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Asymmetric Expression/ Distribution of Connexins is Essential for Contractile Function in the Mammary Gland

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Asymmetric Expression/ Distribution of Connexins is Essential for Contractile Function in the Mammary Gland

By

Rana Mustapha Mroue

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Comparative Biochemistry

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Mina J. Bissell (co-chair)
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Professor George Sensabaugh
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Abstract

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Doctor of Philosophy in Comparative Biochemistry

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Intercellular communication is essential for glandular functions and homeostasis. In the mammary gland, heterotypic interactions between the luminal epithelial cells specialized for milk production and the underlying contractile myoepithelial cells (myoeps) are necessary for the ejection of milk from the alveoli through the ducts and into the nipple at parturition. Recently, the role of the myoepithelial compartment in the development and differentiation of the mammary tissue has gained increasing attention. A growing body of evidence suggests that perturbation of the normal expression pattern of molecules specific to the myoepithelium alters the growth and differentiation of the entire mammary tissue. This is not surprising, since the mammary myoeps, with their location in the gland, are able to integrate multiple signals from neighboring cells and the underlying basement membrane (BM), and in turn, relay these signals to the luminal compartment to control cell growth, differentiation, and to maintain mammary architecture. Thus, studying the signaling pathways and interactions between the luminal and myoepithelial compartments is of major importance for understanding normal mammary development and function. This is highlighted by the important finding from the Bissell laboratory showing that myoeps mediate the polarity of the acinar structures in three-dimension by the production of the BM protein Laminin111, concomitant with another finding from Runswick et al., that desmosomal cadherins between the luminal epithelial cells and myoeps play an essential role in cellular positioning and tissue morphogenesis.

Gap junctions couple cells homotypically and heterotypically and coordinate reciprocal responses between the different cell types. Connexins (Cxs) are the main mammalian gap junction proteins, and their distribution in the heterotypic gap junctions is not always symmetrical; in the mammary gland, Cx26, Cx30 and Cx32 are expressed in the luminal epithelial cells and Cx43 in myoepithelial cells. Expression of all four Cxs peaks during late pregnancy until late lactation
suggesting essential roles for these proteins in functional differentiation of the gland.

In this study, I addressed the pertinent question of how altering cell-specific expression of junctional proteins leads to defective tissue architecture and function. In particular, I investigated the importance of connexin-mediated intercellular interactions, and of cell-type specific expression of connexins in maintaining mammary function. To that end, I used a transgenic mouse where the luminal Cx26 is expressed under the regulation of a Keratin-5 promoter, thus targeting the expression of the protein to the myoep compartmenl otherwise lacking Cx26. K5-Cx26 dams have an unexpected mammary phenotype; transgenic dams are unable to feed their pups to weaning age, leading to litter starvation and demise in early to mid-lactation. I thus asked why ectopic expression of the luminal Cx26 to mammary myoepithelial cells impairs mammary function, and by what mechanism. The mammary glands of K5-Cx26 female mice develop normally but pups are unable to receive milk. This is despite the normal levels of beta casein and whey acidic protein present in the mammary glands of transgenic mice, and suggesting a defect in delivery rather than milk production. Primary mammary organoids, isolated from wild-type FVB females, contracted in culture upon treatment with oxytocin; however, primary mammary organoids from the transgenic mice failed to respond to oxytocin. Interestingly, I found that ectopic expression of Cx26 to myoepithelial cells alters the expression of endogenous Cx43 and inhibits gap junction-mediated dye coupling in myoepithelial cells expressing high levels of Cx26. Inhibition of gap junctional communication or knock-down of Cx43 in wild-type organoids similarly impairs contraction in response to oxytocin in culture, and suggests that the contractile defect in K5-Cx26 dams is caused by Cx26 acting as a dominant negative to Cx43 function in the mammary myoepithelial cells. This is the first report of a trans-dominant-negative effect of ectopic Cx26 on Cx43 expression in vivo and highlights the importance of tissue- and cell-type- specific expression of Cxs for normal mammary gland development and function.
Dedication

This Dissertation is dedicated to my Parents, Leila Serhan and Mustapha Mroue. In addition to showering us with unconditional love, they endured and overcame extreme circumstances to ensure that my sisters and I receive the best education and opportunities in life. They instilled in us a sense of responsibility and duty and taught us how to face adversities with courage and determination. Most importantly, they infused us with love and respect for life.

It is only thanks to them that I have been able to pursue my passions.
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Chapter 1. Introduction
1.1 Overview: Mammary Gland

The mammary gland is an ideal ‘model organism’ for studying tissue-specificity and gene expression in mammals: it is one of the few organs that develop after birth and it undergoes multiple cycles of growth, differentiation and regression during the animal’s lifetime in preparation for the important and unique function of lactation. The basic milk-making ‘functional unit’ in the gland is the mammary acinus, made up of a layer of polarized luminal epithelial cells (LEPs) specialized for milk production, surrounded by myoepithelial contractile cells (myoeps), and the two-layered structure is surrounded by a basement membrane (BM). The mammary acinar unit of function is part of a large epithelial ductal tree-like network and lies within a fat pad rich with adipose cells and stromal components including a variety of cell-types (Schematic. 1). Mammary gland post-natal development occurs in two phases: ductal growth and early alveolar development during puberty, and alveolar growth, expansion and differentiation, which occur with each pregnancy and lactation cycle. Lactation is followed by a period of post-lactational remodeling termed involution, during which, the gland regresses to a pre-pregnant like state in preparation for another cycle of pregnancy and lactation (1). Much knowledge about the regulation of mammary gland development has been acquired from studying the physiology of the gland and of lactation in rodents. Despite differences between the mouse mammary gland and the human breast, the acinar unit of function is largely similar in the two species, making it a suitable surrogate for understanding human breast function.

