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Expression of planar cell polarity genes during mouse tooth development

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ABSTRACT

Objective: Planar cell polarity (PCP) refers to the cell polarity across the tissue plane and controls various cell behaviors and structures. Although the expression of several PCP signaling components has been detected in tooth germs, knowledge of the gene expression patterns of these PCP components during tooth development remains incomplete. The aim of this study is to characterize the temporal and spatial changes in PCP gene expression during tooth development.

Design: Expression of *Celsr1* and *2*, *Fzd3* and *6*, *Vangl1* and *2*, and *Dvl1-3* genes was analyzed in mouse molar germs from the bud to the bell stage using *in situ* hybridization.

Results: At the bud stage, all target genes were expressed in all areas of the tooth bud. In the enamel organ at the cap stage, expression of *Fzd3* was suppressed in the enamel knot, whereas *Fzd6* was strongly expressed there. Expression of *Vangl2* was strongly expressed in the inner dental epithelium from the cap stage onwards. In the inner dental epithelium, strong expression of *Fzd3*, *Dvl2* and *Vangl2* was noted at the early bell stage, and of *Celsr1*, *Fzd3*, *Fzd6*, *Vangl2* and *Dvl2* at the bell stage. Furthermore, differentiated odontoblasts strongly expressed *Celsr1*, *Vangl2*, and *Dvl2*.

Conclusion: The gene expression patterns delineated in this study improve our understanding of the role(s) of PCP components during tooth development.

1. Introduction

Teeth develop from the oral epithelium and underlying neural crest-derived mesenchyme *via* sequential and reciprocal interactions between the epithelium and mesenchyme. During tooth development, paracrine signaling factors from four major families—FGF, Shh, Wnt and BMP—are repeatedly expressed and mediate these tissue interactions. These signaling pathways constitute a complex network that regulates tooth patterning, morphogenesis and odontogenic cell differentiation in concert with other pathways such as the ectodysplasin (Eda) pathway (which mediates cell interactions within the ectoderm) and the juxtacrine signaling Notch pathway (reviewed in Balic & Thesleff, 2015; Bei, 2009; Jheon, Seidel, Biehs, & Klein, 2013; Jussila & Thesleff, 2012).

Planar cell polarity (PCP) refers to the collective alignment of cell polarity across a tissue plane. This controls various cell behaviors and structures including oriented cell division, cell movement, orientation of cytoskeletal elements, and polarized positioning of cilia. PCP-type polarization was first identified and studied in Drosophila, in which most adult cuticular structures show this type of polarization. Subsequent analyses have revealed that this type of polarization is found in both epithelial and mesenchymal cells in vertebrates, and that the signaling pathway manipulating PCP is evolutionarily conserved (reviewed in Devenport, 2014; Wallingford, 2012; Yang & Mlodzik, 2015).

The PCP pathway is one of the non-canonical Wnt signaling pathways and shares several components with the canonical Wnt/β-catenin pathway (van Amerongen & Nusse, 2009). Directional information is provided globally and locally for the molecular system underlying PCP establishment, and this comprises the so-called core PCP system (Yang & Mlodzik, 2015). The global cues identified to date are Wnt5a and Wnt11 in vertebrates (Gros, Serralbo, & Marcelle, 2009; Heisenberg et al., 2000; Qian et al., 2007), and Wg and dWnt4 in Drosophila (Wu, Roman, Carvajal-Gonzalez, & Mlodzik, 2013). The Drosophila core PCP system is composed of three transmembrane proteins, Frizzled (Fz), Flamingo (Fmi), and Van Gogh (Vang), and three cytoplasmic proteins, Dishevelled (Dsh), Diego (Dgo) and Prickle (Pk). These components interact with each other to form a Fz-Fmi-Dsh-Dgo complex on one side and a Vang-Fmi-Pk complex on the opposite side of a cell. Furthermore,

