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ORIGINAL ARTICLE

Retinoic acid effects on in vitro palatal fusion and WNT signaling

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Abstract

Retinoic acid is the main active vitamin A derivate and a key regulator of embryonic development. Excess of retinoic acid can disturb palate development in mice leading to cleft palate. WNT signaling is one of the main pathways in palate development. We evaluated the effects of retinoic acid on palate fusion and WNT signaling in in vitro explant cultures. Unfused palates from E13.5 mouse embryos were cultured for 4 days with 0.5 μM, 2 μM or without retinoic acid. Apoptosis, proliferation, WNT signaling and bone formation were analyzed by histology and quantitative PCR. Retinoic acid treatment with 0.5 and 2.0 μM reduced palate fusion from 84% (SD 6.8%) in the controls to 56% (SD 26%) and 16% (SD 19%), respectively. Additionally, 2 μM retinoic acid treatment increased *Axin2* expression. Retinoic acid also increased the proliferation marker *Pcna* as well as the number of Ki-67-positive cells in the palate epithelium. At the same time, the WNT inhibitors *Dkk1, Dkk3, Wif1* and *Sfrp1* were downregulated at least two-fold. Retinoic acid also down-regulated *Alpl* and *Col1a2* gene expression. Alkaline phosphatase (ALP) activity was notably reduced in the osteogenic areas of the retinoic acid- treated palates. Our data suggest that retinoic acid impairs palate fusion and bone formation by upregulation of WNT signaling.

KEYWORDS
bone formation, palate fusion, retinoic acid, Wnt signaling

INTRODUCTION

Cleft palate is a disruption of the normal orofacial structures caused by a failure in the growth, elevation or fusion of the palatal shelves during embryonic development [1]. Cleft palate etiology is complex and poorly understood, but it is known to involve genetic as well as environmental factors such as vitamin imbalances [2, 3]. Under normal conditions, the palatal shelves grow out vertically from the maxillary prominences, elevate to a horizontal position, adhere forming the medial epithelia seam and then fuse [4]. Fusion may involve the migration, differentiation and/or apoptosis of medial epithelial seam cells [5]. After the fusion of the palatal shelves, bone formation takes place through intramembranous ossification, in which condensed neural crest-derived mesenchymal cells differentiate directly into osteoblasts [6].

Several signaling pathways have been related to palatogenesis and cleft palate including fibroblast growth factor, sonic hedgehog and wingless-INT (WNT) [7–10]. Canonical WNT signaling is activated by the binding of WNT ligands to the Frizzled (FZD) receptors and the low-density lipoprotein coreceptors-related protein 5/6 (LRP5, LRP6) [11]. This

interaction stabilizes the cytoplasmic β-catenin and facilitates its translocation into the nucleus, where it binds to lymphoid enhancer-binding factor 1/T cell-specific transcription factor (TCF/LEF) [12]. In the absence of a WNT ligand, β-Catenin is phosphorylated and degraded in the cytoplasm [13]. Canonical WNT signaling is antagonized by several secreted proteins such as the secreted frizzled related proteins (sFRPs), WNT inhibitory protein 1 (WIF1) and the dickkopf family (DKK) [14]. sFRPs and WIF1 bind to WNT ligands and prevent their interaction with the WNT membrane receptors [15, 16]. sFRPs can also bind to the WNT-binding domain of the FZD receptors [17]. The DKK family of proteins bind to the WNT co-receptors LRP5 or LRP6, and to Kremen1 and Kremen2. This complex is then endocytosed resulting in the depletion of LRP from the plasma membrane [18].

Genetic studies have associated WNT gene mutations with cleft palate in humans such as a homozygous nonsense mutation in WNT3 and several single nucleotide polymorphism near the *WNT6-WNT10a* cluster at the 2q35 region of chromosome 2 [10, 19]. Experiments in mice have also confirmed the role of WNT signaling in cleft palate [20, 21]. For instance, tissue-specific deletions of *Catnb*, *Tcf4* or *Lef1* from the palatal shelves epithelium in mice disrupt medial epithelial seam disappearance resulting in cleft palate [22]. WNT signaling is also involved in bone formation as it promotes the proliferation and differentiation of mesenchymal stem cells (MSC) into the osteogenic lineage [23]. Several in vivo and in vitro studies indicate that WNT signaling can be disrupted by retinoic acid [24–26].

Retinoic acid is the main active metabolite of vitamin A and is crucial for normal pattern formation during embryonic development [27]. Retinoic acid regulates gene expression by binding to cellular retinoic acid-binding proteins (CRABPs) that transport retinoic acid 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 [28]. An overdose of retinoic acid at different embryonic stages induces congenital malformations in both mouse and humans [29–31]. One of these malformations induced by retinoic acid is cleft palate [30, 32, 33]. Depending on the stage of administration, retinoic acid inhibits palatal shelves growth or fusion [30, 34]. Several studies show contradictory effects of retinoic acid on osteogenic differentiation, depending mainly on the dose and embryonic stage of exposure [35, 36]. For instance, retinoic acid decreases the differentiation of osteoblasts [37]. In contrast, retinoic acid induces bone mineralization in mouse embryo limbs [36]. However, the molecular mechanisms underlying the effects of retinoic acid on palate development are not clearly understood yet. Here, we hypothesized that retinoic acid disrupts palate fusion and bone formation by affecting WNT signaling. Explant cultures of the mouse embryonic palate show that retinoic acid disrupts palate fusion and osteogenic differentiation. This coincides with a downregulation of WNT inhibitors and an upregulation of the WNT marker gene *Axin2*.

