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Role of grainyhead-like 2 in the formation of functional tight junctions

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Epithelial cells develop intercellular junctions, including tight junctions (TJs) and adherens junctions (AJs). In epithelial tissues, TJs act as barriers that protect bodies from dehydration, infection and toxic substances. However, the molecular mechanisms regulating the establishment of functional TJs during organogenesis remain largely unknown. Recently, we identified grainyhead-like 2 (Grhl2) as a transcription factor that is specifically expressed in cholangiocytes, which are epithelial cells lining the bile duct tubules in the liver. Using our three-dimensional (3D) culture system of hepatic progenitor cells, we demonstrated that Grhl2 enhanced barrier functions of hepatic progenitor cells by upregulating claudin (Cldn) 3 and Cldn4, thereby promoting epithelial morphogenesis. In addition, we identified Rab25 as another target of Grhl2, which promotes the localization of Cldn4 at TJs. Our results indicate that a transcription factor promotes epithelial morphogenesis by establishing functional TJs by not only regulating the transcription of Cldns but also affecting their localization at TJs.

Keywords: transcription factor, epithelial morphogenesis, epithelial polarity, liver development, bile duct, cholangiocyte, hepatic progenitor cell

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Introduction

Epithelial cells, which originate from tissue-specific stem cells in early developmental stages, mature structurally and functionally later in the develoment. During structural differentiation/maturation, these cells acquire apico-basal polarity and form three-dimensional (3D) tissue structures that are essential for physiological functions of organs. The apico-basal polarization of epithelial cells is paralleled by the establishment of intercellular junctions, including tight junctions (TJs) and adherens junctions (AJs). TJs, which are located in the upper part of the lateral membrane, segregate the plasma membrane into the apical and the basolateral domains. They consist of occludin, claudins (Cldns), junctional adhesion molecules (JAM), tricellulin and proteins associated with those transmembrane proteins. Cldns comprise a multigene family consisting of 27 members.[1] Interestingly, they contribute to not only physical interactions between neighboring cells but also charge- and size-selective transport of solutes by paracellular channel formation.[2] Therefore, depending on the combination of Cldns, TJs permit or restrict the paracellular transport of certain types of ions. For example, Cldn2 forms paracellular channels that inhibit passage of anions but permit passage of cations and water.[3] Depletion of one type of Cldn in mice causes various abnormalities,[4] and mutations of Cldns in humans result in various genetic diseases, including neonatal ichthyosis and sclerosing cholangitis (NISCH) syndrome as well as familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) syndrome.[5] Therefore, Cldns are essential for maintaining homeostasis as they provide barriers functions in epithelial tissues and regulate paracellular permeability. However, there is still little information on how they contribute to the development of tissue structures/organs and how their expression is controlled during organogenesis. We recently found that the transcription factor grainyhead-like 2 (Grhl2) regulates the expression of Cldns during epithelial morphogenesis. In this paper, we summarize recent results that have demonstrated correlations between the formation of TJs and epithelial morphogenesis

