

Erk AND RETINOIC ACID SIGNALING PARTICIPATE IN THE SEGREGATION AND PATTERNING OF FIRST ARCH DERIVED MAXILLA AND MANDIBLE

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Abstract

In vertebrates, the face is mainly formed with neural crest derived neural crest cells by the inherent programs and the interactive environmental factors. Extracellular signaling-regulated kinase (Erk) is one of such programs to regulate the various cellular functions. And retinoic acid (RA) also plays an important role as a regulator in differentiation process at various stages of vertebrate embryogenesis.

We wanted to know that the segregation as well as the patterning of maxillary and mandibular structure is greatly influenced by the maxillomandibular cleft (MMC) and the failure of this development may result in the maxillomandibular fusion (syngnathia) or other patterning related disorder. It has been well documented that the epithelium at this cleft region has significant expression of Fibroblast growth factor (Fgf) 8, and it is essential for the patterning of the first arch derived structures.

By the morphological, skeletal, cell proliferation and apoptotic, and hybridization analysis, we checked the effects of Erk inhibition and/or RA activation onto MMC and could observe that Erk and RA signaling is individually and synergically involved in the facial patterning in terms of FGF signaling pathway via Barx-1. So RA and Erk signaling work together for the MMC patterning and the segregation of maxilla-mandible by controlling the Fgf-related signaling pathways. And the abnormality in MMC brought by aberrant Fgf signaling may result in the disturbances of maxillary-mandibular segregation.

Key words: Erk, Retinoic acid, Patterning, Facial development, Maxillomandibular fusion

I. INTRODUCTION

The syngnathia, also termed as maxillomandibular fusion, is a congenital disease showing the complete bony fusion (syngnathism) or soft tissue (synechia) fusion of mandible (Md), maxilla (Mx) or zygomatic arch, and the gingivas. It is a rare disorder that about 25 cases have been reported so far¹⁾ and its etiology is not clearly understood. Theories proposed to explain this disorder have included the abnormal ectodermal ingrowth for gum fusion²⁾, abnormalities in stapedial artery³⁾, early loss of neural crest cell (NCC)⁴⁾, teratogenic agent or intrauterine trauma⁵⁾.

In order to understand the underlying mechanism of this disease, we first wanted to start at the normal

facial development and the segregation of maxillary and mandibular development. In vertebrates, the face is mainly formed by neural crest (NC) derived neural crest cells (NCC). NCCs become widespread all over the body, and it also plays a major role in craniofacial development⁶⁻⁸⁾. And it has been generally accepted that the developmental fate of these cells is inbuilt by complex programs⁹⁾ as well as by the environmental factors.

NC-derived cells of maxillary and mandibular prominences give rise to the bones, cartilages, and connective tissues of Mx and Md¹⁰⁾. Both prominences and their derived skeletal structures are developed from the same origin of embryonic cells and the structure called as the first pharyngeal arch.

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The maxillary prominence starts to grow out at the anterior portion of mandibular process during the early stage of facial development, simultaneously making the maxillomandibular cleft (MMC) between these processes. The epithelial and mesenchymal cells at this cleft region already have gained the some attention for their ability to sustain and support the proximodistal patterning of the maxillary and mandibular processes.

So our hypothesis was that the segregation as well as the patterning of maxillary and mandibular structure is greatly influenced by the MMC and the failure of this development may result in the syngnathia or other related disease. It has been well documented that the epithelium at this cleft region has significant expression of Fibroblast growth factor (Fgf) 8 and its complex interactive gene expressions and signaling cascades with the underlying mesenchyme are essential for the patterning of the first arch derived structures.

Among the various signaling pathways, mitogen-activated protein kinases (MAPK) have been reported to participate in several patterning related developmental process by the regulation of the cell growth, differentiation, and apoptosis¹¹⁾ or by responses to various extracellular stimuli¹²⁻¹⁶⁾. And extracellular signaling-regulated kinase (Erk), one of MAPK signaling pathways, regulates various cellular functions

in fundamental level by transducing the extracellular signals to the cytoplasm and to the nuclear effectors¹⁷⁾.

FGF signaling is mediated via tyrosine-kinase receptors that act through a number of transcription pathways, including the RAS-Erk MAPK signaling cascade¹⁸⁾, to serve crucial functions during embryogenesis¹⁹⁾. FGF gene expression is reported to be found at the various regions like MMC during the facial development. So Erk was expected by us to play some important roles during the early facial patterning phase at the MMC region.

And Retinoic acid (RA), an active metabolite of vitamin A, is another important signaling molecule during vertebrate development²⁰⁾. Maternal vitamin A deficiency^{21, 22)}, or teratogenic RA excess²³⁾ results in a spectrum of congenital craniofacial abnormalities. It also controls the outgrowth and patterning of the facial prominence²⁴⁾. So RA has been regarded as a good morphogen working at the high level of signaling cascade and Fgf is one of prime targets for regulatory interactions with RA²⁵⁾.

