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หนูปกติและหนู HOMOZYGOUS RSEY ที่มีฟันตัดบนเกิน

นางสาวรุ่งอรุณ เกรียงไกร

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

COMPARISON OF GENE EXPRESSION RELATED TO MAXILLARY INCISOR DEVELOPMENT BETWEEN THE WILD TYPE AND THE HOMOZYGOUS RSEY RATS WITH MAXILLARY SUPERNUMERARY INCISORS

Miss Rungarun Kriangkrai

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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สถาบันวิทยบริการ
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รุ่งอรุณ เกรียงไกร: การเปรียบเทียบการแสดงออกของยีนที่เกี่ยวข้องกับกระบวน การสร้างฟันตัดบนระหว่างหนูปกติและหนู HOMOZYGOUS RSEY ที่มีฟันตัดบนเกิน (COMPARISON OF GENE EXPRESSION RELATED TO MAXILLARY INCISOR DEVELOPMENT BETWEEN THE WILD TYPE AND THE HOMOZYGOUS RSEY RATS WITH MAXILLARY SUPERNUMERARY INCISORS) อ. ที่ปรึกษา: ผศ. ทญ. ดร. สุคนธา เจริญวิทย์ อ. ที่ปรึกษาร่วม: ผศ. ทญ. ดร. ซาชิโกะ อิเซกิ, 113 หน้า
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การศึกษานี้เป็นการศึกษาการสร้างฟันตัดบนของหนูแรทสายพันธุ์เอสดี (SD Sprague-Dawley) และหนูกลายพันธุ์ small eye (*rSey*) *rSey* เป็นการกลายพันธุ์ที่แสดงลักษณะเด่นในยีน *Pax-6* การกลายพันธุ์นี้ทำให้เกิดความบกพร่องของการอพยพของนิวรัลเครสเซลล์กลุ่มที่เกิดจากสมองส่วนกลางส่วนหน้าและเกิดความผิดปกติของการสร้างระบบประสาทส่วนกลาง ความบกพร่องนี้ทำให้หนูกลายพันธุ์โฮโมไซโกสไม่มีตา ไม่มีจมูก เกิดรอยแหว่งบนใบหน้าและมีฟันตัดบนเกินจากปกติ ผลจากการศึกษานี้ได้จากการศึกษาทางจุลกายวิภาคศาสตร์และการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการสร้างฟันในระยะเริ่มต้นโดยเทคนิคอินซิตูไฮบริไดเซชั่นที่ตัดจากส่วนศีรษะของตัวอ่อนของหนูปกติเอสดีและหนูกลายพันธุ์โฮโมไซโกสระยะ 12 วัน ถึง 15 วันหลังจากปฏิสนธิ นอกจากนี้เรายังศึกษาคุณลักษณะของฟันตัดบนเกิน โดยการศึกษาทางจุลกายวิภาคศาสตร์ในตัวอ่อนหนูกลายพันธุ์โฮโมไซโกสระยะ 20 วัน จำนวน 20 ตัวอย่าง พบว่าในหนูปกติการสร้างฟันตัดบนในระยะเริ่มต้นประมาณอายุ 13 วัน ถึง 14 วัน เกิดจากการเชื่อมกันของหน่อฟันเริ่มต้นซึ่งเราเรียกว่าหน่อฟันเริ่มต้นใกล้กลางและหน่อฟันเริ่มต้นด้านข้าง หน่อฟันที่เชื่อมกันแล้วนี้จะกลายเป็นหน่อฟันระยะบัด ณ ตัวอ่อนอายุ 15 วัน ส่วนในหนูกลายพันธุ์โฮโมไซโกสนั้นหน่อฟันเริ่มต้นใกล้กลาง และหน่อฟันเริ่มต้นด้านข้างจะยังคงสภาพที่แยกกันไม่มีการเชื่อมกัน ต่อมาหน่อฟันเริ่มต้นใกล้กลางจะเริ่มแบ่งตัวและสร้างเป็นฟันตัดบน ขณะที่หน่อฟันเริ่มต้นด้านข้างเริ่มแบ่งตัวและประมาณ 25% จะกลายเป็นฟันเกินที่มีรูปร่างคล้ายฟันตัดบน และประมาณ 75% จะแบ่งตัวและหยุดการเจริญอยู่ที่หน่อฟันระยะบัด การเชื่อมกันของหน่อฟันเริ่มต้นนั้นเป็นขณะเดียวกับที่เกิดการเชื่อมกันของใบหน้าซึ่งในตัวอ่อนของหนูกลายพันธุ์โฮโมไซโกสการเชื่อมกันของใบหน้านี้ไม่เกิดขึ้น ผลการศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการสร้างฟันในระยะเริ่มต้นโดยเทคนิคอินซิตูไฮบริไดเซชั่น ได้แก่ *Fgf-8, Pitx-2, Shh, Bmp-4, Msx-1* และ *Pax-9* พบว่ายีนเหล่านี้แสดงออกในระยะเริ่มสร้างฟันตัดบน การแสดงออกของยีนเหล่านี้ก็พบได้ในหน่อฟันเริ่มต้นใกล้กลางและหน่อฟันเริ่มต้นด้านข้างของหนูกลายพันธุ์โฮโมไซโกส จากผลที่ได้จากการศึกษานี้แสดงว่า การกลายพันธุ์ของยีน *Pax-6* ในตัวอ่อนของหนูกลายพันธุ์โฮโมไซโกสนั้นไม่ได้เกี่ยวข้องกับการเกิดฟันเกินที่มีรูปร่างคล้ายฟันตัดหน้าบนโดยผ่านการควบคุมยีน แต่น่าจะเกิดจากการที่มีความผิดปกติของการเชื่อมกันของใบหน้าขณะที่สร้างใบหน้าและการที่ใบหน้าไม่เชื่อมกันนี้ทำให้หน่อฟันเริ่มต้นไม่เชื่อมกันและสามารถสร้างฟันตัดบนและฟันเกินที่มีรูปร่างคล้ายฟันตัดบน

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KEY WORDS: RSEY/ MAXILLARY INCISOR/ SUPERNUMERARY INCISOR

RUNGARUN KRIANGKRAI: COMPARISON OF GENE EXPRESSION RELATED TO MAXILLARY INCISOR DEVELOPMENT BETWEEN THE WILD TYPE AND THE HOMOZYGOUS RSEY RATS WITH MAXILLARY SUPERNUMERARY INCISORS: THESIS ADVISOR: ASST. PROF. SUCONTA CHAREONVIT, Ph.D., THESIS CO-ADVISOR: ASST. PROF. SACHIKO ISEKI, Ph.D., 113 pp.
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This study aims to investigate the developmental process of the maxillary incisor tooth in the wild type SD and in the homozygous rat small eye. Rat small eyes; *rSey*; is inherited a dominant mutation in *Pax-6* gene. The mutation leads to impaired migration of anterior midbrain neural crest cells as well as the developmental defects of central nervous system, resulting in lack of eyes and nose, facial cleft and excess maxillary incisor-like structure in the homozygote. Histological observation of serial head sections and whole mount in situ hybridisation of the genes involved in early tooth development were carried out between E12 and E15 wild type and homozygous embryos. The supernumerary incisor-like structure was characterized by histological analysis of twenty E20 homozygous *rSey* fetuses. In wild type, the initiation of maxillary incisor formation is composed of the fusion of the medial and lateral primary dental placodes (M-PDP and L-PDP) at E13-14 and it develops into bud stage of incisor at E15. In the *rSey* homozygous fetus, the fusion of PDPs does not take place and they remain separated. Eventually, M-PDP starts invagination and proceeds incisor development. L-PDP also starts invagination, then 25% of them develop incisor-like structures while the rests are arrested at bud stage. The PDPs fusion is concomitant with the fusion of facial primodia, which is interrupted in the homozygote. Whole mount in situ hybridization showed that the genes involved in early tooth development (*Fgf-8, Pitx-2, Shh, Bmp-4, Msx-1* and *Pax-9*) are expressed in wild type maxillary incisor at the initiation stage. This expression pattern is also seen in homozygous M-PDP and L-PDP. These results suggest that *Pax-6* mutated in homozygous *rSey* is not associated to the incidence of supernumerary incisor-like structure via gene controlling but rather via the failure of the fusion of facial primodia to form midface in the homozygote. The failure of the fusion leaves the potentiality to develop tooth of the separated PDPs and allows them to develop incisor as well as the supernumerary incisor-like teeth.

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# ABBREVIATIONS

| | |
|---|---|
| ***Barx-1*** | barh-like1 homeobox gene |
| **Bmps** | bone morphogenetic proteins |
| **Bmp-4** | bone morphogenetic protein-4 protein |
| ***Bmp-2*** | bone morphogenetic protein-2 gene |
| ***Bmp-4*** | bone morphogenetic protein-4 gene |
| ***C. elegans*** | Caenorhabditis elegans |
| **Ci** | cubitus interruptus |
| ***Dlx*** | distal-less gene |
| **E12** | embryonic day 12 |
| **FNP** | frontonasal process |
| **FB** | forebrain |
| **Fgfs** | fibroblast growth factors |
| **Fgf-8** | fibroblast growth factor-8 protein |
| ***Fgf-3*(int-2)** | fibroblast growth factor-3 (integration site-2) gene |
| ***Fgf-4* (hst, kFgf)** | fibroblast growth factor-4 (heparin secretory transforming protein, kaposi sarcoma oncogene fibroblast growth factor) gene |
| ***Fgf-7*(KGF)** | fibroblast growth factor-7 (keratinocyte growth factor) gene |
| ***Fgf-8* (AIGF)** | fibroblast growth factor-8 (androgen-induced growth factor) gene |
| **Fhfs** | fibroblast growth factors homologous factors |
| ***Hox*** | homeobox gene |
| **L-DP** | lateral dental placode |
| ***Lef-1*** | lymphoid enhancer binding factor-1 gene |
| **L-PDP** | lateral primary dental placode |
| **LNP** | lateral nasal process |
| ***lacZ*** | beta-galactosidase |
| **MdP** | mandibular process |
| **MNP** | medial nasal process |
| **MB** | midbrain |
| **M-PDP** | medial primary dental placode |
| **MxP** | maxillary process |
| ***msh*** | muscle-segment homeobox gene |
| ***Msx*** | msh homeobox homolog *Drosophila* gene |
| **NBT/BCIP** | nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate |
| ***Pax-6*** | paired box 6 gene |
| ***Pax-9*** | paired box 9 gene |
| ***Pitx*** | pituitary homeobox gene |
| **proRhA** | prorhombomere A |
| **proRhB** | prorhombomere B |
| **proRhC** | prorhombomere C |
| **proRhD** | prorhombomere D |
| **Ptc** | patched protein |
| ***rSey*** | a mutant rat small eye |
| **SD-rat** | Sprague-Dawley rat |
| ***Shh*** | sonic hedgehog gene |
| **Smo** | smoothened protein |

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**TGF-beta** transforming growth factor-beta

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# CHAPTER I

# INTRODUCTION

Tooth develops from reciprocal interactions between oral epithelium and ectomesenchyme. It has been demonstrated that both oral epithelium and ectomesenchyme have the potentiality to pre-assign the type of tooth formation. Maxillary incisor develops in the middle part of the upper jaw derived from the fusion of frontonasal process (FNP) and maxillary process (MxP). The dental epithelial origin of the maxillary incisor is different from the other teeth and the ectomesenchyme of FNP is also populated by different group of cranial neural crest cells to those of the other teeth. The developmental process as well as its genetic controlling of the other teeth especially the mandibular molar has been well studied, however, those of the maxillary incisor have not yet been well defined. In this study, we first characterize the initiation stage of developmental process of maxillary in the SD-rat by histological analysis. The genetic controlling of maxillary incisor development is carried out by analyzing the tooth related gene expression patterns at the stage prior to the dental placode formation to the early bud stage. Second, we study maxillary incisor development associated with the supernumerary incisor formation in the mutant rat with small eye or *rSey*. Besides the supernumerary incisor formation, the homozygous *rSey* has facial cleft due to the defect of facial process fusion. Histological analysis and tooth related gene expression patterns are studied in the homozygous *rSey* comparing to the

wild type to elucidate how the maxillary incisor develops and what might be involved in the formation of the supernumerary incisor.

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## 1.1 Tooth development

### 1.1.1 Tooth morphogenesis

Teeth are highly special structures developed in the jaw. In the early mammalian embryo development, the face and jaw are ultimately derived from the fusion of the three facial processes, the frontonasal process (FNP), and two first branchial arch derivatives, maxillary and mandibular processes (MxP and MdP, respectively). The FNP gives rise to the medial nasal process (MNP) and lateral nasal process (LNP) which consequently develop to the nose. The MxP fuses to the FNP to form the upper jaw while the bilateral mandibular processes (MdP) join to form the lower jaw. Thus, teeth are initiated in the oral epithelium covering the FNP, MxP and MdP. Subsequently, the oral epithelia interact with the underlying mesenchyme in the upper and lower jaws to form teeth.

There are various types of teeth developed in mammalian jaws and we can characterize them into families according to their specific locations in jaws. In the mammalian dental formula, there are four tooth families including three incisors, one canine, four premolars and three molars in each quarter of the jaws. The phylogenic reduction in the number of teeth is found in the rodent. Mouse and rat have only two tooth families; incisor and molar. One incisor and three molars develop in the front and the back of each quarter of jaws separated by the region corresponding to presumptive cuspid and premolar formation (Peterkova et al., 1995).

Mice have been mostly used in studies of tooth development, especially mandibular molar tooth. The mandibular molar develops via a series of reciprocal interaction between the oral epithelium and underlying ectomesenchyme derived from the first branchial arch. There are three stages on the morphological change of mouse mandibular molar formation characterized by the histological analysis. The first stage is an initiation stage that occurs at approximately embryonic day 10.0-11.0 (E10.0-11.0) with the formation of localized thickening of the oral epithelium to form the dental placode in the dental arch of upper and lower jaws. Histology of the dental placode shows that thickening of the oral epithelium is formed by high basal cylindrical cells and several layers of flat superficial cells facing oral cavity (Peterkova et al., 1993). The second stage of tooth development is morphogenesis including bud and cap stage, the dental placode undergoes localized proliferation into underlying mesenchyme to form tooth bud while the underlying mesenchymal cells accumulate around the bud to form tooth germs at E12.0-E13.0. The tooth germ then progresses onto the cap stage at E13.0-E15.0. The dental epithelial compartment forms dental organ and the underlying mesenchyme forms dental papilla. At this stage, stellate reticulum appears between the cells localized in the central of the dental organ, while the peripheral cells are now defines as outer and inner enamel epithelium. Some part of epithelial cells in dental organ develops into enamel knot; signaling center which directs further tooth development. At E15.0-E17.0, the tooth germ develops into bell stage when the beginning of the cusps and outline of the future dentino-enamel junction are formed. Cytodifferentiation stage of tooth development begins at E17.0 onwards. The inner enamel epithelium differentiates into enamel-secreting ameloblasts to form the crown,

while the dental papilla provides pulp and dentine-producing odontoblasts aligning themselves opposite to the ameloblasts (Jernvall et al., 1994; Thesleff et al., 1996; Vaahtokari et al., 1996).

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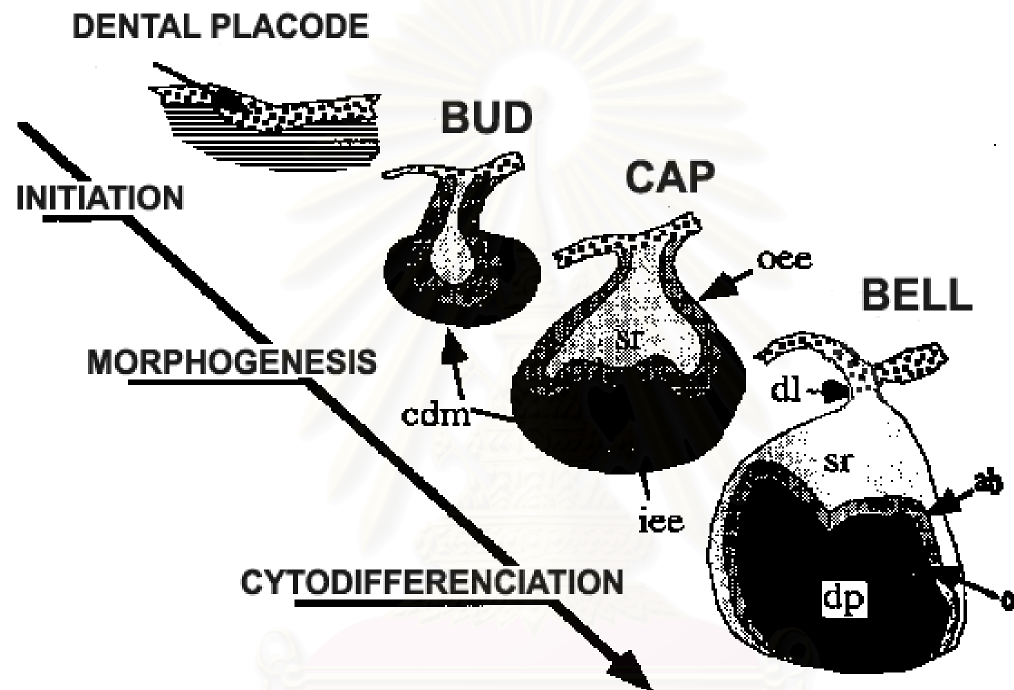


Figure 1.1 Tooth development including of initiation stage shows dental placode formation, morphogenesis stage shows bud and cap stage and cytodifferenciation stage shows ameloblast and odontoblast formation. Abbreviations: cdm, condensing dental mesenchyme; sr, stellate reticulum; iee, inner enamel epithelium; oee, outer enamel epithelium ; ab, ameloblast; ob, odontoblast; dl, dental lingual cord; dp, dental papilla ( adapted from Thesleff et al., 1996).

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### 1.1.2 The cranial neural crest cells involved in tooth development

During craniofacial development, the cranial neural crest cells are released from the fold of neural ectoderm and migrate ventrolaterally to populate and contribute to the formation of frontonasal mass, first branchial arch and the cranial ganglia (Nichols, 1981, 1986; Tan and Morriss-Kay, 1986). Cranial neural crest cells emigrated from forebrain and anterior midbrain contribute to the formation of frontonasal process (FNP) which will develop into anterior facial primodium. The rostral most region of the forebrain does not contribute to the neural crest cell population, but yields the neural head epithelium, including oral epithelium, nasal epithelium, nasal placode and Rathkes's pouch (Couly and Le Douarin, 1985, 1987; Osumi-Yamashita et al., 1994). The cranial neural crest cells emigrating from midbrain and anterior hindbrain migrate to the first branchial arch, which will later develop into the maxillary and mandibular processes (MxP and MdP, respectively) (Matsuo et al., 1993). This behavior suggests the distinct groups of cranial crest cells populated in the ectomesenchyme of FNP (forebrain and anterior midbrain) and the first branchial arch (midbrain and anterior hindbrain).

After cranial neural crest cells migrate to FNP and first branchial arch. The face itself is formed by the fusion of three processes, FNP, MxP, and MdP. The FNP generates MNP and LNP to form nose starting by the invagination of nasal placodes in FNP at E9.0 of mouse embryo (Trasler, 1968). MxP incorporates with MNP and LNP to form mid-face and develop into upper jaw, while MdP itself forms the lower jaw.

Murine tooth development is occurred via a series of the epithelial-mesenchymal interaction between the oral epithelium and the cranial neural crest cells-derived ectomesenchyme in the upper and lower jaws (Tucker and Sharpe, 1999). The fact that ectomesenchyme is derived from the cranial neural crest and participates in the process of mandibular molar tooth development is fully confirmed. Imai et al. show that midbrain crest cells migrate to mandibular process and contribute to development of mandibular molar tooth in rat embryos (Imai et al., 1996). Focal labeling in rat embryos indicates that posterior midbrain crest cells emigrating by the end of the 4-somite stage predominantly migrate to the region where tooth buds normally develop. Moreover, they show that posterior midbrain crest cells contribute to dental mesenchyme of mandibular tooth bud in long-term culture system. Recently, the genetic marker has been used to follow neural crest cells migration and differentiation in the mouse. Transgenic mice generated by conditional gene knock out and exhibit ubiquitous *lacZ* reporter gene expression in the neural crest precursors, have allowed the method of staining and therefore identification of these cells at the later stages of the development. This has clearly demonstrated that cranial neural crest cells-derived ectomesenchyme contribute to the formation of the condensed dental mesenchyme at the initial bud stage and subsequently to formation of the dental papilla and follicle. In addition, the analysis of six week old mice demonstrates definitively that cranial neural crest cells give rise to odontoblast, dental matrix, pulp tissue, cementum and periodontal ligaments of teeth in the adult mouse dentition while dental epithelium component gives rise only to ameloblast (Chai et al., 2000).

### 1.1.3 Signals from dental epithelial-mesenchymal interaction determine presumptive tooth site and tooth type formation in the mandible

There are various signaling molecules produced by the reciprocal interaction between oral epithelium and underlying ectomesenchyme during tooth morphogenesis. Interestingly, prior to any morphological sign of tooth morphogenesis in the mandible, the oral epithelium signaling induces the expression of transcription factors in underlying ectomesenchyme, particularly the homeobox genes that possess an essential role to identify the tooth site and tooth type formation.

Bone morphogenetic proteins (Bmps) are growth factors belonging to the family of transforming growth factor-beta (TGF-beta). *Bmp-4* expression is localized on the oral epithelium and induces expression of the homeobox gene; *Msx-1*; in the underlying ectomesenchyme at distal region of mouse mandibular arch. At proximal region the oral epithelium expresses *Fgf-8* and the expression induces another homeobox gene; *Barx-1*; in the proximal ectomesenchyme. The boundary between *Msx-1*and *Barx-1* is found to correspond to the junction between the expression of *Fgf-8* and *Bmp-4* as shown in Figure 1.2. It has been shown that Fgf-8 beads can induce *Barx-1* expression in the ectomesenchyme of the proximal region when the epithelium has been removed whereas the beads fail to induce *Barx-1* expression in the distal mesenchyme that covered by epithelium expressed *Bmp-4*. In addition, Bmp-4 beads implanted into intact mandible inhibit the endogenous expression of *Barx-1*. These results demonstrate that the restriction of *Barx-1* expression is regulated by epithelial *Bmp-4* expression. This effect is not through the activation of *Msx-1* expression, which is supported by the

investigation of *Barx-1* expression in *Msx-1* mutant mice. No change in the medial-lateral boundary of *Barx-1* is detected in these mutant mice. The inhibition of Bmp-4 signaling early in mandibular development by the action of exogenous Noggin protein results in ectopic *Barx-1* expression in the distal presumptive incisor mesenchyme and changes the tooth identity from incisor to molar. These results show that there are different signaling pathways of epithelial-mesenchymal interactions in presumptive incisor and molar region and the responding of ectomesenchyme pre-assigns the type of tooth prior to the tooth morphogenesis in the mouse mandible. The expression of *Barx-1* induced by epithelial *Fgf-8* and restricted by the epithelial *Bmp-4* marks the presumptive molar tooth whereas the incisor tooth is marked by the mesenchymal expression of *Msx-1* induced by the epithelial expression of *Bmp-4* (Tucker, 1998).

