(奥 羽 大 歯 学 誌
Vol. 42 (4) ; 133−140 2015)

Expression of Myogenic Regulatory Factors in the Pharyngeal Muscle of the Mouse Embryos

Asuka KISHI, Hiroshi SAITO and Akinobu USAMI

Morphogenesis of the pharynx, which is involved in swallowing, remains unclear. In the present study, the expression of myogenic regulatory factors: MyoD, Myf5, myogenin, and MRF4, were immunohistologically examined using mouse embryos. Myf5 protein was expressed in the caudal pharynx at embryonic day 12 (E12), while MyoD, myogenin and MRF4 proteins were expressed at E13. Subsequently, the expressions spread from the caudal to cranial pharynx over time by E15. Thus, muscle precursor cells in the pharyngeal muscles differentiate into mature myotubes during the embryonic stages and they spread throughout the entire pharynx by E15.

Key words : development, myogenic regulatory factors, skeletal muscle, swallow, pharynx

Introduction

Swallowing is a movement to send ingested food to the stomach through the pharynx and esophagus. This movement starts in the embryo[1]. Also, tongue and head movements start in the embryo[2]. The morphogenesis of skeletal muscles of the areas are critical for these movements. The pharyngeal muscles are skeletal muscles involved in swallowing[3]. Several reports have been published on skeletal muscles involved in oral movements[4,5]. Tongue muscles involved in tongue movements complete maturation at birth[4]. The masseter muscles remain immature at birth[5]. On the other hand, few reports have been published on the morphogenesis of the pharyngeal muscles. The timing of the morphogenesis of the pharyngeal muscles remains unclear.

The skeletal muscles are formed through the differentiation and maturation of muscle precursor cells into myoblasts and myotubes. In these processes, myogenic regulatory factors (MRFs), transcription factors that are specifically expressed in the skeletal muscles, play an important role[6]. The MRFs include Myogenic differentiation 1 (MyoD)[7], Myogenic factor 5 (Myf5)[8], myogenin[9,10], and Myogenic factor 6 (MRF4)[11]. MyoD and Myf5 are involved in the differentiation of muscle precursor cells into myoblasts[12,13,14]. Myogenin is involved in the differentiation of myoblasts into myotubes[15,16]. MRF4 expression is involved in myotube maturationtion[17].

In the present study, the development and morphogenesis of the pharyngeal muscles were examined along with expressions of MRFs proteins.

Materials and Methods

Pregnant ICR mice were purchased (Nippon Clea, Tokyo, Japan). ICR mice at embryonic

受付：平成27年12月25日，受理：平成28年２月12日
奥羽大学生体構造学講座口腔解剖学分野

Division of Oral Anatomy, Department of Morphological Biology, Ohu University, School of Dentistry

days 11, 12, 13, 14 and 15 (E11, E12, E13, E14 and E15) were used in this study. Mice in this study were used in accordance the Guidelines for Animal Experiments at Ohu University (2014-14). Mice were deeply anesthetized with dimethyl ether. Mice were euthanized to remove embryos. The embryos were fixed in 4% paraformaldehyde-phosphate buffer (pH7.4) at 4℃ for 24 hours. Then, to observe the midline region, paraffin blocks were made according to routine procedures to prepare serial sections with a thickness of 5 μm in a sagittal direction.

For morphological observation, hematoxylin and eosin (H-E) staining was conducted with an optical microscope (Optiphot-2, Nikon, Tokyo, Japan).

