Disturbed cranial neural crest cell development caused by reduced sonic hedgehog signaling underlie the pathogenesis of retinoic-acid-induced cleft palate

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ABSTRACT
Cleft palate is one of the most common congenital craniofacial anomalies in humans which is caused by genetic and environmental factors. Excessive intake of retinoic acid (RA) or its precursor, vitamin A, during early pregnancy is associated with increased incidence of cleft palate in offspring. However, the pathogenetic mechanism of cleft palate caused by excess RA is not fully understood. In order to investigate detailed cellular and molecular mechanism of retinoic-acid-induced cleft palate, we gave all-trans RA to ICR pregnant mice by gastric intubation from embryonic day 8.5 to 10.5 (E8.5 – E10.5). In RA treated group, we found disturbed expression pattern of *Sox10*, which marks cranial neural crest cells (CNCCs) in the trigeminal region at E9.5. This disruption of CNCCs also existed at the maxillary component of the first branchial arch at E10.5 which is known to give rise into secondary palatal shelves. Moreover, we found significant elevation of apoptotic cell death in *Sox10* positive CNCCs at E9.5 in RA treated group. For investigating possible molecular mechanism underlies this pathogenesis, we focused on Sonic hedgehog (*Shh*) signaling pathway from our previous RNAseq result. Interestingly, *Shh* and its downstream genes *Ptch1* and *Gli1* were perturbed in the developing face at E9.5 of RA-treated embryos. Consistently, the incidence of cleft palate and CNCC apoptosis due to overdose RA was reduced by administration of SAG (*Shh* signaling agonist). Altogether, our results suggest that one of the critical mechanism of retinoic-acid-induced cleft palate associate with elevated cell death of CNCCs through the down-regulation of *Shh* signaling pathway.

KEYWORDS
Retinoic acid, Cleft palate, Cranial neural crest cell, Sonic hedgehog signaling

INTRODUCTION

Isolated cleft palate (CP) is one of the most common birth defects in humans which could occur 1 in 1,000 live births (Cox 2004). Further, 55% of cases with isolated CP are associated with other abnormalities as part of a syndrome (Jones 1988). The mechanism of CP is considered to be multifactorial and various genes and environmental factors (Dixon et al. 2011). Among these factors, retinoic acid (RA) signaling is well known to play critical roles during craniofacial development and disturbance could result in CP both in human and animal model (Lammer et al. 1985, Abbott, Harris and Birnbaum 1989, Ackermans et al. 2011). RA signaling is also well known to be involved in a variety of biological processes and is indispensable for normal embryonic development by interacting with multiple signaling pathways in a temporospatial manner which regulates cellular activity (Ross et al. 2000).

Classic animal experiment has revealed excessive RA signaling during palatogenesis results in CP. Interestingly, depends on the time of RA exposure, different mechanisms are suggested to cause cleft palate. For example, pregnant mice with RA treatment on E10 produce small palatal shelves in their fetuses without contacting with each other by E14, while treatment on E12 results in normal size palatal shelves with contact but not fuse because of disturbance of epithelial structure (Abbott et al. 1989). These results indicate the etiology of CP which induced by excessive RA during embryonic development could be different according to the developmental stage. However, there is still a limited number of research which investigated the earlier stage (younger than E10) effect of RA signaling on the etiological perspective of CP. In the present study, we discovered excessive RA signaling at E8.5 in mice exhibit a high incidence of CP than other later stages with significant elevation of cell death in migrating cranial neural crest cells (CNCCs).

CNCCs is migratory stem cell population which arise from the dorsal part of the developing neural tube and contribute to the majority of the cartilage, bone, connective and peripheral nerve tissue in the head. The rostral CNCCs extensively generate the frontonasal skeleton and the membranous bones of the skull, whereas the caudal CNCCs migrate into the pharyngeal arches where they form the mandible and maxilla, the middle ear, hyoid and thyroid cartilages (Minoux and Rijli 2010). CNCCs development involves a sequence of critical phases, such as formation, migration, and differentiation. Problems in one or more of these events can result in craniofacial abnormalities. For example, Pierre Robin syndrome, which is characterized by cleft palate due to mechanical interference with palatal shelf elevation by a malpositioned tongue and small jaw, is related to defects in the

migration of CNCCs (Poswillo 1988). Additionally, elevated cell death of cranial neural crest cells are known to associate with Treacher Collins syndrome which also exhibits CP (Jones et al. 2008). Furthermore, we confirmed substantial reduction of Sonic hedgehog (*Shh*) signaling during craniofacial development in embryos which treated with RA. Sonic hedgehog (*Shh*) is an important secreted signaling molecule known to function in the patterning of a variety of regions during embryonic development. *Shh* signaling is known to be activated in target cells by binding of ligand to *Ptch1* which derepress the activity of Smoothened (Casali and Struhl 2004). Reduced *Shh* signaling is responsible for causing holoprosencephaly, defects in midline structures, particularly the face and eyes, and a lack of hemisphere separation of the brain (Roessler et al. 1996). Notably, *Shh* is expressed in the notochord and the floor plate of the neural tube during neural crest cell migration. Moreover, inhibiting *Shh* signaling in chick embryo has been shown to lose the structure of pharyngeal arch and reduced head size due to elevated cell death in the neural tube and neural crest cells (Ahlgren and Bronner-Fraser 1999).

