Differentiation (2007) 75:158–165 DOI: 10.1111/j.1432-0436.2006.00122.x

ORIGINAL ARTICLE

Sung-Won Cho · Jae-Young Kim · Jinglei Cai · Jong-Min Lee · Eun-Jung Kim · Hyun-A Lee · Hitoshi Yamamoto · Han-Sung Jung

Temporospatial tissue interactions regulating the regeneration of the enamel knot in the developing mouse tooth

Received April 13, 2006; accepted in revised form July 26, 2006

Abstract The enamel knot (EK), which is a transient signaling center in the tooth germ, regulates both the differential growth of the dental epithelium and the tooth shape. In this study, the regeneration of the EK was evaluated. The EK regions were removed from the E14 and E16 dental epithelia, and the remaining epithelia were recombined with their original dental mesenchymes. All these tooth germs could develop into calcified teeth after being transplanted into the kidney capsule for 3 weeks. One primary EK was regenerated earlier, and two or three secondary EKs were regenerated later in culture. When simply recombined without removing the EK, the tooth germ, which had four secondary EKs and four cuspal areas of the dental papilla, generated one primary EK first and subsequent secondary EKs. These results indicate that the patterning of the EK in all tooth germs always starts from a primary EK independent of the direct epithelial or mesenchymal control. This suggests that neither the dental epithelium nor the dental mesenchyme can dictate the pattern or number of the EK formation, but the interaction between the dental epithelium and the dental mesenchyme is essential for the regeneration and patterning of the EKs.

Key words enamel knot · tooth germ · regeneration · tissue interaction · fate map

Sung-Won Cho · Jae-Young Kim · Jinglei Cai · Jong-Min Lee · Eun-Jung Kim · Hyun-A Lee · Han-Sung Jung (✉)
Division in Anatomy and Developmental Biology
Department of Oral Biology
Research Center for Orofacial Hard Tissue Regeneration
Oral Science Research Center
Brain Korea 21 Project, College of Dentistry, Yonsei Center of Biotechnology
Yonsei University, Seoul, Korea
Tel: +82 2 2228 3065
Fax: +82 2 312 8012
E-mail: hsjung@yumc.yonsei.ac.kr

Hitoshi Yamamoto
Department of Anatomy (Division II)
Nihon University School of Dentistry at Matsudo 2-870-1
Sakaecho-nishi
Matsudo, Chiba 271-8587, Japan

Introduction

The mammalian tooth is an ectodermal organ, in that its development is controlled by reciprocal interactions between the epithelium and the mesenchyme. All ectodermal organs share similar signaling molecules during early morphogenesis but each organ undergoes its own specific pattern of formation later in development (Hogan and Yingling, 1998; Pispa and Thesleff, 2003).

The formation of cusps is also predicated on the formation of species-specific patterns. The enamel knot (EK) is considered to be the most important structure in determining the number of cusps, as well as the shape of an individual tooth. There are two types of EKs during mouse molar tooth development: the primary EK and the secondary EK (Jernvall et al., 2000). Both the primary and secondary EK share the expression of the same genes. Signaling molecules such as *Fgf4*, *9*, *Shh*, *Wnt10a*, *b*, and *Bmp2*, *4*, *7*, and transcription factors such as *Msx2* and *Lef1* are expressed in the primary and secondary EK (Åberg et al., 1997; Keränen et al., 1998; Kettunen and Thesleff, 1998; Sarkar and Sharpe, 1999; Kratochwil et al., 2002; Laurikkala et al., 2003). Among these genes, *Fgf4* and *Slit1* can be used as EK markers because they are exclusively observed in both primary and secondary EKs (Jernvall et al., 1994; Pispa et al., 1999; Loes et al., 2001).