Immunohistochemical staining was routinely conducted to examine the protein expressions of the MRFs. After blocking in 3% hydrogen peroxide methanol at room temperature for 10 minutes, polyclonal rabbit anti-MyoD (1 : 200, Bioss, Boston, MA, USA), polyclonal rabbit anti-Myf5 (1 : 500, Bioss, Boston, MA, USA), monoclonal rabbit anti-myogenin (1 : 200, Abcam, Cambridge, UK), and polyclonal rabbit anti-MRF4 (1 : 500, Bioss, Boston, MA, USA) antibodies were employed as primary antibodies. Staining was conducted at 4℃ overnight. After washing with phosphate-buffered saline (PBS pH7.4), Histofine Simple Stain MAX-PO (R) (Nichirei Biosciences., Tokyo, Japan) was applied as a secondary antibody at room temperature for an hour. Finally, Diaminobenzidine coloring (Nichirei Biosciences., Tokyo, Japan) reaction was conducted for observation with an optical microscope (Optiphot-2, Nikon, Tokyo, Japan).

Results

1. E11

In the E11 mouse, mesenchymal cells scattered throughout the entire pharyngeal subepithelial connective tissue, although they accumulated at a higher density in the caudal pharynx (Fig. 1A). In the E11 mouse, none of the MRFs was positive in the pharynx (Fig. 1B-E).

2. E12

In the E12 mouse, the primordial hyoid bone was found in the cranial larynx. In addition, the primordial first cervical vertebra was noted in the dorsal pharynx. In posterior pharyngeal wall, the cell density was lower in the cranial pharynx, and was higher in the caudal pharynx with cell accumulation (Fig. 2A). In the E12 mouse, Myf5 proteins was positive in the caudal pharynx relative (Fig. 2B-E).

3. E13

In the E13 mouse, the primordial cricoid cartilage was found in the ventral pharynx.

In posterior pharyngeal wall, the cells accumulated around the upper end of the first cervical vertebrae cranially to the reference line. In anterior pharyngeal wall, the cell at a higher density in the caudal pharynx (Fig. 3A). In the E13 mouse, MyoD, Myf5, myogenin, and MRF4 proteins were positive in posterior pharyngeal wall, upper end of the first cervical vertebrae cranially to the reference line. In anterior pharyngeal wall, none of the MRFs protein was positive in the pharynx (Fig. 3B-E).

4. E14

In the E14 mouse posterior pharyngeal wall, the cell density was higher throughout the entire laryngopharynx. In anterior pharyngeal wall the cell density accumulated around the upper of the cricoid cartilage (Fig. 4A). In the E14 mouse posterior pharyngeal wall, MyoD, Myf5, myogenin, and MRF4 proteins were positive throughout the entire laryngopharynx. In the anterior pharyngeal wall, MyoD, Myf5, myogenin, and MRF4 proteins were positive around the upper of the cricoid cartilage (Fig. 4B-E).