In the mouse, there are five pairs of mammary glands located below the skin and extending from the thoracic side (three pairs) to the inguinal side of the mouse (two pairs). Each mammary fat pad has an exterior nipple which connects the ductal network of the gland to the outside to allow the release of milk during lactation. There is a gradient of differentiation across the glands with the inguinal glands being the most differentiated (2). Embryonically, the mammary gland exists as an epithelial ductal rudiment, termed anlage, which originates from the parenchyma. The mammary anlage is quiescent until about 3 weeks of age, at which time it becomes stimulated by ovarian hormones, which enable the invasion of the epithelial rudiment into the fat pad (3). Around this time, a bulbous cluster of epithelial cells termed terminal end buds (TEB) appears at the end of the epithelial ducts; TEBs drive the process of ductal elongation and invasion of the epithelium into the fat pad until about 10-12 weeks of age, when they reach the end of the fat pad and regress. Estrogen guides ductal elongation into the fat pad, while progesterone allows the formation of side branches. During puberty, extensive proliferation and invasion of the epithelial cells results in an arboreal-like structure of epithelial ducts and side branches decorated by small alveolar buds. After the formation of the epithelial tree, the gland becomes relatively quiescent until pregnancy, although minimal stages of alveolar growth and recession occur with each estrous cycle. After conception, and under the action of pregnancy hormones, including progesterone, prolactin and placental...
lactogen, alveolar expansion and differentiation are initiated to prepare the gland for the important function of lactation. By late pregnancy, adipose cells regress to occupy around 30% of the gland making way for the alveoli to become densely packed as they attain their differential ability to synthesize specific milk constituents. At parturition, functional lactogenesis is realized; a dramatic fall in progesterone levels and the presence of prolactin drive the production and secretion of copious amounts of milk proteins by the alveoli for about 3 weeks. At weaning, involution starts and the gland begins a process of tissue remodeling which involves orchestrated apoptosis of mammary epithelial cells, such that the gland regresses to a state somewhat similar, but not identical, to the pre-pregnant gland architecture (4-7). The process of involution takes approximately 2 weeks to complete in rodents, after which the gland is ready to initiate another cycle of pregnancy, lactation, and involution (Schematic. 2).
Schematic. 1. Mammary Unit of Function: Acinus/Alveolus. The mammary acinus or alveolus is a bilayer of luminal epithelial cells (light brown) surrounded by myoepithelial cells (dark brown) and the whole structure is surrounded by a basement membrane (purple) and stromal cells (adipocytes in white and fibroblasts in yellow)
Schematic. 2. Mouse Mammary Glands and Stages of Development (A) representative drawing of the mouse mammary glands: the thoracic pairs (1,2,3) and the inguinal pairs (4,5) are indicated. (B) Representative drawings of the stages of post-natal development of the mammary gland.
1.2 Control of Lactation in the Mammary Gland

Complete and successful lactation depends on multiple factors including structural and functional differentiation of the alveolar units, correct endocrine signaling to prepare, trigger and maintain the production and secretion of milk by the alveoli, and successful removal of milk to ensure that milk making capacity is maintained throughout the lactation period.

Structural and functional differentiation of the alveolar units in the mammary gland occurs by the end of pregnancy. At this time, polarized LEPs become sparsely connected to their myoepithelial counterparts, such that they are in direct contact with the BM on their basal side. The BM signals via a variety of proteins, namely laminins and collagen, to maintain the structural organization and the functional differentiation of the milk-making LEPs. At the apical end of LEPs, tight junctions become impermeable and form a barrier that prevents the leakage of solutes from the cells into the interstitial space. Prolactin and glucocorticoids drive tight junction sealing by late pregnancy and maintain tight junctions in that state after parturition (8-12). At parturition, a fall in the levels of progesterone is essential to initiate lactogenesis, whereas the presence of prolactin, insulin and cortisol is required for maintaining lactation (12-15). Recent evidence suggests that once initiated, and in the presence of the lactogenic hormones, the most important factor for successful lactation is the removal of milk by the suckling pups (16). If milk is not removed, milk accumulation and stasis causes distention of the alveolar lumen and increased pressure on the epithelium. The increase in pressure activates a mechanosensing mechanism in the epithelium which relays a signal to drive the involution process. At this time, tight junctions become leaky, and the intercellular adhesion complexes between luminal cells start to remodel. Concomitantly the signal transducer and activator of transcription 3 (STAT3) is up-regulated and phosphorylated, which initiates and drives programmed cell death in the epithelial compartment (17, 18).

