N = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the controls at each time point.

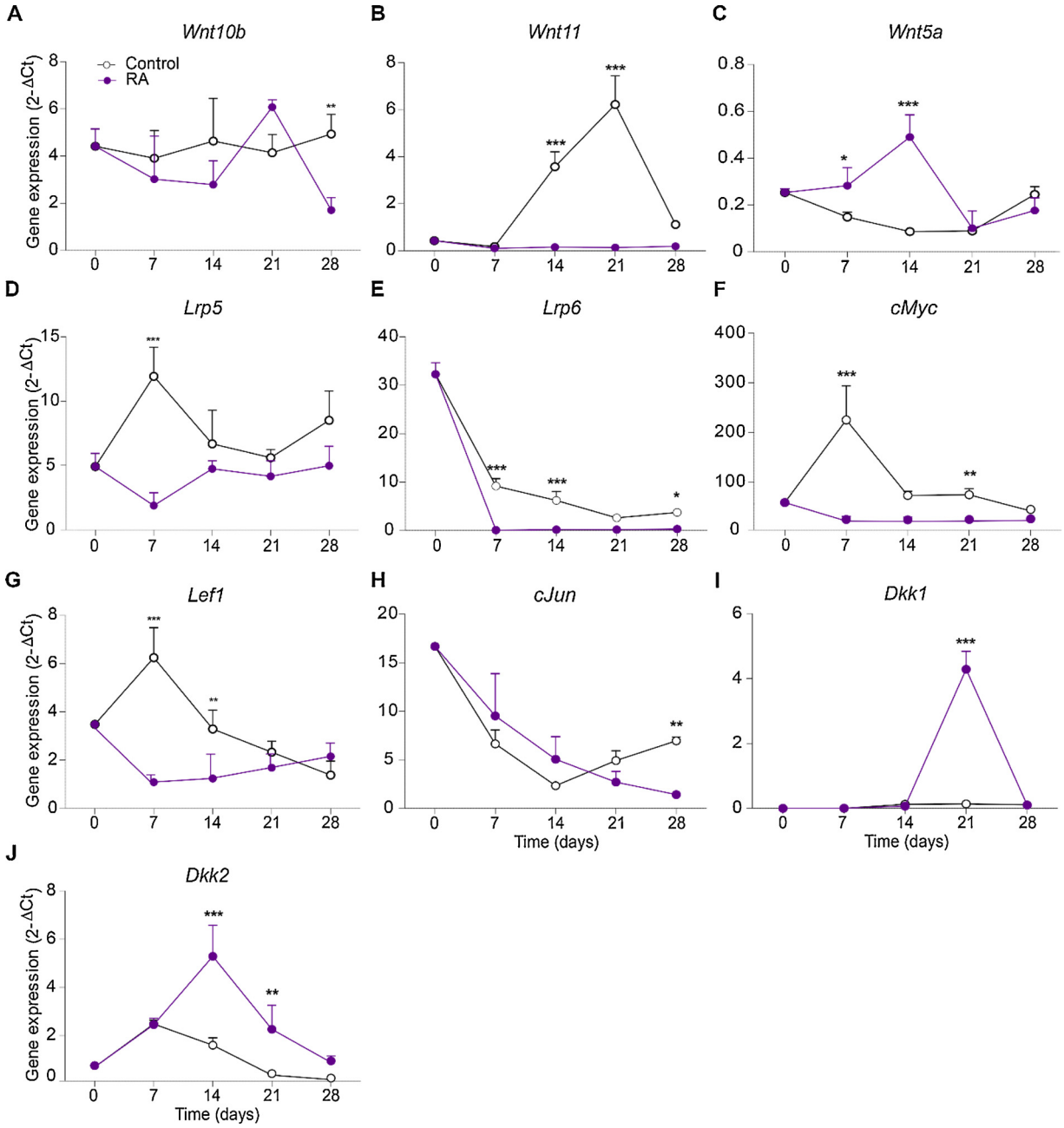

Fig. 4. RA inhibits WNT signaling by down-regulation of WNT components and up-regulation of WNT inhibitors. MC-3T3 cells were cultured with or without (control) 0.5 μM RA for up to 28 days (A, B, C) Wnt ligands: *Wnt10b*, *Wnt11* and *Wnt5a*. (D, E) WNT co-receptors: *Lrp5* and *Lrp6*. (F, G, H) WNT target genes: *cMyc*, *Lef1*, and *cJun*. (I, J) WNT inhibitors: *Dkk1* and *Dkk2*. Data are represented as the average ± SD (N = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the controls at each time point.

between the start and end of the experiment, but RA significantly up-regulated *Wnt5a* at days 7 and14 ($p < 0.05$, Fig. 4C).

The WNT co-receptor *Lrp5* was up-regulated at day 7 in the controls, followed by a down-regulation (Fig. 4D). Compared to the controls, RA inhibited *Lrp5* expression only at day 7 ($p < 0.01$), after which expression levels were similar. *Lrp6* expression in the controls showed a gradual decrease until day 28 (Fig. 4E). In the presence of RA, *Lrp6* expression was further reduced significantly at days 7, 14 and 28 ($p < 0.05$).

