Expression patterns of *Zfhx1a* and *Zfhx1b* during mouse craniofacial development

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Recent studies have demonstrated that *Zfhx1a* and *Zfhx1b* are transcription factors involved in many important signaling pathways. They are known to be essential for neural development, and for the development of other neural-crest-derived tissues. However, much remains to be learned about their expression patterns and functions in the developing tissues of the craniofacial region. We determined the unique expression patterns of *Zfhx1a* and *Zfhx1b* during mouse craniofacial development from embryonic day (E) 13.5 to E16.5. In the epithelium of the circumvallate papilla facing the oral cavity, *Zfhx1a* and *Zfhx1b* were strongly and weakly expressed, respectively. The epithelial component of the submandibular gland expressed *Zfhx1a* and *Zfhx1b*. In the developing eye, *Zfhx1a* and *Zfhx1b* were expressed strongly in the retina, and in the anterior region of the lens at E13.5 and E14.5. At E16.5, transcripts of *Zfhx1a* and *Zfhx1b* were detected in the developing eyelids. These findings demonstrate the spatial and temporal expression patterns of *Zfhx1a* and *Zfhx1b* during mouse craniofacial development.

**Keywords:** *Zfhx1a*, *Zfhx1b*, circumvallate papilla, submandibular gland, eye.

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Introduction
Zfhx1a and Zfhx1b, the two zinc-finger E-box-binding homeobox factors, are two transcription regulators of the vertebrate that are closely associated with Zfh-1 of Drosophila.1,2,3,4,5,6 Zfhx1a is diversely known as Zeb15,7,8, δEF19, and Zfhep10,11; Zfhx1b is also known as Zeb2 and Sip15,8,12. Zfhx1a and Zfhx1b may function as mediators of other signaling pathways13. Zfhx1a is involved in transforming growth factor beta (Tgf-β) signaling in vascular smooth muscle cell differentiation14, and in sonic hedgehog (Shh) signaling during mouse limb development15. Zfhx1a is strongly expressed in the neural tube, brain, mesoderm, and neural-crest-derived tissues such as the limb buds, somites, and branchial arches10,16. In addition, the Zfhx1a-knockout mouse exhibits cleft palate, suggesting its role as a regulator of cell proliferation during secondary palate development16. Zfhx1b is a transcription repressor of the Zfh-1 family that acts as a downstream mediator of Tgf-β and bone morphogenetic protein signaling (BMP)8,12. Zfhx1b is widely expressed in humans and mice, most prominently in the heart and the neural tissues17,18. Mutations causing Zfhx1b haploinsufficiency during embryogenesis is related to Mowat-Wilson syndrome, which is characterized by mental retardation, dysmorphic facial features, microcephaly, seizures19,20,21. Knockout of Zfhx1b in mice is embryonic lethal at embryonic day (E) 9.5–E10.5, with the mice exhibiting developmental defects in neural crest formation22,23,24 that are caused by ectopic expression of E-cadherin. Zfhx1b is expressed in numerous tissues during embryonic development, including the neural crest, neuroepithelium, and limb buds23. However, expression of Zfhx1a and Zfhx1b in the internal organ of craniofacial region was not determined. Here, we determined the unique and overlapping expression patterns of Zfhx1a and Zfhx1b in the developing mouse craniofacial region by in situ hybridization in the circumvallate papilla of the tongue, submandibular gland, and the developing eye at E13.5, E14.5, and E16.5.

Materials and Methods
All experiments were performed according to the guidelines of the Intramural Animal Use and Care Committee of the College of Dentistry, Yonsei University.

Animals
Adult ICR mice were housed in a temperature-controlled room (22°C) under artificial illumination (lights on from 05:00 to 17:00) and 55% relative humidity, with access to food and water ad libitum. The embryos were obtained from time-mated pregnant mice. E0 was designated as the day on which the presence of a vaginal plug was confirmed. Embryos at each developmental stage (E13.5, E14.5, and E16.5) were used in this study.