5. E15

In the E15 mouse posterior pharyngeal wall,

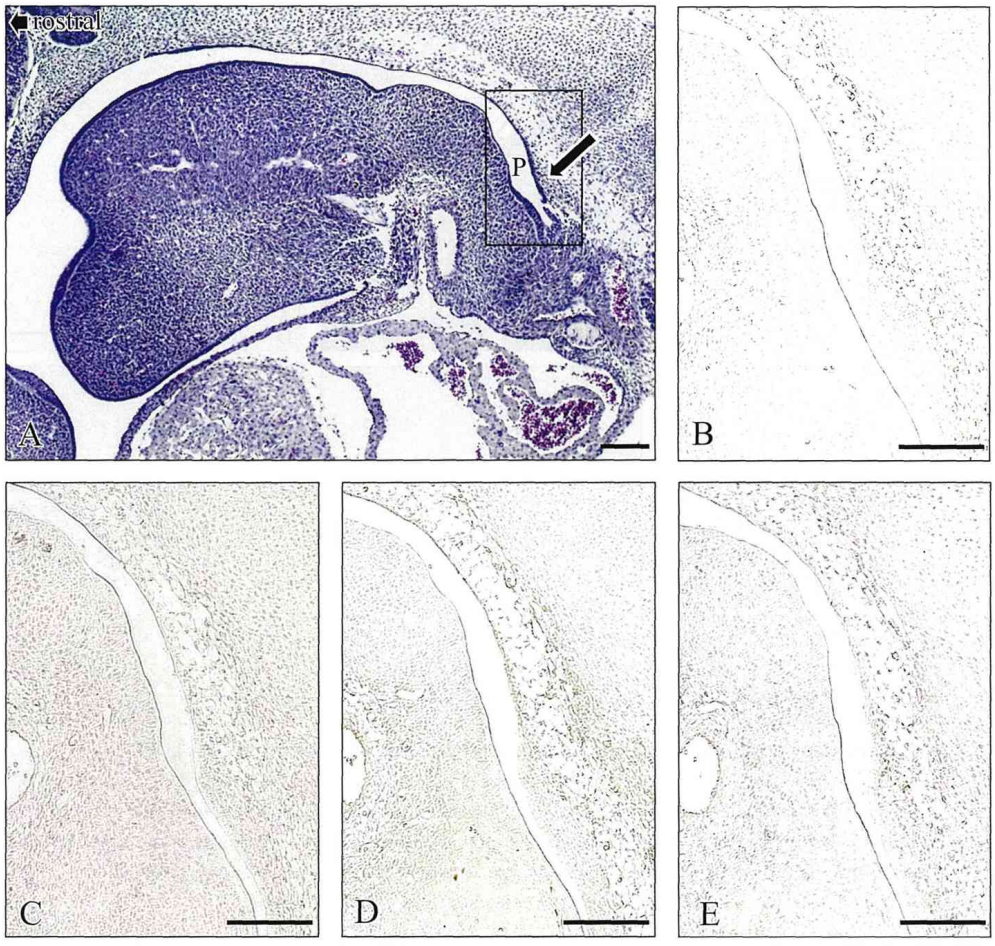

Fig. 1 Histological appearance and expression of MRFs protein in pharyngeal area at E11.
H-E stain (A). Cells accumulated in the caudal pharynx (arrow). Expression of MyoD (B), Myf5 (C), myogenin (D), and MRF4 (E). No positive reaction was observed in (B-E). (B-E) correspond to boxed areas in (A). *P* pharynx. Bar line=100 μm

cells were noted even in the nasopharynx, and the cell density was higher throughout the entire pharynx. In the anterior pharyngeal wall, cell accumulation was higher than E14 mice in the upper pharynx (Fig. 5A). In the E15 mouse, the four MRFs proteins were positive throughout the entire pharynx (Fig. 5B-E). The timing of MRFs expressions is shown in Table 1.

Discussion

In the mouse pharynx, the nasopharynx is located between the nasopharyngeal hiatus and nasal cavity, and the laryngopharynx is located between the nasopharyngeal hiatus and rostral fourth of the plate of the cricoid cartilage[18]. In the E12 mouse, the cell density was lower in the cranial pharynx, and was higher in the caudal pharynx with cell accumulation by the morphological observation. Myf5 protein was positive. Myf5 is involved in the differentiation of muscle precursor cells into myoblasts[12,13,14], suggesting that differentiation into myoblasts starts at E12 in the pharynx. In addition, since the expression site of Myf5 protein