MATERIAL AND METHODS

In vitro palate culture

Seventy-two palatal shelves were dissected in cold fetal bovine serum (FBS) from the mouse fetuses of nine different mothers at embryonic day 13.5 using a stereomicroscope (Leica MZ16). The wild-type mice were from an outbred strain Hsd:ICR (CD1). Experiments were approved by the board for animal experiments of Radboud University, Nijmegen (RUDEC 2015-0080) according to Dutch laws and regulations. The dissected palates were gently put on a 0.8 μm pore MF-Millipore membrane filter (Millipore) on top of a sterilized stainless-steel mesh in a six-well plate (for detailed description, see Figure 1). Generally, three palates were put in one well. Three different media conditions were used. Control: DMEM/F12 medium (Sigma-Aldrich) containing 5% FBS (GIBCO), 200 mM glutamine, 400 mg/ml ascorbate, 1% penicillin – streptomycin. The other two media contained control medium with all-trans-Retinoic acid (Sigma-Aldrich) in a concentration of 0.5 μM retinoic acid or 2 μM retinoic acid, diluted in dimethyl sulfoxide (Sigma-Aldrich). Palates were incubated at 37˚C with 5% CO_2 for 4 days. Medium was changed and photographs were made every 24 h.

RNA isolation and real-time quantitative PCR

Three biological replicates, containing at least six pooled palates (from three different mothers) were used for the RNA isolation using the RNeasy MiniKit (Qiagen) 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). 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 and their sequences are summarized in Table 1.

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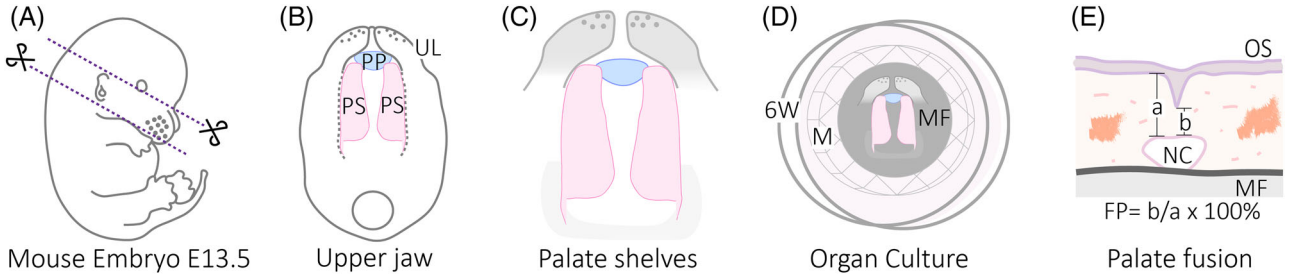

FIGURE 1 Mouse embryo palate dissection and quantification method for palate fusion. **(A)** Under the stereomicroscope, mouse embryos were examined to confirm the embryonic stage E13.5 based on Theiler's stages criteria [96]. Each embryo was placed in one drop of cold PBS in a petri dish. To isolate the upper jaw, two transversal cuts were made; one just below the eye level and over the ears and the second from the oral commissures to below the ears. **(B)** Once the upper jaw is isolated, the oral side is orientated up and the surrounding tissue is carefully removed. *PP*: primary palate. *PS*: Palate shelves. *UL*: Upper lip. **(C)** In order to maintain the in vivo position of the palatal shelves, some tissue anterior and posterior to the shelves was left. **(D)** The dissected palates are carefully transferred to a well in a 6-well plate, containing a stainless mesh with a filter on top and 3.5 ml of medium. *6W*: well from a 6-well plate. *M*: Stainless-steel mesh. *MF*: 8 μM pore MF-Millipore membrane filter. **(E)** Diagram showing the quantification of palate fusion on a histological section. *OS*: Oral side. *b*: Total thickness of the palate. *a*: part of the palate fused. *NC*: Nasal cavity. *FP*: Fusion percentage

TABLE 1 Primer sequences

Gene category	Symbol	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
RA RESPONSIVE	Cyp26b1	GATCCTACTGGGCGAACACC	GGAGAAGACCTTGCGCTTGT
	Crabp2	TGATCTCGACTGCTGGCTTG	TCCCATCGGGTTCCCATAAAG
	Rarb	GAAAACGACGACCCAGCAAG	TTACACGTTCGGCACCTTTC
APOPTOSIS MARKER	Trp53	GGAAGACTCCAGTGGGAACC	CTTCTGTACGGCGGTCTCTC
PROLIFERATION MARKER	Pcna	AGAGCATGGACTCGTCTCA	CCAGCACATTTTAGAATTTTGGACA
WNT TARGET	Axin2	GGTTCCGGCTATGTCTTTGC	CAGTGCGTCGCTGGATAACTC
WNT INHIBITORS	Dkk3	GGCCCACAGTCTTCATCAAT	CCAGAGTGGACAGGTGGTCT
	Dkk1	CGGGGGATGGATATCCCAGAA	ACGGAGCCTTCTTGTCCTTTG
	Kremen1	TGGGTTTCCATGATCCTTGT	GCATGAGGACGGAGTCTACTG
	Sfrp1	TCTAAGCCCCAAGGTACAACC	GCTTGCACAGAGATGTTCAATG
	Sfrp4	ATCATCCTTGAACGCCACTC	TCGAACACAAGTCCCTCTCA
	Wif1	GCATTCTTTGTTGGGCTTTC	CCATCAGGCTAGAGTGCTCA
BONE DIFFERENTIATION MARKERS	Alpl	CCAGCAAGAAGAAGCCTTTG	AACCCAGACACAAGCATTCC
	Col1a2	CCTGGCAAAGACGGACTCAAC	GCTGAAGTCATAACCGCCACTG
	Runx2	CGGACGAGGCAAGAGTTTCA	GGATGAGGAATGCGCCCTAA

Fusion percentage

After four days in culture, six palates from each concentration group were fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm. The sections were stained with hematoxylin and eosin (HE), and photographed with a Zeiss Imager Z1 microscope (Zeiss AxioCam MRc5; Carl Zeiss Microimaging). Measurements were made on every tenth section, of the middle region of each palate using ImageJ software [38]. The fusion percentage (FS) was calculated as the part of the palate that is fuse (*b*), divided by total thickness of the palate, including any remaining medial epithelial seam (*a*), as previously reported (Figure 1E) [39].

