JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 42 (2): 47–53, 2009
doi:10.1267/ahc.08027

Immunohistochemical Localization of Barx2 in the Developing Fetal Mouse Submandibular Glands

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Received August 11, 2008; accepted January 28, 2009; published online March 31, 2009

The development of mouse submandibular gland (SMG) begins at embryonic day 11.5–12 (E11.5–12), during which successive rounds of epithelial clefting and branching create complex epithelial tree-like structures. Homeobox genes regulate place-dependent morphogenesis, including epithelial-mesenchymal interactions, and control the expression patterns of signaling molecules. The Barx2 containing Homeobox exerts several key roles in development. Some studies have shown that the Barx2 plays important roles in the epithelial-mesenchymal interactions of organogenesis. However, the mechanisms of Barx2 associated with the development of SMG are obscure. In this study, we demonstrated for the first time the exact spatial and temporal Barx2 expression pattern in SMG epithelial tissue during development using immunohistochemical staining and Real-Time quantitative PCR. Barx2 was expressed in the nucleus of the epithelial cells located in the proliferative and differentiative regions of the developing SMG during the early development stages (E11.5–E13.5). After the E14.5-time period, the expression gradually decreased, and at E16.5, expression mostly disappeared despite the fact that evidence of cytodifferentiation, such as the appearance of proacinar cells, distinct lumen formation, and secretory products, was beginning to be observed. Results of Real-Time PCR demonstrated that the amount of *Barx2* mRNA expression in SMG was maximal on E14.5, and gradually decreased by E18.5. These results indicate that Barx2 is associated with early stage epithelial tissue development, and can be a useful epithelial marker of the SMG during early developmental stages.

Key words: Barx2, development, mouse, submandibular gland, immunohistochemistry

I. Introduction

Around embryonic day 11 (E11), embryonic mouse submandibular gland (SMG) morphogenesis is initiated with the thickening of mandibular arch oral epithelium. This thickened epithelium grows down into the first brachial (mandibular) arch mesenchyme, which is derived from the cranial neural crest, to form the initial SMG bud. With continued epithelial proliferation and successive rounds of cleft formation, duct elongation, and lumen formation, the SMG primordium becomes more complex and develops a characteristic three-dimensional, tree-like structure [7]. This developmental system is called branching morphogenesis and is observed in many organs, such as the mammary glands, lungs, pancreas, and the kidneys. Some studies have revealed that branching morphogenesis is dependent on epithelial-mesenchymal interactions [4], and that this interaction is regulated by many different signaling molecules, such as bone morphogenetic proteins (BMPs) [6, 13], transforming growth factors (TGFs) [3, 9, 12], fibroblast growth factors [13, 15, 16, 31], epidermal growth factor (EGF) [9, 20, 21], hepatocyte growth factor (HGF) [8], sonic hedgehog (Shh) [14], hormones [9, 11], and extracellular matrix (ECM) [23]. Homeobox genes are thought to be transcription factors that control the activity of other genes. In many

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organs, as well as the SMG [11, 22], the expression of these genes has been well investigated [19, 29]. To date, several homeobox genes including *Six-1* [22], *Pitx-1* [32], *Pax-6* [13] and *Msx-2* [11] have been shown to be differentially and spatially expressed during embryonic development of the SMG. However, not much is known about which molecules are regulated by the products of these genes. Other homeobox genes have been implicated in the control of the expression of cell adhesion molecules [17, 34]. A vertebrate homolog of the *Drosophila Bar* homeobox gene, mouse *Barx2*, was isolated when various factors regulating the gene for the cell adhesion molecules NCAM-L1 were screened [18]. Barx2 appears to be able to both activate and repress the *NCAM-L1* promoter. In mouse embryogenesis, *Barx2* is expressed in the central and peripheral nervous system and the ectodermal lining of craniofacial tissues, regions that express NCAM-L1 and another cell adhesion molecule, Ng-CAM [18]. However, the precise location of *Barx2* in the complex SMG tissue was not identified to date.

This study determined whether Barx2 exerts an effect during SMG development. Using immunohistochemistry the spatiotemporal distribution of Barx2 during the various stages of SMG development was investigated.