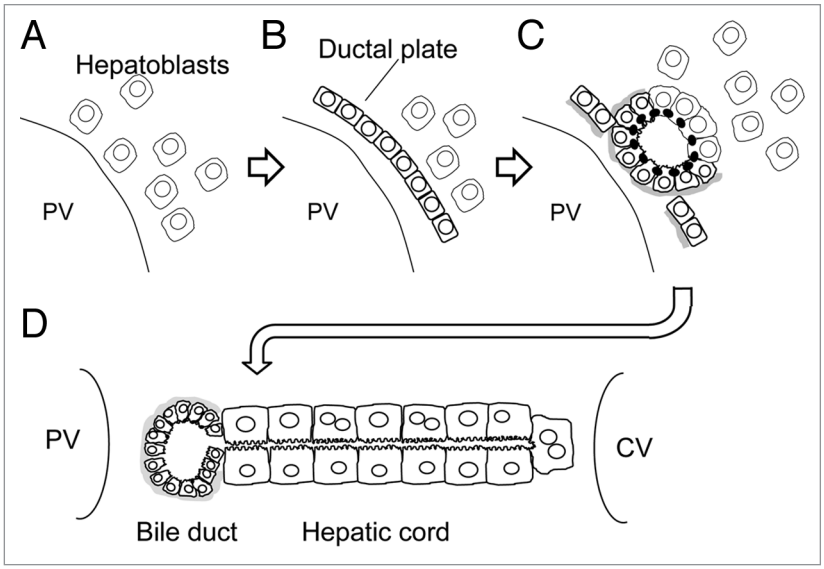

Figure 1. Formation of tight junctions (TJs) and the morphogenesis of bile ducts during liver development. (**A**) Hepatoblasts near the portal vein (PV) receive transforming growth factor (TGF)-β and Notch signals from periportal mesenchymal cells and differentiate into cholangiocytes. (**B**) Cholangiocytes first form a ductal plate. (**C**) The luminal spaces that are surrounded by cholangiocytes and hepatoblasts are observed in late gestation and in the neonatal period. At this stage, TJs recognized by ZO-1 staining are already evident. (**D**) Cholangiocytes eventually form bile ducts next to PV, whereas hepatocytes establish hepatic cords. PV, portal vein; CV, central vein.

and discuss the roles of transcription factors in the formation, maturation and maintenance of functional TJs.

Bile Duct Morphogenesis and TJ Formation

Here we summarize bile duct morphogenesis by describing the findings of a correlation between TJ formation and epithelial morphogenesis. The liver performs many important biological functions, including metabolic reactions, detoxification and production of serum proteins and bile. Hepatocytes are hepatic parenchymal cells that perform most of the metabolic reactions and form the tiny apical luminal space called the bile canaliculus (BC) between neighboring cells. Cholangiocytes are biliary epithelial cells that form tubular structures called bile ducts around portal veins (PV). Hepatocytes produce bile and secrete it into BC, which is connected to the intestine via the bile ducts. Cholangiocytes secrete water and ions into the luminal space of bile ducts to control the flow rate and composition of bile. Because bile is cytotoxic, it is crucial that the bile juice is drained into the intestine without any leakage. Therefore, it is assumed that hepatocytes and cholangiocytes acquire strong barrier functions; in other words, they develop functional/mature TJs while hepatocytes produce and secrete the bile.

In midgestation, hepatoblasts (fetal liver stem/progenitor cells) next to PV are committed to cholangiocytes (**Fig. 1**). Cholangiocytes first form a single cell layer called the ductal plate around PV just after the lineage commitment of hepatoblasts. The ductal plate is reorganized into tubular structures in the perinatal period (**Fig. 1B**).[6] At an early stage of reorganization of the ductal plate, luminal spaces surrounded by cholangiocytes and hepatoblasts are observed.[7,8] At this point, TJs and AJs recognized by ZO-1 and E-cadherin, respectively, are generated (**Fig. 1C**). Therefore, the basic structure of TJs is established at the initial stage of bile duct morphogenesis. Within several weeks after birth, bile duct structures are formed around PV, whereas hepatocytes are polarized to form the hepatic cord (**Fig. 1D**). However, it remains unclear whether the TJs of immature ducts in fetal liver and those of bile ducts in neonatal liver are functional.

Notch and transforming growth factor (TGF) β signals are two major signaling pathways that regulate the development of bile ducts. Abnormalities in one of these pathways result in paucity of bile ducts.[8,9] Sox9, hepatocyte nuclear factor (HNF) 1β and Hes1 have been identified as downstream targets of the Notch pathway.[8,10] Knockout mice lacking any of these transcription factors exhibited abnormalities of bile duct morphogenesis.[7,11,12] However, in contrast to the case in hepatocytes, in which HNF4α positively regulates the levels of gene expression of adhesion molecules, including E-cadherin and occludin,[13] the regulation of the expression of TJ components during bile duct morphogenesis by the Notch signal and its downstream targets remains unknown.

Transcriptional Regulation of Cldns

Given that Cldns have many important roles, their transcription must be strictly controlled in developing and mature organs. Because Cldns confer barrier functions and intercellular interactions in epithelial cells, their loss causes dramatic changes in cellular behavior. It has been reported that Twist1, which is involved in epithelial mesenchymal transition (EMT), binds to the E-box in the promoter region of Cldn4 and downregulates its expression. Furthermore, high levels of Twist1 and low levels of Cldn4 are associated with poor prognosis of esophageal cancer.[14] The E-26 (ETS)-related gene (ERG), which is downregulated by inflammatory stimuli, positively regulates vascular endothelial (VE)-cadherin, endoglin and von Willebrand factor and negatively regulates intercellular adhesion molecule (ICAM)-1 and interleukin (IL)-8 in endothelial cells. It has been recently demonstrated that ERG controls endothelial barrier functions by upregulating Cldn5 in primary human umbilical vein endothelial cells (HUVEC).[15] In immortalized human pancreatic duct cells that were established from normal human pancreatic duct epithelial cells by introducing the human telomerase reverse transcriptase (hTERT) gene, treatment with 12-O-tetradecanoylphorbol 13-acetate (TPA) increases the expression level

