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이차구개 형태분화의 내적 조절유전자 규명

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ABSTRACT

Identification of Intrinsic Regulators in the Secondary Palate Morphogenesis

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이차구개는 발생과정에서 구개선반의 형성과 성장, 거상과 융합의 과정을 통해 형성된다. 이와 같은 이차구개의 형성과정은 미세한 분자유전학적 신호전달기전에 의해 조절되는 것으로 알려져 있어서, 신호전달과정에 관여하는 유전인자의 발현이상이 되면 정상적인 이차구개가 형성되지 못하고 구개파열이라는 선천성 기형이 발생된다. 구개파열의 유발인자들에 대한 많은 연구에도 불구하고 현재까지 정상적인 이차구개의 형성을 조절하는 분자유전학적 기전에 대해서는 명확히 알려져 있지 않다. 따라서 본 연구에서는 이차구개의 형태분화를 조절하는 분자유전학적 기전을 알아보고자, 이차구개 형성의 내적 조절인자 중 핵심유전자로 알려져 있는 *Osr2*가 결손된 생쥐의 이차구개 형성과정에서 정상생쥐에 비해 발현의 변동이 나타나는 유전자를 확인하였다. 유전자 발현의 변동은 발생 14.5일(E14.5)의 구개선반으로부터 추출한 total RNA를 이용하여 ACP-based GeneFishing PCR을 시행하여 확인하였고, 각각의 변동된 유전자를 동정하여 정상생쥐의 이차구개 형성과정에서의 발현양상을 *in situ* hybridization을 시행하여 확인하였다. 총 120쌍의 primer를 이용한 검색을 통해서 정상생쥐의 구개선반에 비해 mutant에서 발현이 변동된 유전자는 7개가 검출되었고, 이들은 모두 정상생쥐에 비해 mutant에서 발현이 증가되는 것으로 확인되었다. 검출된 유전자는 vimentin(*Vim*), β-tropomyosin 2(*Tpm2*), thioredoxin-like 5(*Txnl5*), procollagen type II alpha 1(*Col2a1*), Insulin-like growth factor binding protein 7(*IGFbp7*), Sui 1 homologs(*Sui1*), Defender against cell death 1(*Dad1*)이었다. 검출된 유전자를 동정하여 정상생쥐의 구개 형성과정에서의 발현양상을 알아본 결과, *Col2a1*을 제외한 유전자들은 모두 E13.5의 구개선반에서 특이적으로 발현되고 있었으나 구개선반이 융합된 E15.5에서는 *Vim, Txnl5* 그리고 *Dad1*만이 봉합선을 따라 발현이 지속되고 있었다. 이상의 결과로 보아 검출된 유전자들은 구개선반의 형태분화과정에서 발현되어 이차구개의 형성과정에 관여할 것으로 여겨진다. 또한 이들은 이차구개 형성의 내적조절인자인 *Osr2*의 downstream target으로 구개선반의 성장과 융합과정에 직접적으로 관여하는 유전물질일 것으로 추정된다.

Key Word : *Osr2*, palatogenesis, intrinsic regulator, differential expressed gene, knockout.

I. Introduction

The palate develops from the primary and secondary components. The primary palate formed from the frontonasal and medial nasal processes. The secondary palate arises

from the maxillary processes that develop paired swellings, initially called lateral palatal processes. These processes grow downward, as the lateral palatal shelves, on either side of the tongue before becoming elevated to a horizontal position above the tongue. The medial edge epithelium (MEE) of the two opposing palatal shelves then fuse to form the midline seam, which subsequently degenerates through a combination of epithelial–mesenchymal transformation, cell migration and apoptosis to create an intact palate separating the oral and nasal cavities. The palatal shelves also fuse with the nasal septum dorsally and the primary palate anteriorly[1].

The secondary palate of mammals forms through a highly regulated and complex process characterized by a series of events involving cell proliferation and migration, cell differentiation, production of extracellular matrix, and cell death[1]. Disturbance of the events affecting palatogenesis results in cleft palate, a failure of the palate to close. Cleft palate occurs frequently, affecting approximately 1 in 1500 births; it is usually considered a sporadic occurrence resulting from an interaction between genetic and environmental factors[2]. Mouse models have been widely used to study the etiology of these defects since the morphogenetic and molecular processes of craniofacial development are strikingly similar in the mouse and human. In the mouse, a variety of loss of function mutations that result in isolated cleft palate have been described[3]. Studies with targeted mutations in mice have revealed a growing number of genes play crucial roles in the formations of secondary palate. These genes encode a variety of molecules including transcription regulators[4–9], growth factors[10–11], enzymes for signaling molecule synthesis[12–13], receptors[14–16] and adhesion molecules[17].