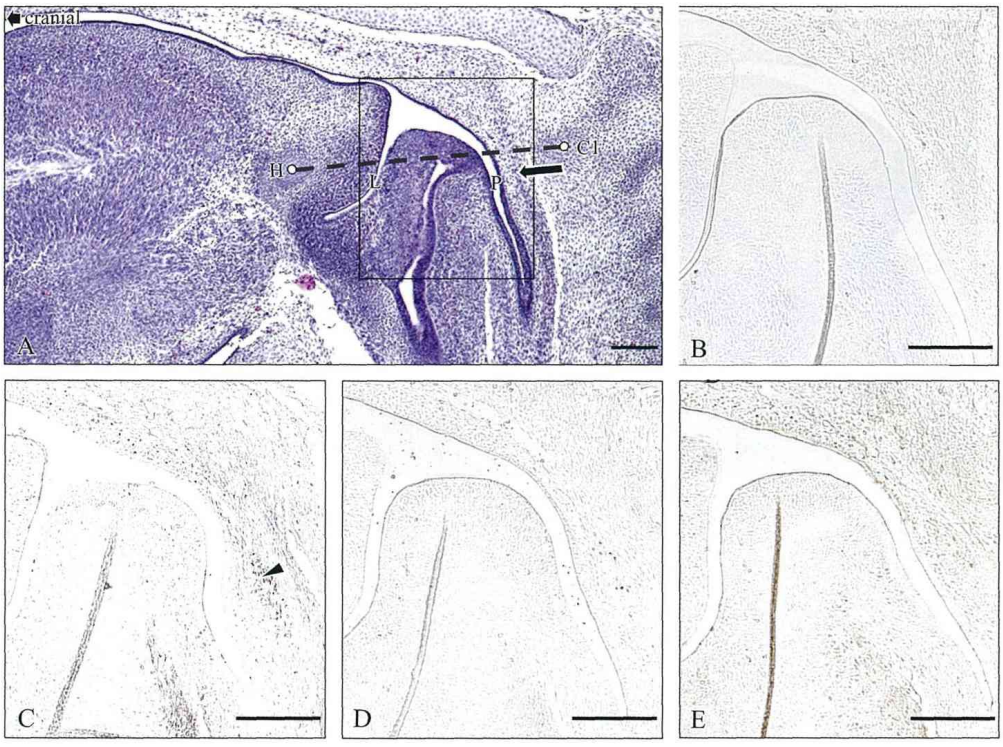

Fig. 2 Histological appearance and expression of MRFs protein in pharyngeal area at E12.
H-E stain (A). Arrow indicates cell aggregation. Expression of MyoD (B), Myf5 (C), myogenin (D), and MRF4 (E). Positive reaction were seem in (C) (arrowhead). No positivity was observerved in (B). (B-E) correspond to boxed areas in (A). A reference line was determined by connecting the primordial hyoid bone and the first primordial cervical vertebra. *P* pharynx, *L* larynx, *H* anlage of hyoid bone, *C1* first primordial cervical vertebra, *Dotted line* reference line. Bar line=100 μm

corresponded to the cell accumulation site in the caudal pharynx, muscle precursor cells may accumulate in the pharynx. Myogenin is involved in the differentiation of myoblasts into myotubes[15,16], and the expression of MRF4 implicates myotube maturation[17]. It is suggested that myoblast differentiation coincides with myotube differentiation and maturation in the pharyngeal muscles. Although MyoD protein was negative at E12, MyoD protein was positive at E13. MyoD and Myf5 are involved in the differentiation of muscle precursor cells into myoblasts[12,13,14]. The expression of myoD mRNA was later than that of Myf5 mRNA during mouse development[19,20,21,22].

At E14 when the primordial pharyngeal cartilage appeared, all the four proteins were positive in the laryngopharynx. At E15, they were positive in the cranial nasopharyngeal region. Thus, pharyngeal cells proliferate from the caudal to cranial regions over time and also in the MRFs- proteins positive regions, suggesting that the pharyngeal muscles are formed from the caudal to cranial regions. The posterior pharyngeal wall is formed earlier than the anterior pharyngeal wall, because the former shows earlier cell accumulation and positive reactions for MRF proteins than the latter.

