

Expression patterns of *Zfmx1a* and *Zfmx1b* during mouse craniofacial development

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Recent studies have demonstrated that *Zfmx1a* and *Zfmx1b* 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 *Zfmx1a* and *Zfmx1b* during mouse craniofacial development from embryonic day (E) 13.5 to E16.5. In the epithelium of the circumvallate papilla facing the oral cavity, *Zfmx1a* and *Zfmx1b* were strongly and weakly expressed, respectively. The epithelial component of the submandibular gland expressed *Zfmx1a* and *Zfmx1b*. In the developing eye, *Zfmx1a* and *Zfmx1b* 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 *Zfmx1a* and *Zfmx1b* were detected in the developing eyelids. These findings demonstrate the spatial and temporal expression patterns of *Zfmx1a* and *Zfmx1b* during mouse craniofacial development.

Keywords: *Zfmx1a*, *Zfmx1b*, 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 *Zeb1*^{5,7,8}, *δEFL*³, and *Zfhep*^{9,10,11}; *Zfhx1b* is also known as *Zeb2* and *Sip1*^{5,8,12}. *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 arches^{10,16}. 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 tissues^{17,18}. Mutations causing *Zfhx1b* haploinsufficiency during embryogenesis is related to Mowat-Wilson syndrome, which is characterized by mental retardation, dysmorphic facial features, microcephaly, seizures^{19,20,21}. 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 formation^{22,23,24} 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 circum-

vallate papilla, except the arch-like structure, but it was localized in the overall mesenchyme underlying the circumvallate papilla (Fig. 1J and K).

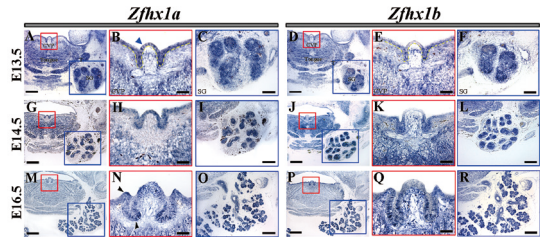


Figure 1. Localization of *Zfhx1a* and *Zfhx1b* in the developing circumvallate papilla and submandibular gland at E13.5, E14.5, and E16.5.

All samples are frontal sections. (A, D, G, J, M, P) Low magnification images of the posterior mandible at the level of the developing circumvallate papilla of the tongue and the submandibular gland. Red and blue boxes are higher magnification images of panel A, D, G, J, M, and P. (B, H, N) Expression of *Zfhx1a* in the circumvallate papilla of the posterior tongue. (E, K, Q) Expression of *Zfhx1b* in the circumvallate papilla of the posterior tongue. (C, I, O) Expression of *Zfhx1a* in the submandibular gland. (F, L, R) Expression of *Zfhx1b* in the submandibular gland. yellow dotted line, epithelium outline in circumvallate papilla. Blue arrowhead, arch-like structure in circumvallate papilla; black arrowhead, strong expression region in apex region and trench wall in circumvallate papilla. CVP, circumvallate papilla; SG, submandibular gland. Scale bars – A, D, G, J: 200 μm; B, C, E, F, H, I, K, L, N, O, Q, R: 50 μm; I, L, N, Q: 100 μm; M, P: 400 μm

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*²⁶⁾. The relationship between *Zfhx1a* and *Shh* signaling has been investigated during mouse limb development¹⁵⁾. *Patched* is the molecular target of *Shh*²⁷⁾, and it is suggested that the Shh signaling pathway plays an important role in the developing circumvallate papilla²⁸⁾. 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 embryo¹⁶⁾, 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 buds²⁹⁾. 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^{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).

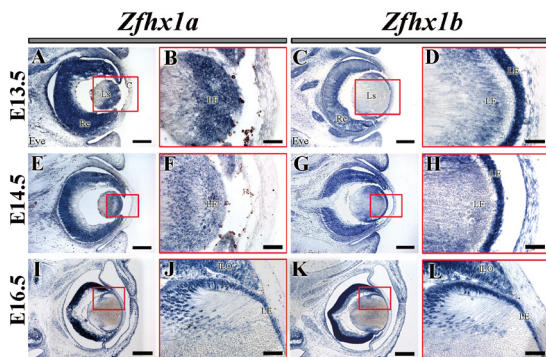


Figure 2. Localization of *Zfhx1a* and *Zfhx1b* in the developing eye at E13.5, E14.5, and E16.5.

All samples are frontal sections. Expression of *Zfhx1a* in the developing eye. (A, C, E, G, I, K) Low magnification images of the developing eye. (B, D, F, H, J, L) Red boxes are higher magnification images of panel. Ls, lens; C, cornea; Re, retina; LF, lens fiber; LE, lens epithelium; ILO, inner layer of optic cup. Scale bars – A, C, E, G: 200 μ m; B, D, F, H, J, L: 50 μ m; I, K: 400 μ m

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^{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.

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한글초록

생쥐 두개 안면 성장 동안 *Zfhx1a*와 *Zfhx1b*의 발현 양상

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최근의 연구에 따르면 *Zfhx1a*와 *Zfhx1b*는 많은 중요한 신호 전달 경로에 관여하는 전사 인자이다. 이 유전자들은 신경 발달 및 신경능선세포로부터 유래되는 다양한 조직의 발생에 필수적인 것으로 알려져 있다. 그러나, 두개 안면 발생 시 *Zfhx1a*와 *Zfhx1b*의 발현 양상과 기능에 대한 연구는 입천장과 치아 발생을 제외하고는 미흡한 편이다. 본 연구에서는 배아 발생 13.5일에서 16.5일까지 생쥐 두개 안면 성장 동안 *Zfhx1a*와 *Zfhx1b*의 발현 양상을 혀의 성곽유두와 턱밑 샘, 눈에서 확인하였다. 발생 중인 혀 성곽유두의 상피에서, *Zfhx1a*와 *Zfhx1b*의 발현 양상을 시기별로 비교하였다. 또한 턱밑샘 발생 중 이 유전자들의 발현 양상 또한 비교 분석하였다. 발생 중인 눈에서의 *Zfhx1a*와 *Zfhx1b* 시공간적 발현 양상도 확인하였다. 이러한 시공간적 발현의 차이는 두개안면 발생 동안 *Zfhx1a* 및 *Zfhx1b*가 중요한 역할을 하고 있음을 시사한다고 할 수 있다.

주제어: *Zfhx1a*, *Zfhx1b*, 성곽유두, 턱밑샘, 눈