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Expression of p63 during Early Craniofacial Development of the Mouse Embryo

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생쥐의 초기 두개악안면 발생 중 p63의 발현 양상

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ABSTRACT : p63 has been demonstrated to localize in stem cells and precursor cells of various epithelial tissues previously, but the localization of p63 throughout tooth formation, particularly during the enamel and root formation stages, remains to be adequately characterized. Therefore, in this study, we have demonstrated, via immunohistochemical methods, that p63 is ubiquitously expressed in the dental epithelium during tooth development. p63 was detected in the basal and suprabasal layers of the epithelia, including the skin, hair follicles, oral mucosa, and submandibular ducts. However, in the tooth region, all cells of the dental lamina, enamel organ, Hertwig's epithelial root sheath (HERS), and epithelial cell rests of Malassez (ERM) evidenced immunoreactivity for p63. These results indicate that p63 may perform different roles, other than stem cell maintenance, in tooth development.

Key words : p63, Tooth development, Dental epithelium, Hertwig's epithelial root sheath, Epithelial cell rests of Malassez.

요 약 : p63은 다양한 상피 조직의 줄기세포와 전구세포에 존재한다는 사실이 잘 알려져 있으나, 치아 형성, 특히 사기질과 뿌리 형성시기에서의 p63 위치는 아직 연구해야 할 과제로 남아 있다. 본 연구에서는 p63이 치아 발생 동안 치아상피에 편재하여 나타나는 것을 면역조직화학 기법을 이용하여 확인하였다. p63은 피부, 모낭, 구강점막 그리고 턱밑샘 도관을 포함하는 상피의 바닥층과 바닥위층에 위치하였다. 그러나 치아 부위에서는 치아판의 모든 세포, 사기질기관, 헤르트비히 뿌리상피집 그리고 말라세쯔 상피잔사에 p63이 관찰되었다. 이 결과는 치아 발생 중 p63이 줄기세포 유지 외에도 다른 기능을 한다는 사실을 보여준다.

INTRODUCTION

p63 has been recognized as a transcription factor belonging to a family of two structurally related proteins, p53 and p73 (Lohrum and Vousden, 2000). The p63-deficient mice showed developmental failures of the stratified squamous epithelium and the attendant appendages, including hair

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follicles, mammary glands, and salivary glands (Bilal et al., 2003; Mills et al., 1999; Yang et al., 1999). In addition, as p63 has been detected in the epithelial basal cells of a variety of tissues, including the skin, glandulae sebaceae, hair follicles, mammary glands, and esophagus (Di Como et al. 2002; Glickman et al., 2001; Yang et al., 1998), this protein is believed to be a stem and/or progenitor cell marker of the epithelium (Chen et al., 2004; Senoo et al., 2007). In the teeth, p63 has been reported to be expressed in the enamel organ from the bud stage to the early bell stage of tooth development (Kock et al., 2005; Laurikkala et al., 2006) and the mutation of this gene in mice induced tooth loss prior to the dental lamina stage (Laurikkala et al., 2006; Rufini et al., 2006). However, as these reports were analyzed only during the early stages of tooth development, the localization of p63 throughout tooth formation, especially at the enamel and root formation stages, remains to be adequately characterized.

p63 exists as multiple protein isoforms of two classes, deltaNp63 and TAp63, with different functional capacities (Yang et al., 1998). These proteins have been implicated in a number of biological processes that contribute to cell proliferation and differentiation in epithelial tissues (King and Weinberg, 2007). DeltaNp63 is thought to be associated with cell proliferation in human cancers as well as in normal skin (Parsa et al., 1999). Additionally, deltaNp63 is required for the initial commitment to cell differentiation, and that its subsequent suppression is also necessary for cell differentiation to proceed (King et al., 2003; Nguyen et al., 2006). The enamel organ of mouse embryo revealed that most of the transcripts were deltaNp63, while TAp63 transcripts showed no expression (Laurikkala et al., 2006). Therefore, it is thought that deltaNp63 is the major splice form in the enamel organ and might be regulate the cell proliferation and differentiation, but the functions of p63 in dental epithelium after birth are still unclear.

