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Negative effects of retinoic acid on stem cell niche of mouse incisor

Jinlei Xi [b], Shijing He [a], Cizhao Wei [a], Wanyao Shen [a], Juan Liu [b], Ke Li [a], Yufeng Zhang [c], Jiang Yue [a], Zheqiong Yang [a,*]

[a] Department of Pharmacology, Wuhan University School of Basic Medical Science, 185 Donghu Road, Wuhan, Hubei 430071, China
[b] Department of Pharmacy, Wuhan University of Science and Technology School of Medicine, 2 West Huangjiahu Road, Wuhan, Hubei 430065, China
[c] The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan 430079, China

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ABSTRACT

The continuous growth of mouse incisors depends on epithelial stem cells (SCs) residing in the SC niche, called labial cervical loop (LaCL). The homeostasis of the SCs is subtly regulated by complex signaling networks. In this study, we focus on retinoic acid (RA), a derivative of Vitamin A and a known pivotal signaling molecule in controlling the functions of stem cells (SCs). We analyzed the expression profiles of several key molecules of the RA signaling pathway in cultured incisor explants upon exogenous RA treatment. The expression patterns of these molecules suggested a negative feedback regulation of RA signaling in the developing incisor. We demonstrated that exogenous RA had negative effects on incisor SCs and that this was accompanied by downregulation of Fgf10, a mesenchymally expressed SC survival factor in the mouse incisor. Supplement of Fgf10 in incisor cultures completely blocked RA effects by antagonizing apoptosis and increasing proliferation in LaCL epithelial SCs. In addition, Fgf10 obviously antagonized RA-induced downregulation of the SC marker Sox2 in incisor epithelial SCs. Our findings suggest that the negative effects of RA on incisor SCs result from inhibition of mesenchymal Fgf10.

1. Introduction

The primary functions of tissue stem cells (SCs) are to replace cells that die due to injury or disease, and to maintain the steady functions of organs (Xin et al., 2016). SCs are capable of self-renewal throughout the lifetime of the organism and can be activated to give rise to fully differentiated cells. The numbers of SCs are usually maintained at stable levels to ensure tissue homeostasis but they may be elevated, at least transiently, during tissue repair (Fuchs et al., 2013). The maintenance of homeostasis of the SCs niche relies on a balance between the gain of SCs through proliferation and the loss of SCs through differentiation or apoptosis (Alenzi et al., 2009; Biteau et al., 2011). Understanding how SCs acquire their fates is a central question in stem cell biology.

Mouse incisors grow continuously to compensate for the wear of the tooth at the apical end. The enamel is deposited asymmetrically on the surface of the incisor, and the labial side of the incisor is covered with enamel, whereas the lingual side has only soft dentin. Therefore, the lingual side is more susceptible to abrasion, which leads to the formation of a cutting edge. The SCs, residing in a structure called the labial cervical loop (LaCL) at the proximal end of the incisor, provide a continuous supply of cells to compensate for the constant abrasion (Harada et al., 1999). The LaCL is composed of loosely arranged stellate reticulum (SR) cells and a single layer of basal epithelial cells (inner and outer enamel epithelium) that surround the SR. The progeny of the SCs are incorporated from the SR into the enamel epithelium. The cells move apically as they proliferate in the transit amplifying (TA) zone and differentiate into enamel secreting ameloblasts (Fig. 1A and B) (Harada et al., 1999; Tummers and Thesleff, 2003).

Retinoic acid (RA), the primary metabolic derivative of vitamin A (retinol), is perceived as a "morphogen" (Lewandoski and Mackem, 2009; Schilling et al., 2012). Numerous studies revealed that a broad range of physiological processes, from embryonic development to adult homeostasis, are regulated by RA. (Blum and Begemann, 2013; Cunningham and Duester, 2015; Maden, 2007; Rhinn and Dolle, 2012). RA plays a critical role in craniofacial development. Endogenous RA is necessary for the initiation of odontogenesis. It is present in the embryonic mouse mandible and reaches a peak concentration at the time of the formation of the dental lamina (Kronmiller et al., 1995b). A 24 h exposure to citral, a known inhibitor of RA synthesis, prior to the formation of the dental lamina (E9), completely blocked tooth formation in mandibles. Tooth formation was restored by treatment with RA during citral exposure (Kronmiller et al., 1995a). On the other hand, an excess of RA was suggested to induce stage-specific inhibition of tooth morphogenesis. When exogenous RA is applied ex vivo before the formation of the dental lamina, it leads to formation of supernumerary incisors and missing molars (Kronmiller and Beeman, 1994; Kronmiller et al., 1995b).

* Corresponding author at: 185 Donghu Road, Wuhan, Hubei 430071, China.
E-mail address: zheqiong_yang@whu.edu.cn (Z. Yang).