Recently, a mouse odd-skipped related 2 (*Osr2*) gene was identified as a homologue of Drosophila pair-rule gene, odd-skipped. The *Osr2* gene encodes a zinc finger protein with extensive sequence similarity to the Drosophila odd-skipped family of putative transcription factors[18]. The *Osr2* gene is specifically activated in the nascent palatal mesenchyme at the onset of palatal outgrowth[18]. During mouse embryogenesis, *Osr2* mRNA expression marks the medial maxillary regions where the initial palatal outgrowths occur and persists strongly in the downward growing palatal shelves. Upon palatal shelf elevation and fusion at the midline, *Osr2* expression in the palate is downregulated[18]. The targeted disruption of *Osr2* gene in mice showed in impairment of palatal shelf growth and causes delay in palatal shelf elevation, resulting in cleft palate[19]. Whereas palatal outgrowth initiates normally in the mutant embryos, significant reduction in palatal mesenchyme proliferation occurs specifically in the medial halves of the downward growing palatal shelves at E13.5, which results in retarded, mediolaterally symmetric palatal shelves before palatal

shelf elevation. These data strongly support that *Osr2* is a key intrinsic regulator of palatal growth and patterning[19]. However, it is not identified that not only the downstream targets of *Osr2* in the developing palatal shelves but also the molecular mechanisms in the inhibition of palatal mesenchymal proliferations. These genes may be regulated by *Osr2* transcription, which could be more direct evidence of cleft palate caused by *Osr2* deletion. In addition, the closure of the secondary palate involves intrinsic factors in the palatine shelves, the nature of which has not been determined yet. Therefore, we purposed to identify the alterations of gene expression profiles in the secondary palate of *Osr2* gene targeted mice to elucidate the molecular genetic regulatory mechanisms of secondary palate formation.

II. Materials and Methods

1. *Animals and tissue preparations*

To generate *Osr2* null mouse embryos, *Osr2* heterozygous mice were used as described before[19]. *Osr2* heterozygous mice were intercrossed for generation of homozygous null embryos in the evening. If a vaginal plug was found, the following morning was designated as days 0.5 of gestation (E0.5). Embryos were taken on E14.5, in which the morphogenesis of palatal shelves were arrested immediately after by the targeted disruption of *Osr2*[19]. Time-scheduled pregnant mice were killed by cervical dislocation and the embryos were isolated free of extraembryonic membranes in phosphate-buffered saline (PBS, pH 7.4). Embryos were genotyped by PCR, using genomic DNA extracted from the yolk sac. PCR with primer 1 (5'-GAT ACG GGT AAG ACA GAA ACT G-3') and primer 2 (5'-CTA CAA GGA TCT AGC ACA TGC TG-3') amplified a product of 490 bp from the wild-type *Osr2* allele. PCR with primer 2 and primer 3 (5'-CTT CTT GAC GAG TTC TTC TGA GG-3') amplified a mutant allele-specific product of 460 bp. Wild type, heterozygous and homozygous null embryos of littermates were used for analysis of gene expression profile. Palatal shelves were carefully dissected from each embryos for total RNA isolation.

2. *Total RNA extraction*

Total RNA was isolated by the standard guanidium isothiocyanate method using Trizol reagent (Invitrogen, Gaitersburg, MD) after homogenization with a polytron homogenizer. RNA was quantified by determination of absorbance at 260 nm. The integrity of RNA was qualified by the ratio of absorbance at 260 nm and 280 nm, which was higher than 1.9.

3. *First-strand cDNA Synthesis*

Total RNAs extracted from samples were used for the synthesis of first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42°C in a final

reaction volume of 20 ㎕ containing 3 ㎍ of the purified total RNA, 4 ㎕ of 5 × reaction buffer (Promega, Madison, WI), 5 ㎕ of dNTPs (each 2 mM), 2 ㎕ of 10 ㎛M dT-ACP1 (5'-CTGTGAATGCTGCGACTACGATIIIIIT(18)-3'), 0.5 ㎕ of RNasin® RNase Inhibitor (40 U/ ㎕ Promega), and 1 ㎕ of Moloney murine leukemia virus reverse transcriptase (200 U/ ㎕ Promega). First-strand cDNAs were diluted by the addition of 80 ㎕ of ultra-purified water for the Gene Fishing™ PCR and stored at -20°C until use.

