Retinoic acid induces abnormal palate during rat embryogenesis; focusing on *Hox* gene and apoptosis

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Retinoic acid induces abnormal palate during rat embryogenesis; focusing on *Hox* gene and apoptosis

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이분들이 아니었다면 지금의 제가 없었을 것입니다. 감사의 글을 쓰고 있는 이 순간 다들 너무 보고 싶네요. 모두모두 사랑하고 감사드려요. 위에 언급한 모든 분들께 다시 감사의 말씀 전하며, 앞으로 건강하시고 행복한 일만 가득하길 기원합니다.

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ABSTRACT

Retinoic acid induces abnormal palate during rat embryogenesis; focusing on *Hox* gene and apoptosis

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The process of palatogenesis is mostly similar in rat and human. In rats, palatal process occurs between gestation day of 13 and 17. Palatal shelves start to grow on the gestation day of 14 and 15 with the downward movement. During gestation day of 16, palatal shelves move upward above the tongue and maintain in horizontal position. At the end of 16^{th} day palatal shelves centered around and contact each other, and then complete palate is formed by gestation day of 17.

Cleft palate is the most widespread congenital facial deformity occurring in 1 or 2 human beings out of 1,000. All-trans retinoic acid (RA) is an endogenous metabolite of vitamin A, which is known to be a necessary substance for normal embryonic development. It is widely used for the treatment of pimple and other skin infections etc. It has been reported that excessive or insufficient RA causes craniofacial malformation, thymic non-formation, heart outflow tract defect and neural tube defect including cleft palate at the early stage of pregnancy. Endogenous RA plays an important role in apoptosis of medial edge epithelium during fusion of palatal shelves. Several reports suggested that exogenous and/or excess RA can act as a strong deformity causing

substance.

Hox genes encode transcription factors that are known to be involved in formation of embryo by expressing at the particular time and particular region during embryogenesis. It is known to affect embryogenesis under the influence by RA and retinoid, a precursor. When *Hoxa*7 was expressed ectopically, morphological changes including cleft palate were detected in head region. Furthermore, this change was similar to that of embryo exposed to RA during pregnancy.

In this study, we compared and analyzed the process of formation of cleft palate by morphological observations in the embryos at different stages after injecting RA during early stage of development (E11) and collected the embryos from 13^{th} to 17^{th} day. We also examined the effect of RA on in vitro cultured palatal tissues (E15.5) to know the *in vitro* effect of RA. We performed molecular studies related to Hoxa7 and apoptosis related gene expression during RA treatment from 13^{th} day (embryonic day 13; E13) of development to the 17^{th} day. We also see the effect of RA by injecting the late stage of palatogenesis (E15) and collected the embryos on 16^{th} and 17^{th} days. We also performed the immunohistochemistry on the same samples using Hoxa7 antibody and TUNEL assay to know the apoptosis in control and RA treated samples.

In the case of embryo exposed to RA, at early stage and late stage of palate development, cleft palate was formed including head development delay, and cleft lip was discovered only when the embryo was exposed to RA at early stage of palate development. As a result of *in vitro* organ culture, RA was confirmed to act directly as deformity causing substance in the formation of palate. In case RA is infused at early stage (E11) of craniofacial development, expression of *Hoxa*7 increased from development 15^{th} day in normal palate tissues, remaining constant until the 17^{th} day

when palate formation ends, however in palate tissues exposed to RA, no increase was made even on the 15th day. In case RA is infused in late stage (E15) of palate development, Hoxa7 expression was weak compared to control group in period of fusion. When RA is infused at early stage of palate development, expression of *Bax*, a pro-apoptotic gene was strong overall from the 13th day to the 17th day, however the expression of RA treatment group showed dramatic decrease from development 16th day. When RA is infused at late stage (E15) of palate development, TUNEL positive cells were observed mostly at epithelium on the side of nasal cavity and oral cavity which form rugae with parts where MEE contact occurs and medial epithelial seam (MES) occurs, and on 17th day when palatal incorporation is nearly reached, TUNEL positive cell was observed at parts where MES disappears. On the other hand, in the case of RA treatment group, it was observed that on 16th day TUNEL positive cells are distributed around the mesenchymal cell and epithelial cell of palatal shelves, and on 17th day they are distributed to parts immediately below palatal shelves epithelial cell.

These results altogether indicate that exposure to RA during palate development process causes facial deformity including cleft palate and cleft lip by modulating the expression of *Hoxa*7 as well as an apoptosis-related gene, *Bax*, and thus malregulating the apoptosis during palatogenesis.

Key words : palatogenesis, cleft palate, retinoic acid, *Hoxa*7 gene, apoptosis.

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I. INTRODUCTION

Palate of mammals prevents mixing between the paths of food and air by separating nasopharynx and oropharynx and enables the baby to suck breast by forming vacuum in the oral cavity, and it is also an important organ for pronunciation of language. Development of palate in humans was divided into two steps, the primary and secondary palate. This developmental process begins from the early 6^{th} week of pregnancy. The important period of palate formation is between late 6^{th} week and early 9^{th} week. The mechanism of palatogenesis involved the formation of primary palate, which leads to formation of the front and middle part of maxilla. The process of primary palate takes place with the development of medial palatal prominence through incorporation of medial nasal prominences between the interior surfaces of the maxilla. Secondary palate consists of hard part with rugae (front) and soft part (back). The process of formation of secondary palate starts with the expansion of mesenchymal

prominences from maxillary prominence in early 6th week of development and also passing through the 7th and 8th weeks of development. Palatal shelves develop at both sides of tongue protrude downward. At the end of 8th week, the palatal shelves heaved up above the tongue for moment and maintain horizontal balance.[1, 2] The palatal shelves of both sides starting with the incorporation at the right center by approaching each other. Both sides of the medial edge epithelia (MEE) at palatal shelve tip contact each other and form midline seam. Finally the complete incorporation occurs as MEE disappear and basal membrane collapses.[3]

To understand the developmental process of palatogenesis, the extensively used animal model to study by researchers was rat model. The process of palatogenesis in rat was mostly similar to palatal development in human. It is known that palatal process occurs between gestation day 13th (E13) and 17th (E17) of development in rats. Palatal shelves start to grow on the gestation day 14th and 15th with the down ward movement. During gestation day 16th (E16), palatal shelves grow from downward to up ward above the tongue and maintain balance of horizontal position. At the end of 16th day palatal shelves center around and contact each other, and finally the complete palate is formed at the gestation day of 17.[4]

Cleft palate is the most widespread congenital facial deformity occurring in 1 or 2 human beings out of 1,000. This occurs more frequently to women than men.[5,6] Cleft palate formed due to growth delay of palatal shelves itself, delay or failure of horizontal array of palatal shelves that were growing vertically for the first time, or contact abnormality of both sides of palate shelves after horizontal array. Cleft palate is also due to the abnormal cell death of MEE, secondary destruction after incorporation, abnormal condensation and differentiation of mesenchymal cells around palate. Furthermore,

hereditary and/or environmental factors are also known to be involved. Among the environmental factors, excessive or shortage of certain vitamin, hormone or drug have been reported to induce cleft palate during embryogenesis.[3]

All-trans retinoic acid (RA) is an endogenous metabolite of vitamin A, which is known to be necessary substance for normal embryo formation during animal development.[7-10] It has been widely used for treatment of pimple and other skin infections etc. It is reported that excessive or insufficient RA causes craniofacial deformity, thymic non-formation, heart outflow tract defect and neural tube defect including cleft palate at the early stage of pregnancy.[8] Endogenous RA plays an important role in apoptosis of MEE during fusion of palatal shelves[11]. Several reports suggested that exogenous and/or excess RA can act as a strong deformity causing substance.[12-14] In another report, exogenous infusion of RA into pregnant mouse causes induction of apoptosis in different cells[15]

Hox genes encode transcription factors that are known to be involved in formation of embryo by expressing at particular time during embryo development and are known to affect formation of early stage anterior-posterior axis.[16] They are known to affect embryogenesis under the influence by RA and retinoid, a precursor.[17,18] When the RA is introduced during vertebrate development homeotic transformations have been induced at the cervical spine, chest, waist and sacral vertebra, etc., due to the changes in *Hox* gene expression. Ectopic expression of *Hoxa*7, *Hoxb*6, *Hoxb*7, *Hoxd*4 has been reported to induce craniofacial malformation including cleft palate.[19-23] Interestingly enough, the morphological changes induced by the ectopic expression of Hox genes were similar to those of embryos exposed to RA. Some reports showed that phenytoin down regulates the *Hoxa*2 expression and caused the deformity at palatal shelves.[24] In the case of

*Hoxa*7 the expression profile was changed in head tissues when RA was treated.[25]

In this study, we compared and analyzed the process of cleft palate formation by morphological observations of different stages of embryos development day 13th (E13) to 17th (E17) following the injection RA at early stage of development (E11). We also examined the effect of RA on palatal tissues (E15.5) cultured *in vitro* to know the *in vitro* effect of RA. To understand the underlying molecular mechanism during cleft palate formation, *Hox* as well as apoptosis related gene expression pattern was analyzed in RA treated embryos (from E13 through E17).We also analyzed the effect of RA on the late stage of palatogenesis by injecting at the embryonic stage E15 and collecting the embryos on the following days (E16 and E17), which were followed by the immunohistochemistry with Hoxa7 antibody and TUNEL assay.