The esophagus adjacent to the pharynx consists of the same foregut-derived tissues as the pharynx. In the esophagus of the embryo,

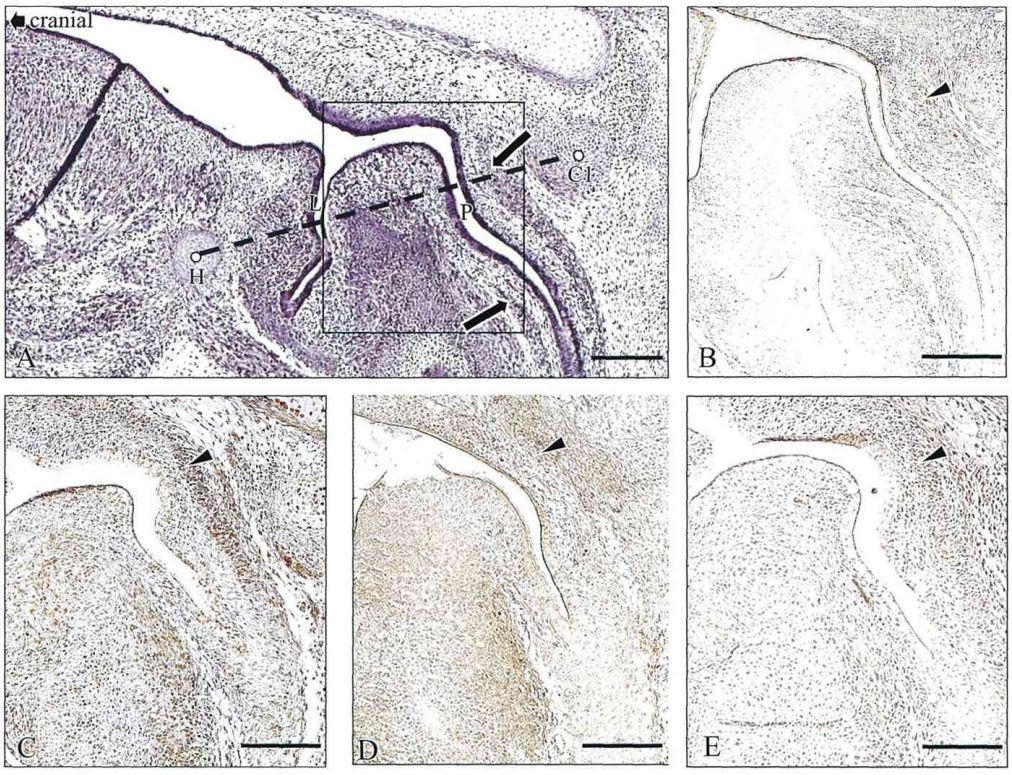

Fig. 3 Histological appearance and expression of MRFs protein in pharyngeal area at E13.
H-E stain (A). Arrow indicates cell aggregation at cranial pharynx. Expression of MyoD (B), Myf5 (C), myogenin (D), and MRF4 (E). Arrowhead indicates a positive reaction in pharyngeal cell. (B-E) correspond to boxed areas in (A). *P* pharynx, *L* larynx, *H* anlage of hyoid bone, *C1* first primordial cervical vertebra, *Dotted line* reference line. Bar line=100 μm

the mRNA expressions of MRFs are first noted in the cranial regions close to the pharynx and subsequently in the caudal regions[19]. Thus, myogenesis in the pharynx and esophagus may start at and spread from their adjacent site. In the esophagus, Myf5 mRNA was expressed first, followed by myogenin, MRF4, and MyoD mRNAs on the following day, suggesting that the esophagus is formed in the same manner as the pharynx.

The expressions of MRFs mRNA in the embryo differ with sites. In the trunk, Myf5 mRNA is first expressed at E8, myogenin mRNA at E8.5, MRF4 mRNA at E10 and 11, and MyoD mRNA at E10.5. In the skeletal muscles of the limb bud, Myf5 mRNA is expressed at E10-12, myogenin mRNA and MyoD mRNA at E10.5, and MRF4 mRNA at E16[20,21,22]. In the skeletal muscles involved in oral functions, MyoD mRNA, Myf5 mRNA, and myogenin mRNA are expressed at E11, and MRF4 mRNA at E13 in the mouse tongue muscles[4]. The movement of the mouse tongue starts at E13-14[2], although the maturation of skeletal muscle was completed at birth in the tongue muscles, but not in the masseter muscles[5]. The initiation of suckling by the tongue muscles differs from that of biting by the masseter muscles[23]. Thus, muscle maturation may be associated with the muscle functions[5].