The WNT target gene *cMyc* showed an up-regulation at day 7 in the controls followed by a marked down-regulation at day 14 (Fig. 4F). In RA-treated cells, *cMyc* expression was down-regulated compared to the controls over the 28 days of treatment ($p < 0.01$ at days 7 and 21). In the controls, *Lef1* showed a peak in expression at day 7 followed by a constant decrease until day 28 (Fig. 4G). RA significantly down-regulated expression at days 7 and 14 compared to the controls ($p < 0.01$). *cJun* expression in the controls progressively decreased until day 14 followed by an increase at days 21 and 28 (Fig. 4H). RA treatment inhibited *cJun* expression significantly at day 28 compared to the controls ($p < 0.01$).

The expression of the Wnt inhibitor *Dkk1 was* low over the 28 days of culture in the controls, but RA induced a marked up-regulation at day 21 ($p < 0.001$, Fig. 4I). *Dkk2* expression in the controls showed a slight increase at days 7 and 14 days followed by a decrease (Fig. 4J). RA-treated cells showed a *Dkk2* up-regulation at days 14 and 21 compared to the controls ($p < 0.01$).

4. Discussion

In humans, normal serum RA concentrations range from 1.99 to 2.7 ng/ml (Zhou and Li, 2018; Liu et al., 2016; Manickavasagar et al., 2015; Moulas et al., 2006). As RA plays an important regulatory role in early embryonic development, an excess (> 2.7 ng/ml) can induce cleft palate, heart defects and blindness among others (Laue et al., 2011; Chenevixtrench et al., 1992; Weston et al., 2003; Bastos Maia et al., 2019). Additionaly, shortage of RA (< 1.99 ng/ml) has been related to fetal loss, clef palate, hydrocephaly, heart malformations and anophthalmia (Maden, 2001; Kostetskii et al., 1996). WNT signaling is involved in bone formation by promoting osteoblast differentiation, and can be negatively regulated by RA as reported in other tissues (Elizalde et al., 2011). We hypothesized that an excessive level of RA (e.g. 150 ng/ml or 0.5 μM) reduces bone formation through inhibition of WNT signaling. Therefore, we have investigated the effects of 0.5 μM RA on the differentiation and mineralization of MC-3T3 pre-osteoblasts as well as the expression of WNT signaling components.

To confirm the activity of RA and the activation of RA signaling, we evaluated the expression of three retinoic acid signaling genes (*Rarb*, *Cyp26b1* and *Crabp2*) known to respond to RA (Yorgan et al., 2016; Spoorendonk et al., 2008). As expected, these RA target genes were strongly induced in cells treated with RA.

In addition, RA strongly inhibited the mineralization process in MC-3T3 pre-osteoblast cultures as shown by the alizarin red staining. Similar effects on mineralization by RA have been found in primary fibroblasts and osteoblasts, and in cell lines as well as cultured bone explants derived from mouse, human or rat (Lind et al., 2013; Bi et al., 2013; Yorgan et al., 2016; Shao et al., 2016; Zhang et al., 2010; Chen et al., 2010). Inhibition of mineralization has also been observed in transfected mouse osteoblasts that constitutively express one of the nuclear receptors of RA signaling, the retinoid-related orphan receptor-β (*Rorβ)* (Theodosiou et al., 2010; Stehlin-Gaon et al., 2003). These cells show an up-regulation of decorin and matrix gla protein, which inhibit TGF*β* and BMP2, reducing the production of ECM proteins (Roforth et al., 2012, 2013). Therefore it seems that mineralization depends on the production of ECM proteins controlled by growth factors such as TGF*β* and BMP2. Additionally, it has been proven that the inhibitory effects of RA on mineralization of osteoblasts are also RAR-dependent in mouse and human (Lind et al., 2013; Green et al., 2018). These data suggest that RA exerts an anti-osteogenic effect by inhibiting normal ECM formation, which is a prerequisite for mineralization.

In conjunction with mineralization, the activity of ALP is often investigated due to its vital role in the regulation of phosphate transport (Green et al., 2017). Our results show that the ALP activity increased over the first two weeks of osteogenic differentiation and then declined, which was not affected by RA. One previous study shows similar results in MC-3T3 cells (Bi et al., 2013) but, generally, ALP activity is induced by RA in a dose-dependent manner. In spite of this, mineralization is reduced in osteoblasts and pre-osteoblast from mouse and human (Shao et al., 2016; Zhang et al., 2010; Liu et al., 2014; Li et al., 2014). Several studies show that RA inhibits the expression and deposition of collagen, the fibrils where the mineralization starts (Mattinzoli et al., 2012; Togari et al., 1991; Pan et al., 1992). Additionally, RA also stimulates the expression of collagenase through nuclear receptors such as RARs (Varghese et al., 1994). Despite the normal ALP activity, the lack of collagen fibrils caused by RA might prevent the deposition of hydroxyapatite crystals.

