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ARTICLES

Connexins Induce and Maintain Tight Junctions in Epithelial Cells

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Abstract Connexins (Cx) are considered to play a crucial role in the differentiation of epithelial cells and to be associated with adherens and tight junctions. This review describes how connexins contribute to the induction and maintenance of tight junctions in epithelial cells, hepatic cells and airway epithelial cells. Endogenous Cx32 expression and mediated intercellular communication are associated with the expression of tight junction proteins of primary cultured rat hepatocytes. We introduced the human Cx32 gene into immortalized mouse hepatic cells derived from Cx32-deficient mice. Exogenous Cx32 expression and the mediated intercellular communication by transfection could induce the expression and function of tight junctions. Transfection also induced expression of MAGI-1, which localized at adherens and tight junction areas in a gap junctional intercellular communication (GJIC)–independent manner. Furthermore, expression of Cx32 was related to the formation of single epithelial cell polarity of the hepatic cells. On the other hand, Cx26 expression, but not mediated intercellular communication, contributed to the expression and function of tight junctions in human airway epithelial cells. We introduced the human Cx26 gene into the human airway epithelial cell line Calu-3 and used a model of tight junction disruption by the Na^+/K^+-ATPase inhibitor ouabain. Transfection with Cx26 prevented disruption of both tight junction functions, the fence and barrier, and the changes of tight junction proteins by treatment with ouabain in a GJIC–independent manner. These results suggest that connexins can induce and maintain tight junctions in both GJIC-dependent and –independent manners in epithelial cells.

Keywords Connexin · Gap junction · Tight junction · Hepatocyte · Airway epithelial cell · Fence function · Barrier function · Protein-protein interaction

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Introduction

Gap junctional channels composed of connexins (Cx) mediate reciprocal exchange of ions and small molecules of less than 1 kDa, including the second messengers cyclic adenosine monophosphate (cAMP), inositol 1,4,5-trisphosphate and Ca^{2+}, between adjacent cells (Saez et al., 1986; Kumar & Gilula, 1996). Gap junctional intercellular communication (GJIC) is thought to play a crucial role in development, cell growth and cell differentiation (Loewenstein, 1979; Yamasaki & Naus, 1996; Trosko & Ruch, 1998). Moreover, as epithelial cells differentiate, small gap junctional plaques appear within the networks of tight junction strands in freeze-fracture replicas (Elias & Friend, 1976; Decaens et al., 1996; Kojima et al., 2001b). In recent studies, several connexins have been shown to interact with PDZ domains containing the tight junction protein ZO-1 (Giepmans, 2004; Laird, 2006).

Tight junctions are the apicalmost components of intercellular junctional complexes in epithelial cells and endothelial cells. They separate the apical and basolateral

cell surface domains (the fence function) and inhibit solute and water flow through the paracellular space (the barrier function) (Gumbiner, 1993). They are formed by the integral membrane proteins claudins, occludin and junctional adhesion molecules (JAMs), as well as many peripheral membrane proteins including the membrane-associated guanylate kinase (MAGUK) family, ZO-1, ZO-2, ZO-3, membrane-associated guanylate kinase with inverted orientation 1 (MAGI-1), cell polarity molecules ASIP/PAR-3, PAR-6 and atypical protein kinase C (PKC) (Tsukita, Furuse & Itoh, 2001; Tsukita & Furuse, 2002; Sawada et al., 2003; Schneeberger & Lynch, 2004). Occludin was the first reported integral membrane protein of tight junctions and is the most ubiquitously expressed at the apicalmost basolateral membranes (Furuse et al., 1993). Furthermore, occludin is directly involved in tight junction barrier and fence functions (Tsukita et al., 2001), and the long carboxy-terminal domain is rich in serine, threonine and tyrosine residues and a coiled-coil, which interact with several proteins associated with kinase function, c-Yes, PKC-ζ and the p85 regulatory subunit of phosphatidylinositol 3-kinase, as well as the gap junction protein Cx26 (Nusrat et al., 2000). Cx26-mediated GJIC also plays a crucial role in enhancing the barrier function of tight junctions in human colonic Caco-2 cells (Morita et al., 2004).

Connexins are considered to be associated with adherens and tight junctions. In addition, this review describes how expression of Cx32 or Cx26 contributes to induction and maintenance of the expression and function of tight junctions in epithelial cells, hepatic cells and airway epithelial cells.

