# Developmental Regulation of Gap Junctions and Their Role in Mammary Epithelial Cell Differentiation

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Gap junctions play a critical role in the development and differentiation of many tissues. Formed by the joining of two connexons on opposing membranes of two cells, gap junctions permit passage of ions and small molecules. Six connexins (Cx) belonging to a family of closely related tetraspan transmembrane proteins form a connexon. Connexin expression peaks in lactation, and those identified in the gland, thus far, are Cx26, Cx30, Cx32, and Cx43. Cx43 associates with myoepithelial cells, while others associate with epithelial and ductular cells. In vitro, assembly of functional gap junctions appears to be essential for differentiation of mammary epithelial cells. However, the role of gap junction intercellular communication (GJIC) in differentiation and growth remains unclear. Recent evidence challenges the view that gap junctions are simply pore-forming proteins and suggests that cell adhesion-associated proteins interact with the cytosolic carboxy-terminus of connexins and participate in signaling events. The possible implications on mammary cell function are discussed.

**KEY WORDS:** connexin; differentiation; gap junction; mammary.

## GAP JUNCTIONS: AN OVERVIEW

Gap junctional intercellular communication (GJIC) is critical in diverse cell and tissue functions, including regulation of growth, differentiation, and developmental signaling. Based on X-ray diffraction studies a three-dimensional model of the gap junction channel predicts that each apposed cell contributes a hemichannel (connexon) to the formed aqueous pore. Each hemichannel is constructed from six protein subunits called connexins. Connexins, a multigene family comprising over 20 distinct connexin genes that code for homologous proteins, are temporally and spatially distributed throughout the body. Sequence analysis of connexin cDNAs has shown that they all share a similar structural topology, with four hydrophobic regions, presumably the membrane spanning domains, two extracellular loops, thought to be involved in initiating the interactions between two opposing connexons, and one cytoplasmic loop. Both carboxy and amino-terminal domains of the protein are located on the cytosolic side. Sequence comparison revealed that, within the connexin gene family, the N-terminus, the extracellular loops, and the transmembrane regions

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are highly conserved. The cytoplasmic loop and C-terminus are highly divergent, both in length as well as in sequence. These sequence differences are likely to be responsible for many of the connexin-specific functional properties, including sensitivity to different stimuli and second messenger molecules, and the recruitment of other associated proteins to the junctional complexes. Connexins oligomerize intracellularly and are then inserted into the plasma membrane in a series of assembly processes. They are synthesized and inserted into the membrane of the endoplasmic reticulum (ER) where they must fold correctly prior to oligomerizing into hemichannels, an event occurring as they traffic from the ER to the Golgi. Connexons are then transported to the membrane, where they remain in a closed hemichannel configuration until they form channels with other connexons on opposing cell membranes. Recent evidence for connexons' hemichannel functions has been described. Complete channel formation is accomplished upon the docking of neighboring connexons via disulfide bridges between the cysteine residues on extracellular loops of opposing connexins. Recently, it has become clear that connexins, via their cytosolic domain, interact with a variety of structural and signaling molecules, including tight junction-associated proteins such as ZO-1, tetraspan trans-membrane proteins occludin and claudins, or Src proteins, as well as adherens junction-associated proteins, such as cytoskeletal proteins and catenins. The most commonly used nomenclature for connexins was first introduced by Beyer *et al.* (1) and is based on the molecular mass predicted from the cDNA. An alternate nomencalture ($\alpha, \beta,$ and $\gamma$) based on the length of the cytoplasmic loop has also been used. Connexin expression displays organ, tissue, and cell-type specificity, and in many cases multiple members of the connexin family are expressed at the same location. Hemichannels may assume various configurations; they may be homomeric, comprising six identical connexin subunits, or heteromeric, comprising more than one isoform of connexins. Channels may also be homotypic when connexons are identical or heterotypic when the two connexons are different. The intercellular channels cluster and aggregate in the plane of the membrane to form plaques that are known as gap junctions (Reviewed by 2–5).

The intercellular channels allow diffusion of ions and small molecules (up to 1.5 kDa) between the cytosols of two adjacent cells, therefore creating an ionic and metabolic syncytium in the compartments of interacting cells. In excitable cells, such as cardiomyocytes and neurons, the ionic conductance allows for the rapid intercellular spread of action potentials. However in nonexcitable cells, many different small molecules and second messengers might diffuse from cell to cell. One consequence of the molecular diversity in the connexin family is that the channels display selective permeability with respect to molecular mass and/or charge that will permit the discrimination of second messengers like cAMP, cGMP, IP$_3$, or Ca$^{2+}$ (5). The basic function of gap junctions can be electrically regulated by transmembrane voltage and/or chemically via changes in cytoplasmic pH and calcium ions (6). Hormones, growth factors, second messengers, cell–cell adhesion molecules, and cell–extracellular matrix (ECM) interactions also potentially regulate GJIC function. In addition, the stability of connexin proteins is an important factor in regulating GJIC function, since connexins have a high turnover rate with very rapid disassembly. Musil *et al.* (7) have shown that reducing gap junction degradation is coupled to an upregulation in GJIC.

The expression of connexins can be developmentally or metabolically regulated at both transcriptional and post-translational levels. Typically, connexin genes are made of two exons separated by an intron. Promoter regions have been sequenced, and several putative regulatory sites have been examined for function. Of those connexins expressed in the mammary gland, the promoter sequences for *Cx32* gene were found to be differentially regulated in several tissues (8). The human chorionic gonadotropin (hCG) hormone has been shown to up-regulate *Cx26* in rat mammary and uterine tissues by increasing the binding of Sp1 and Sp3 transcription factors to the promoter sequence (9). Estrogen has been shown to increase *Cx43* transcription by affecting the estrogen responsive-elements in the *Cx43* gene promoter region (10). Except for *Cx26*, the only non-phosphoprotein connexin, post-translational modification via phosphorylation plays a central role in gap junction formation, regulation of channel permeability, and degradation. Connexin phosphorylation by Protein Kinase A occurs mostly on the serine residues that reside on the COOH-tail, increasing GJIC. Basal phosphorylation is needed for proper trafficking and assembly of gap junctions. Once formed, gap junction gating is regulated by phosphorylation (Reviewed by 11,12). Phosphorylation has been shown to decrease or inhibit GJIC, as in the cases of epidermal growth factor and platelet-derived growth factor causing *Cx43* serine phosphorylation via a MAPK (13,14) and *Cx32* serine phosphorylation via protein kinase C in rat liver epithelial cells (15), respectively. On

the other hand, Atkinson *et al.* (16) reported serine/threonine phosphorylation of Cx43 by kinases in turn increasing GJIC. A schematic diagram of the current knowledge of connexin/connexon structure is presented in Fig. 1.

