

# Excess vitamin a might contribute to submucous clefting by inhibiting WNT-mediated bone formation

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## Abstract

**Objectives:** Cleft lip and/or palate (CLP) is a common craniofacial birth defect caused by genetic as well as environmental factors. The phenotypic spectrum of CLP also includes submucous clefts with a defect in the palatal bone. To elucidate the contribution of vitamin A, we evaluated the effects of the vitamin A metabolite all-trans retinoic acid (ATRA) on the osteogenic differentiation and mineralization of mouse embryonic palatal mesenchymal cells (MEPM).

**Setting and Sample Population:** MEPM cells were isolated from the prefusion palates of E13 mouse embryos from three different litters.

**Materials and Methods:** MEPM cells were cultured with and without 0.5  $\mu$ M ATRA in osteogenic medium. Differentiation was analysed by the expression of osteogenic marker genes and alkaline phosphatase (ALP) activity after 1, 2, and 7 days. The expression of Wnt marker genes was also analysed. Mineralization was assessed by alizarin red staining after 7, 14, 21, and 28 days.

**Results:** The bone marker genes *Sp7*, *Runx2*, *Alpl*, and *Col1a1* were inhibited  $10\% \pm 2\%$ ,  $59\% \pm 7\%$ ,  $79\% \pm 12\%$  and  $57\% \pm 20\%$  ( $P < .05$ ) at day 7. ALP activity was inhibited at days 1 and 7 by  $35 \pm 0\%$  ( $P < .05$ ) and  $23 \pm 6\%$  ( $P < .001$ ). ATRA also inhibited mineralization at 3 and 4 weeks. Finally, expression of the universal Wnt marker gene *Axin2* was strongly reduced, by  $31 \pm 18\%$  ( $P < .001$ ), at day 7.

**Conclusion:** Our data indicate that ATRA (vitamin A) inhibits bone formation by reducing Wnt signalling. This might contribute to the molecular aetiology of submucous clefting.

## KEYWORDS

mineralization, mouse embryonic palatal mesenchymal cells, osteoblast differentiation, retinoic acid, WNT signalling

## 1 | INTRODUCTION

A common craniofacial birth defect in humans is cleft lip and/or palate (CLP), with an occurrence rate of 1/700 live births.<sup>1</sup> CLP aetiology includes both genetic and environmental factors, but the

mechanisms of clefting of the primary (hard) palate that involve the lip and/or palate seem to be different from those of the secondary (soft) palate.<sup>2</sup> Use of medication, tobacco, or alcohol, or a vitamin imbalance during pregnancy, increases the risk of CLP.<sup>1</sup> CLP can involve the palate, alveolus, and/or lip, and can be divided into unilateral or

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bilateral CLP, which can vary in severity.<sup>1</sup> CLP can further be non-syndromic or syndromic.<sup>1</sup> A submucosal cleft only involves defects in the bone and muscles of the palate, while the etiological risk factors appear to overlap partially with CLP and CP.<sup>3</sup>

The formation of the secondary palate involves downward growth of the palatal shelves, elevation above the tongue, and fusion in the midline.<sup>4</sup> Subsequently, intramembranous ossification occurs. In humans, the palate is formed between embryonic weeks 6 and 12 and is similar in all mammals.<sup>4</sup> Hence, mice are often used in research on palatogenesis and CLP.<sup>4</sup> In mice, the palate develops from embryonic day 11.5 to day 15.5.<sup>5</sup> During ossification, mesenchymal cells differentiate into osteoblasts initiated by, among others, the transcription factors runt-related transcription factor 2 (RUNX2) and SRY-box transcription factor 9 (SOX9).<sup>6</sup> Further differentiation and mineralization are regulated by factors such as osterix (SP7), osteocalcin (BGLAP), and wingless-type MMTV integration site family (Wnt) signalling.<sup>6</sup>

Wnt proteins bind to the frizzled receptor and co-receptor LRP5/6, causing  $\beta$ -catenin to translocate to the nucleus and activate transcription factors of the T cell-specific transcription factor/lymphoid enhancer binding factor (TCF/LEF) family, which control the expression of target genes.<sup>7</sup> Wnt signalling generally increases bone formation in rats and mice by stimulating osteoblast differentiation.<sup>8</sup>