Structures of Gap and Tight Junctions in Primary Cultures of Rat Hepatocytes

In the liver and hepatocytes, well-developed gap and tight junctions are observed (Kojima et al., 2001a, 2003). In freeze-fracture analysis of primary rat hepatocytes cultured with dimethyl sulfoxide (DMSO)/glucagons (Kojima et al., 1997, 2001b), typical large gap junction plaques containing many particles are observed (Fig. 1a). The ultrastructure of tight junctions is quite different from that of gap junctions. In freeze fractures, tight junctions appear as a set of continuous, anastomosing strands in the P-fracture face, with complementary grooves in the E-face. Tight junctional strands encircling bile canalicular cysts form well-developed networks (Fig. 1a). Furthermore, several small gap junctional plaques are also observed within tight junction strand networks (Fig. 1a). It is possible that the small gap junction plaques within tight junction strand networks may play a crucial role in the expression and function of tight junctions.

Cx32 Expression and Mediated Intercellular Communication Regulate Expression of Tight Junctions in Primary Cultures of Rat Hepatocytes

In primary rat hepatocytes cultured with DMSO/glucagons (Kojima et al., 2001b), Cx32-, occludin- and claudin-1-immunoreactive lines are strongly observed at cell borders (Fig. 1c). Cx32, but not Cx26, is colocalized with occludin and claudin-1 at the apicalmost regions of the membranes (Kojima et al., 2001b). Cx32 is detected in coimmunoprecipitates using occludin, claudin-1 and ZO-1 antibodies (Fig. 1b). Downregulation of occludin and claudin-1 is observed after treatment with the GJIC blockers 18β-glycyrrhetinic acid and oleamide in immunocytochemistry and Western blotting (Fig. 1c,d). Furthermore, when Cx32 expression is knocked down by short interfering RNA (siRNA) in hepatocytes, downregulation of claudin-1 and upregulation of claudin-2 and phospho-mitogen-activated protein kinase (MAPK) are observed in reverse-transcriptase polymerase chain reaction (RT-PCR) and Western blotting (Fig. 1e,f). These results suggest that endogenous Cx32 expression and mediated intercellular communication regulate the expression of tight junction proteins in primary cultures of rat hepatocytes.

Cx32 Expression and Mediated Intercellular Communication Regulate Tight Junctions in an Immortalized Mouse Hepatic Cell Line

We introduced the human Cx32 gene to an immortalized mouse hepatocyte derived from Cx32-deficient mice and examined the expression and function of tight junctions (Kojima et al., 2002). In Western blotting, expression of Cx32 protein was strongly observed in three transfectants (termed 32-1, 32-2 and 32-3) but not in parental cells (Fig. 2a). Expression of occludin, claudin-1 and ZO-1 proteins in all of the transfectants was significantly increased compared to that of parental cells (Fig. 2a). Expression of occludin and claudin-1 induced by the transfection was inhibited by treatment with 18β-GA (Kojima et al., 2002).

We examined whether the induction of tight junction proteins by transfection with Cx32 resulted in enhanced fence and barrier functions of tight junctions. By labeling the apical cell surfaces of parental cells and transfectants with BODIPY-sphingomyelin, we examined the changes of fence function. In transfectants, the fluorescent probe was effectively retained in the apical domain accompanied by a weakly labeled basolateral surface, while in parental cells, the probe diffused through the tight junctions, strongly labeled the basolateral and basal surfaces and appeared to penetrate the cells (Fig. 2b). In the barrier function, a