Immunohistochemistry

Mouse palate shelves cultured in vitro during four days, six palates from each concentration group, were fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm. Briefly, the sections were deparaffinized with xylene and rehydrated with a graded series of ethanol. Next, endogenous peroxidase activity was inhibited in 30% H_2O_2 in phosphate buffered saline (PBS) in the dark at room temperature for 20 min. The sections for DKK3 and KI-67 staining were boiled with 0.1 M citrate buffer (pH 6.0) in a microwave oven for 10 min and left at room temperature for 20 min to cool down. To reduce non-specific binding, the samples were

incubated in 10% normal donkey serum in PBS for 20 min in the dark. After washing with PBS, the primary antibody against DKK3 and KI-67 (both from Proteintech) was applied and incubated overnight at 4˚C. The sections for WIF1, sFRP4 and AXIN2 received a trypsin treatment for 1 min at 37˚C (all from abcam). Then, the primary antibody was applied and labelled with a streptavidin-biotin immunoperoxidase method using a commercial kit (Vectorlabs). Antibody binding was visualized using diaminobenzidine (DAB) as a chromogene to produce a brown color. Counterstaining was performed with Mayer's hematoxylin. The slides were mounted with DPX mounting medium and photographed with the Zeiss Imager Z1 microscope (Zeiss AxioCam MRc5; Carl Zeiss Microimaging).

Alkaline phosphatase staining

The palate sections were deparaffinized with xylene and rehydrated with a graded series of ethanol. Then, they were washed with MilliQ water and incubated at 37˚C with preheated ALP solution of pH 9.5 (100 mM NaCl, 100 mM Tris–HCl, 50 mM MgCl2, 4.5 μl/ml nitroblue tetrazolium and 3.5 μl/ml 5-bromo-4-chloro-3-indolyl phosphate) for 60 min and washed again in MilliQ water. Sections were counterstained with natrium acetate 0,1 M (pH 5.1) for 15 min followed by 0.1% Methylgreen in natrium acetate (pH 5.1). Finally, the sections were mounted in Kaisers gelatin and photographed with the Zeiss Imager Z1 microscope (Zeiss AxioCam MRc5; Carl Zeiss Microimaging)

Statistical analysis

The Shapiro-Wilk test showed that all data were normally distributed. Each culture experiment was performed in triplicate, and the results are presented as mean ± SD. Differences between the groups were evaluated by one-way ANOVA. Post-hoc comparisons were made using the Tukey's multiple comparison test. Differences were considered significant if $p < 0.05$. All statistical tests were performed with Graphpad Prism version 8.2.1.

RESULTS

Retinoic acid effect in palate fusion

Palates shelves were dissected from mouse embryos at E13.5 and cultured with 0.5 μM and 2 μM retinoic acid for up to 4 days. At day 1, the shelves from the control group had already grown and contacted in the midline (Figure 2A). The palatine rugae also started to be visible (data not shown). At day 1 in the 0.5 μM and 2 μM retinoic acid groups, the anterior region of the shelves was not in contact (Figure 2A). From 2 to 4 days in culture, no evident changes occurred in the palate shelves from the control group. However, in the 0.5 μM retinoic acid group a persistent gap was observed in the anterior region. The length of the palate shelves was measured between the yellow dotted lines (Figure 2A, bottom row). The palates treated with 2 μM retinoic acid showed a progressive reduction in length up to 34% after 4 days in culture, compared to the controls.

Histological sections were evaluated after 4 days in culture. The controls showed limited persistence of medial epithelial seam, molars buds (Figure 2B, M) and mesenchymal condensation (Figure 2B, black arrows). Retinoic acid treatment (0.5 and 2 μM) showed a decreased number of samples with molar buds (data not shown), persistence of the medial epithelial seam (Figure 2B, red arrows) or reduced contact between the palatal shelves (data not shown). Mesenchymal condensations were absent (Figure 2B). Then, to quantify the fusion of the palatal shelves, defined as the disappearance of the medial epithelial seam, the percentage of fusion was measured using image analysis. After 4 days in culture, the control group showed 84% ± 6.8% of fusion (Figure 2C) while in the 0.5 μM retinoic acid group this had decreased to 56 ± 26% (not significant, Figure 2C). The fusion percentage was significantly reduced (16 ± 19%) in the 2 μM retinoic acid group when compared with the control and the 0.5 μM retinoic acid group ($p < 0.001$ and $p < 0.05$, respectively; Figure 2C).

Retinoic acid effects on apoptosis, proliferation and WNT signaling

To confirm the functionality of retinoic acid, we determined 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 (*Rarb*) that are involved in retinol-dependent signaling. As expected, retinoic acid up-regulated the expression of two retinoic acid-responsive genes (Figure 3A). *Cyp26b1* gene expression showed a 2-fold increase in the 0.5 μM retinoic acid group ($p < 0.05$) and a 4-fold increase in the 2-μM retinoic acid treated group ($p < 0.01$). *Rarb* expression showed a 50-fold increase in the 2 μM retinoic acid group compared to the controls ($p < 0.01$). *Crabp2* expression only showed a trend towards increased expression.

We also determined the expression of an apoptosis marker and a proliferation marker (*Trp53* and *Pcna*). After 4 days in culture, no differences were observed in the expression of *Trp53* (Figure 3B). *Pcna* expression showed a 3.2-fold increased expression only in the palate cultures treated with 2 μM retinoic acid ($p < 0.05$, Figure 3B). Positive cells for the proliferation marker Ki-67 were located in the plate epithelium, and the number increased from 129 in the controls, to 239 and 303 in the 0.5 and 2 μM retinoic acid treated groups respectively ($p < 0.05$, Figure 3C, D).