All-trans retinoic acid (ATRA), the active derivate of vitamin A, is a crucial regulator of embryonic development, including palatogenesis.<sup>9</sup> Intracellular ATRA can be degraded by Cyp26b1; alternatively, it binds to cellular retinoic acid-binding proteins (CRABPs) and is then translocated to the nucleus.<sup>10</sup> There, ATRA binds to retinoic acid receptors (RARs) such as Rarb, which form a heterodimer with retinoid X receptors (RXRs) to induce target gene expression.<sup>10</sup> Epidemiological studies show an increased risk of CLP when excessive or insufficient amounts of vitamin A are used by the pregnant mother.<sup>11,12</sup> In vivo studies in rats and mice also show a causative role for ATRA in cleft palate.<sup>13,14</sup> In vitro studies on chondroprogenitor cells, mouse adipose-derived mesenchymal cells, and mouse embryonic palatal mesenchymal cells (MEPM cells) show an inhibition of cell proliferation, differentiation, and mineralization by ATRA.<sup>15-18</sup> Interestingly, studies in certain non-primary cell lines indicate this might be caused by inhibition of Wnt signalling.<sup>19,20</sup> An in vivo study in mice also shows that Wnt signalling in the developing palate is inhibited by ATRA and causes CLP.<sup>21</sup> Since ATRA interacts with Wnt signalling in several in vivo and in vitro models, we set out to investigate this in primary MEPM cells isolated from mouse embryos just before the fusion stage. We hypothesized that ATRA inhibits the osteogenic differentiation and mineralization of MEPM cells by reducing Wnt signalling.

## 2 | MATERIAL AND METHODS

### 2.1 | Cell culture

MEPM cells were isolated from E13.5 fetuses from three pregnant mice. E13.5 is the embryonic stage just before the fusion of the palatal shelves. The experiments were approved by the board for animal

experiments of Radboud University, Nijmegen (RUDEC 2015-0080). Cells were isolated from the palates by enzymatic digestion and plastic adherence. All cells were mixed and frozen in liquid nitrogen after two passages. For the dose-response study, MEPM cells were cultured in proliferation medium composed of minimum essential medium  $\alpha$  (MEM  $\alpha$ ; Gibco) with 10% fetal bovine serum (FBS; Gibco), 10 000 U/mL penicillin, 10 000  $\mu$ g/mL streptomycin (2% Pen Strep, Gibco) and cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Next, the cells were distributed in 24-well plates at a density of 25 000 cells/well and grown until confluent. The medium was then switched to osteogenic medium composed of MEM  $\alpha$  (Gibco), 10% FBS, 2% Pen Strep, 10 mM  $\beta$ -glycerophosphate (Sigma-Aldrich), 50  $\mu$ g/mL vitamin C (Sigma-Aldrich) with 0, 0.1, 0.5, or 1  $\mu$ M ATRA (Sigma-Aldrich), and a final concentration of 0.05% dimethyl sulfoxide (DMSO). As no significant differences between control groups with and without DMSO were observed, only data from the control group with DMSO are shown. The medium was changed every other day for up to 7 days. Cultures for the analysis of gene expression were grown in triplicate.

In the main study, MEPM cells were distributed in 24-well plates as described above. Upon confluency, the medium was switched to osteogenic medium with 0.0 or 0.5  $\mu$ M ATRA. Only the control group with DMSO is shown, because no significant differences between groups with and without DMSO were observed. Cells were cultured for up to 28 days. Cultures for the alkaline phosphatase (ALP) activity assay and gene expression analyses were grown in triplicate and cells were harvested after 1, 2, and 7 days. Cultures for the mineralization assay were performed in duplicate and cells were harvested on days 7, 14, 21, and 28.

### 2.2 | Alkaline phosphatase activity assay

ALP activity was measured with a colorimetric assay to quantify the hydrolysis of p-nitrophenol (p-NP). Cells were washed once with Dulbecco's phosphate-buffered saline (DPBS) (Gibco), then lysed with two freeze-thaw cycles in 1 mL of water and stored at -20°C. Next, 100  $\mu$ L of standard (0-25 nM serial dilutions of 4-NP (Sigma-Aldrich)) or 80  $\mu$ L of sample mixed with 20  $\mu$ L of buffer solution (0.5 M 2-amino-2-methyl-1-propanol, pH 10.3; Sigma-Aldrich) was mixed with 100  $\mu$ L of substrate solution (5 mM p-nitrophenyl phosphate (Sigma-Aldrich) in a 96-well plate and incubated at 37°C for 60 min. The reaction was stopped by adding 100  $\mu$ L of 0.3 M NaOH, and absorbance was measured in a Synergy HTX Multi-Mode Microplate Reader at 405 nm. Total DNA was determined using the Quantifluor dsDNA kit (Promega). ALP activity values were expressed as nmol 4-NP/h/ng DNA.