4. *ACP-based Gene Fishing™ PCR*

Differentially expressed genes were screened by ACP-based PCR method[20] using the Gene Fishing™ DEG kits (Seegene, Seoul, Korea). Briefly, second-strand cDNA synthesis was conducted at 50°C during one cycle of first-stage PCR in a final reaction volume of 20 ㎕ containing 3-5 ㎕ (about 50 ng) of diluted first-strand cDNA, 1 ㎕ of dT-ACP2 (10 ㎛M), 1 ㎕ of 10 ㎛M arbitrary ACP, and 10 ㎕ of 2 × Master Mix (Seegene). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, followed by a 5 min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide.

5. *Direct Sequencing*

The differentially expressed bands were re-amplified and extracted from the gel by using the GENCLEAN™II Kit (Q-BIO gene, Carlsbad, CA), and directly sequenced with ABI PRISM™3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA).

6. *Cloning and synthesis of cRNA probes*

The differentially expressed PCR products were simultaneously cloned into pGEM-T Easy vector (Promega) and sequenced with ABI PRISM™3100-Avant Genetic Analyzer (Applied Biosystems). Information of each clone to synthesize cRNA probe was listed in Table 1.

The RNA probes for in situ hybridization were generated by in vitro transcription from each cDNA templates extended the SP6 or T7 RNA polymerase promotor sequences. The plasmids were linearized with the corresponding restriction enzymes and used as a template to synthesize sense or anti-sense RNA transcripts labeled with digoxigenin-UTP (Roche Diagnostics GmbH, Mannheim, Germany).

7. *Whole mount in situ hybridization*

C57BL/6 mice were used at embryonic stages. The age of the mouse embryos was determined according to the vaginal plug (embryonic day 0.5; E0.5) and confirmed by morphological criteria. Animals were killed by cervical dislocation and the embryos were carefully dissected free of extraembryonic

Table 1. Clones of differently expressed genes (DEGs)

DEG No.	Name of Gene	Insert size (bp)	enzyme for linearization	RNA Polymerase
DEG1	Vimentin (Vim)	1020	Sal I	T7
DEG2	beta tropomyosin 2 (Tpm2)	460	Pst I	T7
DEG3	Thioredoxin-like 5 (Txnl5)	590	Sac II	SP6
DEG4	Procollagen type II alpha1 (Col2a1)	650	BamH I	T7
DEG5	Insulin-like growth factor binding protein 7 (IGFbp7)	340	Sac II	SP6
DEG6	Sui1	540	Sal I	SP6
DEG7	Defender against cell death 1 (Dad1)	640	Pat I	T7

membranes in phosphate-buffered saline (PBS, pH 7.4) under the stereomicroscope from E13.5 to E15.5. Dissected heads including palate were fixed in 4% paraformaldehyde in PBS for overnight at 4°C. Organs were rocked gently throughout the procedure on a mechanical rocking platform unless otherwise indicated. The organs were washed three times with PBS containing 0.1% Tween 20 (PBT), dehydrated into 100% methanol and stored in absolute methanol at −20°C until analysis by whole mount in situ hybridization.

Dissected organs were thawed and treated with 5:1 methanol/30% hydrogen peroxide for 3 to 5 hrs at room temperature. After several washes with methanol, the organs were rehydrated through a methanol-PBT series and washed three times in PBT. Organs were treated with 10 μg/ml proteinase K in PBT for 2～20 min at room temperature depending on the age of embryos, followed by two washes for 5 min each with PBT containing 2 mg/ml glycine, and then two washes with PBT. The organs were refixed in 0.2% glutaraldehyde and 4% paraformaldehyde in PBS for 20 min at room temperature and then washed three times with PBT. Organs were prehybridized for 1 hr at 70°C in hybridization buffer (50% formamide, 0.75 M NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 100 g/ml tRNA, 0.05% heparin, 0.1% BSA, 1% SDS). The hybridization buffer was replaced, and single stranded RNA probes labelled with digoxigenin were added to 1 g/ml, and the embryos were hybridized overnight at 70°C. The organs were washed through three changes of Wash I (0.3 M NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 1% SDS), followed by two washes for 30 min