In addition to the fact that each Erk and RA signaling is individually involved in the facial patterning in terms of FGF signaling pathway, there are some evidences that they work synergically to enhance the developmental process. ERK activation by mitogen-activated protein / ERK kinase (MEK) was neces-

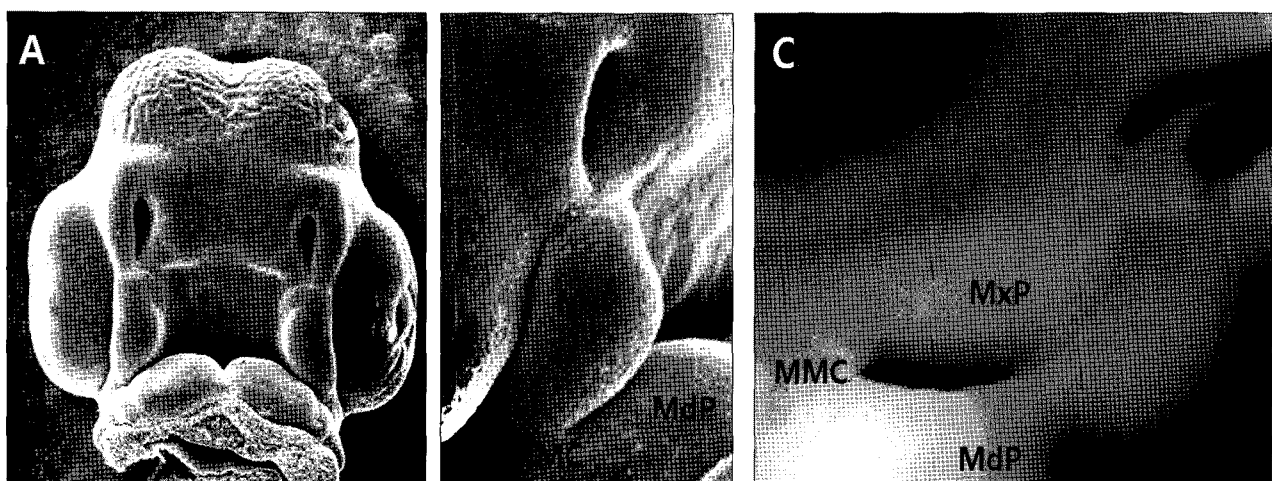


Fig. 1. Early facial appearance of chicken embryo with MMC and Fgf 8 expression. A frontal (A) and oblique lateral (B) SEM images of normal embryo at embryonic day 4.5 (H-H stage 24) showed the normal maxillary and mandibular prominence developing from first pharyngeal arch and the maxillomandibular cleft (designated as MMC at B & C) between them. And an external morphology of in-situ hybridized embryo (C) revealed the epithelial Fgf8 expression at the maxillomandibular cleft region.

sary for RA-induced differentiation in a study using PD98059 to block MEK phosphorylation²⁶. And it is the reverse case that FGFs could regulate the retinoic acid receptor (RAR) expression, confirming that they may be closely linked altogether for the facial morphogenesis²⁷.

So it motivated us another hypothesis that RA and Erk signaling work together or may be closely related to the MMC patterning and the segregation of Mx-Md, by controlling the Fgf and its downstream signaling pathways. Here we showed that Erk and RA signaling through Fgf signaling pathway participate and control the facial developmental patterning of the first arch and the segregation of their derived Mx and Md.

II. MATERIALS AND METHODS

1. Embryos and a bead implantation

Fertilized White Leghorn chick eggs were incubated at 38°C in a non-rotating incubator (Vision, Korea) until the desired stage was reached. Embryos were staged according to Hamburger and Hamilton (H-H) stage²⁸. AG1-X2 beads (Bio-Rad, USA; formate form, 100~125µm diameter) were soaked in concentration PD98059 50µM, 0.1mg/ml of all-*trans* RA (Sigma, USA) dissolved in dimethylsulfoxide (DMSO; Sigma, USA) for 20 minute (min) at room temperature (RT) and then were rinsed in Dulbecco's Modified Eagle Medium (DMEM; GIBCO, Grand Island, NY, USA). The beads for control were soaked in DMSO.

2. Injections of DiI

DiI (1,1'-diiododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes Inc., USA) were injected with micro-injector into the proximal region of the first pharyngeal arch of H-H stage 15 embryos immediately after a PD98059/RA or DMSO soaked bead was implanted. After the injection, the embryos were incubated for 48 hr to reach H-H stage 24, sacrificed, and fixed in 4% paraformaldehyde with 0.25% glutaraldehyde. The embryos with the labeled cells were taken with

MZ16FA Leica fluorescence stereo microscope (Texas Red filter set) and DFC300 FX Leica camera (Leica, Germany).

3. BrdU assay

PD98059/RA or DMSO treated embryos at H-H stage 15 were treated after 24 hr with 100µl of 5mg/ml BrdU (5-Bromo-2'-deoxyuridine; Sigma, USA) dissolved in sterile PBS, incubated for 2 hr, sacrificed and fixed with 4% PFA in PBS for 16 hr at 4°C, followed by the paraffin embedding. Deparaffinized and rehydrated of slides were blocked with 3% hydrogen peroxide in methanol for 10 min at RT and 5% horse serum in PBS for 10 min at RT, incubated for 1 hr at 37°C with anti-BrdU (1:200; Becton-Dickinson, USA), followed by biotinylated anti-mouse IgG (1:500; Vector Laboratories, Inc. Burlingame, USA) for 1 hr at 37°C and HRP-conjugated streptavidin (1:200; Vector Laboratories, Inc. Burlingame, USA) for 1 hr at RT. Detection was performed with DAB peroxidase substrate kit (Vector Laboratories, Inc. Burlingame, USA).

4. TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end-labeling) assay was performed on paraffin sections of embryos after 48 hr of the bead implantation (approximately H-H at stage 24) using the apoTACS-DAB in situ apoptosis detection kit (Trevigen) following manufacturer's protocol.

5. Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as previously described. The embryos after 48hr of the bead implantation (approximately at H-H stage 24) were fixed in 4% PFA in PBS for 16 hr at 4°C, processed to 100% methanol, and stored at -70°C until use. Specimens were rehydrated and treated with 3% H₂O₂ in PBT (PBS + 0.1% tween 20). Antisense riboprobes were labeled with digoxigenin-UTP and hybridization was performed at 65°C. Detections of the label was performed with ALP-con-

jugated anti-digoxigenin antibody (Roche Diagnostics, Germany) at 4°C for 16 hr followed by incubation with the substrate for ALP (BCIP and NBT; Promega, USA).

III. RESULTS

1. Early morphological changes at mandibular and maxillary process by an Erk inhibitor / RA to the first pharyngeal arch

Chick embryos were implanted with PD98059 (50 μ M) / RA (0.1mg/ml) soaked bead into the first arch at H-H stage 15 (Fig. 2). After 2, 3 and 4 days of incubation, DMSO treated embryos did not show any abnormal morphological changes (Fig. 2A, B, C). But PD98059 treated embryos showed that there was a depression around the bead at the mandibular arch, while the maxillary arch was less affected (Fig. 2D, E, F).

RA treated embryos showed the shortened MMC (gray arrowhead in Fig. 2G) with the enlarged maxillary arch in anterior-posterior dimension and the decreased size of mandibular arch with some depression (Fig. 2G, H, I). And the embryos treated with simultaneous PD98059 and RA were observed to have the similar external morphologies, as compared with those of RA treated embryos. But the striking differences from RA embryos were a newly formed

bulging just posterior to maxillary arch (white arrow-heads in Fig 2K). And there were also reduced sized and abnormal shaped maxillary and mandibular arch (Fig. 2J, K, L).

2. Long-term external and skeletal morphological changes after the treatment

The embryos at H-H stage 38, 12 days after bead implantation, were checked for the external morphological change (Table 1). Embryos treated with PD98059 and cleared for the skeletal patterning (Fig. 3A-F) showed that the proximal end of quadratojugal bone and its counterpart of quadrate were deformed to make a smaller and sharp end (Fig. 3C). And the cartilaginous defects were observed in nasal conchae (Fig. 3A) to have some extra cartilages and pleurospenoid (Fig. 3D, E) to make smaller size.

Treatment of chick embryos with RA resulted in the deformation of skeletal patterns in the upper and lower beak (Fig. 3G-I). The quadratojugal bone (Fig. 3G) got thicker than control side with some extra bone. And the jugal bone was elongated at the proximal end toward squamosal bone. The squamosal bone showed some, but prominent elongation toward jugal bone (Fig. 3G, H).

The embryos simultaneously treated with PD98059 and RA showed much more prominent skeletal and

Table 1. Changes of skeletal pattern at H-H stage 38 by exogenous implantation of Erk inhibitor PD98059 and/or all-trans RA at the first pharyngeal arch of H-H stage 15.

Bone & Cartilage	Deformity	DMSO	PD98059	PD98059 + RA
Quadrate	Supernumerary	0 (0)	0 (0)	38 (95)
Quadrato-jugal	Small / abn. Shaped	0 (0)	16 (40)	35 (87.5)
Jugal	Small / abn. Shaped	0 (0)	1 (2.5)	33 (82.5)
Maxillary Bone	Small / abn. Shaped	0 (0)	0 (0)	38 (95)
Palatine	Small / abn. Shaped	0 (0)	0 (0)	0 (0)
Pterygoid	Small / abn. Shaped	0 (0)	1 (2.5)	39 (97.5)
Nasal Conchae	Small / abn. Shaped	0 (0)	9 (22.5)	0 (0)
Pleurospenoid	Small / abn. Shaped	0 (0)	12 (30)	40 (100)
Cerato/Epi branchial	Small / abn. Shaped	0 (0)	7 (17.5)	4 (10)
Supra-angular	Small / abn. Shaped	0 (0)	3 (7.5)	40 (100)
Total (n)		40	40	40