### 2.3 | Quantitative real-time PCR

Total RNA was isolated using the miRNeasy Micro Kit (Qiagen). Equal amounts of RNA from each sample were reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). RT-qPCR reactions were



TABLE 1 Primer sequences

Gene category	Symbol	Forward primer (5'-3')	Reverse primer (5'-3')
Housekeeping genes	<i>Gapdh</i>	GGCAAATTCACGGCACA	GTTAGTGGGGTCTCGCTCCTG
	<i>Actb</i>	CGGTTCCGATGCCCTGAGGCTCTT	CGTCACACTTCATGATGGAATTGA
	<i>18S rRNA</i>	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
Osteogenic markers	<i>Runx2</i>	CGGACGAGGCAAGAGTTTCA	GGATGAGGAATGCGCCCTAA
	<i>Sp7</i>	CCTACCCAGGAAGAAGCTCAC	TGGTTTTGGGGGCTGAAAGG
	<i>Alpl</i>	GCACCTGCCTTACCAACTCT	GTGGAGACGCCCATACCATC
	<i>Col1a1</i>	AAGGTGCCAATGGTGCTC	ACCAGTGTCTCCTTTGTTGC
	<i>Bglap</i>	CCGGGAGCAGTGTGAGCTTA	CCATACTGGTCTGATAGCTC
ATRA-responsive genes	<i>Rarb</i>	GAAAACGACGACCCAGCAAG	TTACACGTTCCGGCACCTTTC
	<i>Cyp26b1</i>	GATCCTACTGGGCGAACACC	GGAGAAGACCTTGCGCTTGT
	<i>Crabp2</i>	GCTAAAAGCTCTGGGGGTGAA	TGATCTCGACTGCTGGCTTG
Wnt components	<i>Axin2</i>	GGTTCGGCTATGTCTTTGC	CAGTGCCTCGCTGGATAACTC
	<i>Myc</i>	GGTTTGCCTCTTCCACAG	TCCTGTACCTCGTCCGATTC
	<i>Lef1</i>	TATGAACAGCGACCCGTACA	TCGTCGCTGTAGGTGATGAG
	<i>Lrp5</i>	ACGTCCCCTAAGGTTCTCTTC	GCCAGTAAATGTCGGAGTCTAC
	<i>Lrp6</i>	TGGGGCAAGCACACTGATAAAAA	TGGGGAGAAGTGCCAAAGATAGAAC

carried out in 25  $\mu$ L containing 5  $\mu$ L diluted cDNA, 4.5  $\mu$ L RNase-free water, 3  $\mu$ L of 2.5  $\mu$ M forward and reverse primers, and 12.5  $\mu$ L SYBR Green Supermix (Bio-Rad). The amplifications were performed in a CFX96 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*, *Actb*, and 18S rRNA). Relative expression was calculated according to the  $2^{-\Delta C_t}$  method. Primer (Sigma-Aldrich; Biologio) sequences are shown in Table 1.

## 2.4 | Mineralization assay

Staining of the cells was performed with the Osteogenesis Quantitation Kit (Merck KGaA). Pictures were taken to monitor mineralization. To quantify mineral deposition, incorporated alizarin red was solubilized and absorbance was compared with a standard curve of alizarin red concentrations (0–2 mM). Alizarin red data are expressed as mM.

## 2.5 | Statistical analysis

Each culture experiment was performed in triplicate (except for the mineralization assay) and the results are presented as mean  $\pm$  SD. Differences between the groups in the dose–response study were evaluated at day 7 by one-way analysis of variance (ANOVA), with the control group set at 100%. Differences between the groups in the main study were evaluated by two-way ANOVA (except for the mineralization assay, where N was too small). Post-hoc comparisons were made using the Bonferroni test. Differences were considered

significant for values of  $P < .05$ . All statistical tests were performed with Graphpad Prism 5 software (Graphpad Software).