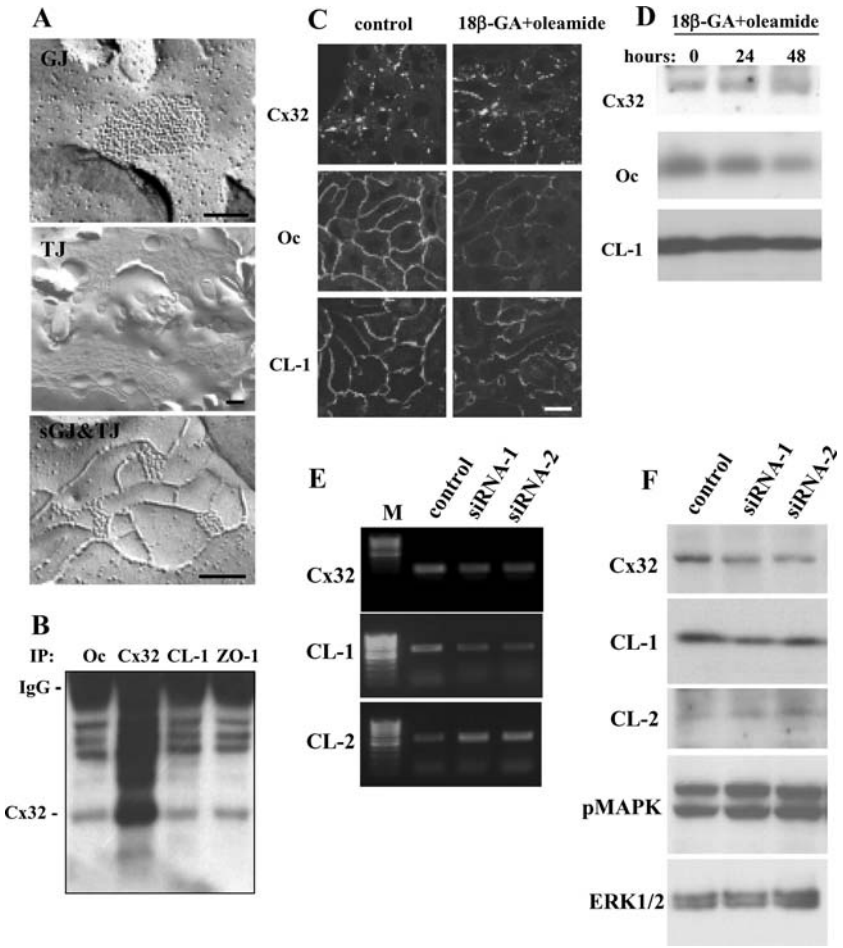

Fig. 1 (**a**) Freeze-fracture replicas of a large gap junction plaque (*GJ*), tight junction strands (*TJ*) and small gap junction plaques (*sGJ*) in primary cultures of rat hepatocytes. Bars = 100 nm. (**b**) Coimmunoprecipitation and Western blotting for rat hepatocytes using anti-Cx32, occludin, claudin-1 and ZO-1 antibodies. Immunocytochemistry (**c**) and Western blotting (**d**) for Cx32, occludin and claudin-1 in rat hepatocytes after treatment with 18β-GA and oleamide. Bar = 20 μm. RT-PCR (**e**) and Western blotting (**f**) for Cx32, claudin-1, claudin-2 and phospho-MAPK in rat hepatocytes after treatment with siRNAs of Cx32. siRNA-1, 5′-GUCUUCUUUCAUCUGUAAC-3′ and 5′-GUUACAGAUGAAAGAAGA-3′; siRNA-2, 5′-GGAAAAGGAGGACACAUCU-3′ and 5′-AGAUGUGUCCUCCUUUUCC-3′

significant increase of transepithelial resistance (TER) in the transfectants was observed compared to that of parental cells (Fig. 2c). These results suggest that exogenous Cx32 expression and mediated intercellular communication also regulate the expression and function of tight junctions in the immortalized mouse hepatic cell line.

To investigate the mechanisms of induction of tight junctions by transfection with Cx32, we performed cDNA microarray analysis of Cx32 transfectants compared to parental cells derived from Cx32-deficient hepatocytes (Murata et al., 2005). In cDNA microarray analysis, a 2.5-fold increase in expression of MAGI-1, which is known to be localized at adherens and tight junction regions (Dobrosotskaya, Guy & James, 1997; Ide et al., 1999; Nishimura et al., 2000), was observed. High expression of MAGI-1 in Cx32 transfectants was confirmed by RT-PCR and Western blotting (Fig. 2d,e). MAGI-1 was colocalized with occludin, claudin-2, ZO-1 and F-actin but not with E-cadherin on the apicalmost regions at cell borders of Cx32 transfectants, similar to JAM-1, which may play a crucial role in the formation and assembly of tight junctions (Murata et al., 2005). Treatment with 18β-GA did not affect expression of MAGI-1 and JAM-1 in Cx32 transfectants (Murata et al., 2005). These results suggest that exogenous Cx32 expression is in part related to induction of tight junctions through modulation of MAGI-1 expression in the immortalized mouse hepatic cell line.