1.3 The role of Myoepithelial Cells in Mammary Gland Development and Lactation

Myoeps in the mammary tissue are positioned at the boundary between the milk-making LEPs and the extracellular milieu, and mediate the reciprocal interactions between the epithelium and the connective tissue around it. In addition, myoeps express various receptors for soluble and cell surface-associated signaling molecules and produce and secrete major components of the BM, namely laminins (19). Previously, myoeps were thought to be only important for mechanical functions in the gland, including structural support of the luminal cells, and contraction during lactation. However, recent evidence suggests that myoeps are essential regulators of mammary gland development and differentiation, and that the perturbation of the expression pattern of molecules specific to the myoepithelium alters the differentiation of the entire tissue.
Differentiated myoeps are contractile cells with smooth-muscle attributes; they contain large amounts of microfilaments and smooth-muscle-specific contractile proteins including smooth-muscle-alpha actin (a-SMA). However, unlike smooth muscle cells, they are ‘epithelial’ in nature and are derived from the ectoderm rather than the mesoderm. As such they also express proteins that are common to the basal cells from stratified epithelia. In particular, they express basal-type cytokeratins 5, 14 (K5 and K14, respectively), cytokeratin 17, P-cadherin and high levels of DNp63 (20-22).

Myoepithelial and luminal cells from the pseudostratified mammary epithelium are functionally and phenotypically distinct. Segregation of the two cell types into distinct compartments begins early during embryonic development. Most cells from the mammary buds of the mouse embryos at E15 express basal K5, and many cells stain positive for both, K5 and luminal cytokeratin 8 (K8). Only the expression of the basal cell marker p63 is restricted to the most basal cell layers. By E18, expression of K5 is higher in basal cells, and K8 displays the opposite pattern of expression. Few mammary basal cells from E18 mouse embryos express the smooth muscle-specific protein, a-SMA. In fact, the expression of smooth muscle-specific contractile and cytoskeletal proteins is acquired gradually by myoeps as the cells differentiate. When the myoeps mature, they turn on the expression of various contractile proteins and adhesion molecules, including integrins, and they start producing major extracellular matrix (ECM) and BM components, namely laminin (23).

The expression of smooth muscle markers and basal cytokeratins is spatio-temporally regulated in the mammary gland in post-natal development; ductal and alveolar myoepithelial cells at different stages of mammary development have distinct patterns of expression of these proteins. In addition, myoeps have different architectural organizations depending on their location within the gland; around the ducts they form a continuous layer of cells, whereas around the alveoli they form a basket like structure and assume a stellate shape (20). Ductal myoeps are derived from the cap cells at the basal end of the TEBs; these cells express a-SMA, P-Cadherin, and p63, but are weakly positive for K5 and K14. The heterogeneity in protein expression might reflect differences in the degree of differentiation of myoeps, and in the functional properties and characteristics of these cells (23).

One of the essential functions of myoeps is contraction in response to the pulsatile release of oxytocin during lactation. Oxytocin is released from the pituitary gland upon suckling and binds to its promiscuous G-protein 7-transmembrane pass receptor on the surface of the myoepithelial cell to drive the contractile activity of myoeps (24). Oxytocin receptor engagement activates a signaling cascade which culminates in the phosphorylation of myosin light chain 2 (MLC2), similarly to other types of smooth-muscle cells. Binding of oxytocin to its receptor triggers the release of Ca\(^{2+}\), which then binds to Calmodulin to activate Ca/CaM/MLCK signal cascade. Phosphorylation of the MLC2 by MLCK
induces myosin ATPase activity and results in the binding of myosin to actin which causes the initial contraction (25). Subsequently, MLC2 is dephosphorylated by a specific phosphatase (MLCP) leading to relaxation in preparation for a second contractile pulse. Recently, it has been shown that the RhoA/ROCK signaling cascade is essential for the oxytocin-induced contraction of myoeps (26). ROCK inhibition was found to completely prevent the contractile response of myoeps. Whereas MLCK directly phosphorylates MLC2, ROCK activation was found to be necessary to initially inhibit the activity of MLCP. The balance between the activity of MLCK and MLCP is important to sustain contractility; if MLCK is not activated, contraction does not occur but if MLCP is inhibited, MLC2 dephosphorylation is prevented and relaxation is impeded causing myoeps to be in a state of sustained contraction. It has been recently shown that activation of FAK/Rac/PAK pathway by α3β1 integrin signaling is required for inhibition of MLCK and dephosphorylation of MLC2 and relaxation. Cultured mammary myoeps depleted of α3β1 contract in response to oxytocin, but are unable to maintain the state of post-contractile relaxation. The expression of constitutively active Rac or its effector PAK, or treatment with MLCK inhibitor rescues the relaxation capacity of mutant cells, strongly suggesting that α3β1-mediated stimulation of the Rac/PAK pathway is required for the inhibition of MLCK activity, permitting completion of the myoep contraction/relaxation cycle and successful lactation (26).

Differentiation of myoeps into contraction-competent smooth-muscle like cells depends on the presence of serum response factor (SRF), a transcription factor that activates expression of smooth-muscle genes such as a-SMA and heavy chain myosins. The expression of SRF-dependent genes requires the presence of transcription co-activators that belong to the family of myocardin-transcriptional co-activators, the myocardin-related transcription factors Mkl1 and Mkl2 (27, 28). Recently, it has been shown that the mammary glands of MKL1-deficient mice develop normally, but the dams fail to feed their litters due to impaired contraction of the myoeps. In the absence of Mkl1, mammary myoeps contain only very low amounts a-SMA, myosin heavy chains, tropomyosin, transgelin, caldesmon and myosin light chain kinase. Mkl2 is upregulated in the tissue of MLK-1 deficient tissue, but fails to compensate for the loss of MKL1(27, 28). a-SMA is the most abundant actin isoform in mammary myoeps. A recent study showed that a-SMA was necessary for the generation of the contractile force required for milk ejection. Mice lacking a-SMA presented lactation failure, despite the normal development of their mammary glands and the ability to make milk proteins (29).