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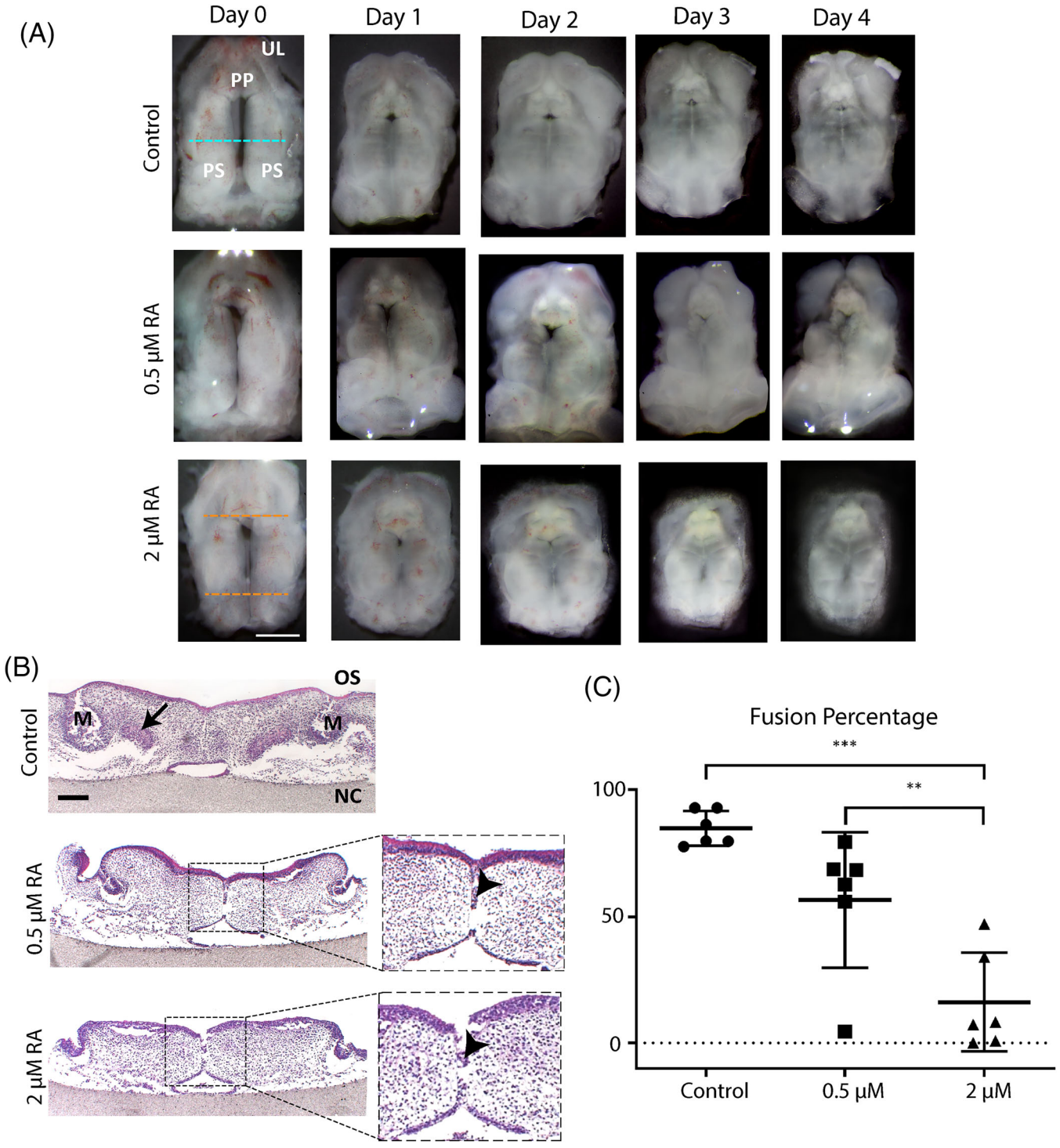

FIGURE 2 Retinoic acid disrupts palate fusion in mouse palate cultures. Palates were isolated from E13.5 mouse embryos from nine different mothers, cultured oral-side up for 4 days with 0.5–2 μM or without retinoic acid, fixed, and stained with HE. **(A)** Representative daily pictures of palates in culture. *Green dotted line*: middle region. *Red dotted line*: palate shelves length. *PP*: primary palate. *UL*: upper lip. *PS*: palate shelves. Scale bars: 1 mm. **(B)** HE staining of frontal sections. Representative pictures of the palates from the middle region, stained with HE. *M*: molar. *OS*: oral side. *NC*: nasal cavity. Arrowheads: medial epithelial seam. Scale bars: 200 μm. **(C)** Palate shelves fusion percentage. Six palates from each concentration group were used for the measurements. Ten consecutive sections from the middle of each palate were analyzed (* $p < 0.05$, *** $p < 0.001$)