## SPATIAL AND TEMPORAL EXPRESSION OF CONNEXINS DURING MAMMARY GLAND DEVELOPMENT

### Gap Junctions in the Rodent Mammary Gland

A variety of factors are involved in mammary gland development and differentiation. These include soluble factors, cell–ECM interaction, and direct cell–cell interaction. In the latter, gap junctions are perceived as "modulators of cellular differentiation" and "coordinators of cellular function" (17–20).

Using electron microscopy techniques, Pitelka *et al.* (21) was the first to describe gap junctions between epithelial cells of the mammary gland in virgin, pregnant, and lactating mice. Later, Berga *et al.* (22) demonstrated gap junctional intercellular communication or coupling between alveolar cells by microinjection of lucifer yellow dye into lobules of lactating mammary glands. The development of specific anticonnexin antibodies and the cloning of individual Cx isoforms permitted characterization of the spatial and temporal expression of Cx isoforms within rodent and human mammary gland. Monaghan *et al.* (23) reported Cx26 as the dominant isoform expressed in lactating mouse mammary gland epithelium and suggested its involvement in synchronous activity of epithelial cells. Cx26 was not detected in virgin mice mammary glands; however, its levels increased at early stages of pregnancy, localized to the luminal epithelial cells of the duct system, peaked during lactation in alveolar cells, and then declined during involution. Cx32, 40, and 43 were absent or expressed below detection levels.

Pozzi *et al.* (24) and Perez-Armendez *et al.* (25) have identified Cx26 and Cx32 within the luminal cell population. The temporal expression patterns for these connexins in both studies were not in agreement. Pozzi *et al.* (24) reported the detection of Cx26 and Cx32 transcripts using RT-PCR and proteins using immunolocalization in the luminal epithelium of the BALB/c mouse and Sprague-Dawley rat mammary gland only during lactation. No Cx26 staining was evident in nonpregnant, pregnant, or postweaning rodents, although Cx26 mRNA was present in nonpregnant mouse but not in rat. Using immunolocalization studies, Perez-Armendez *et al.* (25) reported that Cx26 and Cx32 were evident in the virgin gland and during all stages of mammary development in CD1 mice and Wistar rats.

Locke *et al.* (20) demonstrated that Cx26 and Cx32 were differentially expressed throughout pregnancy, with the latter being detected only during lactation. Interestingly, immunolocalization, freeze fracture, and differential centrifugation studies showed that both Cx26 and Cx32 can organize as homomeric and heteromeric connexons and localize to the same junctional plaques. Similar results were reported by Yamanaka *et al.* (26). Such plaques were larger during lactation; however, whether gap junctions were more numerous in these plaques was not determined.

In a recent study (Talhouk *et al.*, 2001), it has been shown that in addition to Cx26 and Cx32 (both expressed and confined to cell borders of luminal epithelial cells in all stages of mammary development), Cx30, a novel Cx expressed in mouse skin and closely related (77% sequence homology) to Cx26 (4,27,28), was detected after day 15 of gestation and peaked at the onset of lactation.

Within the myoepithelial cells, Cx43 is the only identified isoform. Cx43 was detected in virgin, pregnant, lactating, and postweaning rodent mammary gland. The intensity of Cx43 immunolabeling and the phosphorylation state of Cx43, which is a prerequisite for the assembly and/or maintenance of Cx43 in gap junction plaques (29), were shown to increase during early lactation and mammary differentiation (24,30, and Talhouk *et al.*, submitted). At parturition and in response to oxytocin, a dramatic and transient induction of Cx43 mRNA occurs that rapidly declines thereafter, while increased Cx43 protein persists in the gland (31, and Talhouk *et al.*, submitted). It has been suggested that the increased expression of Cx43 in myoepithelial cells leads to increased communication and thus coordinated contraction and milk ejection into the ducts (18).

Heterocellular communication between luminal and myoepithelial cell compartments has been suggested earlier by Berga (22). Woodward *et al.* (19), reported that both mammary epithelial cells and fibroblasts readily assemble Cx43-positive gap junction plaques when cocultured with an intermediate cell type (i.e. a cell type with both epithelial and fibroblastic characteristics), but not when cocultured together. Whether this cell type has any resemblance to a myoepithelial cell in vivo was not discussed. Preliminary studies in our laboratory demonstrated GJIC, as
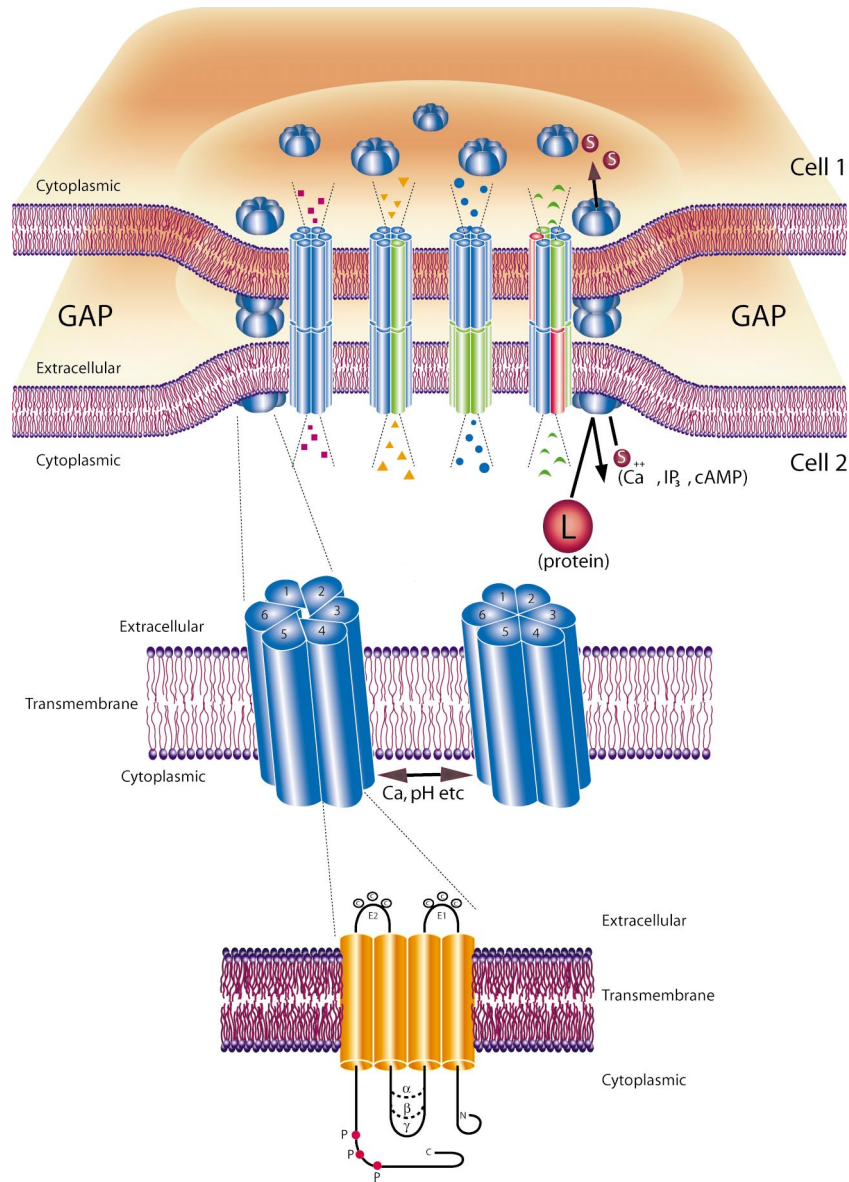
**Fig. 1.** A schematic diagram of a gap junction plaque joining the cytoplasm of two adjacent cells (Cell 1 and Cell 2; top panel). The opposed phospholipid bilayers are traversed by connexons that cluster and aggregate in the plane of the membrane to form a gap junction plaque. Connexons from two adjacent cells form an intercellular channel that allows the passage of small (s) molecules (up to 1.5 kDa), such as $Ca^{++}$, $IP_3$, and cAMP, but not large (L) molecules such as proteins, from the cytoplasm of one cell to the other. Depending on the type of connexin (for ex. black, grey, light grey), the connexon formed could be homomeric or heteromeric and consequently the gap junction could be homotypic or heterotypic, with selective permeability (■ ▲ ● ✔). Each connexon (middle panel) is made of six connexins (1, 2, 3, 4, 5, and 6). Tangential twisting of the connexon mediates gap junction gating that opens (left connexon) or closes (right connexon) the hemichannel. Changes in cytoplasmic pH and calcium (Ca) ion concentration, among other things, regulate gap junction function. A connexin (lower panel) has four hydrophobic transmembrane domains, two extracellular loops (E1 and E2), one cytoplasmic loop of different lengths (indicated by connexin iso-forms $\alpha$, $\beta$, $\gamma$), and N- and C-termini, both cytoplasmic. The main variation between the different connexins resides in the C-terminal domain and the cytoplasmic loop. P, indicates phosphorylation sites (Adapted from (4)).