Among the osteogenic genes, the lineage factor *Runx2* was markedly up regulated by RA. Normally, osteoprogenitor cells undergo commitment induced by *Runx2* before mineralization (Komori, 2002). Subsequently, *Runx2* expression drops, which allows the start of the mineralization (Komori, 2008; Adhami et al., 2014; Liu et al., 2001; Takarada et al., 2013). Over-expression of *Runx2* in mice impairs ECM production by the down regulation of bone matrix proteins such as type I collagen. These transgenic mice also show a reduction in mineralization, osteopenia and fragile bones (Liu et al., 2001; Adhami et al., 2015; Komori, 2010). In conclusion, the upregulation of *Runx2* by RA impairs the normal process of osteoblast differentiation and mineralization.

Our data also indicate an inhibition of WNT signaling by RA through the increased expression of WNT inhibitors and a reduced expression of WNT target genes. It is demonstrated that the promoter region of the *Runx2* gene contains a functional TCF regulatory element responsive to canonical WNT signaling (Gaur et al., 2005). *Runx2* therefore is a direct target of canonical WNT signaling (Reinhold and Naski, 2007; Cai et al., 2016). This allows pre-osteoblasts to differentiate (*Runx2* up-regulation) and mineralize (*Runx2* down-regulation). Our results suggest that RA disrupts this regulation of *Runx2* through inhibition of WNT signaling.

Differently from *RunX2*, the expression of the early osteogenic marker *Sp7* was notably down regulated by RA. Similar results have been shown in skull mesenchymal tissue and mouse calvaria osteoblasts from β-catenin conditional knockout mice and heterozygous LRP5 knockout mice, respectively (Hill et al., 2005; Li et al., 2018). SP7 is essential for embryonic osteoblast differentiation and bone formation (Nakashima et al., 2002). Sp7 mutations in human, mice and zebrafish generate several defects in bone growth and mineralization (Nakashima et al., 2002; Lapunzina et al., 2010; Kague et al., 2016). These and our data suggest that inhibition of WNT signaling by RA delays osteoblast differentiation and mineralization through upregulation of *Runx2* and suppression of *Sp7*. Apparently, osteoblast commitment is increased but the terminal differentiation and mineralization of the cells is decreased.

RA also inhibited the expression of specific WNT components such as low density lipoprotein receptor-related protein 5 and 6 (*Lrp5* and *Lrp6*), two well-known canonical WNT signaling co-receptors. Loss-of-function mutations in *Lrp5* in mice result in decreased bone mass (Boyden et al., 2002; Akhter et al., 2004). To activate canonical WNT signaling, WNT ligands bind to a complex formed by LRP5 or LRP6 with Frizzled protein. The reduced expression of *Lrp5* and *Lrp6* induced by RA might contribute to reduced WNT signaling, and inhibition of differentiation and subsequent mineralization. In addition, our data show an RA-induced up-regulation of the inhibitors *Dkk1* and *Dkk2* that act as soluble inhibitors of canonical WNT signaling (Pinzone et al., 2009). These inhibitors bind to *Lrp5* and *6* leading to the internalization and degradation of the co-receptors (Kim et al., 2013). It has also been shown that deletion of *Dkk1* in mice increases bone mass (Morvan et al., 2006), while over-expression of *Dkk1* leads to osteopenia and skeletal deformities (Li et al., 2006). These results suggest that RA negatively regulates canonical WNT signaling by reducing the expression of Wnt components and stimulating the expression of WNT inhibitors.

Cross-talk between WNT and RA signaling has been reported before in different cellular and animal models. Lrp6 knockout mouse embryos show an ectopic and increased expression of the RA-synthesizing enzyme Raldh3 in the palate and enhanced RA signaling (Song et al., 2009). This led to bone defects in the limb and palate. Another study also indicate that WNT inhibitors play an importand role in the RA-WNT cross–talk. Raldh1-3 knockout mice showed an excessive WNT signaling by a down regulation of DKK2 and an up-regulation of Axin2 (Kumar and Duester, 2010). Activation of RA signaling by RAR agonists, also inhibited WNT signaling in mouse osteoblasts by an increased expression of Sfrp4, and reduced β-catenin and Axin2 expression. This delayed differentiation and mineralization of the osteoblasts (Green et al., 2017). Similarly, RA treatment can inhibit WNT signaling by reducing β-catenin translocation to the nucleus in human hepatic cancer cells, pancreatic mouse cells and mouse embryonic stem cells (Osei-Sarfo and Gudas, 2014; Zhu et al., 2015; Xiao et al., 2015). These data show that also in other models increased RA signaling can impair WNT signaling and bone formation through the activation of WNT inhibitors.

In summary, this study shows that an excess of RA strongly disrupts the differentiation and mineralization of MC-3T3 pre-osteoblasts. Our data in combination with previous studies indicate that this is

effectuated through inhibition of canonical WNT signaling by a down-regulation of WNT components and an up-regulation of WNT inhibitors. This mechanism might also explain the already known inhibitory effects of RA on bone formation in animal models and congenital bone disorders related to vitamin A excess.

Declaration of Competing Interest

None.

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