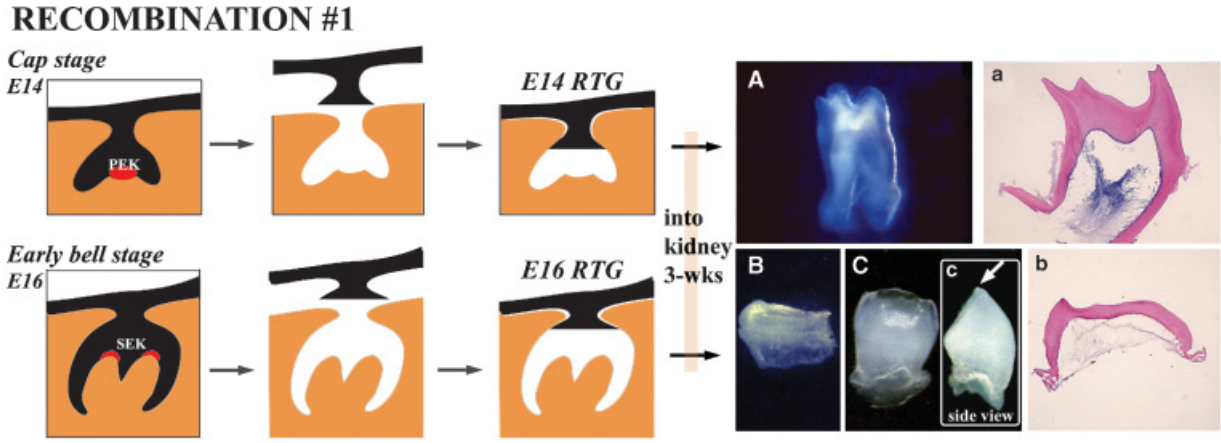

Fig. 1 Recombination method and the calcified teeth from the recombinant tooth germs (RTGs). (Recombination #1) The remaining E14 and E16 epithelia were recombined with each original dental mesenchyme to form an E14 RTG and E16 RTG, respectively. After three weeks in kidney, calcified teeth were formed from the E14 and E16 RTGs. The calcified teeth had a different shape according to the various RTGs. (**A**,**a**) The E14 recombinant tooth also exhibited a molar shape with cusps. (**B**,**b**) The E16 recombinant teeth had an abnormal molar shape with a low cusp. (**C**,**c**) The E16 recombinant teeth also formed a human incisor-like tooth, which shows incisal edge (arrow) in its side view.

Many investigators have suggested that the primary EK acts as a signaling center, which may also regulate crown morphogenesis (Jernvall et al., 1994; Vaahtokari et al., 1996). The signaling molecules expressed in EKs have been shown to stimulate cell division in both the dental epithelium and the dental papillae. However, there are no apparent fibroblast growth factor (FGF) receptors in EK cells, and these cells do not undergo mitosis when exposed to FGF (Kettunen et al., 1998, 2000). The cyclin-dependent kinase inhibitor, *p21*, which is involved in apoptosis, is also expressed in the primary EK (Keränen et al., 1998). Therefore, it was suggested that both cell death in the primary EK and cell proliferation in the epithelium adjacent to the primary EK coordinate epithelial folding and the transition of the bud to the cap stage (Jernvall et al., 1994, 2000).

This study examined the phenomena occurring after removing these epithelial signaling centers. The regeneration of EKs was investigated in the E14 and E16 Recombinant Tooth Germs (RTGs), which had the primary and secondary EKs removed, respectively. The precursor of the regenerated EK and the EK-regeneration mechanism were investigated using recombination and cell labeling methods.

Materials and methods

Removal of the EK region from the tooth germ at different stages

The tooth germs at the cap stage (E14) and early bell stage (E16) were carefully isolated from the mandibles of ICR mouse embryos. The anterior and posterior portions of the first molar in the E14 and E16 tooth germs were initially excised. The dental epithelium and dental mesenchyme of the first molars were separated from each other after 12 min incubation in dispase II® (Boehringer Mannheim, Indianapolis, IN). The apical half of the dental epithelium, including the signaling centers, was mechanically removed using fine tungsten needles. The remaining half of the dental epithelium at E14 was referred to as the remaining E14 epithelium. The remaining half of the dental epithelium at E16 was referred to as the remaining E16 epithelium.

Recombination #1

The RTG, which consisted of the remaining E14 epithelium and the E14 dental mesenchyme, was designated the E14 RTG. The remaining E16 epithelia were recombined with each original dental mesenchyme to form the E16 RTGs (see Recombination #1 in Fig. 1). These recombinant tooth germs were cultured for 12 hr, 1, 2, or 4 days *in vitro*. Ten tooth germs were cultured for each culturing time.

Recombination #2

After the dental papilla had been labeled with lipophilic dye, the remaining E14 epithelium and E14 dental mesenchyme were recombined (see Recombination #2 in Fig. 4). These recombinant tooth germs were cultured for 2 days *in vitro*. Ten tooth germs were cultured.

Recombination #3

After being separated from the E16 dental mesenchyme, the E16 dental epithelium was recombined with the E16 dental mesenchyme without any mechanical removal of the EKs (see Recombination #3 in Fig. 4). These Recombined Tooth Germs (RdTGs) were cultured for 12, 18, or 24 hr *in vitro*. Ten tooth germs were cultured for each culturing time.

Transplantation of RTGs into the subcapsular layer of mouse kidney

In total, 43 RTGs (22 E14 RTGs and 21 E16 RTGs) were transplanted into the subcapsular layer of an adult ICR mouse kidney and cultured for 3 weeks. Seven or eight RTGs were transplanted in a kidney. All surgical procedures were performed under anesthesia administered intra-peritoneally. No immuno-suppressive medication was used.

Whole mount *in situ* hybridization

In vitro cultured tooth germs were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). *In situ* hybrid-