evidenced by calcein dye transfer between mammary epithelial cells and an $\alpha$-actin-staining mammary myoepithelial-like cell population (unpublished data).

It is apparent that connexins are abundant in the lactating gland; however, there is no agreement on the temporal expression of connexin isoforms during the different stages of mammary gland development. This is attributed to either species differences in the development of the mammary gland (32,33) or the fact that discriminating antibodies and cDNA for all connexin isoforms have only recently become available. In support of this notion, more recent studies have demonstrated some concordance and reported Cx expression throughout mammary gland development and most notably during lactation (9,26, and Talhouk *et al.*, submitted), consistent with early EM studies of Pitelka *et al.* (21).

## Gap Junctions in the Human Mammary Gland

Given the difficulty of obtaining normal human breast tissue at different time points of mammary development, the expression of specific gap junction proteins in normal human epithelium is not well documented. Wilgenbus et al. (34), described gap junctions in mammary cells obtained from reduction mammoplasties and showed a decrease in gap junction proteins in breast cancers. In another study, normal mammary cell lines, capable of transferring Lucifer yellow dye, expressed the twoconnexin genes, *Cx26* and *Cx43*. Regulation of Cx26, in contrast to Cx43, was cell cycle dependent. Mammary tumor cell lines, on the other hand, did not express either Cx26 or Cx43 and failed to show dye transfer. Screening of normal and tumor human cell lines for expression of Cx31.1, Cx32, Cx33, Cx37, and Cx40 showed that none of these connexins were expressed under the culture conditions tested (35).

Monaghan and Moss (17), using both in vivo and in vitro studies, have attempted to attribute specific gap junction proteins to individual cell types in the human breast tissue. Ultrastructural studies described gap junctions between myoepithelial (basal) cells in ducts and alveoli, but rarely between secretory (luminal) cells. No gap junctions were observed between myoepithelial and secretory cells. Immunofluorescence studies performed on breast samples obtained from reduction mammoplasties re-

ported Cx43 between myoepithelial cells in major ducts and to a lesser extent within alveolar structures. Using immunolabeling, Cx26, although less abundant in breast epithelium, was detected between secretory cells in major ducts but to a lesser extent in alveolar structures. In addition, PCR analysis of breast mRNA identified messages for Cx26 and Cx43. To date, however, it is not yet certain if Cx32 is expressed at any time during human breast development.

Homologous and heterologous communication between normal human mammary cells was reported by Tomasetto *et al.* (36). Three types of cells were used: normal mammary epithelial cells (NMEC) expressing both *Cx26* and *Cx43* genes, mammary tumor epithelial cells (TMEC) where neither gene is expressed but into which *Cx26* or *Cx43* gene were transfected, and normal human mammary fibroblasts (NHMF) where only *Cx43* protein is synthesized. Calcein dye transfer quantitated by flow cytometry showed that NMEC and NHMF communicate effectively and that all three cell types expressed strong homologous communication. However, no heterologous gap junctional intercellular communication was detected between Cx26- and Cx43-transfected TMEC, suggesting that heterotypic channels do not form or that Cx26/Cx43 channels do not permit dye transfer.

There is increasing interest in the role gap junctions play in breast cancer. A correlation has been drawn in many instances between loss of gap junction communication and a transformed phenotype. The majority of these studies speculate that the absence or decrease in GJIC in highly metastatic cells may enhance tumor proliferation, detachment from the primary site, tumor invasion, and possible formation of tumor metastasis (Reviewed by 37). However, direct association between aberrant gap junctional intercellular communication and a true malignant phenotype is not yet well established. For example, Jamieson *et al.* (38) observed an upregulation of Cx26 and Cx43 in about two-thirds of the invasive human breast carcinomas studied. A similar observation has been reported in mouse skin cancer (39). According to Jamieson *et al.* (38), the increase in connexin expression in breast carcinoma cells may enhance tumor progression and establish distant metastases. This notion is further supported by the fact that highly metastatic rat mammary adenocarcinoma cells communicate with vascular endothelium in vitro to a higher extent than their low metastatic counterparts (40). It is worth noting, however, that such contrasting

reports do not contradict the hypothesis of GJIC's role in tumor suppression. We speculate that at the primary site of tumors, heterologous GJIC is downregulated, facilitating detachment and intravasation. However, at the secondary site of tumor, where distant metastases will be established, GJIC between tumor and target cells is up-regulated facilitating extravasation (41, and Bazarbachi *et al.*, submitted).