Ⅱ. MATERIALS AND METHODS

1. Animal management and RA treatment

Rat strain Spargue-Dawley (SD) was used in this study. Adult SD rats were maintained at room temperature under dark/lights cycle of 12/12. The rats were mated and the presence of a vaginal smear was defined as embryonic day 0. All-trans Retinoic acid (RA) (R-2625, sigma, St. Louis, Missouri, USA) was used in this study. The rats were divided into the experimental group and control group and the experimental group mixed RA of 100mg/ml concentration in the sesame oil (S-3547, Sigma, St. Louis, Missouri, USA) and pregnant rats were injected intraperitoneally with 100mg/kg all-trans RA[26] mixture at embryonic day 11 (E11) or embryonic day 15 (E15) by intraperitoneal administration. In the case of control, sesame oil including DMSO was injected into abdominal cavity.

2. Dissection of embryos and morphological observation

Embryos were collected on the gestation day 13^{th} and 17^{th} from control and RA injected group by using standard ethical animal procedures. The mandible region was removed from the embryos by using surgical tools. The collected embryos were dipped in autoclaved PBS and analyzed under the optical microscope (M10, Leica, Switzerland). The palate samples were collected following the standard methods[25] for different experimental purposes. In order to perform the immunohistochemistry, the maxillary region was dissected and fixed in 4% paraformaldehyde (pH 7.4).

3. Total RNA isolation and RT-PCR

For isolation of total RNA, RNA Zol B (LPS industries inc., New Jersey, NY, USA) was used. Complementary DNA (cDNA) was prepared by reverse transcriptase (RT) was performed using 2 μg of total RNA in a reaction volume of 25μl. From this cDNA, 1 μl was used for the subsequent PCR. Amplification of cDNA was done by using rat *Hoxa*7, *Bax* and β-*actin* specific primers sets. Beta actin was used as an internal control. PCR reaction conditions were depend upon the primer sequences, and each conditions were written the text. PCR products were quantified using 1.5% agarose gel, visualized under UV light, and photographed with Dolphin-DOC (Wealtec gel documentation system).

4. *In vitro* organ culture

Palatal shelves were separated from E15.5 embryos and cultured on 1 μm Millipore filter membrane and a metal grid in a falcon organ culture dish with serum free DMEM/F12 (Welgene, Daegu, Korea) containing 20 μg/ml ascorbic acid (A0278, sigma, St. Louis, Missouri, USA) and 1% penicillin/streptomycin, and then the medium was changed every 24 hours.[27] The primary palate and the palatal shelves were remaining to maintain the precise physiological distance between the shelves in the organ culture. RA was used in culture medium at 20 μM final concentration. The culture dish was placed in a 5% CO_2 and 37℃ humidified incubator for 60 hours. Tissues were then fixed and processed for hematoxylin-eosin (H-E) staining.

5. Sequence analysis of *Hoxa*7 protein through *in silico*

The protein sequences of *Hoxa*7 were collected from the NCBI database (http://www.ncbi.nlm.nih.gov/). The amino acid sequences of *Hoxa*7 protein sequences from different species (human, mouse, and rat) were compared using the multiple sequence alignment software (CLUSTALW; http://align.genome.jp/).

6. Gene cloning

*Hoxa*7 gene was amplified by PCR using *Hoxa7* specific primers (Forward: 5′ – ccc aag ctg gtc aaa att, Reverse: 5′ – gat atc ctg gcc ctt tac). The amplified product (720 bp) was ligated with the pGEM-T easy vector (promega, Fitchburg, Wisconsin, USA) and then transformed in to the *E.coli* DH5-Alpha. After confirming the presence of 720bp insert with restriction enzymes, the gene was sub-cloned into pCDNA3 (Invitrogen, Carlsbad, California, USA) vector following digestion of *Not* Ⅰ restriction enzyme.

7. Cell culture and transfection

The COS-7 (monkey kidney cell line) cells were maintained in Dulbecco's Modified Eagle's Medium (Welgene, Daegu, Korea) with 10% of FBS (JBI, Seoul, Korea) and 1% penicillin-streptomycin (Welgene, Daegu, Korea) at 37℃ in a 5% CO_2 incubator. For transfection, $2x10^4$ cells per chamber slide were seeded. After 24 hours incubation, cells were transfected with pCDNA:*Hoxa*7 using Lipofectamine 2000

reagent (Invitrogen, Carlsbad, California, USA) according to manufacturer's instructions. Immunocytochemistry was performed with *Hoxa*7 antibody (sc-17152, Santa cruz biotechnology, Delaware, USA) to detect the expression of *Hoxa*7 in the cells 48hours after transfection.

8. Immunohistochemistry and TUNEL assay

After fixation, the tissues were embedded in OCT compound (Triangle Biomedical Science, Durham, N.C., USA) for frozen sections using standard protocol. Using microtome (12 μm) of the tissue sections were prepared and then incubated at 4°C overnight with the primary goat polyclonal antibody against *Hoxa7* (sc-17152, Santa Cruz Biotechnology, Delaware, USA). All Slides were washed with PBS, the specimens were allowed to react with biotinylated rabbit antigoat immunoglobulins as a secondary antibody and streptavidin peroxidase at room temperature for 10 minutes. The specimens were visualized using a 3, 3'- diaminobenzidine (DAB) reagent kit (Zymed, South San Francisco, CA, USA).

In order to analyze the apoptosis, TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assay was performed using *in situ* cell death detection kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The 12 μm thick sections were treated with 0.1% triton x-100 in sodium citrate for 2 minutes on ice. The samples were incubated with the labeling reaction mixture at 37°C for 1 hour. The slides were observed under the fluorescent microscope (Olympus 1X70, Olympus corp., Melille, NY, USA))

Ⅲ. RESULTS

1. Effect of retinoic acid on craniofacial and palate development

A. *In vivo* effect of RA on palate formation

In order to study the effect of RA on the morphology of embryos during palatal development, the embryos from control group and RA treated groups were compared. The RA was injected on the 11^{th} day of pregnancy (early stage of palate development), and then embryos were dissected from the 13^{th} to 17^{th} day, and analyzed under optical microscope. Embryos obtained from 3 pregnant rats, were analyzed at each stage.