Since roles for RA in palatogenesis have been observed in a stage-dependent manner and various molecules are involved in regulating palate development, the teratogenic mechanisms of RA-induced cleft palate remain to be fully elucidated. In this study, we show that mouse fetuses with RA at E8.5 were the most sensitive time point to cause CP during early gestation stage and there was a reduction in the survival of CNCCs in the trigeminal ganglion, which led to defects of the first pharyngeal arch structures. Importantly, we have also proved this theory with reduced incidence of CP and CNCC apoptosis by reintroducing *Shh* signaling. Altogether, we have discovered novel molecular and cytological etiology of CP with elevated RA signaling which in turn disturbs *Shh* signaling and survival of CNCCs.

MATERIALS AND METHODS

Animals, RA treatment and SAG rescue

Pregnant female ICR mice (CLEA, Japan) were given by gastric intubations of all-trans RA (25 mg/kg b.w.) (Sigma-Aldrich) and the RA (25 mg/ml in dimethylsulphoxide) was diluted 1/10 in corn oil just before use. Control animals were given the equivalent volume of the carrier. The gastric intubations were performed once a day at different time points of early gestation stages (from E8.5 to E10.5) (Fig.1E).

For the SAG rescue experiments, SAG (Enzo) was administered once a day by gastric intubations at E9.5 and/or E10.5 after RA-treatment at E8.5. SAG solution was prepared in fine suspension in 0.5% methylcellulose/ 0.2% Tween 80 at 1.5 mg/ml and given to pregnant mice at 100 µl per 10 g body weight (Frank-Kamenetsky et al. 2002).

Bone and cartilage staining

E18.5 embryos were skinned and eviscerated. The embryos were fixed in 100% ethanol overnight and then stained for 24 hours in Alcian Blue (150 µg/ml in 20ml glacial acetic acid and 80ml of 95% ethanol). After washing in 100% ethanol, soft tissues were dissolved in 2% KOH overnight and stained in Alizarin Red (50 µg/ml in 1% KOH) overnight. Stained embryos were kept in 20% glycerol/ 1% KOH until skeletons became clearly visible. Embryos were stocked in 50% glycerol/ 50% water.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed using a digoxigenin (DIG)-UTP (Roche) labeled antisense RNA probes corresponding to the sequence of *Sox10*, *Shh*, *Ptch1*, *Gli1* (all previously reported in Allen Brain Atlas). Signal was detected using an alkaline-phosphatase-conjugated anti-DIG antibody and NBT/BCIP substrate (Roche). Whole mount hybridization was carried out on E9.5-E10.5 embryos as described (Wilkinson 1992) with slight modifications. For all *in situ* hybridization analyses, a minimum of three embryos of each genotype were examined per probe.

DAPI staining and analysis of apoptosis and cell proliferation

For whole mount DAPI staining to visualize palate tissues, E15.5 and E17.5 embryos were fixed in 4% PFA, rinsed in PBS. Upper jaws were isolated from lower jaws, incubated overnight in 1:1000 DAPI dilution (Dojindo), and scanned using an Olympus SZX16 microscope.

For analysis of apoptosis and cell proliferation, E9.5 embryos were dissected, fixed in 4% PFA, rinsed in PBS and then labeled whole or after sectioning. Frozen sections were cryoprotected through a graded sucrose series (10, 20, and 30%) and an overnight step in 50% sucrose/ 50% OCT (Tissue-Tek), and embedded in OCT compound. Transverse sections were cut at 8 µm. Detection of apoptotic cells was performed using the in situ cell death detection kit (www.Roche.com) following manufacture´s instructions. For detection of proliferation, samples were incubated with mouse anti-phosphohistone H3 antibody (1:200, Millipore) at 4℃ overnight followed by secondary Alexa488 donkey anti-mouse IgG (1:200, Invitrogen) for 6h at room temperature for sections and overnight at 4℃ for the whole mount, respectively. Samples were counterstained for CNCCs with goat anti-*Sox10* antibody (5 µg/ml**,** R&D Systems) at 4℃ overnight, followed by secondary antibody (Alexa546 donkey anti-goat IgG, 1**:**200**,** Molecular Probes) for 6h at room temperature for sections and overnight at 4℃ for the whole mount, respectively. Samples were also stained for nuclei with DAPI, mounted with fluorescence mounting medium (Dako), and

scanned using a Leica TCS SP8 microscope. Projected Z-stacks were flattened for the whole mount. pHH3(+)*Sox10*(+) cells (yellow), TUNEL(+)*Sox10*(+) cells (yellow), and *Sox10*(+) cells (red) were counted in trigeminal ganglia and the ratio of pHH3(+)/*Sox10*(+) and TUNEL(+)/*Sox10*(+) were evaluated on sections. At least five adjacent sections were counted in each assay. Statistical significance assessed using two-tailed Student´s *t*-test.