each at 65°C in Wash I. The embryos were washed twice with Wash II (50 mM NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 0.1% SDS), then once with the same for 30 min at 60°C. The wash buffer was replaced by two changes of RNase buffer (0.5 M NaCl, 10 mM PIPES pH 7.2, 0.1% Tween 20). The organs were incubated with 100 μg/ml RNase A and 100 U/ml RNase T1 in RNase buffer twice for 30 min at 37°C. The organs were washed twice with RNase buffer, twice with Wash III (50% formamide, 300 mM NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 1% SDS), then once at 50°C for 30 min in Wash III. This was followed by two changes through Wash IV (50% formamide, 150 mM NaCl, 10 mM PPIES pH 6.8, 1 mM EDTA, 0.1% Tween 20) after which the embryos were washed at 50°C for 30 min in Wash IV. The embryos were washed twice with Wash V (500 mM NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 0.1% Tween 20), then placed in a heating block at 70°C for 20 min to inactivate endogenous alkaline phosphatases.

The organs were incubated for 1 hr at room temperature with 10% normal goat serum (heat-inactivated just before use at 70°C for 30 min) and 2 mM levamisole (fresh) in TBST (137 mM NaCl, 25 mM Tris-HCl pH 7.6, 3 mM KCl, 0.1% Tween 20). Sufficient embryo acetone powder was heat-inactivated just before use in a small volume of TBST at 70°C for 30 min. Anti-digoxigenin antibody coupled to alkaline phosphatase (Roche Diagnostics GmbH) at a 1/5,000 dilution was preabsorbed for 30 min at 4°C with 1% (w/v) embryo acetone powder in TBST containing 1% heat inactivated normal goat serum and 2 mM levamisole. Debris was removed from the antibody by centrifugation at 10,000 g. Organs were incubated with the preabsorbed antibody overnight at 4°C. The organs were washed three times at room temperature with TBST containing 2 mM levamisole, followed by five to seven washes for 1 hr each at room temperature. The last wash was followed by three changes of NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM MgCl2, 0.1% Tween 20) containing 2 mM levamisole. The colour reaction was initiated by washing the organs into NTMT containing 2 mM levamisole, 4.5 μl/ml NBT (75 mg/ml nitroblue tetrazolium salt in 70% dimethylformamide) and 3.5 μl/ml BCIP (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidine salt in 100% dimethylformamide). Staining was allowed to proceed overnight in the dark at 4°C for moderately prevalent messages, or at room temperature for rare messages. When staining was satisfactory, the organs were washed three times with TBST, then dehydrated through 30, 50, 70 and 100% methanol to intensify the reaction product. The organs were rehydrated and cleared in 50 and 80% glycerol and photographed under a Nikon stereomicroscope using bright field or dark field optics. Composite images were manipulated with Adobe Photoshop software.

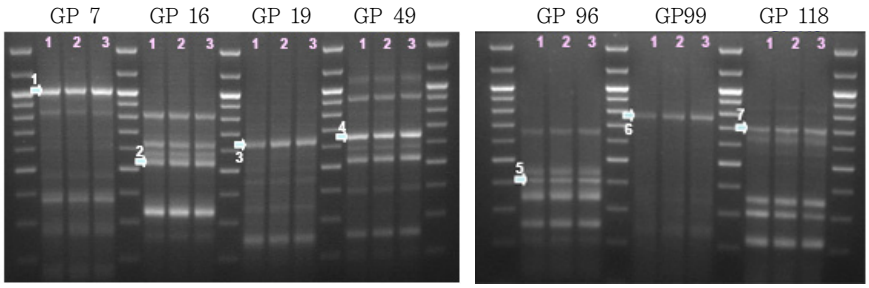

그림 1. Re-amplified DEGs among the *Osr2* wild type (1), heterozygote (2) and homozygotes (3) result from ACP based GeneFishing PCR with 120 pairs of primer sets. Seven DEGs are identified with GP7, GP16, GP19, GP49, GP96, GP99, GP118 primer sets. All DEGs are expressed higher in the homozygotes than heterozygotes and wild type mice.

III. Results

1. Differently Expressed Genes (DEGs) in the palatal shelves among the *Osr2* wild type, heterozygous and homozygous mice at E14.5

To identify the downstream target genes of *Osr2* in palatogenesis, the author took out dissected palatal shelves of wild type, heterozygous and homozygous mice at E14.5, which is a time point of just before palatal shelf elevation, and compare the differently expressed genes in the palatal shelves by the genotype using ACP based GeneFishing PCR methods.