In the present study, we demonstrate that p63 is specifically localized within all dental epithelial cells throughout the tooth formation process. Observations on cell differentiation during the enamel formation process were made by using amelogenin-specific antibody. In addition, in order to compare the pattern of p63 localization with that in other tissues, the head regions of embryonic mice were also assessed via p63 immunohistochemistry.

MATERIALS AND METHODS

All experiments were conducted in accordance with the strict guidelines established by the Intramural Animal Use and Care Committee of the Yonsei University College of Dentistry. The mouse heads were collected at embryonic day (E) 13~17 and postnatal day (PN) 2~14, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours at 4℃. These specimens were demineralized with 10% EDTA (pH 7.4) for 2 weeks at 4℃, then paraffin-embedded. Sagittal and frontal sections (5-㎛ thickness) were processed for immunohistochemistry using mouse monoclonal antibody against human p63 (4A4, NeoMarkers, CA, USA) and rabbit polyclonal antibody against bovine amelogenin (Hokudo Co., Sapporo, Japan) for 12 hours at 4℃. After incubation with biotynylated goat antibody against mouse IgG (Dako Cytomation, CA, USA) or rabbit IgG (Zymed Laboratories Inc., CA, USA), they were then allowed to react with horseradish peroxidase (HRP)-conjugated streptavidin (Zymed Laboratories Inc.). During these procedures, the sections were subjected to autoclaving (Cell Marque, CA, USA), while being immersed in citric acid buffer (pH 6.0; Zymed Laboratories Inc., CA, USA), at 121℃ for 15 minutes for antigen retrieval. The immune complexes were then visualized using diaminobenzidine (Liquid DAB Substrate kit; Zymed Laboratories Inc), and the sections were counterstained with hematoxylin.

RESULTS AND DISCUSSION

During embryogenesis, p63 was localized in many kinds of epithelial cells in the heads of the mice. At E13, p63

positive cells were detected in the skin epithelium and oral mucosa of the tongue and palate. These epithelia are classified as simple epithelium, and all cells of these epithelia evidenced p63 immunoreactivity (not shown). At E17, epithelial tissue of the skin, oral mucosa, nasal mucosa, and tongue became multilayered, and p63 positive cells were confined to the basal and suprabasal layers of these tissues (Fig. 1a~c). This immunoreactivity was also observed in the outer root sheath and hair matrix cells of the hair follicle (Fig. 1a). Additionally, ducts of the palatine glands evidenced p63 immunoreactivity, as did also the epithelium of the mucous membrane in the hard and soft palate (Fig. 1b~c). Basal duct cells of submandibular and sublingual glands (Fig. 1d) and myoepithelial cells of submandibular gland (Fig. 1e) tested positive for p63, but luminal duct cells and acinar cells exhibited no immunoreactivity (Fig. 1d~e). As has been noted in previous reports (Bilal et al., 2003; Tsujita Kyutoku et al., 2003; Yang et al., 1999), p63 was detected in the basal and suprabasal layers of the epithelium in the head region; however, all cells of the dental lamina evidenced this immunoreactivity (Fig. 1f).