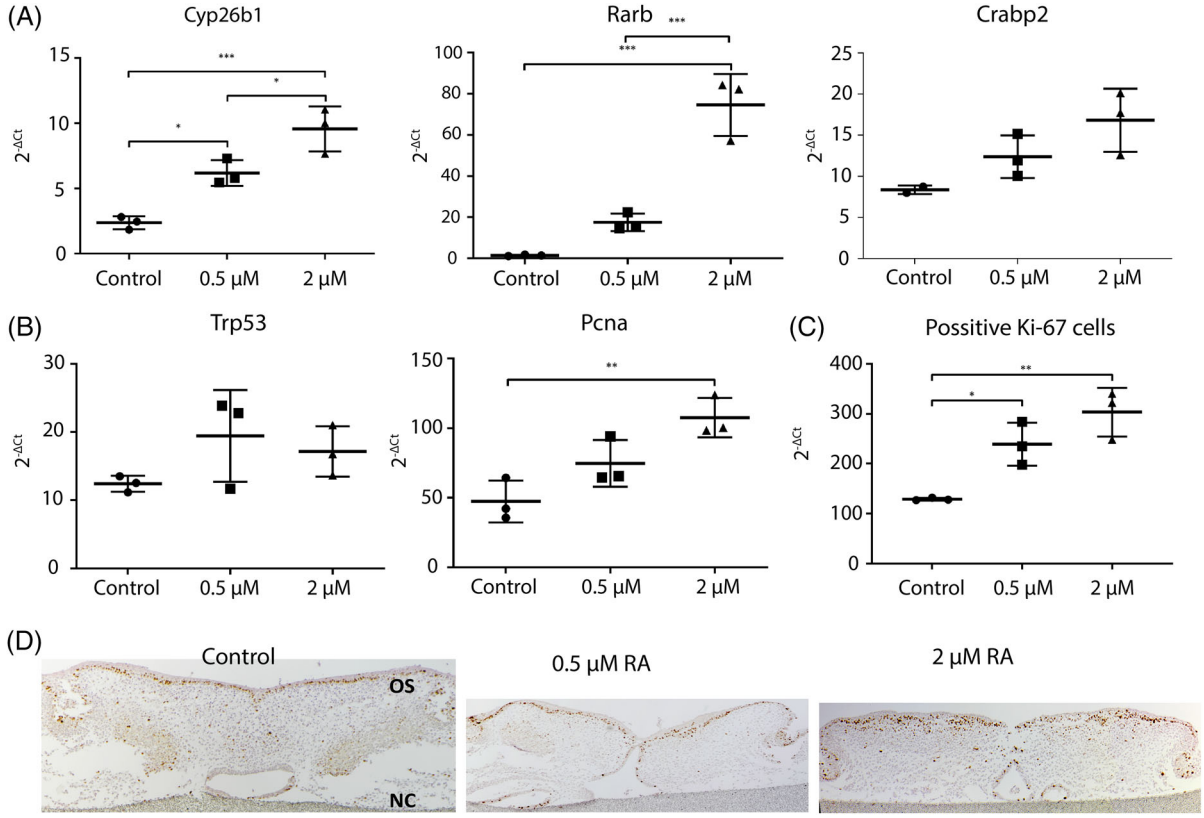

FIGURE 3 Gene expression analysis. Quantitative real-time PCR was performed with RNA isolated from mouse palates cultured for 4 days with 0.5–2 μM or without retinoic acid. **(A)** Expression of retinoic acid-responsive genes. Data are represented as the mean ± SD (N = 3). * $p <$ 0.05, *** $p <$ 0.001, compared with the controls. **(B)** *Trp53* and *Pcna* gene expression. Data are represented as the mean ± SD (N = 3). ** $p <$ 0.01. **(C)** Proliferation marker KI-67 positive cells. Data are represented as the mean ± SD (N = 3. * $p <$ 0.05, ** $p <$ 0.01, compared with the controls. **(D)** KI-67 immunohistochemistry. Representative pictures of the staining. *OS*: oral side. *NC*: nasal cavity

To evaluate whether the expression of *Axin2, a* WNT marker gene, was affected by retinoic acid during palate fusion, we analyzed the gene expression of *Axin2* (40, 41). *Axin2* gene expression did not show significant changes in the 0.5 μM treated group. 2 μM retinoic acid treatment induced a 4.9-fold increase in *Axin2* expression, compared with the control group ($p < 0.05$; Figure 4A). In the controls, AXIN2 protein expression was located mainly in the oral epithelium and the condensed mesenchyme of the palate (Figure 4B). In the 0.5 μM retinoic acid-treated group, AXIN2 was almost absent in the mesenchyme but the staining intensity was increased in the oral and nasal epithelium (Figure 4B). In the 2 μM retinoic acid-treated group, AXIN2 expression was stronger all over the mesenchyme and the oral epithelium (Figure 4B).

WNT inhibitor expression in palate shelves treated with retinoic acid

We have shown earlier that retinoic acid induces the expression of WNT inhibitors during osteogenic differentiation of MC-3T3 preosteoblasts cultured in vitro (37). To investigate whether the activation of WNT signaling was caused by inhibition of expression of WNT inhibitors, we evaluated the expression of six WNT inhibitors after 4 days of retinoic acid treatment. The expression of dickkopf-related protein 1 (*Dkk1*) and dickkopf-related protein 3 (*Dkk3*) was significantly down-regulated compared to the control group ($p < 0.001$, Figure 5A). The kringle containing transmembrane protein 1 (*Kremen1)* did not show differences in expression (Figure 5A). The WNT inhibitor factor 1 (*Wif1*) was down-regulated in the retinoic acid–treated palates ($p < 0.05$, Figure 5A). The gene expression of the WNT inhibitor secreted frizzled related protein 1 (*Sfrp1*) showed a 2-fold decrease in the palates treated with 0.5 μM retinoic acid ($p < 0.01$, Figure 5A). *Sfrp4* was significantly up-regulated in the two retinoic acid-treated groups ($p < 0.01$, Figure 5A).

To localize the WNT inhibitors DKK3, SFRP4 and Wif1 in the cultured palates we used immunostaining. Palates from the control group showed DKK3 protein expression in the oral and nasal epithelium and in the mesenchymal areas lateral to the middle (Figure 5B, DKK3-control). However, in the retinoic acid treated groups, mesenchymal expression was

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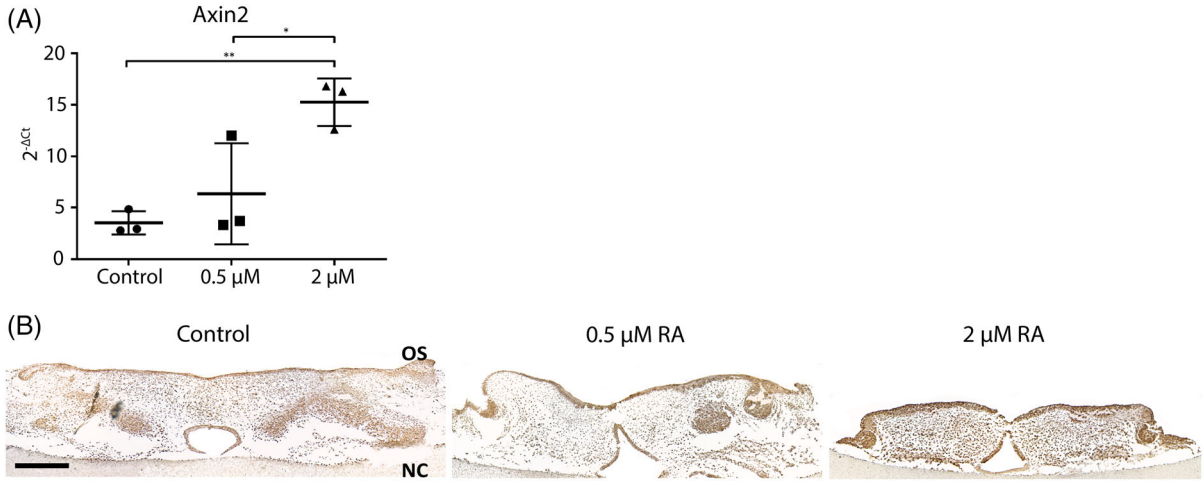