RNAseq analysis

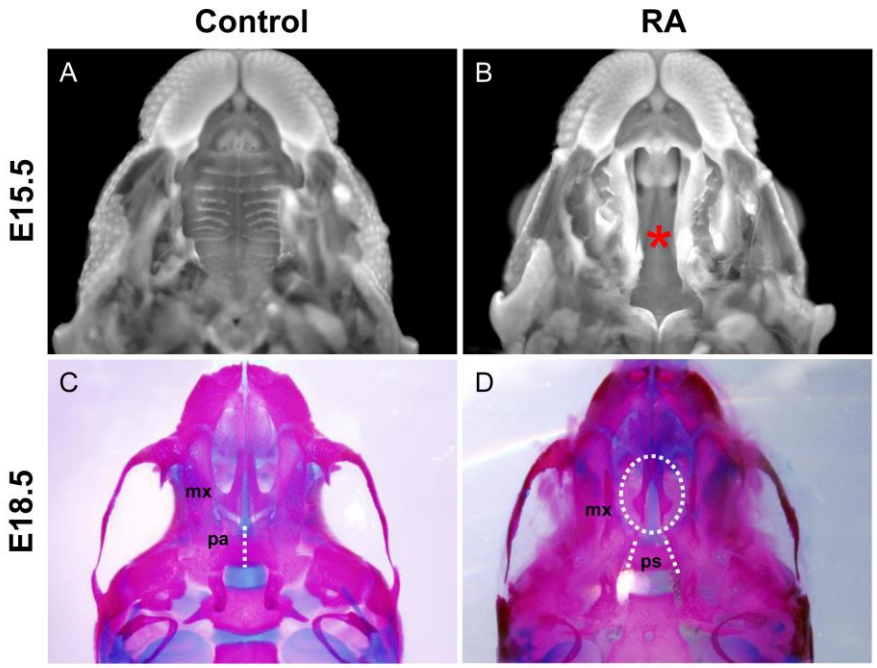
Medial nasal, lateral nasal and maxillary processes were separated from E11.5 control ($n = 3$) and RA-treated embryos ($n = 3$). RNAseq analysis was performed as previously described (Kurosaka et al. 2017).

RESULTS

Excessive RA intake induce cleft palate in different probability according to its embryonic stage

The etiology of overdose RA in cleft palate has been investigated in a stage-dependent manner (Abbott et al. 1989, Pennimpede et al. 2010). In the present study, we oral gavaged 25 mg/kg weight RA in pregnant mice at different embryonic time point in order to investigate the embryonic stage dependent effect of excessive RA signaling for secondary palate development (from E8.5 to E10.5) (Fig. 1E). Relative to control embryos, all of the RA gavaged samples in any time points from E8.5 to E10.5 exhibited cleft palate in a certain ratio. Among all of these experiments, we specifically found the samples with excess RA exposure which include E8.5 exhibit a higher probability of cleft palate than other groups. Based on these data, we hypothesized overdose RA signaling at E8.5 is causing the critical cellular event to cause cleft palate in a higher ratio. Thus we chose RA exposure (25 mg/kg maternal weight) at E8.5 for further investigation of cleft palate in an attempt to understand the embryological basis of this anomaly.

Dissection of the E15.5 embryos revealed that RA-treated embryos had a complete cleft of the secondary palate (Fig.1B). Skeletal staining demonstrated that both the maxillary and palatine shelves exhibited complete cleft (Fig. 1D). As a result, the presphenoid bone was visible in the ventral view (Fig.1D) while the palatal bones were fused with each other and the maxillary processes located at the midline in the control embryos (Fig.1C). We also observed the other cranial neural crest cells (CNCCs) derived skeletal abnormalities, including zygomatic bone, sphenoid bone, tympanic ring, and mandible defects in RA-treated embryos, indicating that RA-induced craniofacial anomalies are relevant to CNCCs.



E

Exposure day	Incidence of CP
E8.5	83.18% (n=107)
E8.5, E9.5	95.56% (n=45)
E9.5, E10.5	55.00% (n=40)
E8.5, E10.5	97.73% (n=44)
E8.5, E9.5, E10.5	100% (n=91)

Fig.1.

Cleft palate (CP) in RA-treated embryos. (A,B) DAPI-stained upper jaws of control (A) and RA-treated (B) embryos at E15.5 is shown. The asterisk in B marks the CP in RA-treated embryos. (C,D) Skeletal preparations of E18.5 control (C) and RA-treated (D) embryos stained with alizarin red for mineralized bone and alcian blue for cartilage. The palatal shelves in the maxilla (mx) of RA-treated embryos with complete CP (oval dashed line in D) failed to grow toward the midline. Fusion of the bilateral palatal bones (pa) is observed in the control embryos (dashed line in C) but the presphenoid bone (ps) of the cranial base is fully exposed in the RA-treated embryo skeleton due to CP (dashed lines in D). (E) The incidence of CP with all-trans RA (25 mg/kg body weight) treatment at different embryonic days. n, number of embryos analyzed; %, percentage relative to the number of embryos analyzed.