## ROLE OF GAP JUNCTIONS IN MAMMARY DEVELOPMENT AND DIFFERENTIATION

### Status of Studies With Connexin Knockout Animals

Knock out animal models and in vitro mammary cell culture models have been used to address the contribution of different mammary connexins (*Cx26, 30, 32,* and *43*) to development and differentiation of the mammary gland. Unfortunately no studies on the phenotype of the mammary gland in *Cx30* and *Cx32* knock out animals have been reported, while *Cx26* and *Cx43* knockouts have been found to be lethal (42,43). *Cx26*-deficient mice die during midgestation due to insufficient nutrient uptake through the placenta from the maternal blood stream to the embryo (43). Cx43 knockouts die within a few hours after birth due to gross abnormalities in the heart tissue (42).

Homozygous mutant $Cx30^{(-/-)}$ knockouts that develop normally were fertile, but exhibited a severe hearing impairment. The Cx30 protein was reported in the brain, skin, lung, kidney, and uterus. However, no obvious histological abnormalities were detected in the organs of Cx30-deficient mice, but *Cx30* missense mutations have been reported to cause hidrotic ectodermal dysplasia or Clouston syndrome (Reviewed by 4,44).

Cx32-null mice, a model for human patients who suffer from Charcot–Marie–Tooth (X type) disease, exhibit enhanced vulnerability to global ischemia-induced neuronal death, consistent with a role for Cx32 gap junctions in neuroprotection against ischemia-induced cell death (45).

The fact that homozygous $Cx30^{(-/-)}$ and $Cx32^{(-/-)}$ knockouts can be generated, and apparently can mate, reproduce, and maintain a litter suggests, contrary to most reported studies (18,20,30), that the role of these connexins in regulating mammary function may be compensated by connexin redundancy (46).

## Gap Junction Cross Talk With Adhesion-Related Molecules

### Interplay Between ECM and Gap Junction

The role of the microenvironment in governing mammary phenotype and differentiation has long been recognized. Initially, the work of Emmerman and Pitelka in 1977 (47) highlighted the critical role of the microenvironment, embodying cell–cell and cell/ECM interactions, in regulating differentiation of mammary cells in culture. Subsequently, Bissell *et al.* (48) suggested that the cell's interaction with its microenvironment dictates cell function and phenotype, which in turn dictate the makeup of the microenvironment. The term "dynamic reciprocity" was coined for this concept, and much effort since then has emphasized the importance of cell/ECM interactions in dictating the differentiated phenotype of the mammary epithelial cell. The majority of studies have suggested that cell–cell interactions enhance mammary cell differentiation, but in a matrix-dependent manner (Reviewed by 49). A study by Streuli *et al.* (50) demonstrated that casein production was synergistically elevated upon cell–cell interaction. A consequence of such cell–cell interaction could be intercellular communication through gap junctions (30).

Molecules involved in cell–ECM interactions are implicated in GJIC regulation. Endothelial cells of the aorta and pulmonary artery fail to communicate via gap junctions except when cultured on ECM (51). ECM has also been shown to increase GJIC in thymic cells (52) and keratinocytes by increasing the assembly of Cx43 into Triton-X insoluble junctions (53). In alveolar type II epithelial cells ECM, specifically fibronectin, regulated the expression of Cx43 and Cx26. Cx43 protein expression was up-regulated and deposited on the cell membrane, while Cx26 protein expression was reduced (54,55). A recent study showed that parathyroid hormone treatment of MC3T3-E1 osteoblast cells increased mineralization of ECM, coinciding with a high expression of Cx43 and GJIC (56).

The complex interplay between prolactin signaling, cell/ECM interactions, and GJIC in mediating optimal mammary differentiation was noted by Miyoshi *et al.* (57). The authors reported that mammary glands of both STAT-5 or prolactin receptor (PrlR) null mice failed to develop normally and that Cx32 was not detectable in the secretory epithelia. Studies in our laboratory showed that CID-9 cells, a mammary cell strain that differentiates and expresses β-casein in an ECM- and prolactin-dependent

manner, modulated the expression of their connexins in response to growth factors (58) and to ECM (30). In contrast to CID-9 cells cultured on plastic, those cultured on EHS-matrix possess functional GJIC, as measured by lucifer yellow dye transfer assays. When cultured in the presence of EHS-matrix, but not when cultured on plastic, Cx26, 32, and 43 localized predominantly to the plasma membrane. Inhibition of GJIC of cells on EHS-matrix with $18\alpha$ glycyrrhetinic acid resulted in reversible down-regulation of $\beta$-casein expression. In contrast, enhancing GJIC for cells on plastic by treatment with cAMP, in the absence of an exogenously provided basement membrane, up-regulated Cx43 and Cx26 protein levels and increased $\beta$-casein expression. Both Cx43 and $\beta$-casein were down-regulated when these cells were treated with $18\alpha$ glycyrrhetinic acid. In addition, cells plated on a nonadhesive substratum (PolyHEMA) or on plastic and supplemented with function-blocking anti-$\beta_1$ integrin antibodies, both under conditions that enhance GJIC, maintained $\beta$-casein expression, suggesting that cell–ECM interaction may induce differentiation through formation of functional gap junctions. These events are downstream of ECM signaling due to the fact that enhanced GJIC induced partial differentiation in mammary epithelial cells in the absence of an exogenously provided basement membrane and in a $\beta_1$-integrin and cell–ECM adhesion-independent manner (30).

## Connexin-Associated Proteins

Historically, the function of gap junctions in cellular communication has been limited to their role as aqueous pores that traverse the cell membranes of two adjacent cells. In contrast to occluding junctions and anchoring junctions, evidence that gap junctions interact with other cellular or cytosolic components was scarce. However, recently many reports have demonstrated that this is a limited view of a rather complex gap junction structure, and that connexins, via their cytosolic domain, interact with a variety of structural and signaling molecules, including tight junction-associated proteins, such as zonula occluden-1 (ZO-1), occludin, claudins and Src proteins, and adherens junction-associated proteins, such as cytoskeletal proteins and catenins (Reviewed by 2). The majority of studies describe the association of the cytosolic structural and signaling molecules with Cx43, since it is the most studied connexin and the primary connexin subtype found in many tissues, including fibroblasts, myoepithelial cells, and myocytes (4).