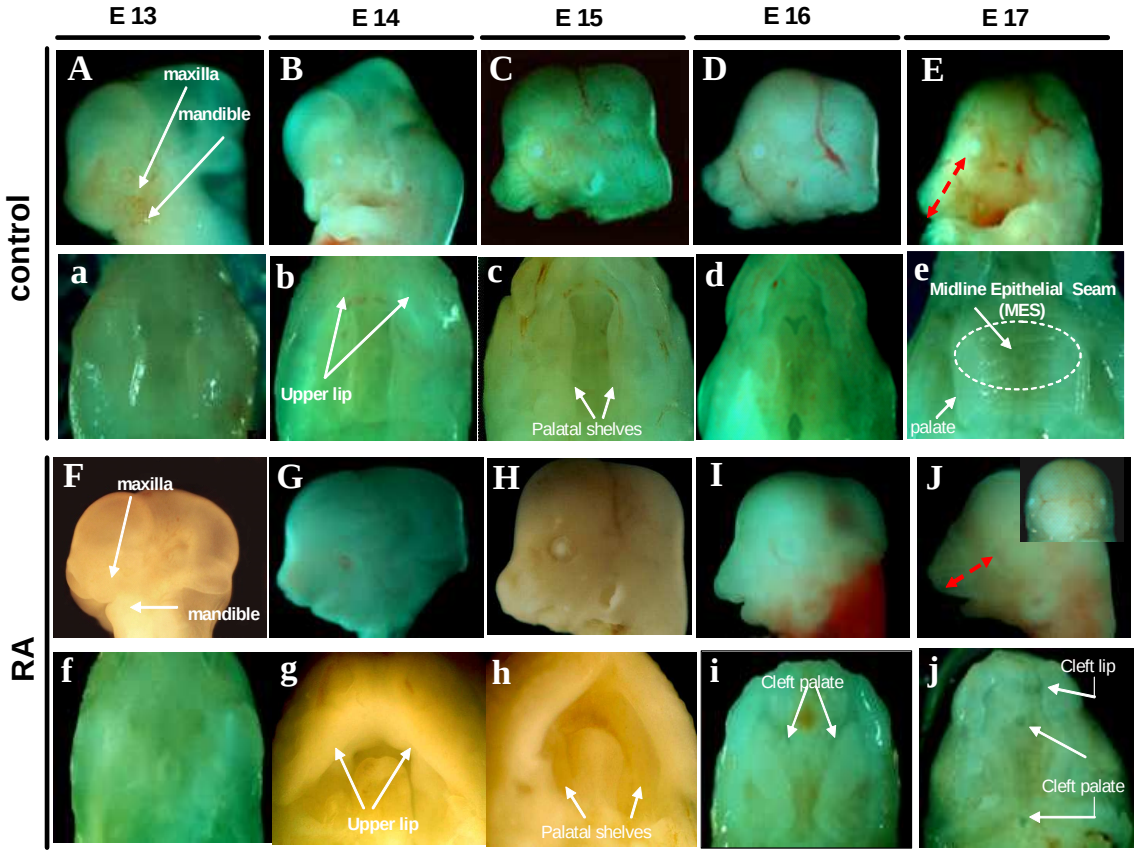


Fig. 1. Morphology of head and palate between control and RA treated groups. Retinoic acid was injected into the abdominal cavity of rat at embryonic day 11. The craniofacial morphology of control (A-E) and RA-treated group (F-J) embryos were shown. The palate morphology of control (a-e) and RA-treated group (f-j) were shown also.

Developments progressed both in control group and RA treated group, however RA treated group showed retardation of overall craniofacial development. In addition, the length from jaw to eye was shortened (Fig.1J red arrow) compared to that of the control group (Fig. 1E). Also the eye development seemed to be delayed in RA treated group. (Fig.1I, J, D and E)

We tried to find out palate morphology after removing mandible. The result revealed that the medial edge epithelia (MEE) of palatal shelves tip contacted each other

and fused completely on the 16th and 17th day (Fig.1d, e) by forming MES in the case of control group, whereas RA-treated group showed no contact of MEE. (Fig. 1i) Sometimes delayed fusion of palate with partially formed MES and cleft lip as well were also detected (Fig.1j).

B. *In vitro* effect of RA on palate formation through organ culture

To see the effect of RA on palate formation *in vitro*, we performed *in vitro* organ culture of palate (Fig.2A), The palate was dissected out at the embryonic stage of E15.5 and cultured for 60 hours in the presence or absence of RA (20 μM) to reach the *in vivo* stage of E17, and then compared the palatal shelves after sectioning and H-E staining. As shown in Fig. 2, the palate of control group has been fused (Fig.2B, 2a & 2c), whereas RA treated group showed no fusion of palate (Fig. 2C, 2b & 2d). This result further proved that the morphological changes induced by RA are almost similar both *in vivo* and *in vitro*.

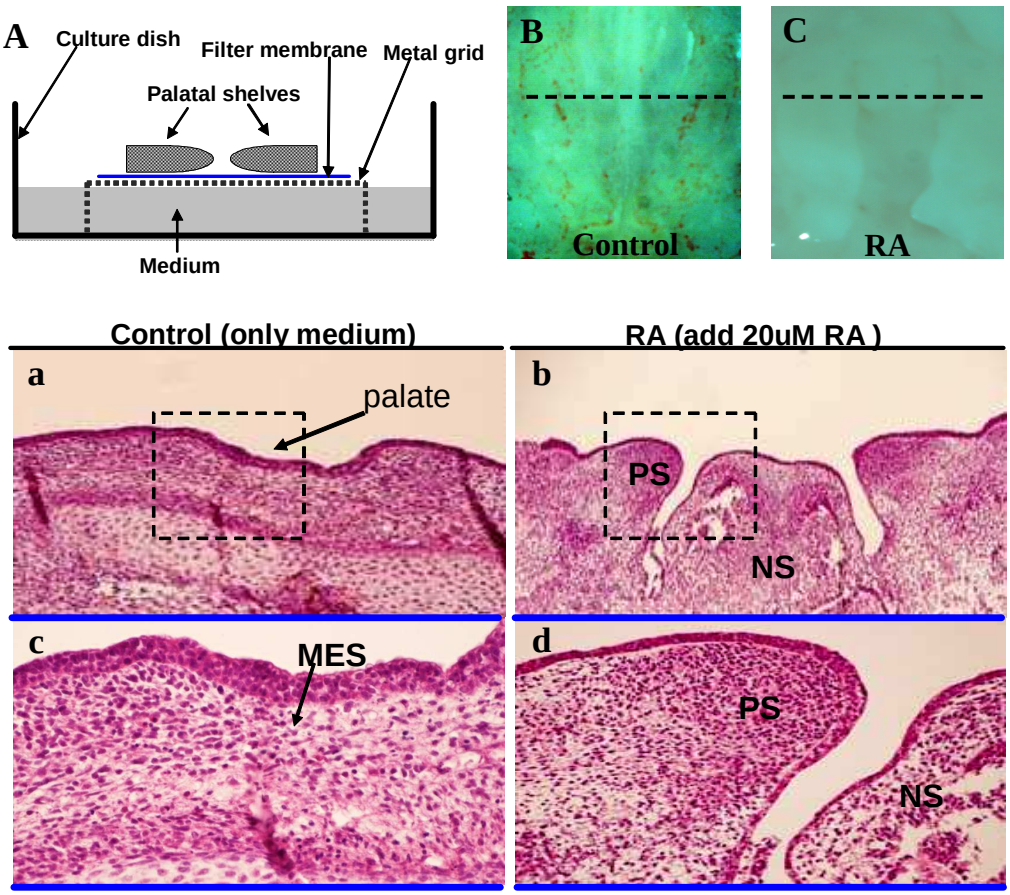


Fig.2 Morphology observation of palatal shelves in control and RA treated group of in vitro organ culture. Schematic of *in vitro* organ culture system (A), The palate of control group (B, a, c) and RA-treated group was culture for (C, b, d) was seen in the photograph. PS: palatal shelves, MES: midline epithelium seam, NS: nasal septum. Blue line: 1㎛ filter membrane, The black dotted line is sectioning direction

C. The effect of RA on the late stage of palatogenesis (E15)

Previous organ culture experiment indicated that the administration of RA at the late stage of palatogenesis (E15) still caused the malformation of palate, we injected RA at this stage (E15), harvested the embryos at the stage of E17, and analyzed the

morphology. As shown in Fig. 3, no fusion was observed in the palate of RA group (Fig. 3B, a′, b′, and c′). On the other hand, control group showed normal palate formed (Fig. 3A, a, b, and c). These results altogether indicate that RA causes severe effect on palatogenesis during embryo development irrespective of the day of injection.