Impaired CNCC development contributes to RA-induced cleft palate

Because CNCCs have the ability to give rise to the majority of the skeletal elements of the head, including palatal bones (Santagati and Rijli 2003) and the stage which we gave RA to mice is E8.5, the critical time CNCCs leave the neural tube of mouse embryos (Trainor 2005), we hypothesized that RA-induced cleft palate resulted from CNCCs defects. In order to test this hypothesis, we performed *In situ* hybridization using a probe which could detect *Sox10* for detecting migrating CNCCs at E9.5 (Marmigere and Ernfors 2007). In the control embryo, *Sox10* expression condenses to the cranial ganglia, which migrates into the branchial arches at both E9.5 and 10.5 (Fig.2A, C). On the other hand, RA-treated embryos, showed disturbed *Sox10* expression in the trigeminal ganglia (Fig.2B, arrowhead) and the maxillary component of the first branchial arch at E10.5 (Fig.2D, arrowhead). These results indicated that overdose RA signaling around E8.5 disturb CNCCs development which in turn result in cleft palate phenotype.

We further investigate possible mechanisms of disturbed CNCCs by using immunohistochemistry with antibodies against *Sox10* and PHOSPHORYLATED HISTONE H3 (pHH3) or TUNEL staining in order to assess cell proliferation and cell death in migrating CNCCs. Similar to the finding from *In situ* hybridization, substantial reduction of *Sox10* expression could be detected in E9.5 trigeminal region (Fig.2 E-L). The distribution as well as number of dividing CNCCs in control and RA treated trigeminal region did not show significant differences (Fig2E-H and M). On the other hand, notable elevation of cell death could be detected in RA treated trigeminal region compared to the control group (Fig2 I and K white arrowhead). This result was also confirmed by transverse sections and quantitative analysis of TUNEL staining with immunolabeling of *Sox10* (Fig.2J, L and N; P=0.008). These results strongly suggest that overdose RA signaling at E8.5 results in elevation of cell death in migrating CNCCs which could contribute to a wide variety of craniofacial defects including a high incidence of cleft palate.

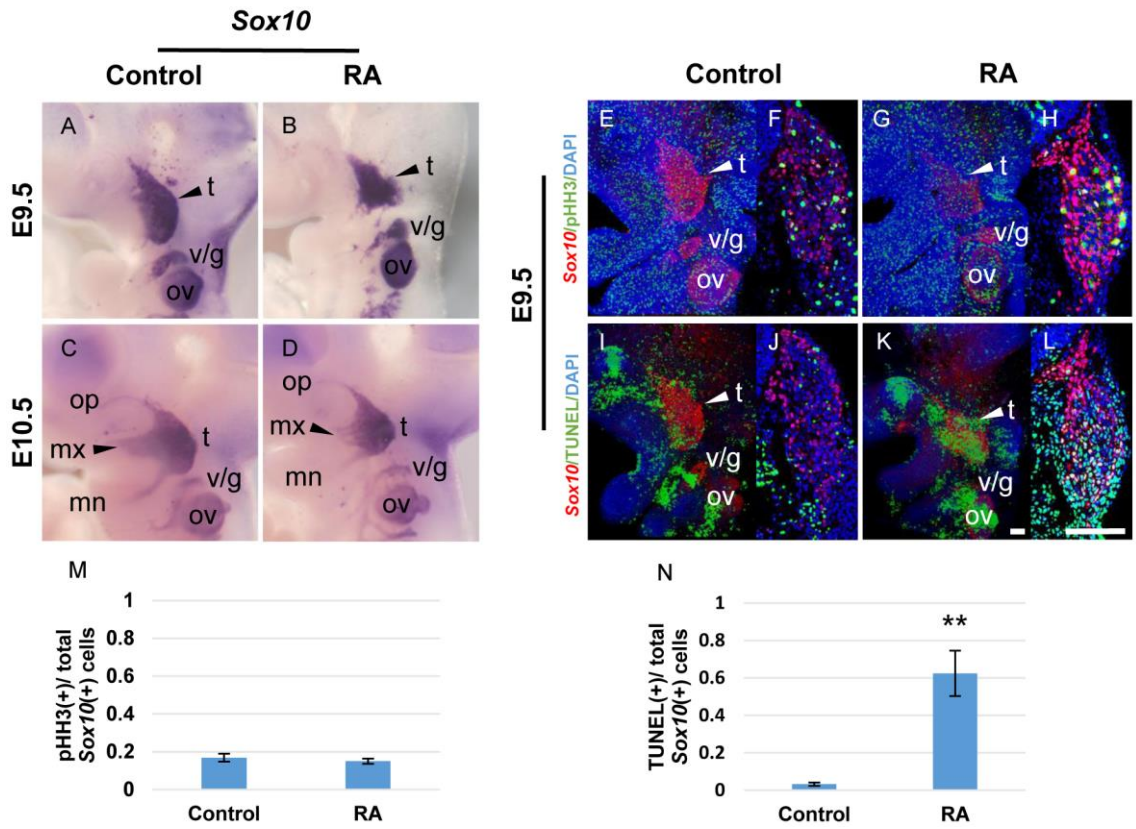


Fig.2.

