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ABSTRACT OF DISSERTATION

Title	Iroquois homeobox 3 regulates odontoblast proliferation and differentiation mediated by Wnt5a expression （ホメオボックス型転写因子 Irx3 は Wnt5a の発現に関与し象牙芽細胞の増殖と分化に調整する）
Author's Name	Anrizandy Narwidina

Background: Tooth development occurs in a sequential process, which can be morphologically classified to describe the process. During this process, communication occurs between epithelial and mesenchymal cells, which regulates the spatiotemporal expression of various growth factors, such as Wnt and bone morphogenetic proteins (Bmps). Wnt5a is an important secreted protein that plays a crucial role in tooth development. Although many Wnt molecules are expressed during tooth development, the mechanism underlying Wnt5a expression regulation in dental mesenchymal cells remains unclear. Iroquois homeobox (Irx) genes are TALE- class homeobox genes that are evolutionary conserved across species and have multiple critical cellular functions in fundamental tissue development processes. Previous studies have shown that Irxs genes are expressed during tooth development. However, the precise roles of genes in teeth remain unclear. Therefore, understanding the regulation of Wnt5a and Irxs expression in dental mesenchymal cells may provide insights into the molecular mechanisms underlying tooth development and inform the development of novel dental therapies.

Purpose: To clarify the expression and function of Irx3 in odontogenic mesenchymal cells during tooth development.

Methodology: To analyze gene expression, RT-PCR and quantitative PCR were performed. The localization of IRX3 was examined by immunohistochemistry. mDP and M3H1 cell lines were used to assess cell proliferation and differentiation by Alizarin Red S staining. To silence Irx3 gene expression, siRNA experiments were used. All animal experiments were conducted in accordance with the guidelines for animal experiments, and the Institutional Animal Care and Use Committee of the University of Tokushima approved the study (T30-59).

Results: cDNA synthesized from postnatal day 1 (P1) tooth germs was used to investigate the expression of Irx genes (Irx1-Irx6) by RT-PCR. The results showed that all genes except Irx4 were expressed in the tooth tissue. Irx1-Irx3 were expressed in the dental epithelial cell line M3H1 cells, while Irx3 and Irx5 were expressed in the dental mesenchymal cell line mDP cells. Notably, Irx3 was

expressed in both undifferentiated cell lines. Immunostaining revealed the presence of IRX3 in the dental epithelial cells and mesenchymal condensation. Inhibition of endogenous Irx3 by siRNA blocked the proliferation and differentiation of mDP cells. Furthermore, quantitative PCR analysis showed that Wnt3a, Wnt5a, and Bmp4, factors involved in odontoblast differentiation, were highly expressed in mDP cells. Interestingly, the expression of Wnt5a, but not Wnt3a or Bmp4, was suppressed by Irx3 siRNA.

Discussion: Irx homeobox genes are expressed in a specific and coordinated manner in tissues, with Irx1, Irx2, Irx3, and Irx5 showing similar expression patterns. We found that all Irx genes except Irx4 are expressed in teeth. Irx genes are organized into two clusters, IrxA and IrxB, characterized by the orientation of their transcription. Bidirectional transcription, where genes are transcribed in opposite directions, is an efficient gene expression strategy that promotes rapid and sophisticated development. The opposite transcriptional directions of Irx genes in clusters A and B suggest an important role in tissue development, including tooth tissue. The role of Irx homeobox genes in tooth development was investigated using two odontogenic cell lines. Irx1, Irx2, and Irx3 were found to be expressed in undifferentiated M3H1 cells, while only Irx3 and Irx5 were expressed in undifferentiated mDP cells. Only Irx3 was found to be specifically expressed in dental epithelial and mesenchymal cells in the E13.5 tooth germ, indicating its important role in initial tooth development. Knockdown of Irx3 expression in mDP cells led to suppressed cell proliferation, suggesting that Irx3 regulates the proliferation of dental mesenchymal cells during tooth development. Among Iroquois homeobox genes, Irx5 is associated with human disease Hamamy syndrome with craniofacial dysmorphism. However, although Irx5-deficient mice were grossly normal. Although Irx3-deficient mice also showed normal development of the craniofacial region, double knockout mice of Irx3 and Irx5 showed several similarities with Hamamy syndrome. Furthermore, deletion of osteoblast-specific Irx3 with Osterix-Cre in Irx5-deficient mice showed abnormal craniofacial mineralization and decreased gene expression of the osteogenic regulators, indicating that Irx3 and Irx5 can cooperatively regulate cranial bone formation. In addition, genome-wide association studies (GWAS) have shown that Irx3 is a target for fat mass and obesity-associated genes (Fto) and plays an important role in determining body size and composition. Fto is known to promote adipocyte differentiation, and its suppression has been reported to promote osteoblast differentiation of bone marrow-derived mesenchymal stem cells. These findings suggest that the function of Irx3 involves the regulation of cell fate determination in mesenchymal stem cells and plays a role in skeletal homeostasis and disease. Finally, we found that the knockdown of Irx3 based on siRNA transfection suppressed the expression of Wnt5a in mDP cells, suggesting that Irx3 regulates the expression of Wnt5a in dental papilla cells.