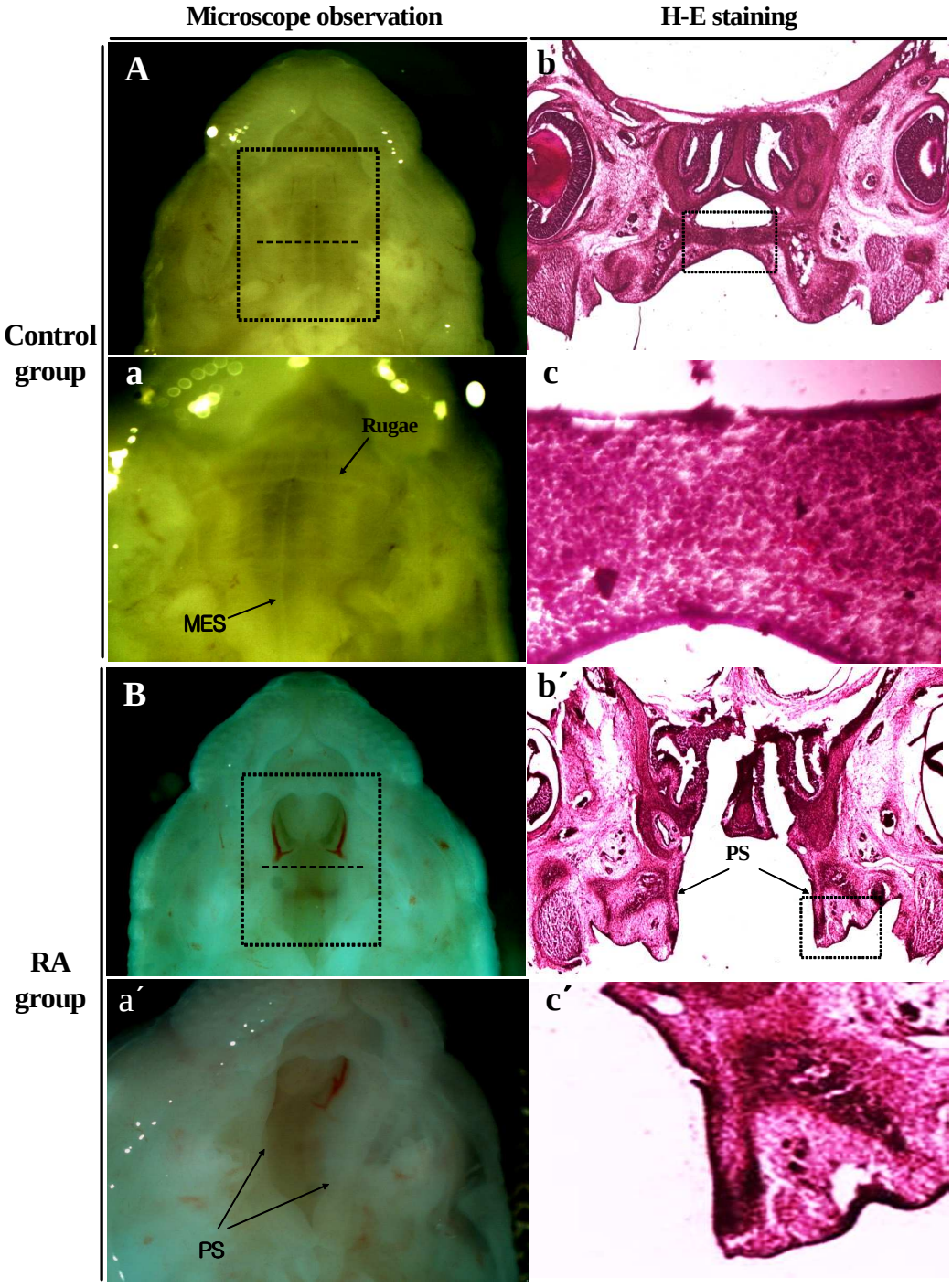
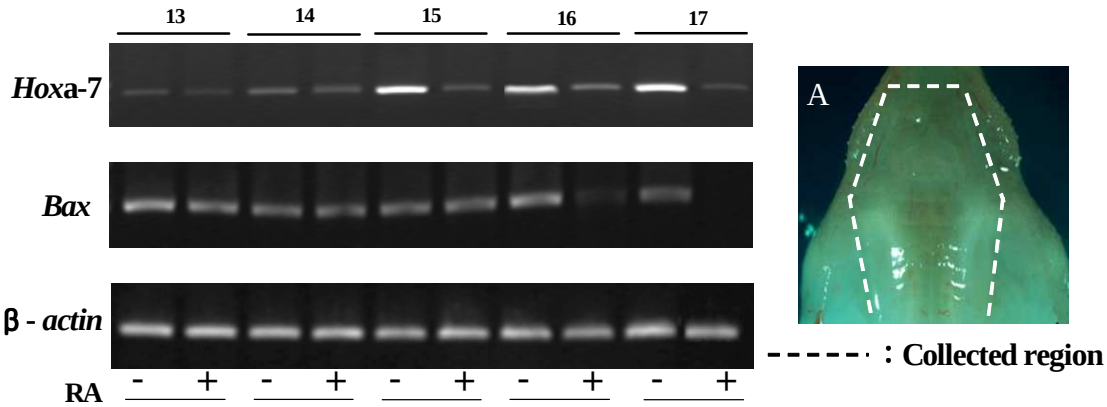


Fig. 3. Different shape of closure of palatal shelves in embryo of control and RA treated group (RA injected at the embryonic stage of E15). The morphology of E17 palate following the injection of RA on gestation day E15. Control group, palatal shelves were completely fused (A, a, b, c) whereas RA group, cleft palate was formed (B, a', b', c'). PS: palatal shelves, MES: medline epithelial seam

2. The effect of RA on *Hox* and apoptosis-related gene expression during palatogenesis

To find out whether the expression of *Hoxa*7 and *Bax* genes are modified by RA administration, total RNAs were isolated from palate region of E13 to E17 embryos, and then RT-PCR was performed with the gene specific primers listed in materials and methods after synthesizing cDNA. *Hoxa*7 expression was increased from gestation day 15, where the palatal shelves grow vertically and remained constant until E17, on the other hand, Hoxa7 expression was not increased throughout the stage E17 in the embryos exposed to RA (Fig. 4).

To understand the relationship between the cleft palate formation and apoptosis, we performed semi quantitative PCR using *Bax* primers on cDNA of control and RA treated group. We found that the *Bax* gene expression was observed in both control and RA treated group. The expression levels of *Bax* gene in control and RA were almost similar from gestation day of 13 to 15 (Fig.4), but on the 16^{th} and 17^{th} day, dramatic decrease in expression of Bax gene was observed in RA treated groups. This indicates the role of apoptosis in palatogenesis (Fig. 4).



RT – PCR conditons

primer	product size	sequence	annealing temp. / cycle
Hoxa-7	425 bp	5'-CCCAAGCTGGTCAAAATT-3' 5'-GATATCCTGGCCCTTTACTC-3'	56℃ / 28
Bax	496 bp	5'-GCTCTGAACAGATCATAAG-3' 5'-GCCACTCCTGCTCGAAG-3'	50 ℃/30
β - *actin*	327 bp	5'-CATGTTTGAGACCTTCAACACCCC-3' 5'-GCCATCTCCTGCTCGAAGTCTAG-3'	55℃ / 28

day	Control	RA
13	8 embryos	9 embryos
14	10 embryos	8 embryos
15	10 embryos	11 embryos
16	10 embryos	9 embryos
17	7 embryos	14 embryos

Fig. 4. Semi-quantitative expression analysis of *Hoxa*7 and *Bax* genes in control and RA group during palate development. The palate tissues (A) only were collected from each stage (E13, 14, 15, 16, & 17) of embryos following the injection of RA (+) or control (-) at the embryonic stage E11. Total numbers of embryos used for synthesizing cDNAs are indicated in the box below right. RT-PCR was performed with appropriated primers and conditions written in the box below left.

3. The Effect of RA on *Hoxa*7 expression pattern during palatogenesis: immunohistochemistry

In order to analyze the expression pattern of *Hoxa*7 during palate development, immunohistochemistry technique was applied. Since the antibody used here were raised against human origin, Hoxa7 (N-18), we compared the amino acid sequences of *Hoxa*7 from rat, mouse and human. As shown in Fig 5, the N-terminal sequences of all these three Hoxa7 proteins were identical.

To confirm whether the antibody against human Hoxa7 is really working with rat *Hoxa*7, we cloned rat *Hoxa*7 gene into the eukaryotic expression vector pcDNA3. As shown in Fig. 6, the *Hoxa*7 gene was confirmed to be cloned successfully into the target site (Fig.6A), when the orientation of *Hoxa*7 gene insert was analyzed by restriction endonucleases (Fig. 6B).

After cloning, the subcellular localization of Hoxa7 was analyzed after transfecting the pcDNA3:*Hoxa*7 into COS-7 (monkey kidney cell line) cells for 48 hours. Over expression of *Hoxa*7 was analyzed by performing immunocytochemistry using Hoxa7 antibody as well as RT-PCR (Fig. 7). Hoxa7 turned out to be located in the nucleus (Fig. 7A, B, and C). RT-PCR showed the over expression of Hoxa7 in transfected cells (Fig.7D).