Myoeps are in direct contact with the BM and as such, they relay signals from the ECM to the epithelial compartment in the mammary gland. The BM is rich with laminins and collagen IV. Mammary myoeps express a number of integrin proteins that mediate the contact with the basement membrane components. Early immunohistochemical studies revealed the expression of various integrin dimers,
including collagen receptors α1β1, α2β1 and fibronectin receptor α5β1, αvβ3 integrin and high levels of laminin receptors α3β1, α6β1 and α6β4, in rodent and human mammary myoep (30-33). Mice lacking the α1 or α2 integrin chains are viable, but ablation of the α2 integrin gene results in a slight decrease in mammary ductal branching in virgin mice. A lack of α3β1 or α6β4 integrins leads to perinatal lethality. Mammary epithelium deficient for α3 or α6 integrin chains (i.e. depleted of α3β1 or α6β1 and α6β4 integrin dimers), when transplanted into cleared mouse mammary fat pads, produced ducts and alveoli similar to those developed from control tissue, suggesting that these integrins are dispensable for mammary morphogenesis. One possible explanation for these results is the functional redundancy of the α3β1, α6β1 and α6β4 integrins.

Like other epithelial cells, mammary basal myoep form junctional complexes, including desmosomes and adherens junctions, between them and with luminal cells. Of note, numerous cell-cell-adhesion molecules are expressed differentially in myoep and LEPs of the mammary epithelium. For example, desmosomal cadherin subtypes, desmocollin 3 and desmoglein 3 are restricted to the myoepithelium. Runswick and coworkers provided evidence that desmosomes play an important role in the establishment and maintenance of the bilayer organization (34, 35), and that perturbation of the cell-cell interactions involving these myoep-specific molecules interfere with the cell type-specific positioning of luminal and basal mammary epithelial cells.

### 1.4 Overview: Gap Junctions

Gap Junctions (GJs) are intercellular channels that allow the diffusion of ions and small molecules (up to 1 kDa in size) between the cytoplasms of the neighboring cells (36, 37). Most cells of the normal tissues, except skeletal muscle cells, erythrocytes and circulating lymphocytes, generally communicate via these junctions. GJs are specialized regions of the cell membrane formed by the docking of two hemi-channels termed connexons from neighboring cells to form a channel with a narrow 2-3nm gap. The hemichannels are composed of connexins (Cx), highly related transmembrane proteins consisting of at least 13 members encoded by 20 distinct genes in the mouse genome and 21 genes in the human genome (38, 39). All Cxs share a similar structural topology with four hydrophobic membrane-spanning regions (M1-M4), two extracellular loops (E1 and E2) mediating the interactions between two apposing connexons, and one cytoplasmic loop. The carboxy and amino-terminal domains are both located on the cytosolic side (CT and NT respectively). Whereas the Cx N-terminus, the extracellular loops, and the transmembrane are highly conserved, the cytoplasmic loop and the C-terminus are highly divergent. These sequence differences are likely to be responsible for many of the Cx-specific functional properties, including sensitivity to different stimuli, second messenger molecules, and recruitment of other associated proteins with the junctional complexes (40,
Most Cx genes share a common structure: a first exon which comprises the 5’ untranslated region (5’-UTR) followed by an intron with variable length and a second exon containing the remaining 5’-UTR, the coding sequence and the 3’-UTR (42).

Cxs display organ, tissue and cell type specific, but overlapping patterns of expression. Hemichannels may assume various configurations; they may be homomeric, comprising six identical subunits, or heteromeric, comprising more than one isoforms of Cxs. Channels are described as 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 GJs (43).

The formation of GJs involves a stepwise series of assembly processes. Cxs are inserted into the endoplasmic reticulum (ER) membrane where they must fold correctly prior to oligomerization into the connexon hexameric channels. This event occurs as they traffic from the ER to the Golgi via intermediate compartments. Cxs delivered to the plasma membrane then align and dock with partners in neighboring cells to generate the gap-junction channel. However, it is thought that an assembly signal regulating principal Cx subunit recognition may be located in the C-terminal portion (preferentially third transmembrane domain) of the Cx peptide, while a selectivity signal regulating specific assembly of heterotypic connexons is located in the amino terminal portion (NH2-terminal, first transmembrane or first extracellular domain) of the Cx polypeptide sequence (44-48) (Schematic. 3).

The principal process leading to assembly of GJs involves the co-translational insertion of Cx proteins into the endoplasmic reticulum, followed by their rapid oligomeric association into homo- or heteromeric connexons that are trafficked via the Golgi apparatus to the plasma membrane. Oligomerisation is a high-fidelity process that determines connexon channel stoichiometry and conductance characteristics. A large number of mutations in Cx26 and Cx32 detected in genetic diseases have emphasized the requirement for precise oligomerisation of Cxs into hexameric connexons that traffic to the plasma membrane. Mutations in Cx43 are rare, and in the cardiovascular system, where it is the dominant Cx, disease changes are linked to its abundance and to GJ remodeling. Cxs with short carboxyl tails may also be post-translationally inserted as oligomeric channels directly into plasma membranes. This mechanism of channel assembly is highly dependent on microtubule integrity and may allow cells to rapidly modulate Gap junctional cross talk (46, 49-52).