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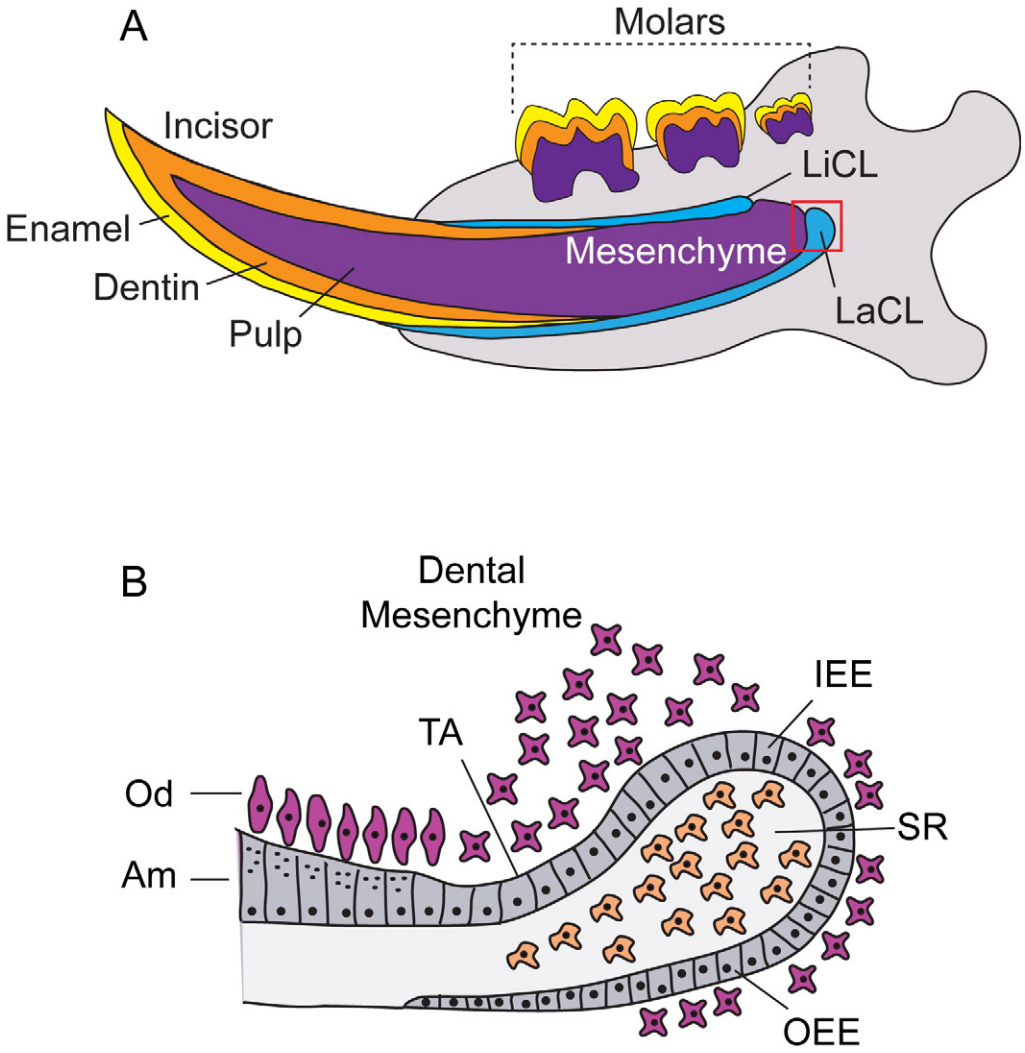


Fig. 1. Schematic illustrations of the mouse incisor. (A) Sagittal cross section of mandible. (B) Higher magnification of LaCL of incisor in a sagittal section. Am, ameloblasts; Od, Odontoblasts; LaCL, labial cervical loop; LiCL, lingual cervical loop; TA, transient amplified cells; IEE, inner enamel epithelium; OEE, outer enamel epithelium; SR, stellate reticulum.

Fgf10 is a crucial growth factor for incisor development. Fgf10 is expressed in the mesenchyme surrounding the LaCL, and binds to FGFR2b, which is expressed in the LaCL epithelium (Harada et al., 1999). Fgf10-null embryos exhibit hypoplastic incisors. Ex vivo culture of developing incisors in the presence of Fgf10-blocking antibodies causes regression of the LaCL, suggesting that Fgf 10 acts as a survival factor for SCs in the LaCL (Harada et al., 2002). Conversely, the loss-of-function of Fgf antagonists Spry2 and Spry4 results in thickened LaCL and formation of huge tusk-like incisors (Charles et al., 2011).

Interactions between epithelial and mesenchymal tissues play crucial roles in the development of epithelial organs (Balic and Thesleff, 2015; Biggs and Mikkola, 2014; Ribatti and Santoiemma, 2014; Shaker and Rubin, 2010), and they also exert key functions in the maintenance of epithelial SCs during organ renewal (Balic and Thesleff, 2015; Sennett and Rendl, 2012; Shaker and Rubin, 2010; Tummers and Thesleff, 2009). Like tooth morphogenesis, the maintenance of the cervical loop SC niche also relies on reciprocal interactions between epithelial and mesenchymal compartments (Balic and Thesleff, 2015; Jussila and Thesleff, 2012; Tummers and Thesleff, 2009). The conserved signaling pathways, such as Wnt, Fgf, Tgfβ, Bmp, and Shh, mediate interactions between the mesenchymal and epithelial cells within the SC niche and form integrated networks during the renewal of the epithelial tissue in organs such as hair, intestine and teeth (Balic and Thesleff, 2015; Harada et al., 1999; Le Guen et al., 2015; Sennett and Rendl, 2012).

In this study, we have explored the effects of exogenous RA on the homeostasis of mouse incisor SC niche in cultured incisor explants. The expression profiles of several key molecules in the RA signaling pathway following RA treatment suggest a negative feedback regulation of the RA signal in the developing mouse incisor. We discovered that exogenous RA has negative effects on the SC niche and that it up-regulated apoptosis and down-regulated proliferation and the expression of the SC marker Sox2 in the LaCL. This was accompanied by inhibition of Fgf10, an important mesenchymal signaling molecule in the incisor. The effects of RA on incisor SCs could be completely blocked by exogenous Fgf10, implying that the negative effects of RA on the incisor SC niche result from downregulation of Fgf10.

2. Materials and methods

2.1. Animals

NMRI mice were used at postnatal day 2 (P2). Plug day was taken as embryonic day 0 (E0). Sox2-GFP mice, in which eGFP expression is

under the control of a 5.5 kb fragment of the upstream regulatory element of the Sox2 promoter, were obtained from Jackson Laboratories (stock no. 017592). All protocols were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and conformed to Regulations for the Administration of Affairs Concerning Experimental Animals (Hubei Province, China 2005).

2.2. Organ culture

The incisors were dissected from lower jaws at P2 in Dulbecco's phosphate buffered saline (PBS) pH 7.4, and cultured in Trowell-type organ culture dishes (RA treatment) or as hanging drops (all other treatments) as previously described (Narhi and Thesleff, 2010). A minimum of biological and technical triplicates were used for each treatment. RA (Sigma) was added in standard medium (DMEM/F12 + 10% FCS) to obtain three final concentrations, 1 μM, 3 μM and 10 μM. To block the effects of RA, 100 ng/ml Fgf10 (R&D) was added with 3 μM RA in medium.