To analyze the Hoxa7 expression in palate, embryos of E16 and E17 were collected after injection of RA on gestation day of 15, and then the immunohistochemistry was performed with Hoxa7 antibody (Fig. 8). Control group of E16 has Hoxa7 expression on both sides of palate (Fig. 8A, a), not where MEE is conjugated, whereas E17 showed the expression at the central part of palate where midline seam is formed (Fig. 8B, b). In the case of RA injected group, low amount of Hoxa7 expression was detected (Fig. 8C, c, D, and d).

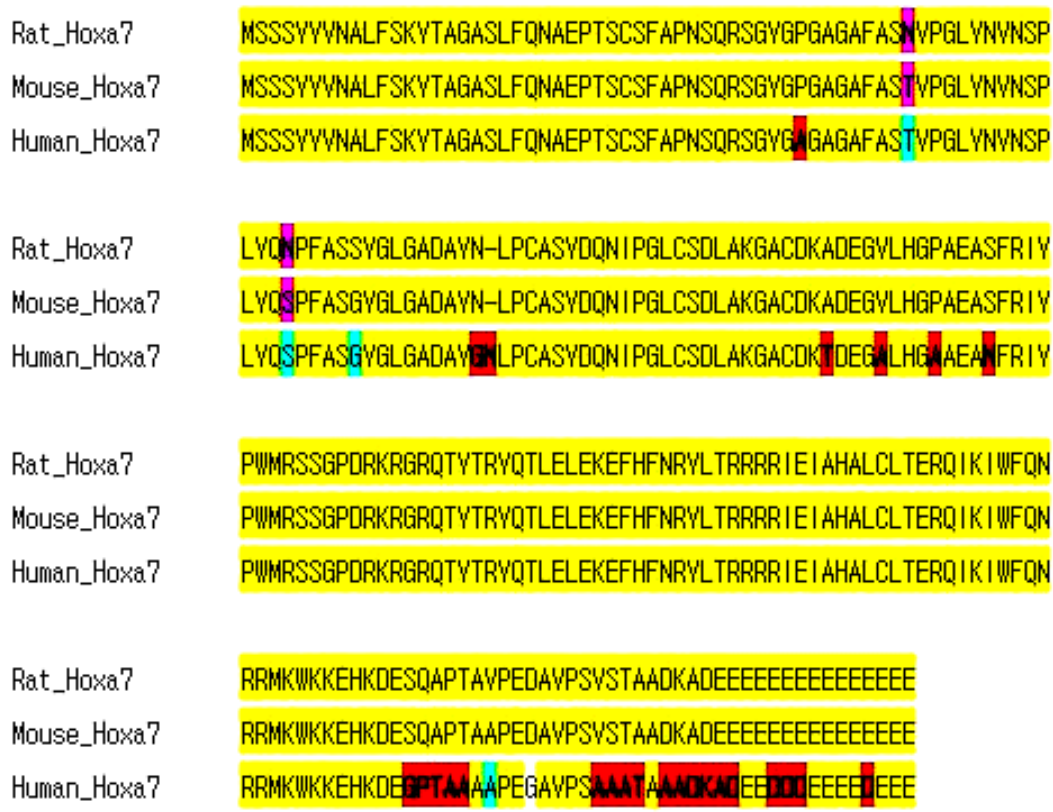


Fig.5. Comparison of *Hoxa*7 protein sequences in Human, Mouse and Rat

Sequence 1: rat Hoxa7 229 amino acid (a.a.), Sequence 2: mouse Hoxa7 229 a.a., Sequence 3 : human Hoxa7 230 a.a.

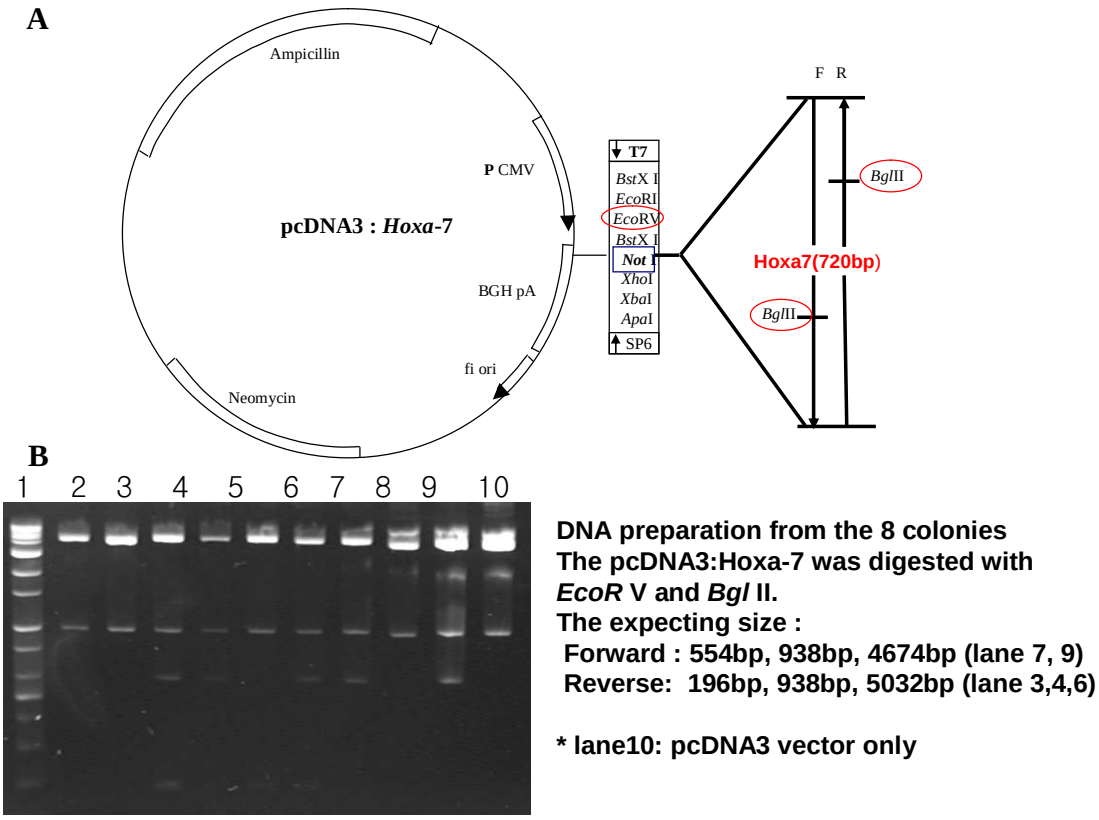


Fig. 6. Cloning of hoxa7 into pcDNA3: For expression of *Hoxa*7 gene in eukaryotic cells, pcDNA3:*Hoxa*7 construct and orientation was checked by using agarose electrophoresis. A: pcDNA3:*Hoxa*7 construct, B: the orientation of *Hoxa*7 gene insert

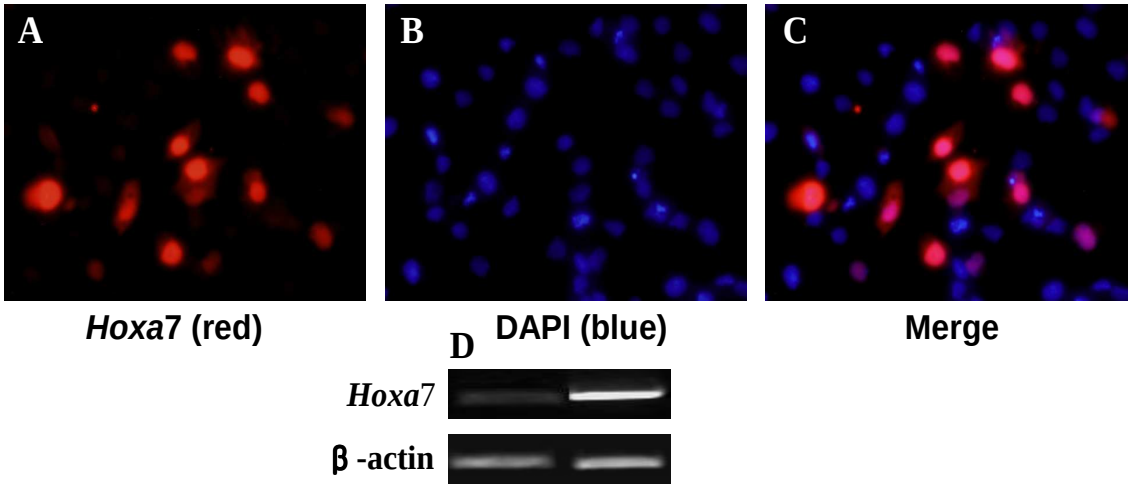


Fig.7. pcDNA3:*Hoxa*7 was tranfected into COS-7 cells and localization of *Hoxa*7 was shown by performing immunocytochemistry.