() : percentage

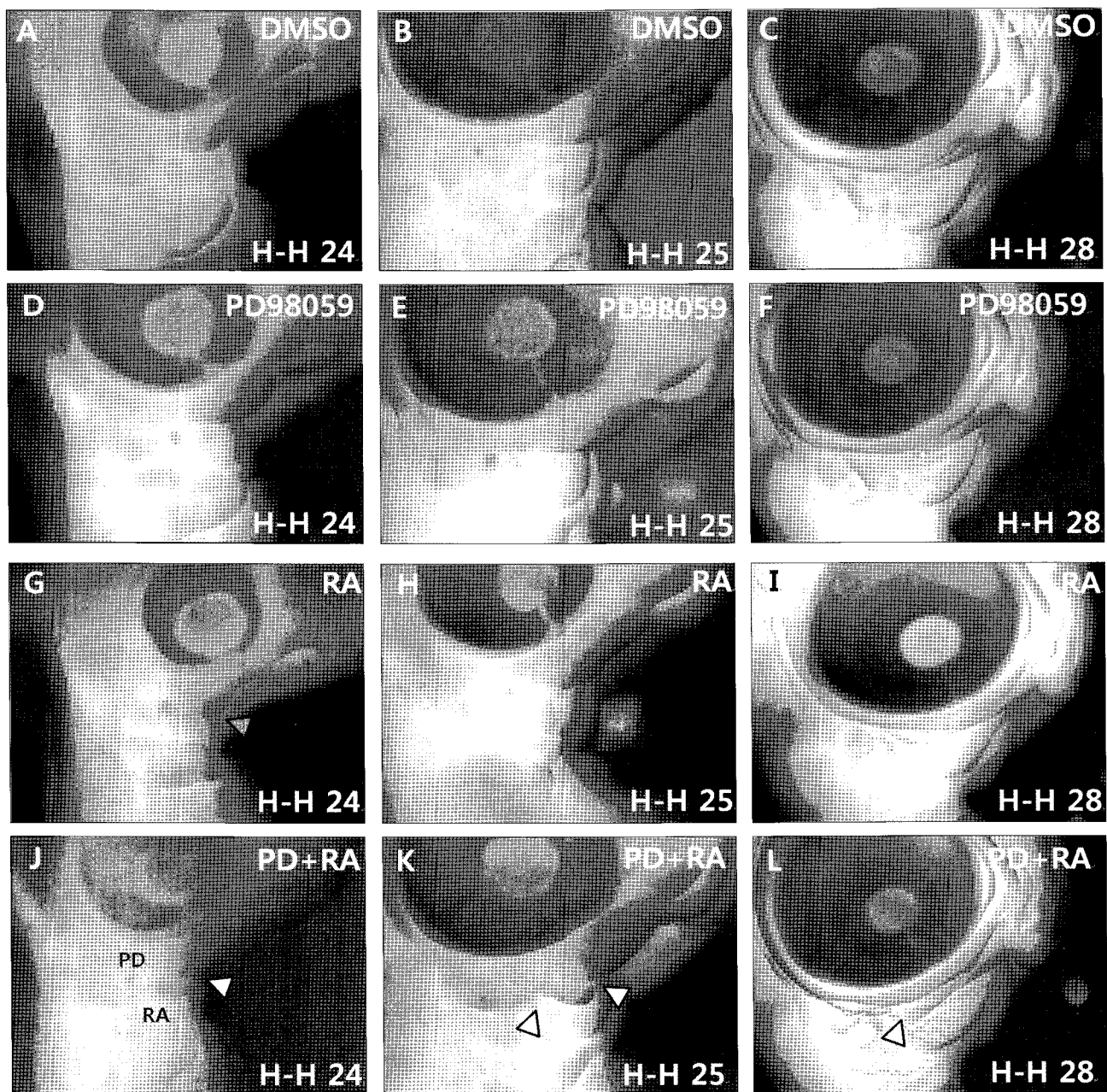


Fig. 2. Early morphological changes of pharyngeal arches and MMC region after the inhibition of Erk and/or RA at the sequential developmental stages of H-H st24, 25 and 28.

Embryos treated with DMSO showed the normal maxillary and mandibular region (A-C). External morphology of Erk inhibitor (PD98059 50 μ M) treated embryos was changed on the treated side of mandibular arch of surface (D-F). RA treated embryos showed the decreased size of maxillary arch and the distally advanced MMC (G, gray arrowhead) (G-I). Embryos treated with PD98059 and RA showed the reduced maxillary and mandibular region (white arrowheads) and newly formed an extra prominence in MMC regions (J-L). (MMC, maxillomandibular cleft)