On the other hand, a single epithelial cell has epithelial cell polarity like multiple epithelial cells *in vitro* and may form a linear circle of adherens junctions at the plasma membrane (Baas et al., 2004). We examined whether Cx32 expression affected formation of single epithelial cell polarity of hepatic cells. In Cx32 transfectants ($Cx32^{+}$), ZO-1, MAGI-1 and JAM-1, but not occludin and claudin-1,

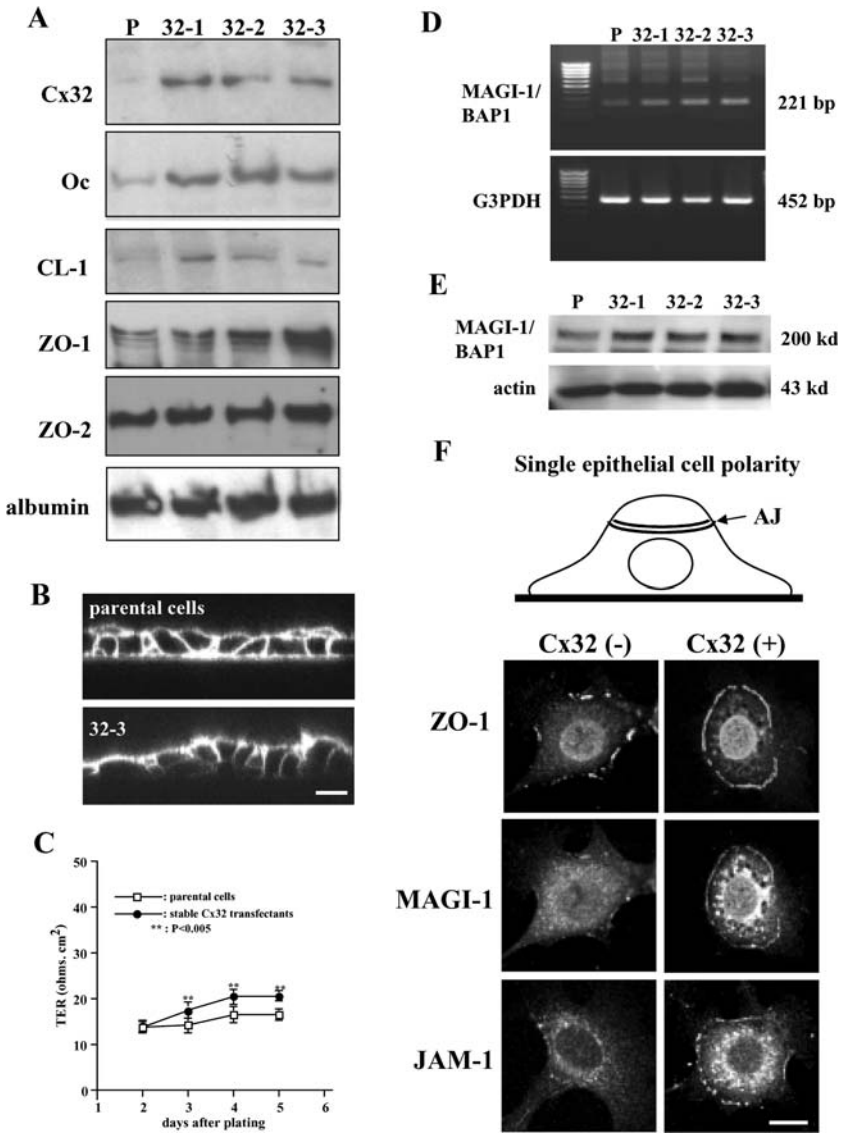

Fig. 2 (**a**) Western blotting for Cx32, occludin, claudin-1, ZO-1, ZO-2 and albumin in stable Cx32 transfectants. Fence function (**b**) and barrier function (**c**) of tight junctions in stable Cx32 transfectants. Bar = 20 μm. RT-PCR (**d**) and Western blotting (**e**) for MAGI-1 in stable Cx32 transfectants. (**f**) Immunocytochemistry for ZO-1, MAGI-1 and JAM-1 in parental cells and stable Cx32 transfectants. *AJ*, adherens junction

were localized to form a linear circle at the plasma membrane, whereas parental cells ($Cx32^{-}$) were localized to form a dotted circle (Fig. 2f). It is possible that hemichannels of Cx32 may be related to the formation of single epithelial cell polarity.