of TJ proteins through the activation of protein kinase C (PKC) and on activation of PKC, Cldn7 becomes a target of the transcription factor ELF3.[16] The Wnt/β-catenin pathway, one of the most important signaling pathways regulating multiple developmental events, has been implicated in the increased expression of Cldns. It has been reported that the promoter of Cldn1 has binding sites for Cdx1, Cdx2 and GATA4.[17] Interestingly, activated β-catenin further induces the Cdx2-dependent transcription of Cldn1.[17] Another group has shown that the inhibition of glycogen synthase kinase (GSK) 3β, which negatively regulates the Wnt/β-catenin pathway, results in increased permeability with downregulation of Cldn1, occludin and E-cadherin in a epithelial cell line derived from the intestine.[18] These results indicate that the transcriptional regulation of Cldns is important for regulating cellular behavior in normal and pathological conditions. However, given that there are more than 20 Cldns, more experimental evidence must be provided to clarify the entire process of the transcriptional regulation of Cldns.

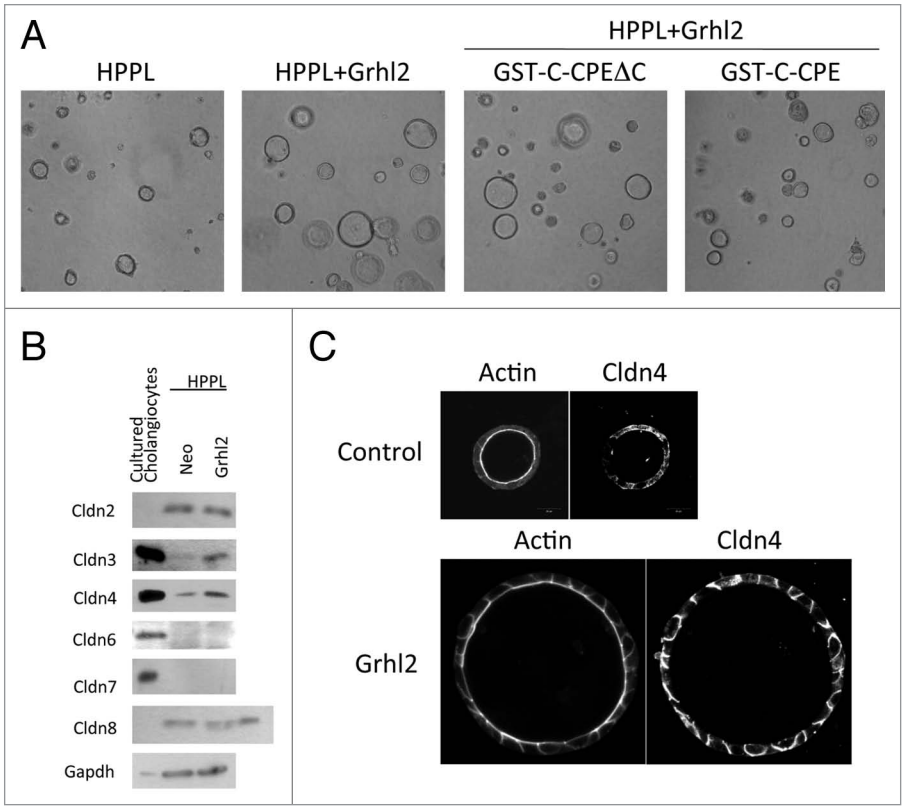

Figure 2. Grainyhead-like 2 (Grhl2) promotes epithelial morphogenesis by upregulating claudin (Cldn) 3 and Cldn4. (**A**) Grhl2 expands the luminal space of cysts derived from hepatic progenitor cells, HPPL. Because the glutathione S-transferase and the C-terminal half of *Clostridium perfringens* enterotoxin (GST-C-CPE) peptide blocks lumen expansion, the functions of Cldns are important for the effects of Grhl2. GST-C-CPEΔC, which lacks C-terminal 5 amino acids and cannot bind to Cldns, does not affect the size of cysts. (**B and C**) Indeed, Grhl2 upregulates Cldn3 and Cldn4 proteins.