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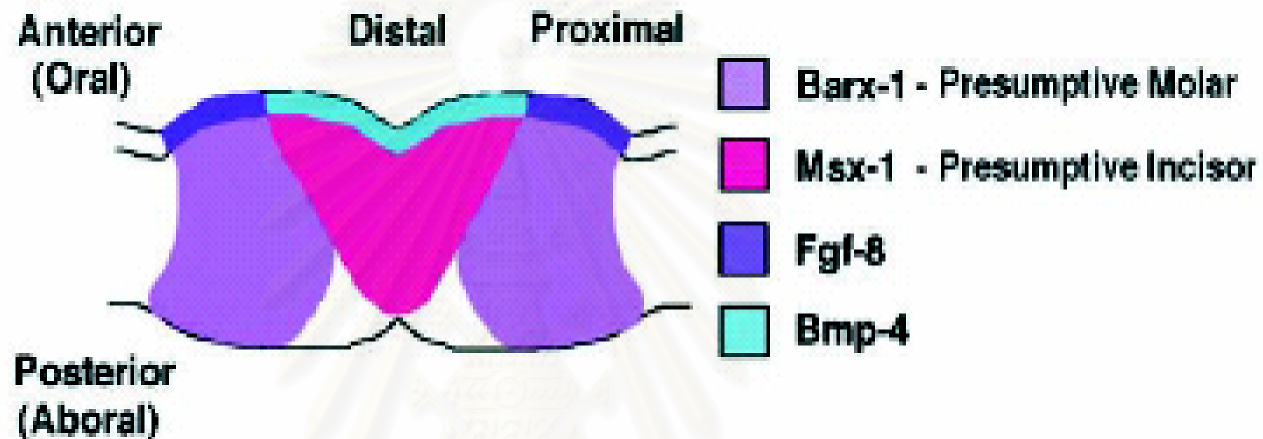


Figure 1.2 Schematic representation of the expression domain of *Barx-1*, *Msx-1*, *Bmp-4* and *Fgf-8* at the early development prior to dental placode formation in the mandibular arch. *Barx-1* and *Msx-1* are expressed in the ectomesenchyme indicated presumptive molar and incisor, respectively. The boundary between *Barx-1* and *Msx-1* expression is found to correspond to the junction between *Fgf-8* and *Bmp-4* expression in the overlying oral epithelium. Epithelial *Bmp-4* and *Fgf-8* expression are to induce *Msx-1* and *Barx-1*expression, respectively. *Bmp-4* is found to have the inhibitory effect on the endogenous ectomesenchymal *Barx-1* expression at the distal region that *Msx-1* is expressed to proceed incisor (Tucker et al., 1998).

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*Pax-9* expression is first identified in ectomesenchyme of mandible around E10.5, which is the stage prior to the thickening of the dental epithelium. At E10.0 to E10.5, only two lateral patches of *Pax-9* expressing cells are detected in the presumptive molar tooth region. The earlier onset of *Pax-9* expression in the molar domains is consistent with studies demonstrating that initiation of molar tooth development precedes that of the incisors (Ruch, 1984; Lumsden and Buchanaan, 1986). Furthermore, the separation of the *Pax-9*-positive prospective molar and incisor mesenchyme by a region in which *Pax-9* is expressed at a lower level is consistent with morphological studies suggesting that molars and incisors develop from separate field (Gaunt, 1964). *Pax-9* expression is induced and regulated by signal from overlying dental epithelium. *Fgf-8* induces the expression of *Pax-9* whereas *Bmp-4* and *Bmp-2* inhibit. Thus, the antagonistic signals of *Fgf-8* and *Bmp-4/2* are part of the molecular machinery for positioning the sites of tooth formation (Figure 1.3).

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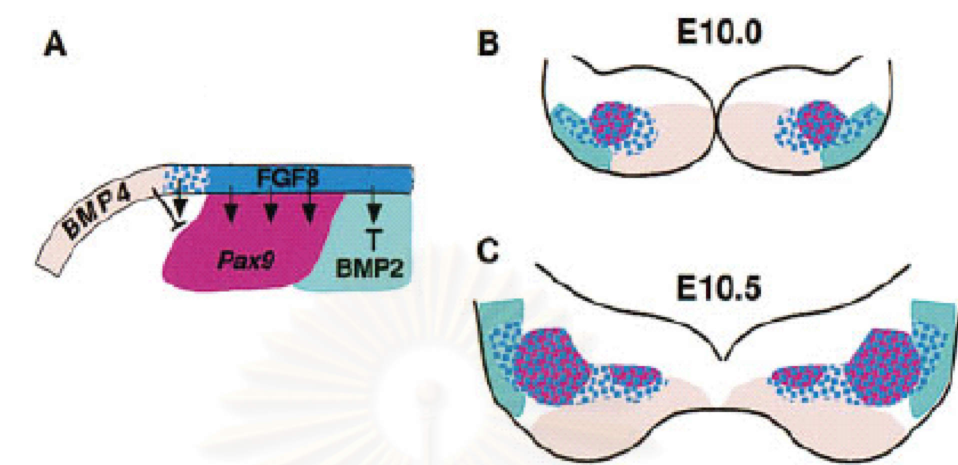


Figure 1.3 A mechanism for positioning the sites of tooth formation

(A) Proposed roles of Fgf-8 and Bmp-4 signaling in defining the sites of *Pax-9* expression, an early marker of tooth formation. Fgf-8 produced in the cranial mandibular ectoderm induces *Pax-9* expression in the underlying mesenchyme. Bmp-2 and Bmp-4 prevent this induction. The net result is that *Pax-9* expression is induced only in the regions where Fgf-8 is present and Bmp-4 is not.

(B and C) Schematic diagrams illustrating the expression domains of *Fgf-8, Bmp-2, Bmp-4* and *Pax-9* at E10.0, the stage at which *Pax-9* is first induced in the prospective molar mesenchyme, and at E10.5, when *Pax-9* expression is the first induced in prospective incisor mesenchyme (Neubuser et al., 1997).

**1.1.4 Different ectomesenchymal homeobox genes responding to the oral epithelial induction in the maxilla and mandible**

Distal-less genes incorporate a six-gene family of mammalian homeobox genes (*Dlx 1, 2, 3, 5, 6* and *7*) that also exhibit highly nested domains of the expression in the branchial arches during early development (Bulfone et al., 1993; Qiu et al., 1997; Depew et al., 1999). Within the mammalian genome, these genes are arranged in the convergent pairs, with each pair having similar domains of expression (*Dlx1/2; Dlx3/7; Dlx 5/6*) (Qiu et al., 1997). In particular, along the rostral-caudal axis of the branchial arches *Dlx1/2* are expressed more-or-less continuously, whilst the expression domain of *Dlx3/7* and *Dlx5/6* are found to be progressively more restricted in the caudal direction (Bulfone et al., 1993; Qiu et al., 1997; Depew et al., 1999). In the first branchial derivative, *Dlx1/2* are similarly expressed in the epithelial mesenchymal cells of both the maxilla and mandible. *Dlx 5/6* are expressed in the nest manner in the mandible, but not in the maxilla. Targeted disruption of *Dlx1/2* in the transgenic mice results in absence of maxillary molar teeth but retain all other teeth. In the absence of *Dlx1/2*, maxillary ectomesenchyme is re-programmed to chondrogenic fate (Qiu et al., 1997; Thomas et al., 1998). The evidences suggest that the restricted expression patterns of the homeobox gene in the ectomesenchyme of the first branchial arch derivative are responsible for patterning of the dentition. This leads to the question of how these domains are established. It might be established from the neural crest cells prior to their arrival in the first branchial arch or might be resulted from the interaction of the cells with the oral ectoderm after their migration. Early embryonic tissue recombination

experiments between E9.0 and E12.0 have hinted the latter hypothesis that oral epithelium of the mandibular arch when combined with the ectomesenchyme that does not normally participate in the tooth development can induce tooth formation. In contrast, recombinants between the non-oral epithelium and mandibular ectomesenchyme participating in the tooth development do not undergo the tooth morphogenesis (Mina and Kollar, 1987 a,b; Lumsden, 1988). These data indicate that tooth formation is initiated by the signals produced from the mandibular oral epithelium. The underlying ectomesenchyme has not yet been specified for the tooth development earlier than E12.0. By E12.0 onward, the oral epithelium has apparently lost of the ability to induce the tooth formation, in contrast and the ability shifts to the underlying ectomesenchyme. When specified for tooth formation, the ectomesenchyme can induce non-oral epithelium to undergo tooth formation and differentiation into ameloblasts (Kollar and Baired, 1970 a,b ; Mina and Kollar, 1987; Lumsden, 1988). The results also suggest that the ability to participate in odontogenesis is not an exclusive feature of the neural crest cells that migrated into the first branchial arch.

Prior to the first morphological sign of tooth formation, removal of the oral epithelium leads to rapid loss of almost all ectomesenchymal homeobox gene expression (Ferguson et al., 2000). Importantly, addition of exogenous Fgf-8 onto these explants can restore the expression of many of these genes, consistent with the finding that Fgf-8 is one of the signals responsible for patterning the early mandible (Ferguson et al., 2000). However, this response of mandibular ectomesenchyme to Fgf-8 is highly dynamic. Prior to E10, all mandibular ectomesenchyme equally competent to respond and homeobox genes can be ectopically induced in close proximity to the source of Fgf-

8. By E10.5, whilst removal of the oral epithelium still results in downregulation of homeobox gene expression in isolated ectomesenchyme, addition of exogenous Fgf-8 can only restore gene expression in the original domains. However, removal of oral epithelium at E11.0 onward does not effect gene expression, at this stage the ectomesenchymal expression domain are established and importantly independent to the oral epithelial signals (Ferguson et al., 2000). These findings have ultimately provided a molecular explanation for the results of previous recombination experiments. Thus, in the mouse prior to the bud stage the oral ectoderm is able to induce odontogenesis and determine tooth type (Miller, 1969; Lumsden et al., 1988; Kollar and Mina, 1991). However, the recombination of the dental tissue taken from later stages of development has demonstrated that after bud stage, the necessary information required for determination of tooth shape resides in the ectomesenchyme of the dental papilla (Kollar and Baired, 1969). These findings suggest that the maxillary and mandibular ectomesenchyme respond differently to oral epithelial signaling for certain genetic pathways (Ferguson et al., 2000). Fgf-8 can induce the expression of *Dlx2* and *Dlx5* in isolated mandibular ectomesenchyme, but only *Dlx2* can be induced in the maxillary ectomesenchyme. These observations are consistent with the observed expression patterns of these two genes, *Dlx 2* is expressed in the ectomesenchyme of the maxillary and mandibular processes whereas *Dlx 5* is essentially expressed in only mandibular ectomesenchyme (Qiu et al., 1997). However, maxillary epithelium is capable of inducing *Dlx 5* expression in mandibular arch ectomesenchyme, implying that the ectomesenchyme of maxillary and mandibular primodia behave fundamentally differently to each other. Further, reciprocal transplantation experiments demonstrate

that between E9.5 and E10.25 the ectomesenchyme of the mandible and maxilla do not take on the expression characteristic of their host, they appear to be intrinsically different in their response to oral epithelial signaling. These finding invite speculation as to how a seemingly homogenous population of cranial neural crest cells that migrate into the maxillary and mandibular primordia acquire subtle difference in their ability to respond to instructive signals from the oral epithelium (Ferguson et al., 2000). Clearly, some degree of pre-patterning may be present in the neural crest cells.

Previous studies have demonstrated a special inductive potential of the oral epithelium to initiate tooth formation that interacts with the ectomesenchymal tissue during the tooth development. There are different signaling pathways of epithelial-mesenchymal interactions in presumptive incisor and molar region and the responding of ectomesenchyme cells pre-assigns the type of tooth prior to the tooth morphogenesis in the mouse mandibular. Thus, prior to the bud stage, mouse oral epithelium is able to induce odontogenesis and determine tooth type. The restricted patterns of the homeobox gene expression in the ectomesenchyme of mandible are responsible for the tooth patterning. Moreover, the maxillary and mandibular ectomesenchyme are different in their ability to respond to instructive signals from the oral epithelium for certain genetic pathways in the molar tooth formation. These findings suggest that the homogenous population of cranial neural crest cells migrating into the maxillary and mandibular primordia presents a different pre-patterning in homoeobox gene responding to oral epithelial signals to develop teeth. Thus, the oral epithelium and underlying ectomesenchyme derived from the FNP, MxP and MdP might have different genetic controlling pathways to determine the tooth type in the upper and lower jaws.

Since maxillary incisor develops from fusion of FNP and MxP. The dental epithelial origin of the maxillary incisor is not derived from the first branchial arch and different group of cranial neural crest cells populated the ectomesenchyme of FNP. Thus, the tooth morphogenesis and genetic controlling in the maxillary incisor might differ from the other teeth, which require further investigation.

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### 1.1.5 The tooth related gene expression patterns in mandibular molar tooth morphogenesis

#### Bone morphogenetic proteins 4 (*Bmp-4*)

Bone morphogenetic proteins (Bmps) are growth factors belonging to the family of transforming growth factor-beta (TGF-beta). Bmps regulate bone and cartilage development and individual Bmps have been shown to contribute to the shaping of various bone and skeletal elements. Bmps regulate bone and dentin formation, and they have therapeutic potentiality in reparative osteogenesis and odontogenesis. Bmps also act as inductive signals between tissue layers in the embryo and regulate the expression of several transcriptional factors, including homeobox-containing genes. Bmp-4 has been identified as an epithelial inductive signal in tooth development. As it is produced by early dental epithelium and regulates tooth specific gene expression in the dental mesenchyme, such as *Msx-1* demonstrating its important signal for the initiation of tooth development.

At initiation stage, the expression of *Bmp-4* is localized in dental placode, dental mesenchyme and oral epithelium. At this stage, epithelial *Bmp-4* expression induces the mesenchyme to express *Msx-1*, *Msx-2* and *Dlx-2* (Bei et al., 1998). Epithelial *Bmp-4* expression decreases at bud stage and it shifts from dental epithelium to dental mesenchyme. At this stage, Bmp-4 epithelial signaling can induce *Msx-1* and its own expression in the dental mesenchyme (Vainio et al., 1993). Moreover, the present of *Bmp-4* expression in the dental mesenchyme requires the function of mesenchymal

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*Msx-1* that is demonstrated by the deletion of *Msx-1* and *Bmp-4* expression in the *Msx-1* mutant mice. *Bmp-4* expression in dental mesenchyme is reduced in the *Msx-1* mutant and recombinant Bmp-4 cannot induce its own expression in the mutant mice. However, the epithelial *Bmp-4* expression is preserved in the *Msx-1* mutant epithelium (Chen et al., 1996). These results indicate that *Msx-1* is required for the expression of *Bmp-4* in the dental mesenchyme, therefore *Bmp-4* functions downstream of *Msx-1* in the dental mesenchyme. On the controversy, epithelial *Bmp-4* expression is not required *Msx-1* for its expression and act upstream of *Msx-1* in oral epithelium.

The transitional *Bmp-4* expression from dental epithelium to underlying mesenchyme at this stage is correlated to the transition of tooth formation inductive potentiality from dental epithelium to dental mesenchyme, indicating that *Bmp-4* may constitute one component of the odontogenic potentiality. Indeed, *Bmp-4* in the dental mesenchyme can induce the morphological change and the expression of a number of genes including the transcription factor *Msx-1, Dlx-2, Lef-1* and *Bmp-4* itself (Vaino, 1993; Bei et al., 1998; Chen, 1996; Kratochwil, 1996), thus mimicking an effect on the early dental epithelium (Figure 1.4). The expression of *Bmp-4* in the mesenchyme is believed to exert its function upon the dental epithelium as a feedback signal to further the tooth development (Chen et al., 1996). This is demonstrated by the ability of the exogenous *Bmp-4* that can partially rescue the tooth development from bud to cap stage in *Msx1* mutant mice, which have the arrested tooth development at the bud stage (Satokata and Maas, 1994; Chen et al., 1996). At the cap stage, the expression of *Bmp-4* is observed in the enamel knot, dental papilla and dental sac but not detected in the outer and inner enamel epithelium and stellate reticulum (Jernvall et al., 1998). *Bmp-4*

is expressed in the later stage of tooth formation in the dental papilla and pre-odontoblasts. At differentiation stage, there is *Bmp-4* expression in the enamel-secreting ameloblasts and dentine-producing odontoblasts (Aberg et al., 1997).

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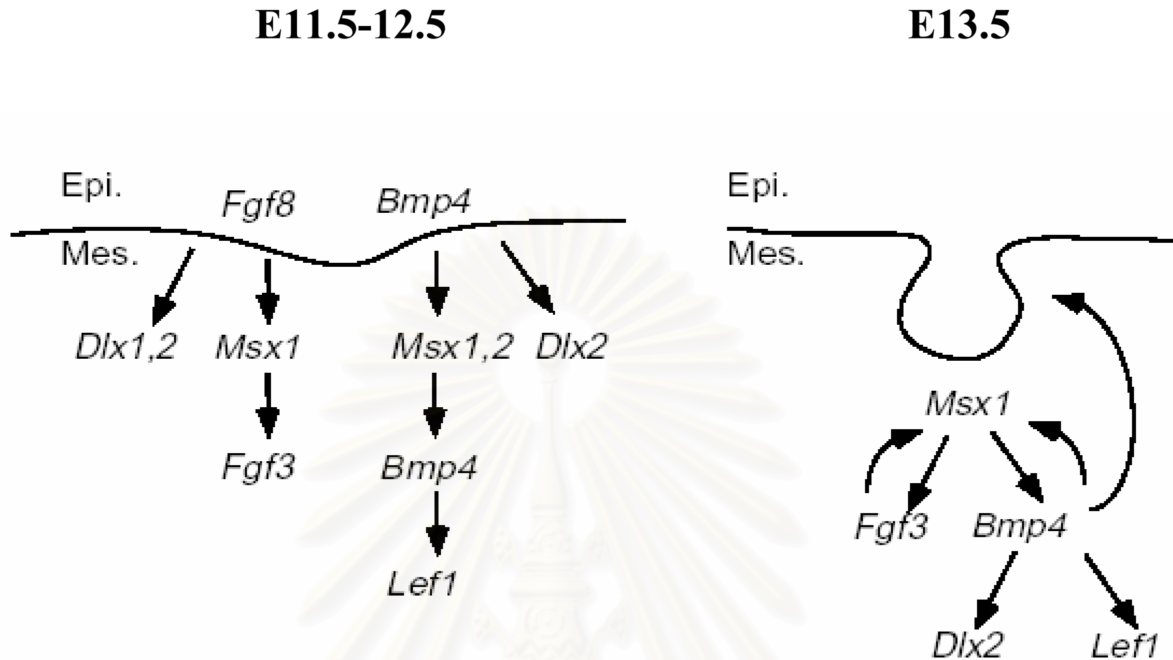


Figure 1.4 A genetic pathway for early tooth morphogenesis. At E11.5 epithelial Bmp-4 and Fgf-8 require *Msx-1* to induce expression of member of their own gene families in the dental mesenchyme. While both Bmp-4 and Fgf-8 can induce *Msx-1*, only Bmp-4 can induce *Msx-2* expression. In addition, while Bmp-4 and Fgf-8 both induced *Dlx-2* expression in the dental mesenchyme, only Fgf-8 can induce *Dlx-1* expression. Bmp-4 cannot induce *Fgfs*, and Fgfs cannot induce *Bmp-4* expression, suggesting that Bmp-4 and Fgf-8 act through separate *Msx-1* dependent pathways to induce expression of these downstream of genes. At the bud stage of development, *Fgf-3* is placed downstream of the mesenchymal *Msx-1* since its expression is reduced in the mutant dental mesenchyme. *Dlx-2* is place downstream of mesenchymal *Bmp-4* because Bmp-4 induced *Dlx-2* expression even in the absence of *Msx-1* and *Dlx-2* expression is reduced in the *Msx-1* mutant dental mesenchyme (Bei and Maas, 1998).

**Fibroblast growth factor-8 (*Fgf-8*)**

Fibroblast growth factors (Fgfs) are heparin binding growth factors, which promote the growth and differentiation of cells originated from endoderm, mesoderm and neuroectoderm *in vitro* (Wilkie et al., 1995). Currently 10 Fgfs and 4 FgF homologous factors (Fhfs) are identified (Mason, 1994; Smallwood et al., 1996; Yamasaki et al., 1996). Out of the Fgfs, *Fgf-3* (int-2), *Fgf-4* (hst, kFgf), *Fgf-7* (KGF), and *Fgf-8* (AIGF) mRNAs have been detected in the developing tooth (Wilkinson et al., 1989; Niswander and Martin, 1992; Finch et al., 1995; Neubuser et al., 1997).

The *Fgf-8* expression pattern has been investigated during the course of mandibular molar formation. At E11.0, the intent expression of *Fgf-8* is observed in the thickening presumptive dental epithelium or dental placode. This expression continues throughout the onset of epithelial budding. The expression of *Fgf-8* is down regulated when the tooth bud develops into bud stage at E12.0-E13.0. At E13.0, *Fgf-8* expression is mainly detected in the distal oral epithelium of the tooth bud whereas only weak signal is observed in the mesial part. No *Fgf-8* expression is detected in the developing tooth after bud stage.

Target genes of *Fgf-8* signaling from dental epithelium are observed in the *Msx-1* mutant mice. *Fgf-8* expression is maintained in E11.5 dental epithelium in the mutant embryos, indicating that epithelial expression of *Fgf-8* does not require *Msx-1*. It has been demonstrated that Fgf-8 beads can induce *Fgf-3* in the dental mesenchyme in normal condition. In the *Msx-1* mutant mice, tooth development is arrested at E13.5 and *Fgf-3* expression is not detectable in the deficient molar tooth mesenchyme. The

data indicate that *Fgf-8* requires *Msx-1* to induce *Fgf-3* expression in the dental mesenchyme. In normal condition, both Bmp-4 and Fgf-8 can induce *Msx-1* expression, which is required for signaling the expressions of *Fgf-3* and *Bmp-4* itself (Bei and Maas, 1998). The relation between the two proteins has been investigated. It is found that Fgf-8 beads cannot induce the dental mesenchyme to express *Bmp-4* and Bmp-4 beads also cannot induce the expression of *Fgf-3*. Thus, the pathways of Bmp-4 and Fgf-8 signaling are independent with respect to the induction of *Bmp-4* and *Fgf-3* expression in the dental mesenchyme (Figure 1.4).

**Muscle segment homeobox-1 (*Msx-1*)**

Homeobox genes constitute a large, highly conserved, multigene family of developmentally regulated transcription factors. *Msx-1* and *Msx-2* (formally *Hox-7* and *Hox-8*, respectively) are two members of the diverged homeobox family homologous to the *Drosophila*, muscle-segment homeobox *(msh)* (Hill et al., 1989). *Msx-1* and *Msx-2* expression within the neural tube extents caudally from the forebrain and they are expressed in the branchial arches including presumptive tooth germ (MacKenzie et al., 1991a; MacKenzie et al., 1991b). MacKenzie et al. (1991a, b, 1992) proposed that the initiation of tooth formation and the subsequent ability of condensed ectomesenchyme to induce tooth formation are related to the expression of the *Msx-1* and *Mxs-2* genes. They are first expressed in the early maxillary and mandibular processes with anteroposterior gradient within the ectomesenchyme for *Msx-1* and both epithelium and mesenchyme for *Msx-2* (MacKenzie et al., 1991b; MacKenzie et al., 1992). *Msx* genes are also implicated in the epithelial-mesenchymal interactions involved in tooth development. The *Msx-1* is strongly expressed in the dental mesenchyme throughout the dental placode, bud, cap, and bell stages of the mandibular molar odontogenesis (MacKenzie et al., 1991a, 1992; Maas et al., 1996). The expression of *Msx-1* in the dental mesenchyme at the initiation stage is induced by the dental epithelial signals; *Bmp-4* and *Fgf-8* (Vainio et al., 1993; Chen et al., 1996; Bei and Maas, 1998; Kettunen and Thesleff, 1998) and is subsequently maintained by the *Bmp-4* expression in the mesenchyme (Chen et al., 1996; Tucker et al., 1998). In return, the *Msx-1* controls the expression of *Bmp-4* and *Fgf-3* and several other genes, including *syndecan, Lef-1* and

*Ptc* in the dental mesenchyme (Chen et al., 1996; Bei and Maas, 1998; Tucker et al., 1998; Zhang et al., 1999). Thus, one function of the *Msx-1* in tooth development is regulation of the expression of inductive signaling molecules, such as *Bmp-4* and *Fgf-3*, which may exert their functions in the further tooth development. The *Msx-2* expression is initially restricted to the mesenchyme directly beneath the prospective dental placode, thereafter localizing to the dental papilla mesenchyme and to the epithelial enamel knot (MacKenzie et al., 1992). The involvement of the *Msx* gene function in tooth development is demonstrated by *Msx-1* knocked out mice, which exhibit a highly penetrated arrest at the bud stage of the molar tooth development (Satokata and Maas, 1994).

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**Paired box 9 (*Pax-9*)**

*Pax* genes encode a family of transcription that plays key roles during embryogenesis. They are required for the development of a variety of organs including the nervous system, muscular system, skeleton, eye, ear, kidney, thymus, and pancreas. During embryogenesis, *Pax-9* is expressed in somites, pharyngeal pouches, mesenchyme involved in craniofacial, tooth, and limb development.