Cx26 Expression, but not Mediated Intercellular Communication, Prevents Downregulation of Barrier and Fence Functions of Tight Junctions by the Na^+/K^+-ATPase Inhibitor Ouabain in the Human Airway Epithelial Cell Line Calu-3

We introduced the Cx26 gene into the human airway epithelial cell line Calu-3 and used a disruption model of tight junctions employing the Na^+/K^+-ATPase inhibitor ouabain. In parental Calu-3 cells, gap junction proteins Cx32 and Cx43, but not Cx26, were detected by RT-PCR (Fig. 3a). After introduction of the human Cx26 gene into parental cells, Cx26 protein was detected in four transfectants (termed 26-1, 26-5, 26-8, 26-6) by Western blotting (Fig. 3b). Expression of claudin-14 protein was increased in three Cx26 transfectants compared to parental cells, whereas no changes in expression of occludin, JAM-1, ZO-1 or claudins 1, 2 or 3 were observed (Fig. 3c; Go et al., 2006). The BODIPY-sphingomyelin of parental cells was effectively retained in the apical domain (Fig. 3d). The probe of 26-1 cells was effectively retained in the apical domain at 4 h after treatment with 100 μM ouabain, whereas the probe of parental cells diffused through the tight junctions (Fig. 3d). No significant change in TER of 26-1 cells was observed after treatment with 100 μM ouabain, whereas TER of parental cells was markedly decreased (Fig. 3e). Furthermore, treatment with 18β-GA or

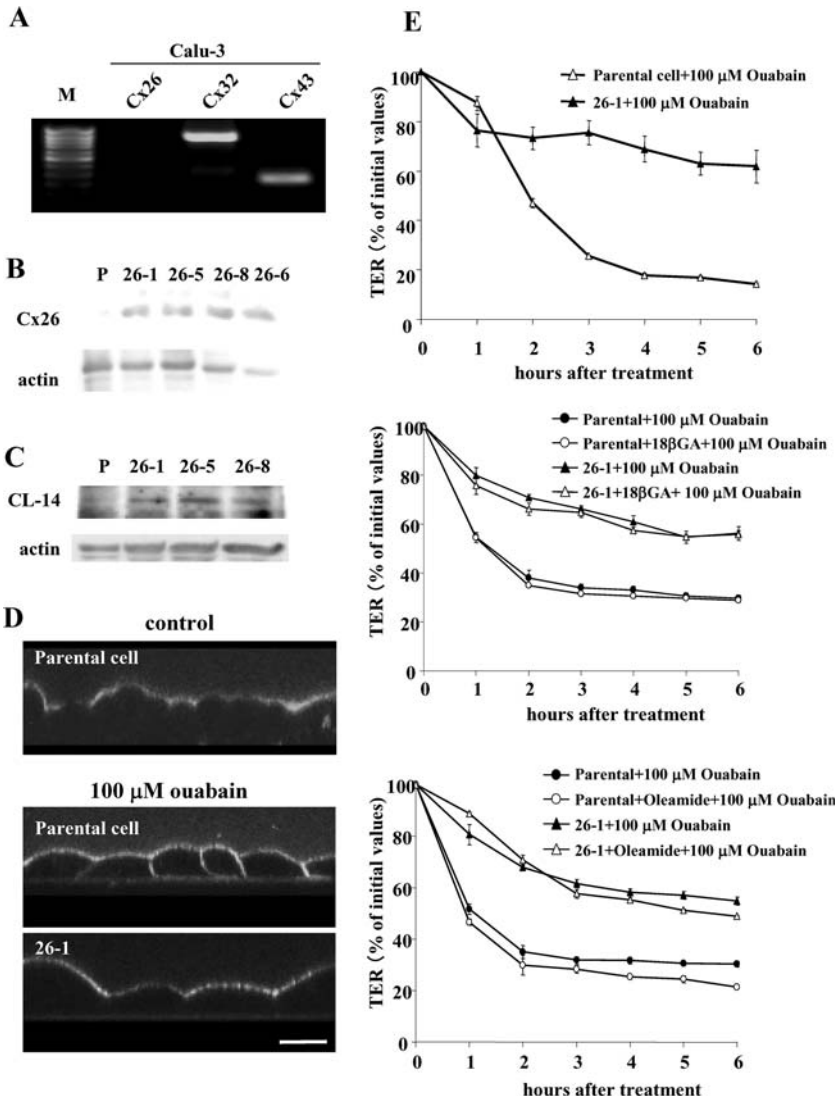

Fig. 3 (**a**) RT-PCR for Cx26, Cx32 and Cx43 in Calu-3 cells. *M*, 100-bp ladder DNA marker. Western blotting for Cx26 (**b**) and claudin-14 (**c**) in stable Cx26 transfectants. (**d**) Fence function of tight junctions in a stable Cx26 transfectant (26-1) after treatment with ouabain. Bar = 20 μm. (**e**) Barrier function of tight junctions in a stable Cx26 transfectant (26-1) after treatment with ouabain with or without GJIC blockers, 18β-GA or oleamide

oleamide did not affect TER of 26-1 cells and parental cells after treatment with 100 μM ouabain (Fig. 3e). These results suggest that Cx26 expression, but not mediated intercellular communication, regulates tight junction barrier and fence functions in the human airway epithelial cell line Calu-3.