Tight junction associated-proteins, such as ZO-1, and transmembrane occludin and claudins, might be involved in the trafficking or organization of gap junctions. Cx43 associates with ZO-1, a member of the membrane-associated guanylate kinase (MAGUK) family of proteins, which contain up to three distinct amino acid sequence motifs that mediate protein–protein interactions, the PDZ domains, a Src homology 3 (SH3) domain, and a guanylate-kinase domain (59,60). The interaction between Cx43 and ZO-1 has been identified in the cardiac myocytes at the intercalated discs (61,62), testis, rat-1 fibroblast, lung epithelial cells (63), and osteoblastic cells (59). The C-terminal residues of Cx43 interact with the second PDZ domain of the ZO-1 protein, which may recruit regulatory proteins into gap junctions (59,61,62). More recently, Cx45 was also shown to associate with ZO-1; indeed, Cx45 appears to interact with PDZ domains of ZO-1 via a short terminal PDZ binding motif, namely the last four amino acids (SVWI) of the C-terminus (59,64). Multiple alignment studies showed that Cx46 could also display a ZO-1 binding motif similar to that of Cx43. Moreover, Cx32, which ends with hydrophobic amino acids, could potentially have a ZO-1 binding motif. Kojima *et al.* (65,66) reported that Cx32 but not Cx26 associates with tight junction proteins, such as ZO-1, ZO-2, occludin, and claudin-1, in primary culture of rat hepatocytes, and thus modulates cell polarization. In contrast, occludin was reported to interact with Cx26 in human intestinal cells, T84 (67), suggesting that interactions of such proteins with connexins may be cell type specific. The functional relevance of the association between connexins and tight junction proteins, such as ZO-1, occludin, and claudin is still poorly understood. These proteins may play a role in targeting or localizing connexins to specialized plasma membrane domains or in connexon assembly and stability. ZO proteins associated with gap junctions could serve to recruit signaling molecules involved in the regulation of intercellular communication and/or provide a linkage between connexins and other crucial elements in the cell through ZO-1's ability to associate with cytoskeletal components (eg. actin).

Src, a tyrosine kinase that localizes to the cytoplasmic side of the plasma membrane and contains SH2 and SH3 domains, is another signaling molecule reported to associate with gap junctions. Increasing evidence indicates that v-Src and c-Src tyrosine kinases can bind directly to and phosphorylate the Cx43 C-terminal tail via SH2 and SH3 domain interactions (63,68). It has been reported that tyrosine

phosphorylation (Tyr 265) of Cx43 is required for the pp60v-Src-induced inhibition of GJIC in paired oocytes (69). Subsequently, it was shown that v-Src, via its SH2 and SH3 domains, interacts with the proline-rich region of Cx43 and phosphorylates neighboring Tyr 247 and 265 at its C-terminus (70–72). The phosphorylation of Tyr$^{265}$ has been shown to be important for the binding of the oncogene v-Src to Cx43, as has the second of two proline-rich putative SH3 binding domains in the C-terminus of Cx43 (68,72). Multiple alignment studies conducted in our laboratory showed that such a proline-rich site is also present in Cx46, bringing forth the possibility of an interaction between Cx46 and c-Src (unpublished data). c-Src has also been thought to modulate the interaction between Cx43 and ZO-1. Different studies reported that Tyr$^{265}$ phosphorylation by c-Src is most likely involved in the regulation of the interaction of Cx43 with ZO-1 (63,72,73). This finding suggests a role for c-Src in the regulation of the protein complex composition at the Cx43 plaque. c-Src could thus act by affecting trafficking of connexins to the plasma membrane (gap junctional plaque) through regulation of the connexin and ZO-1 interaction (Reviewed in 2).

Coimmunoprecipitation experiments using the C-terminal tail of connexin fused to glutathione-S-transferase showed that the Cx43 tail binds directly to tubulin via a binding sequence $^{234}$KGVKDRVKGK$^{243}$ (74). Alignment studies revealed that a tubulin-binding site was present at least in connexins 41 and 46 in addition to Cx43 (unpublished data); thus, we speculate that Cx43 is not the only member of its family to bind or anchor microtubules. Indeed, it is reasonable to assume that the anchoring of gap junctions to the cytoskeleton would be of crucial importance for cell function by providing a structural link to the cytoskeleton. Such a link might lead to highly regulated control of the cell's responses to various substances that are exchanged through the junctions by physically modulating many downstream events. Recent reports have also described the association of $\beta$-catenins (75) and p120 catenins with Cx43 (76). However, the significance of this interaction in the signaling cascade is not yet clear.

## MODEL FOR GAP JUNCTION ROLE IN MAMMARY DEVELOPMENT AND DIFFERENTIATION

Available data suggest that GJIC play an important role in regulating mammary development and
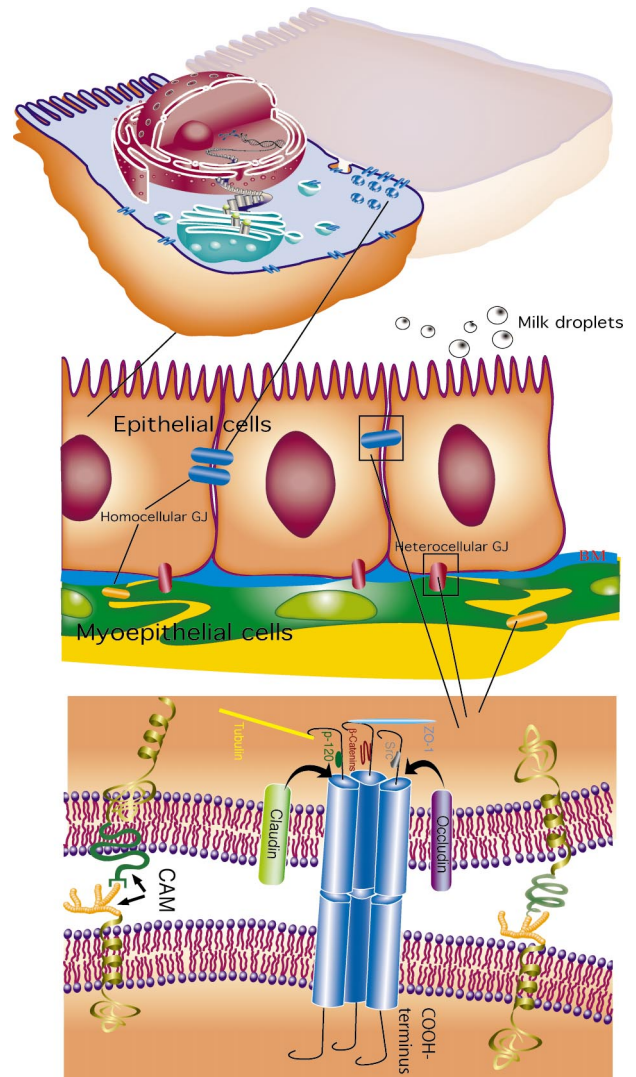