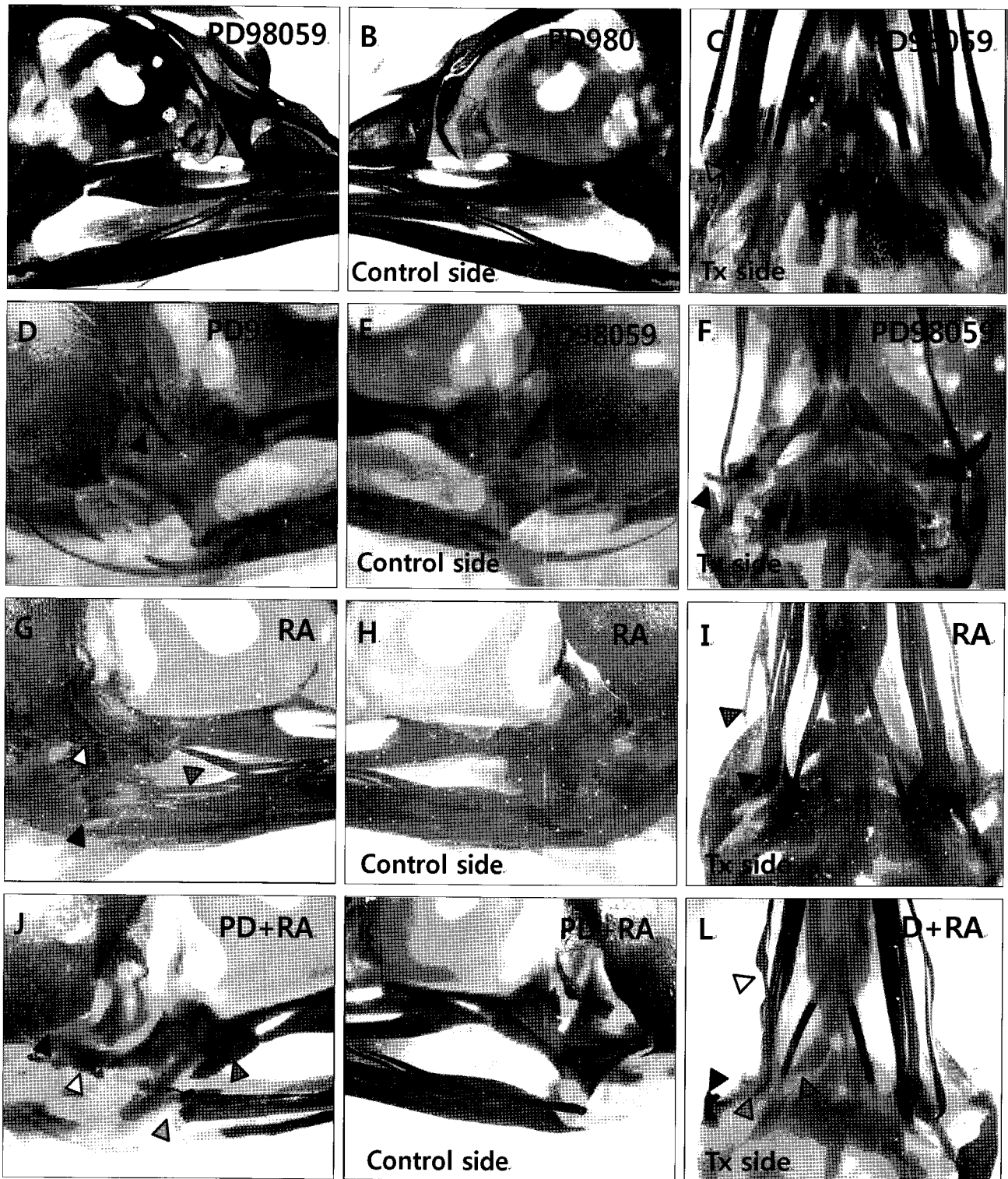


Fig. 3. Morphological evaluation of skeletal pattern for embryos at H-H stage 38. Embryos treated with PD98059 (A-F) at the H-H stage 15 resulted in the various facial skeletal defects, including deformation at nasal conchae (A), a decrease in the size of pleurosphenoid (D; black arrowhead) and deformed quadratojugal bone (C; gray arrowhead). Embryos treated with RA showed the skeletal deformation in upper and lower beaks (G-I), mainly at quadratojugal (G, I; gray arrowhead), jugal (G, I; black arrowhead) and squamosal bone (G; white arrowhead). The skeletal staining of PD98059 and RA treated embryos also showed the deformed squamosal bone (J, K; gray arrowhead), quadrate (J; white arrowhead), jugal bone, which was shortened in length and deformed in shape. Some missing bones were found at quadratojugal, supra-angular (J; gray arrowhead) and pterygoid bone (J, K; blue arrowhead).

morphological changes in the facial regions (Fig. 3J–L). The fusion of maxillary and mandibular bone at the MMC region was also evident that resulted in the shorter MMC (Fig. 3L). In the skeletal staining of them, the proximal part of maxillary and mandibular bone (like quadrate, quadrato-jugal, supra-angular bone, and Meckel's cartilage) were missing or deformed (Fig. 3J). The jugal bone was deformed in shape and decreased to make a fusion at the proximal end with supra-angular bone (Fig. 3L). In addition, the squamosal bone showed the smaller size with deformation, and the pterygoid got thicker and shorter (Fig. 3J).

3. Cell migration patterns observed by Dil injection, and cell proliferation and apoptosis in the first branchial arch by BrdU and TUNEL.

Dil injections were made onto the ectomesenchyme of MMC at stage 15 after the bead implantation at the first pharyngeal arch of H-H stage 15 (Fig. 4A, D, G, and J). The labelled cells from the MMC were shown to migrate into the proximal Mx and Md in the embryos treated with DMSO (Fig. 4A). But those treated with PD98059 50 μ were shown to have labeled cells mainly at the proximal maxillary region, staying away from the proximal mandibular region where the bead was implanted (Fig. 4D). Those embryos treated with RA had the labeled cells at the proximal maxillary region, similar to PD98059 treated embryos (Fig. 4G). And simultaneous PD98059 and RA treated embryos also showed the labeled cells at the proximal Mx, which was the same as those from PD98059 or RA treatments (Fig. 4J).

The BrdU labeling showed relatively general spreading of positively-stained cells at mandibular and maxillary process with the proximal maxillary region except at the MMC at H-H stage 24 (Fig. 4B) in the DMSO treated embryos. Embryo with PD98059 was seen to have the decreased number of positively stained cells in the mandibular process as well as MMC region (Fig. 4E). In the RA treated embryos, BrdU positive cells were shown at proximal Mx and Md and especially at the MMC region (Fig. 4H). The simultaneous PD98059 and RA treated embryos showed the decreased number of positive

stained cells at proximal Md and Mx distal to the implanted bead, while more cells were found at the proximal region to the bead (Fig. 4K).

In the TUNEL assay to detect the programmed cell death at the H-H stage 24, there was no noticeable increased or decreased number of positive staining from DMSO, PD98059, and/or RA treated embryos in the first branchial arch (Fig. 4C, F, I, and L).

4. Gene expression patterns during the first arch development.

At H-H stage 20 (Fig. 5A–D) and stage 24 (Fig. 5E–H), the gene expression patterns of mesenchymally expressed *Barx1* and epithelially expressed *Fgf8* genes were checked. DMSO treated embryos showed that *Barx1* was expressed mainly at the mandibular and less at maxillary process at H-H stage 20 (Fig. 5A). By stage 24, the expression of *Barx1* was extended mainly to the maxillary and mandibular process per se and less to the proximal mandibular process (Fig. 5E). In addition, there was small coverage of *Barx1* expression at the MMC.

PD98059 treated embryos showed the less expression domain of *Barx1* around the bead and less at the MMC and proximal mandibular arch at H-H stage 20 and 24 (Fig. 5B and F). The expression from RA treatments was somewhat different from those from DMSO or PD98059 in that more expressions were noted at the MMC with less at the proximal mandibular process at H-H stage 24 (Fig. 5G). The embryos from the simultaneous PD98059 and RA treatment were observed to have the similar to that of RA at the extended MMC expression, but they had less or none expression at the maxillary process and the proximal maxillary and mandibular arch region (Fig. 5D and H).