A: *Hoxa*7 antibody, B: DAPI stain, C: Merge, D: transfection check using PCR (left side: control)

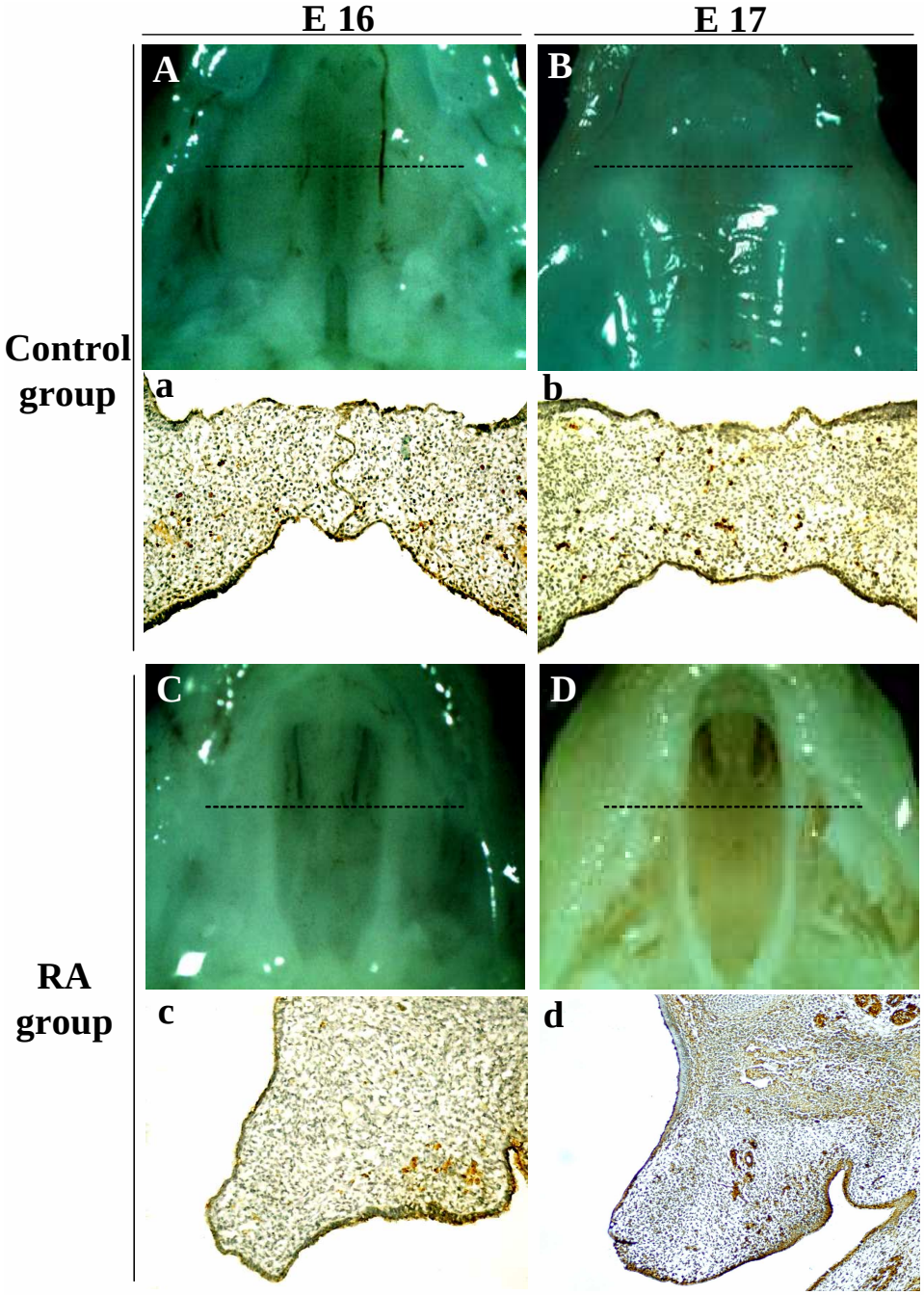


Fig. 8. Immunohistochemistry using *Hoxa*7 antibody on developing palate

On the palate in control group E16 (Fig. 8. A, a), expression of *Hoxa*7 was shown mostly at both sides of palate, and on E17 (Fig. 8. B, b), expression was shown mostly mesenchymal cells at central part of palate that forms midline seam. However, the palate of embryos to which RA is infused, expression was in small amount compared to control group at mesenchymal cell (Fig. 8. C, D, c, d)

4. Effect of RA on apoptosis during palatogenesis: TUNEL assay

To analyze the effect of RA on apoptosis during palatogenesis, TUNEL staining was performed in the embryos (E16 and E17) treated or non-treated with RA at the embryonic stage of E15. As shown in Fig. 9 the both sides of MEE of palatal shelves contacted each other in E16 control group, and the TUNEL positive cells were observed mostly at the epithelium surrounding nasal cavity and oral cavity which forms rugae, whereas E17 when palatal incorporation is nearly reached, TUNEL positive cell was observed at the region where MES disappears (Fig. 9A, B). In the case of RA treated group, TUNEL positive cells were distributed in the mesenchymal cells and epithelial cells of palatal shelves in E16 embryos. In E17, apoptotic cells were distributed in the areas immediately below the epithelium of palatal shelves (Fig. 9C, D).

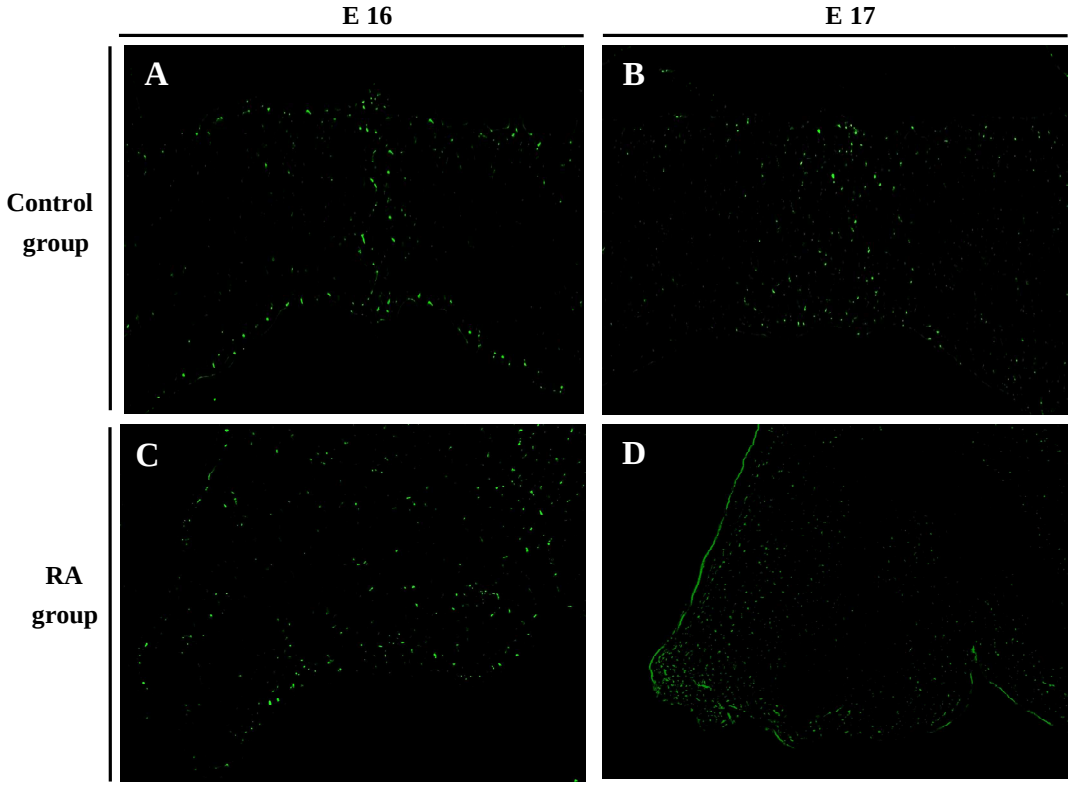


Fig. 9. Apoptosis patterns during palatogenesis by TUNEL assay.