**Fig. 2.** Schematic diagram showing the synthetic pathway of connexins and the assembly route into homocellular (between epithelial cells or between myoepithelial cells) or possible heterocellular (between epithelial and myoepithelial cells) gap junctional (GJ) structures, and their known binding partners. Connexins traffic from the ER to the Golgi. Connexons pinch off in vesicles from the Golgi apparatus and are transported to the plasma membrane where they align and dock with other connexons from the neighboring cell to form a gap junctional channel. The precise localization and manner of how connexin binding partners (i.e. Src, $\beta$-catenin, p-120 catenin, claudin, occludin, and tubulin) associate with the C-terminus of connexin is not clear yet. Optimal mammary epithelial cell differentiation in a complex tissue environment requires the proper assembly of gap junctional plaques with their associated proteins in addition to proper cell/ECM interactions, cell/cell adhesion, and soluble factor signaling. BM, basement membrane.

differentiation by mechanisms that are still largely unclear. The following summary outlines the "state of our knowledge" on the involvement of gap junctions in mammary development and differentiation. First, gap junctions have been detected at all stages of mammary development, with Cx26, 30, 32, and 43 being the main connexins described in the rodent mammary gland. In human breast tissue only Cx26 and Cx43 are described. Second, mammary epithelial cell differentiation in culture is partially dependent on the membrane assembly and functionality of gap junctions. GJIC signaling is downstream of ECM signaling (30) and may be a crucial regulatory step in differentiation. Whether homocellular (epithelial–epithelial) or heterocellular (epithelial–myoepithelial) communication is essential to GJIC-induced differentiation is not yet clear. Third, the emerging literature strongly suggests that gap junctions are not simply transmembrane channels mediating the passage of soluble molecules between cells, but rather are actively involved in cell signaling by association of Cxs with Src, catenins, occludins, and other molecules. More importantly, recent findings strongly suggest that gap junctions are in fact anchored to the cytoskeleton of the cell not only through microtubules (74) but also perhaps through actin (77), and that their assembly and proper functioning is modulated by cell/ECM interactions (30) (Fig. 2). In conclusion, a comprehensive understanding of how GJIC-mediated signaling generates tissue-specific gene expression leading to complex phenotypes is still lacking. Furthermore, an additional hierarchy of control is imposed by complex cell and tissue architecture. Interpreting how this hierarchy affects cross talk between cells, cells and their ECM, and soluble signaling factors in complex tissue environments is only beginning to be addressed.

## REFERENCES

1. E. Beyer, D. Paul, and D. Goodenough (1987). Connexin43: A protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* **105**(6 Pt. 1)**:**2621–2629.

2. H. Duffy, M. Delmar, and D. Spray (2002). Formation of the gap junction nexus: Binding partners for connexins. *J. Physiol. Paris* **96**(3–4)**:**243–249.

3. G. M. Kidder and A. A. Mhawi (2002). Gap junctions and ovarian folliculogenesis. *Reproduction* **123**(5)**:**613–620.

4. K. Willecke, J. Eiberger, J. Degen, D. Eckardt, A. Romualdi, M. Guldenagel, *et al.* (2002). Structural and functional diversity of connexin genes in the mouse and human genome. *Biol. Chem.* **383**(5)**:**725–737.

5. D. A. Goodenough and D. L. Paul (2003). Beyond the gap: Functions of unpaired connexon channels. *Nat. Rev. Mol. Cell. Biol.* **4:**285–294.

6. P. Martin, J. Steggles, C. Wilson, S. Ahmad, and H. Evans (2000). Targeting motifs and functional parameters governing the assembly of connexins into gap junctions. *Biochem. J.* **394:**281–287.

7. L. S. Musil, A. C. Le, J. K. Van Slyke, and L. M. Roberts (2000). Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J. Biol. Chem.* **275:**25207–25215.

8. I. M. Neuhaus, G. Dahl, and R. Werner (1995). Use of alternate promoters for tissue-specific expression of the gene coding for connexin32. *Gene* **158**(2)**:**257–262.

9. Z. Jin Tu, R. Kollander, and D. T. Kiang (1998). Differential up-regulation of gap junction connexin 26 gene in mammary and uterine tissues: The role of Sp transcription factors. *Mol. Endocrinol.* **12:**1931–1938.

10. W. Yu, G. Dahl, and R. Werner (1994). The connexin43 gene is responsive to oestrogen. *Proc. R. Soc. Lond. B. Biol. Sci.* **255**(1343):125–132.

11. J. C. Saez, V. M. Berthoud, A. P. Moreno, and D. C. Spray (1993). Gap junctions. Multiplicity of controls in differentiated and undifferentiated cells and possible functional implications. *Adv. Second Messenger Phosphoproteins Res.* **27:**163–198.

12. P. D. Lampe and A. F. Lau (2000). Regulation of gap junctions by phosphorylation of connexins. *Arch. Biochem. Biophys.* **384**(2)**:**205–215.

13. A. F. Lau, M. Y. Kanemitsu, W. E. Kurata, S. Danesh, and A. L. Boynton (1992). Epidermal growth factor disrupts gap-junctional communication and induces phosphorylation of connexin43 on serine. *Mol. Biol. Cell.* **8:**865–874.

14. M. Z. Hossain, A. B. Jagdale, P. Ao, and A. L. Boynton (1999). Mitogen-activated protein kinase and phosphorylation of connexin43 are not sufficient for the disruption of gap junctional communication by platelet-derived growth factor and tetradecanoylphorbol acetate. *J. Cell. Physiol.* **179**(1)**:**87–96.

15. P. Ren, P. P. Mehta, and R. J. Ruch (1998). Inhibition of gap junctional intercellular communication by tumor promoters in connexin43 and connexin32-expressing liver cells: Cell specificity and role of protein kinase C. *Carcinogenesis* **19:**169–175.

16. M. M. Atkinson, P. D. Lampe, H. H. L. R. Kollander, X. R. Li, and D. T. Kiang (1995). Cyclic AMP modifies the cellular

distribution of connexin43 and induces a persistent increase in the junctional permeability of mouse mammary tumor cells. *J. Cell. Sci.* **108:**3079–3090.

17. P. Monaghan and D. Moss (1996). Connexin expression and gap junctions in the mammary gland. *Cell. Biol. Int.* **20:**121–125.

18. D. Locke (1998). Gap junctions in normal and neoplastic mammary gland. *J. Pathol.* **186:**343–349.

19. I. L. Woodward, M. A. Sia, O. W. Blaschuk, J. D. Turner, and D. W. Laird (1998). Deficient epithelial-fibroblast heterocellular gap junction communication can be overcome by co-culture with an intermediate cell type but not by E-cadherin transgene expression. *J. Cell. Sci.* **111:**3529–3539.

20. D. Locke, N. Perusinghe, T. Newman, H. Jayatilake, W. H. Evans, and P. Monaghan (2000). Developmental expression and assembly of connexins into homomeric and heteromeric gap junction hemichannels in the mouse mammary gland. *J. Cell. Physiol.* **183**(2)**:**228–237.