Excess RA leads to defects of *Sox10*-positive cranial neural crest cells(CNCCs). (A-D) Whole-mount in situ hybridization for *Sox10* at E9.5 (A, B) and E10.5 (C, D) control (A, C) and RA-treated (B, D) embryos. The arrowheads in A and B represent *Sox10* expression domain in the trigeminal ganglia at E9.5. (B) *Sox10* staining pattern in RA-treated embryos is smaller. (C, D) The arrowheads show CNCCs migration into the maxillary component in the first branchial arch. The stream of RA-treated embryos (D) is disturbed. (E-N) Unaltered proliferation but increased apoptosis in *Sox10*-positive (red) trigeminal ganglia (arrowheads) at E9.5 after RA treatment. Whole-mount immunofluorescent detection of pHH3 (green; E, G) and TUNEL (green; I, K) in profile views of control (E, I) and RA-treated (G, K) E9.5 embryos. Immunostaining of pHH3 (green; F, H) and TUNEL (green; J, L) of transverse sections of control (F, J) and RA-treated (H, L) E9.5 embryos at the level of the trigeminal ganglia. Scale bars: 100μm. Quantification of proliferation (pHH3+, M) and apoptosis (TUNEL+, N) in *Sox10* positive CNCCs from control and RA-treated embryos in the trigeminal ganglia regions. T-test ***P*<0.01, n=5. Data are mean ± s,e.m. n=3 for each stage and experiment. t, trigeminal ganglion; v/g, vestibulo-cochlear/geniculate ganglia; ov, otic vesicle; op, ophthalmic branch; mn, mandibular branch; mx, maxillary branch.

RA overdose affects Sonic hedgehog (SHH) signaling in the craniofacial region

In order to understand the potential molecular causes underlying the RA-induced CNCCs defects and cleft palate, we performed RNA sequencing analysis using E11.5 maxillary complex including medial nasal process (giving rise to the primary palate), lateral nasal process, and maxillary process (giving rise to the secondary palate) (Bush and Jiang 2012). With comparison of control and E10.5 RA treated embryos, several genes showed significant difference in their expression level including *Sonic hedgehog* (*Shh*) and *Sim2* (Table S1). *Shh* is known to play critical roles in craniofacial development by regulating NCC survival, proliferation and patterning (Ahlgren and Bronner-Fraser 1999, Jeong et al. 2004). Therefore we examined the expression patterns of *Shh* and its transcriptional target *Patched1* (*Ptch1*) (Goodrich et al. 1997) and *Gli1* (Lee et al. 1997) in relation to the distribution of CNCCs. At E9.5, *Shh* is expressed in the floor plate of the neural tube (Fig.3A-B, black arrowhead) up to the ventral forebrain neuroepithelium (Fig.3A-B, white arrowhead). In E9.5 RA-treated embryos, *Shh* expression is reduced in these two regions (Fig.3C-D, arrowheads). *Ptch1* and *Gli1* expression in the ventral central nervous system and the mesenchyme surrounding the notochord (Bai et al. 2002), adjacent to the *Shh* expression domain were noticeably lower in E9.5 RA-treated embryos (Fig.3E-L). These results suggests that excessive RA signaling at E8.5 could cause reduction of *Shh* in the floorplate and forebrain which impacts the survival of CNCCs and results in craniofacial anomalies such as cleft palate.

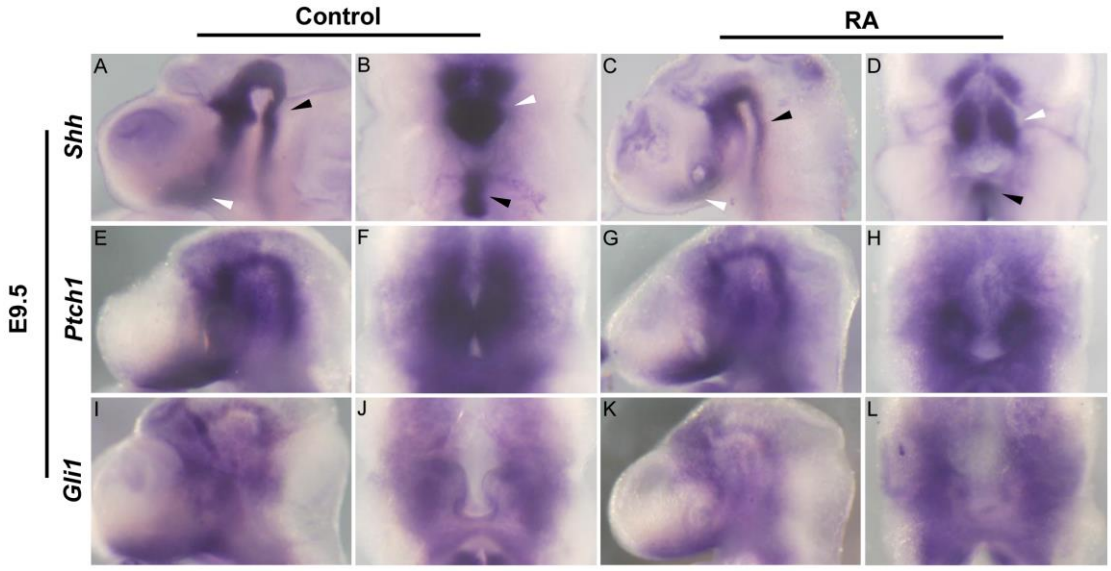


Fig.3.