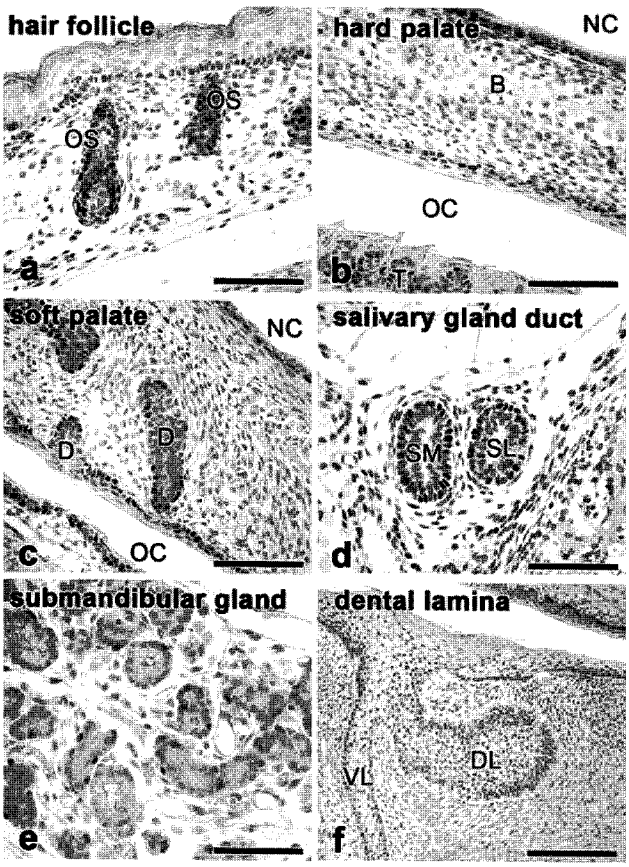

Fig. 1. Immunohistochemical staining of p63 in the hair follicle (a), hard palate (b), soft palate (c), salivary gland duct (d), submandibular gland (e), and dental lamina (f) at E17. Figures "b~c, e~f" and "a, d" show the sagittal and frontal sections, respectively. B, bone; D, duct of palatine gland; DL, dental lamina; LD, luminal duct; NC, nasal cavity; OC, oral cavity; OS, outer root sheath; SL, sublingual duct; SM, submandibular duct; T, tongue; VL, vestibular lamina. Scale bars = 150 ㎛ (f), 80 ㎛ (a~e).

In molar tooth germ, p63 was localized in the dental epithelium, but not in the dental mesenchyme at the bud stage (E13). This immunoreactivity was observed within the cell nucleus of these epithelial cells (Fig. 2a). At the cap stage (E14), the enamel organ can be divided into three portions of the epithelia, including the outer dental epithelium, the inner dental epithelium, and the stellate reticulum. These cells evidenced positive immunoreactivity, but the outer dental epithelium and stellate reticulum evidenced more intense p63 staining than was observed in the inner dental epithelium (Fig. 2b). At the early bell stage (E17), the stratum intermedium formed between the inner dental epithelium and stellate reticulum, and p63 was localized within this tissue. The same intensity of p63-immunostaining was observed in all dental epithelial cells (Fig. 2c). Amelogenin localization and expression occur within enamel, ameloblasts, preameloblasts, and even only temporarily in preodontoblasts (Hu et al., 2001; Karg et al., 1997). At the late bell stage (PN2), as the cells of the inner layer of the enamel organ were immunopositive for amelogenin without enamel formation, these cells had differentiated into preameloblasts from inner dental epithelium (Fig. 2i). In this stage, p63 was localized within these preameloblasts, in addition to the inner dental epithelium, outer dental epithelium, stellate reticulum, and stratum intermedium (Fig. 2d~f). At PN10, thick enamel was formed and ameloblasts aligned with the surface of