2.3. Immunohistochemistry

Immunostaining was conducted on whole explants. After culture, explants were fixed overnight (O/N) in 4% paraformaldehyde (PFA) at 4 °C. Nonspecific staining was blocked by incubation O/N in 10% normal donkey serum and 0.5% TritonX-100 in PBS at 4 °C. Explants were incubated with primary antibody Caspase 3 (1:400, Cell Signaling Technology, RRID: AB_331441) at 4 °C for two days. Subsequently they were incubated O/N at 4 °C with Alexa Fluor conjugated secondary antibody (1:300, Life Technologies) and counterstained with Hoechst 33343. Samples were mounted with Vectashield (Vector Laboratories) and visualized using a Zeiss LSM 700 confocal microscope. Images were analyzed with Imaris 7.2.3 software (Bitplane).

2.4. EdU incorporation

In ex vivo experiments, after 2 days culturing explants were incubated with 10 μM EdU for 2 h, and then fixed in 4% PFA O/N at 4 °C. EdU incorporation into DNA was detected in whole explants by using Click-iT EdU Alexa Fluor Imaging Kit (Life Technologies) according to the manufacturer's protocol. The samples were counterstained with Hoechst 33343 at 4 °C O/N. The tissues were mounted with Vectashield (Vector Laboratories) and visualized using a Zeiss LSM700 confocal microscope. Images were analyzed with Imaris 7.2.3 software (Bitplane).

2.5. RNA isolation and quantitative RT-PCR (qRT-PCR)

After 24 h culturing, total RNA of the cultured explants, which includes the LaCL and LiCL as well as the mesenchyme in between, was isolated with the RNeasy Plus Micro Kit (Qiagen) according to the manufacturer's instructions. First strand cDNA was synthesized using the SuperScript III Reverse Transcriptase (Life Technologies). The quantitative PCR (qPCR) reactions were conducted with LightCycler DNA Master SYBR Green I (Roche). The qPCR thermal cycler conditions were: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C. Data were normalized against GAPDH expression. A minimum of triplicate samples was assayed each time. The primers sequences were listed in Table S1.

2.6. In situ hybridization

Whole mount in situ hybridization was performed according to the protocol described by Wilkinson (1992) with modifications. Briefly, the explants were fixed O/N in 4% PFA at 4 °C, bleached with 6% H_2O_2 in PBT (PBS/0.1% Tween), pretreated with proteinase K (Sigma), refixed in fresh 4% PFA/0.2% glutaraldehyde in PBST, prehybridized for 1 h and then hybridized O/N with digoxigenin-labelled Shh probe (Kim et al., 1998) at 70 °C in a hybridization buffer. After hybridization, tissues were washed in high-stringency conditions and preblocked in antibody blocking solution, and then incubated with preabsorbed antibody O/N at 4 °C. Color development was performed in BM Purple AP Substrate Precipitating Solution (Boehringer Mannheim). The tissues were postfixed in 4% PFA at 4 °C for 1 h before photography.

2.7. Statistical analysis

One-Way ANOVA was used for statistical analysis of relative expression of RARs, RXRs, CYP26s, RALDHs, area measurement of the LaCL and fluorescence intensity of GFP in the LaCL. The proliferating and apoptotic cell numbers and relative expression folds of Bcl2 and Fgf10 were tested with the Student *t*-test. The results are presented as mean ± SEM. In all comparisons, $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1. The effects of exogenous RA on the expression of RARs, RXRs, RADLHs and CYP26s in cultured incisor explants

In order to study the expression patterns of key molecules in the RA signaling pathway in the LaCL upon treatment with exogenous RA, we turned to our tissue culture system. The proximal end of the P2 mouse incisors, which include the LaCL and LiCL as well as the mesenchyme in between, was dissected and cultured ex vivo. RA was diluted in DMSO and used with 1 μM, 3 μM or 10 μM concentration. The controls were supplemented with the equivalent amount of DMSO. The whole explants were collected after 1 day culture for total RNA extraction.

We measured the expression of two RA receptor families, retinoic acid receptors (RARs: RARα, RARβ and RARγ) and retinoid X receptors (RXRs: RXRα, RXRβ and RXRγ). In addition, the expression levels of two enzyme families, RA synthetases retinaldehyde dehydrogenases (RALDHs) and RA metabolic enzymes Cytochrome P450 26 (CYP26s) were analyzed.

Our quantitative PCR data indicated that exogenous RA induced a marked downregulation of RARα, RARγ, RXRα and RXRβ (10 μM, $p < 0.05$), whereas RARβ transcription was increased significantly upon RA treatment (1 μM, 3 μM and 10 μM, $p < 0.05$). RA showed no visible effect on RXRγ expression (Fig. 2A).

In the RALDH family, the expression of all three isoforms (RALDH1, RALDH2 and RALDH3) decreased upon RA treatment (RALDH1: 3 μM, $p < 0.05$; 10 μM, $p < 0.01$) (RALDH2: 1 μM, 3 μM and 10 μM, $p < 0.01$) (RALDH3: 1 μM, $p < 0.05$; 3 μM and 10 μM, $p < 0.01$) (Fig. 2B). Among three isoforms of the CYP26 family (CYP26A1, CYP26B1 and CYP26C1), CYP26A1 and CYP26B1 transcription was significantly enhanced by RA (CYP26A1: 10 μM, $p < 0.01$) (CYP26B1: 1 μM, $p < 0.05$; 3 μM and 10 μM, $p < 0.01$) (Fig. 2B). The RA-induced upregulation of CYP26B1 appeared to be dose dependent (not statistically significant). The effects of RA on CYP26C1 were opposite to that of the other two CYP26 members. The expression of CYP26C1 was significantly inhibited by RA (3 μM and 10 μM, $p < 0.01$) (Fig.2B).