A, B : palatal shelves in control group, C, D: palatal shelves in RA treated group

Ⅳ. DISCUSSION

Palate is composed of two parts: a hard palate at the front containing rugae and a soft palate at the back, which is formed when secondary palate occurred. Morphologically palate forms ceiling of oral cavity and floor of nasal cavity. Palate development begins when the neural crest cells of the 1st branchial arch move to primitive oral cavity and interact with craniopharyngeal ectoderm, thus forming palatal process inside the maxillary prominence. Hard palate is formed with the front part of maxilla through combination with both sides medial nasal prominence and front nasal prominence due to the mesenchymal prominence maxilla prominence on both sides of soft palate. During development of soft palate, palatal shelves continue to grow as the palatal shelves of both sides of tongue stick out, and with the growing palatal shelves maintaining horizontal balance by rising to above the tongue, the palatal shelves that grew up form midline epithelial seam (MES) by contacting and fusing each other at medial edge epithelium. This MES, in the first form, is formed in several cell layers, however they eventually break down and disappear gradually.[1-3] After all, they come to extinction by forming monocellular layers, which leads to fusion of both sides palatal shelves and nasal septum, and after through separation of oral cavity and nasal cavity, palate is formed completely.

Such palate development process begins on about the 13th day in rat (in the case of human, in about the 6th week of development), and on the 16th and 17th days (in the case of human, in about the 9th week), contact and fusion of palatal shelves take place.[4] In this process, signaling molecules like transforming growth factor beta-3 (*TGFb*-3), fibroblast growth factors (*FGF*s) and receptors of these factors are expressed along the

mesenchyme. It has been reported that *TGFb*-3 deficient mice, had a cleft palate without any other deformity, and *FGFr*2 mutation mice developed craniofacial deformity including cleft palate.[28-33] In addition, transcription factors like R-*twist* and *snail* were reported to be expressed during platogenesis.[34,35]

Cleft palate occurring by the abnormality of elongation or elevation and fusion of palatal shelves during embryogenesis, has been reported to be developed by the various causes. Reportedly, cleft palates are involved with hereditary or environmental factors either individually or collectively; hereditary factors include chromosomal anomaly and Treacher Collins syndrome, etc[36] and environmental factors include excessive or shortage of certain vitamin and hormone or drug, smoking or alcohol consumption etc., during embryogenesis.[37,38] Among those, RA, most widely known as palatal development inhibiting substance, is known to be involved in embryogenesis as active derivative of vitamin A. According to the animal research, exogenous and /or excess RA causes cleft lip, extremely little toe syndrome, microphthalmia and unascended kidney etc in addition to the cleft palate. When 13-cis-RA and all-trans-RA induced a RA isomer in rat system were administered at early stage of pregnancy, all-trans RA has shown to have more teratogenic effects, forming the strongest facial deformity at the concentration of 100mg/kg concentration[26]: fusion occurs by occurrence of apoptosis while the epithelial cells at MEE are conjugated.[39] If they are not fused by RA, it becomes cleft palate. During palatogenesis, RA also has been reported to prohibit the elevation of palatal shelves by causing abnormal apoptosis to tongue and generate cleft palate by arresting G1/S progression in palatal mesenchymal cells.[40] Also, it was reported that RA prohibits transformation of MEE cells that should be present during fusion of palatal shelves to mesenchymal cell.[11,41,42] In addition to that, *Wnt*11 signaling

molecule also reported to lead the complete fusion of palatal shelves by causing apoptosis during palatal development.[43] there is still other report that the apoptosis MEE is not a prerequisite for fusion of palatal shelves.[44]

It is known that *Hox* gene, a development regulatory gene, is expressed at particular time and particular region during early embryogenesis in both vertebrates and invertebrates. *Hox* is the master transcription factor involved in body patterning along the anterior to posterior axis.[45,46] The expression of Hox gene is reported to be influenced by RA and retinoid, influenced by RA and retinoid, its precursor. Particularly, if *Hox* gene expression region is modified by RA in vertebrates, homeotic transformation occurs at that region.[7,47-49]

In order to understand the effects of all-trans-RA on palate development, RA was injected into the abdominal cavity on the 11^{th} or 15^{th} day of pregnancy, and then the embryos in the following days were taken and analyzed morphologically as well as molecular biologically.

Morphological analysis showed that the embryos exposed to RA harbored cleft palate in which palatal shelves fusion did not occur (Fig. 1i, j). In the case of normal embryo, palatal shelves were elevated on the development 15^{th} day (Fig. 1c), contact and fusion of palatal shelves started on the 16^{th} day (Fig. 1d) forming complete palate on the 17^{th} day (Fig. 1e). Interesting thing is that embryo with cleft palate and/or cleft lip was observed when RA is infused on development 11^{th} day, but the group to which RA is infused on the 15^{th} day of development showed only cleft palate (Fig. 3B, a´, b´, c´). Such cleft lip and cleft palate occur while this process is delayed by RA on the 11^{th} day of development, when the maxillary prominence forms outside upper border of stomatodeum by growing toward the center and being separated by groove from lateral

nasal prominence and nasal pit starts to be visible from outside of embryo. However if RA was injected on the 15th day of development, when the two palatal shelves were projected from inside maxillary prominence after this process completes, on cleft palate was observed (Fig. 3). *In vitro* organ culture experiment also exhibited that RA treatment prohibited the development of palatal shelves, which resulted in the open palatal shelves. (Fig. 2) Here we also attempted to analyze the palate morphology after birth following the infusion of RA at the concentration of 100 mg/kg at both stage of E11 and E15. In both cases, however, embryo could not come into birth and this could be due to the concentration of RA (100 mg/kg), which is a lethal concentration making the birth of embryo hard.

In order to understand the molecular mechanism underlying the palatogenesis, *Hox* as well as apoptosis related gene expression pattern was analyzed with the dissected palate. The embryos injected with RA (100mg/kg) at the developmental stage of E11 or E15 were dissected out in the following days (E13 to E17), and then the palate was isolated. After purifying total RNA, cDNA was synthesized, and then RT-PCR was performed with Hoxa7 as well as apoptosis-related gene specific primer sets. When RA was injected in E11, expression of *Hoxa*7 was increased in 15 and the increased expression remained constant until E17. However in RA treated group, the *Hoxa*7 expression level was not changed until E17 (Fig. 4).

Immunohistochemistry analysis with embryos injected RA in E15 has revealed that Hoxa7 expression was reduced in the experimental group compared to that of at the stages of E16 & E17. Before immunohistochemistry performing, we confirmed whether the antibody against human Hoxa7 is really working with rat *Hoxa*7. We cloned successfully rat *Hoxa*7 gene into the eukaryotic expression vector pcDNA3 (Fig.6A).

And then, the subcellular localization of Hoxa7 was analyzed after transfecting the pcDNA3:*Hoxa*7 into COS-7 (monkey kidney cell line) cells. Hoxa7 turned out to be located in the nucleus (Fig. 7). These results implies that Hoxa7 protein, probably plays an important role during contact and fusion of palatal shelves (Fig. 4, 8).

In general, endogenous RA has been reported to play an important role during programmed cell death occurred in MEE when palatal shelves are fused. However, exogenous RA can act as a strong teratogen depending on the dosage as well as development stages during palatogenesis.[12-14] It was reported that in pregnant mouse, apoptosis has been induced in various cells when RA is infused from outside. Furthermore, it was shown that anti-apoptotic genes such as *Bcl-2* and *Bcl-xl* prohibit apoptosis induced by RA, [52-54] and *Bax*, a pro-apoptotic gene, induces apoptosis.[55] In previous study performed with total head RNA, *Bax* gene expressed consistently at high level form E13 to E17. Whereas in RA treated group, the expression pattern of *Bax* gene was gradually increased from developmental stage of E15 throughout E17[24]. In this study, however, *Bax* expression was reduced when RA was treated in E11 (Fig. 4). TUNEL assay also showed that RA treatment inhibited apoptosis (Fig. 9). In the case of control group, TUNEL assay revealed that the TUNEL positive cells were mostly observed at the region where MES is forming and at the epithelial cells surrounding nasal cavity as well as oral cavity where rugae is made in E16 (Fig.9A). In E17, TUNEL positive cells were observed at the region where MES is incorporated (Fig. 9B). In the case of RA treated group, TUNEL positive cells were found in both epithelial and mesenchymal cells in palatal shelves at the stage of E16 (Fig. 9C), while E17 palate has shown TUNEL positive cells only in/near epithelial cells of palatal shelves (Fig. 9D). Previously, it has been reported that the apoptosis is required for palate formation,

especially at the marginal MEE during palate development[39, 41]. If apoptosis does not occur properly, cleft palate can be formed because complete fusion is not accomplished although palatal shelves may go through conjugation. The results here indicate that the RA treatment inhibited apoptosis whereas previous result strongly implied the induction of apoptosis during palatogenesis 25. The discrepancy could be explained partially by the dosage of RA administered at the stage of E11, or by the tissues analyzed: total RNAs isolated from total head region were used in previous study, whereas only the palatal tissues were used here.

