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Hey1 and *Hey2* are differently expressed during mouse tooth development

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ABSTRACT

The Hey family (also known as Chf, Herp, Hesr, and Hrt) is a set of Hairy/Enhancer of Split-related basic helix-loop-helix type transcription factors. *Hey1*, *Hey2*, and *HeyL* have been identified in mammals. Although Hey proteins are known to regulate cardiovascular development, muscle homeostasis, osteogenesis, neurogenesis, and oncogenesis, their roles in tooth development have been largely obscure. Therefore, this study aimed to clarify detailed spatiotemporal expression patterns of *Hey1* and *Hey2* in developing molars and incisors of mice by section *in situ* hybridization. *Hey1* and *Hey2* were not significantly expressed in tooth germs at epithelial thickening, bud, and cap stages during molar development. In the dental epithelium in molars at the bell stage and incisors, *Hey2* transcripts were restricted to the undifferentiated inner enamel epithelium and down-regulated in preameloblasts and ameloblasts. On the other hand, *Hey1* was mainly expressed in preameloblasts and down-regulated in differentiated ameloblasts. Both genes were not significantly expressed in other dental epithelial tissues, including the outer enamel epithelium, stellate reticulum, and stratum intermedium cells. In the dental mesenchyme, *Hey1* was intensely transcribed in the subodontoblastic layer of the dental pulp in both molars and incisors, whereas *Hey2* was barely detectable in mesenchymal components. Our data implied that *Hey2* function is restricted to transient amplifying cells of the ameloblast cell lineage and that *Hey1* plays a role in the composition of the subodontoblastic layer, in addition to ameloblast differentiation. These findings provide novel clues for the better understanding of tooth development.

1. Introduction

Tooth germs consist of the enamel organ, derived from the oral ectoderm, and the dental mesenchyme, derived from cranial neural crest cells. Tooth development proceeds through reciprocal and reiterative interactions between these two components, mediated by signaling molecules, such as fibroblast growth factor (Fgf), bone morphogenetic protein (Bmp), sonic hedgehog (Shh), and Wnt (Peters and Balling, 1999; Tucker and Sharpe, 2004; Jussila and Thesleff, 2012). As odontogenesis progresses, developing tooth germs exhibit characteristic morphologies, namely epithelial thickening, bud, cap, and bell stages (Nanci, 2013). Dental placodes in the oral epithelium invaginate into the underlying mesenchyme at the epithelial thickening stage. At the bud stage, neural crest-derived dental mesenchymal cells condense and surround the epithelial tooth bud. Subsequently, the dental epithelium takes a cap-like shape, termed the enamel organ, consisting of the inner and outer enamel epithelium (IEE and OEE, respectively) and the stellate reticulum (SR), while the underlying dental mesenchyme divides into the dental papilla and the dental sac at the cap stage. At the following bell stage, ameloblasts and stratum intermedium (SI) cells differentiate from the IEE in the enamel organ, whereas some of the dental papilla cells just underneath the IEE differentiate into odontoblasts. Other remaining dental papilla cells form the dental pulp. In parallel to tooth morphogenesis, differentiated ameloblasts and odontoblasts commence depositing enamel matrix and dentin matrix, respectively. Periodontal tissues, such as the cementum, alveolar bone, and periodontal ligament are all derived from the dental sac. Although numerous studies on tooth development have accumulated, entire molecular mechanisms of odontogenesis are not yet fully understood.

Transcription factors positively or negatively regulate downstream gene expression to control embryogenesis. Basic helix-loop-helix (bHLH) transcription factor family members, such as Ascl (Mash), Hand, Hif, Id, MyoD, and Twist, commonly possess a conserved bHLH domain (Massari and Murre, 2000; Skinner et al., 2010). The basic region functions as a DNA binding domain and the HLH region is employed for protein-protein interactions to form either homo- or heterodimers. The Hey family (also known as CHF, Herp, Hesr, and Hrt) was identified as Hairy/Enhancer of Split-related novel bHLH transcription factors, and three *Hey* genes, *Hey1*, *Hey2*, and *HeyL*, are known in mammals (Leimeister et al., 1999; Nakagawa et al., 1999). Hey