To summarize, the expression profiles of these key molecules suggest a negative feedback regulation of RA signaling in the developing incisor.

3.2. Exposure of LaCL to exogenous RA ex vivo results in thinning of the LaCL

RA plays a critical role during embryonic development in many organs (Cunningham and Duester, 2015). Though RA has a known role in tooth morphogenesis (Gibert et al., 2015; Kronmiller et al., 1995a,b), its effects on the incisor SC niche are not known. To explore the effects of RA on the incisor SC niche, we cultured the proximal parts of P2 Sox2-GFP reporter mouse incisors with different RA concentrations (1 μM, 3 μM or 10 μM). Sox2-GFP is expressed specifically in the LaCL (Juuri et al., 2012). The size of the LaCL and the

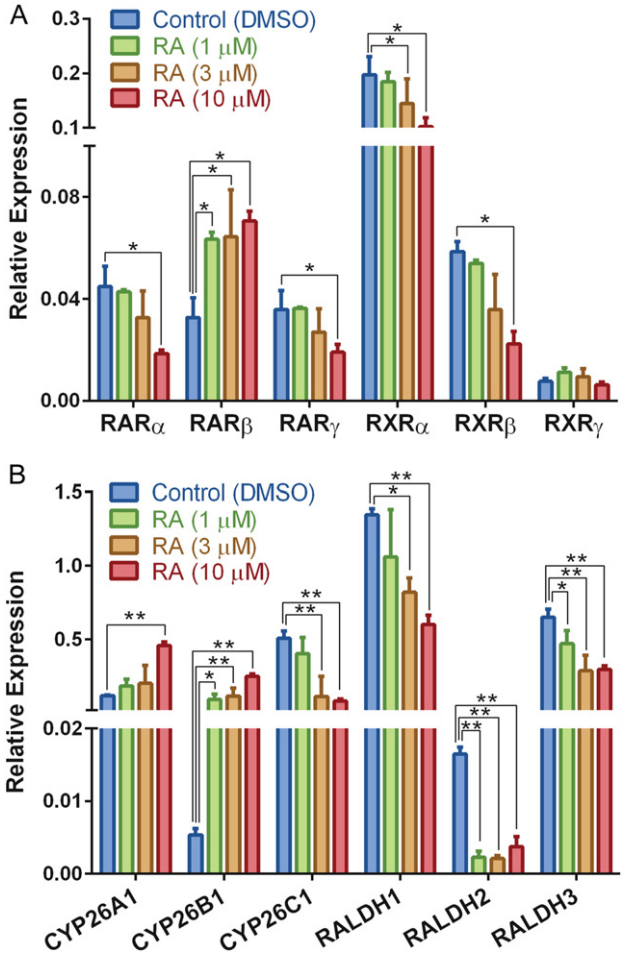


Fig. 2. The effects of exogenous RA on the expression of RARs, RXRs, RALDHs and CYP26s in P2 mouse incisor cultured ex vivo. The proximal ends of P2 mouse incisors were cultured with 1, 3, and 10 μM RA respectively for 24 h, and the controls were supplemented with the equivalent amount of DMSO. Total RNA was extracted from the cultured explants after 1 day culture. The transcripts of the isoforms of RARs (α, β and γ) and RXRs (α, β and γ) (A), RALDH1As (1, 2 and 3) and CYP26s (A1, B1 and C1) (B) were measured by qRT-PCR, and the data are shown as relative expression compared to GAPDH levels. (*$p < 0.05$, **$p < 0.01$).

fluorescence intensity of GFP in the LaCL were measured after 1 and 2 days of culture. Already after 1 day, the LaCLs cultured with RA were significantly thinner than controls. The thinning was more evident after 2 days ($p < 0.01$) (Fig. 3A and B). Moreover, the inhibitory effects of RA on LaCL size were concentration-dependent - the higher the RA concentration in the culture was, the thinner the LaCLs were (1 day, $p < 0.05$; 2 days, $p < 0.01$) (Fig. 3A and B). The intensity of Sox2-driven GFP expression in the LaCL was also remarkably inhibited by all three concentrations of RA after 1 and 2 days of culture ($p < 0.01$) (Fig. 3A and C).

3.3. RA induces apoptosis and inhibits proliferation in the LaCL

To discover the mechanism of RA-induced thinning of the LaCL, we next examined cell proliferation and apoptosis using EdU incorporation and immunostaining of activated Caspase-3, respectively. In the above-described experiments, all three RA concentrations triggered a clear, suppressive effect on the size of the LaCL. Therefore, in this part, we chose the medium dose (3 μM) to examine RA effects on cell proliferation and apoptosis.

After 2 days of culture, RA inhibited proliferation in the LaCL epithelium, particularly in the SR compartment (Fig. 4A). By using Imaris software (Bitplane), confocal 2D image stacks were reconstructed to 3D stereo and the proliferating cells were counted. The data showed that the number of proliferating cells in the SR compartment was remarkably decreased after 2 days treatment with RA ($p < 0.01$) (Fig. 4B, Video S1 and S2).

It has been shown that an apoptotic cell population is located in a specific area of the SR, close to the outer enamel epithelium (Yang et al., 2015). We counted the Caspase-3 positive apoptotic cells from the reconstructed 3D stereo images of the LaCL epithelium. We observed that the region harboring the apoptotic cells had significantly expanded in the RA treated explants ($p < 0.05$) (Fig. 4C and D, Video S3 and S4). In addition, we found that the expression of Bcl2, an anti-apoptotic molecule, was significantly inhibited by RA (Fig. 4E). These results were consistent with previous reports that RA-induced apoptosis was associated with downregulation of Bcl-2 in umbilical cord stem cells and breast cancer cell lines (Pratt et al., 2003; Saraee et al., 2014).