Previous findings excluded cadherins and catenins from the GJ plaques, but reports indicated that the cadherin-catenin cell adhesion system may be involved in the formation of GJs (53). In fact, cell adhesion molecules facilitate the formation of GJs by establishing and stabilizing small GJ contact spots; they narrow the gap between the membranes and they protect newly formed channels
from membrane ruffling. Several reports have confirmed that cell adhesion molecules can affect GJ formation, for example cell lines deficient in cadherins showed diminished Gap junctional communication, corrected by the transfection of the appropriate adhesion molecule (54, 55). However, it is important to note that GJs can in turn regulate adhesion molecules (56).

**Schematic. 3. Connexin to Connexon to GJ.** Representative drawing of the structure of a Cx protein with the N- and C- termini indicated and the cytoplasmic and extracellular loops shown. 6 Cxs oligomerize into a homomeric or a heteromeric connexons, and two connexons join to form homotypic or heterotypic channels.
Most cells communicate with their immediate neighbors through the exchange of cytosolic molecules such as ions, second messengers and small metabolites via GJs. Cx channels participate in the regulation of signaling between developing and differentiated cell types by a variety of mechanisms. In fact, unique ionic- and size-selectivities are determined by Cx identities within GJ channels; moreover, the establishment of intercellular communication is defined by the expression of compatible Cxs. Different signals mediate opening and closing of the channels depending on cellular needs. In fact, intercellular communication through GJs is regulated by different gating mechanisms such as calcium concentration, pH, transjunctional membrane potential and protein phosphorylation. It has been shown that GJs close in response to high concentration of calcium ion, which suggests that calcium-dependent cellular processes and events might be regulating Gap Junctional functionality. Moreover, GJ gating was shown to be modulated by intracellular pH (57-63). In fact, regulatory sites that respond to pH levels within GJs were found in the intracellular loop and CT domains of the Cx proteins, a region which shows little sequence homology between different Cxs. Hence, differential composition of the GJ channels is an important determinant of the response to pH in different cells and tissues. GJ conductance and permeability were also shown to respond to transmembrane voltages whereby large transjunctional voltages were shown to close the channels (57, 64, 65). This tight regulation mechanism suggests that GJs are not simply aqueous channels but that they participate in coordinated signaling events between neighboring cells which influence cellular development and differentiation.

GJ intercellular communication is essential at different stages of cell, tissue and organism development. At the most basic level, GJs perform a highly specialized and unique role in the organism: they provide direct, yet selective, intercellular communication routes that allow the propagation of coordinated responses across cells, such that tissues are linked as a unit, electrically and metabolically (66). Electrical coupling via GJs is essential for synchronous contraction of cardiomyocytes and the rhythmic pumping of heart, uterine smooth muscle contraction during labor and the unidirectional beating of tracheal cilia to expel fluids from the airway passages. (67-72). In non-excitable tissues, gap junction intercellular communication (GJIC) has been shown to promote embryonic growth, bone modeling, alveolar differentiation and mammary epithelial differentiation among others (73). Many studies highlighted the role of GJIC in promoting differentiation. For example, it was found that restoration of GJIC in colon cancer cells leads to re-establishment of a differentiation phenotype (74). In another study using mouse mammary epithelial cells it was shown that enhanced GJIC induces partial differentiation of mammary epithelial cells in the absence of an exogenously provided BM (75). GJ mediated communication can exist between different cell types. Homocellular GJs are assembled between cells of similar phenotype and heterocellular GJs form between cells of different types. Many reports have characterized heterocellular GJs in vivo and in culture, and attributed important regulatory roles for heterocellular GJIC. For example, a
study revealed an important role of heterocellular gap junctional communication between Type I and Type II epithelial alveolar cells to promote epithelial differentiation in the rat lung (76). Previous studies have also reported the importance of heterocellular GJIC between cardiac myocytes and surrounding fibroblasts (77, 78), macrophages and intestinal cells (79). Heterocellular GJs have been reported in various tissues between different cell types. In fact, previous studies showed that heterocellular GJs exist between neuronal and glial cells in the nervous system and that heterocellular GJIC between the two cell types is important for their function and for information processing (79). In addition to the above, hetero-cellular GJIC was recently found to be important for interaction between grafted and host cells in tissue grafts. In one study, the interaction via GJs of grafted neural stem cells and host cells was found to facilitate the integration of the grafted cells into the neural circuitry of the host (80). In other studies the importance of gap junctional coupling was highlighted in cell transplantation after myocardial injury, where engrafted skeletal muscle cells over-expressing Cx43 were found to establish better communication with host cardiomyocytes and this interaction favored their integration and differentiation in the host tissue (77).

Unapposed hemi-channels, termed connexons have been described to occur independently in multiple cell types and in cultured cells including embryonic stem cells, where they act as paracrine conduits that spread signals to surrounding cells. Cx hemi-channels have been shown to mediate the release of ATP, glutamate, NAD+ and prostaglandin E2, from cells (81). ATP acts on purinergic receptors on adjacent cells and activates intercellular Ca2+ release possibly to complement the Ca2+ release signals occurring more directly via GJs. In addition, hemichannels are involved in the movement of NAD+ into and out of cells, reversibly, which may regulate Ca2+ concentrations through the CD38 transmembrane glycoprotein (82). Hemichannels have also been shown to exist in heart ventricular myocytes, where they have an osmo-regulatory role, with both negative and positive potential impacts with respect to myocardial infarcts and cardiac physiology (83-85). Furthermore, hemichannels, similarly to GJs, play a role in spreading cell survival and cell death signals such as during ischemic injury. For example, it was reported that Cx43 hemichannels enhanced and accelerated cell death following ouabain drug treatment (86). In contrast, Cx43 hemichannels have been shown to play a role in the transduction of survival signals. Osteocytes and osteoblasts treated with bisphosphonates activated Src Kinase and ERK by the hemichannel pore activity and the C-terminal domain of Cx43 led to ERK activation and attenuation of osteoblast cell death (87).