21. D. R. Pitelka, S. T. Hamamoto, J. G. Duafala, and M. K. Nemanic (1973). Cell contacts in the mouse mammary gland. I. Normal gland in postnatal development and the secretory cycle. *J. Cell. Biol.* **56:**797–818.

22. S. E. Berga (1984). Electrical potentials and cell-to-cell dye movement in mouse mammary gland during lactation. *Am. J. Physiol.* **247:**C20–C25.

23. P. Monaghan, N. Perusinghe, G. Carlile, and W. H. Evans (1994). Rapid modulation of gap junction expression in mouse mammary gland during pregnancy, lactation, and involution. *J. Histochem. Cytochem.* **42**(7)**:**931–938.

24. A. Pozzi, B. Risek, D. T. Kiang, N. B. Gilula, and N. M. Kumar (1995). Analysis of multiple gap junction gene products in the rodent and human mammary gland. *Exp. Cell. Res.* **220**(1)**:**212–219.

25. E. M. Perez-Armendariz, J. Luna, C. Aceves, and D. Tapia (1995). Connexins 26, 32 and 43 are expressed in virgin, pregnant and lactating mammary gland. *Dev. Growth. Differ.* **37:**421–431.

26. I. Yamanaka, A. Kuraoka, T. Inai, T. Ishibashi, and Y. Shibata (2001). Differential expression of major gap junction proteins, connexins 26 and 32, in rat mammary glands during pregnancy and lactation. *Histochem. Cell. Biol.* **115**(4)**:**277–284.

27. J. Lautermann, W. J. ten Cate, P. Altenhoff, R. Grummer, O. Traub, H. Frank, *et al.* (1998). Expression of the gap-junction connexins 26 and 30 in the rat cochlea. *Cell Tissue Res.* **294**(3)**:**415–420.

28. J. I. Nagy, D. Patel, P. Y. Ochalski, and G. L. Stelmack (1999). Connexin30 in rodent, cat and human brain: Selective expression in gray matter astrocytes, co-localization with connexin43 at gap junctions and late developmental appearance. *Neuroscience* **88:**447–468.

29. L. S. Musil, B. A. Cunningham, G. M. Edelman, and D. A. Goodenough (1990). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell. Biol.* **111** (5 Pt 1)**:**2077–2088.

30. M. El-Sabban, A. Sfeir, M. Daher, N. Kalaany, R. Bassam, and R. Talhouk (2003). Gap junctional communication induces $\beta$-casein expression in mammary epithelial cells in the absence of cell/ECM interaction. *J. Cell. Sci.* **116:**3531–3541.

31. I. Yamanaka, A. Kuraoka, T. Inai, T. Jshibashi, and Y. Shibata (1997). Changes in the phosphorylation states of connexin43 in myoepithelial cells of lactating rat mammary glands. *Eur. J. Cell. Biol.* **72:**166–173.

32. M. Sibilia and E. F. Wagner (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* **269**(5221)**:**234–238.

33. S. J. Weber-Hall, D. J. Phippard, C. C. Niemeyer, and T. C. Dale (1994). Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland. *Differentiation* **57**(3)**:**205–214.

34. K. K. Wilgenbus, C. J. Kirkpatrick, R. Knuechel, K. Willecke, and O. Traub (1992). Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues. *Int. J. Cancer* **51:**522–529.

35. S. W. Lee, C. Tomasetto, D. Paul, K. Keyomarsi, and R. Sager (1992). Transcriptional down regulation of gap junction proteins blocks junctional communication in human mammary tumor cell lines. *J. Cell Biol.* **118:**1213–1221.

36. C. Tomasetto, M. J. Neveu, J. Daley, P. K. Horan, and R. Sager (1993). Specificity of gap junction communication among human mammary cells and connexin transfectants in culture. *J. Cell Biol.* **122:**157–167.

37. J. E. Trosko and C. C. Chang (2001). Mechanism of up-regulated gap junctional intercellular communication during chemoprevention and chemotherapy of cancer. *Mutat. Res.* **480–481:**219–229.

38. S. Jamieson, J. J. Going, R. D'Arcy, and W. D. George (1998). Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours. *J. Pathol.* **184:**37–43.

39. M. J. Sawey, M. H. Goldschmidt, B. Risek, N. B. Gilula, and C. W. Lo (1996). Perturbation in connexin 43 and connexin 26 gap-junction expression in mouse skin hyperplasia and neoplasia. *Mol. Carcinog.* **17**(2)**:**49–61.

40. M. E. El-Sabban and B. U. Pauli (1991). Cytoplasmic dye transfer between metastatic tumor cells and vascular endothelium. *J. Cell Biol.* **115:**1375–1382.

41. M. E. El-Sabban and B. U. Pauli (1994). Adhesion-mediated gap junctional communication between lung-metastatatic cancer cells and endothelium. Invasion Metastasis **4:**164–176.

42. A. G. Reaume, P. A. de Sousa, S. Kulkarni, B. L. Langille, D. Zhu, T. C. Davies, *et al.* (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science* **267**(5205)**:**1831–1834.

43. H. D. Gabriel, D. Jung, C. Butzler, A. Temme, O. Traub, E. Winterhager, *et al.* (1998). Transplacental uptake of glucose is decreased in embryonic lethal connexin 26-deficient mice. *J. Cell Biol.* **140:**1453–1461.

44. B. Teubner, V. Michel, J. Pesch, J. Lautermann, M. Cohen-Salmon, G. Sohl, *et al.* (2003). Connexin30 (Gjb6)-deficiency causes severe hearing impairment and lack of endocochlear potential. *Hum. Mol. Genet.* **12**(1)**:**13–21.

45. K. Oguro, T. Jover, H. Tanaka, Y. Lin, T. Kojima, and N. Oguro (2001). Global ischemia-induced increases in the gap junctional proteins connexin 32 (Cx32) and Cx36 in hippocampus and enhanced vulnerability of Cx32 knock-out mice. *J. Neurosci.* **21:**7534–7542.

46. R. Bruzzone, T. W. White, and D. L. Paul (1996). Connections with connexins: The molecular basis of direct intercellular signaling. *Eur. J. Biochem.* **238:**1–27.

47. J. T. Emerman and D. R. Pitelka (1977). Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In Vitro* **13:**316–328.

48. M. J. Bissell, H. G. Hall, and G. Parry (1982). How does the extracellular matrix direct gene expression? *J. Theor. Biol.* **99:**31–68.

49. C. D. Roskelley, A. Srebrow, and M. J. Bissell (1995). A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. *Curr. Opin. Cell. Biol.* **5:**736–747.

50. C. H. Streuli, N. Bailey, and M. J. Bissell (1991). Control of mammary epithelial differentiation: Basement membrane induces tissue-specific gene expression in the absence of cell–cell interaction and morphological polarity. *J. Cell Biol.* **115:**1383–1395.