At E11.5, *Pax-9* is detected in the dental mesenchyme adjacent to the dental placode. At E12.0-E13.5, *Pax-9* is detected at a high level in the dental mesenchyme surrounding the epithelial invagination. At later stage, the expression of *Pax-9* is observed in the dental papilla throughout the bell stage (Neubuser et al., 1997; Peters et al., 1998).

*Pax-9* mutant mice demonstrate the role of *Pax-9* in tooth development. Inspection of both jaws of newborn mutant mice shows the absence of all teeth. By morphological criteria, tooth development is initiated normally in the mutant and is indistinguishable from wild type until E12.5. At E13.5, the dental epithelia of both mutant and wild type have invaginated to form epithelial buds, but the condensation of mesenchymal cells around the bud is less prominent in the mutant. At E14.5, tooth development reaches the cap stage in wild type embryos whereas only a rudimentary bud is present in mutants. The later stages reveal that tooth development never proceed beyond the bud stage. Thus, *Pax-9* function is required in all developing teeth before or at the bud stage.

**Pituitary homeobox gene (*Pitx*)**

The mammalian *Pitx* gene family consists of three bicoid-related homeobox genes, with related to *Drosophila* gene *bicoid*. Each gene has an important role in the development of multiple organs (Gage et al., 1999a). *Pitx-2* was first identified in human (Semina et al., 1996). The mutations of the *Pitx-2* are one of the causes of Rieger syndrome (RGS) in human, a phenotypically and genetically dominant disorder. RGS is characterized by abnormalities of eye, tooth, umbilicus and occasionally heart defects (Semina et al., 1996). The phenotype of mice with reduction of *Pitx-2* function mimics Rieger syndrome. Mice heterozygotes for null (*Pitx-2*) allele have low frequency of eye and tooth abnormalities, consistent with RGS. Null homozygotes exhibit severe defect in the same organs that are mildly affected in Rieger patients (Gage et al., 1999b; Kitamura et al., 1999). Development of heart, eyes and teeth are also profoundly disrupted in the mutant mice, and they die by E14.5. Analysis of *Pitx-2* null mice established the critical role of *Pitx-2* in the development of craniofacial structures such as eyes, teeth and multiple organs including the pituitary, heart and lungs (Gage et al., 1999b).

The expression of *Pitx-2* is identified in developing mouse tooth germ. At E10.5, the expression of *Pitx-2* prefigures the site of the future odontogenesis and is restricted to the epithelium. As development of the lamina stage defined as dental placode formation (E11.5), the expression of *Pitx-2* becomes more confined to the dental placodes, which now are seen as local thickenings of the epithelium. At bud stage, the expression of *Pitx-2* remains restricted to the dental epithelium of both molar

and incisor. Later at cap stage, *Pitx-2* is strongly expressed in the dental organs as well as the enamel knot of both incisor and molar teeth. By E17.5, when the differentiation of cells begins, strongly expression of *Pitx-2* is found in the inner enamel epithelium of both incisor and molar teeth (Mucchielli et al., 1997; St Amand et al., 2000).

These evidences are well defined in mandibular tooth formation. In maxilla, the expression of *Pitx-2* is observed in the oral epithelium at E11.0 and this expression becomes restricted to the dental placodes of maxillary incisor and molar teeth at E12.0 (Keranen et al., 1999). However, the further studies of the role of this gene and its regulation to the maxillary incisor tooth formation have not yet been investigated.

**Sonic hedgehog (*Shh*)**

*Shh* is one of secreted signaling factors involved in growth and patterning of a number of organs developing from the epithelial-mesenchymal interaction, including the tooth. During initiation of tooth development, *Shh* expression is localized on each developing tooth germ and becomes restricted to a small population of molar epithelial cells locating at the tip of the tooth bud. This gene expression pattern suggests roles of *Shh* in initiation of the tooth development. Consistent with this hypothesis, *Shh*-coated bead inserted into mandibles before tooth formation causes an initiation of the ectopic epithelial thickening (Hardcastle et al., 1998). From its expression pattern in dental placode and tooth bud, it is suggested that *Shh* may regulate the proliferation of dental epithelial to form dental placode as well as the ingrowth of dental placode to form tooth bud. The *Shh* is temporary down regulated in the early bud stage and becomes up regulated again at the enamel knot in the stage between bud and cap stages, implicating the role of *Shh* in patterning the cusp of tooth. Prior to the cytodifferentiation, the *Shh* expression domain broadens to encompass the inner enamel epithelium and is maintained in differentiating ameloblast. Therefore, *Shh* has performed a role in regulating the cytodifferentiation of the inner enamel epithelium. When the activity of *Shh* is removed shortly after the ingrowth of the dental epithelium, the tooth is severely disrupted in cap stage. The overall size of the disrupted tooth is reduced, however, the enamel knot is still present. At birth, the size and shape of the tooth are severely affected and the polarity as well as the organization of the ameloblastic and the

odontoblastic layers are disrupted (Dassule et al., 2000). These studies demonstrate that *Shh* plays important role in regulating growth and morphogenesis of the tooth.

The hedgehog signaling pathway is a tale of two transmembrane proteins. Patched (Ptc), a twelve-pass membrane protein binds hedgehog ligand. Smoothened (Smo), a seven pass-membrane protein is a signal transducer. In absence of hedgehog ligand, Ptc interacts with and inhibits Smo, either directly or indirectly. This repression culminates in a transcription factor acting as a transcriptional repressor. When hedgehog binds to Ptc that normally represses Smo, the inhibition is released allowing Smo to activate the transcription of the downstream target genes via Gli transcriptional factors (Chen et al., 1996; Ruiz i Altaba, 1999; McMahon, 2000). Transcription factor Gli in vertebrates or cubitus interruptus (Ci) in *Drosophila* exhibit both positive and negative regulation of this pathway. The assay to evaluate the role of Shh signaling pathway in tooth development is investigated by *Gli2* and *Gli3* mutant models. *Gli2* mutants were generated by deletion of zinc fingers 3, 4 and 5 of *Gli2* gene from the mouse genome. *Gli2* heterozygotes have no abnormality, whereas the homozygotes have defects in the development of the teeth, palate, limbs, sternum, vertebral column and skull (Mo et al., 1997). *Gli2* mutant mice are found to have tooth defects predominantly associated with maxillary incisors, which manifest as a partial fusion of the two maxillary incisors, resulting in one large, central malformed tooth. In addition, the combination of the $Gli2^{-/-}$ $/Gil3^{+/-}$ mutant mice shows the abnormality in tooth development. They have small molars and mandibular incisor, whereas the maxillary

incisor development is arrested as a rudimentary epithelial thickening (Hardcastle et al., 1998).

Interestingly, the mutations of *Shh* in human and mouse lead to the abnormal pattern of neural plate and result in the holoprosencephaly and the cyclopia. In the mild form of the holoprosencephaly, there is a loss of the maxillary incisor. In the more severe form, there is no maxillary incisor development, while the other teeth are not affected (Cohen and Sulik, 1992; Roessler et al., 1996). It is likely that the *Shh* signaling pathway plays an essential role in the development of the maxillary incisor. However, the roles and the expression pattern of *Shh* during the tooth morphogenesis of maxillary incisor have not yet been investigated.

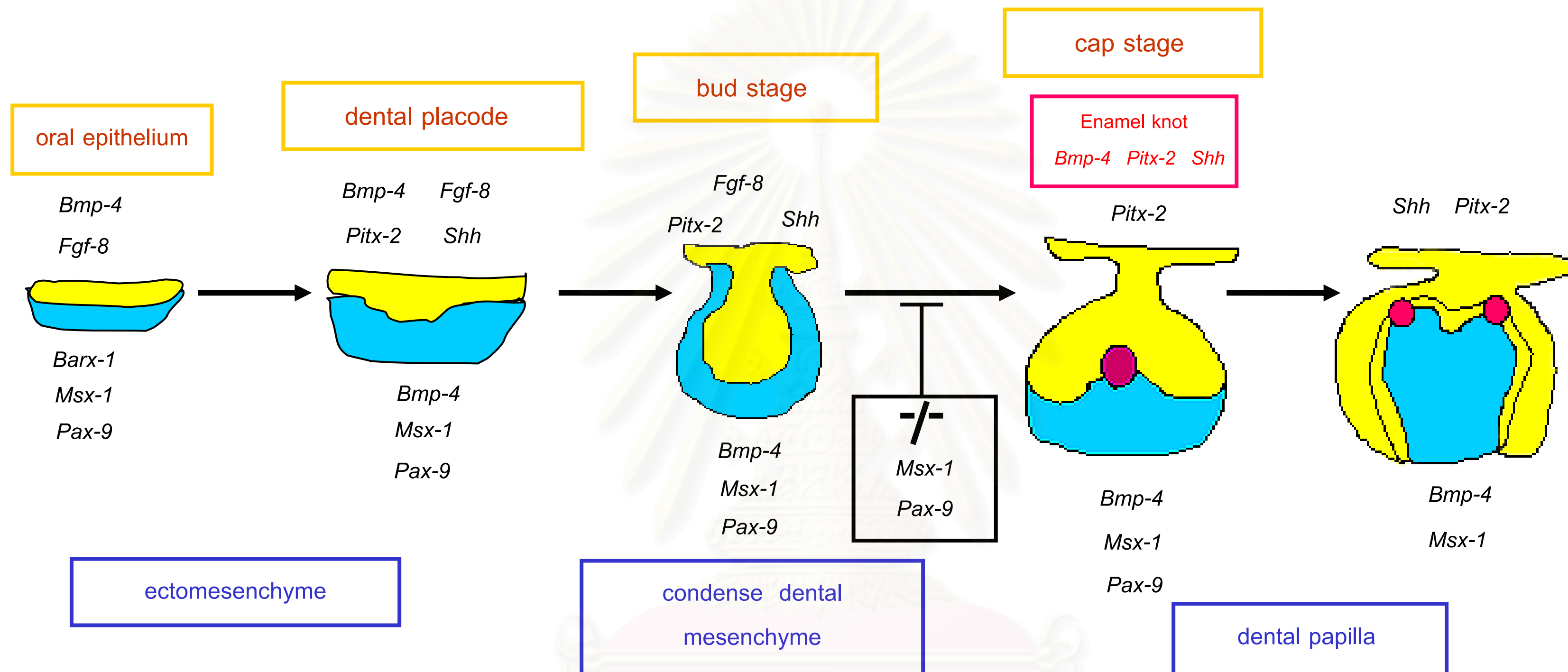


Figure 1.5 Schematic drawing demonstrates the expression pattern of tooth-related genes (*Bmp-4, Barx-1, Fgf-8, Msx-1, Pax-9, Pitx-2* and *Shh*) at initiation stage to bell stage. Prior to dental placode formation, oral epithelial *Bmp-4* and *Fgf-8* induce ectomesenchymal *Barx-1*, *Msx-1* and *Pax-9* indicating tooth type and tooth site formation. Subsequently, dental placode forms and expresses *Bmp-4, Fgf-8, Pitx-2* and *Shh* inducing the expression of *Bmp-4, Msx-1* and *Pax-9* in ectomesenchyme to proceed bud, cap and bell stage of the incisor or molar tooth. *Msx-1* and *Pax-9* mutant mice demonstrate the role of these genes in tooth development. The tooth development can not proceed beyond the bud stage in these mutant mice.

## 1.2 A mutant rat small eye or *rSey*

### 1.2.1 Craniofacial development in *rSey*

A mutant rat small eye or *rSey* is an autosomal dominant mutation in rat spontaneously arisen in a colony of Sprague-Dawley (SD) rat. Heterozygotes (*rSey/+*) have small eyes, whereas homozygotes (*rSey/rSey*) lack of eyes and nose formation (Fujiwara et al., 1994; Osumi-Yamashita et al., 1997). The first sign of abnormal morphology in the homozygote is seen at embryonic day 11 (E11.0), roughly corresponding to E9.0 in mouse embryos. At this time, histological analysis of the heterozygous or wild type embryos demonstrates that lens and nasal placodes are formed and optic vesicle grows out to contact the lens placode. On E12.0-13.0, the lens placode of heterozygous or wild type embryos invaginates to form the lens pit and subsequently the lens vesicle. Similar morphological changes are observed in the developing nose rudiment of heterozygous or wild type embryos, the nasal placode invaginates to form nasal pit and nasal cavity, resulting in protrusion of LNP and MNP. In contrast, the eyes and nose rudiments fail to development in homozygous embryos. On E11.0 homozygotes have the optic vesicle, which has an abnormal structure as well as the lens and nasal placodes were not developed at the corresponding regions. In addition, the neuroepithelium of the forebrain and optic vesicle degrade in homozygotes. At this stage, the homozygous embryos are distinguishable from wild type or heterozygous embryos by the more transparent eye rudiments when viewed under a dissecting microscope. These abnormalities result in a complete lack of eyes

and nasal cavity by E13.0. It is noted that the LNP appears to be missing in homozygotes, while the FNP protrudes to make an appearance of the MNP.

Tissue recombination studies indicate that ectoderm from homozygous embryos never differentiates into lens tissue even if it is cultured with normal optic vesicles from wild type or heterozygous embryos. In contrast, lens differentiation occurs when ectoderm from wild type or heterozygous embryos is cultured with optic vesicle from homozygous as well as wild type or heterozygous embryos. These results suggest that the failure of lens formation is resulted from defects in the underlying mesenchyme before the optic vesicle grows out to contact the head ectoderm (Fujiwara et al., 1994).

Morphological differences are not recognized between wild type and heterozygous embryos until the eye structure is fully developed. Heterozygous adults have smaller eyes than wild type but do not have nasal abnormalities. Microscopic observation discloses a localized island of retinal dysplasia and vacuolar degeneration of the iris stroma, with a normal span of the iris in the heterozygote adults (Mastuo et al., 1993).

Homozygous *rSey* is perinatal death and does not affect any other cranial regions including the maxillary, mandible, hyoid arch and otic vesicles. Interestingly, facial cleft and supernumerary incisor development are observed in the newborn homozygous *rSey*, however, both of the abnormalities are described with out the further studies.

### 1.2.2 *Pax-6* gene in *rSey*

*Pax* (paired like homeobox-containing) genes are a mammalian multigene family and share a conserved sequence motif called paired box which code a protein domain with DNA ability, possibly regulating other developmental genes. Nine *Pax* genes are identified and designated as *Pax-1* to *Pax-9*. Mutations in some of the *Pax* genes have been found in different kinds of developmental mutant phenotype in mouse. Moreover, some human genetic diseases have been shown to have mutations in human counterpart of the *Pax* genes. The mutations of *Pax-6* are associated with the "small eye" phenotype (*Sey*) in mouse, *rSey* in rat and aniridia (absence of iris) in human. *Sey* in mouse resembles *rSey* in rat in phenotype which shows a complete failure of lens induction and absence of the nasal pit (Walther and Gruss, 1991; Gruss and Walther, 1992). The rat *Pax-6* gene is quite similar to mouse gene (95% homology at the nucleotide level) and the rat Pax-6 amino acid sequence was identical to the mouse gene product (Matsuo et al., 1993).

The genetic analysis of *Pax-6* gene in *rSey* demonstrates that at genomic level, a single base, glutamine "G" insertion in an exon of the genomic DNA gives rise to a new sequence, "GT", which serves as an abnormal 5' splice site. This event results in an internal deletion of about 600 basepairs in the serine/threonine-rich domain of the messenger RNA (Matsuo et al., 1993). A novel small eye rat strain (*rSey*$^2$) is spontaneously identified in the SD-rat colony with the phenotypes similar to rat small eye (*rSey*). Analysis of the *Pax-6* gene reveals one base; cytosine(C); insertion in an exon encoding the region downstream of the paired box of the *Pax-6* gene, resulting in

an abnormal stop codon at the down stream of the insertion site due to the frame-shift (Osumi-Yamashita et al., 1997). This observation reveals that *rSey* and *rSey*$^{2}$ contain the abnormal truncated Pax-6 protein.

The eye and nasal defects in homozygous *rSey* embryos relate to the defects of *Pax-6* expression during eye and nasal development. In normal development of eye, there are two principal components of the early eye; the neural ectoderm of the optic vesicle and the overlying surface ectoderm. The former develops into the retina, and the latter forms the lens and cornea. *Pax-6* mRNA expression is found in a broad domain of frontonasal ectoderm during early development. The expression is subsequently down regulated from the ectoderm but specifically maintained in the developing lens placode. Moreover, other tissues that express *Pax-6* are frequently able to transdifferentiate into lens (Grindley et al., 1995). Thus, the phenotype together with its expression suggests that *Pax-6* might play some roles in lens determination. Early homozygous *rSey* optic vesicles are abnormally broad and fail to constrict proximally. These defects occur prior to the time of lens placode formation so it is probably that normal formation of optic vesicle is required for the lens formation. The results from the tissue recombination studies demonstrate that the lens development does not depend on the optic vesicle but rather depend on the Pax-6 protein since the homozygous optic vesicle can induce lens formation when combined with the ectoderm that produces normal Pax-6 proteins (Fujiwara et al., 1994).

Like the lens, the frontonasal ectodermal placodes express *Pax-6* mRNA, subsequently invaginate to produce the MNP and LNP and eventually the nasal cavities are formed. In homozygous *rSey*, LNP does not develop and imperforated snouts are

formed. Lack of LNP, the rest of facial processes including MNP and MxP fail to fuse to form the midface resulting in cleft between the midface and maxilla. The defect of the fusion in homozygous *rSey* shows severe craniofacial abnormality resembling the facial cleft between MNP and MxP in human.

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### 1.2.3 Cranial neural crest cells in *rSey*

Mammalian cranial neural crest cells emigrate from the neuroepithelium in the rostral neural plate. There are seven morphological units identified from anterior to posterior of rostral neural plate, forebrain (FB), anterior midbrain (MB), posterior MB, prorhombomere A (proRhA) or rostral hindbrain, prorhombomere B (proRhB) or preotic hindbrain, prorhombomere C (proRhC) and prorhombomere D (proRhD).

In mouse and rat, cranial neural crest cells start migration at 5-6 somite stage when the neuroepithelium become closer to form the neural tube. The first groups of cranial crest cell emigrate from FB, MB and proRhA. The most anteriorly situated facial primodia is the FNP underlying olfactory placode, to which crest cells from both the forebrain and midbrain migrate. Caudally, the first pharyngeal (branchial) arch appears, later developing into the MxP and MdP, which are the primodia of upper jaw and lower jaw, respectively. Crest cells from the posterior midbrain and proRhA migrate to the first arch, those from the proRhB migrate to the second arch, and those from proRhC and proRhD migrate to the third and fourth arches, respectively (Osumi-Yamashita et al., 1994).

In homozygous *rSey* embryos, migration of anterior midbrain crest cells into FNP is specifically impaired, though crest cells from other regions migrate normally. Anterior midbrain crest cells migrated towards the FNP and nasal placodes are formed and invaginate to produce MNP and LNP in normal development. These crest cells are further traced with respect to formation of the LNP. In homozygous *rSey*, aggregated anterior midbrain crest cells are observed at the dorsal region of eye primodium in the

early emigration and can not further migrate into the FNP to contribute to LNP formation (Figure 1.6).

The affect of *Pax-6* gene mutation in an impaired anterior midbrain crest cell migration in homozygous *rSey* is determined and clearly shown by labeling normal midbrain crest cells from wild type embryos with DiI. The labeled cells are orthotopically injected into host *rSey* embryos. Migration of the donor cells into the LNP is abnormal in the homozygous host embryos, while they migrate normally in wild type or heterozygous embryos. These data suggest that the cranial defects in homozygous *rSey* embryos are due to inappropriate substrate for crest cells migration towards the LNP. *Pax-6* is not expressed in these crest cells themselves, but is expressed in the frontonasal ectoderm which might play a role in conducting migration of these neural crest cells (Osumi-Yamashita et al., 1997).

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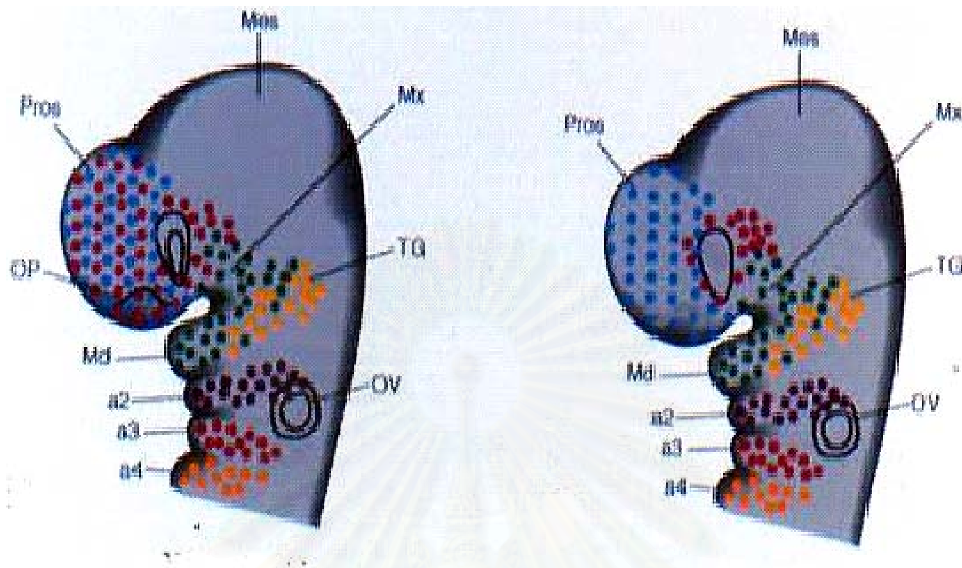


Figure 1.6 Cranial crest cells migration patterns in the wild type and the homozygous *rSey* embryos. Wild type embryo at the developmental stage in which migration of crest cells is nearly complete, the normal cranial neural crest were observed, at the most anteriorly situated facial primodium is the frontonasal prominence underlying the olfactory placode (OP), to which crest cells from both forebrain (blue dot) and anterior midbrain (red dot) migrate. Caudally, the first branchial arch appears, later developing into the maxillary (MxP) and mandibular (MdP) arch. Situated further caudally are the second, third, and the fourth pharyngeal arches (a2, a3, and a4). Crest cells derived from posterior midbrain (green dot) and ProRhA (orange dot) migrate to the first arch, those from the ProRhB (purple dot) to the second arch, and those from proRhC (red dot) and proRhD (orange dot) to the third and fourth arches, respectively. The homozygous *rSey* embryos, migration of anterior midbrain crest cells into the frontonasal region is specifically impaired indicated by the red dot accumulated behind the eyes remnant, though crest cells from other regions migrate normally. Abbreviations: Pros, prosencephalon; Mes, mesencephlon; OV, otic vesicle; TG, trigerminal ganglion (Osumi-Yamashita et al., 1994).

**1.3 Problem and hypothesis**

An initiation of the tooth development involves the reciprocal interactions between oral epithelium and underlying ectomesenchyme. Most of the teeth in the dentition develop in either maxillary or mandibular processes which both are derived from first branchial arch. The maxillary incisor is the only tooth developing in the frontonasal process in rat. Development of the maxillary incisor is come to our interested since the origins of both dental epithelium and ectomesenchyme are different from the other teeth. Moreover, the study of maxillary incisor development is still limited comparing to the mandibular molar development. The cascade of the gene expression during maxillary incisor development has also not yet been investigated.

There are reports that homozygote of a *Pax-6* mutant rat; rat small eye (*rSey)*; develops supernumerary maxillary incisor. The homozygous *rSey* has craniofacial abnormalities including of no eye, no nose and facial cleft between the MxP and FNP. The cleft is resulted from impaired migration of anterior midbrain crest cells toward the FNP leading to absence of LNP. The FNP protrudes and has the appearance of MNP where the supernumerary maxillary incisor has been observed. It has been demonstrated that mutation of *Pax-6* involves in gene controlling in the specification of subtypes of hindbrain motor neurons. We then investigate forward the hypothesis that the *Pax-6* gene may also involve in tooth-related gene controlling in maxillary incisor development and mutation of the gene causes supernumerary maxillary incisor.

To give some insight into maxillary incisor development and how the supernumerary incisor develops in the homozygous *rSey*, we analyze histology and expression pattern of tooth-related genes in the maxillary incisor of both wild type SD-rat and homozygous *rSey* fetuses.