FIGURE 4 WNT signaling target gene expression. **(A)** *Axin2 gene expression*. Data are represented as the mean ± SD (N = 3). * $p < 0.05$, ** $p < 0.01$. **(B)** AXIN2 immunohistochemistry. Representative pictures of the staining. Scale bars: 200 μm. *OS*: oral side. *NC*: nasal cavity

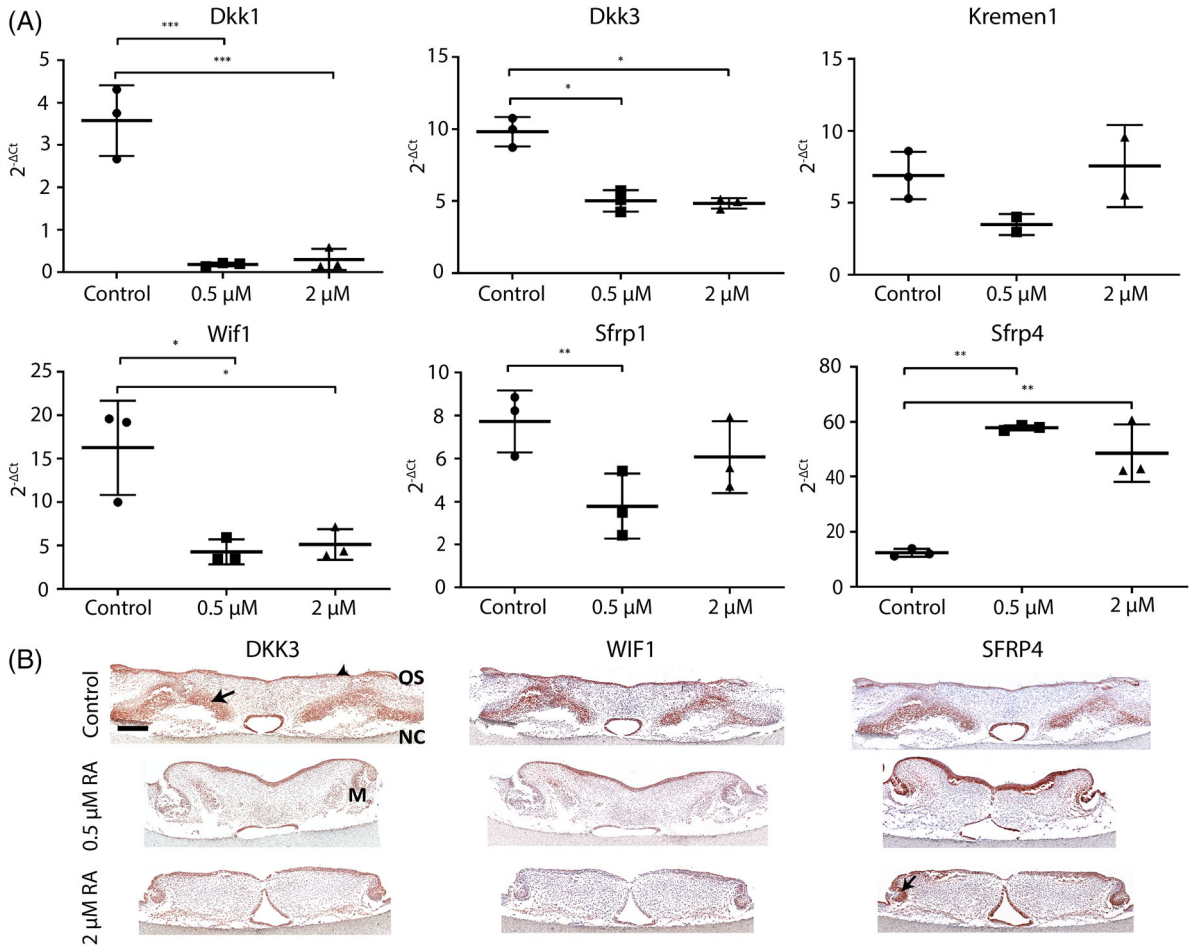

FIGURE 5 Retinoic acid affects the expression of WNT inhibitors. Palates were isolated from E13.5 mouse embryos from nine different mothers, cultured oral-side up for 4 days with 0.5–2 μM or without retinoic acid and then processed for qPCR or fixed for histology. **(A)** Relative WNT inhibitors gene expression. Data are represented as the mean ± SD (N = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *M*: molar. *OS*: oral side. *NC*: nasal cavity **(B)** Immunohistochemistry of WNT inhibitors. Representative pictures of the staining of each group. *Black arrows*: protein expression. Scale bars: 200 μm