Role of Grhl2 in Maturation of Epithelial Barriers of Bile Ducts

To study the epithelial morphogenesis of bile ducts, we generated a bipotential liver progenitor cell line, named HPPL, derived from Dlk1$^+$ mouse hepatoblasts[19] and established a 3D culture system of these cells in which they formed cysts, which are spherical structures lined by polarized epithelial cells.[20] In addition, we established a similar culture system of primary cholangiocytes isolated from adult livers based on the expression of the epithelial cell adhesion molecule (EpCAM). On comparing the cysts derived from HPPL and those from the cholangiocytes, we realized that the cholangiocytes formed remarkably larger cysts than HPPL. We also found that the value of transepithelial electric resistance (TER) of HPPL was much lesser than that of the cholangiocytes. These results suggested that the TJs formed by HPPL were not fully functional. We considered that genes upregulated in cholangiocytes confer epithelial characteristics on liver progenitor cells during bile duct morphogenesis. To identify such molecules, we compared the expression profiles of Dlk1$^+$ hepatoblasts isolated from E14.5 livers and EpCAM$^+$ cholangiocytes from neonatal livers by microarray analysis. We then selected transcription factors upregulated in the neonatal cholangiocytes. Subsequently, we examined whether each gene would affect the luminal structure of HPPL in 3D culture. As a result, we observed that Grhl2 remarkably expanded the lumen of cysts.[21]

We examined the process of lumen expansion by live cell imaging and found that the lumens of cysts derived from HPPL-Grhl2 expanded without cell proliferation. In 2D culture, Grhl2 increased TER and decreased the efflux of 4-kDa fluorescein isothiocyanate (FITC)-Dextran from the apical to the basal side. Based on these results, we considered that Grhl2 promoted epithelial morphogenesis by enhancing the barrier function of HPPL. Therefore, we focused on the expression of Cldns. Although we detected small increases in mRNAs, Cldn3 and Cldn4 proteins were significantly upregulated in HPPL-Grhl2 (**Fig. 2**). To evaluate the biological functions of Cldn3 and Cldn4 in expanding the size of the lumen, we used the fusion protein of glutathione S-transferase (GST) and the C-terminal half of *Clostridium perfringens* enterotoxin (C-CPE), which binds to these proteins and inhibits their functions. Indeed, C-CPE significantly decreased the size of the lumen of cysts derived from HPPL-Grhl2 compared with the nonactive C-terminal truncated form of the peptide (**Fig. 2A**). We found that overexpression of Cldn3 increased lumen size. On the other hand, overexpression of Cldn4 alone did not show a phenocopy to that of Ghrl2 and, actually, Cldn4 was not localized at TJs in HPPL-Cldn4. This result strongly suggested that another Grhl2 target

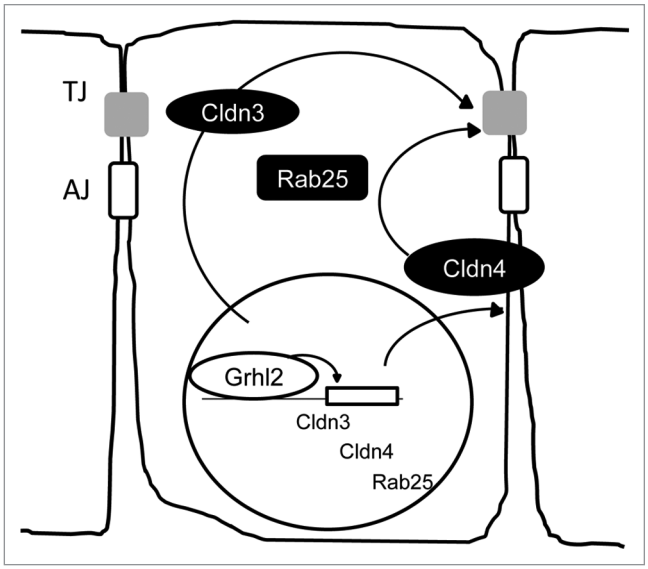

Figure 3. Molecular network of Cldn3, Cldn4 and Rab25 downstream of Grhl2. Grhl2 upregulates expression levels of mRNAs of Cldn3, Cldn4 and Rab25. Cldn3 is localized at TJs, whereas Cldn4 localized at the basolateral domain is redistributed to TJs by Rab25.

enhanced the TJ localization of Cldn4. We used the sequence database of proximal promoters derived from mouse and human and identified several potential targets of Grhl2. Among them, we particularly focused on Rab25, a member of the Rab11 family, involved in vesicle transport and epithelial polarization.[22,23] Indeed, the overexpression of Rab25 increased the TJ localization of Cldn4 and expanded the lumen. Altogether, these findings suggested that Grhl2 positively regulated Cldn3, Cldn4 and Rab25 to establish mature TJs and promoted bile duct morphogenesis (**Fig. 3**).