II. Materials and Methods

Sample preparation

All animal experiments were performed according to the guidelines for the care and use of animal issued by Ohu University. Timed-pregnant female mice (strain ICR) were purchased from Clea Japan (Tokyo, Japan). The plug day was considered to be the day of gestation initiation. Pregnant mice were anesthetized with diethyl ether and sacrificed by cervical dislocation on days 11.5, 12.5, 13.5, 14.5, 16.5, and 18.5 of gestation (E11.5, 12.5, 13.5, 14.5, 16.5, and 18.5, respectively). Embryos were dissected in cold phosphate-buffered-saline (PBS, Takara Bio Inc., Shiga, Japan) and were used for the immunohistochemical analysis. Mouse embryos at E11.5–13.5 were fixed in 10% phosphate-buffered neutral formalin (pH 7.4) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), dehydrated in an ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) series, washed in xylene, and then embedded in paraffin. Embryos at E14.5–18.5 were decalcified in 0.5 mol/l EDTA (pH 7.5) (Wako Pure Chemical Industries) for 5 days at 4°C prior to ethanol dehydration.

Immunohistochemistry

Immunohistochemical staining was performed as described previously [28].

Rabbit polyclonal antibody to mouse Barx2 and NCAM-L1 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). These antibodies were used in the immunohistochemical analysis to detect Barx2 or NCAM-L1, respectively.

Some sections were stained with hematoxylin and eosin (H&E), and the other sections were stained using immunohistochemical techniques to identify Barx2. Tissue sections (4–5-μm-thick) were mounted on poly-L-lysine coated slides. For staining, the slides were dewaxed with xylene, rehydrated with descending grades of ethanol, and rinsed with Tris-Buffered-Saline (TBS, Takara Bio Inc.). After washing with TBS, endogenous peroxidase was blocked by 0.3% hydrogen peroxide at room temperature for 10 min. Then, the slides were washed with TBS and treated with 0.1% trypsin solution (Nichirei Biosciences Inc, Tokyo, Japan) for 10 min. Subsequently, all slides were washed with TBS and blocked using endogenous mouse immunoglobulin for 30 min at 4°C with goat serum. After washing with TBS, the primary antibodies were diluted, and the slides were incubated overnight at 4°C. Negative control slides were incubated in diluent buffer alone. The slides were then washed with TBS and incubated for 10 min at room temperature with biotinylated goat anti-rabbit secondary antibody (Histofine SAB-PO(R) kit; Nichirei Biosciences Inc.). After washing with TBS, the sections were incubated with peroxide-conjugated streptavidin for 5 min at room temperature (Nichirei Biosciences Inc.). After washing with TBS, the DAB substrate kit (Nichirei Biosciences Inc.) was applied to the slides for 6 min at room temperature. Next, all of the slides were dehydrated and mounted. The experiments were repeated at least 5 times to negate sex differences between the specimens.

RNA isolation and Real-Time PCR

Total RNA was extracted from E12.5~18.5 SMG using EASYPrep RNAKit (Takara Bio Inc.), and treated with DNase 1 (Toyobo Co., LTD. Osaka, Japan). First-stand cDNA was synthesized from total RNA (400 ng) using RT-PCR kit (Takara Bio Inc.) and random primers. Subsequently 1 μg of cDNA was used as a template for the second step of Real-Time quantitative PCR (RT-qPCR). Primers was used at 5 μM with 12.5 μl of SYBR Green Premix (Takara Bio Inc.) in a final volume of 25 μl.

SYBR Green PCR amplification and real-time fluorescence detection were performed using the Smart Cycler II System (Takara Bio Inc.). Rapid PCR cycling conditions were as follows: 95°C for 10 seconds (denaturation), followed by 45 cycles at 95°C for 5 seconds and 60°C for 20 seconds (annealing/extension). The primer sequencese were as follows: β-*actin*, 5'-ATT CCG ACA GGA TGC GCA GA-3' and 5'-GAG TAC TTG CGC TCA GGA GGA-3'; *Barx2*, 5'-CAG AAG TCG CAC CAT CTT CAC C-3' and 5'-CTT CAC TTG CAG CTG AGT GAG TCC-3'. Measurements were taken at the 60°C extension step in each cycle, and the second-derivative method was used to calculate the threshold cycle. Melt curve analysis showed a single sharp peak for all samples. Target gene expression level was normalized to beta-actin expression in each sample. Each PCR product was analyzed by 3% agarose gel electrophoresis, and ethidium bromide-stained PCR products were evaluated.