3.4. Fgf10 rescues the RA-induced thinning of the LaCL via antagonizing downregulation of cell proliferation and upregulation of apoptosis

Previous studies have demonstrated that Fgf10 is indispensable for epithelial SC survival in the incisor (Harada et al., 2002). This molecule furthermore antagonizes the proapoptotic effects of mesenchymal Wnt activation in the LaCL (Yang et al., 2015). We analyzed mRNA levels of several signaling factors in P2 LaCL explants cultured for 1 day in the presence or absence of RA, and found that Fgf10 expression was inhibited remarkably by RA ($p < 0.05$) (Fig. 5A). Therefore, we speculated that Fgf10 might be involved in the negative regulatory effects of RA on the LaCL. To examine the potential of Fgf10 to counteract the effects of RA, we added Fgf10 (100 ng/ml) to culture medium of P2 Sox2-GFP incisor explants that either contained or did not contain RA. After 2 days of culture, we measured the sizes of LaCLs and the fluorescence intensity of GFP in LaCLs. Expectedly, RA induced a remarkable thinning of the LaCL ($p < 0.01$) and a significant decrease in the expression of Sox2-GFP in the LaCL ($p < 0.01$), whereas Fgf10 increased the size of the LaCL ($p < 0.01$) (Fig. 5B, C and D). When the explants were co-cultured with Fgf10 and RA, the thinning of the LaCL and the downregulation of Sox2 expression were rescued and resembled the control (Fig. 5B, C and D). This indicates that Fgf10 blocked the negative effect of RA on the LaCL.

The effects of Fgf10 on apoptosis and proliferation in the LaCL were analyzed after 2 days of culture. Enhanced apoptosis was detected in the SR compartment of the RA treated LaCLs, whereas it was almost completely blocked by Fgf10 treatment (Fig. 5E). The inhibition of Cell proliferation in the LaCL induced by RA was obviously antagonized by Fgf10, particularly in the SR compartment (Fig. 5E). To confirm that the blocking effect of Fgf10 on RA was specific, we also examined the intervention effect of some other key signaling molecules known to be involved in maintenance of the SC niche. Our data showed that addition of Fgf8 or Noggin to the culture medium did not block the negative effects of RA (Fig. S1). Noggin is a Bmp inhibitor and Bmp4 is a negative regulator of SC niche of mouse incisor (Wang et al., 2007).

3.5. RA induced the downregulation of Shh in the transient amplifying (TA) zone

Besides apoptosis and proliferation, we also examined the effects of RA on SC differentiation in cultured incisor explants. Our qPCR results showed that the expression of Sonic hedgehog (Shh), which marks cells that are differentiating along the ameloblast lineage, was remarkably downregulated after the treatment with exogenous RA in cultured incisor explants. Furthermore, our

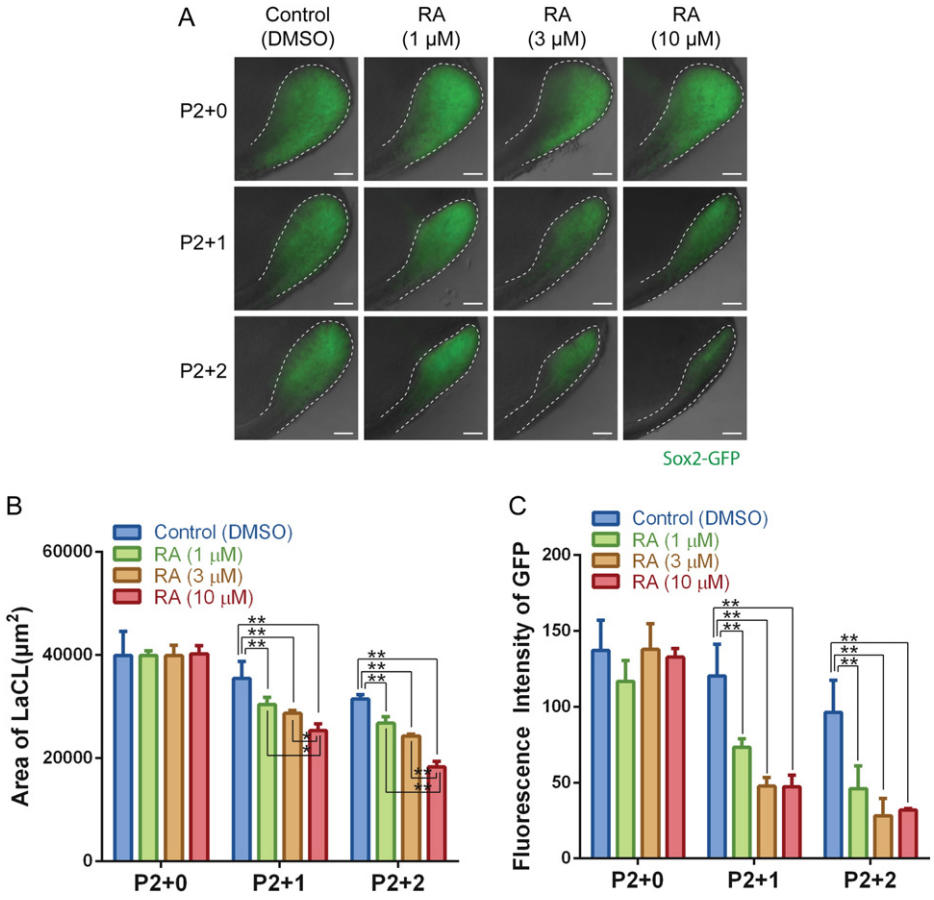


Fig. 3. RA treatment results in thinning of the LaCL. The proximal end of P2 Sox2-GFP reporter mouse incisor was dissected and cultured with 1, 3, and 10 μM RA respectively for 24 h and 48 h. The controls were supplemented with the equivalent amount of DMSO. The size of LaCLs and the fluorescence intensity of GFP were measured after the culturing. All three concentrations (1, 3 and 10 μM) of RA caused the significant thinning of the LaCL ($p < 0.01$) and the remarkable downregulation of Sox2-driven GFP expression ($p < 0.01$). The effects of RA on LaCL size is in a concentration-dependent manner (P2 + 1, $p < 0.05$; P2 + 2, $p < 0.01$). (A) Representative images of LaCLs treated with RA. Quantification of LaCL size (B) and GFP fluorescence intensity (C) of RA treated explants. Scale bar = 50 μm.

in situ hybridization data displayed the shrinking of the TA zone consisting of Shh positive pre-ameloblast cells from the proximal end of the incisor (Fig. 6). Fgf10 has been proven to be vital for maintaining Shh expression in the developing incisor (Klein et al., 2008; Rice et al., 2004), therefore, the suppressive effects of RA on differentiation of incisor SCs could be secondary to the inhibition of Fgf10.