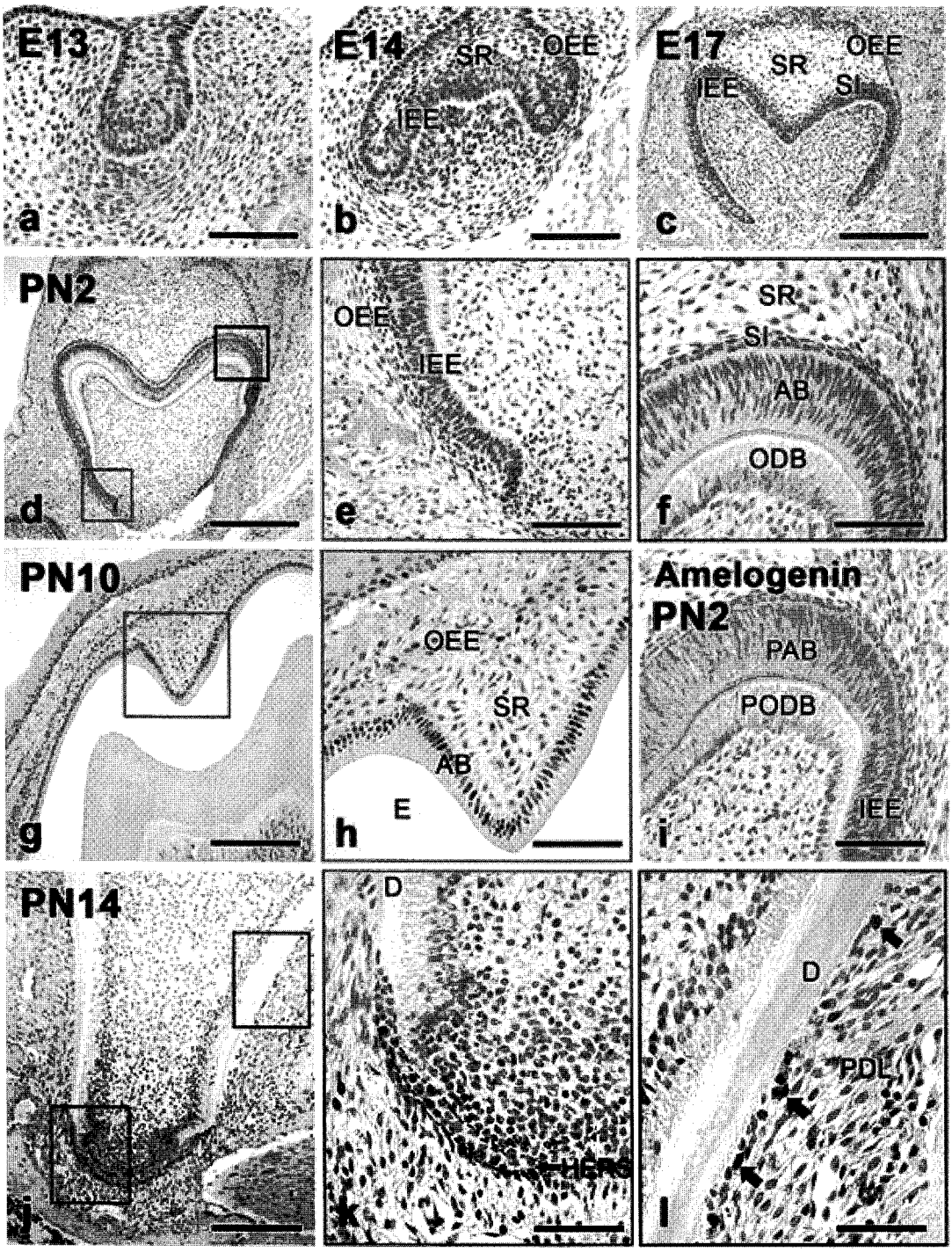

Fig. 2. Immunohistochemical staining of p63 (a~h, j~l) and amelogenin (i) in the mandibular first molar at E13 (a), E14 (b), E17 (c), PN2 (d~f, i), PN10 (g~h), and PN14 (j~l). Higher magnifications of the rectangles in "d", "g" and "j" are shown in "e~f", "h" and "k~l", respectively. AB, ameloblast; D, dentin; E, enamel space; HERS, Hertwig's epithelial root sheath; IEE, inner dental epithelium; OEE, outer dental epithelium; PAB, preameloblast; PDL, periodontal ligament tissue; PODB, preodontoblast; SI, stratum intermedium; SR, stellate reticulum. Scale bars = 250 ㎛ (d, j), 180 ㎛ (c, g), 80 ㎛ (a~b), 60 ㎛ (e~f, h~i, k~l).

this tissue. Ameloblasts are terminally-differentiated cells of the inner dental epithelium and the expression of deltaNp63 isoform disappears in these cells from the inner dental epithelium (Laurikkala et al., 2006), but p63 localization was observed in the ameloblasts (Fig. 2g~h). The dental papillae and dental follicle evidenced no immunoreactivity (Fig. 2b~h). Therefore, during crown formation, p63 was localized in all cell types of dental epithelium, regardless of the state of epithelial cell differentiation.

After the crown formation stage, the inner and outer dental epithelia at the lower edge of the enamel organ proliferate and form the Hertwig's epithelial root sheath (HERS). The HERS migrate between the dental papillae and dental follicle, and this structure rupture as root formation progress. This ruptured epithelium is generally referred to as the epithelial cell rests of Malassez (ERM). At the root formation stage (PN14), p63 positive cells were confined to the root apex and root surface (Fig. 2j). In the root apex, some HERS cells were intensely positive for p63, but other HERS cells evidenced weak immunoreactivity (Fig. 2k). In the upper region of the periodontal space, p63 immunostaining could be detected in the ERM cells (arrows in Fig. 2l), which were also immunopositive for the epithelial cell marker, cytokeratin (not shown). In both regions, no immunoreactivity for p63 was noted, with the exception of that associated with the dental epithelium (Fig. 2j~l).