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## Hypothesis

1. There are the same cascade of developmental process and the tooth-related gene expression patterns in the maxillary incisor and the mandibular molar.

2. There are differences in the patterns and the expression of tooth-related genes of the maxillary incisor development between the wild type SD-rat and the homozygous *rSey* associated with the incidence of the maxillary supernumerary incisor.

## 1.4 Specific Aims

1. To study the developmental process of rat maxillary incisor by histological analysis in wild type SD-rat at the stage day 12.0 to day 15.0.
2. To study the cascade of gene controlling of maxillary incisor development in wild type SD-rat by whole mount in situ hybridisation of tooth-related genes.
3. To investigate the process and incidence of supernumerary maxillary incisor in homozygous *rSey* by histological analysis compared to wild type-SD rat at stage day 12.0 to day 15.0 and stage day 20.0.
4. To investigate the role of *Pax-6* gene in controlling of maxillary incisor development by comparing the tooth-related gene expression patterns between the wild type SD-rat and the homozygous *rSey* by whole mount in situ hybridisation.

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## 1.5 Benefits

These studies might receive new beneficial evidences in order to understand the process and genetic control in the maxillary incisor development in rat.

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# CHAPTER II

# THE HISTOLOGICAL STUDY AND TOOTH-RELATED GENE EXPRESSION PATTERNS OF MAXILLARY INCISOR IN THE WILD TYPE RAT EMBRYOS

## 2.1 Introduction

Teeth develop through a series of reciprocal interactions between oral epithelium and neural crest-derived mesenchyme (ectomesenchyme). Tissue recombination experiments show that the oral epithelium isolated from the mandibular arch of mouse embryos between E9.0-E12.0 can induce non-dental mesenchyme to form tooth. After E12.0, this so-called odontogenic potentiality shifts from the epithelium to the mesenchyme. At later stage the mesenchyme when combined with a non-dental epithelium can induce tooth formation, whereas the epithelium has lost this ability. These data indicate that the signals initiating the tooth formation are produced by the oral epithelium and the signals shift to the underlying mesenchyme from E12.0 onward (Mina and Kollar, 1987; Lumsden, 1988).

During epithelial-mesenchyme interaction of tooth formation, various signaling molecules such as transcriptional factors particular the homeobox genes have been investigated in mouse mandibular molar. Prior to tooth dental placode formation at E9.5, *Bmp-4* expression is localized on the oral epithelium at the distal region of mandibular arch. It induces adjacent distal mesenchyme to express *Msx-1*, which marks

presumptive incisor region and also regulates the restriction of *Barx-1* expression in the proximal underlying mesenchyme, which marks presumptive molar region. When Bmp-4 signaling is inhibited by exogenous Noggin protein, *Barx-1* is expressed ectopically in the distal mesenchyme and transformed the tooth identity from incisor to molar. Moreover, epithelial Bmp-4 signaling has an antagonistic effect with epithelial *Fgf-8* expression on marking the tooth site formation of incisor and molar via the mesenchymal *Pax-9* expression. Fgf-8 signaling induces *Pax-9* expression that is inhibited by Bmp-4 signaling (Neubuser et al., 1997).

*Fgf-8* is one of the signaling molecules observed at early stage of tooth development. The expression of *Fgf-8* is intensely observed in the dental placode and continues in the epithelial bud and becomes down regulated in the dental epithelium at bud stage. After bud stage, *Fgf-8* expression is not detected in the developing tooth. The epithelial expression of *Fgf-8* induces mesenchymal *Msx-1* expression that is required for *Fgf-3* expression in the dental mesenchyme (Bei and Maas, 1998).

*Shh* and *Pitx-2* are expressed strongly in the dental epithelium (Hardcastle et al., 1998, Mucchielli et al., 1997; St. Amand et al., 2000). *Shh* expression regulates dental epithelial proliferation to form dental placode and also regulates the ingrowth of dental placode to develop the tooth bud (Dassule et al., 2000). *Bmp-4* is also expressed intensely in the epithelium and induces the underlying mesenchyme to express *Msx-1, Msx-2* and *Dlx-2* (Bei and Maas, 1998). Subsequently, the epithelial *Bmp-4* expression decreases and shifts from dental epithelium to the mesenchyme at early bud stage. At this stage, Bmp-4 epithelial signaling induces *Msx-1* and its own expression in the mesenchyme.

Mesenchymal homeobox genes, *Msx-1* and *Pax-9*, which respond to the epithelial signal show the essential role in the tooth formation. *Msx-1* and *Pax-9* are expressed strongly in the dental mesenchyme at initiation stage throughout the bell stage. *Msx-1* and *Pax-9* knocked out mice which exhibit a highly penetrated arrest at bud stage of molar tooth development indicate the involvement of these genes in tooth development (Satokata and Maas, 1994; Peter et al., 1998).

Development of different types of teeth such as molars and incisors enables the role of epithelial mesenchymal interactions in pattern formation to be studied. It has been suggested that determination of the tooth type may be pre-assigned according to the origin site of the oral epithelium and the ectomesenchyme. Since the maxillary incisor develops in the frontonasal process, the origins of the epithelium and the ectomesenchyme are different from the other teeth developed in the first branchial arch. Moreover, the site and onset of maxillary incisor development are overlapping with the fusion of the medial nasal process (MNP) and maxillary process (MxP) to form the midface. We then explore the developmental process of maxillary incisor formation in rat embryos by histological analysis. Expression pattern of tooth-related genes (*Fgf-8, Pitx2, Shh, Bmp-4, Msx-1* and *Pax-9*) studied in mouse molar tooth formation that is a standard model of developing tooth is also investigated.

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## 2.2 Materials and Methods

### Embryo collection and Histology

The embryos of the wild type SD-rat were used in this study. The intercourse of wild type SD-rats was arranged, and the appearance of the vaginal plug was designated as day 0 of embryogenesis (E0). The stages of rat embryos used are E10 onwords in SD which corresponding to E8 in mouse embryos.

The heads of embryos at various stages were fixed in Bouin's fixative solution, washed in phosphate-buffered solution (PBS) and decalcified in 10 % ethylene diamine tetra-acetic acid disodium (EDTA-2Na) solution, pH 7.6. The samples were then dehydrated through a graded series of ethanol and embedded in paraffin. The sections of 5-6 μm thick were frontally cut using a microtome and stained with haematoxylin and eosin.

### In situ hybridisation

### Probes

The following probes were used: (1) the 1.0-kb fragment of mouse *Bmp-4* (from 6.5 embryo cDNA library ) cloned into pSP72 vector ; (2) the 0.67-kb fragment of mouse *Fgf-8* cloned into pBSCT/AIGF vector; (3) the 0.6-kb EcoRI fragment of mouse *Msx-1* cloned into pTZ19 vector; (4) the 1.7-kb fragment of mouse *Pax-9* cloned into

pBluescript II SK (+) (Stratagene); (5) the 1.8-kb of mouse *Pitx-2* cloned into pBluescript KS (+) (Stratagene); (6) the 0.64-kb EcoRI fragment of mouse *Shh* (from B. Hogan E 8.5 embryo cDNA library) cloned into pBluescript II SK (-) (Stratagene). To generate antisense and sense transcripts, the plasmids were linearized and transcribed by using T3, T7 or Sp6 RNA polymerase with digoxigenin RNA mixture (Roche Molecular Biochemicals). All transcripts, excepted *Shh* and *Msx-1* were reduced to average size of 200 bases by limited alkaline hydrolysis. Probe size and yield were checked by electrophoresis on 1% agarose gel. All digoxigenin-labeled riboprobes were detected with alkaline phosphatase-coupled anti-digoxigenin antibody using NBT/BCIP (Roche Molecular Biochemicals) as a color of substrate.

**Whole mount in situ hybridisation**

The embryos were fixed in 4 % paraformaldehyde (PFA) in PBS, washed in 0.1 % Tween 20 in PBS (PBT), dehydrated and rehydrated in graded series of methanol. The embryos were washed in PBT and then treated in 10 μg/ml proteinase K in PBT for 10-15 min, rinsed in 2 mg/ml glycine in PBT and washed in PBT. We refixed the embryos in 0.2% glutaraldehyde (GA)/4 % PFA in PBS, washed in PBT and incubated in hot PBT at 70 °C for 30 min. After bleaching in 6 % $H_2O_2$ in methanol, samples are rinsed in PBT and incubated in a pre-hybridization mixture (50 % formamide, 5x SSC, 50 μg/ml yeast RNA, 50 μg/ml heparin and 1 % SDS) for more than 60 min. The samples were hybridised at 70°C overnight in the hybridisation solution with the probe.

The hybridised samples were washed twice in a solution I (50 % formamide, 5x SSC, and 1 % SDS) at 70°C for 30 min, and then washed twice in solution II (50 % formamide, 2x SSC) at 65°C for 30 min. The samples were then rinsed in 0.1 % Tween 20 in Tris-buffered saline (pH 7.4, TBST), blocked in 1.5% blocking solution in TBST for 60 min, and incubated in pre-absorbed anti-DIG antibody (1:2,500) in 1.5% blocking solution in TBST at 4°C overnight. Before the immunodetection, the samples were intensely washed with TBST five times per hour and incubated in NTMT for 30 min. The color reaction was performed using NBT/BCIP (Roche Molecular Biochemicals) as a color of substrate and stop reaction by washing twice in the NTMT for 10 min and stored in PBT at 4°C. The samples were bleached in 50% and 100% ethanol in PBT, washed in PBT, subsequently preserved in the 25% and 50% glycerol in PBT and taken photographs. For the section of whole mount in situ hybridsation, the samples were trimmed and transferred in the 25% and 50% glycerol in PBT and washed in the PBT and PBS and immersed in 20% sucrose in PBS. The samples were incubated overnight in gloop solution (mixed solution of 0.75% gelatin in the PB solution with 30% sucrose and 47% albumin in the PB solution) at 4°C and embedded in the gloop activated by 2% glutaldehyde appropriated for cutting with vibrotome.

## 2.3 Results

### Dental placode of rat maxillary incisor was composed of two primary dental placodes

We studied the histology of rat maxillary incisor from E12.0 to E15.0. At E12.0, the facial process, medial nasal process (MNP) and maxillary process (MxP), started fusion to form the midface and oral epithelium of the maxilla. At this stage, there was no appearance of epithelial thickening of maxillary dental placode (data not shown). At E13.0, the oral epithelial thickening was observed aligned around the boundary of MNP-MxP fusion (Figure 2.1A, E). Subsequently, two developed maxillary primary dental placodes were identified medio-laterally to the MNP-MxP fusion site and we designated them as the medial and lateral primary dental placodes, (M-PDP and L-PDP) (Figure 2.1B, F). They eventually approached each other and fused to develop the single maxillary dental placode at E14.0 (Figure 2.1C, G). Maxillary incisor dental placode proliferated and invaginated into the underlying mesenchyme to form tooth bud at E15.0 (Figure 2.1D). These results showed that two primary dental placodes formed at the boundary of MNP-MxP fusion site and subsequently fused into a single maxillary dental placode. The time course of primary dental placodes fusion overlapped with the fusion of the medial nasal process (MNP) and maxillary process (MxP) to form the midface.

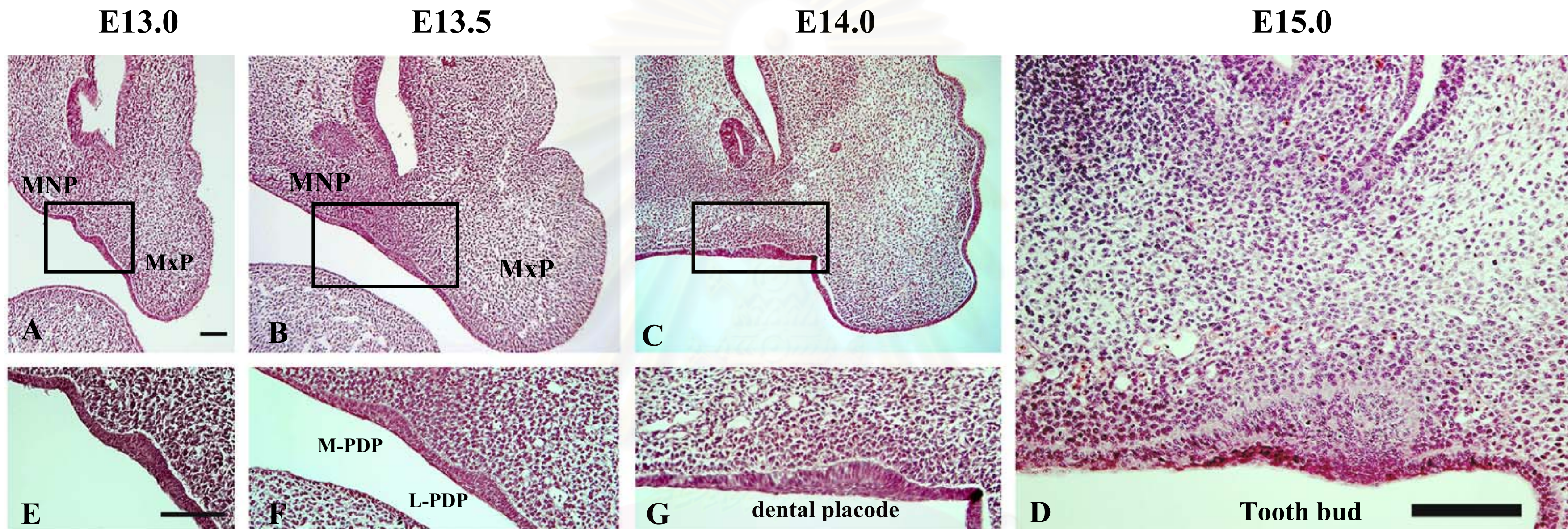


Figure 2.1 Histological analysis of maxillary incisor formation of SD-rat embryos from E13.0 to E15.0. The oral epithelial thickening was observed at the boundary of MNP and MxP fusion at E13.0 (A, E). The M-PDP and L-PDP developed and approached each other at E13.5 (B, F). Subsequently, the two primary dental placodes fused to make a single maxillary incisor dental placode at E14.0 (C, G). The dental placode developed into tooth bud at E15.0 (D) Scale bar = 100 µm. Abbreviations: MNP, medial nasal process; MxP, maxillary process; M-PDP, medial primary dental placode; L-PDP, lateral primary dental placode.

**Tooth-related gene expression patterns of maxillary incisor formation**

***Fgf-8, Bmp-4, Msx-1, Pax-9* and *Shh* were expressed in MNP prior to the maxillary incisor dental placode formation**

We investigated *Fgf-8, Bmp-4, Msx-1, Pax-9* and *Shh* expression at E12.0-12.5; the stage prior to formation of maxillary incisor dental placode; by whole mount in situ hybridisation. At E12.0, we found the expression of *Fgf-8* in the epithelium of MNP and the expression of *Pax-9* in the underlying mesenchyme at the prospective site of tooth formation (Figure 2.2A, B). The expression of *Bmp-4* was observed in the epithelium of MNP at the domain relatively exclusive to the expression of *Pax-9* and *Fgf-8* at this stage (Figure 2.2C). At E12.5, *Fgf-8* expression was decreased and restricted in the oral epithelium of MNP (Figure 2.3A asterisk). The expression of *Bmp-4* was increased in the epithelium overlapping the area of mesenchymal *Pax-9* and *Msx-1* expression in the MNP (Figure 2.3B, C, D asterisks). At this stage, the beginning of *Shh* expression was observed in the oral epithelium of MNP (Figure 2.3E asterisk). Furthermore, we also found the expression of *Bmp-4* and *Msx-1* at the boundary of MNP-MxP fusion (Figure 2.3B and D arrowheads). The expression of *Fgf-8* and *Pax-9* was intensely observed in the dental epithelium and mesenchyme of MxP respectively, indicating the presumptive site of molar tooth formation (Figure 2.3A, C arrow).

At E13.0, weak expression of *Fgf-8* was detected at this stage (Figure 2.4A). *Shh* and *Pitx-2* were expressed along the oral epithelium of the upper arch (Figure 2.4B,

C). The epithelium and mesenchyme of MNP and MxP expressed *Bmp-4* and mesenchymal expression of *Msx-1* was also observed in these regions (Figure 2.4D, E). *Pax-9* was consistently expressed in mesenchyme of MNP region (Figure 2.4F).

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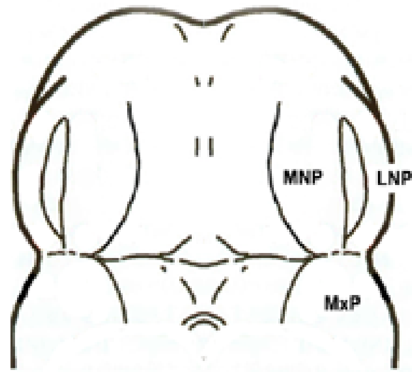
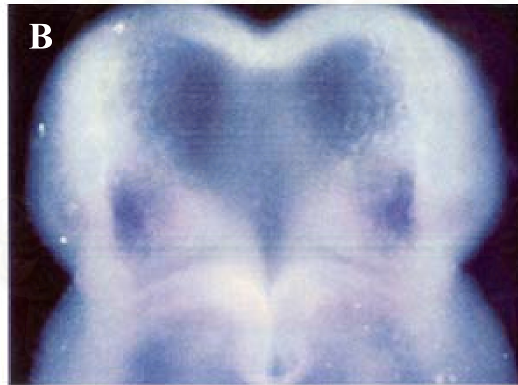


Schematic drawing shows facial processes,
MNP, LNP and MxP, of rat embryo at E12.0

***Fgf-8*** ***Pax-9*** ***Bmp-4***



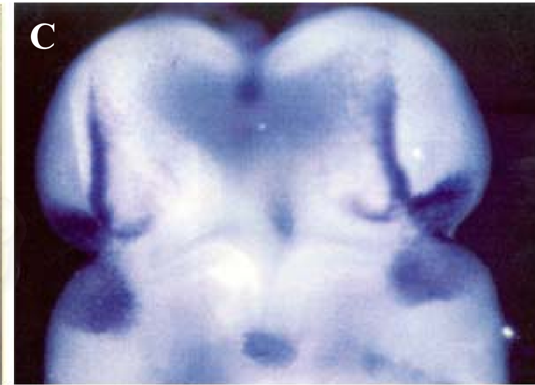




Figure 2.2 Schematic drawing and whole mount in situ hybridisation of E12.0 SD-rat embryos using digoxigenin-labeled riboprobes showed the expression pattern of *Fgf-8* (A), *Pax-9* (B) and *Bmp-4* (C) in the maxillary arch. Abbreviations: MNP, medial nasal process; LNP, lateral nasal process; MxP, maxillary process.

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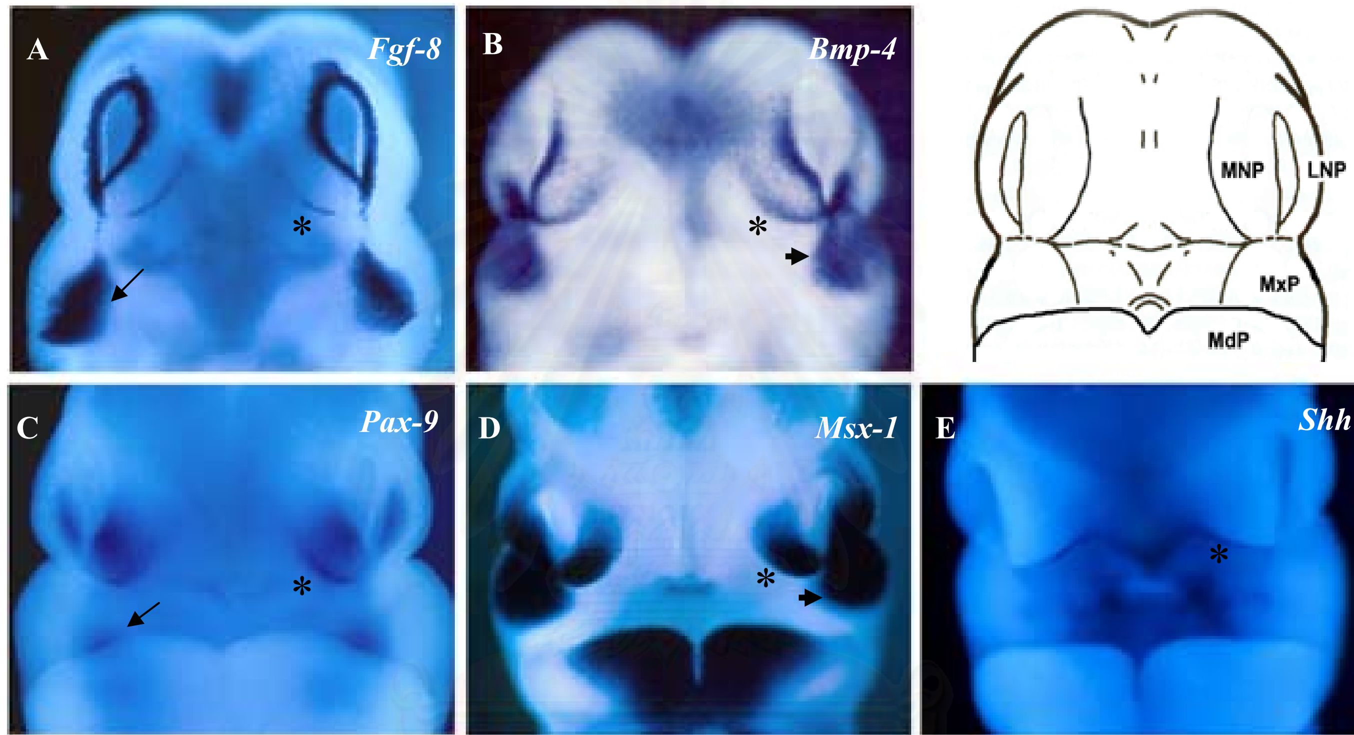


Figure 2.3 Schematic drawing and whole mount in situ hybridisation of E12.5 SD-rat embryos showed the expression pattern of *Fgf-8* (A), *Bmp-4* (B), *Pax-9* (C), *Msx-1* (D) and *Shh* (E) in the maxillary arch. Asterisks indicated the presumptive maxillary incisor; Arrows indicated the presumptive maxillary molar; Arrowheads in B, D indicated the expression of *Bmp-4* and *Msx-1* at the boundary of MNP and MxP. Abbreviations: MNP, medial nasal process; LNP, lateral nasal process; MxP, maxillary process; MdP, mandibular process.