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proteins mediate Notch signaling as transcriptional repressors (Nakagawa et al., 2000; Iso et al., 2003). Whereas *Hey1*-null mutant mice exhibit no apparent developmental defects (Fischer et al., 2004), *Hey2* knockout mice die in the neonatal period owing to ventricular septum and cardiac valve defects (Donovan et al., 2002; Gessler et al., 2002; Sakata et al., 2002). Early embryonic lethality of *Hey1/Hey2* double homozygous mutant mice indicates their redundant roles in cardiovascular development (Fischer et al., 2004; Kokubo et al., 2005). Hey proteins also function in skeletogenesis, neural development, and oncogenesis (Weber et al., 2014).

Although *HeyL* expression in the developing tooth germ has been briefly described (Leimeister et al., 2000), no data is available to date for *Hey1* and *Hey2* involvement during tooth development. Therefore, this study aimed to examine detailed spatiotemporal mRNA expression patterns of *Hey1* and *Hey2* in developing molars and incisors of mice.

2. Results

2.1. Early developmental stages of embryonic molar germs

Hey1 and *Hey2* were not expressed at the epithelial thickening stage at embryonic day (E) 12.5 (Fig. 1B and C). At the bud stage at E13.5, *Hey1* was transiently expressed on the buccal side of the dental mesenchyme surrounding the tooth bud (arrow in Fig. 1E), at a similar staining level observed in osteoblasts (asterisk in Fig. 1E). *Hey2* was hardly detectable in the serial section. The expression of *Hey1* and *Hey2* were almost negative, or at a quite low level, at the early (E14.5) and late (E15.5) cap stages (Fig. 1H, I, K, L), compared with the later developmental stages (see Figs. 2 and 3). To address whether transient *Hey1* expression at the bud stage is in the neural crest-derived dental mesenchymal cells, *in situ* hybridization analysis of *Pax9*, a known dental mesenchymal marker (Peters et al., 1998), was carried out using serial sections. The result demonstrated co-expression of *Hey1* and *Pax9* in the same cell population on the buccal side (arrow in Fig. 1N, O).

2.2. Late developmental stages of embryonic molar germs

During the bell stage from E16.5 to E18.5, *Hey1* expression in the IEE and preameloblasts became gradually stronger and reached a peak at E18.5 (arrowhead in Fig. 2F', J'). On the other hand, *Hey2* expression was widely expanded in the undifferentiated IEE at E16.5 (Fig. 2C), most intense at E17.5 (Fig. 2G'), and already peaked out at E18.5 (Fig. 2K'). Both *Hey1* and *Hey2* were not significantly expressed in other enamel organ components, including the OEE, SR, and SI cells. The *Sonic hedgehog* (*Shh*) gene, which encodes a secretory signaling protein, is known as a differentiation marker for ameloblasts and SI cells and plays a critical role in amelogenesis (Dassule et al., 2000; Gritli-Linde et al., 2002; Seidel et al., 2010). *Shh* expression overlapped with *Hey1* and *Hey2* in the IEE and preameloblasts, but not in SI cells (Fig. 2D, H, L). In the dental mesenchyme, *Hey1* expression was recognized in the pulp horn region (arrow in Fig. 2B, F', J'). The most intense expression of *Hey1* in the mesenchyme was noted in the undifferentiated dental pulp cells beneath the odontoblasts (arrow in Fig. 2J'). *Hey2* expression was absent, or at a very low level, in the dental mesenchyme (Fig. 2C, G', K').