In situ hybridization
In situ hybridization on whole mouse embryos was performed as previously described25 in paraffin wax sections by using standard protocols. Briefly, embryos were fixed in 4% PFA, embedded in paraffin wax and sectioned at 7 μm. Sections were incubated at 60°C, dewaxed in xylene, re-hydrated through a graded series of alcohol washes and post-fixed in 4% PFA. Sections were prehybridized in a humid
chamber containing 50% formamide in 2× saline sodium citrate buffer at 58°C for 30 min. Digoxigenin (DIG)-labelled RNA probes were prewarmed to 85°C and hybridized to sections overnight at 58°C. Mouse DNA Zfhx1a and Zfhx1b plasmids were used as templates for the synthesis of DIG-labeled RNA probes.

Results and Discussion

Expression patterns of Zfhx1a and Zfhx1b in the developing circumvallate papilla of the tongue and the submandibular gland

The expression patterns of Zfhx1a and Zfhx1b were examined on sections of developing mouse circumvallate papilla region of the tongue (Fig. 1). At E13.5, Zfhx1a was expressed strongly in the epithelium of the circumvallate-papilla-forming region, including the arch-like structure (i.e., the epithelium of the circumvallate papilla facing the oral cavity; Fig. 1A and B). Zfhx1a was weakly expressed in the mesenchyme underlying the epithelium of the circumvallate papilla (Fig. 1A and B). Zfhx1b was expressed weakly in the mesenchyme of the underlying circumvallate papilla (Fig. 1D and E). Interestingly, Zfhx1b was strongly expressed in the epithelium where the trench of the circumvallate papilla was developing, but weakly in the arch-like structure of the circumvallate papilla (Fig. 1D and E). At E14.5, Zfhx1a was expressed strongly in the overall epithelium, including the arch-like structure of the circumvallate papilla, but it was not observed in the mesenchymal cells underlying the epithelium of the circumvallate papilla (Fig. 1G and H). Zfhx1b transcripts were detected in the epithelium of the circumvallate papilla, except the arch-like structure, but it was localized in the overall mesenchyme underlying the circumvallate papilla (Fig. 1J and K).

At E16.5, the developing circumvallate papilla underwent prominent morphological changes, resulting in a more bulbous shape, deeper location of the floor epithelium of the trench, and uplifting of the arch-like region (apex) of the trench-wall epithelium (Fig. 1N and Q). At this stage, Zfhx1a was expressed throughout the epithelium of the circumvallate papilla (Fig. 1M and N). It was strongly expressed in the arch-like region of the trench-wall epithelium and
the floor epithelium of the trench of the circumvallate papilla (Fig. 1N). Zfhx1b was expressed in the epithelium and mesenchyme of the circumvallate papilla region, but not in the arch-like structure of the circumvallate epithelium at E16.5 (Fig. 1Q).

The expression pattern of Zfhx1a in the developing circumvallate papilla region (Fig. 1H and N) was similar to that of Patched, while that of Zfhx1b (Fig. 1K and Q) was similar to that of Shh. The relationship between Zfhx1a and Shh signaling has been investigated during mouse limb development. Patched is the molecular target of Shh, and it is suggested that the Shh signaling pathway plays an important role in the developing circumvallate papilla. Therefore, we suggest that Zfhx1a and Zfhx1b, in association with the Shh signaling pathway, are involved in the morphogenesis and pattern formation of the circumvallate papilla. Both Zfhx1a and Zfhx1b were also expressed in the muscle fibers of the tongue below the developing circumvallate papilla region at E13.5, E14.5, and E16.5 (Fig. 1B, E, H, K, N and Q).

These results are in agreement with the expression patterns of Zfhx1a and Zfhx1b found in the muscle cells of the developing mouse embryo, and suggest that Zfhx1a and Zfhx1b are involved in the development of the muscle fibers in the circumvallate papilla region. In addition, Zfhx1a and Zfhx1b were expressed in the mylohyoid muscle and the digastric muscle at E13.5 and E14.5, but their levels were diminished at E16.5 (Fig. 1A, D, G, J, M and P).