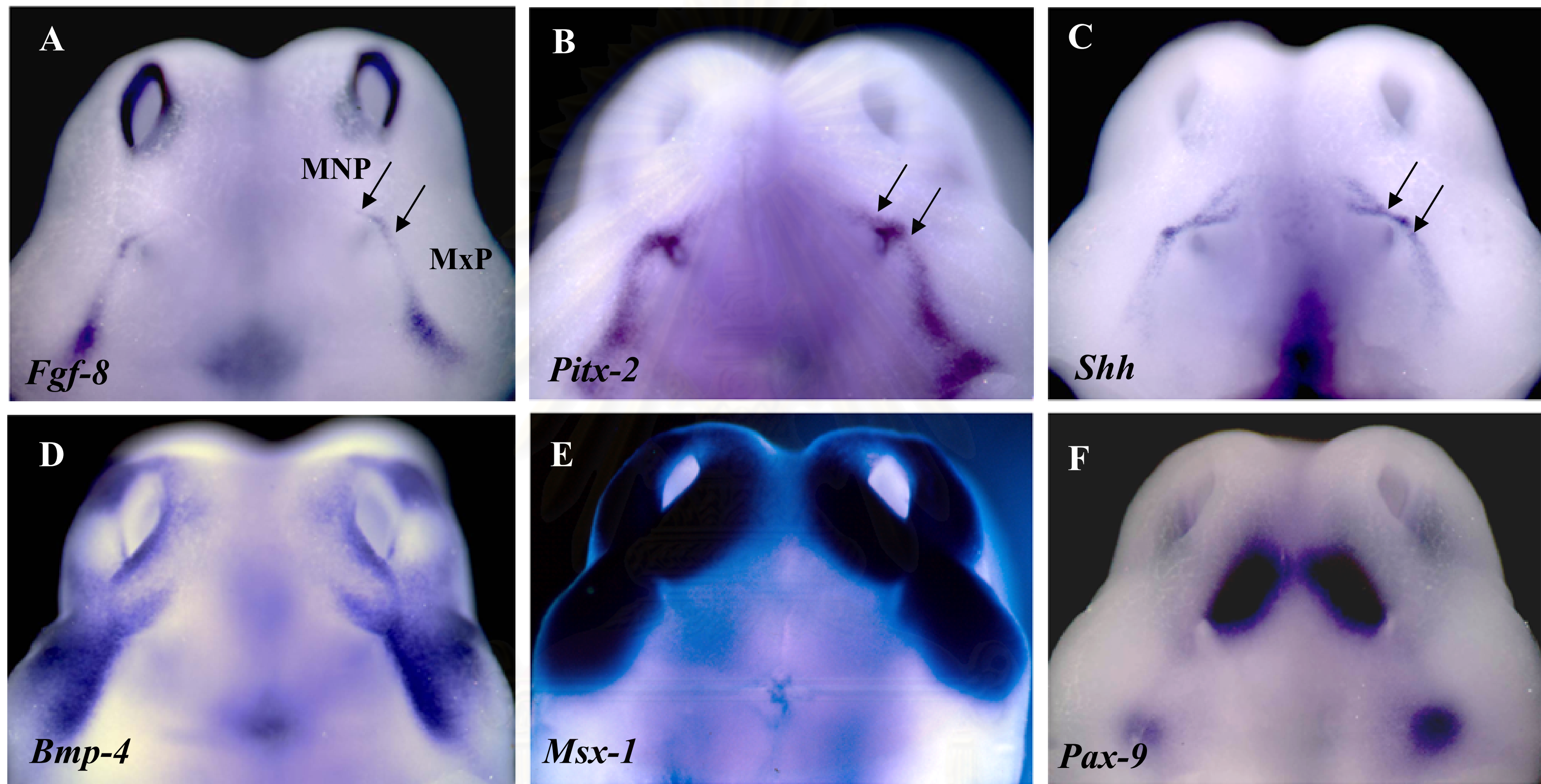


Figure 2.4 Whole mount in situ hybridisation of E13.0 SD-rat embryos showed the expression pattern of *Fgf-8* (A), *Pitx-2* (B), *Shh* (C), *Bmp-4* (D), *Msx-1* (E) and *Pax-9* (F) in the maxillary arch. The expression of *Fgf-8* was faintly seen while *Pitx-2* and *Shh* were expressed intensely in the presumptive maxillary incisor dental placode (arrows in A-C). *Bmp-4* was expressed in epithelium and mesenchyme of MNP and MxP (D). *Msx-1* and *Pax-9* were consistently expressed in mesenchyme of MNP and MxP (E, F).

***Shh, Pitx-2 and Fgf-8* indicated the medial and lateral primary dental placode formation and *Bmp-4, Msx-1 and Pax-9* were expressed during maxillary incisor dental placode formation**

To investigate the tooth-related gene expression in the maxillary incisor dental placode formation at E13.5-14.0, we carried out whole mount in situ hybridisation of these genes on the maxillary part and cut the section frontally by vibrotome. The whole mount in situ hybridisation showed the expression of *Shh* and *Pitx-2* in the oral epithelium at E13.0. At E13.5, they became restricted to the M-PDP and L-PDP aligning medio-laterally to the primary choana which indicated the previous place of MNP-MxP fusion (Figure 2.5B, C arrows). *Fgf-8* expression was faintly seen in the dental epithelium of M-PDP and L-PDP (Figure 2.5A, D) while *Bmp-4* was observed in both dental epithelium and mesenchyme of the two PDPs in Figure 2.5 G. Because of the plane of section that can not demonstrate the *Bmp-4* expression of both PDPs in the same section, *Bmp-4* expression was observed only in L-PDP (Figure 2.5J). *Msx-1* and *Pax-9* were strongly seen in the underlying mesenchyme of the maxillary incisor dental placode (Figure 2.5H, I, K, L). At E14.0, the expression of *Shh* and *Pitx-2* were observed in a single maxillary incisor dental placode where the expression of *Fgf-8* was faintly found (Figure 2.6A-C arrows, D-F). *Bmp-4* was expressed in both the dental placode and its underlying mesenchyme whereas expression of *Msx-1* and *Pax-9* were intensely observed at the underlying mesenchyme (Figure 2.6G-L).

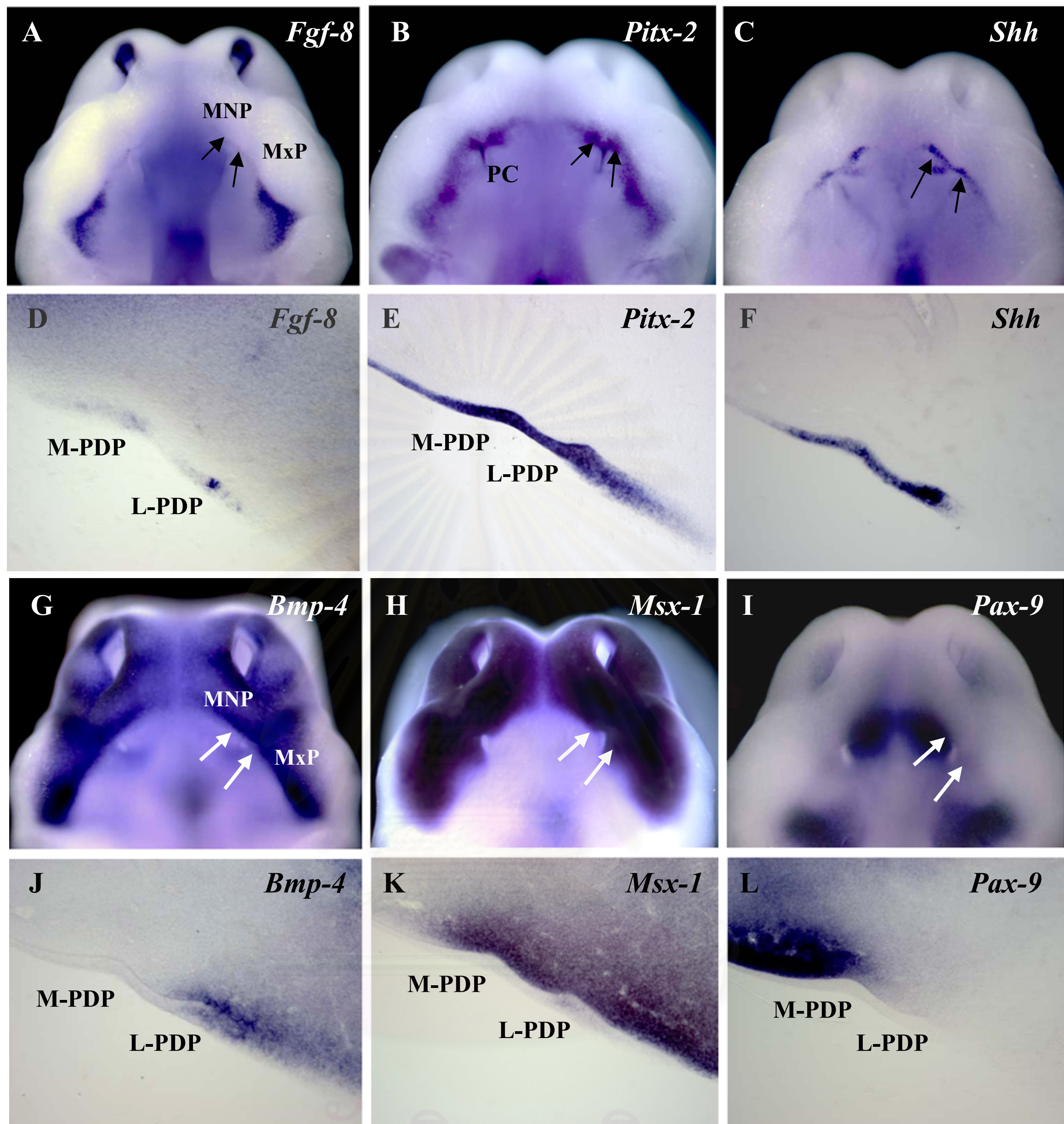


Figure 2.5 Whole mount in situ hybridisation and frontal sections of E13.5 SD-rat embryos showed the expression pattern of *Fgf-8* (A, D), *Pitx-2* (B, E), *Shh* (C, F), *Bmp-4* (G, J), *Msx-1* (H, K), and *Pax-9* (I, L) in the maxillary arch and the M-PDP and L-PDP of maxillary incisor. The M-PDP and L-PDP expressed *Fgf-8, Pitx-2* and *Shh* (A-F) and the underlying mesenchyme expressed *Bmp-4, Msx-1* and *Pax-9* (arrows in G-L). Abbreviations: M-PDP, medial primary dental placode; L-PDP, lateral primary dental placode; PC, primary choana (arrowhead). Arrows in A-C and G-I indicated primary dental placode of maxillary incisor.

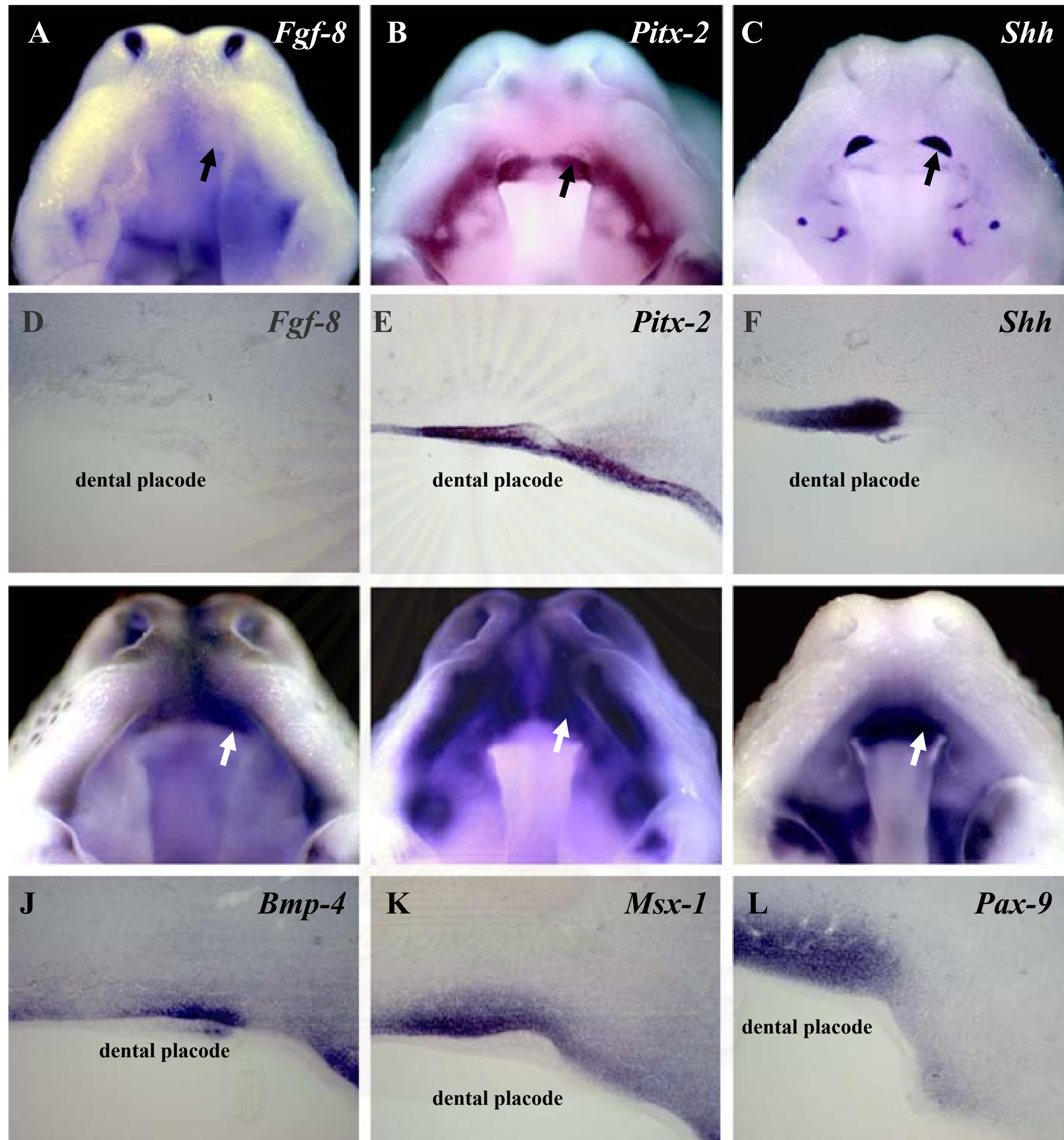


Figure 2.6 Whole mount in situ hybridisation and frontal sections of E14.0 SD-rat embryos showed the expression pattern of *Fgf-8* (A, D), *Pitx-2* (B, E), *Shh* (C, F), *Bmp-4* (G, J), *Msx-1* (H, K) and *Pax-9* (I, L). The single dental placode developed and expressed *Fgf-8, Pitx-2* and *Shh* in the wild type embryos (A-F). The declination of *Fgf-8* expression was observed. *Bmp-4* was expressed in both the dental placode and its mesenchyme (G, J) and the intense mesenchymal expression of *Msx-1* and *Pax-9* was observed at this stage (H, I, K, L). Arrows in A-C and G-I indicated maxillary incisor dental placode.

## 2.4 Discussion

### Rat maxillary incisor developed from fusion of two primary dental placodes

During early stage of mouse maxillary incisor formation, several embryonic maxillary dental placodes have been identified. Strassburg et al. reported in 1970 that there are three maxillary dental placodes formed but only the middle one developed into a functional maxillary incisor while the other two regressed during the early stage of development. Recent data showed that five maxillary incisor dental placodes formed and fused to develop into a one functional maxillary incisor (Peterkova et al., 1993). Our study in rat maxillary incisor showed that dental placode developed from two primary dental placodes which subsequently approached each other and fused into a single maxillary incisor dental placode. This developmental procedure occurred at the boundary of facial process fusion between MNP-MxP, however, the origins of the dental epithelium given rise to maxillary incisor dental placode has not yet been well defined. Our result supports the previous study in mouse demonstrated that the place where primary dental placodes form as well as the period when they fused relates to those of MNP-MxP fusion (Peterkova et al., 1993). It seems that the maxillary incisor dental placode development might require involvement of not only MNP but also MxP. This makes it more complicated than other teeth developed in the first branchial arch since the fusion of facial processes has to be considered.

***Bmp-4, Fgf-8, Msx-1* and *Pax-9* expression involve in tooth site and tooth type of maxillary incisor formation**

Previous studies in mouse mandibular tooth has been demonstrated the interaction of Fgf-8, Bmp-4, Msx-1 and Pax-9 signaling involved in prospective tooth site and tooth type formation. Prior to dental placode formation, *Bmp-4* is expressed in oral epithelium and induces the adjacent mesenchyme to express *Msx-1* at distal region of mandibular arch designated as presumptive mandibular incisor. This interaction is specific to incisor but not molar tooth development (Tucker et al., 1998). Fgf-8 produced in mandibular epithelium induced *Pax-9* expression in the underlying mesenchyme while Bmp-4 prevented this induction. *Pax-9* expression induced by the presence of Fgf-8 and absence of Bmp-4 is used as an early marker for mandibular incisor and molar tooth formation (Neubuser et al., 1997). In our studies, all the genes expression in mouse mandibular incisor are observed in maxillary incisor formation as we summarize as an illustration in Figure 2.7. The epithelial *Bmp-4* expression was observed overlapping to the mesenchymal expression of *Msx-1* in the MNP. The results correspond to previous study in the mandibular incisor that Bmp-4 and Msx-1 signaling interact to determine the incisor tooth type formation. The expression patterns of *Bmp-4, Fgf-8, Pax-9* and *Msx-1* in the MNP region suggest that these genes interact to pre-assign the site of maxillary incisor formation in the same way as the mandibular incisor. In addition, the expression of *Bmp-4* and *Msx-1* are also observed in the epithelium and mesenchyme around the boundary of MNP-MxP fusion. Together with the histological investigation that the primary dental placode is formed at the boundary of MNP-MxP

fusion, it is suggested that this region might involve in the maxillary incisor formation during the facial process fusion (Figure 2.3B, D arrowheads).

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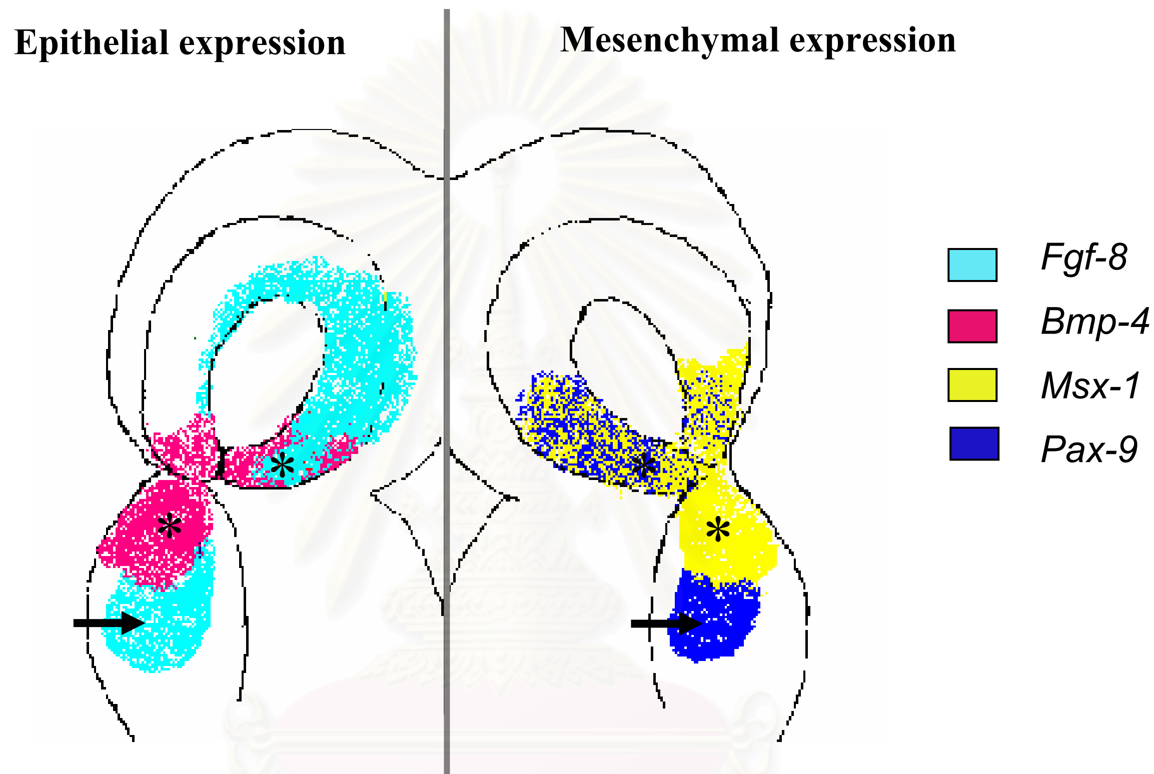


Figure 2.7 Schematic illustration of tooth-related gene expression patterns, *Fgf-8* (light blue) and *Bmp-4* (red) in the epithelium and *Msx-1* (yellow) and *Pax-9* (blue) in the mesenchyme of maxilla at the presumptive incisors (asterisks) and molars regions (arrows). Epithelial *Fgf-8* expression overlapped mesenchymal *Pax-9* expression represented presumptive incisor and molar tooth while the epithelial *Bmp-4* expression overlapped mesenchymal *Msx-1* expression specific to presumptive incisor formation.

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**Maxillary incisor dental placode expressed tooth-related genes at the early stage of development**

*Shh*, *Pitx-2* and *Fgf-8* were expressed in dental placode at early stage of odontogenesis and thus were used as early markers for dental placode formation. Our studies show the expression of *Shh, Pitx-2* and *Fgf-8* in the M-PDP and L-PDP. These expressions were also seen when the single maxillary dental placode developed. These results supported the histological investigation indicating primary dental placode formation at the early stage of maxillary incisor formation. Furthermore, maxillary incisor dental placode and its underlying mesenchyme expressed the tooth-related genes, *Bmp-4*, *Msx-1* and *Pax-9* in the same way as the early stage of mandibular molar tooth.

As summarized in Figure 2.8, our results showed that the expression of *Pax-9* and *Msx-1* in the mesenchyme at the stage prior to maxillary dental placode formation indicated the region of presumptive maxillary incisor formation in the same way as the mandibular incisor. It has been reported that the region marked for the presumptive mandibular molar tooth expressed *Pax-9* and *Barx-1*. Apart from the genes determining of tooth type, it seems that all dental placodes express identical cascade of tooth-related gene expression even though the origins of epithelium and mesenchyme of maxillary incisor differ from those of the other teeth. The results suggested that the determination of tooth type might be pre-assigned by the signaling molecules produced by the epithelial-mesenchymal interaction more than the origin of the oral epithelium or mesenchyme. Subsequently, the dental placode and its underlying mesenchyme of all teeth had the identical tooth-related gene expression patterns to develop incisor or molar

tooth. Interestingly, the mechanism of maxillary incisor dental placode formation was distinct from the other teeth. It developed by fusion of multi-dental placode origins and it might require the mechanism of facial process fusion to achieve one functional incisor.

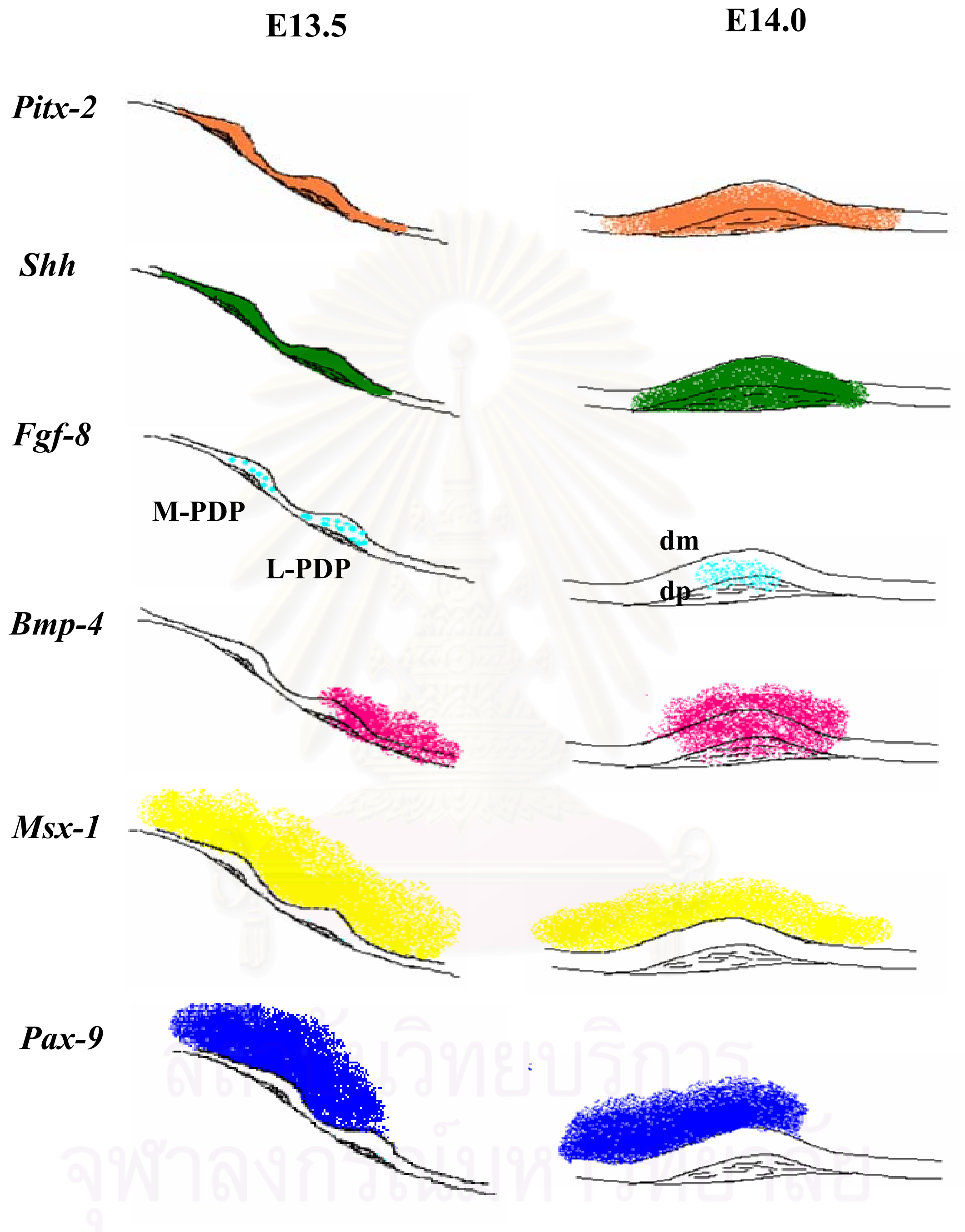


Figure 2.8 Schematic illustration of frontal sections of tooth germs at E13.5 (left column) and E14.0 (right column) showed the expression patterns of *Pitx-2, Shh, Fgf-8, Bmp-4, Msx-1* and *Pax-9* at the initiation stage of maxillary incisor formation. Abbreviations: M-PDP, medial primary dental placode; L-PDP, lateral primary dental placode; dp, dental placode; dm, dental mesenchyme.