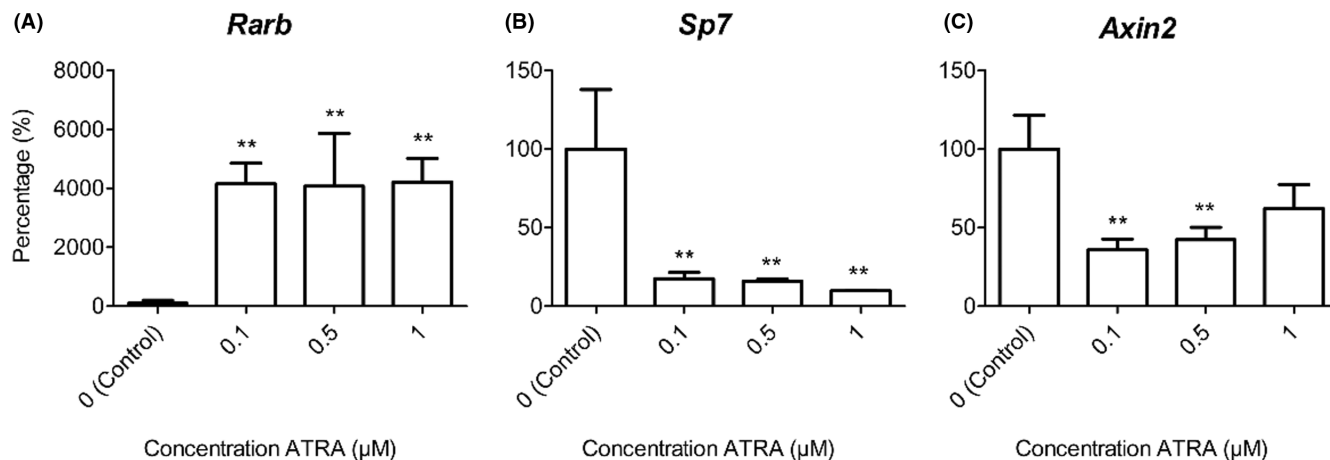
## 3 | RESULTS

### 3.1 | Dose–response effects of ATRA

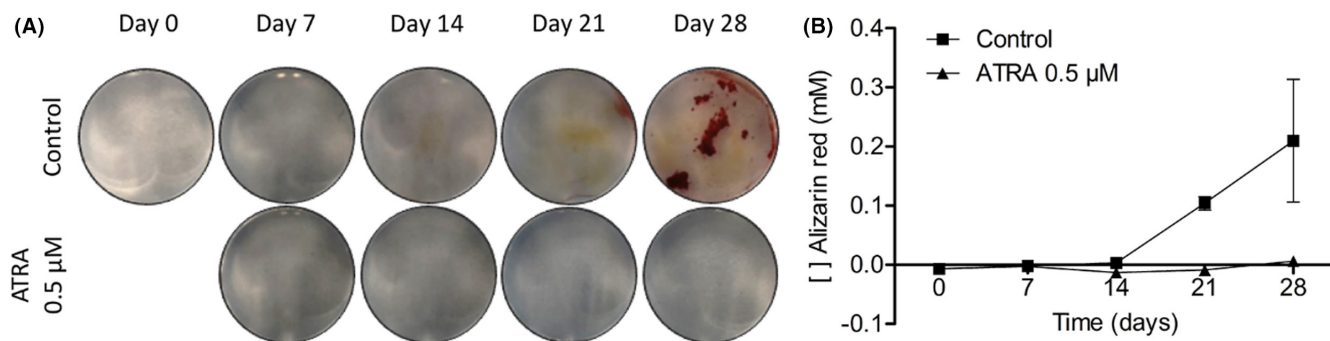
To decide which concentration to use for the main study, we analysed the gene expression of a retinoic acid–responsive gene, an osteogenic differentiation marker, and a Wnt signalling marker gene (retinoic acid receptor beta [*Rarb*], *Sp7*, and axis inhibition protein 2 [*Axin2*], respectively) using 0.1, 0.5, and 1  $\mu$ M ATRA. The expression of *Rarb* was upregulated about 40-fold in all three ATRA-treated groups as compared with the controls ( $P < .01$ , Figure 1A). In contrast, the expression of *Sp7* was downregulated about 6-fold in the three ATRA-treated groups ( $P < .01$ , Figure 1B). The expression of *Axin2* was downregulated more than 2-fold only in the groups treated with 0.1 and 0.5  $\mu$ M ATRA ( $P < .01$ , Figure 1C). There were no significant differences between the control groups with and without DMSO for any of the genes and there were no significant differences between the three ATRA concentrations. We therefore chose the average concentration of 0.5  $\mu$ M ATRA for the main experiments.

### 3.2 | ATRA inhibits MEPM cell mineralization

MEPM cells were cultured with and without 0.5  $\mu$ M of ATRA in osteogenic medium for up to 28 days and stained with alizarin red to examine mineralization. The controls show a clear increase in alizarin red staining after 28 days, whereas the treated cells remained



**FIGURE 1** Effects of different ATRA concentrations on gene expression. MEPM cells were treated with 0, 0.1, 0.5, or 1  $\mu\text{M}$  ATRA for 1 week and compared with the control group; expression of *Rarb* (A), *Sp7* (B), and *Axin2* (C) was analysed. Data are presented as mean  $\pm$  SD in percentages of the control groups ( $N = 3$ ). \*\* $P < .01$ , compared with the control group at day 7



**FIGURE 2** Mineralization of MEPM cells. MEPM cells were cultured with and without 0.5  $\mu\text{M}$  ATRA for up to 28 d. Representative photographs of the alizarin red staining (A) and quantitative analysis of the alizarin red staining (B) on days 7, 14, 21, and 28 are shown. Data of the quantitative analysis are presented as mean  $\pm$  SD ( $N = 2$ )

unstained (Figure 2A). After 4 weeks, extraction of the dye also showed an increase of alizarin red staining in the controls, whereas the cells treated with ATRA showed no staining (Figure 2B).