Perspectives

The barrier and fence functions of tight junctions may be regulated by various factors (Tsukita et al., 2001; Tsukita & Furuse, 2002; Schneeberger & Lynch, 2004). Claudin-4 is significantly increased in human colonic Caco-2 cells stably expressing Cx26, and Cx26-mediated GJIC prevents downregulation of the barrier function of tight junctions by oleic acid and taurocholic acid (Morita et al., 2004). In our experiments using epithelial cells, hepatic cells and airway cells, transfection with Cx32 or Cx26 induced expression of ZO-1, MAGI-1, occludin and claudins 1 and 14. Furthermore, upregulation of claudin-2 was also observed by knockdown of Cx32. These findings suggest that the interactions of connexins with tight junction proteins may be cell type-specific and that tight junction function may in part be controlled by connexins via both GJIC-dependent and -independent mechanisms in epithelial cells (Fig. 4). However, it is still unclear what molecules in GJIC induce adherens and tight junctions (Fig. 4).

It is known that the MAGUK family, ZO-1, ZO-2 and β-catenin bind to Cx43 (Laird, 2006). Cx32 also interacts with one member of the MAGUK family containing the PDZ domain, Discs Large homolog 1 (Dlgh1), but not claudin-1, in yeast two-hybrid complementation; and the interaction was confirmed by coimmunoprecipitation and immunocytochemistry using mouse livers (Duffy et al., 2007). Cx26 interacts with a coiled-coil domain of occludin

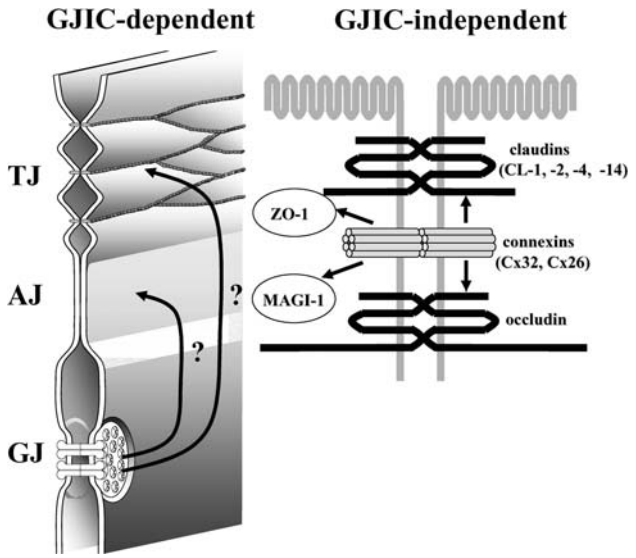

Fig. 4 Diagram showing interaction of intercellular junctions in GJIC-dependent and -independent manners. *TJ*, tight junction; *AJ*, adherens junction; *GJ*, gap junction

(Nusrat et al., 2000). However, direct interaction between connexins and claudins that can form tight junction strands is not found.

Although gap and tight junctions perform very different functions, there are numerous points at which their functions overlap. Our findings indicate the possibility for either coordinate or reciprocal regulation of macromolecular complexes containing gap and tight junction proteins. Expression of connexins including hemichannels may play a crucial role in the induction and maintenance of tight junctions in epithelial cells. Cx26 and Cx32 are closely associated with function of tight junctions, belonging to the β subgroup of connexin genes (Willecke et al., 2002). Cx31.9, Cx36, Cx43, Cx45, Cx46, Cx47 and Cx50 belong to the α or γ subgroup and interact with ZO-1 (Giepmans, 2004). Although there is currently no evidence, it is possible that the other members of the β subgroup, Cx30, Cx30.3, Cx31 and Cx31.1, which are mainly expressed in skin, may also be associated with function of tight junctions. Studies of protein-protein interactions and of coordinate and subordinate regulation of gene families are expected to soon disclose the intricacies of inter- and intracellular signaling and the regulatory mechanisms of the epithelial barriers formed by gap and tight junctions.

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