Following the ACP based GeneFishing PCR with 120 pairs of primer sets, seven PCR products – GP7, GP16, GP19, GP49, GP96, GP99, GP118 – were differently amplified among the genotypes (그림 1). Interestingly, all of differently expressed genes were more strongly expressed in the palatal shelves of homozygous mice more than those of the heterozygous mice, and which were more stronger than those of wild type mice. These results mean that all of differently expressed genes are overexpressed by the inactivation of *Osr2* in the palatal shelves around E13.5. Overexpression of these genes cause of the disturbance of growth in the palatal shelves, result in the failure of palatal elevation and fusion.

The amplified PCR products of differently expressed genes were directly sequenced to identify the genetic nature. Sequence homology searchings were performed in the Basic Local Alignment Search Tool (BLAST) of NCBI with the produced sequences. All of differently amplified PCR products (GP7, GP16, GP19, GP49, GP96, GP99, GP118) were fragments of previously identified genes, which were vimentin (*Vim*), beta tropomyosin (*Tpm2*), thioredoxin-like 5 (*Txnl5*), procollagen type II alpha 1 (*Col2a1*), insulin-like growth factor binding protein 7 (*IGFbp7*), Riken cDNA 1500010M16 (*Sui1*), and defender against cell death 1 (*Dad1*), respectively (Table 2). However, none of these differently expressed genes

have not known to be involved in the process of palatal growth and morphogenesis until now.

2. Cloning and Expression Analysis of DEGs during the Palatogenesis

To analyze the expression patterns of DEGs in the palatogenesis, each of PCR products were reamplified and cloned into vectors. Expression patterns of each DEGs were analyzed to confirm their involvement in the palatogenesis. Except DEG4 (*Col2a1*), six DEGs were specifically expressed in the

Table 2. Lists of DEGs by sequence homology searchings the Basic Local Alignment Search Tool (BLAST) of NCBI

DEG No.	Sequence homology search
DEG1	·>gi|58476927|gb|BC089335.1| _ Mus musculus vimentin, mRNA (cDNA clone MGC:102095 IMAGE:64156929), complete CDs Length=1888 Score=1074 bits (542), Expect=0.0 Identities=550/554 (99%), Gaps=0/554 (0%) Strand=Plus/Plus
DEG2	·>gi|202162|gb|MB1086.1|MUSTROB _ Mouse skeletal muscle beta tropomyosin mRNA, complete cds Length=2097 Score=872 bits (440), Expect=0.0 Identities=447/448 (99%), Gaps=1/448 (0%) Strand=Plus/Plus
DEG3	·>gi|20988064|gb|BC030344.1| _ Mus musculus thioredoxin-like 5, mRNA (cDNA clone MGC:40618 IMAGE:3673521), complete cds Length=763 Score=1088 bits (549), Expect=0.0 Identities=558/560 (99%), Gaps=1/560 (0%) Strand=Plus/Plus
DEG4	·>gi|30353887|gb|BC052326.1| _ Mus musculus procollagen, type II, alpha 1, mRNA (cDNA clone MGC:58872 IMAGE:6315379), complete cds Length=4856 Score=1063 bits (536), Expect=0.0 Identities=536/536 (100%), Gaps=1/536 (0%) Strand=Plus/Plus
DEG5	·>gi|6253129|gb|BC092538.1| _ Mus musculus insulin-like growth factor binding protein 7, mRNA (cDNA clone MGC:107287 IMAGE:30284911), complete cds Length=1279 Score=593 bits (299), Expect=1e-166 Identities=315/318 (99%), Gaps=2/318 (0%) Strand=Plus/Plus
DEG6	·>gi|2131215B|ref|NM_026892.1| _ Mus musculus RIKEN cDNA 1500010M16 gene (1500010M16Rik), mRNA Length=885 Score=1098 bits (554), Expect=0.0 Identities=568/575 (98%), Gaps=0/575 (0%) Strand=Plus/Plus
DEG7	·>gi|37046924|gb|BC058116.1| _ Mus musculus defender against cell death 1, mRNA (cDNA clone MGC:69625 IMAGE:5683424), complete cds Length=666 Score=1150 bits (580), Expect=0.0 Identities=609/616 (98%), Gaps=3/616 (0%) Strand=Plus/Plus