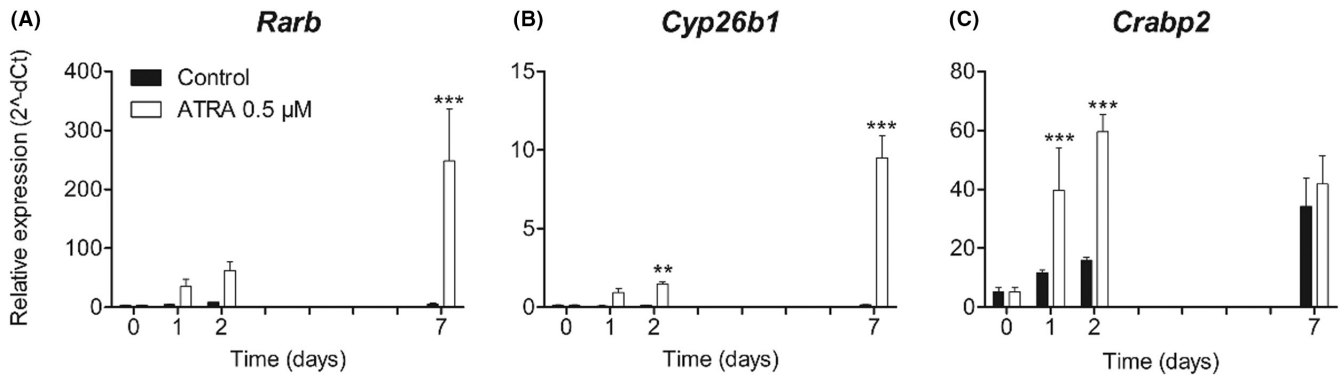
### 3.3 | ATRA upregulates ATRA-responsive genes

We evaluated the effects of ATRA on the expression levels of the genes *Rarb*, cytochrome P450 family 26 subfamily B member 1 (*Cyp26b1*), and cellular retinoic acid binding protein 2 (*Crabp2*), which are involved in retinol-dependent signalling. The expression of *Rarb* in the controls was constantly low, whereas it increased up to 50-fold in the ATRA group after 7 days ( $P < .001$ , Figure 3A). Similar effects were observed for *Cyp26b1* expression in the controls, whereas it increased about 13-fold in the ATRA group at day 2 ( $P < .01$ , Figure 3B). After 7 days, *Cyp26b1* expression was 70 times higher than in the controls ( $P < .001$ , Figure 3B). The expression of *Crabp2* in the controls increased linearly during the 7 days of culture. In the ATRA-treated group, *Crabp2* expression showed significant upregulation at days 1 and 2 ( $P < .001$ ), but was normalized at day 7

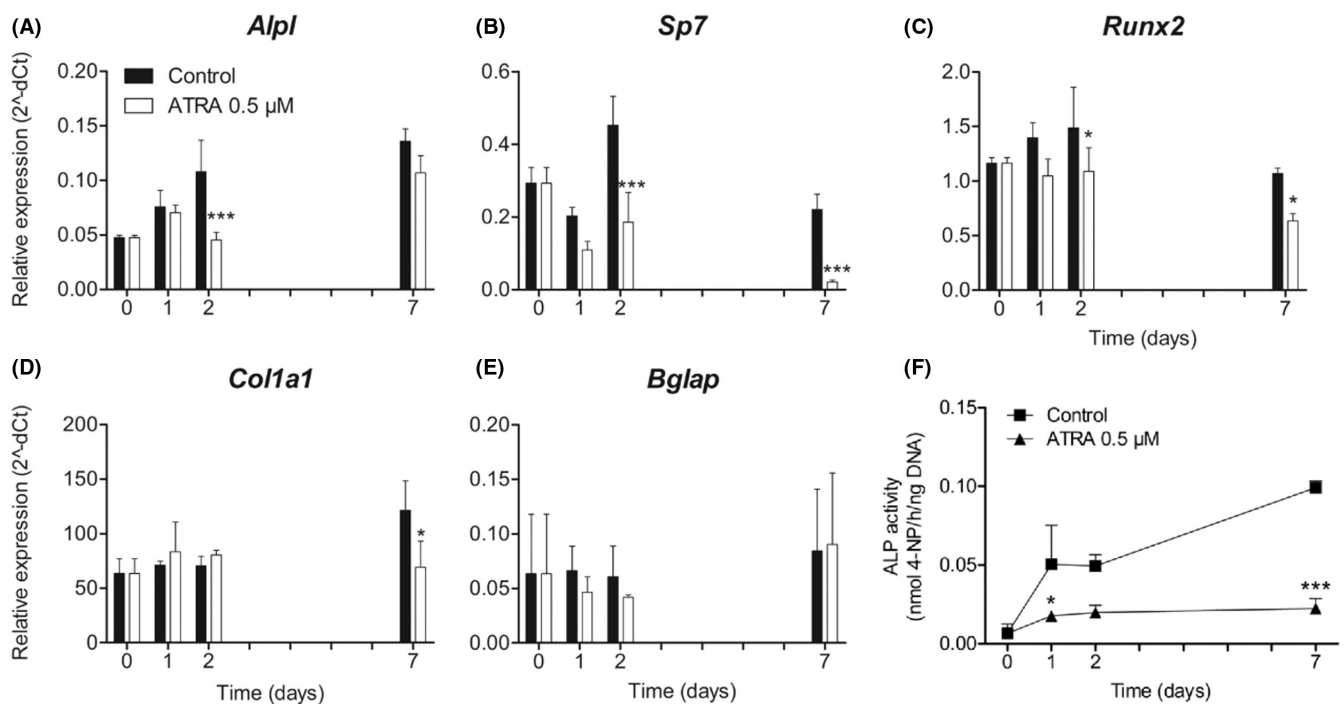
(Figure 3C). There were no significant differences between the control groups with and without DMSO for any of the genes (data not shown).