4. Discussion

In this study, we analyzed the expression profiles of several key molecules in the RA signaling pathway upon exogenous RA treatment in cultured incisor explants, two RA receptor families (RARs and RXRs) and two enzyme families that produce RA (RALDHs) and degrade it (CYP26s). We explored the effects of exogenous RA on the homeostasis of mouse incisor SCs, and found that via inhibition of mesenchymal Fgf10, RA exerted negative effects on the incisor SC niche, which included upregulation of apoptosis and downregulation of proliferation in LaCL epithelial cells.

The mechanisms by which RA induces apoptosis have not been fully elucidated. The existing evidence elucidating the proapoptotic and antiproliferative activities of RA is mostly limited to the mechanism of RA anti-cancer action (Noy, 2010). In breast cancer cell lines, RA was shown to induce mitochondrial apoptosis accompanied by downregulation of the antiapoptotic protein Bcl2 and to trigger growth inhibition by inducing cell cycle arrest (Danforth, 2004; Niu et al., 2001). We demonstrated that in the continuously growing mouse incisor, RA negatively regulated the homeostasis of the mouse SC niche by promoting apoptosis and restraining proliferation in LaCL epithelial cells, and that these effects were accompanied by inhibition of Bcl2 expression.

As a crucial survival signal in the mouse incisor, Fgf10 is known to be expressed in the mesenchyme surrounding the LaCL (Harada et al., 2002; Zhao et al., 2011). We discovered that RA suppressed the expression of Fgf10 and the negative effects of RA on mouse incisor SCs were blocked by addition of Fgf10 to the incisor cultures. Our findings suggest a possible coordination between RA and Fgf10 during incisor development. In addition, we observed a negative feedback regulation of the RA signal in the developing mouse incisor. This negative feedback loop is likely a protective mechanism built in the SC niche, which could protect the SCs from excess RA signaling, and maintain the proper level of survival molecule Fgf10. RA signaling was also reported to be linked to Fgf signaling during morphogenesis of other organs. The increase of RA levels, caused by the absence of CYP26B1, suppressed Fgf10 expression in the bend region of the middle palatal shelves in the developing palate and was accompanied by decreased cell proliferation (Okano et al., 2012). In the developing mammalian inner ear, disruption of the FGF10 signal was induced by excess RA within the otic epithelium, leading to downregulation of the Fgf10 downstream gene Dlx5/Dlx6 expression (Frenz et al., 2010). RA and Fgf10 signaling are also tightly connected during the initial stages of lung morphogenesis. RA was