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Fz-Fmi on a given cell interacts with Vang-Fmi expressed on its neighbors. In this way, the directional information is locally provided from a cell to its neighbors to localize the core PCP components asymmetrically within a cell and in a uniform pattern along the tissue axis (reviewed in Devenport, 2014; Wallingford, 2012; Yang & Mlodzik, 2015). In vertebrates, multiple orthologues for each of the six core PCP components exist and function redundantly or distinctly. The Fz receptor is a seven-transmembrane domain (7TMD) protein that binds Wnt ligands (Bhanot et al., 1996; Vinson, Conover, & Adler, 1989). Among ten mouse orthologues, at least five *Fzds* (*Fzd1*, *2*, *3*, *6*, and *7*) are known to participate in PCP (Hua, Chang, Wang, Smallwood, & Nathans, 2014; Yu et al., 2010; Yu, Ye, Guo, & Nathans, 2012). Fmi, which is also known as starry night (stan), is an atypical cadherin with 7TMD receptor features and is capable of homophilic binding (Usui et al., 1999). The mouse genome has three orthologues of *Fmi*, known as *Celsr1-3* (Formstone & Little, 2001; Hadjantonakis, Formstone, & Little, 1998). Vang is a four-pass transmembrane protein (Wolff & Rubin, 1998), and its mouse orthologues are named *Vangl1* and *2*. Dsh, a cytoplasmic protein containing DIX, PDZ, and DEP domains (Klingensmith, Nusse, & Perrimon, 1994), is required not only for PCP but also for activating the canonical pathway (Boutros & Mlodzik, 1999). Mouse orthologues of *Dsh* are *Dvl1–3* (Klingensmith et al., 1996; Sussman et al., 1994; Tsang et al., 1996). Pk is a cytoplasmic protein with three LIM domains and a PET domain (Gubb et al., 1999). In the mouse, *Prickle1* and *2* are closely related to Drosophila *Pk* (Katoh & Katoh, 2003), whereas two other orthologues (*Prickle3* and *4*) are more distantly related. Dgo is a cytoplasmic ankyrin repeat protein whose mouse orthologues are *inversin* and *diversin* (Jones et al., 2014; Watanabe, 2003).

The significance of non-canonical Wnt signaling pathways to tooth development is not well understood, whereas that of the canonical pathway has been abundantly demonstrated (Balic & Thesleff, 2015; Jheon et al., 2013; Jussila & Thesleff, 2012; Liu & Millar, 2010). Human Robinow syndrome is characterized by shortened limb dwarfism with some dental anomalies (Beiraghi et al., 2011; Patton & Afzal, 2002) and is thought to be caused by disruption of PCP signaling (Wang, Sinha, Jiao, Serra, & Wang, 2011). In fact, both Wnt5a and its receptor Ror2, which activates the non-canonical pathway (Oishi et al., 2003), have been shown to be responsible for Robinow syndrome (Afzal et al., 2000; Person et al., 2010; van Bokhoven et al., 2000). During tooth development, *Ror2* is expressed both in the dental mesenchyme and the enamel organ (Cai et al., 2011). In *Ror2* knockout mice, retarded tooth growth and defective development of odontoblasts and ameloblasts are evident at birth, whereas the tooth germs appear normal at embryonic day 16.5 (Lin et al., 2011; Schwabe et al., 2004). Wnt5a is also expressed both in the dental mesenchyme and in a part of the enamel organ (Cai et al., 2011; Lin et al., 2011; Sarkar & Sharpe, 1999). In *Wnt5a*-deficient mice, teeth are smaller and have abnormal cusp patterns, and odontoblast differentiation is delayed (Lin et al., 2011). Furthermore, addition of exogenous Wnt5a to wild-type tooth germs also generates smaller teeth (Cai et al., 2011). Recently, the development of small and malformed teeth following siRNA-mediated inhibition of *Vangl2* revealed for the first time the importance of PCP components in tooth morphogenesis (Wu et al., 2016).