It is now uncontested that Cxs have channel-independent roles (55, 88). Cxs have been shown to regulate gene expression partly via Cx-responsive elements (CxRE), where Cxs and GJIC induce differential recruitment of sp1 and sp3 transcription factors to the CxRE through the ERK/PI3K pathway and regulates CxRE-mediated gene expression. Cxs can also regulate cell differentiation in a GJIC-independent manner (89, 90). It has been shown that the C-terminal domain of Cx45.6 stimulates lens cell differentiation and that Cx43, through its
C-terminal tail is crucial for preventing premature neuronal differentiation during embryonic brain development (41, 91). Cxs have been also localized to the nucleus and Cx43 has been shown to contain a putative nuclear targeting sequence in its C-terminal domain. In support of this observation, both full length Cx43 or its C-terminus by itself have been localized to the nucleus, where they inhibit cell growth (92). The precise function and mechanism of action of Cxs in the nucleus require further study.

Cxs can also regulate signaling pathways, notably via the engagement of Cx-associated proteins en route to, or at, the membrane as part of the GJ complex. We don’t know as yet whether these interactions are important for GJ channel function or whether they are just involved in regulating Cx targeting to the GJ, however, much evidence points to the assembly of Cx-interacting proteins at the GJ interface such that a GJ signaling complex is formed.

Most strategies to study the mechanisms of Cx-interactions have made use of culture systems due to the difficulty of probing interactions in real time in vivo. This is further complicated by the transient nature of these interactions which accommodate the highly dynamic life cycle of Cxs. Using real time imaging and fluorescently labeled Cx-fusion proteins, many studies were able to gage the interaction of Cxs with their trafficking modulators such as the interaction between onnexin and microtubules (51). Other studies showed that Cx43 governs directional neural crest cell migration (93), while siRNA knockdown of Cx43 reduced the cell-surface distribution of N-cadherin, suggesting that Cx43 regulates the status of N-cadherin and possibly cell adhesion in a cadherin-dependent mechanism (94). Interestingly, binding of β-catenin to cadherins (E- or N-) (95, 96) is essential for down regulation of TCF/LEF-gene expression and inhibition of cell proliferation; thus, Cx43, along with cadherins seems to be important for down regulation of β-catenin-dependent signaling and growth inhibition. This was also shown in a study using HEK293 cells (97). Moreover, binding of β-catenin to Cx43 at cell–cell contact areas in cardiac myocytes was also shown to be important for down-regulation of β-catenin-dependent gene transactivation (98).

1.5 Gap Junctions in the Mammary Gland

The role of the microenvironment in governing mammary phenotype and differentiation has long been recognized. The term “dynamic reciprocity” (99, 100) was coined for the concept of dynamic interchange of signals from the tissue microenvironment to the nucleus for gene expression, and much effort since then has emphasized the importance of cell/ECM interactions in modulating the differentiation and function of the mammary epithelial cell. Initially, the work of Emmerman and Pitelka in 1977 (101) highlighted the critical role of cell–cell interactions, in regulating differentiation of mammary cells. The majority of studies have suggested that cell–cell interactions enhance mammary cell differentiation, but in a matrix-dependent manner (102). However, a study by Streuli et al. (1991) demonstrated that production of the milk protein, β-casein,
occurs in isolated single mammary epithelial cells, but is synergistically elevated upon cell–cell interaction (103). Much evidence suggests that among intercellular interactions, direct cell-cell communication mediated by GJs, the “modulators of cellular differentiation”, is an important determinant of mammary function (75).

GJs were first described in the mammary gland in the work of Pitelka (1973) following visualization of the channels with freeze fracture and electron microscopy (104). Four Cx’s have since been identified in the rodent mammary epithelium; Cx26, Cx30, and Cx32 are expressed in the luminal epithelial cells whereas Cx43 is the only isoform expressed in the myoepithelium (105-107). The expression of all four subtypes in the mammary epithelium is temporally regulated and highest during late pregnancy and lactation, suggesting that Cxs are important for functional differentiation of the gland. Cx26 and Cx32 are the dominant luminal isoforms expressed in the lactating mouse mammary epithelium; Cx26 levels are low in the mammary glands of nulliparous mice but levels increase as of early pregnancy and peak at parturition and lactation, and then decline during involution (108-111). Freeze-fracture and differential centrifugation studies showed that Cx32 and Cx26 can organize as either homomeric or heteromeric connexons and localize to the same junctional plaques. The stoichiometry of their association is regulated during lactation such that Cx32-Cx32 GJs replace Cx32-Cx26 GJs as the latter are sensitive to, and inhibited by the accumulation of taurine during this stage (107, 112, 113). Interestingly, the myoep Cx43 was shown to be hyper-phosphorylated at parturition (114). Previous studies have reported that Cx43 is important for the differentiation and contractile activity of smooth muscle cells (71). GJs made of Cx43 have been shown to account for the increased electrical coupling in the uterine myometrium and a mouse model with a mutant Cx43 had a defect in parturition, implying a role of Cx43 in contraction of myometrial cells (72). Recently, a number of transgenic and knock-in mouse models of Cxs have shed better light on the specific and shared functions of the different Cx isotypes. Knock-in mice where the coding region for Cx43 was replaced by that of Cx26, Cx32 and Cx40 have been generated and their mammary gland phenotypes described. While no mammary defects are observed when Cx40 replaces Cx43, Cx43KICx26 and Cx43KICx32 both exhibited mammary lactation defects (115). Mammary glands of Cx43KICx26 female mice were shown to have impaired mammary gland growth and differentiation (116), and mammary glands of Cx43KICx32 female mice developed normally but were unable to feed their pups presumably due to a defect in milk ejection (115). No lactation defects were observed in heterozygous Cx43+/− suggesting that the defects observed with the knock-in models are not only due to decreased levels of Cx43 but by a dominant-negative effect of the substituting Cxs. Recently a mouse model of oculodentodigital dysplasia expressing a mutant Cx43, GJa1 (Jrt/+), was described whereby the mutant Cx43 caused impaired alveolar development and milk ejection to the pups of the GJa1 (Jrt/+) dams (117). The limited functional
overlap between Cx subtypes confirms the importance of context-specific expression of Cxs in mediating tissue function.