Expression of SHH signaling in relation to facial development in control and RA-treated embryos. Lateral views (A, C, E, G, I, K) and ventral views (B, D, F, H, J, L) of whole-mount in situ hybridization for *Shh* (A-D), *Ptch1* (E-H) and *Gli1* (I-L) in E9.5 control (A, B, E, F, I, J) and RA-treated (C, D, G, H, K, L) embryos. (A-D) *Shh* expression in the floor plate of the neural tube (black arrowheads) and the ventral forebrain neuroepithelium (white arrowheads) is reduced in RA-treated embryos. *Ptch1* (E-H) and *Gli1* (I-L) expression in the ventral central nervous system and the mesenchyme surrounding the notochord, adjacent to the *Shh* expression domain is slightly lower in RA-treated embryos.

RA-induced cleft palate and CNCC apoptosis are partially rescued by exogenous SAG (*shh* agonist) *in utero*

To further confirm whether disturbed *Shh* signaling by excess RA signaling underlies the etiology of cleft palate (exposed at E8.5), we tried to re-administrate *Shh* signaling by supplementing Smoothened activator SAG to RA treated embryos. Interestingly, administration of SAG after RA-treatment resulted in a reduced frequency of cleft palate at E18.5 (Fig. 4C). Rescued individual maxillary process growth to midline, and the fusion of the bilateral palatal bones were indistinguishable from those of control embryos (Fig. 4A, D and F). Notably, the frequency of cleft palate altered depending on the timing of SAG administration (Fig.4G). In RA-treated embryos, apoptosis of CNCCs in the trigeminal ganglion increased at E9.5 and CNCCs contribution to the maxillary arch disturbed at E10.5. Therefore, we predicted that rescue of cleft palate would require SAG at or around E9.5. We gave vehicle or SAG (12.5 mg/kg body weight) to RA-treated (E8.5) pregnant mice at E9.5 and/or E10.5 by oral gavage. The incidence of cleft palate was significantly reduced in the administration of SAG at E9.5 and E9.5-E10.5 ($P<0.01$, Fig. 4G). Giving SAG at E10.5 also reduced the incidence of cleft palate however there was no statistical difference compared with that of RA-treated group (Fig. 4G). Furthermore, we analyzed the changes in CNCC survival rate of the trigeminal ganglion after exogenous SAG treatment. Whole mount and transverse section TUNEL staining have shown significant increased level of cell death in the trigeminal ganglia of RA-treated embryos at E9.5 (Fig.4J, K). We found that the addition of exogenous SAG resulted in a reduction of CNCC apoptosis after RA-treatment (Fig.4L, M). Statistical analyses revealed that exogenous SAG was able to restore RA-induced survival rate of CNCCs in the trigeminal ganglion, and almost to control levels (Fig.4N). Taken together, these results further confirmed that disrupted *Shh* signaling around E9.5 to E10.5 by overdose RA signaling underlies the pathology of cleft palate.