2.3. Postnatal molar germs

Hey1 was expressed in the pulp horn region of the molar dental pulp at postnatal day (P) 1, 3, and 7 (Fig. 3B, F, J). More specifically, *Hey1* was intensely transcribed in the subodontoblastic layer (arrow in Fig. 3B', F', J'), but not expressed in differentiated odontoblasts. *Dentin sialophosphoprotein* (*Dspp*), a marker for differentiated odontoblasts, was expressed complementarily to *Hey1* in the pulp horn region (Fig. 3D', H', L'). However, interestingly, *Hey1* and *Dspp* expression overlapped with each other in preameloblasts (arrowhead in Fig. 3B, D, F, H), where *Dspp* is known to be transiently expressed (Ritchie et al., 1997). Similar expression patterns of *Hey1* and *Dspp* were observed in the frontally sectioned lower incisor (arrow in Fig. 3B, D). By contrast, *Hey2* transcripts were barely identified by *in situ* hybridization in postnatal molar germs, both in the dental epithelium and mesenchyme (Fig. 3C, G, K).

2.4. Quantitative analysis

To further confirm our histological observations, quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses were performed, using cDNA derived from the total RNA of molar germs obtained at E17.5 and P3 (n = 3 each). To clarify whether *Hey1* and *Hey2* expression are tooth-specific, cDNA derived from the tip of the tongue obtained at E17.5 was also used as a representative of non-dental oral tissues (n = 3). In accordance with the *in vivo* data, *Hey1* expression at P3 was significantly higher than at E17.5 (Fig. 4A) and *Hey2* expression at E17.5 was significantly higher than at P3 (Fig. 4B). *Hey2* expression at P3 may reflect a low level of transcription in tooth germs which was undetectable by *in situ* hybridization or transcripts in the supporting tissue surrounding the tooth germ. Both *Hey1* and *Hey2* expression levels in tooth germs at E17.5 were significantly higher than in the tip of the tongue at the same stage (Fig. 4A and B).

2.5. Incisor germs

Different from all human teeth and rodent molars, rodent incisors grow continuously throughout their lifetime. Epithelial stem cells exist in the posterior end of the incisor, termed the apical bud (Ohshima et al., 2005), to produce all dental epithelial cell lineages, including ameloblasts and SI cells, whereas mesenchymal stem cells give rise to odontoblasts and the subodontoblastic layer. Thus an advantage of analyzing rodent incisors is that all of the differentiation processes of ameloblasts and odontoblasts can be observed in a sagittally sectioned plane. The expression patterns of *Hey1* and *Hey2* in incisors almost mimicked those in molars at the bell stage. In the lower incisor at E18.5 and the upper incisor at P9 and P8w, intense *Hey1* transcripts were observed in the subodontoblastic layer beneath differentiated odontoblasts (arrow in Fig. 5B4, F2, J2), while *Hey1* expression was not identified in undifferentiated mesenchymal cells near the apical bud (asterisk in Fig. 5B2). *Hey1* was expressed only slightly in preameloblasts (arrowhead in Fig. 5B4), but not significantly expressed in other dental epithelial cells. By contrast, *Hey2* expression was restricted to the undifferentiated IEE in the apical bud (arrowhead in Fig. 5C3, G1, K1) and barely detectable in preameloblasts, ameloblasts, and mesenchymal cells. *Shh* was expressed in differentiating ameloblast lineage cells (Fig. 5D, H, L). Of particular interest, and different from molars, *Hey2* and *Shh* did not overlap well with each other except for a narrow intermediate region between the apical bud and preameloblasts (asterisk in Fig. 5C3, D3, G1, H1, K1, L1).

3. Discussion

The present study clearly demonstrated that *Hey1* and *Hey2* are differently expressed during mouse tooth development, suggesting that these genes play distinct roles in odontogenesis. An interesting difference between *Hey1* and *Hey2* expression first appears during ameloblast differentiation. Ameloblasts are highly specified epithelial cells and responsible for enamel formation. Ameloblast differentiation consists of several morphological and functional changes (Zeichner-David et al., 1995; Bei, 2009). The cells of the amplifying IEE (called transient amplifying cells) exit from the cell proliferation cycle, elongate, and polarize to become preameloblasts. Subsequently, preameloblasts turn into ameloblasts, which commence secreting enamel proteins toward the underlying dentin. Our data suggest that *Hey2* plays a role in the undifferentiated IEE, while *Hey1* mainly functions in preameloblasts. A