There is increasing interest in the role GJs play in breast cancer. A correlation has been drawn in many instances between loss of GJ 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 (118, 119). However, direct association between aberrant gap junctional intercellular communication and a true malignant phenotype is not yet well established. For example, Jamieson et al. (1998) observed an upregulation of Cx26 and Cx43 in about two-thirds of the invasive human breast carcinomas studied (120). 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 (121). It is worth noting, however, that other studies have linked gap junctional communication with tumor suppression. This contrasting hypothesis does not contradict the findings that GJIC is increased in metastatic tumors, but point to possible dual roles of GJIC and of Cxs in regulation of tumors. In fact, it has been suggested 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 (106, 122).

Homocellular and heterocellular communication between normal human mammary cells has been reported; 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 quantified by flow cytometry showed that NMEC and NHMF communicate effectively and that all three cell types expressed strong homocellular communication. However, no heterocellular gap junctional 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 (123). Heterocellular communication between luminal and myoep compartments has been suggested by Berga (1984) (124). Woodward et al. (1998), reported that both mammary epithelial cells and fibroblasts readily assemble Cx43-positive GJ plaques when co-cultured with an intermediate cell type (i.e. a cell type with both epithelial and fibroblastic characteristics), but not when co-cultured together (124, 125).
1.6 Context-Dependent Expression of Cxs

Despite the fact that many tissues express two or more members of the Cx family and that two Cxs can be co-expressed in the same cell, there is little functional overlap between Cx subtypes. This is not surprising given the differences in the functional domains on the Cx protein, which regulate Cx–Cx interactions, Cx interactions with other proteins and trafficking and localization of Cxs within the cell and on the membrane. One example of the stark difference in the structural domains and function of two Cx isoforms is the distinction between Cx26 and Cx43; both isoforms are co-expressed in many tissues and in cultured cells, but to date they have not been shown to assemble into heteromeric connexons (126, 127). The reason for this apparent incompatibility is not fully understood, however many studies suggested that the differences in Cx26 and Cx43 structural domains and post translational trafficking and modifications account for their inability to interact. More precisely, Cx26 has a very short carboxy-tail compared to other Cx isoforms and in contrast to Cx43 is neither phosphorylated nor glycosylated and hence might be trafficked to the membrane via an alternate pathway independent of the common secretory pathway used by modified proteins targeted to the membrane (62, 128). One hypothesis suggests that Cx26 is spatially segregated from Cx43 and cannot oligomerize with it en route to the membrane (47, 48). However separate studies using different cell culture systems found conflicting results about Cx26 and Cx43 trafficking. A study using Cos and HeLa cells showed that targeting of Cx43-GFP to GJs was inhibited in cells treated with Brefeldin A, a drug that disassembles the Golgi network. However, GJs constructed of Cx26-GFP were only minimally affected by Brefeldin A, but were sensitive instead to Nocodazole treatment, suggesting that Cx26 shuttles to the membrane via microtubules rather than through the secretory pathway and confirming the proposed hypothesis of alternate routing (46). However, another study using HEK and HeLa cells found that pharmacological treatment with Brefeldin A or nocodazole affected both Cx26 and Cx43 similarly suggesting that both isoforms followed similar routes of cellular trafficking and assembly into GJs (126). In contrast yet another study using NRK cells revealed that disruption of microtubules with nocodazole inhibited the recruitment of Cx43-GFP into GJs but had limited effect on the transport and clustering of Cx26-YFP into GJs within the photobleached regions of cell–cell contact suggesting that Cx43 shuttles using the MT network, which is plausible given that Cx43 has a tubulin binding site (49-52). The conflicting findings from these studies point to a more complex regulation of these two proteins in the different cell systems and culture conditions examined, and suggest that there are other factors involved in the trafficking control of these two proteins. Another striking feature of these two Cx isoforms is that their co-expression in the same cell was found to reduce the total intercellular junctional conductance to a little more than 10% of that in cells expressing only a single Cx (either Cx26 alone or Cx43 alone) (126). The presence of incompatible connexons might be behind the observed reduction of GJIC by preventing functional docking of apposing hemi-channels and causing the
accumulation of uncoupled hemi-channels at the intermembrane junction. Alternatively, the lowered conductance might be caused by negative interference between the adjacent connexons which recruit gating regulators away from the nearby channels resulting in uncooperativity between the distinct channels at the GJ plaque region.