### 3.4 | ATRA downregulates osteogenic marker genes and ALP activity

We investigated the effects of ATRA on the expression of early (alkaline phosphatase, liver/bone/kidney [*Alpl*], *Sp7*, and *Runx2*) and late (collagen type I, alpha 1 [*Col1a1*] and *Bglap*) osteoblast marker genes. *Alpl* expression increased gradually over 7 days in the controls and in the ATRA-treated group, but ATRA downregulated *Alpl* significantly at day 2 compared with its expression in the controls ( $P < .001$ , Figure 4A). *Sp7* did not show a consistent expression pattern (Figure 4B). At days 2 and 7, ATRA reduced *Sp7* expression compared with the control levels ( $P < .001$ , Figure 4B). *Runx2* expression was more or less constant in the controls, but was decreased in the ATRA-treated groups at days 2 and 7 ( $P < .05$ , Figure 4C). *Col1a1* expression showed a gradual increase over 7 days in the control group,



**FIGURE 3** Expression of ATRA-responsive genes. MEPM cells were cultured with and without 0.5  $\mu\text{M}$  ATRA for up to 7 days. Expression of the genes *Rarb* (A), *Cyp26b1* (B) and *Crabp2* (C) is shown. Data are presented as mean  $\pm$  SD (N = 3). \*\* $P < .01$ , \*\*\* $P < .001$ , compared with the control group at each time point



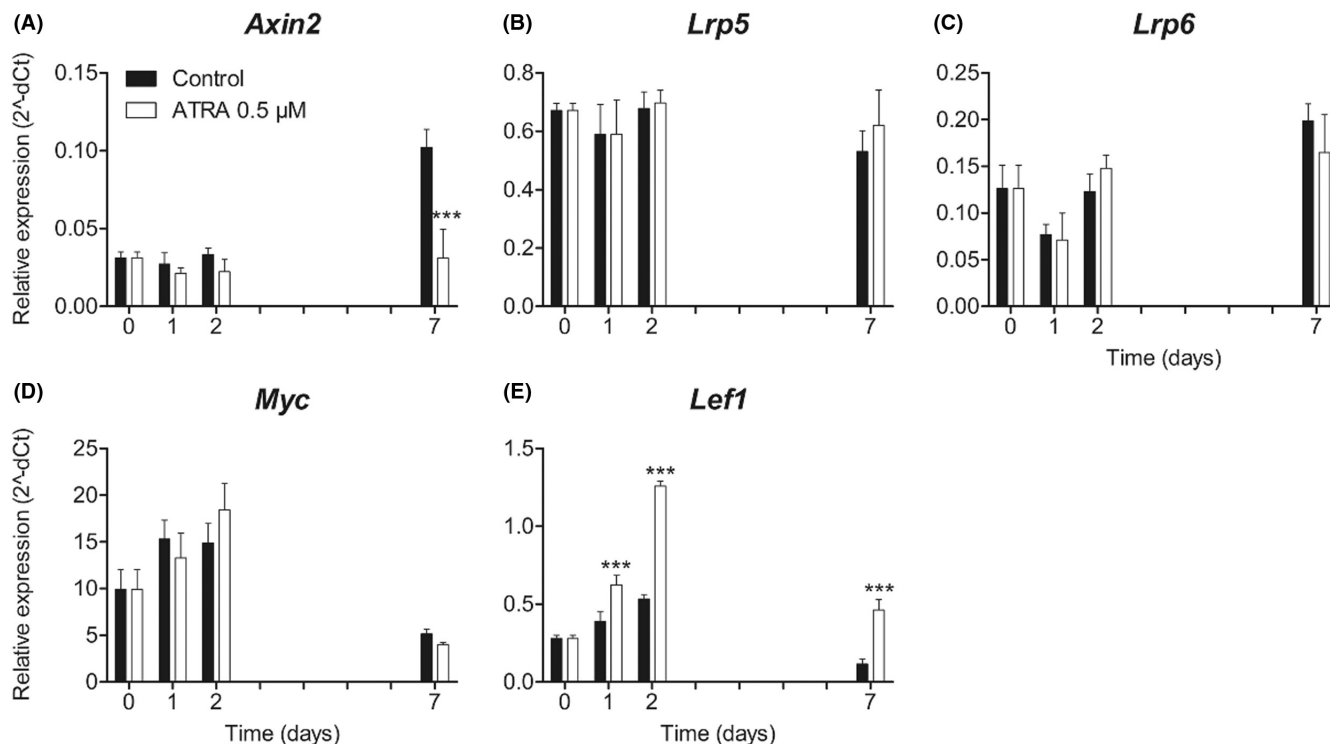
**FIGURE 4** Expression of osteogenic differentiation genes and ALP activity. MEPM cells were cultured with and without 0.5  $\mu\text{M}$  ATRA for up to 7 d. Expression of the early osteogenic marker genes *Alpl* (A), *Sp7* (B) and *Runx2* (C) and late osteogenic marker genes *Col1a1* (D) and *Bglap* (E) is shown. Data are represented as mean  $\pm$  SD (N = 3). \* $P < .05$ , \*\*\* $P < .001$ , compared with the control group at each time point. ALP activity in MEPM cells with and without 0.5  $\mu\text{M}$  ATRA for up to 7 d (F). Data are presented as mean  $\pm$  SD (N = 3). \* $P < .05$ , \*\*\* $P < .001$ , compared with the control group at each time point