But *Fgf8* was mainly expressed at the MMC epithelial region at the DMSO treated embryos (Fig. 5I) and its pattern was the same in the embryos with PD98059 and/or RA treatments (Fig. 5J–L).

IV. DISCUSSION

While much attention has been paid to the embryonic origin of the facial structures or pathways of

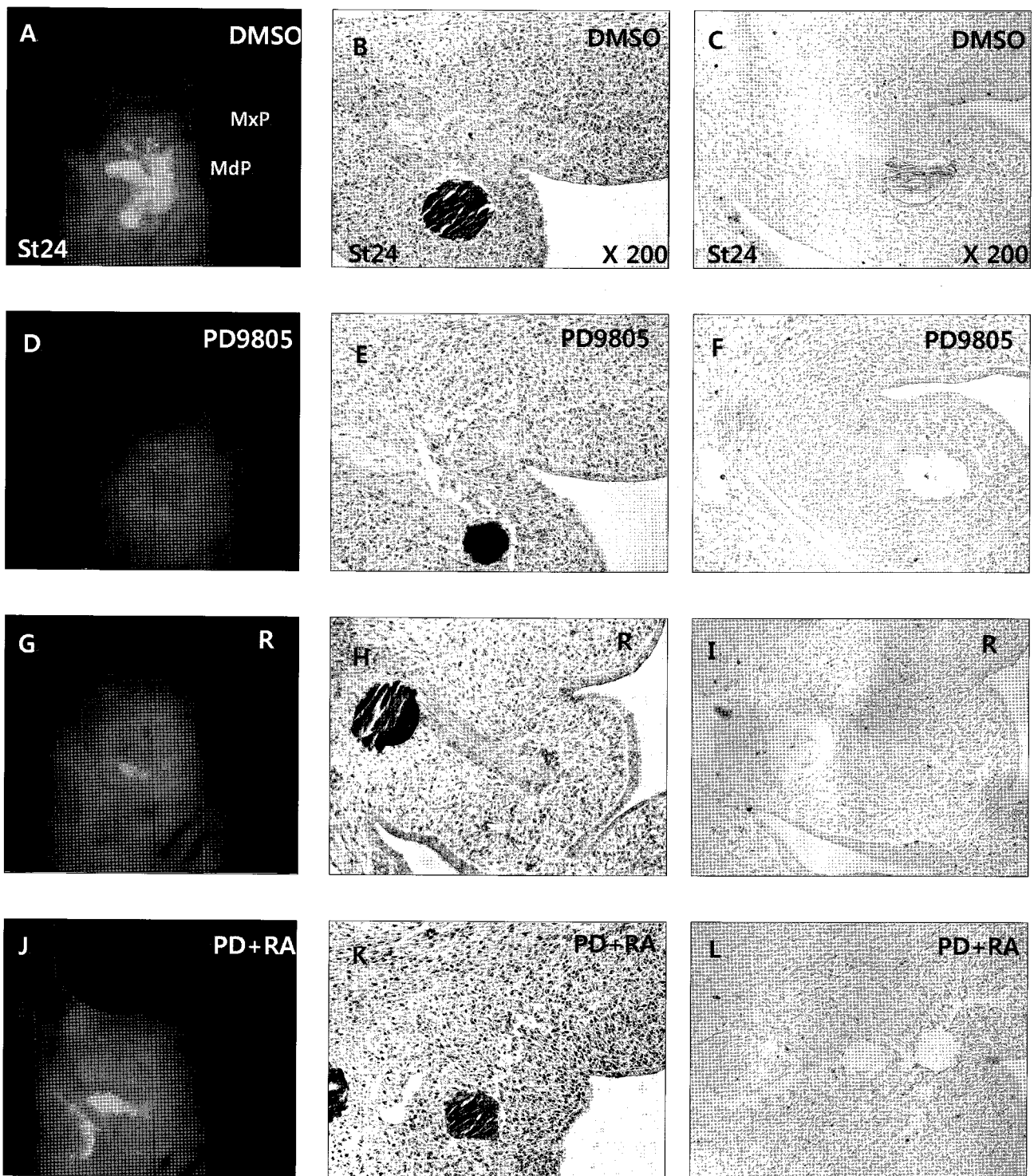


Fig. 4. Patterns of cell migration, cell proliferation, and cell death at MMC at H-H stage 24.

On Dil labeling experiments, DMSO treated embryos had a normal wide spreading pattern of cells toward the proximal Mx and Md (A), while some reduced in number at proximal Mx and Md in PD98059 treatments (D) and areas close to proximal Mx in RA treated embryos (G). And the simultaneous PD98059 and RA treated embryos showed the cells at proximal maxilla (J).

On BrdU assay, embryos treated with DMSO were seen to have the positive labeled cells extensively at the mandibular and maxillary process, and proximal Mx (B). But PD98059 and/or RA treated embryos showed the different pattern of proliferating cells (E, H, K). In addition, TUNEL assay revealed the normal cell death pattern in all embryos (C, F, I, and L).

neural crest migration²⁹⁻⁴⁰), little is known about the mechanisms which control the patterning of the facial primordia. The maxillary and mandibular primordia are mainly made up of the neural crest derived cells from several distinct populations which, on the whole, remain segregated to give rise to distinct skeletal structures. The neural crest cells typically forms the skeletal and connective tissues of the arches^{41,42}), and the mesoderm do the musculature and the endothelial cells^{37,41,43,44}).