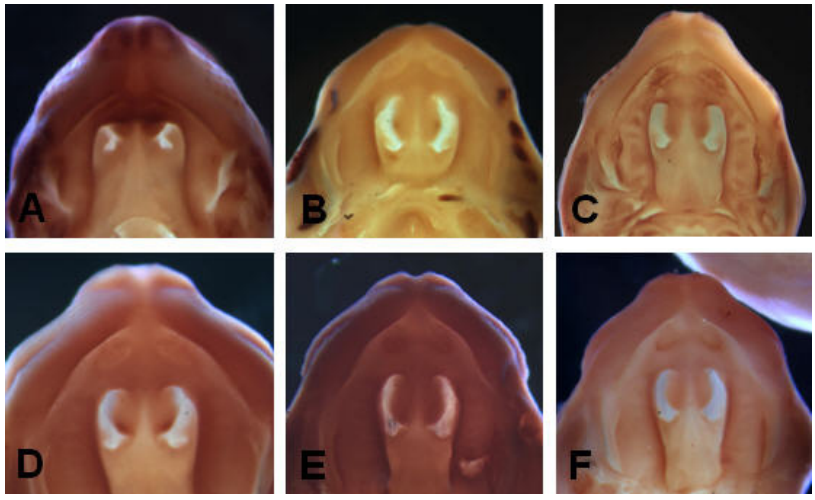

그림 2

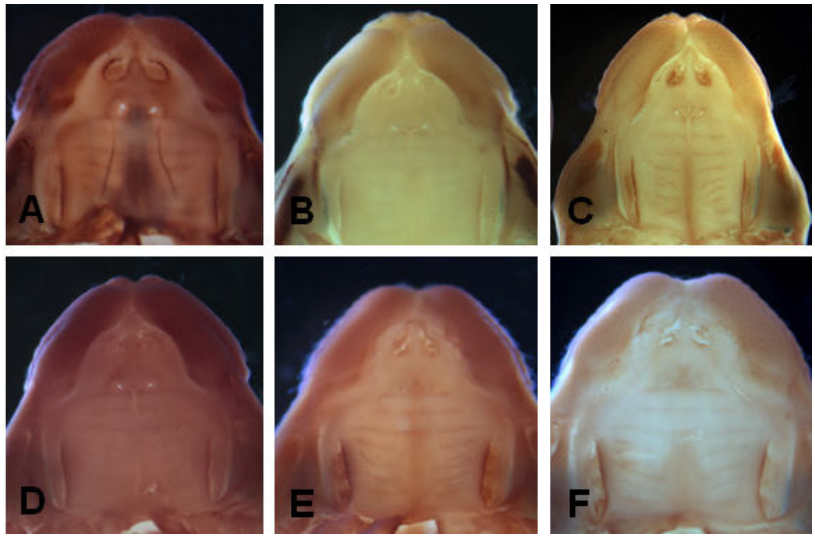

그림 3

그림 2~3. Expression patterns of DEGs in the developing palatal shelves of mouse at E13.5 (Fig. 2) and E15.5 (Fig. 3). All of DEGs are specifically expressed in the growing medial edge of palatal shelves at E13.5 whereas some of them, such as *Tpm2* and *IGFbp7*, are not expressed in the median epithelial edge at E15.5 . A: *Vim*, B: *Tpm2*, C: *Txnl5*, D: *IGFbp7*, E: *Sui1*, F: *Dad1*.

palatal shelves and ridges of E13.5, a time point of palatal growth, which was affected by *Osr2* transcription (그림 2).

At E15.5, immediately after the fusion of secondary palate, expression of *Vim*, *Txnl5*, *Sui1* and *Dad1* were observed around the

midline of palate among the DEGs but those of *Tpm2*, *IGFbp7* and *Col2a1* were disappeared in the palate (그림 3). *Vim* was diffusely expressed in the mesenchyme surrounding the medial edge epithelium of palate whereas *Txnl5* and *Sui1* were clearly expressed in the medial edge epithelium. *Dad1* was specifically expressed in the posterior part of medial edge epithelium, where the process of the palatal fusion was still ongoing. In addition, all of DEGs were expressed in the palatal ridges and growing septal cartilage with variation of their intensity. *Vim, Txnl5, Sui1* and *Dad1* were also expressed in the developing tooth germs both of E13.5 and E15.5 (그림 2, 3).