51. R. R. Shivers, F. E. Arthur, and P. D. Bowman (1988). Induction of gap junctions and brain endothelium-like tight junctions in cultured bovine endothelial cells: Local control of cell specialization. *J. Submicrosc. Cytol. Pathol.* **20:**1–14.

52. L. A. Alves, O. K. Nihei, P. C. Fonseca, A. C. Carvalho, and W. Savino (2000). Gap junction modulation by extracellular signaling molecules: The thymus model. *Braz. J. Med. Biol. Res.* **33**(4)**:**457–465.

53. P. D. Lampe, B. P. Nguyen, S. Gil, M. Usui, J. Olerud, and Y. Takada (1998). Cellular interaction of integrin alpha3beta1with laminin 5 promotes gap junctional communication. *J. Cell Biol.* **143**(6)**:**1735–1747.

54. Y. Guo, C. Martinez-Williams, C. E. Yellowley, H. J. Donahue, and D. E. Rannels (2001). Connexin expression by alveolar epithelial cells is regulated by the ECM. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* **280**(2)**:**L191–L202.

55. A. I. Alford and D. E. Rannels (2001). Extracellular matrix fibronectin alters connexin43 expression by alveolar epithelial cells. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* **280**(4)**:**L680–L688.

56. P. C. Schiller, G. D'Ippolito, W. Balkan, B. A. Roos, and G. A. Howard (2001). Gap-junctional communication mediates parathyroid hormone stimulation of mineralization in osteoblastic cultures. *Bone* **28**(1)**:**38–44.

57. K. Miyoshi, J. M. Shillingford, G. H. Smith, S. L. Grimm, K. U. Wagner, T. Oka, *et al.* (2001). Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. *J. Cell Biol.* **155**(4)**:**531–542.

58. R. Talhouk, F. Maa'ni, N. Kalaa'ni, G. S. Zoubian, C. J. Simaa'n, M. Abi-Sai'd, *et al.* (2001). Partial purification and characterization of growth factors involved in ovine mammary development. *Domest. Anim. Endocrinol.* **3:**143–159.

59. J. G. Laing, R. N. Manley-Markowski, M. Koval, R. Civitelli, and T. H. Steinberg (2001). Connexin45 interacts with zonula occludens-1 in osteoblastic cells. *Cell Adhes. Commun.* **8**(4–6)**:**209–212.

60. L. L. Mitic, E. E. Schneeberger, A. S. Fanning, and J. M. Anderson (1999). Connexin-occludin chimeras containing the ZO-binding domain of occludin localize at MDCK tight junctions and NRK cell contacts. *J. Cell Biol.* **146**(3)**:**683–693.

61. T. Toyofuku, M. Yabuki, K. Otsu, T. Kuzuya, M. Hori, and M. Tada (1998). Intercellular calcium signaling via gap junction in connexin-43-transfected cells. *J. Biol. Chem.* **273**(3)**:**1519–1528.

62. B. N. Giepmans and W. H. Moolenaar (1998). The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. *Curr. Biol.* **8**(16)**:**931–934.

63. T. Toyofuku, Y. Akamatsu, H. Zhang, T. Kuzuya, M. Tada, and M. Hori (2001). C-Src regulates the interaction between connexin 43 and ZO1 in cardiac myocytes. *J. Biol. Chem.* **276:**1780–1788.

64. P. J. Kausalya, M. Reichert, and W. Hunziker (2001). Connexin45 directly binds to ZO-1 and localizes to the tight junction region in epithelial MDCK cells. *FEBS Lett.* **505**(1)**:**92–96.

65. T. Kojima, Y. Kokai, H. Chiba, M. Yamamoto, Y. Mochizuki, and N. Sawada (2001). Cx32 but not Cx26 is associated with tight junctions in primary cultures of rat hepatocytes. *Exp. Cell Res.* **263**(2)**:**193–201.

66. T. Kojima, D. C. Spray, Y. Kokai, H. Chiba, Y. Mochizuki, and N. Sawada (2002). Cx32 formation and/or Cx32-mediated intercellular communication induces expression and function of tight junctions in hepatocytic cell line. *Exp. Cell Res.* **276:**40–51.

67. A. Nusrat, J. Chen, C. Foley, T. Liang, J. Tom, M. Cromwell, *et al.* (2000). The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. *J. Biol. Chem.* **275**(38)**:**29816–29822.

68. L. Zhou, E. M. Kasperek, and B. J. Nicholson (1999). Dissection of the molecular basis of pp. 60(v-src) induced gating of connexin 43 gap junction channels. *J. Cell Biol.* **144**(5)**:**1033–1045.

69. K. I. Swenson, H. Piwnica-Worms, H. McNamee, and D. L. Paul (1990). Tyrosine phosphorylation of the gap junction protein connexin43 is required for the pp60v-src-induced inhibition of communication. *Cell Regul.* **1**(13)**:**989–1002.

70. L. Loo, J. Berestecky, M. Kanemitsu, and A. Lau (1995). pp60src-Mediated phosphorylation of connexin 43, a gap junction protein. *J. Biol. Chem.* **270**(21)**:**12751–12761.

71. M. Kanemitsu, L. Loo, S. Simon, A. Lau, and W. Eckhart (1997). Tyrosine phosphorylation of connexin 43 by v-Src is mediated by SH2 and SH3 domain interactions. *J. Biol. Chem.* **272**(36)**:**22824–22831.

72. E. Y. Lin, A. V. Nguyen, R. G. Russell, and J. W. Pollard (2001). Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J. Exp. Med.* **193**(6)**:**727–740.

73. B. N. Giepmans, T. Hengeveld, F. R. Postma, and W. H. Moolenaar (2001a). Interaction of c-Src with gap junction protein connexin-43. Role in the regulation of cell–cell communication. *J. Biol. Chem.* **276**(11)**:**8544–8549.

74. B. N. Giepmans, I. Verlaan, T. Hengeveld, H. Janssen, J. Calafat, M. M. Falk, *et al.* (2001b). Gap junction protein connexin-43 interacts directly with microtubules. *Curr. Biol.* **11:**1364–1368.

75. Z. Ai, A. Fischer, D. Spray, A. Brown, and G. Fishman (2000). Wnt-1 regulation of Connexin 43 in cardiac myocytes. *J. Clin. Invest.* **105**(2)**:**161–171.

76. X. Xu, W. Li, G. Huang, R. Meyer, T. Chen, Y. Luo, *et al.* (2002). Modulation of mouse neural crest cell motility by N-cadherin and connexin 43 gap junctions. *J. Cell Biol.* **154**(1)**:**217–229.

77. C. Theiss and K. Meller (2002). Microinjected anti-actin antibodies decrease gap junctional intercellular commmunication in cultured astrocytes. *Exp. Cell Res.* **281**(2)**:**197–204.