FGF signaling is mediated via tyrosine-kinase receptors (FGFR) which act through a number of transcription pathways, including the Ras-Erk mitogen-activated protein kinase (MAPK) signaling cascade⁴⁵). Erk regulates various cellular functions in

fundamental level by transducing the extracellular signals to the cytoplasm and to the nuclear effectors. Mutations in members of the Erk and FGF pathways have demonstrated their importance in development of extraembryonic tissues⁴⁶⁻⁴⁹). The activated Erk is also reported to be concentrated at the adhesion sites and to regulate cell spreading and migration⁵⁰). And Erk2 activation is probably an early necessary process of the RA-induced cellular response²⁶).

The molecular events linking cell surface receptors to activation of ERKs are complex. Erk-1/2 can be activated by a number of pathways including those that involve tyrosine kinase receptors (RTK). Epidermal growth factor (EGF), platelet derived growth factor (PDGF) and FGF are activators of

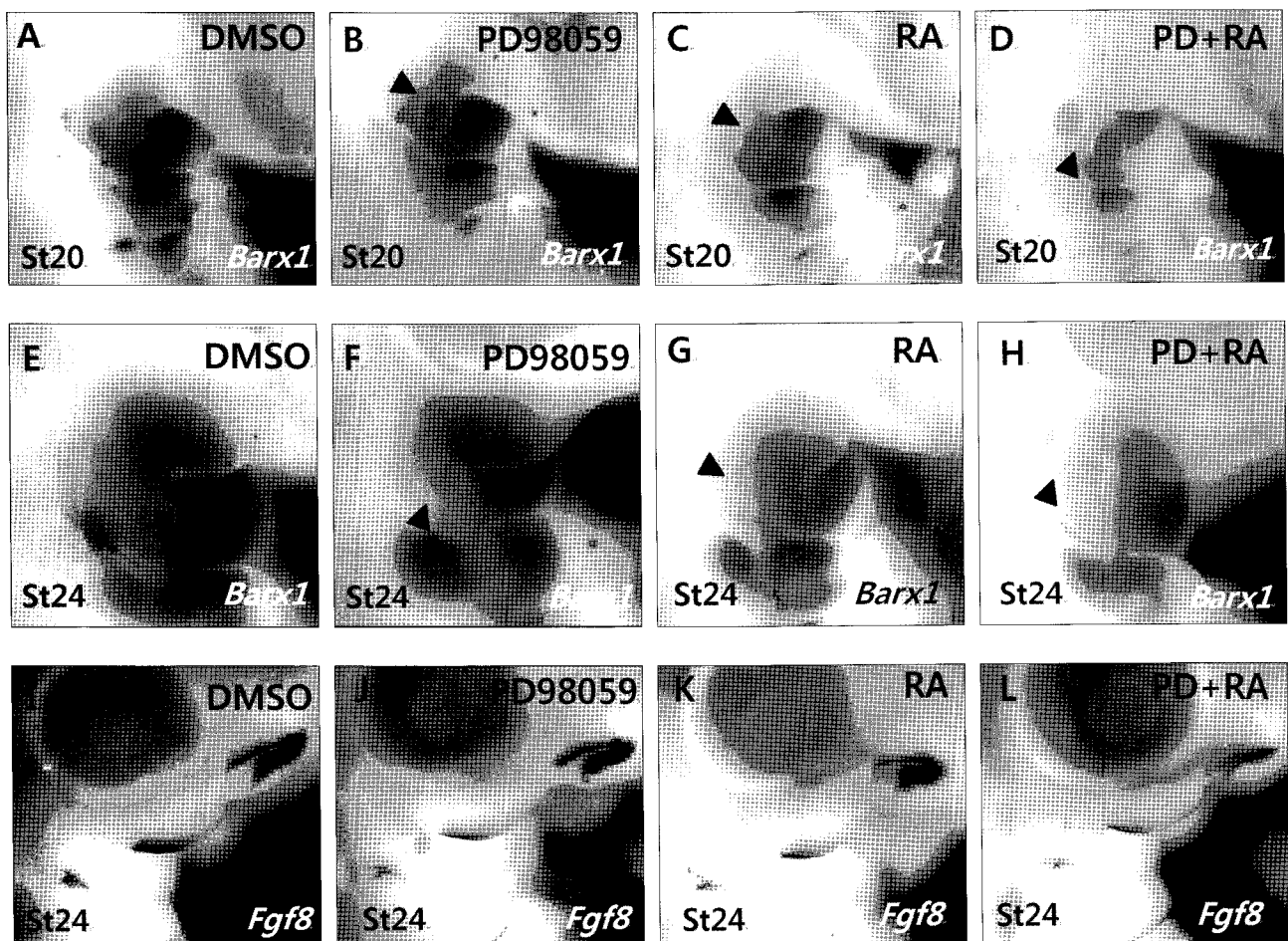


Fig. 5. Whole mount in situ hybridization showing the expression of *Barx1* at stages 20 (A-D) and 24 (E-H), *Fgf8* (I-L) at stages 24 respectively at the developing chick face. Changed gene expression patterns were prominent at the embryos with PD98059 and/or RA treatment.

Erk. In the intracellular pathway, some kinases were shown to be involved in the activation of Erks, including Ras GTP-binding proteins or Raf-1⁵¹⁾. ERK activity reflects the competing actions of upstream activator kinases and inhibitory MAP kinase phosphatases. PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase (MEK) in vitro and in vivo, thereby inhibiting the phosphorylation and the activation of ERK⁵²⁾. The participation of phospho-(P-) Erk1/2 was confirmed by exposure of the cells to PD98059, which directly prohibits Erk1/2 phosphorylation and inhibited cell migration⁵³⁾.