Roles of Grh Transcription Factors in Adhesion Molecule Expression

Grh is an evolutionarily conserved transcription factor. Nematodes and flies have one Grh, while mice and humans have three Grhl factors. Drosophila Grh has been studied extensively, and it has been described as a gene that regulates early embryonic development, epidermal barrier maturation, tracheal tube size control, neural proliferation and subtype specification, wing hair polarity and tissue repair.[24] Therefore, Grh is considered a crucial factor in multiple processes of epithelial morphogenesis. In mice, functions of Grhl family members were investigated by generating knockout mice. Grhl1 regulates hair anchorage and epidermal differentiation.[25] Grhl3, also known as Get1, regulates skin permeability by modulating the barrier function of the epidermis, urothelial differentiation and dorsal closure of the neural tube.[26,27] In addition, Grhl3 regulates keratinocyte migration and thereby plays a pivotal role in wound healing. Recently, Grhl2 has proven to be a key transcription factor in the completion of neural tube closure[28,29] and in modulation of the nature of cell-cell contacts by promoting the formation of the apical junctional complex.[28] In addition to E-cadherin and Cldns, cell adhesion molecules including desmoglein 2, desmocolin 2, desmoplakin and epithelial cell adhesion molecule (EpCAM) have been identified as targets of Grhl2 by comparing mutant mice with controls.[29] However, the roles of these molecules in epithelial morphogenesis have not been elucidated.

We also found that Grhl2 increased mRNA expression of Cldn3 and Cldn4. However, increase in the protein levels was much more prominent. This fact raised the question of whether another target of Grhl2 might further upregulate the protein levels of target genes. Recently, Bhandari et al. have reported that Grhl3 directly binds to the promoter of miR21 and suppresses its expression.[30] The miR21 gene has been shown to downregulate MSH2 in transformed cells with low expression levels of the RNA-binding protein DND1 and promote tumor formation. In addition to their function of promoting degradation of target mRNAs, microRNAs could stabilize mRNAs or promote translation. Therefore, Grhl2 may regulate the expression of microRNAs and further increase the protein levels of Cldns.

Because mice lacking Grhl2 die around E13.5 before the formation of most epithelial organs, the role of Grhl2 in epithelial morphogenesis in vivo remains largely unknown. Recent studies using in vitro culture systems suggest that Grhl2 may control TJ formation and epithelial morphogenesis during the development of hepatic bile ducts and other epithelial tissues and organs.[21,31]

Perspective

Epithelial tissues are often associated with luminal structures. The establishment of apico-basal polarity and the transport of apical components initiate the formation of the lumen.[23,32] On the other hand, fluid accumulation is an important factor in the promotion of lumen formation. Recent studies, including ours, have indicated that fluid accumulation into the apical luminal space is highly correlated with paracellular transport and the barrier functions regulated by Cldns. In zebrafish, Cldn15 and Cldn15-like b are necessary for formation of the single lumen during intestine development[33] and for formation of the tubular network of bile ducts,[34] respectively. During the formation of mouse blastocysts, Cldn4 and Cldn6 establish an epithelial barrier, which is necessary for fluid accumulation in the blastocyst cavity. Our results indicate that Cldn3 and Cldn4 are important for the enlargement of the apical lumen during bile duct development.

One transcription factor can be critical in the developmental process by simultaneously regulating a number or a group of genes.[35] Interestingly, Grhl2 not only upregulates the transcription of Cldns but also regulates their localization by upregulating another target, Rab25. Thus, a new

aspect of transcription factors is that one transcription factor regulates multiple types of genes in order to contribute to the efficient promotion of developmental processes.

Gene targeting in mice is a powerful method to study the biological functions of a gene of interest. Although Grhl2 knockout mice die early, which makes it difficult to analyze the functions of Grhl2 in epithelial morphogenesis, the generation of tissue/organ-specific knockout mice may provide crucial information. We have demonstrated that organotypic culture systems could contribute to revealing the roles of transcription factors that affect the formation of TJs and epithelial morphogenesis. It is a simple strategy to compare cystic structures and gene expression profiles between controls and those expressing a target gene (or knocking down a gene). Using the culture system, we can follow the entire process of morphogenesis under a microscope. To understand in vivo tissue formation, it may be necessary to establish new protocols to induce more complex morphogenetic processes, including tube formation. In addition, measuring TER and examining the efflux of small molecules are still the quickest and easiest ways to examine the barrier functions of epithelial cells. Thus, the combination of the available in vitro and in vivo methods would be essential to clarify the molecular mechanisms linking the formation of functional TJs and epithelial morphogenesis during the development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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