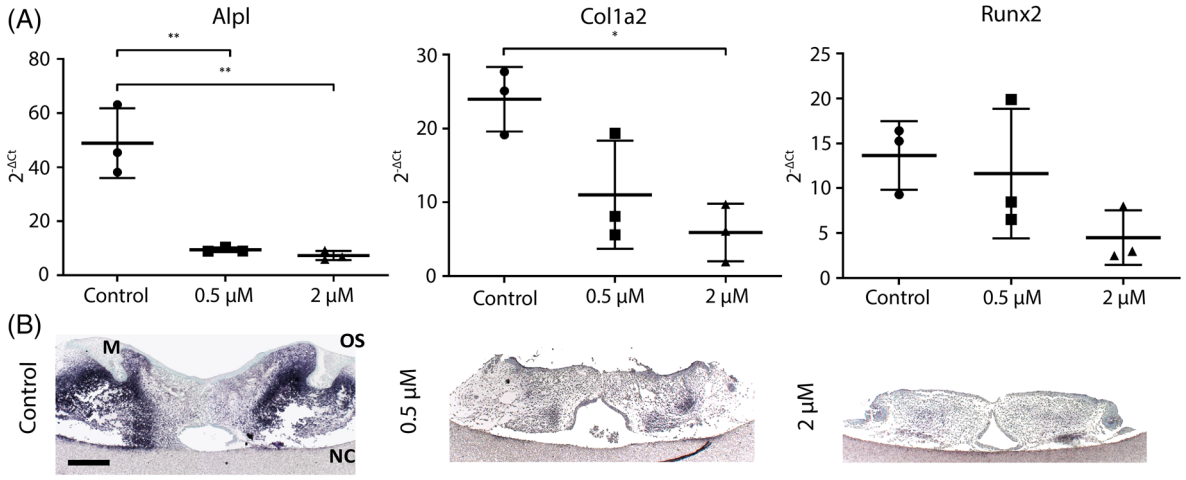

FIGURE 6 Retinoic acid inhibits the expression of osteogenic markers. After four days in culture RNA from the palates was isolated for qPCR. Additionally, palates were fixed in 4% paraformaldehyde for ALP staining. **(A)** Relative osteogenic markers gene expression. Data are represented as the mean ± SD ($N = 3$). * $p <$ 0.05, ** $p < 0.01$. **(B)** Alkaline phosphatase staining. Representative pictures of each group. Dark blue staining indicates ALP activity. *M*: molar tooth bud. *OS*: oral side. *NC*: nasal cavity Scale bar: 200 μm

almost absent and the signal in the epithelial tissues was reduced (Figure 5B, DKK3).

Palates from the control group, showed WIF1 expression in the nasal and oral epithelium, and in the lateral mesenchymal tissue (Figure 5B, WIF1-control). In the 0.5 μM retinoic acid-treated group, oral epithelial WIF1 expression was restricted to the intermolar region, and the mesenchymal expression was strongly reduced. In the 2 μM RA-treated group, a weak WIF1 expression was present in the lateral parts of the oral epithelium. Palates from the control group, showed a strong SFRP4 protein expression in the nasal epithelium but not in the oral epithelium. The lateral mesenchymal areas were also stained (Figure 5B, SFRP4-control). Interestingly, the expression in the oral epithelium was greatly increased in the 0.5 μM retinoic acid-treated palates, which continued into the medial epithelial seam and the nasal epithelium. Additionally, SFRP4 was highly expressed in the molar buds and the surrounding condensed mesenchyme but absent in the lateral mesenchyme. The 2 μM retinoic acid-treated group showed slightly increased expression in the oral and nasal epithelium, and the molar buds (Figure 5B, SFRP4).

Osteogenic differentiation in palatal shelves treated with retinoic acid

As WNT signaling is related to bone formation, we also analyzed the expression of three marker genes for osteogenic differentiation. The gene expression of the early osteogenic marker alkaline phosphatase (*Alpl*) was significantly downregulated in the palates treated with 0.5 μM and 2 uM retinoic acid ($p < 0.01$, Figure 6A). The extracellular matrix protein collagen type I alpha 2 (*Col1a2*) was significantly downregulated in the 2 μM retinoic acid-treated palates ($p < 0.05$, Figure 6A). Retinoic acid did not significantly inhibit *Runx2* expression, but showed a clear trend ($p >$ 0.04, Figure 6A).

To localize ALP activity, we used an enzymatic staining on histological sections. In the controls, ALP activity was located in the lateral sides of the palatal shelves and around the molar tooth buds. ALP activity was completely absent in the two retinoic acid treated groups except a few small lateral spots in the 0.5 μM retinoic acid-treated palates (Figure 6B).

DISCUSSION

The complex molecular and cellular regulation of palate development is susceptible to disruptions, which may lead to c left palate [42]. In humans, retinoic acid is known to increase the risk of cleft palate if the serum concentration is outside the normal range of 0.004–0.009 μM [43–45]. In addition, retinoic acid is known to interact with WNT signaling, which has an essential role during embryogenesis [46, 47]. We hypothesized that retinoic acid disrupts palate development by affecting WNT signaling. Therefore, we cultured palates from mouse embryos (E13.5) to study the effect of retinoic acid on palate fusion and the underlying mechanism.

Our results show that retinoic acid reduces palate fusion in vitro as measured by the reduced disappearance of the medial epithelial seam. Previous studies show similar effects of retinoic acid both in vitro and in vivo, which was related to the inhibition of apoptosis and cell migration in the medial epithelial seam [48–50]. However, in our data, the expression of the apoptosis marker Trp53 was not affected by retinoic acid. As the medial epithelial seam is only a small part of

the total palatal shelves tissue, we might not have picked up an inhibition of Trp53 expression. In addition, we show reduction in the growth of the palatal shelves by retinoic acid. Similarly, studies in pregnant mice exposed to retinoic acid show that the growth of the palatal shelves was reduced because of an inhibition of mesenchymal cell proliferation leading to cleft palate [51–54]. Conversely, our results show increased Ki-67 expression by retinoic acid, which seems to be mainly located in the epithelium of the treated palates. Contradictory effects of retinoic acid on cell proliferation have been also reported earlier. While retinoic acid treatment induces proliferation of irradiated murine fetal liver-derived stromal cells, it also has anti-proliferative effects on human renal and breast cancer cells [55–57].