One of the major pathways activated by Fgf signals is the conserved protein cascade referred to as MAP kinase module (Ras - Raf - MEK - ERK or Ras - MAPK pathway) as explained above. And Fgf signals are critical for the patterning and outgrowth of the first arch. For example, Erk has been shown to regulate apoptosis which showed the changed cell migration pattern in maxillomandibular region⁵⁴⁾.

In order to understand the patterning role of Erk for the MMC development, we tried the loss of function with an ERK inhibitor, PD98059 implanted to the mandibular process near MMC. In the morphological analysis of early arch development at H-H stage 24, 25, and 28, as well as the late development at H-H 38-40, embryos showed the relatively minor morphological changes including the quadratojugal bone, pleurophoid, and nasal conchae (Fig 2D-F and 3A-F). The proximal end of quadratojugal bone was deformed to make a smaller and sharp end (Fig 3C), and the pleurospenoid (Fig. 3D, E) was smaller. The distribution of above mentioned affected bone (Fig 3), the region of external depression around the bead (Fig 2E), and the pattern and area of the changed expression of Barx1 (Fig 5F) matches well for the area of deformation caused by the inhibition of ERK signaling. And it also matches well with the domain of Fgf expression in MMC. So we could believe that the inactivation of some part in the downstream of Fgf signaling was affected by the PD98059 treatment and its effect was the inhibitory regulation of Fgf signaling cascade.

Another candidate signaling, RA is an active metabolite of retinol can regulate the activity of a

number of signal transduction pathways to be a very important signaling molecule for a vertebrate development²⁰⁾. It has been well documented about the role of RA in the MMC and first arch patterning during the early avian development.

In the morphological analysis of RA treated embryos, we could find the phenotypical and skeletal changes in MMC as well as maxillary and mandibular process (Fig 2G-I, Fig. 3G-I). And these areas of morphological changes matched well with Barx-1 expression pattern, as a well known downstream target gene of Fgf8. More extended Barx-1 expression at the MMC and less expression at the proximal mandibular process were evident at H-H stage 24 (Fig 5G). So we could assume that the ectopic RA inhibited the Barx-1 expression at MMC to have more extended expression domain. And it resulted in the aberrant skeletal patterning of proximal maxillary and mandibular bones.

We could prove the cooperation or connection of RA and Erk signaling for MMC and first arch patterning. First, their connection is deduced from the fact that Erk is a key candidate for the observed response to RA. The treatment with RA can regulate Erk-1/2 activity via transcriptional changes in mouse embryonic stem cells and human leukemia cells^{55,56)}. In addition, Raf activation depends on MEK, RARa, and RXR activation. RA signaling thus causes sequential MEK, ERK2 and then RAF activation⁵⁶⁾.

RA has been demonstrated to augment EGF signaling by the increase of the expression of the EGF receptor⁵⁷⁾ or to elevate the binding efficiency of the EGF receptor⁵⁸⁾. And RA is also known to interact with G-protein cascades at many levels. The Gs and Gi classes of G-proteins are known regulators of cell proliferation via Erk-1/2^{51,59,60)}. RA induces a decrease in mesenchymal cell proliferation, likely mediated via Gi G-proteins and Erk-1/2.

The embryos treated with the double RA and PD98059 showed the enhanced morphological and skeletal pattern changes, as compared with the single RA or PD98059 treatments. They showed that the proximal part of maxillary and mandibular bones were missing or deformed as seen in Fig 3J and their early external shapes matched well with the skeletal changes, showing the shorter MMC with the promi-

ment depression around the beads at the mandibular arch and its resultant extra-prominence. In addition, this proximal part of maxillary and mandibular process, where the bones were severely missing or deformed, matches well with that region of less or none expression of Barx-1 of the same region as seen Fig 5H.

From these results, we could observe that Erk and RA signaling is individually and synergistically involved in the facial patterning in terms of FGF signaling pathway via Barx-1. We could also confirm that they work in a line of signaling to have a MMC patterning through Barx-1. So the segregation as well as the patterning of maxillary and mandibular structure can be influenced by MMC and the abnormality in MMC brought by aberrant Fgf signaling may result in the disturbances of maxillary-mandibular segregation, similar to syngnathia. However it is not yet clear in this study that which signaling pathway is exactly laid for RA and Erk in the MMC patterning. Further studies are anticipated to make this point clear.

V. CONCLUSION

In vertebrates, the face is mainly formed by neural crest derived neural crest cells and they are inbuilt by complex programs as well as by the environmental factors. Extracellular signaling-regulated kinase (Erk) is one of such programs to regulate the various cellular functions including the cell proliferation, survival, and differentiation.

And retinoic acid (RA), which is known to be one of the development morphogen, plays an important role as a regulator in differentiation process at various stages of vertebrate embryogenesis. In addition to the fact that each Erk and RA signaling is involved in the facial patterning in terms of Fgf signaling pathway, they may cooperatively work in a line of signaling to enhance the developmental process, especially at MMC of the first pharyngeal arch to segregate and sustain the maxillary and mandibular development.

By the morphological, skeletal, proliferating and apoptotic cell, and in-situ analysis, we checked the effects of changed Erk and/or RA signaling to observe that Erk and RA signaling is individually involved in

the facial patterning in terms of FGF signaling pathway via Barx-1. And RA and ERK signaling work together or are closely related in the MMC patterning, which is important for the segregation of Mx-Md, by controlling the Fgf related Barx-1 expressions. So RA and Erk signaling work together for the MMC patterning and the segregation of Mx-Md development by controlling the Fgf and its downstream signaling pathways.

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