Expression of several core PCP components has been detected by *in situ* hybridization (Sarkar & Sharpe, 1999; Tissir & Goffinet, 2006) and microarray gene expression analyses (Pemberton et al., 2007) in developing mouse molars, and by immunohistochemistry in mouse molars (Wu et al., 2016) and rat incisors (Nishikawa and Kawamoto, 2012), suggesting their involvement in tooth development. However, developmental changes in the gene expression pattern of these components are poorly characterized, except for *Fzd6*, which is expressed in the dental epithelia from the epithelial thickening to the early bell stage (Sarkar & Sharpe, 1999). In this study, we analyzed gene expression of six members of three transmembrane component families (*Celsr1* and *2*, *Fzd3* and *6*, *Vangl1* and *2*) together with three members of a cytoplasmic component (*Dvl1–3*) in the mouse molars at the stages from embryonic day (ED) 13.5 to 18.5 (from the bud to bell stage).

2. Materials & methods

2.1. Animals

Embryos were obtained by mating male and female ICR mice. The morning of the appearance of the vaginal plug was designated as day 0 of embryonic development (ED0). All procedures were approved by the Animal Ethics and Research Committee of Health Sciences University of Hokkaido and complied with the Guidelines for the Care and Use of Laboratory Animals of the university.

2.2. Tissue preparation

Tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C, and then immersed for 6 h at 4 °C in phosphate-buffered saline (PBS) containing 20% sucrose, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and frozen with a Cryon Spray Freezer (Oken Shoji, Tokyo). The frozen tissues were kept in a freezer at −80 °C until use.

2.3. In situ hybridization

Frozen sections cut at a 10-μm thickness were picked up on silane-coated slide glasses, air-dried for 30 min at room temperature, and processed for *in situ* hybridization. Briefly, sections were immersed in absolute ethanol for 5 min and then in 0.2 N HCl for 20 min, and subsequently washed twice in PBS for 5 min each time. Next, the sections were treated with 20 μg/ml of proteinase K (Takara Bio Inc., Otsu, Shiga, Japan) at 37 °C for 15 min, washed in PBS, and re-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min. After having been washed twice in PBS and once in distilled water for 5 min each time, the sections were air-dried and hybridized. Hybridization was performed at 65–67 °C for 14–16 h with a digoxigenin-labeled RNA probe (500–1000 ng/ml) in a hybridization solution containing 50% formamide, 5 × SSC, 10 mM EDTA, 5% dextran sulfate, 1 × Denhardt's solution, 250 μg/ml yeast tRNA, 500 μg/ml fish sperm DNA (Roche diagnostics GmbH, Mannheim, Germany) and 0.02% SDS. Hybridized sections were washed three times in 0.2 × SSC at 65 °C for 30 min each time, immersed in PBS for 10 min at room temperature and then incubated with 20% Block Ace (DS Pharma Biomedical, Osaka, Japan) in PBS for 60 min at room temperature. Subsequently, they were incubated overnight at 4 °C with alkaline phosphatase-conjugated anti-DIG Fab fragments diluted 1:500 in PBS containing 1% bovine serum albumin. After three washes in TBST (0.1 M Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.5), chromogenic reactions were carried out using NBT/BCIP (Roche Diagnostics GmbH, Mannheim, Germany).

The sections were examined under an ECLIPSE 80i microscope with Nomarski differential interference equipment (Nikon Instruments, Tokyo, Japan), and the images were acquired with a digital camera DXM1200F (Nikon Instruments, Tokyo, Japan).

2.4. Hybridization probes

Complementary DNA fragments of mouse planar cell polarity genes were generated by the reverse transcription polymerase chain reaction (RT-PCR) using total RNA extracted from mouse embryos at embryonic day 11, and sub-cloned into pT7/T3α-18 (Life Technologies, Grand Island, NY, USA). Sense and antisense digoxigenin-labeled RNA probes were synthesized in the presence of DIG-UTP (DIG RNA labeling mix; Roche Diagnostics GmbH, Mannheim, Germany) using T7 RNA polymerase or T3 RNA polymerase with linearized templates. Primer sequences used for preparation of cDNAs are shown in Table 1.