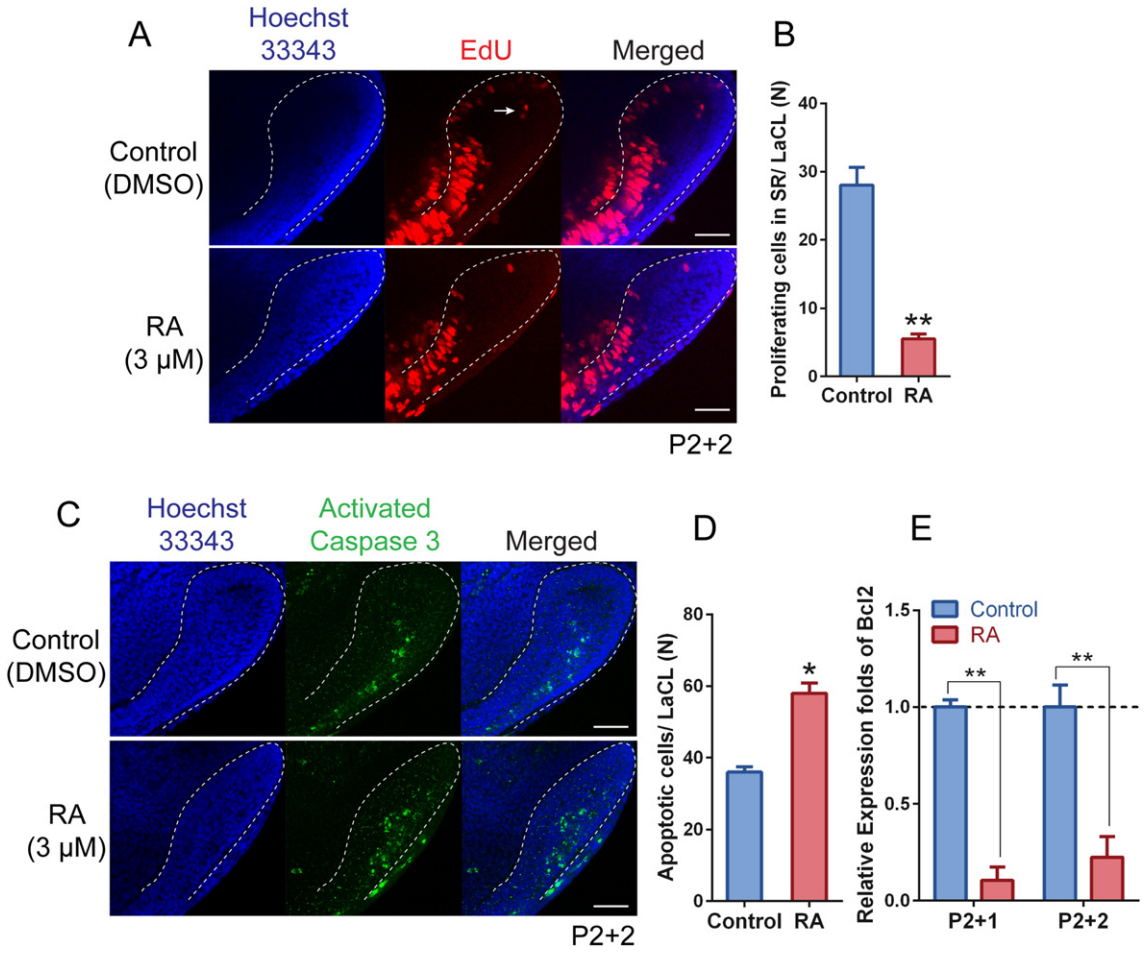


Fig. 4. RA induces apoptosis and inhibits proliferation in the LaCL. (A, B) After 2 days of culture RA inhibited proliferation in LaCL epithelium of P2 explants, especially in SR ($p < 0.01$). The white arrow points to the proliferating cell in SR of the control explants. Scale bar = 50 μm. (C, D) Apoptosis (Activated Caspase 3) was up-regulated by RA in the LaCL of P2 explants after 2 days of culture ($p < 0.05$). Scale bar = 50 μm. (E) P2 explants were cultured with RA for 24 and 48 h, and used for quantitative PCR. Marked downregulation of anti-apoptotic protein Bcl2 was observed after RA treatment ($p < 0.01$).

found to influence early lung pattern formation by suppressing local expression of Fgf10 (Malpel et al., 2000).

In early lung development, Wnt signaling controls Fgf10 expression during the induction of the primordial mouse lung. The activation of Wnt signaling required for lung formation was dependent on local repression of its antagonist, Dickkopf homolog 1 (Dkk1), by endogenous RA. Analysis of mouse Dkk1 genomic sequences revealed one RA-responsive element (RARE) 1212 bp upstream of the translation start site suggesting Dkk1 is a direct target of RA in the lung mesenchymal cells (Chen et al., 2010). We found that Wnt/beta-catenin in mesenchyme was activated upon RA treatment in cultured BATgal mouse incisor explants (Fig. S2). It was previously reported that activation of Wnt/beta-catenin signaling in the mesenchyme surrounding the LaCL inhibited mesenchymal Fgf10 expression, and resulted in massive apoptosis in incisor epithelial SCs (Yang et al., 2015). Therefore, in the developing incisor, RA may regulate Fgf10 expression indirectly through Wnt signaling.

5. Conclusion

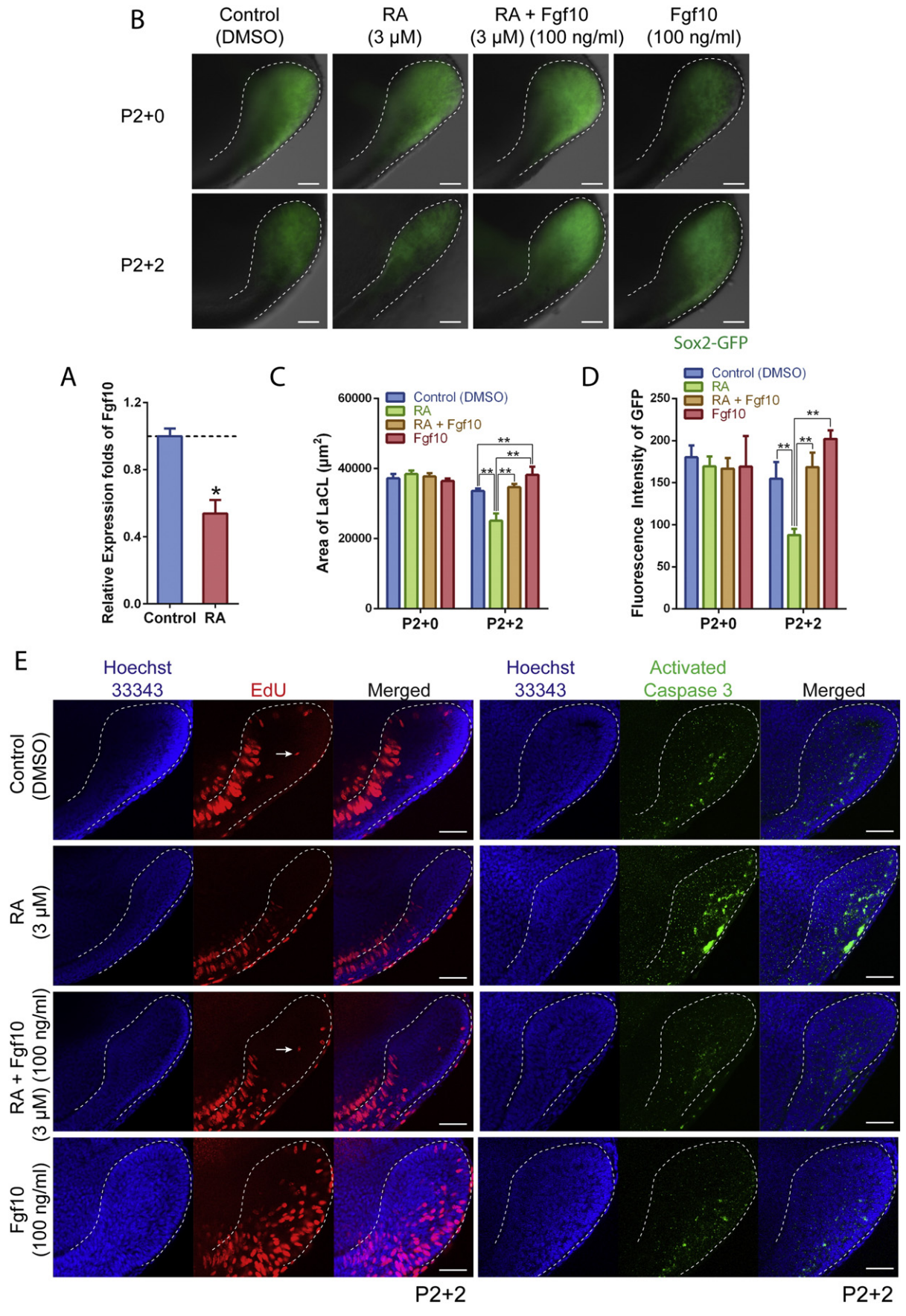
This is the first time that the function of RA signaling has been addressed in the incisor SC niche. We showed that RA signaling is subject to negative feedback regulation in the developing incisor. We demonstrated that exogenous RA exhibits negative effects on the homeostasis of incisor SCs and inhibits proliferation and increases apoptosis. Since the exposure to RA was accompanied by downregulation of mesenchymal Fgf10 expression and exogenous Fgf10 blocked the effects of RA, we conclude that the effects of RA on the incisor SC niche are likely mediated by inhibition of Fgf10 (Fig. 7).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2016.09.030.