IV. Discussion

Present study demonstrates that seven DEGs – *Vim, Tpm2, Txnl5, Col2a1, IGFbp7, Sui1, Dad1* – are identified as candidate genes of *Osr2* downstream targets in palatogenesis using the ACP–based GeneFishing method, following sequencing and expression analysis. These results suggest that DEGs may participate as direct targets of transcriptional regulation by *Osr2* in the signaling pathways regulating intrinsic palatal morphogenesis and growth.

1. *Palatogenesis*

In palate development, mesenchymal cells from the neural crest migrate to the primitive oral cavity forming the maxillary processes in association with the oral ectoderm. The primary palate arises from the fusion of two medial nasal prominences that form the intermaxillary segment. After the formation of primary palate, secondary palate initiates to grow from the paired maxillary processes. At a precise developmental time, the palatal shelves elevate to the horizontal position above the tongue and subsequently adhere and fuse with each other at the midline to form the intact palate. Disturbance of palatal shelf growth, elevation, adhesion, or fusion results in cleft palate[1)].

The development of secondary palate is a dynamic process has been arbitrarily split into three stages by the events of palatal shelf[21)]. Stage I of secondary palate development is characterized by formation of the palatal shelves from the maxillary processes. These shelves are orientated vertically, either side of the developing tongue. At stage II, these vertical palatal shelves elevate to a horizontal position above the dorsum of the tongue. This event occurs rapidly, possibly in a matter of hours. Stage III of secondary palate development involves fusion of the medial edge epithelium (MEE) of the approximating palatal shelves with each other via numerous desmosome contacts to form a midline palatal seam. This then separates the oral and nasal cavities. Keratin fibrils and desmosomes are upregulated in the medial edge epithelium seam at this point,

presumably to strengthen the bond between the newly adherent medial edge epithelium cells. This seam rapidly degenerates, a process characterized by loss of complex cytokeratins and basement membrane components such as laminin and desmosomes, and by an increase of vimentin-rich connective tissue, tenascin, proteoglycan and collagen expression[1]. The increase in vimentin is particularly interesting as it is generally found as a mesenchymal intermediate filament. Medial edge epithelium degeneration allows mesenchymal cells to flow across the new intact horizontal palate. An alternative view is that medial edge epithelium cells migrate nasally and orally out of the medial edge epithelium seam and become incorporated into the oral and nasal epithelia on the palatal surface[22]. Transforming growth factor-β3 is known to be abundantly expressed by rodent medial edge epithelial cells; TGF-β3-null mice exhibit an incompletely penetrant failure of the palatal shelves to fuse, leading to cleft palate[11,23]. Rescue experiments have demonstrated the importance of TGF-β3 in palate development, possibly through the induction of filopodia on the surface of medial edge epithelial cells[24].

Cleft palate may result from disturbances at any stage of palate development: defective palatal shelf growth, delayed or failed shelf elevation, defective shelf fusion, failure of medial edge epithelium cell death, post-fusion rupture and failure of mesenchymal consolidation and differentiation, have all been postulated[1]. Sun et al[25] suggested that lack of intimate palatal shelf contact after elevation is one possible cause, and recent research into palate development has concentrated on fusion of the shelves rather than elevation. Ferguson[26], on the other hand, took the view that failure of palatal shelf elevation may be responsible for 90% of palatal cleft. However, there is only a little comments on the defective growth of secondary palate itself as a cause of cleft palate.

Recently, a mouse odd-skipped related 2 (*Osr2*) gene has been cloned as a homologue of Drosophila pair-rule gene, odd-skipped[18] and the targeted disruption of *Osr2* showed impairment of palatal shelf growth in mice, causes delay in palatal shelf elevation, resulting in cleft palate[19]. Whereas palatal outgrowth initiates normally in the mutant embryos, significant reduction in palatal mesenchyme proliferation occurs specifically in the medial halves of the downward growing palatal shelves at E13.5, which results in retarded, mediolaterally symmetric palatal shelves before palatal shelf elevation[19]. However, it has not been identified that not only the downstream targets of *Osr2* in the developing palatal shelves but also the molecular mechanisms in the inhibition of palatal mesenchymal proliferations, which might be caused by the alterations of its downstream targets.