# CHAPTER III

# THE HISTOLOGICAL STUDY AND TOOTH-RELATED GENE EXPRESSION PATTERNS OF MAXILLARY INCISOR IN THE HOMOZYGOUS *RSEY* EMBRYOS WITH SUPERNUMERARY INCISOR LIKE-STRUCTURES

## 3.1 Introduction

A mutant rat small eyes or *rSey* is an autosomal dominant mutation which is found in the course of breeding in a colony of Sprague-Dawley (SD) rat. Heterozygotes (*rSey*/+) have small eyes while homozygotes (*rSey/rSey*) lack eye and nose formation. Homozygous embryos are firstly distinguishable from wild type or heterozygous embryos on E11.0 by their more transparent eye rudiments viewed under the dissecting microscope. This abnormal morphology is caused by failure of lens placodes formation concomitant with regression of neuroepithelium of the forebrain and optic vesicle. In addition, nasal placodes are failed to form in the frontonasal region of the homozygotes and subsequently no lateral nasal process (LNP) appears. Thus, the frontonasal process protrudes to make an appearance of the medial nasal process (MNP). Lack of LNP causes the facial processes; MNP and MxP; fail to fuse and develop separately resulting in complete facial cleft in a new born homozygous *rSey* (Fujiwara et al., 1994, Osumi-Yamashita et al., 1997). At the MNP derived midface of the new born, supernumerary maxillary incisor has been reported without any further investigations. In this study, we then investigate the histology of maxillary incisor development in the homozygous *rSey*

embryos at E12.0 to E.15.0. The incidence of supernumerary tooth formation is also carefully investigated in homozygous fetuses at E20.0.

Genetic analysis has been demonstrated that there are at least two genotypes identified in the *Pax-6* gene mutation of rat small eye, *rSey* and *rSey*$^2$. Both of the mutations exhibit indistinguishable abnormal phenotypes as described above. Matsuo et al. report a single base insertion in an exon of DNA at 5´ downstream to homeodomain which serves as an abnormal 5´ splice site in the messenger RNA in *rSey*. Later another single base insertion in an exon 3´ downstream to paired box causing abnormal stop codon at down stream of the insertion site has been reported in *rSey*$^2$. Both of these single base pair mutation reveal that *rSey* and *rSey*$^2$ contain an abnormal truncated Pax-6 protein (Matsuo et al., 1993; Osumi-Yamashita et al., 1997). *Pax*-6 is known as a morphogenetic gene with activities in patterning and cell-type specification in early development. Involvement of *Pax-6* in formation of eyes and nose have been reported in many species such as eyeless in *Drosophila*, *vab-3* mutant in *C. elegans*, small eye (*Sey*) in mouse and aniridia in human. In addition, it is known that *Pax-6* mutation in homozygous *rSey* alters the gene controlling in the specification of subtypes of motor neurons in hindbrain. However, involvement of *Pax-6* in gene controlling of tooth development particular the maxillary incisor has never been reported. To clarify the effect of the truncated Pax-6 protein on gene controlling of the maxillary incisor formation and the supernumerary incisor formation, the expression patterns of tooth-related genes (*Fgf-8, Pitx-2, Shh, Bmp-4, Msx-1* and *Pax-9*) are investigated in homozygous *rSey*. Both the histological analysis and gene expression patterns are compared stage-by-stage to those of the wild type SD-rat.

**3.2 Materials and methods** (refers to the material and methods in the chapter 2)

Homozygous *rSey* embryos provided by the Safety Research Laboratories (Yamanouchi Pharmaceutical Co. Ltd., Japan) were used in this study. Since it died immediately after birth, homozygous *rSey* was obtained by intercourse of heterozygous *rSey* parents. The day of the vaginal plug observed was designated as day 0 of embryogenesis (E0). The homozygous *rSey* embryos distinguished by their morphologies were collected from stage E12.0-E15.0 and E20.0. The samples were further investigated by histological analysis and whole mount in situ hybridisation of tooth-related genes (*Fgf-8, Pitx-2, Shh, Bmp-4, Msx-1* and *Pax-9*) using the same experimental procedures explained in the materials and methods in the chapter 2.

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## 3.3 Results

### The separated M-PDP and L-PDP were developed in homozygous *rSey* rat with facial cleft

A new born homozygous *rSey* has the abnormalities in facial formation including the facial cleft and the absence of eyes and nose formation. The defect of facial process fusion; facial cleft; is caused by missing of LNP during facial formation. Subsequently, MNP and MxP failed to fuse and developed separately (Figure 3.1E, F asterisks).

Histological analysis at the initial stage of maxillary incisor formation of homozygous *rSey* showed that two areas of epithelial thickening designated as the M-PDP and L-PDP were observed in MNP region at E13.0 (Figure 3.2H, L arrows). However, the distance between the PDPs was wider than that in the wild type (Figure 3.2A, E). At E13.5, the distance in the homozygous *rSey* (Figure 3.2I, M arrows) was still wider than that observed in wild type (Figure 3.2B, F). Controversy to the wild type, the PDPs did not fuse at E14.0. Instead, the M-PDP and L-PDP developed into medial and lateral dental placode; M-DP and L-DP; respectively (Figure 3.2J, N arrows). The separated M-PDP and L-PDP individually developed into medial and lateral incisor tooth buds at E15.0 (Figure 3.2K) which both were smaller than the single maxillary incisor tooth bud developed in the wild type embryo (Figure 3.2D). The lateral tooth bud was smaller than the medial tooth bud. We also investigated the development of the separated tooth buds at E20 and found that 25% of the lateral tooth

bud could develop into supernumerary incisor like-structures in the MNP (Figure 3.1F-H).

During the course of this study, we also found the rudiment of primary dental placodes in the MxP at the prospective site of facial process fusion. However, we did not find any tooth bud developed from these PDPs (Figure 3.2 I, J arrowheads).

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**The incidence of supernumerary tooth-like structures in homozygous *rSey***

To elucidate the developmental progression of homozygous medial and lateral tooth bud, histological analysis of twenty E20 homozygous fetuses was carried out. It was found that all of homozygous medial tooth buds developed maxillary incisors smaller than those observed in wild type. However, they showed the enamel-forming ameloblasts and the dentine-forming odontoblasts secreting pre-dentine at labial side (Figure 3.1F, G arrowheads). About 25% of homozygous lateral tooth buds developed the inverted unilateral or bilateral supernumerary incisor-like structures (Figure 3.1 F, G arrows) while 75% of them stopped developing at tooth bud formation (data not shown). The developed supernumerary incisor-like structures showed the enamel-forming ameloblasts and dentine-forming odontoblasts like-cells. However, the dental papilla of these tooth structures contained haematopoietic cells and their inverted position marked the incomplete incisor formation (Figure 3.1H).

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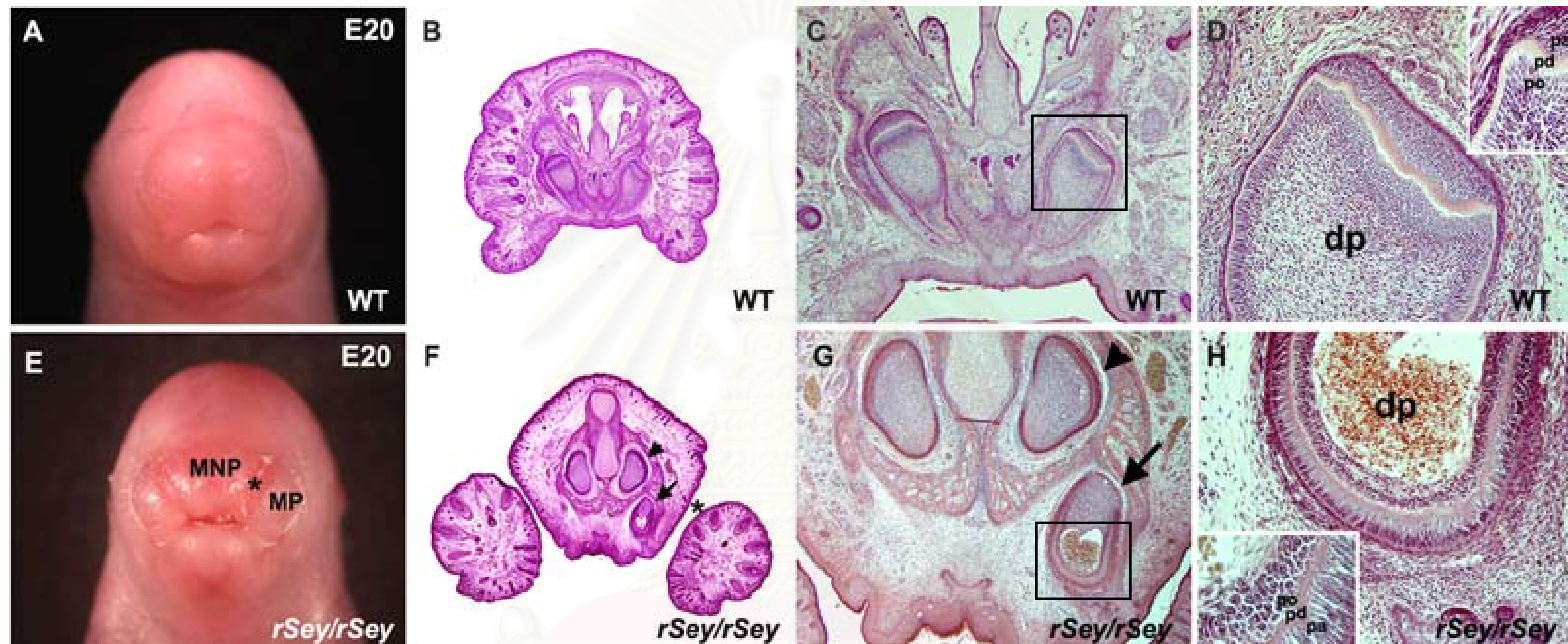


Figure 3.1 Frontal views of facial morphogenesis and histological sections of maxillary incisor formation at E20 of wild type (A-D) and homozygous *rSey* (E-H) embryos. Homozygous *rSey* embryo showed the absence of eyes and nose formation, facial cleft between MNP and MxP indicating by asterisk (E) and supernumerary incisor like-structure developed lateral to maxillary incisor in the MNP region (F-H). Arrows indicated the supernumerary incisor like-structure and arrowheads indicated the maxillary incisor of the homozygous embryo. Abbreviations: pa, pre-ameloblast; pd, pre-dentine; po, pre-odontoblast; dp, dental papilla.

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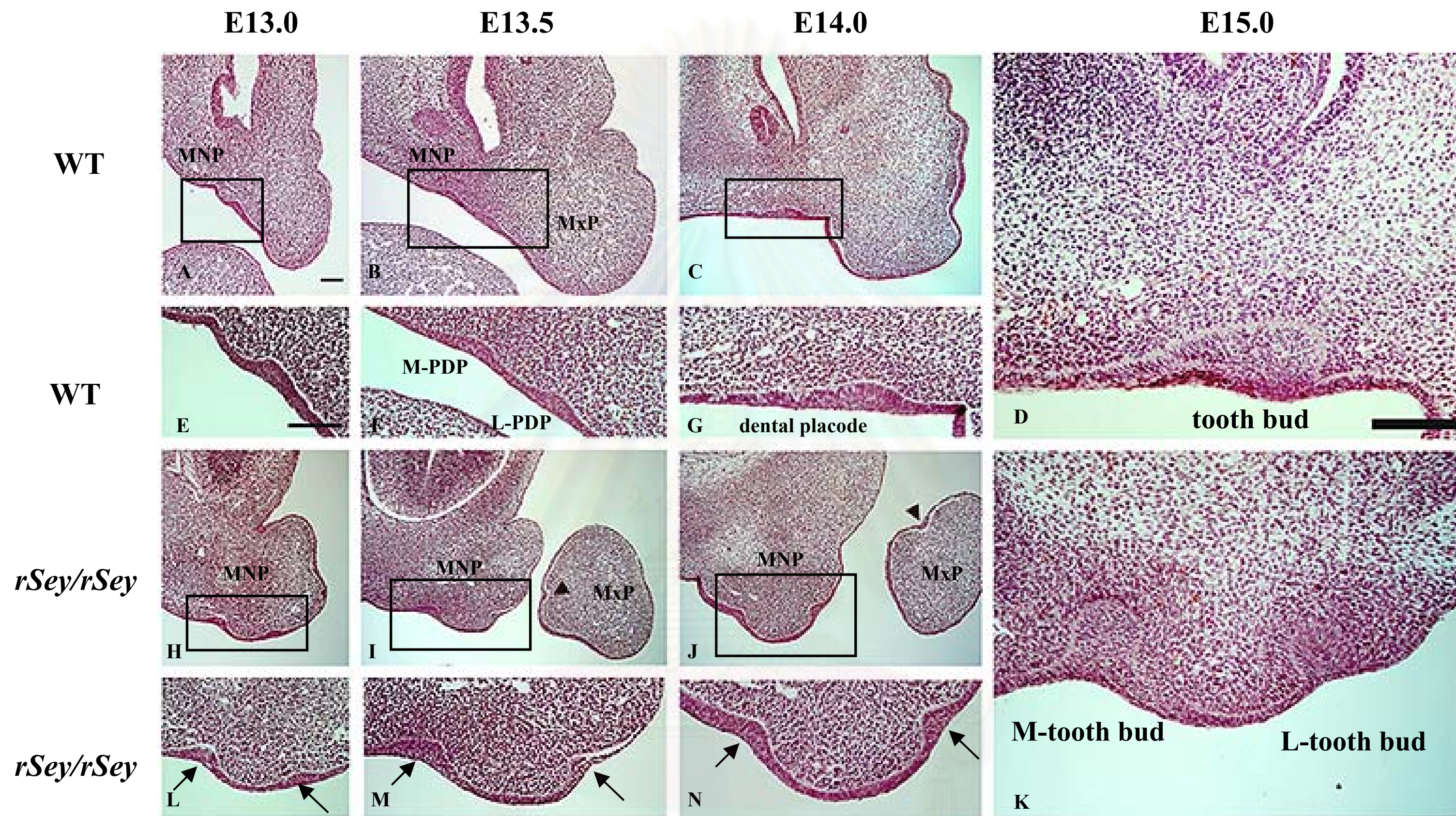


Figure 3.2 Histological analysis of the maxillary incisor formation of wild type (A-G) and homozygous *rSey* (H-N) embryos at E13.0-15.0. Wild type embryos, oral epithelial thickening were seen at the boundary of MNP and MxP fusion at E13.0 (A, E) and developed M-PDP and L-PDP approximately at E13.5 (B, F). These dental placodes fused into the single dental placode at E14.0 (C, G). Homozygous *rSey* embryos showed the separated M-PDP and L-PDP in MNP at E13.0-14.0 (H-J, arrows in L-N) contrasted to wild type embryos and they consequently developed into M-tooth bud and L-tooth bud at E15.0 (K). The remnant dental placodes were found in the MxP of homozygous *rSey* embryos (arrowheads in I, J) Scale bar = 100 µm. Abbreviations: MNP, medial nasal process; MxP, maxillary process; M-PDP, medial primary dental placode; L-PDP, lateral primary dental placode.

**Expression of *Fgf-8, Bmp-4, Pax-9, Msx-1* and *Shh* in MNP prior to the maxillary incisor dental placode formation in the homozygous *rSey***

We investigated the expression of *Fgf-8, Bmp-4, Msx-1, Pax-9* and *Shh* in the homozygous *rSey* embryos at the stage prior to maxillary incisor dental placode formation. At E12.0, strong expression of *Fgf-8* was observed in the epithelium of MNP covering the underlying mesenchymal *Pax-9* expression. The area was designated as the presumptive maxillary incisor formation (Figure 3.3D, E). In the same way as that observed in wild type, *Bmp-4* expression was dispersedly observed in the epithelium of the MNP (Figure 3.3F) which was relatively exclusive to that of *Pax-9* and *Fgf-8* expression at this stage (Figure 3.3D,E). At E12.5, two discrete *Fgf-8* expressions were observed in the epithelium of homozygous MNP (Figure 3.4B asterisks) while the single expression of *Fgf-8* was decreased and restricted to the MNP oral epithelium in the wild type embryos (Figure 3.4A asterisk). The discrete *Fgf-8* expressions locating on medial side was slightly stronger than that located on the lateral side. The expression of *Pax-9* was observed in the mesenchyme of MNP (Figure 3.4D asterisks). Obviously, the expression of *Fgf-8* and *Pax-9* indicating the presumptive molar tooth formation observed in the wild type was also intensely observed in the homozygotes (Figure 3.4 arrows in A-B, C-D). The expression of *Bmp-4* increased in the epithelium overlapping the area of *Msx-1* expression in the MNP of both wild type and homozygote (Figure 3.4 E-H asterisks) and we also found the expression of *Bmp-4* and *Msx-1* at the boundary of MNP-MxP fusion (Figure 3.4 E-H arrowheads). At this

stage, faint expression of *Shh* was firstly observed in the oral epithelium of MNP of both wild type and homozygous embryos (Figure 3.4I, J asterisks).

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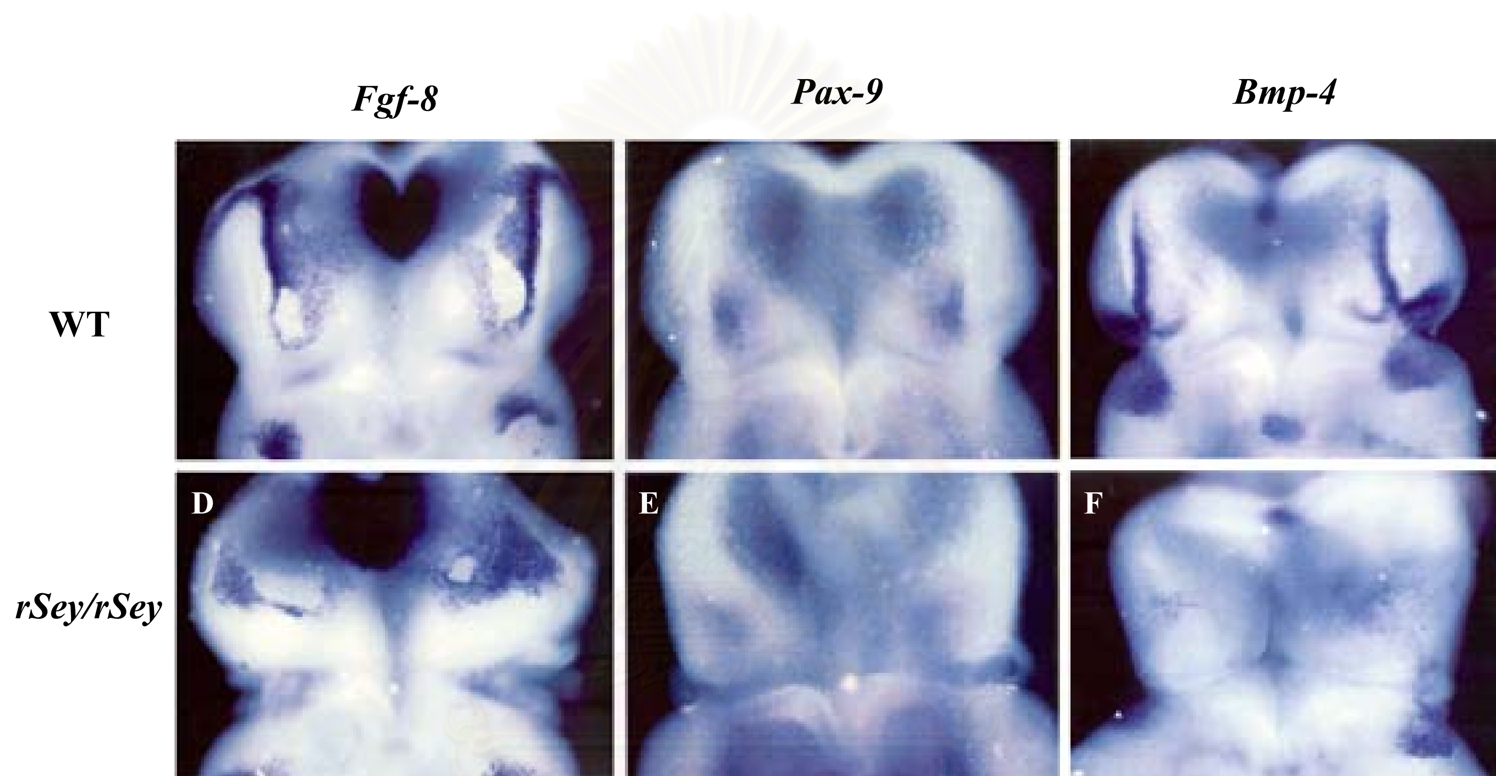


Figure 3.3 Whole mount in situ hybridisation showed the expression pattern of *Fgf-8, Pax-9* and *Bmp-4* in the maxillary arch of wild type and homozygous *rSey* embryos at E12.0.

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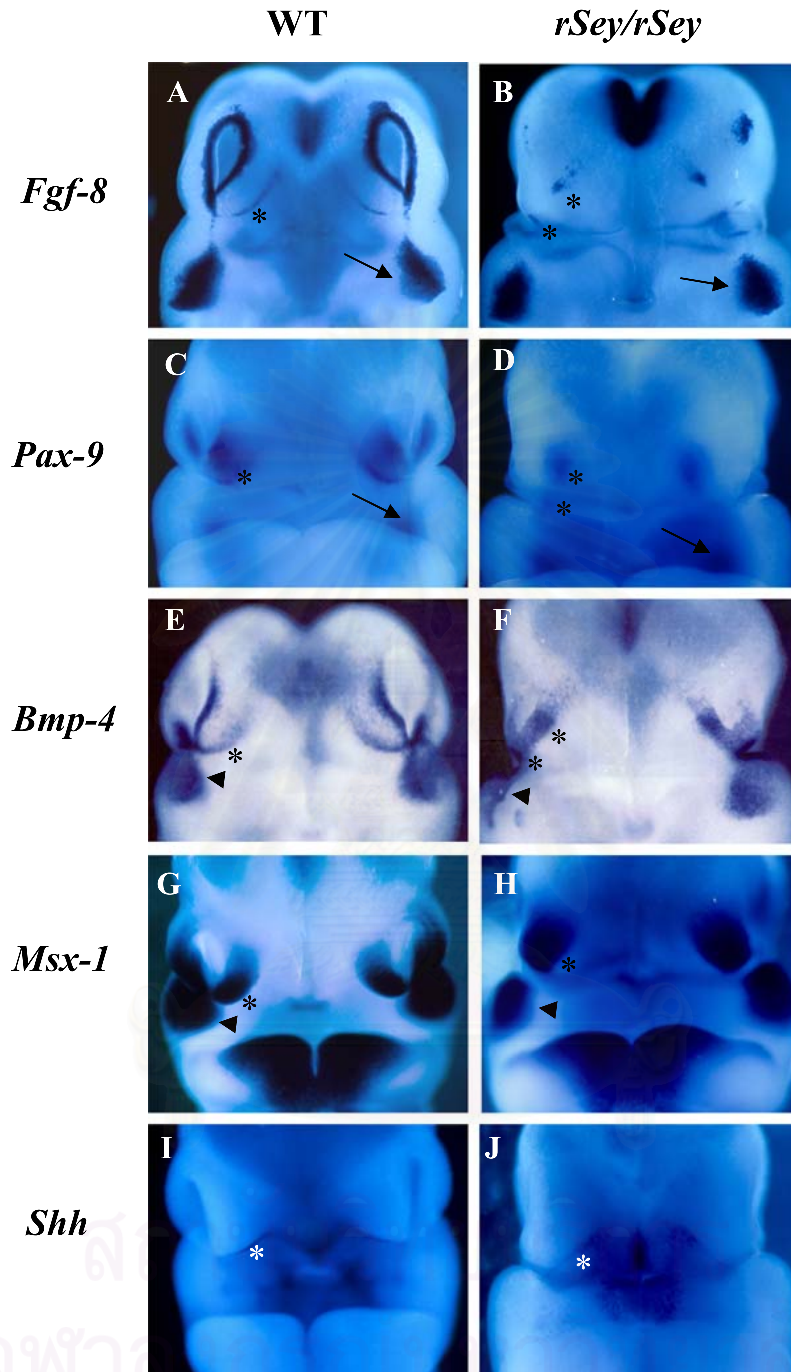


Figure 3.4 Whole mount in situ hybridisation showed the expression pattern of *Fgf-8, Pax-9, Bmp-4, Msx-1* and *Shh* in the maxillary arch of wild type and homozygous *rSey* embryos at E12.5. Asterisks indicated the presumptive maxillary incisor; Arrows in A-D indicated the presumptive maxillary molar; Arrowheads in E-H indicated the expression of *Bmp-4* and *Msx-1* at the boundary of MNP and MxP.