while ATRA significantly downregulated *Col1a1* at day 7 ( $P < .05$ , Figure 4D). *Bglap* expression was more or less stable over 7 days and was not affected by ATRA (Figure 4E). There were no significant differences between the control groups with and without DMSO for any of the genes (data not shown). We also evaluated the effects of ATRA on ALP activity in the cultured MEPM cells after 7 days to assess early osteoblast differentiation (Figure 4F). ALP activity in the control group increased gradually up to day 7 ( $0.10 \pm 0.00$  nmol 4-NP/h/ng DNA). Similar results were observed in the MEPM cells treated with DMSO only (data not shown). MEPM cells treated with

ATRA showed slightly increased ALP activity, but it was significantly reduced at days 1 ( $P < .05$ ) and 7 ( $0.02 \pm 0.00$  nmol 4-NP/h/ng DNA,  $P < .001$ ) compared with the controls.

### 3.5 | ATRA inhibits Wnt signalling

To assess the effects of ATRA on Wnt signalling during osteogenic differentiation, the expression of several Wnt-related genes was analysed. *Axin2* was upregulated more than 2-fold over 7 days in the



**FIGURE 5** Expression of Wnt-related genes. MEPM cells were cultured with and without 0.5 μM ATRA for up to 7 d. The expression of the Wnt-related gene *Axin2* (A), the Wnt co-receptors *Lrp5* (B) and *Lrp6* (C), the Wnt target gene *Myc* (D), and the Wnt signalling transcription factor *Lef1* (E) are shown. Data are presented as mean ± SD (N = 3). \*\*\**P* < .001, compared with the control group at each time point

control group, while ATRA significantly inhibited *Axin2* expression at day 7 (*P* < .001, Figure 5A). There was no difference in the expression of the Wnt co-receptors *Lrp5* and *Lrp6* between the ATRA-treated groups and the control groups (Figure 5B and C). The expression of *Lrp5* was more or less constant over time in the controls and ATRA-treated groups, while *Lrp6* expression showed a slight increase over time with a dip at day 1. In the first 2 days, expression of the Wnt target gene *Myc* was upregulated, and by day 7 it had declined (Figure 5D). ATRA had no influence on *Myc* expression. The expression of *Lef1* in the controls was upregulated in the first 2 days, but declined by day 7 (Figure 5E). ATRA significantly upregulated the expression of *Lef1* at 1, 2, and 7 days compared with its expression in the controls (*P* < .001). There were no significant differences between the control groups with and without DMSO for any of the genes (data not shown).

## 4 | DISCUSSION

ATRA is a key regulator of embryonic development. Epidemiological and animal studies indicate that either an excess or a shortage of ATRA can induce CLP and cleft palate (CP).<sup>22,23</sup> We investigated the effects of ATRA on the osteogenic differentiation and mineralization of primary MEPM cells to clarify the molecular aetiology of submucous clefting. We hypothesized that ATRA inhibits osteoblast differentiation and mineralization by disrupting Wnt signalling.