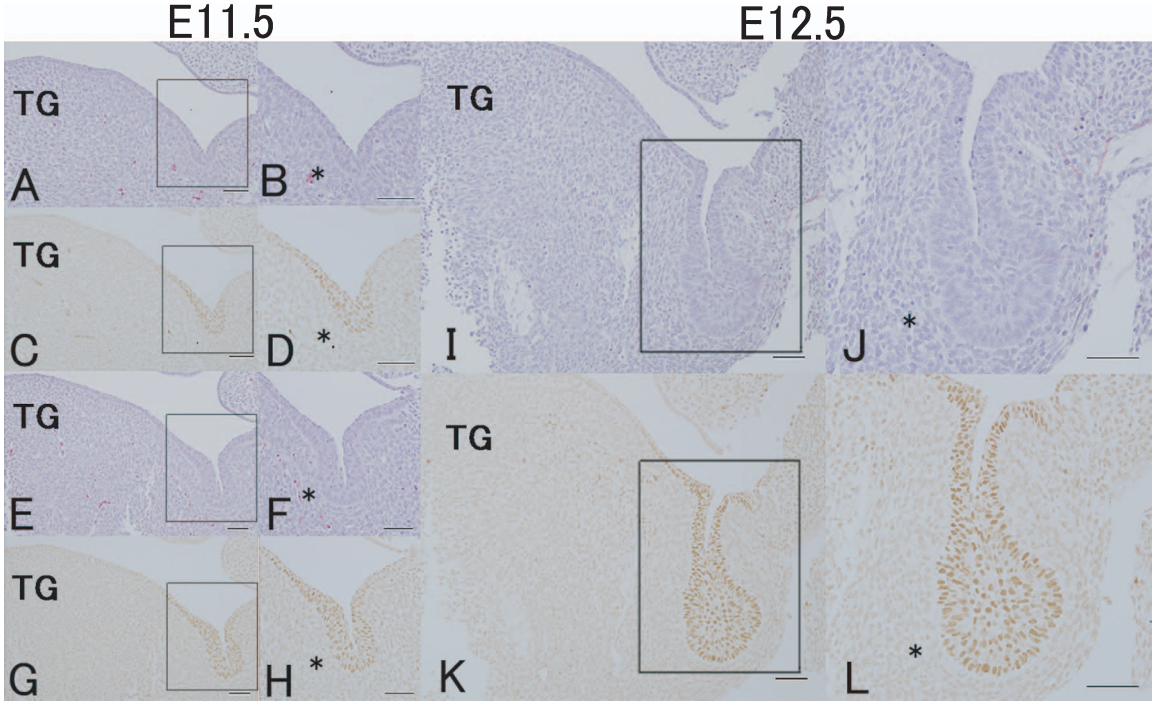

Fig. 1. Immunohistochemical staining for Barx2 expression during embryonic submandibular gland (SMG) development. **A, B, E, F, I**, and **J**: Hematoxylin and eosin (H&E) staining during SMG development. **C, D, G, H, K**, and **L**: Barx2 expression in the developing SMG. **B, D, F, H, J**, and **L**: Higher magnification of the boxed section in **A, C, E, G, I**, and **K**. **A–D**: At early E11.5, Barx2 is primarily found in the thickening oral epithelium. **E–H**: At late E11.5, Barx2 is primarily immunolocalized in the oral epithelium, the epithelial stalk, and the epithelial bulb. **I–L**: At E12.5, Barx2 is immunolocalized in the epithelium stalk and the end-bulb. Bar=100 μm. TG: tongue. *: mesenchymal tissue.

III. Results

Barx2 localization in SMG

At early E11.5, the oral epithelium proliferates to form a thickened epithelium. This thickened epithelium grows down into the underlying mesenchyme, which is derived from cranial neural crest cells, and forms a complex SMG structure (Fig. 1A and B). The expression of Barx2 was observed in the nucleus of this thickened oral epithelium. Positive staining was not observed in the other areas of the oral epithelium (Fig. 1C and D). During the late E11.5 stage, the thickened epithelium had deeply invaded into a condensed mesenchyme and solid epithelial stalk, at this time, a terminal bulb was first observed (Fig. 1E and F). The positive staining of Barx2 was observed in the nucleus of the oral epithelium around the ductal foramen, epithelial stalk, and epithelial bud (Fig. 1G and H). At E12.5, the epithelial stalk and terminal bulb were clearly observed, and the initial formation of the sublingual gland was observed next to the SMG primordia (Fig. 1I and J). The expression of Barx2 was observed clearly throughout SMG epithelial components, such as the stalk and the terminal-bulb (Fig. 1K and L). At E13.5, the solid cord of epithelium elongated and formed the presumptive duct and terminal buds (Fig. 2A). The expression of Barx2 in the nucleus of the branched epithelium of SMG was still high at E13.5 (Fig. 2B). At E14.5, an increased number of epithelial lobules and complex presump-