These results altogether implies that exposure to RA during palate development causes facial deformity including cleft palate and cleft lip by modulating the expression of *Hoxa7* as well as an apoptosis-related gene, Bax, and thus malregulating the apoptosis during palatogenesis.

Ⅴ. CONCLUSION

In order to understand the effects of all-trans-RA on palate development, RA was injected into the abdominal cavity on the 11^{th} or 15^{th} day of pregnancy, and then the embryos in the following days were taken and analyzed morphologically including in vitro organ culture by palatal shelve were separated on E15.5 as well as molecular biologically.

1. When RA was administered at early stage (E11) or late stage (E15) during palate development, the overall craniofacial development was retarded. The length from jaw to eye was shortened, and compared to that of normal control group. Also the eye development seemed to be delayed. Especially, cleft lip was found only when the embryo was exposed to RA at early stage (E11) during palatogenesis.

2. *In vitro* palate culture experiment has shown that, RA is a potent teratogen causing a cleft palate.

3. When RT-PCR with palate RNA was performed early stage administration of RA (E11) during palate development inhibited the upregulation of *Hoxa*7 expression at E15 to E17. Whereas in control group, high level of *Hoxa*7 expression was detected in the palate of E15 to E17. In the case of *Bax*, the expression was decreased from E16, while constant in control.

4. When RA is infused at late stage (E15) of palate development, TUNEL positive cells

were detected in the mesenchymal cells as well as epithelial cells of palatal shelves of E16, and in E17 palate, at the region below palatal shelves epithelial cell. On the other hand, TUNEL positive cells were observed mostly at the epithelium around the nasal cavity and oral cavity where rugae is made after MEE contact and MES is made. When palatal incorporation is nearly reached in E17, TUNEL positive cells were observed at the region where MES disappears.

It is concluded that exposure to RA during palate development causes facial deformity including cleft palate and cleft lip by modulating the expression of *Hoxa*7 as well as an apoptosis-related gene, Bax, and thus malregulating the apoptosis during palatogenesis.

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국문요약

배자 발생시 레티노산에 의해 유도된 구개열에서 *Hox* 유전자의 발현 양상과 세포사멸의 변화에 관한 연구

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백서에서 구개발생은 발생 13일에서 17일 사이에 구개 돌기가 발생되고 융합된다. 발생 14일째에 입천장 선반이 아래쪽으로 자라기 시작하고 15일째에는 혀 양쪽 아래쪽으로 입천장 선반이 자라고 16일째에는 아래쪽으로 자라던 입천장선반이 혀 위쪽으로 이동하여 수평을 이루게 되고 중앙을 중심으로 입천장 선반의 가운데 부분부터 접근, 병합을 시작하며 17일째가 되면 앞뒤부분까지 융합이 완료된 완전한 구개가 형성된다. 구개열은 1000명 중 1, 2명의 비율로 일어나는 매우 흔한 선천성 안면기형이다. 레티노산은 비타민 A의 내인성 대사산물로서 동물의 발생 과정 동안 정상적인 형태형성을 하기 위해 필요한 물질로 알려져 있다. 그리고 피부의 여드름 등을 치료하는 데에 많이 쓰이는데, 임신 초기에 레티노산에 과도하게 노출되거나 부족하면 구개열을 포함한 두개안면기형, 흉선 무형성, 심장유출로 결손증, 신경관 결손 등의 기형을 갖는 아이가 생긴다는 보고가 있다. 내인성 레티노산은 입천장선반의 병합시 중앙가장자리상피 세포의 세포사멸에 중요한 역할을 하지만, 외부에서 주입되거나 과도한 레티노산은 강력한 기형유발물질로 작용할 수 있는 것으로 알려져 있다. 배자 발생 과정 중 특정 시간에 그리고 배자의 특정한 위치에서 발현하여 배자의 형태를 형성하는데 관여한다고 알려져 있는 전사조절인자인 *Hox*유전자는 RA와 전구물질인 레티노이드의 영향을 받아 배자 발생에 영향을 미치는 것으로 알려져 있다. 특히, *Hoxa7*유전자의 ectopic expression은 구개열을 포함한 안면기형이 일어나는 형태적 변화를 유도하였으며 이는 RA에 노출된 배자의 형태와 유사하다는 보고가 있었다.

본 연구에서는 백서를 이용하여 두개안면형성이 일어나는 발생 초기(E11)에 RA(100 mg/kg)를 주입한 다음 각 단계의 배자를 분리하여 구개열의 형성과정을 형태학적으로 비교 분석하였고, 특히 입천장 선반이 올라오는

시기인 E15에 구개 조직을 RA가 처리된 배지에 배양하여 *in vitro* 상에서도 레티노산이 구개발생에 특이적으로 영향을 미치는지 알아보았다. 또 구개 발생 과정 중 각 시기 별로 얻은 구개 조직에서 추출한 총 RNA를 이용하여 *Hoxa*7이 구개조직에서 발현하는지 알아보았고, 세포사멸 관련 유전자인 *Bax*의 발현의 변화를 확인하였다. 또한 구개 발생의 후기인 E15에 레티노산을 주입한 다음 16, 17일의 배자를 분리하여 형태를 확인하고, *Hoxa*7 단백질 특이적인 항체를 이용하여 면역조직화학염색을 수행하였으며, 세포사멸의 패턴을 분석하기 위해 TUNEL assay를 수행하였다.

레티노산에 노출된 배자의 경우 구개 발생 초기와 구개 발생 후기 모두에서 머리 발생 지연을 포함하여 구개열이 형성되었으며, 구순열의 경우에는 구개발생 초기에 RA에 노출된 경우에만 관찰되었다. 입천장 선반을 가지고 *in vitro* organ culture를 수행한 결과 RA는 구개형성에 있어서 직접적으로 기형유발 물질로 작용함을 확인하였다. 안면 발생 초기(E11)에 레티노산을 주입한 경우, *Hoxa*7의 발현은 정상 구개 조직에서는 발생 15일째부터 발현이 증가되어 구개형성이 끝나는 17일까지 유지되었으나, 레티노산에 노출된 구개조직에서는 15일째가 되어도 증가되지 않았다. 구개 발생 후기(E15)에 레티노산을 주입한 경우, 입천장 선반이 접촉, 병합되는 시기에 *Hoxa*7의 발현은 대조군에 비해 약하게 발현하였다. 구개 발생 초기에 레티노산을 주입하여 세포사멸에 관여하는 pro-apoptotic한 유전자인 *Bax*의 발현양상을 보면 대조군에서는 13일에서 17일까지 전체적으로 강하게 발현하는 반면, 레티노산 처리군은 발생 16일부터 급격히 줄어들었다. 구개 발생 후기(E15)에 레티노산을 주입하여, 16일과 17일의 구개 조직을 이용하여 TUNEL assay를 한 결과, 대조군은 MEE가 접촉하는 시기인 16일째에는 MES가 형성되는 부위와 구강과 비강 쪽의 상피세포에서 주로 TUNEL 양성 세포가 관찰되었고, 병합이 마무리되는 17일에는 MES가 완전히 병합되는 부위에서 TUNEL양성 세포가 관찰되었다. 반면 레티노산 처리군은 16일에 중간엽 세포쪽에서 TUNEL 양성 세포가 관찰되고 17일에는 입천장 선반의 상피세포 근처에 주로 TUNEL 양성 세포가 관찰되었다.

이상의 결과로 미루어 볼 때, 구개 발생 과정 중에 레티노산에 노출될 경우 구개열을 포함한 안면기형을 유발하며, 레티노산은 *Hoxa*7유전자와 세포사멸을 조절하여 기형을 유발하는 것으로 유추된다.

핵심 되는 말: 구개 발생, 구개열, 레티노산, *Hoxa*7 유전자, 세포사멸