In the present study, MRF4 protein was positive at E13, indicating myotube maturation,

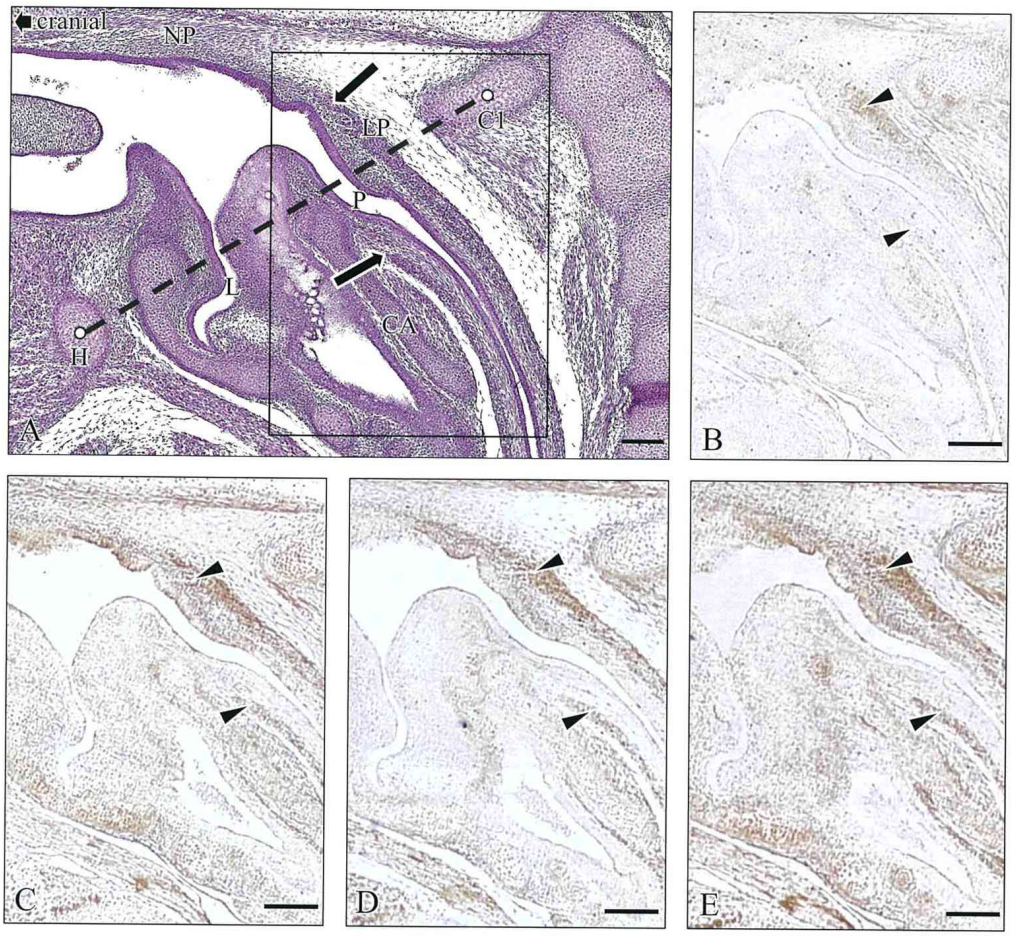

Fig. 4 Histological appearance and expression of MRFs protein in pharyngeal area at E14.
H-E stain (A). Arrow indicates cell aggregation. Expression of MyoD (B), Myf5 (C), myogenin (D), and MRF4 (E). Arrowhead indicates a positive reaction in pharyngeal cells. (B-E) correspond to boxed areas in (A). *P* pharynx, *L* larynx, *NP* nasopharynx, *LP* laryngopharynx, *H* anlage of hyoid bone, *C1* first primordial cervical vertebra, *Dotted line* reference line. Bar line=100 μm

Table 1. MRFs protein expression in pharyngeal area by embryo days.