**Expression of *Fgf-8, Pitx-2*, *Shh, Bmp-4, Msx-1* and *Pax-9* in medial and lateral primary dental placode formation proceeding the medial and lateral tooth bud in the homozygous *rSey***

At E13.0, homozygous *rSey* obviously showed facial cleft between MNP and MxP associated with the discretion of *Fgf-8*, *Pitx-2* and *Shh* expression in the MNP and MxP (Figure 3.5 D-F). In contrast, wild type embryos showed expression of these genes continually along the MNP and MxP. The declination of epithelial *Fgf-8* expression was observed whereas *Pitx-2* and *Shh* became intensely expressed in the oral epithelium (Figure 3.5 A-C). At this stage of homozygous embryos, histological investigation showed that the two discrete oral epithelium thickenings, the M-PDP and L-PDP, developed in the MNP (Figure 3.2L arrow) corresponding to the place of the dental epithelial *Fgf-8*, *Pitx-2* and *Shh* expression (Figure 3.5 D-F arrows). The expression of *Pax-9* in mesenchyme as well as the expression of *Fgf-8*, *Pitx-2* and *Shh* in the oral epithelium marked two separated regions in the MNP that subsequently developed into the separated M-PDP and L-PDP (Figure 3.5L arrows). In addition, the *Bmp-4* expression coincided with the mesenchymal expression of *Msx-1* was found in the boundary of MNP-MxP fusion and in MNP covering the separated M-PDP and L-PDP formation in the homozygous embryos (Figure 3.5J, K).

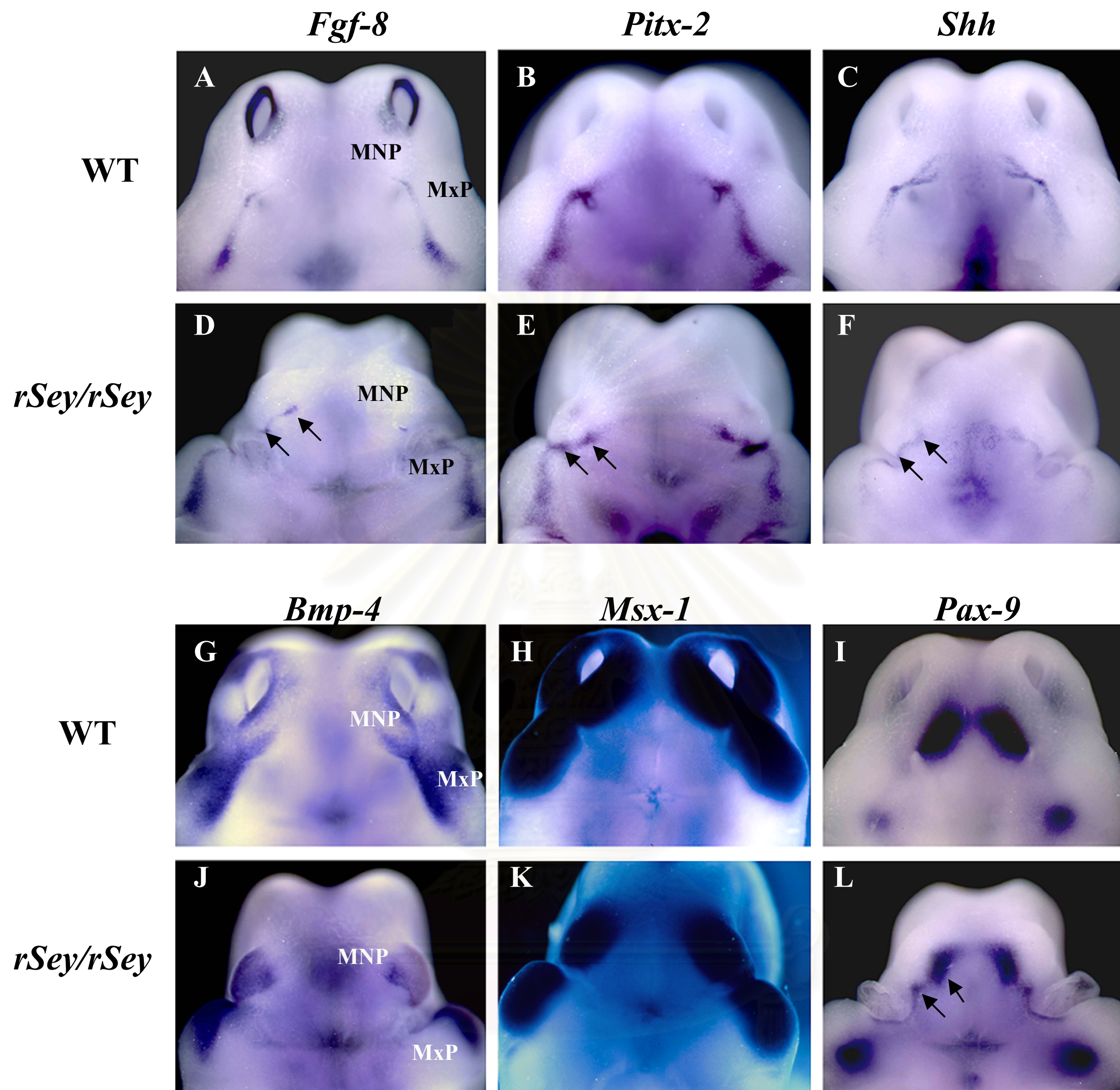


Figure 3.5 Whole mount in situ hybridisation of the wild type (A-C, G-I) and homozygous *rSey* (D-F, J-L) embryos at E13.0 showed the expression pattern of *Fgf-8, Pitx-2, Shh, Bmp-4, Msx-1* and *Pax-9* in the maxillary arch. The expression of *Fgf-8* was faintly seen and the *Pitx-2* and *Shh* were expressed in the oral epithelium in the wild type embryos. Homozygous *rSey* embryos showed the *Fgf-8, Pitx-2, Shh* and *Pax-9* expression indicating the separated M-PDP and L-PDP in the MNP (arrows in D-F, L) and *Bmp-4* and *Msx-1* were expressed in the mesenchymal covering the M-PDP and L-PDP (J, K).

We further investigated and compared these gene expression patterns at E13.5-E14.0 between wild type and homozygous *rSey* embryos by whole mount in situ hybridisation and the tissues at the incisor tooth germ were cut frontally. At E13.5, the M-PDP and L-PDP developed at the boundary of MNP-MxP fusion in the wild type embryos (Figure 3.2B, F) and the expression of *Fgf-8* was faintly seen in these dental placodes (Figure 3.6A, D). The *Pitx-2* and *Shh* were strongly expressed at these dental placodes indicating the M-PDP and L-PDP formation of maxillary incisor (Figure 3.6B, C, E, F). In the homozygous *rSey* embryos, the intense expression of *Fgf-8, Pitx-2* and *Shh* were observed at the separated M-PDP and L-PDP in the MNP region (Figure 3.6 G-L). Interestingly, we found the expression of *Fgf-8*, *Pitx-2* and *Shh* in dental epithelium at the prospective site of facial process fusion in MxP which indicated that this region was the rudiment of primary dental placodes (Figure 3.6G-I short arrows).

The expression of *Bmp-4, Pax-9* and *Msx-1* in dental mesenchyme was observed in the regions marked for the formation of M-PDP and L-PDP in wild type embryos (Figure 3.7A-F). We clearly showed that these expression patterns were also observed in the separated M-PDP and L-PDP in the homozygous embryos (Figure 3.7G-L).

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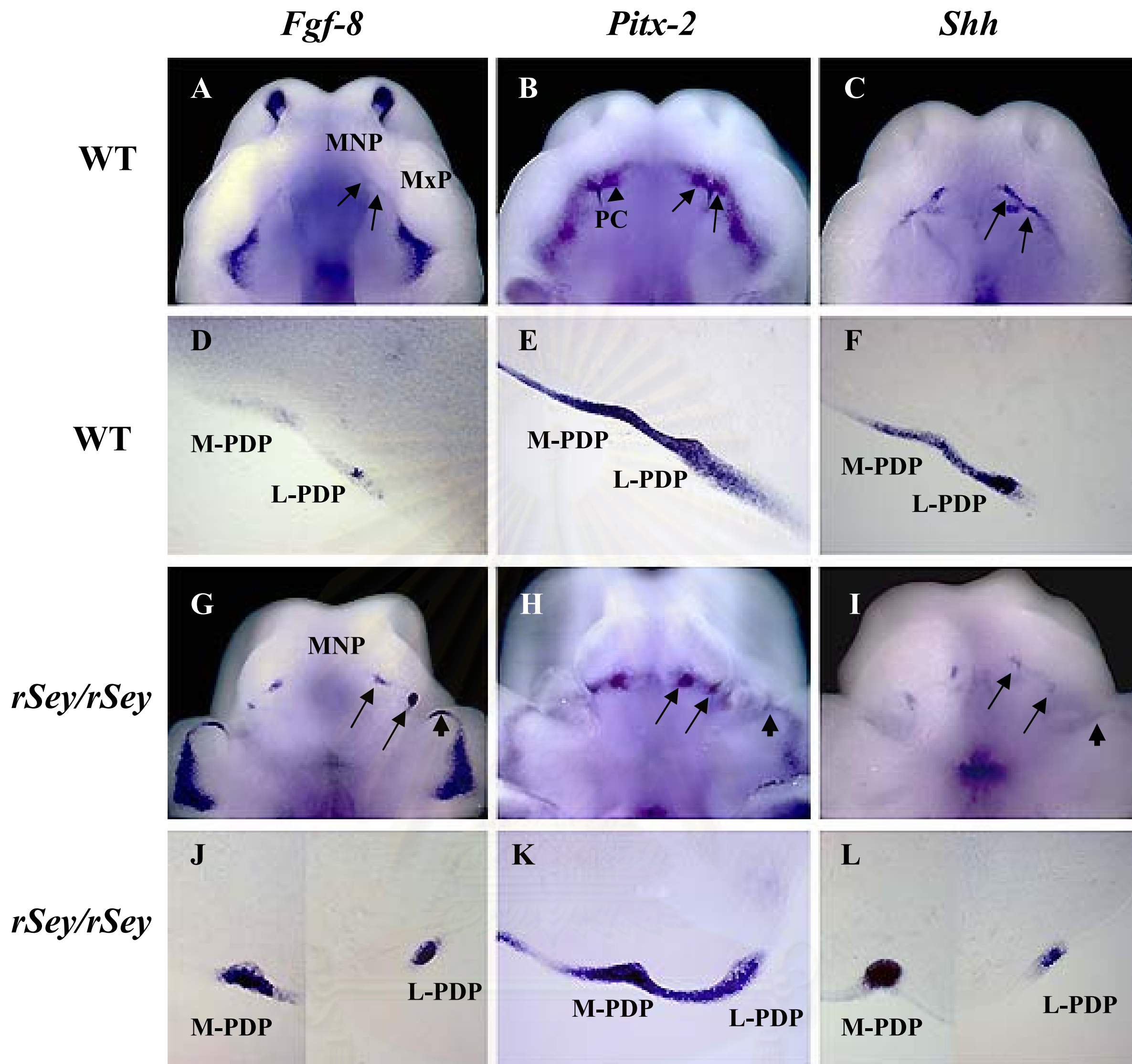


Figure 3.6 Whole mount in situ hybridisation and frontal sections showed the expression pattern of *Fgf-8, Pitx-2* and *Shh* in the wild type (A-F) and homozygous *rSey* (G-L) embryos at E13.5. The M-PDP and L-PDP expressed *Fgf-8, Pitx-2* and *Shh* (arrows in A-C, D-F) in the wild type embryos. The expression of *Fgf-8, Pitx-2* and *Shh* indicated the separated M-PDP and L-PDP developed in the distance in the homozygous embryos (arrows in G-I, J-L) compared to those of wild type embryos. Short arrows indicated the expression of *Fgf-8, Pitx-2* and *Shh* in the remnant dental placodes in the MxP of homozygotes. Arrowheads indicated the primary choana. Abbreviations: MNP, medial nasal process, MxP, maxillary process; M-PDP, medial primary dental placode; L-PDP, lateral primary dental placode; PC, primary choana.

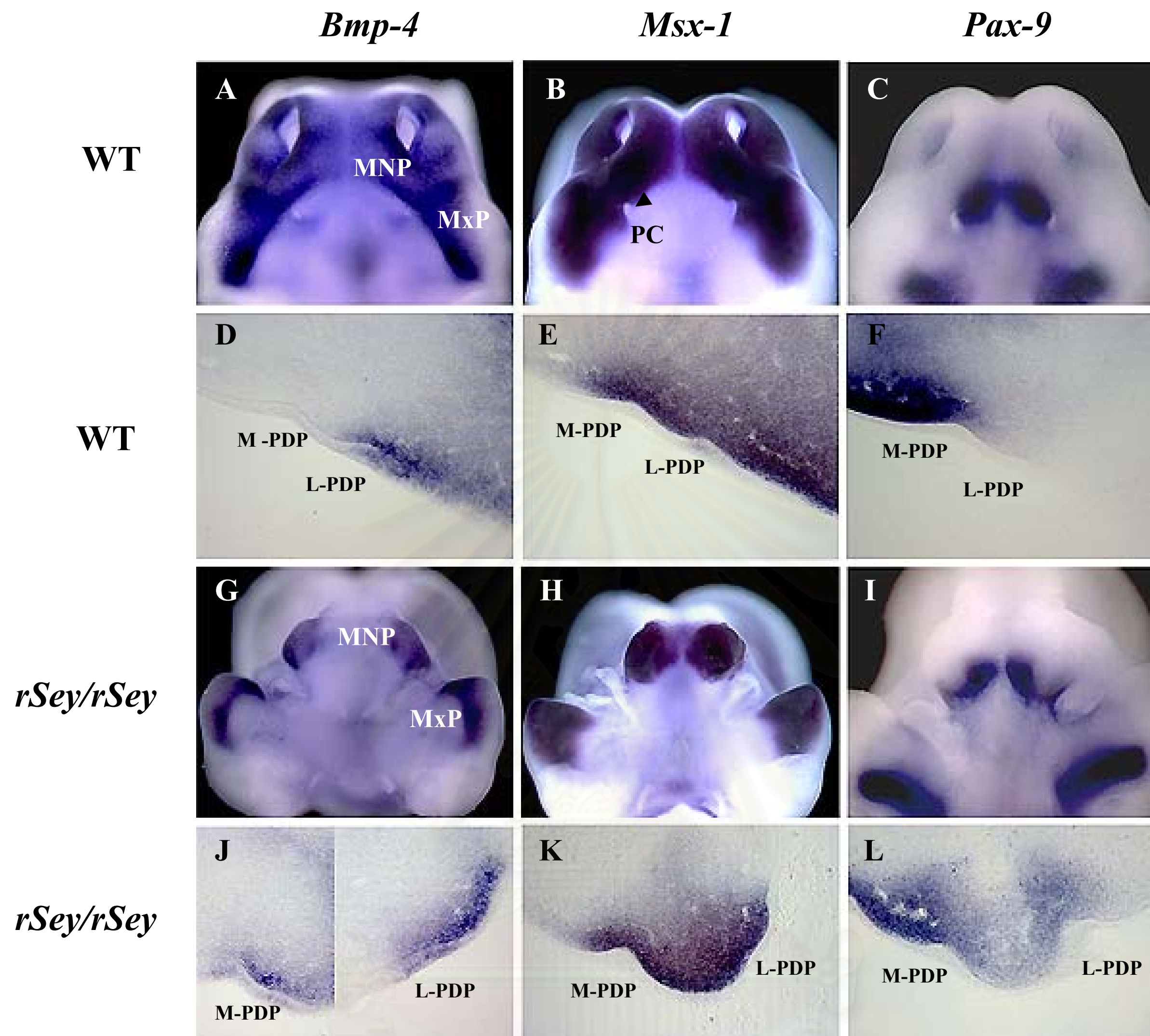


Figure 3.7 Whole mount in situ hybridization and frontal sections showed the expression pattern of *Bmp-4, Msx-1* and *Pax-9* of wild type (A-F) and homozygous *rSey* (G-L) embryos at E13.5. The underlying mesenchyme of the separated M-PDP and L-PDP of the homozygous *rSey* embryos expressed these genes as those observed in the wild type embryos. Abbreviations: MNP, medial nasal process, MxP, maxillary process; M-PDP, medial primary dental placode; L-PDP, lateral primary dental placode; PC, primary choana.

At E14.0, the PDPs fused and developed into the single dental placode of maxillary incisor in the wild type embryos (Figure 3.2C, G). The weak expression of *Fgf-8* and strong expression of *Pitx-2* and *Shh* were observed at the single dental placode formation in wild type embryos (Figure 3.8A-F). Expression of *Bmp-4* was observed in both the dental placode and the underlying mesenchyme whereas intense expression of *Pax-9* and *Msx-1* were observed in the dental mesenchyme (Figure 3.9A-F). The expression of *Fgf-8, Pitx-2*, *Shh, Bmp-4, Pax-9* and *Msx-1* were observed in the separated M-DP and L-DP developed in the homozygous embryos (Figure 3.8 and 3.9, G-L). Remarkably, the expression of *Fgf-8* in L-PD was stronger than that of M-DP (Figure 3.8 G, J) while weaker expression of *Pax-9* was observed in underlying mesenchyme of L-PD (Figure 3.9L).

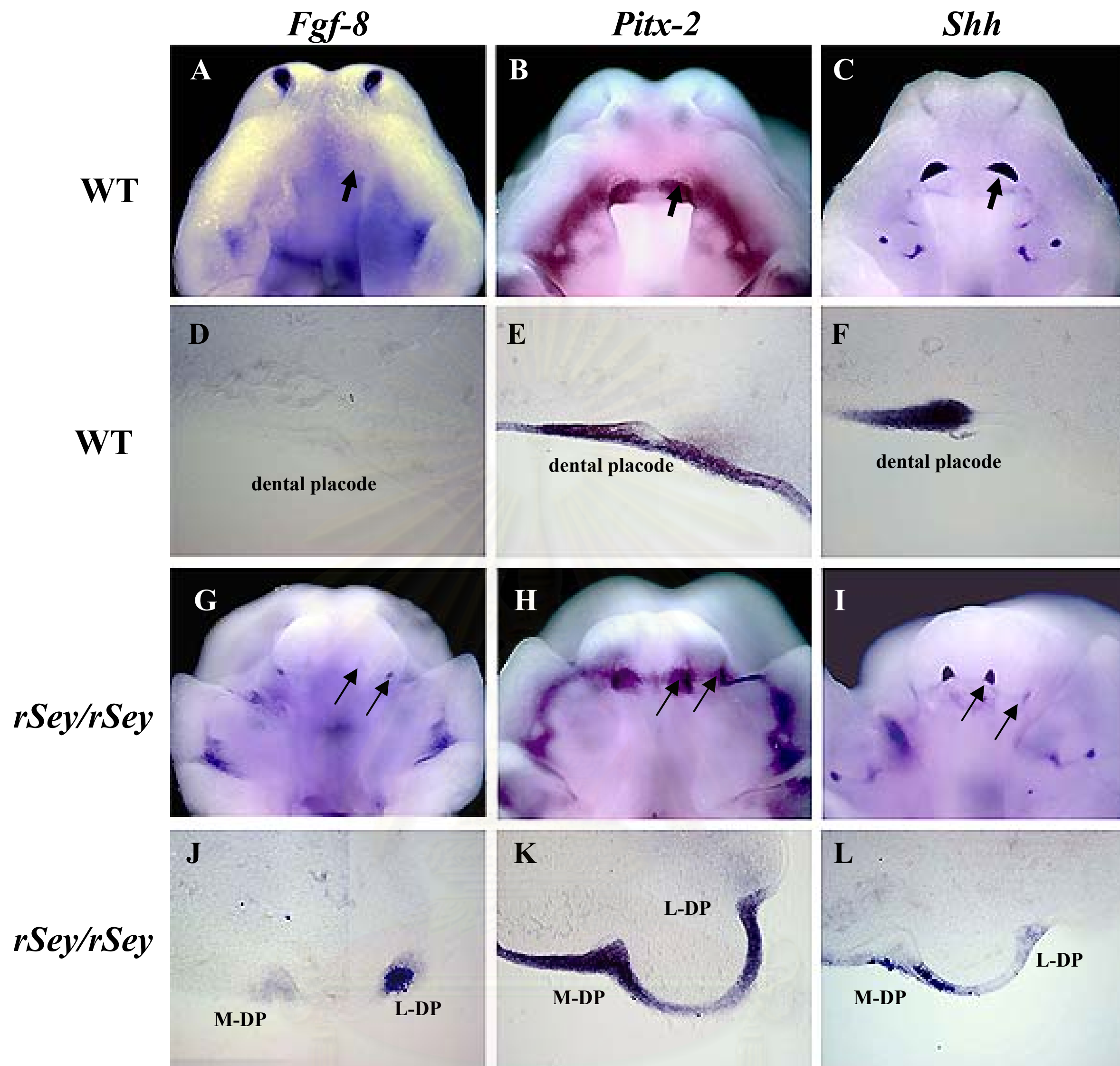


Figure 3.8 Whole mount in situ hybridisation and frontal sections showed the expression pattern of *Fgf-8, Pitx-2* and *Shh* of the wild type (A-F) and homozygous *rSey* (G-L) embryos at E14.0. The single dental placodes developed in the wild type embryos while as the M-DP and L-DP were kept separated in the homozygous *rSey* embryos. Thick arrows indicated the single dental placode in the wild type embryos; Arrows indicated the separated M-DP and L-DP in the homozygous *rSey* embryos. Abbreviations: M-DP, medial dental placode; L-DP, lateral dental placode.