To clarify the mechanism of the retinoic acid effects, we evaluated the activity of WNT signaling. WNT signaling plays an important role during development, controlling both proliferation and differentiation processes [58]. Disruption of WNT signaling during mouse palate development can lead to cleft lip and/or palate [47]. For instance, conditional inactivation of WNT/β-catenin signaling in the mouse palate epithelium leads to cleft lip and/or palate due to failed palate fusion [59]. It has also been shown that LiCl-induced WNT activation during palate development inhibits palate fusion inhibition and ossification [60]. Our results show that retinoic acid-treatment upregulated the expression of the WNT marker gene *Axin2*. *Axin2* is recognized as a good marker gene in vertebrates for WNT signaling as it acts in a negative feedback loop to limit and fine-tune Wnt signaling [61–64]. Studies in mouse mesenchymal stem cells and human fetal palatal chondrocytes also showed that retinoic acid stimulated WNT signaling by an increase in the β-Catenin level during osteogenic induction [65, 66]. However, studies in several types of cancer cells and mouse pre-osteoblasts have shown that retinoic acid can also down-regulate WNT signaling [37, 67–69]. Interestingly, another study reports suppression of Wnt signaling and cleft palate in mouse embryos in vivo by retinoic acid [53]. However, they included tongue tissue in the expression analyses, which might have affected their results as it has been reported that WNT signaling is required to induce proliferation of epithelial cells and differentiation of muscle progenitor cells in the tongue [70]. Our results also showed that the increased AXIN2 expression is mainly localized in the palate epithelium along with an increase in the proliferation marker KI-67. Enhanced proliferation related to WNT signaling activation has also been reported in mouse and human cardiomyocytes, and human ocular epithelial cells [71, 72]. Our results indicate that increased WNT signaling in retinoic acid-treated palatal shelves induces proliferation of epithelial cells.

The increased activity of WNT signaling might also explain the persistence of the medial epithelial seam in the retinoic acid-treated palates. Several studies in the cancer field show that WNT signaling inhibits apoptosis, one of the crucial cellular processes for medial epithelial seam disappearance [73–75]. For instance, inactivation of the WNT inhibitor *DKK1* in human breast cancer cells increases WNT signaling and inhibits apoptosis [75]. In addition, reduced expression of *DKK2* in human and mouse breast cancer cells has also been related to inhibition of apoptosis and cell migration [76].

Our results also show that retinoic acid down-regulates the expression of the WNT inhibitors *Dkk1*, *Dkk3* and *Wif1* in the cultured palates. Similar results were found in mouse bone marrow stem cells in which retinoic acid stimulated WNT target gene expression by downregulation of *Dkk1* [77]. This was also reported in human neuroblastoma cells and in mouse cerebrovascular development where retinoic acid is required to suppress *Dkk* and *Sfrp* expression [78, 79]. Also, during lung development, a retinoic acid-WNT network seems to maintain lung progenitor cell fate by a retinoic acid-dependent *Dkk1* suppression leading to increased WNT signaling [80]. Together, the data suggest that retinoic acid stimulates WNT signaling through repression of WNT inhibitors. However, further work is needed to clarify the exact mechanism through which retinoic acid downregulates WNT inhibitors in palate development.

Different from the other WNT inhibitors, Sfrp4 gene and protein expression was increased in the palates treated with retinoic acid. This was also shown in pancreatic cancer cells, where retinoic acid induced *Sfrp4* expression as well as decreased WNT signaling [81]. Interestingly, SFRPs can also function as WNT enhancers favoring the transport of WNT ligands to the FZD receptor in *Xenopus* embryos, MDCK cells and *Drosophila* S2 cells [82, 83]. Similarly, a simultaneous increase in SFRP4 and WNT expression has been shown in mouse and human skin affected by systemic sclerosis [84]. Based on these reported mechanisms of SFRP4, we suggest that it functions as an agonist of WNT signaling after retinoic acid exposure of the palate.

In normal palate development, bone formation starts around the time of fusion in the lateral areas of the palatal shelves [4]. Our results show a reduction in expression of the osteogenic marker *Alp* by retinoic acid, a trend of reduction in *Runx2* and a pronounced decrease in ALP activity in the mesenchyme. Also, in vivo retinoic acid inhibits the development of the palatine and maxillary bones in mouse embryos [85]. In general, increased WNT signaling is related to an increased bone mass in mouse and rat studies [86, 87]. This evidence suggests that retinoic acid reduces WNT signaling and subsequent osteogenesis in palate development. In mesenchymal stem cells, WNT signaling is required for their commitment to the osteoblast lineage, and inhibition of the adipogenic and chondrogenic cell fate [23, 88]. Once commitment is established, canonical WNT signaling is essential for osteoblast precursor proliferation and differentiation [89]. It has also been shown that too low as well as too high serum retinoic

acid levels can contribute to poor bone health and skeletal fragility in humans [90]. Moreover, long-term exposure to retinoic acid impedes osteoblast differentiation and prevents mineralization of mouse pre-osteoblasts, bone marrow stromal cells and calvarial bone cultures [91–93]. Additionally, it has been suggested that retinoic acid inhibits the differentiation of osteogenic progenitor cells, leading to a marked reduction in the expression of osteogenic markers (*Runx2, Alpl, Sp7)* as also shown in our results [94, 95]. We suggest that long-term retinoic acid exposure inhibits bone formation trough down-regulation of osteogenic genes.

In summary, this study shows that retinoic acid significantly reduces palate fusion and osteogenic differentiation. Our data suggest that this is correlated to an increased WNT signaling caused by a reduced expression of WNT inhibitors. Further in vivo experiments, for instance using reporter mouse lines, are needed to validate our in vitro findings.

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CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Laury Roa, Hans Von den Hoff; **Investigation**: Laury Roa, Marjon Bloemen. **Writing – original draft**: Laury Roa, Hans Von den Hoff. **Writing-review and editing**: Laury Roa, Hans Von den Hoff, Frank Wagener, Carine Carels. All authors approved the final manuscript.

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