Embryo days	11	12	13	14	15
MyoD	−	−	+	+	+
Myf5	−	+	+	+	+
myogenin	−	−	+	+	+
MRF4	−	−	+	+	+

+ positive, − negative

and spread throughout the entire pharynx at E15, suggesting that all pharyngeal muscle tissues matured at E15 or later depending on the timing of swallowing in the embryo.

Conclusion

The morphogenesis of the pharyngeal muscles starts in the proximal part of the esophagus, and all pharyngeal muscles mature by E15, as revealed by the expression of MRF4. These findings may be associated with the start of swallowing.

Acknowledgements

This study was supported by the Young Researcher Award from Ohu University.

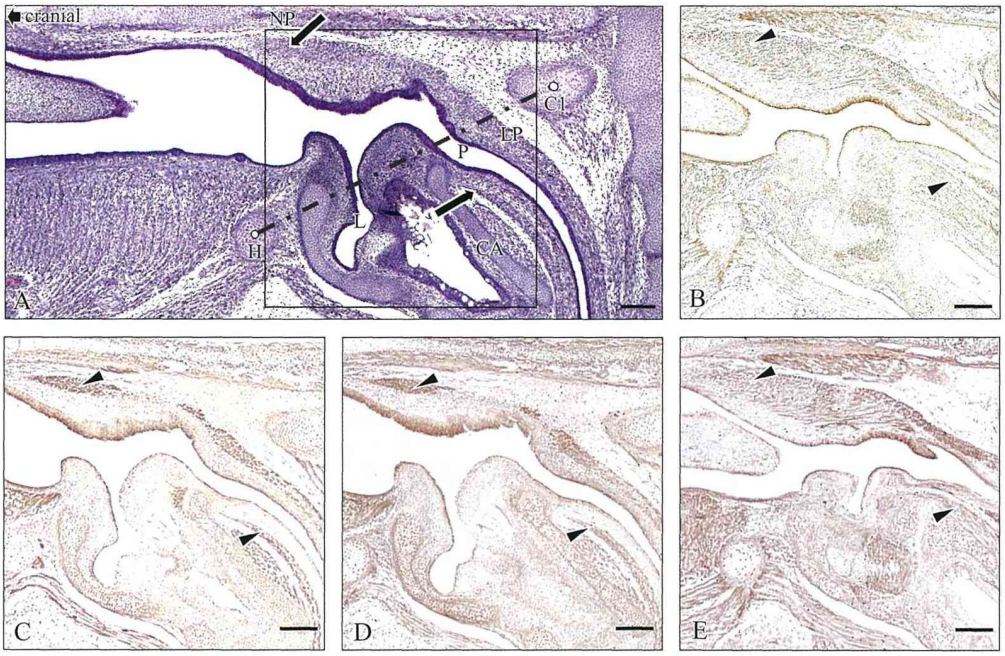

Fig. 5 Histological appearance and expression of MRFs protein in pharyngeal area at E15.
H-E stain (A). Arrow indicates cell aggregation. Expression of MyoD (B), Myf5 (C), myogenin (D), and MRF4 (E). Arrowhead indicates a positive reaction in pharyngeal cells. (B-E) correspond to boxed areas in (A). *P* pharynx, *L* larynx, *NP* nasopharynx, *LP* laryngopharynx, *H* anlage of hyoid bone, *C1* first primordial cervical vertebra, *Dotted line* reference line. Bar line=100 μm