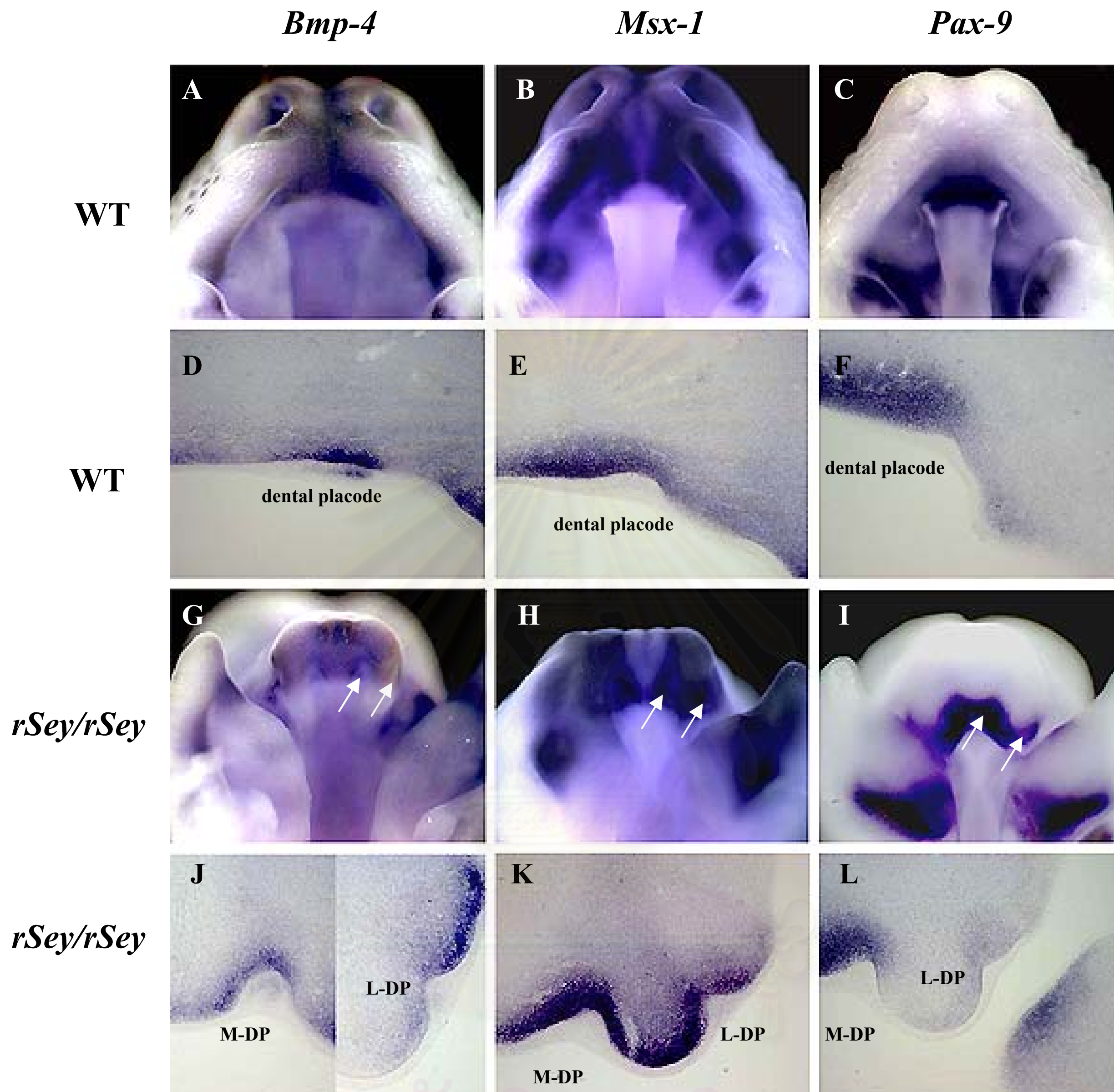


Figure 3.9 Whole mount in situ hybridisation and frontal sections showed the expression pattern of *Bmp-4, Msx-1* and *Pax-9* of the wild type (A-F) and homozygous *rSey* (G-L) embryos at E14.0. These gene expressions indicated the single dental placodes of maxillary incisor developed in the wild type embryos and the separated M-DP and L-DP developed in the homozygous *rSey* embryos. Arrows indicated the separated M-PD and L-PD in the homozygous *rSey*. Abbreviations: M-DP, medial dental placode; L-DP, lateral dental placode.

### 3.4 Discussion

**The defect of facial process fusion, facial cleft, led to the separation of primary dental placode formation of maxillary incisor in the homozygous *rSey***

Histological analysis showed that morphogenesis of maxillary incisor at the initial stage in the homozygous *rSey* was different from that of the wild type. The primary dental placodes, M-PDP and L-PDP, were observed in the MNP. In contrast to the wild type, the fusion of M-PDP and L-PDP did not take place. Consequently, the M-PDP and L-PDP developed into the medial and lateral tooth bud separately. Moreover, we found the remnant of primary dental placode developed in the MxP at the prospective site of MNP and MxP fusion. Our previous work interpreted that the M-PDP and L-PDP developed at the boundary of the facial process fusion in the wild type embryo and the period of the primary dental placode fusion was concomitant with the facial process fusion. It implied that the maxillary incisor has two odontogenic origins which were pre-assigned in the dental epithelium of MNP and MxP. The mechanism of facial process fusion might contribute to the fusion of pre-assigned primary dental placodes to develop into a single maxillary incisor dental placode. Failure of facial process fusion in homozygous *rSey* might result in the separation of primary dental placodes. According to the size of homozygous primary dental placode developed in the MNP and MxP, L-PDP was smaller than the M-PDP and the remnant of primary dental placode in the MxP was the smallest. Expression of *Pitx-2* or *Shh* at the boundary of primary choana marked the origin of L-PDP developed immediately at the

MNP and MxP fusion site (Figure 3.6 B, C). We assumed that at the early development, MxP fused to the MNP and might give rise to L-PDP at the fusion site. Consequently, the M-PDP in the MNP and L-PDP merged and fused each other relying on the mechanism of MNP and MxP fusion. Thus, the failure of MNP and MxP fusion resulted in the separated primary dental placode formation in the homozygous *rSey* (Figure 3.10).

Our results were supported by the studies of the defect of facial process fusion; cleft lip and palate; in monkey and human. In monkey, normal development of maxillary lateral incisor was found in the monkeys with the cleft lip and palate induced with cyclophosphamide. The defect of cleft lip and cleft palate could not effect on the abnormality in primary and permanent maxillary lateral incisor formation because the origin of maxillary lateral incisor derived from the dental placode immediately lateral to fusion area between the MNP and MxP in the normal condition (Wei et al., 2002). Thus, the defect of facial process fusion influenced the abnormal dental formation which was originated in the facial process fusion at the early development.

The human with the cleft lip and palate led to the developmental defect on the primary and permanent maxillary lateral incisors (Bohn, 1950, 1963; Tsai et al., 1998; Hensen K and Mehdinia M, 2002). The previous studied showed that the origin of maxillary lateral incisor dental placode correlated to the MNP and MxP fusion at early development (Ferenczy, 1958; Oeé 1958; Lisson and Kjær, 1997). The defect of MNP and MxP fusion leading to the cleft lip and palate interrupted the maxillary lateral incisor development in the human. Four distribution patterns of the dentition in cleft patients were classified according to the number and location of the maxillary lateral

incisor between the cleft side as shown in Figure 3.11. These patterns showed the absence or the supernumerary lateral incisor developed in the medial and/or lateral to the cleft side (Tsai et al., 1998). The authors explained that there might be two odontogenic tissues located in the MNP and MxP and each of them could develop into individual tooth if they had adequate amount of the tissues. Classic experiments also demonstrated that tooth bud could be divided surgically and develop into two teeth (Glasstone, 1965). If the tooth bud was unequally divided, only the larger part developed into tooth. In our studies, there were three PDPs formed in the non-fused MNP and MxP in homozygous *rSey*. All of the largest placode; M-PDP; developed successfully into maxillary incisor whereas the smallest one in MxP never developed further into bud stage. One-fourth of the medium sized placodes: L-PDP; developed into incisor-like structure. Thus, the size of the dental placode implying the amount of odontogenic tissues might be involved in the potentiality to develop into the functional tooth of them.

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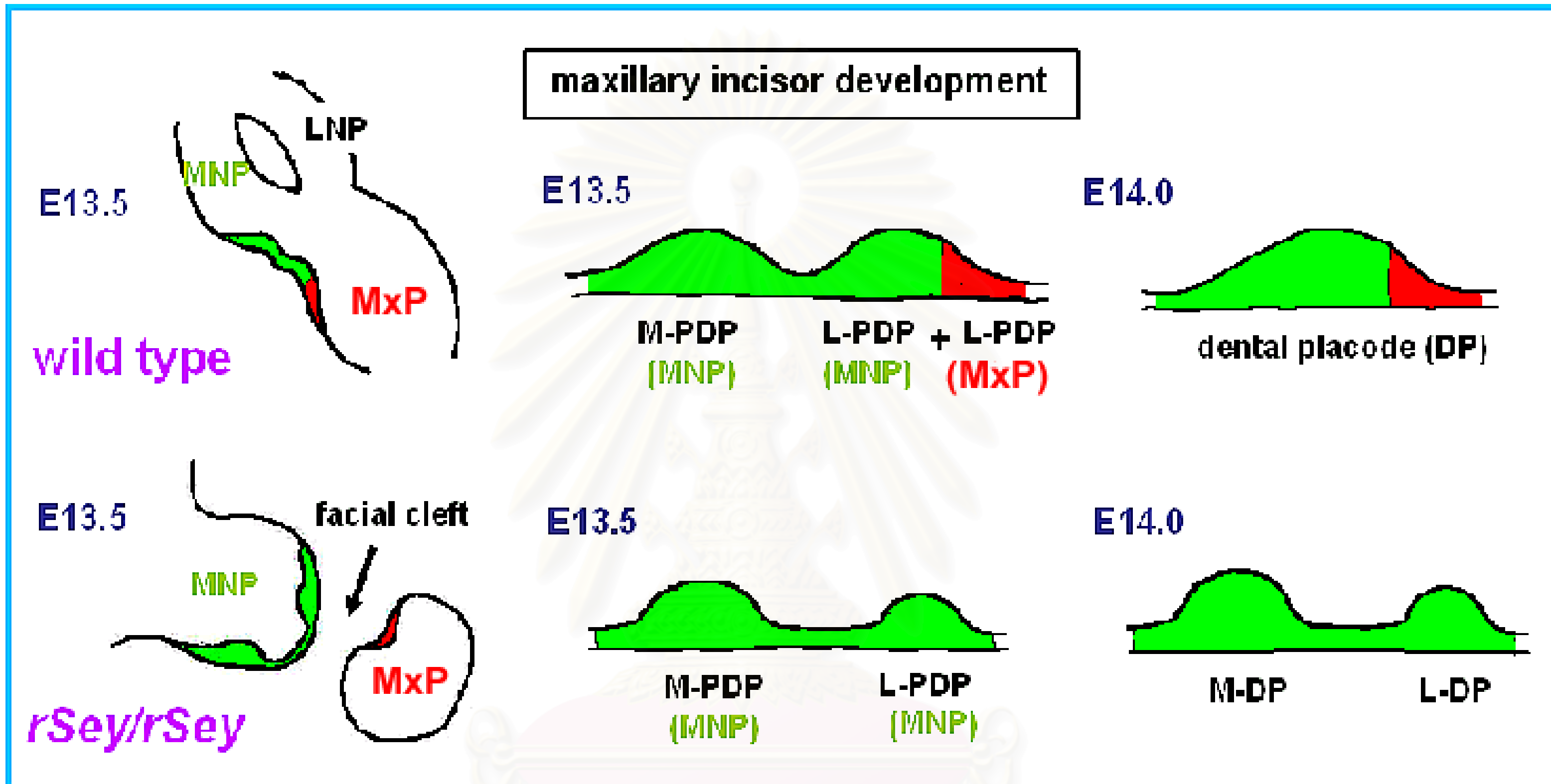


Figure 3.10 Schematic illustration of maxillary incisor formation of wild type and homozygous *rSey* embryos at E13.5-14.0. Green color indicated the epithelium derived from MNP and the red color indicated the epithelium derived from MxP. Wild type showed the M-PDP and L-PDP formed at the boundary of MNP-MxP fusion. L-PDP origin might locate relatively to epithelial bridge between the MNP and MP (green and red color). The M-PDP and L-PDP subsequently fused to develop into maxillary incisor dental placode concomitantly with the MNP-MxP fusion. The defect of facial process fusion, MNP and MxP, in the homozygous rSey might not only inhibit the contribution of MxP (red color) to L-PDP in the MNP but also leave the pre-assigned M-PDP and L-PDP developed separately in the MNP.

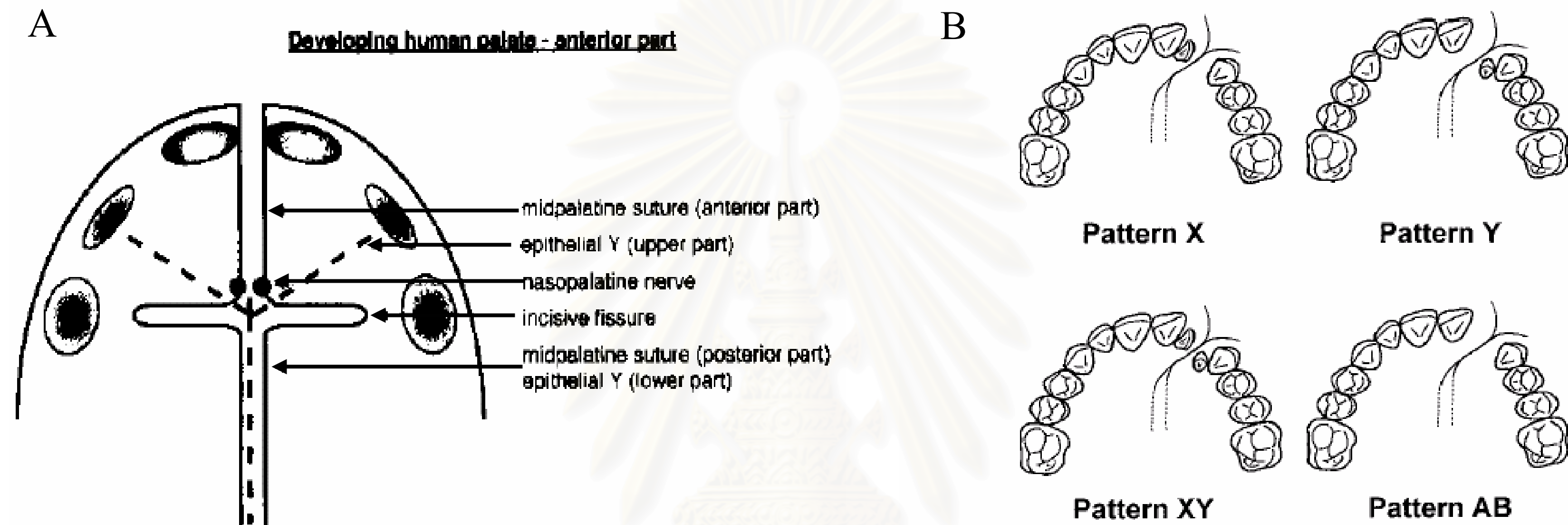


Figure 3.11 **A**: Schematic illustrates the stages in the normal development of the anterior part of the human palate. Broken lines indicate the position of epithelial Y, the epithelial fusion line between the soft tissue palatal shelves derived from MxP and premaxillary mucosa derived from MNP, in the early stage. Unbroken lines indicate the position of the incisive fissure and mid palatine suture at the later development. Note that the anterior extensions of epithelial Y are direct toward the lateral incisors, while the incisive fissures are directed toward the canines. In cleft palate patients, the cleft of the palate followed the broken lines (Lisson and Kjaer, 1997).

**B**: Four distribution patterns of cleft lip and palate patients affected the maxillary lateral incisor are identified by using the number and location of the teeth between the cleft side maxillary central incisor and the canine. Pattern X, in which one tooth is mesial to the alveolar cleft; Pattern Y, in which one tooth is distal to the alveolar cleft; Pattern XY, in which one tooth is located on either side of the alveolar cleft; Pattern AB, in which there is no tooth between the maxillary central incisor and the canine on the cleft side (Tsai et al., 1998).

**Tooth-related gene expressions marked the presumptive maxillary incisor and supernumerary incisor like-structure formation in the homozygous *rSey***

Prior to maxillary incisor dental placode formation at E12.0-12.5, the expression of *Fgf-8, Bmp-4, Pax-9* and *Msx-1* were observed in the MNP region of the wild type. These expressions were also observed in the homozygous *rSey*. The results indicated that at this stage there was no difference between the presumptive maxillary incisor formation of the wild type and the homozygote. At E13.0, the histological investigation demonstrated that the M-PDP and L-PDP developed separately in the MNP of the homozygous *rSey* while the oral epithelial thickening just started at the boundary of MNP and MxP region of the wild type. The results from the previous chapter demonstrated that MNP and MxP started fusion to make the mid-face and might supply all oral epithelium of the upper arch at E13.0. Subsequently, the pre-assigned dental epithelium developed into the M-PDP and L-PDP at E13.5. Thus, lack of the facial process fusion in the homozygote might lead to earlier development of the M-PDP and L-PDP in MNP. Obviously, the intense expression of *Fgf-8* was observed at the M-PDP and L-PDP region in the homozygote at this stage while the expression of this gene seemed to be down regulated in the wild type. Moreover, we found the *Pax-9* expression in the mesenchyme beneath the epithelial *Fgf-8* expression. It suggested that Fgf-8 signaling in the dental epithelium might be inhibited during primary dental placode fusion of wild type maxillary incisor while this signaling might be required for dental epithelium proliferation and induction of the *Pax-9* expression in formation of separated primary dental placodes in the homozygote. Subsequently, the expression of

*Fgf-8, Shh* and *Pitx-2* in the dental epithelium and expression of *Bmp-4, Pax-9* and *Msx-1* in the mesenchyme were observed at the site of M-DP and L-DP formation as well as at the remnant dental placode in the MxP of the homozygous embryos at E13.5-14.0. These results supported the histological analysis indicating that the primary dental placodes separately developed in the MNP and MxP of the homozygous *rSey*. The M-DP of the homozygote and the single dental placode in the wild type expressed the same cascade of tooth-related genes whereas inconsistent expression patterns of tooth-related genes were observed in the L-DP. These results demonstrated that the M-PDP might have adequate odontogenic potentiality to develop into normal incisor, while L-PDP might require more potentiality derived from the MNP-MxP fusion since only one-fourth of the L-PDP succeeded to develop into incisor-like structure. Most of the L-PDPs as well as all the remnant dental placodes in the MxP were arrested at early stage of tooth development. It suggested that both MNP and MxP might supply the odontogenic tissues of these dental placodes to develop into the functional maxillary incisor.

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# CHAPTER IV

# CONCLUSION AND FUTURE STUDIES

The histological study of developmental process in wild type rat embryos demonstrated that the single functional maxillary incisor developed from fusion of two odontogenic origins. The formation of the maxillary incisor was initiated by invagination of two regions of oral epithelium in the frontonasal process to form PDPs which eventually fused to form a single dental placode. The dental placode then proceeds the developmental process through bud, cap, bell and cytodifferentiation to form the functional maxillary incisor.

The expression of the tooth related genes; *Bmp-4, Fgf-8, Msx-1* and *Pax-9*; observed in mandibular molar were also observed in maxillary incisor. Previous studies demonstrated that specific genes expressed in dental epithelium and ectomesenchyme determined the type of tooth formation prior to dental placode formation. The presumptive mandibular incisor expressed *Msx-1* and *Pax-9* while the presumptive mandibular molar expressed *Barx-1* and *Pax-9*. The gene expression patterns in presumptive maxillary incisor in this study were correlated to those observed in the mandibular incisor. However, we could not conclude that the expression patterns were different from those of the molar since we did not investigate the expression of *Barx-1* in this study. At later stage when the type of tooth had been determined, it seemed that dental placodes of maxillary incisor and mandibular molar expressed the same cascade

of tooth-related genes; *Fgf-8, Pitx-2, Shh*, *Bmp-4, Msx-1,* and *Pax-9*; which were studied here.

The data reported in chapter 2 supported our first hypothesis that maxillary incisor and mandibular molar had the same cascade of developmental process and the tooth-related gene expression patterns in the stage of dental placode formation onward. Interestingly, prior to its formation, the maxillary incisor dental placode seemed to be more complicated structure than that of the other teeth in dentition. Histological investigation and expression of *Pitx-2* and *Shh* revealed that two primary dental placodes, M-PDP and L-PDP, were formed at the beginning. Subsequently, they merged and fused to develop into a single dental placode of the maxillary incisor. Furthermore, the course of development of the M-PDP and L-PDP related to that of the MNP and MxP fusion. We then presumed that the maxillary incisor dental placode was multi-odontogenic epithelial origins that might be derived from MNP and MxP epithelium and the fusion of M-PDP and L-PDP to make a single maxillary incisor dental placode might rely on the mechanism of facial process fusion. Further studies are needed to proof the contribution of MxP to the dental placodes.

The effect of facial process fusion on the formation of the single dental placode via fusion of the PDPs was investigated in homozygous *rSey* embryos which had facial cleft between the MNP and MxP as described in chapter 3. Histological investigation and the expression patterns of tooth related genes demonstrated that the homozygous *rSey* embryos developed separated dental placodes in the MNP and MxP associated with the facial cleft. We presumed that the M-PDP and L-PDP in the wild type embryos developed at the boundary of MNP and MxP and they merged as the results of

MNP-MxP fusion. Failure of facial process fusion in the homozygous *rSey* embryos resulted in separation of the pre-assigned primary dental placode in each process. M-PDP and L-PDP developed in the MNP and remnant dental placode had odontogenic potentiality and developed in the MxP. These evidences suggested that the MxP might assemble with MNP to form the primary dental placode. However, there was no direct evidence demonstrating that maxillary process was actually involved in the maxillary incisor dental placode formation. Further investigation such as labeling MxP before the MNP-MxP fusion and tracing for their destination should be done to confirm the contribution of MxP in the dental placode.

It was demonstrated that neural crest cells populated in the frontonasal process emigrated from forebrain and anterior midbrain whereas the first branchial arch was populated by neural crest cells emigrating from midbrain and anterior hindbrain. There was evidence demonstrating that posterior midbrain crest cells contributed to the mandibular molar dental mesenchyme (Imai et al., 1996). Our studies described that the maxillary incisor dental placode might be developed by the epithelial combination of both MNP and MxP which implied that the dental mesenchyme contributed to the maxillary incisor formation might possibly be derived from the forebrain, midbrain and/or anterior hindbrain. In the homozygous *rSey,* there was impaired migration of anterior midbrain crest cell towards frontonasal process while forebrain neural crest cells normally migrated and populated in the MNP. Subsequently, frontonasal process protruded to make the MNP without LNP formation. Lack of LNP, the MNP failed to fuse to MxP, resulting in the separation of PDPs. All of the separated M-PDP proceeded to form the maxillary incisor whereas 25% of the L-PDP developed into

supernumerary incisor-like structure. It implied that the forebrain crest cells might contribute to the odontogenic potentiality in the maxillary incisor formation of homozygous *rSey*. It seemed that the M-PDP obtained enough potentiality to develop into normal incisor while the L-PDP did not. Classic experiment reported that the tooth germ could be divided surgically and would develop into two teeth. If it was unequally divided, the bigger part would develop into a single tooth whereas the smaller one would not. It seemed that L-PDP which developed just immediately to the MNP-MxP fusion site might require assembly dental epithelial and ectomesenchyme from the MxP to gain the potentiality to develop into incisor.

The study of the expression patterns of tooth-related genes in homozygous *rSey* did not meet our hypothesis. We hypothesized that the mutation of *Pax-6* gene which caused the truncated Pax-6 protein in homozygous *rSey* might alter the gene signaling in tooth development and the alteration of the gene expressions might cause supernumerary tooth formation. In the homozygous *rSey*, all PDPs including the remnant of dental placode in MxP expressed all the genes studied; *Bmp-4, Fgf-8, Msx-1, Pax-9, Pitx-2* and *Shh*; in the same cascade as those of the wild type did. We then concluded that the mutation of *Pax-6* genes was not directly associated to the formation of supernumerary incisor-like structure via gene controlling. It is more likely that the formation of the supernumerary tooth was caused by the failure of the fusion between MNP and MxP, which might be resulted from the impaired migration of neural crest cells populating in the ectomesenchyme of the frontonasal process.

Unlike other teeth in dentition, maxillary incisor developed from oral epithelium mostly derived from frontonasal process and the ectomesenchyme populated by

forebrain and midbrain crest cells whereas all the other teeth developed from those derived from first branchial arch. However, the developmental process as well as the cascade of gene expression patterns of the tooth related gene of the presumptive maxillary incisor seemed similar to those of the mandibular molar. Thus, we concluded that signaling control of tooth development did not depend on the origins of the oral epithelium and ectomesenchyme.

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