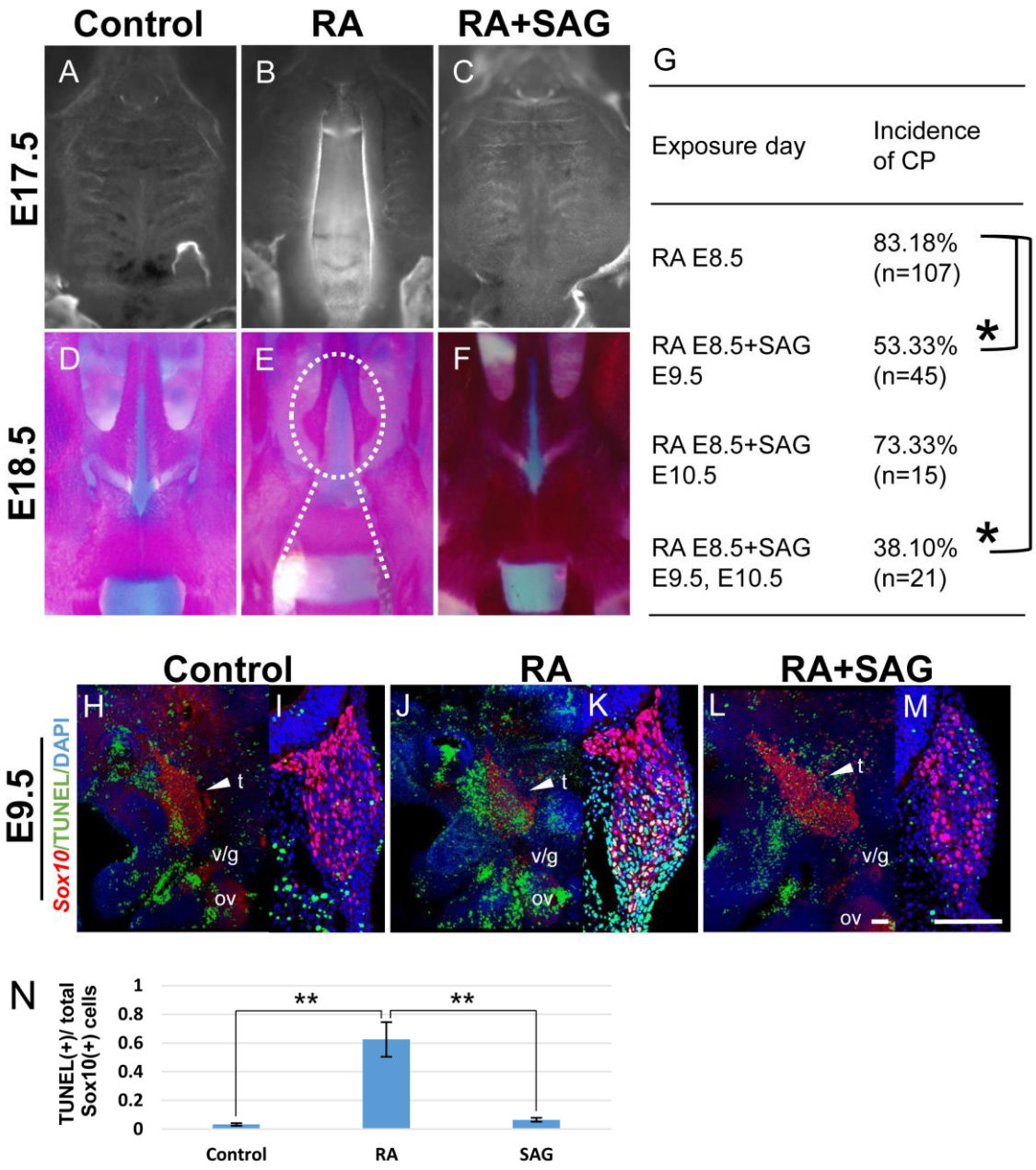


Fig.4.

Exogenous SAG (*Shh* agonist) partially rescues RA-induced cleft palate and CNCC apoptosis *in utero*. (A-C) Ventral views of DAPI-staining on palates in E17.5 control (A), RA-treated (B) and RA-treated embryos with the addition of the SAG (RA+SAG) by oral gavage (C). (D-F) Alizarin red (bone) and alcian blue (cartilage) stained skeletal preparations of control (D), RA-treated (E) and RA+SAG treated embryos at E18.5. The failure of maxillary process growth toward the midline (oval dashed line in E) and the presphenoid bone exposure (dashed lines in E) due to CP in RA-treated embryos are

rescued by the SAG administration (F). (G) The incidence of CP with the SAG (12.5 mg/kg body weight) exposed at E9.5 and/or E10.5 after all-trans RA (25 mg/kg body weight) treatment at E8.5. Fisher´s exact test was used for statistical analysis. *P<0.01. n, number of embryos analyzed; %, percentage relative to the number of embryos analyzed. (H-N) Increased apoptosis in *Sox10*-positive (red) trigeminal ganglia (arrowheads) at E9.5 after RA treatment is rescued by SAG administration. TUNEL (green) detection of whole-mount in profile views (H, J, L) and transverse sections (I, K, M) at the level of the trigeminal ganglia of control (H, I), RA-treated (J, K) and SAG-treated (L, M) E9.5 embryos. Quantification of apoptosis (TUNEL+, N) in *Sox10* positive CNCCs from control, RA-treated and SAG-treated embryos in the trigeminal ganglia regions. T-test **P<0.01, n=5. Data are mean±s,e.m. Scale bars: 100μm**.**

DISCUSSION

In this study, we provide molecular and cytological evidence those how early gestation stage (from E8.5 to E10.5) RA exposure could result in cleft palate. Specifically, we revealed that RA treatment of pregnant mice at E8.5 is the most sensitive time point to result in cleft palate with defects in CNCC-derived head skeleton. Furthermore, we showed excess RA signaling disturbs SHH signaling pathway in the craniofacial region and in turn elevate the apoptosis of CNCC which contribute to the maxillary component. Thus, we demonstrate that RA has a critical function in regulating CNCC-derived craniofacial skeleton development through SHH signaling pathway.

Stage-dependent effects on RA-induced cleft palate

Since excess RA was shown to be one of teratogens to cause cleft palate in conjunction with other congenital malformations in rodents (Kochhar and Johnson 1965), numerous studies have elucidated stage specific effect of overdose RA signaling in pathogenesis of cleft palate. Excess RA exposure at E9 elevated apoptosis in cell population derived from the first branchial arch which reduced the volume of secondary palate component (Sulik et al. 1987), whereas mouse fetuses treated with RA at E10 decreased mesenchymal proliferation resulting in that small palatal shelves did not contact with each other (Abbott and Birnbaum 1990). Meanwhile, later treatment (E11.5-E12.5) have been shown to prevent palatal shelf elevation by affecting extracellular matrix composition and hydration and/or tongue withdrawal by disturbing tongue muscle development (Okano, Suzuki and Shiota 2007, Degitz, Francis and Foley 1998, Okano, Sakai and Shiota 2008). We herein show that the incidence of cleft palate was higher in pregnant mice treated at E8.5 (83.18%) and timing with E8.5 (E8.5&E9.5, 95.56%; E8.5&E10.5, 97.73%; E8.5&E9.5&E10.5, 100%), while time points without E8.5 (E9.5&E10.5) led to reduced cleft

palate incidence to 55% (Fig. 1E). These results strongly suggest excessive RA signaling at E8.5 play critical roles for inducing cleft palate. It is well known that around E8.5 the migration of CNCCs initiate from the dorsal side of the neural tube which is a critical step for craniofacial development (Trainor 2005). Therefore we hypothesized excessive RA signaling at E8.5 could influence CNCC development.