In our initial dose-response experiments, ATRA (0.1, 0.5, and 1 μM) strongly upregulated the expression of the well-known ATRA-responsive gene *Rarb*. Additionally, we found that ATRA strongly downregulated the expression of the osteoblast transcription factor *Sp7* and the Wnt marker gene *Axin2*. *Sp7* is an important transcription factor for osteoblast differentiation,<sup>24</sup> while *Axin2* is a universal marker gene for Wnt signalling in vertebrates.<sup>25</sup> The effects were maximal even at the lowest concentration of ATRA, confirming that the MEPM cells are highly sensitive to ATRA.

Studies on other pre-osteoblasts have generally used ATRA concentrations in the range of 0.004 to 10 μM.<sup>26–29</sup> At low ATRA doses (1–10 nM) the osteogenic gene expression response seems to reach a plateau. In contrast, more supra-physiological doses of ATRA (≥10 μM) can produce opposite effects.<sup>10,30</sup> Higher concentrations (up to 100 μM) of ATRA generally show clear dose-dependent effects.<sup>16,29,31</sup> A study with intermediate concentrations of ATRA (10–50 nM) also reported a dose-dependent effect on osteogenic gene expression in chondroprogenitor cells.<sup>15</sup> Our data might not show a clear dose-response effect because at the lowest concentration of 0.1 μM the ATRA effect had already reached a plateau.

MEPM cultures started to mineralize after about 3 weeks, and this was completely inhibited by ATRA. In addition, ALP activity at day 7 was also reduced. This indicates that the differentiation of MEPM cells was strongly inhibited by ATRA. Similar results for mineralization and ALP activity are reported for osteoblasts and chondroprogenitor cells from humans, rats, and mice treated with ATRA.<sup>17,19,26,29,32</sup> Together these data show that ATRA strongly





reduces the differentiation and mineralization of MEPM cells and several other types of pre-osteoblasts.

The enhanced expression of three ATRA-responsive genes (*Rarb*, *Cyp26b1*, and *Crabp2*) confirms the activation of the ATRA signaling pathway. Intracellular ATRA levels are regulated by the synthesis and degradation of ATRA. If ATRA is not degraded by *Cyp26b1*, it can bind to cellular retinoic acid-binding proteins (CRABPs) and be translocated to the nucleus, where it activates nuclear receptors such as *Rarb* to induce transcription of target genes.<sup>10</sup>

As expected, the early osteoblast differentiation genes *Alpl*, *Sp7*, and *Runx2* were inhibited by ATRA, as been reported in other osteoblast precursor cells.<sup>17,19,26,29</sup> We also found that expression of the late differentiation gene *Col1a1* was downregulated in the first week, but *Bglap* expression was unchanged. Other studies report downregulation of *Bglap* in the second week in osteoblast precursor cells,<sup>19,20,26,29</sup> but we analysed gene expression only in the first week. These data indicate that osteoblast differentiation is strongly inhibited by ATRA in MEPM cells, which is in line with the observed inhibition of ALP activity and mineralization.

A further finding was that ATRA strongly reduced the expression of *Axin2*, which is considered a universal Wnt marker gene in vertebrates.<sup>25</sup> In contrast, the expression of *Lef1* was stimulated. *Lef1* is a transcription factor from the TCF/LEF family that binds to  $\beta$ -catenin in the nucleus after Wnt activation and then induces the transcription of Wnt target genes.<sup>25</sup> *Lef1* is also known to activate the expression of epithelial-mesenchymal transformation-related target genes independently from  $\beta$ -catenin in cancer cells, increasing their proliferation and invasiveness.<sup>33</sup> Moreover, in pre-osteoblast-like MC-3 T3 cells, overexpression of *Lef1* inhibits terminal differentiation.<sup>34</sup> The overexpression of *Lef1* induced by ATRA in MEPM cells may thus enhance the  $\beta$ -catenin-dependent inhibition of terminal differentiation. The expression of more indirectly Wnt-related genes (*Lrp5*, *Lrp6*, *Myc*) was not affected by ATRA.

## 5 | CONCLUSIONS

In summary, our data indicate that ATRA inhibits the differentiation of MEPM cells into osteoblasts by inhibiting Wnt signalling. In vivo, this might contribute to vitamin A-dependent CLP and CP in both animal models and humans. More specifically, the bone-related effects of vitamin A might contribute to the aetiology of submucous clefts. However, the exact molecular mechanisms of vitamin A excess and deficiency in the aetiology of CLP and CP remain to be established.

## AUTHOR CONTRIBUTIONS

CK: performed experiments, data analysis, figures, discussion of data and manuscript writing. LR: Did experimental design and isolated the cells. MB: performed part of the experiments. JH: Did experimental design, discussion of data and manuscript writing. All authors contributed to the article and approved the submitted version.

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## CONFLICT OF INTEREST

All authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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