References

1) Morikawa, Y., Yoshimura, M., Okada, T. and Ohishi, I. : Growth-factor-like substance in amniotic fluid in the rat : effect on the development of fetal colonic goblet cells. Biol. Neonate. **66** ; 100-105 1994.
2) Tsunekawa, N., Arata, A. and Obata, K. : Development of spontaneous mouth/tongue movement and related neural activity, and their repression in fetal mice lacking glutamate decarboxylase 67. Eur. J. Neurosci. **21** ; 173-178 2005.
3) Jones, B., editor. : Normal and abnormal swallowing : Imaging in diagnosis and therapy 2nd Ed. ; xvii-viii Springer-Verlag ; New York 2003.
4) Yamane, A., Mayo, M., Shuler, C., Crowe, D., Ohnuki, Y., Dalrymple, K. and Saeki, Y. : Expression of myogenic regulatoy factors during the development of mouse tongue striated muscle. Arch. Oral Biol. **45** ; 71-78 2000.
5) Yamane, A., Ohnuki, Y. and Saeki, Y. : Delayed embryonic development of mouse masseter muscle correlates with delayed MyoD family expression. J. Dent Res. **79** ; 1933-1936 2000.
6) Megeney, L. A. and Rudnicki, M. A. : Determination versus differentiation and the MyoD family of transcription factors. Biochem. Cell Biol. **73** ; 723-732 1995.
7) Davis, R. L., Weintraub, H. and Lassar, A. B. : Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51** ; 987-1000 1987.
8) Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H. H. : A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO. J. **8** ; 701-709 1989.
9) Wright, W. E., Sassoon, D. A. and Lin, V. K. : Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell **56** ; 607-617 1989.
10) Edmondson, D. G. and Olson, E. N. : A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. **3** ; 628-640 1989.

11) Rhodes, S. J. and Konieczny, S. F. : Identification of MRF4 : a new member of the muscle regulatory factor gene family. Genes Dev. **3** ; 2050-2061 1989.
12) Rudnicki, M. A., Braun, T., Hinuma, S. and Jaenisch, R. : Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell **71** ; 383-390 1992.
13) Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H. and Jaenisch, R. : MyoD or Myf-5 is required for the formation of skeletal muscle. Cell **75** ; 1351-1359 1993.
14) Braun, T., Rudnicki, M. A., Arnold, H. H. and Jaenisch, R. : Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell **71** ; 369-382 1992.
15) Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J.M., Olson, E. N. and Klein W. H. : Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature **364** ; 501-506 1993.
16) Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. and Nabeshima, Y. : Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature **364** ; 532-535 1993.
17) Zhang, W., Behringer, R. R. and Olson, E. N. : Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. Genes Dev. **9** ; 1388-1399 1995.
18) Nakao, T. and Muto, H. : Anatomical observations in the pharynx of the mouse with special reference to the nasopharyngeal hiatus (Wood Jones). Acta Anat. **121** ; 147-152 1985.
19) Zhao, W. and Dhoot, G. K. : Skeletal muscle precursors in mouse esophagus are determined during early fetal development. Dev. Dyn. **219** ; 10-20 2000.
20) Ott, M. O., Bober, E., Lyons, G., Arnold, H. and Buckingham, M. : Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. Development **111** ; 1097-1107 1991.
21) Sassoon, D., Lyons, G., Wright, W. E., Lin, V., Lassar, A, Weintraub, H. and Buckingham, M. : Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. Nature **341** ; 303-307 1989.
22) Bober, E., Lyons G. E., Braun, T., Cossu, G., Buckingham, M. and Arnold, H. H. : The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. J. Cell Biol. **113** ; 1255-1265 1991.
23) Kubota, K., Narita, N., Ohkubo, K., Shibanai, S., Nagae, K., Kubota, M., Odagiri, N. and Kawamoto, T. : Morphological studies of the neuromuscular mechanism shifting from sucking to biting of mice. Acta Anat. **133** ; 200-208 1988.

著者への連絡先：岸 飛鳥，(〒963-8611) 郡山市富田町字三角堂31-1 奥羽大学生体構造学講座口腔解剖学分野
Reprint requests : Asuka KISHI, Division of Oral Anatomy, Department of Morphological Biology, Ohu University, School of Dentistry
31-1 Misumido, Tomita, Koriyama. 963-8611, Japan