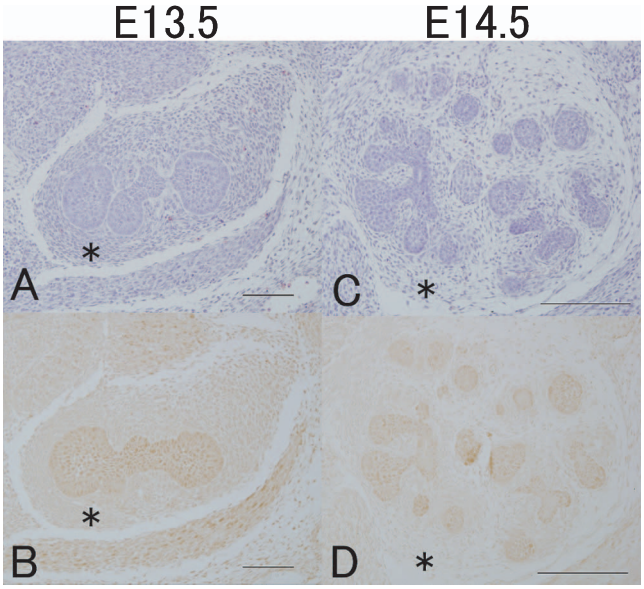

Fig. 2. Immunohistochemical analysis of Barx2 expression during embryonic SMG development. **A** and **C**: H&E staining of the developing SMG. **B** and **D**: Barx2 expression in the developing SMG. **A** and **B**: At E13.5, Barx2 is seen throughout the branching epithelia. **C** and **D**: At E14.5, Barx2 is found only in some epithelial buds and ductal epithelia. Bar=200 μm. *: mesenchymal tissue.

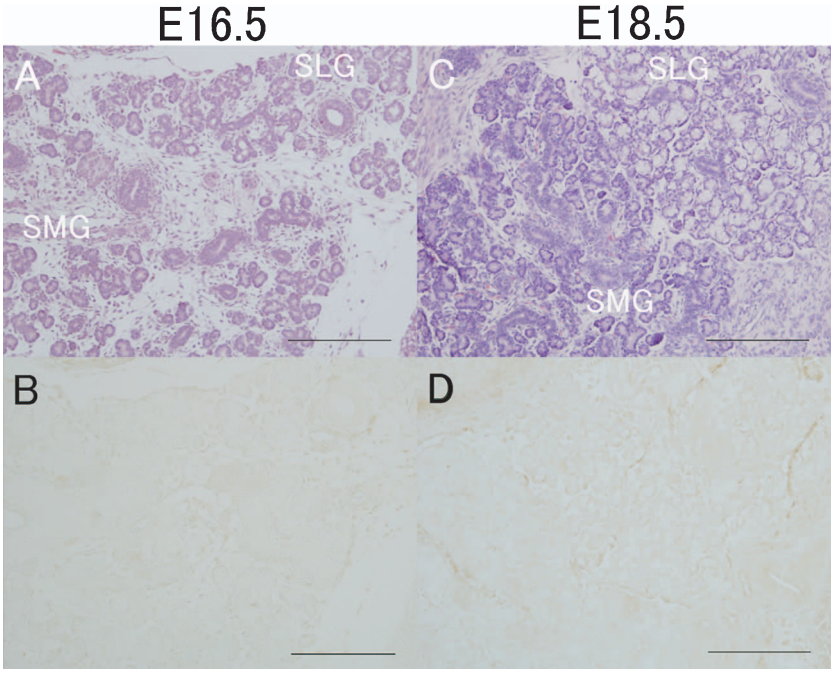

Fig. 3. Immunohistochemical analysis of Barx2 expression during embryonic SMG development. **A** and **C**: H&E staining of the developing SMG. **B** and **D**: Barx2 expression in the developing SMG. **A** and **B**: At E16.5, no expression of Barx2 is found. **C** and **D**: At E18.5, no expression of Barx2 is found. Bar=200 μm. SMG: submandibular gland. SLG: sublingual gland.