2. *Differently Expressed Genes (DEGs)*

Genes expressed differently in the growing palatal shelves among wild type, *Osr2* heterozygous and homozygous mice were detected by the ACP-based GeneFishing PCR methods as downstream targets of *Osr2*. Identified DEGs were seven, all of them were expressed higher in the palatal shelves of homozygous mutants than in those of the wild type mice. These DEGs may be direct causes of cleft palate result from the loss of function in the growing palatal shelves of *Osr2* mutants. It is confirmed that identified DEGs were specifically expressed in the growing palatal shelves of wild type mice embryos at E13.5.

1) *Vimentin (Vim)*

Vimentin is known as mesenchymal intermediate filament. It has been reported that vimentin is normally increased in the palatogenesis, specially during the fusion of each palatal shelves[27]. Following the epithelial fusion, MEE degeneration allows mesenchymal cells to move across the intact horizontal palate. However, expression level of vimentin was higher in the palatal shelves of *Osr2* mutants than in those of wild type mice. These results are difficult to understand the disturbance of growth in palatal shelf of the *Osr2* mutants, because vimentin should be down-regulated following differentiation of the palatal mesenchyme. From these findings, it is suggested that upregulated vimentin in the mesenchyme of palatal shelves may induce the interference of cellular polarity, which inhibits the horizontal elevation of palatal shelves.

2) *beta-tropomyosin (Tpm2)*

beta-tropomyosin, one of tropomyosin multigene family, is widely expressed nonmuscle cells as well as muscles during embryogenesis[28]. During the murine palatogenesis, muscle precursors and markers for muscular differentiation were seen as predominant features for elevation of palatal shelves. However, the expression of *Tpm* was also upregulated in the palatal shelves of *Osr2* mutants, which were not elevated by the defective growth itself. Therefore, it is hard to understand the cause of upregulation of *Tpm2* before confirming their expression in situ. Detailed expression analysis for *Tpm2* may explain the cause of upregulation of this gene in the palate of mutants.

3) *Thioredoxin-like 5 (Txnl5)*

A newly identified gene encoding the 14-kDa thioredoxin (Trx)-related cytosolic protein, Trp14[29]. As like other redoxin such as thioredoxin (Trx1), Trp regulates the intracellular redox. Substrates for the disulfied reductase also include several transcription factors such as nuclear factor-κB (*NF-κB*). Trx functions as a carrier that transfer electrons to enzymes involved in DNA synthesis and protein disulfied reduction : ribonucleotide reductase, methionione sulfoxide

reductase. Transcription factors such as *NF-κB*, *p53*, *PEB32*, and *AP-1* have been shown to be regulated by Trx[30]. Although no report about the functions of Trx in the developmental processes, increased expression of *Txnl5* in the palatal shelf of *Osr2* mutants supported that *Txnl5* would participate in the growing and morphogenesis of secondary palate. These results suggested that *Osr2* regulates the transcription of *Txnl5*, which involved in the transcription of downstream targets such as *NF-κB*, *p53*, *PEB32*, and *AP-1*, etc. To confirm the precise roles of *Txnl5* in the palatogenesis, further studies are necessary.

4) Insulin-like growth factor binding protein 7 (IGFbp7)

A 30 kDa cystine-rich glycoprotein, also known as mac25, angiomodulin or insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1), controls diverse cellular functions, such as cellular growth, adhesion, migration and synthesis of extracellular matrix[31]. IGFbp7 may possibly modulate the prefusion of the palatal shelves to bind to IGF-II, which is known to be expressed in the palatal mesenchyme. Among the IGF superfamily, IGF II not IGF I was specifically expressed in the mesenchyme of horizontal prefusion palate at E14 mouse embryo[32]. Although no direct evidences about the regulation pathways of *Osr2* against IGF system until now, potential actions of IGF-II might be blocked by the binding to the IGFbp7 in the palatal mesenchyme of *Osr2* homozygotes, which result in cleft palate.

In summary, seven genes were identified as a differently expressed genes from the palatal shelves among the wild type, *Osr2* heterozygous and homozygous mice. Those were *Vim, Tpm2, Txnl5, Col2a1, IGFbp7, Sui1* and *Dad1*, all of which were specifically expressed in the growing palatal shelves of wild type mice. Therefore, it is strongly suggested that *Osr2* may play key roles in the palatal growth and morphogenesis by way of transcriptional regulation of these downstream target genes. Further studies about the molecular genetic pathways between these molecules are required for understanding the precise mechanisms underlying the secondary palate completion.

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