As the rodent incisor erupts continuously throughout the life of the animal, the rodent incisor is considered to be a useful sample by which all stages of ameloblast differentiation can be observed in the one tooth. At the apical region of the incisor, epithelial stem cells are believed to exist in the stratum intermedium of the apical bud (Harada et al., 1999; Harada et al., 2004). These stem cells in the labial side proliferate and differentiate into preameloblasts and secretary ameloblasts, as the degree of differentiation progresses. At the apical region, p63 was detected in the dental epithelium on the lingual and buccal sides (Fig. 3a). This immunoreactivity was almost identical to that in the

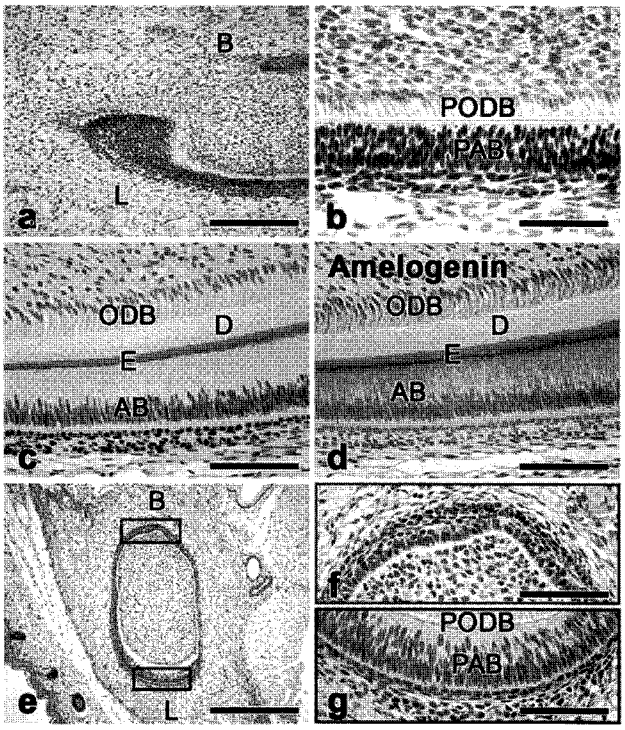

Fig. 3. Immunohistochemical staining of p63 (a~c, e~g) and amelogenin (d) in the mandibular incisor at PN2. Higher magnifications of the rectangles in "e" are shown in "f~g". Figures "a~d" and "e~g" show the sagittal and frontal sections, respectively. AB, ameloblast; B, buccal side; D, dentin; E, enamel; L, lingual side; ODB, odontoblast; PAB, preameloblast; PODB, preodontoblast. Scale bars = 300 ㎛ (e), 200 ㎛ (a), 80 ㎛ (f~g), 70 ㎛ (b~d).

preameloblast (Fig. 3b); however, the ameloblasts secreting enamel matrix (Fig. 3d) evidenced weaker staining than preameloblasts and other enamel epithelial cells (Fig. 3c). In the frontal section of the incisor, all dental epithelial cells proved immunopositive for p63 without any distinction with regard to labial or buccal orientation (Fig. 3e~g).

In the orofacial region, p63 was expressed in a broad variety of epithelial tissues, including the skin, oral mucosa, tongue, and submandibular gland (Fig. 1a~e). These positive cells localized in the epithelial basal layers are referred to as the stem and progenitor cell niche (Pellegrini et al., 2001; Yang et al., 1999). However, all cells of the dental lamina (Fig. 1f) and the enamel organ (Fig. 2a~h, 3) evidenced p63 immunoreactivity. In addition, HERS and

ERM cells were also positive for p63 (Fig. 2j~l), thereby indicating that p63 does not represent epithelial stem cells in the tooth. It is believed that the function of p63 involves the maintenance of the proliferative and differentiative potentials of epithelial stem cells (Koster et al., 2004; Truong et al., 2006). Cells in the enamel organ, HERS, and ERM evidence proliferative potential and are able to differentiate into other type of dental epithelial cells, but ameloblasts exhibit no proliferation. Further research into the role of p63 in tooth formation will clearly be required.

In conclusion, all epithelial cells in the tooth evidenced immunoreactivity for p63 throughout the process of tooth formation. This localization pattern is not consistent with other epithelial tissues in the orofacial region. Therefore, p63 may perform functions separate from the maintenance of stem cell ability in the tooth.

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