tive ducts that consisted of multilayered epithelial cells were observed. Lamina formation was clearly observed into the presumptive ducts (Fig. 2C). The expression of Barx2 gradually decreased in all epithelial structures overtime (Fig. 2D). At E16.5, the duct system was clearly identified, and the terminal bud lumina had increased in size. The initial stages of glandular cytodifferentiation into serous acina and mucous acina were observed (Fig. 3A). The expression of Barx2 was barely detectable at this stage (Fig. 3B). Continuing in this vein, the expression of Barx2 at E18.5 was negative (Fig. 3C and D). Control slides that were incubated in the absence of primary antibody were found to have no specific staining (data not shown).

Real-time quantative PCR

In order to confirm the expression of *Barx2* in the developing SMG, we used RT-qPCR to evaluate the expression level of *Barx2*. The expression level of *Barx2* was higher in the E14.5 and gradually decreased until E18.5 (Fig. 4).

NCAM-L1 lodcalization in SMG

At E12.5, the expression of NCAM-L1 was localized in the epithelial tissues at cell-cell surface (Fig. 5B). At E14.5, NCAM-L1 was expressed in the epithelium at cell-cell surface of the branched epithelium (Fig. 5D). The expression of NCAM-L1 at E18.5 was observed but decreased in the epithelial tissues (Fig. 5F).

IV. Discussion

In this study, the expression of Barx2, one of the homeobox genes, during the development of fetal mouse SMG was investigated. The results of the present study show that Barx2 is expressed in the epithelium of the SMG during the early developmental stage. These results suggest that Barx2 may be a critical factor in the development of SMG. To the best of our knowledge, this is the first report that shows the exact spatial and temporal immunohistochemical expression of Barx2 during SMG development.

Homeobox genes play important roles in the organogenesis and the morphogenesis that occurs during vertebrate

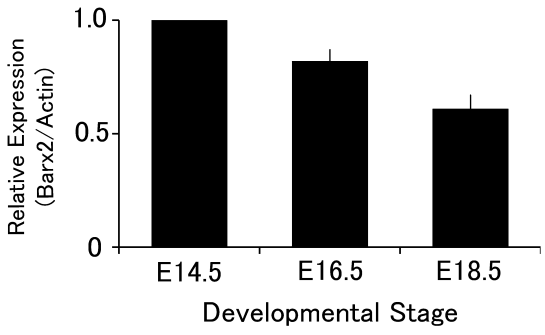

Fig. 4. RT-qPCR analysis of *Barx2* expression during fetal mouse SMG development. *Barx2* expression levels are higher at E14.5 and as developmental stages are progressed, the expression was decreased until E18.5.