The development of the mouse submandibular gland is initiated between E11.5 and E12.5. By E13.5, the epithelial bud begins to cleft and branch. Branching morphogenesis occurs continuously in the immature submandibular gland, resulting in the formation of multiple cords by E14.5. Finally, at E17, differentiation and lumenization occur in the ducts and terminal buds. The expression patterns of Zfhx1a and Zfhx1b on sections of developing submandibular gland are presented in Fig. 2. At E13.5, Zfhx1a and Zfhx1b were expressed strongly in the nascent epithelial bud of the developing submandibular gland, but weakly in the surrounding mesenchyme (Fig. 1A, C, D and F). At E14.5, Zfhx1a and Zfhx1b were strongly expressed in the proliferating and clefting epithelial bud of the embryonic submandibular gland. However, they were weakly expressed in the mesenchyme of the submandibular gland (Fig. 1C and F). At E16.5, Zfhx1a and Zfhx1b were strongly expressed in the epithelial buds that will form the submandibular acini (Fig. 1M, O, P and R). Weaker expressions were found in multiple epithelial cords that will form the submandibular ducts (Fig. 1M, O, P and R).

Expression patterns of Zfhx1a and Zfhx1b in the developing eye

The expression patterns of Zfhx1a and Zfhx1b on sections of the developing mouse eye are presented in Fig. 2. In the lens, Zfhx1a was expressed in the anterior half of the lens fibers at E13.5, and gradually decreased at E14.5 (Fig. 2A, B, E and F). On the other hand, Zfhx1b was expressed strongly in the lens epithelium at E13.5 and E14.5 (Fig. 2C, D, G and H). Despite the expression patterns of Zfhx1a and Zfhx1b differing at E13.5 and E14.5, these genes were both expressed in the same region at E16.5, the region of cell elongation (Fig. 2I, J, K and L). Transcripts of Zfhx1a and Zfhx1b were also observed in the mesenchyme in the edges of the upper and lower
developing eyelids at E16.5 (Fig. 2I and K). Zfhx1a and Zfhx1b are known to be crucial factors in neural development\textsuperscript{10,11,18,24}, and we found that Zfhx1a and Zfhx1b were also strongly expressed in the nervous tunic layer, including the retina, at E13.5, E14.5, and E16.5 (Fig. 2A, C, E, G, I and K).

In summary, this study has demonstrated the unique expression patterns of Zfhx1a and Zfhx1b in the craniofacial region from E13.5 to E16.5. Zfhx1a and Zfhx1b are known to be important in ectoderm-derived organ and the development of neural-crest-derived tissues\textsuperscript{10,11,18,24}. Zfhx1a (but not Zfhx1b) was expressed in the arch-like epithelial layer of the circumvallate papilla facing the oral cavity. The epithelial component of the submandibular gland, but not the mesenchymal cells, expressed Zfhx1a and Zfhx1b (Fig. 1). In the developing eye, strong expression of Zfhx1a and Zfhx1b were found in the retina and the anterior region of the lens (Fig. 2). These findings improve the spatial and temporal understanding of the expressions of Zfhx1a and Zfhx1b during mouse craniofacial development.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) Grant funded by the Korea Government (MSIP) (NRF-2016R1A5A2008630).

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한글초록

생쥐 두개 안면 성장 동안 Zfhx1a와 Zfhx1b의 발현 양상

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최근의 연구에 따르면 Zfhx1a와 Zfhx1b는 많은 중요한 신호 전달 경로에 관여하는 전사 인자이다. 이 유전자들은 신경 발달 및 신경세포에서 유래되는 다양한 조직의 발생에 필수적인 것으로 알려져 있다. 그러나 두개 안면 발생 시 Zfhx1a와 Zfhx1b의 발현 양상과 기능에 대한 연구는 입안장과 치아 발생을 제외 하고는 미흡한 편이다. 본 연구에서는 배아 발생 13.5일에서 16.5일까지 생쥐 두개 안면 성장 동안 Zfhx1a 와 Zfhx1b의 발현 양상을 혀의 성곽유두와 턱밑샘, 눈에서 확인하였다. 발생 중인 혀 성곽유두의 상피에서, Zfhx1a와 Zfhx1b의 발현 양상을 시기별로 비교하였다. 또한 턱밑샘 발생 중 이 유전자들의 발현 양상 또한 비교 분석하였다. 발생 중인 눈에서의 Zfhx1a와 Zfhx1b의 시공간적 발현 양상도 확인하였다. 이러한 시공간적 발현의 차이는 두개안면 발생 동안 Zfhx1a 및 Zfhx1b가 중요한 역할을 하고 있음을 시사한다고 할 수 있다.

주제어: Zfhx1a, Zfhx1b, 성곽유두, 턱밑샘, 눈