Previous in vitro and in vivo studies have reported that proper RA signaling is essential for CNCC development. Targeted inactivation of *Aldh1a2*, the enzyme responsible for early embryonic RA synthesis, in the mouse embryo leads to lack of posterior branchial arch (BA3-6) (Niederreither et al. 2003). Additionally, post-otic neural crest cells fail to establish segmental migratory pathways and are misrouted caudally. On the other hand, early-stage (9.0 days post coitum) RA treatment in rats induced an ectopic caudal migration of the anterior hindbrain (rhombomeres (r) 1 and 2) crest cells into the second branchial arch and acousticofacial ganglion (Lee et al. 1995). Our study also shows that excess RA signaling results in reduced CNCC condensation to trigeminal ganglia and disturbed CNCC population of the first branchial arch. Moreover, in RA-treated embryos, we detected significant elevation of cell death in migrating CNCC at E9.5. These data highlight one of the mechanisms of palatogenesis defect in embryos treated with RA at E8.5 *in utero* is dysregulated CNCC development. One of a possible explanation for the higher ratios of E8.5 with E9.5 and/or E10.5 is combined effects at respective time points.

Proper RA signaling is required for SHH signaling in the craniofacial region

Previous studies have shown that *Shh* and RA signals interact and influence many developmental processes. Especially at the craniofacial region, local synthesis of RA in the chick rostral head enables patterned outgrowth of the forebrain and frontonasal process by maintaining *Shh* expression (Schneider et al. 2001). Additionally, decreased *Shh* expression in *Raldh2*$^{-/-}$ embryos contributes to the defects in both neural and mesodermal patterning and differentiation (Ribes et al. 2009). Present work revealed disturbed expression of *Shh* and which read out genes *Ptch1* and *Gli1* in the floor plate of the neural tube up to the ventral forebrain neuroepithelium in E9.5 RA-treated embryos. Disturbed *Shh* signaling is well known to associate with a variety of craniofacial defects including facial cleft (Kurosaka et al. 2014, Kurosaka 2015). Additionally, it has been reported that proper *Shh* signaling is essential for survival of CNCCs (Ahlgren and Bronner-Fraser 1999, Moore-Scott and Manley 2005). From these results, it is indicated that at least part of the reason of elevated CNCCs cell death in present RA treated embryos is caused by reduced *Shh* signaling and likely contribute to the etiology of cleft palate.

In order to further confirm this possible etiology, we reintroduced *Shh* signaling in RA treated embryos by supplementing SHH agonist (SAG) which directly binds Smoothened (SMO) receptor and activate *Shh* signaling pathway. Previous studies using $Shh^{+/-}$ or $Shh^{-/-}$ embryos show that the midline defects are at least partly rescued by SAG treatment (Frank-Kamenetsky et al. 2002). It has also been shown that SAG activates *Shh* signaling pathway in neurogenesis and augment neuronal survival (Bragina et al. 2010). Importantly, we show that exogenous SAG (12.5 mg/kg) at E9.5 and E10.5 significantly reduced the incidence of cleft palate which induced by overdose RA. Interestingly, administrating SAG at only E10.5 did not show statistical difference in the prevalence of cleft palate which indicates there are temporal requirement of *Shh* signaling for normal palate development. These results again emphasize the significance of reduced *Shh* signaling and followed increased cell death in CNCCs in the etiology of cleft palate. However, not all cleft palate were rescued by this SAG supplementation. One possible explanation for the partial rescue is that RA may also mediate some other genes to result in cleft palate, e.g. *Sim2*, which downregulated in RA-treated embryos according to RNA sequencing analysis. Mice homozygous for the disrupted allele ($Sim2^{-/-}$) exhibit a cleft of the secondary palate and some other craniofacial malformations (Shamblott et al. 2002). Since RA signal is well known to affect many signaling pathways, further investigation is required in order to reveal other molecular cause or mechanism which result in cleft palate by overdose RA signaling.

Taken together, We demonstrate the relations between reducing *Shh* signaling and cleft palate resulting from RA exposure, by showing: (1) the down-regulation of *Shh* and two *Shh* direct target genes, *Ptch1* and *Gli1* after RA treatment; (2) *Shh* signaling is likely to be responsible for RA-induced CNCC apoptosis increasing, which contributes to the reduction of maxillary component including palatal bones; (3) exogenous *Shh* agonist (SAG) partially rescued RA-induced cleft palate *in utero*.

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SUPPLEMENTARY INFORMATION

Table S1. Differentially expressed transcripts in the medial nasal process, the lateral nasal process, and the maxillary process of RA-treated embryos at E11.5. Transcripts were selected by identifying those having a fold change Log2FC < -1 or Log2FC > 1 in RA-treated versus control embryos, as described in the Methods section.

down – regulated gene	Log2FC (Log2FC < -1 or Log2FC > 1)
Sim2	-2.39
Ugt2a1	-1.95
Cyp26c1	-1.54
Sult1e1	-1.12
mt-Tl1	-1.09
Shh	-1.06
Gm129	1.02
Gm14506	1.26

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