Disclosure of potential conflicts of interest

None.

Fig. 5. Fgf10 rescues the thinning of LaCL induced by RA via antagonizing the effects of RA on cell proliferation and apoptosis in the LaCL. (A) The expression of Fgf10 in P2 explants was significantly inhibited by RA after 24 h culture ($p < 0.05$). (B–D) The P2 Sox2-GFP explants were cultured with Fgf10 (100 ng/ml) in the presence or absence of RA (3 μM). The sizes of LaCLs and the fluorescence intensity of GFP in the LaCL were measured after 2 days of culture. (B) Representative images of LaCLs under the treatment with RA or Fgf10 or co-cultured with RA and Fgf10. (C) Marked thinning of LaCL ($p < 0.01$) and (D) significant downregulation of GFP fluorescence intensity ($p < 0.01$) were observed upon RA treatment, (C) while Fgf10 moderately increased the size of the LaCL ($p < 0.01$). Combined supplement of Fgf10 and RA in medium resulted in no visible change in (C) the size of the LaCL and (D) the fluorescence intensity of GFP compared to the controls. These results suggest that Fgf10 rescued the effect of RA on LaCL size and the expression of Sox2. Scale bar = 50 μm. (E) Addition of Fgf10 to the cultures blocked the up-regulation of activated Caspase 3 and down-regulation of cell proliferation in the LaCL induced by RA. The white arrow points to the proliferating cell in SR of Fgf10 treated and control explants. Scale bar = 50 μm.


B
Control (DMSO)
RA (3 μM)
RA + Fgf10 (3 μM) (100 ng/ml)
Fgf10 (100 ng/ml)
P2+0
P2+2
Sox2-GFP
A
Relative Expression folds of Fgf10
Control
RA
C
Area of LaCL (μm²)
Control (DMSO)
RA
RA + Fgf10
Fgf10
P2+0
P2+2
D
Fluorescence Intensity of GFP
E
Hoechst 33343
EdU
Merged
Activated Caspase 3
Control (DMSO)
RA (3 μM)
RA + Fgf10 (3 μM) (100 ng/ml)
Fgf10 (100 ng/ml)
P2+2
P2+2

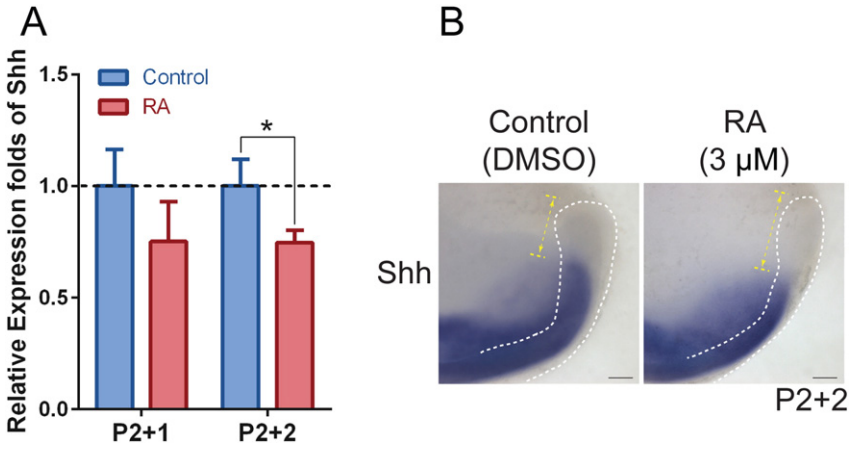


Fig. 6. RA inhibits Shh expression in transient amplifying (TA) zone. The proximal end of P2 mouse incisor was cultured with 3 μM RA for 24 h and 48 h, and the controls were supplemented with the equivalent amount DMSO. The expression of Shh in the cultured explants measured by qPCR at 24 h and 48 h was evidently inhibited by RA (A), and TA zone marked by Shh expression, which was examined by in situ hybridization at 48 h (B), was shrunk from the proximal end of the mouse incisor. Scale bar = 50 μm.

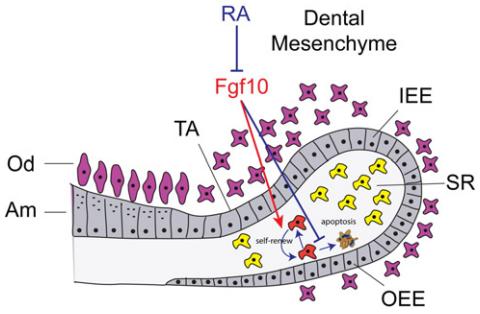


Fig. 7. Schematic presentation of the negative effects of RA on epithelial SCs in the mouse incisor. Exogenous RA negatively regulates the homeostasis of epithelial SC niche of the mouse incisor by down-regulating proliferation and up-regulating apoptosis in LaCL epithelial cells. The effects of RA on LaCL epithelium is mediated by its negative control of mesenchymal Fgf10.

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