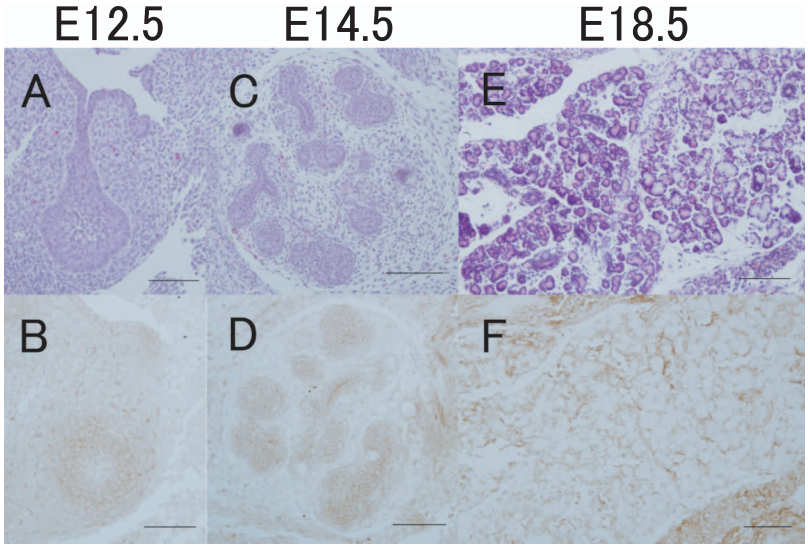

Fig. 5. Immunohistochemical analysis of NCAM-L1 expression during embryonic SMG development. **A, C** and **E**: H&E staining of the developing SMG. **B, D** and **F**: NCAM-L1 expression in the developing SMG. **B**: At E12.5, NCAM-L1 is immunolocalized in the cell-cell surface of the epithelium stalk and the end-bulb. **D**: At E14.5, NCAM-L1 is seen throughout the cell-cell surface of the branching epithelia. **F**: At E18.5, weak expression of NCAM-L1 is found in the epithelial tissues. Bar=100 μm.

development [19, 22]. Previous studies dealing with SMG development have revealed that the knockout of *Six1 or Pax6* results in SMG malformation [13, 16] and that the knockout of *Pitx1* induces a lack of SMG formation [32]. A study using *in situ* hybridization noted that *Msx2*, *Barx1* and *Barx2* are involved in SMG development [11, 27, 33]. Especially, Barx1 was observed in the mesenchymal tissue of the developing SMG between E11.5 and E15.5. After the period, the expression mostly disappeared [33]. *Barx1* and *Barx2* both belong to the *Bar* class homeobox genes, and they have a complementary expression pattern during the development of craniofacial structures [18]. These studies support our findings that Barx2 is intimately correlated with SMG development.

In this report, using immunhistochemical techniques, the expression of Barx2 was observed mainly in the nucleus of the epithelial cells that form the buds, stalk, and ducts during E11.5–14.5, which is considered the early developmental stage. After this time period, the expression of Barx2 was reduced, and its expression was barely detected until E16.5, at which time the epithelial cells began to express secretory gland characteristics [10]. We also used RT-qPCR techniques to confirm the expression of *Barx2* in the developing SMG and found that the expression of *Barx2* correlated with the results of immunohistochemical staining. These results suggest that Barx2 may be required for the initiation of epithelial thickening, initial bud formation, and the earliest epithelial branching, but that it may not be involved in the epithelial differentiation that occurs during the late stage. It has been demonstrated that branching morphogenesis is mediated by an epithelial-mesenchymal interaction controlled by certain growth factors, homeobox genes and cell adhesive molecules. However, the role of Barx2 and of the signaling cascades that are downstream of the Barx2 remains unclear with respect to SMG development. To date, there is evidence that Barx2 is a protein that binds to a regulatory element that is common to the genes related to two neural cell adhesion molecules (NCAMs), Ng-CAM and NCAM-L1 [18, 30]. NCAM mediates cell-cell adhesion not only in the neuronal system but also in a variety of embryonic tissues, such as the teeth, kidneys, pancreas, and hair follicles [1, 2, 5, 24, 26]. These organs, as well as the SMG, are structured by epithelial-mesenchymal interaction. In this paper, we also observed NCAM-L1 expression in the SMG epithelial tissues. This suggests that Barx2 may control the morphogenesis of the early developmental stage of SMG by controlling the expression of cell adhesion molecules such as NCAM and NCAM-L1 on the epithelial side [25, 33].

In conclusion, we demonstrated that the expression of Barx2 was restricted to the epithelial cells during the early developmental stage of the fetal mouse SMG (Fig. 6). This

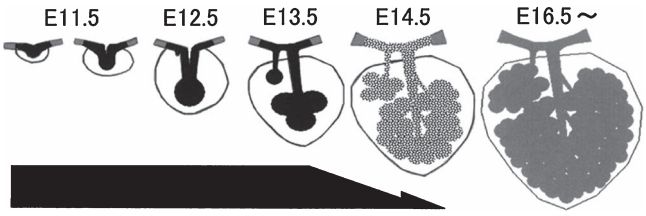

Fig. 6. Summary of the changes in the expression of Barx2 during fetal mouse SMG development. A high level of Barx2 binding was observed in the developing fetal mouse SMG epithelial cells between E11.5 and E13.5. The expression of Barx2 gradually decreased and almost completely disappeared by E16.5.

suggests that the homeobox gene, Barx2, plays physiological roles in developing SMG development. These data contribute to the fundamental studies of SMG development and morphogenesis.

V. Acknowledgements

This study was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan (20791393).

VI. References

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