Insight from new transgenic and knock-in mouse models support the findings that Cx26 and Cx43 functions do not overlap. This was elegantly shown in a knock-in mouse model where the coding region of Cx43 is replaced by that of Cx26 (Cx4343/26), such that Cx26 is either solely expressed (homozygous mice, Cx4343/26) or co-expressed (homozygous mice, Cx4343/26) in cells expressing Cx43 endogenously. Cx26 in this case exerted a dominant negative effect on Cx43 (perhaps due to what was mentioned earlier about negative cooperativity) in many tissues and altered proper tissue-function. For example, Cx4343/26 mice had dysfunctional reproductive organs and slowed ventricular conduction in the heart, abnormalities that were similar to the ones found in Cx43-deficient mice (116). In addition, Cx4343/26 female heterozygous mice had impaired mammary gland growth and differentiation suggesting that Cx26 could not functionally substitute for Cx43 in the mammary gland. Cx26 and Cx43 have distinct expression patterns in the gland, whereby Cx43 is localized mainly to the myoepithelial contractile cells, while Cx26 is exclusively luminal epithelial. In another model where Cx32 substituted for Cx43 expression, the mammary gland developed normally, but milk ejection was impaired suggesting that Cx32 could substitute in part for Cx43 function but that their roles do not overlap, at least, in the mammary gland (115). The mammary phenotypes associated with knock-in of Cx26 and Cx32 in place of Cx43 were not similar to those observed in heterozygous Cx43 (GJa1±) female mice (117), suggesting that the effect is not only mediated by decreased Cx43 levels, but by a dominant-negative effect of the substituting Cxs. However, a mouse model of oculodentodigital dysplasia expressing a mutant Cx43, GJa1.1rt/+ has a comparable mammary gland defect to the knock-in models described for Cx32 and Cx26 whereby alveolar development and milk delivery to the pups are impaired (117, 129). Surprisingly, Cx32 and Cx26 have been shown to co-localize in connexons and at GJs, and the stoichiometry of their association in the mammary gland is regulated temporally such that Cx32–Cx32 GJs replace Cx32–Cx26 GJs at lactation since Cx26 is sensitive to taurine, which is synthesized at lactation, and can induce Cx26 channel closure (107). This differential oligomerization is yet another testament to the tight regulation of Cx expression and its profound effect on tissue function. It is obvious then that dynamic changes in Cx expression alter the physiology within a tissue. This was further highlighted in a study where the conditional knockout of Cx26 at different developmental time points in the mouse mammary gland, altered the normal differentiation status of the mammary gland and lead to early apoptosis of epithelial cells (130). Cx26 loss during late pregnancy did not have significant effects on the differentiation program of mammary cells, perhaps because at this exact time point in the gland, Cx32 or Cx30, a newly identified isoform in the mammary tissue (105) can functionally compensate for
Cx26 loss? Knock-in strategies have paved the way for a better understanding of tissue and stage specific functions of Cxs but we still need to understand what regulates this cell-specificity of Cx expression and function. Ultimately, these strategies should not only study the interchangeability of different Cxs in a tissue, but also identify the mechanisms behind observed differences. Why can some Cxs replace others but others cannot? More importantly, how is it that interchangeability between Cxs had different consequences in distinct tissues and across development within the same tissue? For example, why can Cx40 replace Cx43 without causing mammary gland functional impairment, but cause ventricular dysfunctions and arrhythmias (115)? Is it due to channel-dependent or channel independent functions? And what is the role of Cx-interacting proteins in regulating cell and tissue-specific function, especially in light of recent data pointing to a role of Cx-interacting proteins in regulating tissue and cell selective Cx expression and Cx-Cx pairing? To address these questions it is possible to take the knock-in strategy to a different level, such as temporally controlling the substitution between Cxs, or using Cx fragments in place of a full Cx protein to reveal what parts of the protein are required for different functions. Engineering Cxs with interchanged peptide sequences such as fusing the C-terminus of Cx43 to Cx26 might yield important information about the functional domains in the proteins and possible interacting partners. Studying normal context dependent expression of Cxs and Cx functional interactions is essential for understanding how mis-expression of the GJ proteins can lead to diseases including cancer. In the next section, we will discuss the conflicting roles of Cxs in cancer progression and shed some light on how these different functions are mediated.

The limited functional overlap between Cx subtypes confirms the importance of context-specific expression of Cxs in mediating tissue function. Using a transgenic mouse model designed to study the of Cx26 overexpression in the skin and presenting with a mammary gland phenotype, I asked what are the consequence of ectopically expressing a luminal Cx in the myoepithelial layer? Interestingly, female transgenic mice are unable to feed their pups, which, ultimately die of starvation by day four post-partum independent of their genotype. Pups born to transgenic females and nursed by wild type mothers are able to thrive and survive until weaning age, suggesting that the defect is in the mammary gland of the K5-Cx26 dams. The lactation malfunction in the transgenic animals is not due to impaired mammary development or defective milk protein expression but rather to milk delivery to the pups. We show that mammary glands of K5-Cx26 mice and primary mammary organoids isolated from transgenic animals have an altered response to oxytocin ex vivo and in culture, implying a deficit in milk ejection and delivery. We also show that the impaired milk delivery is due to a contractile defect consistent with the targeting of the transgene to the K5 expressing myoep. Surprisingly, ectopic expression of Cx26 seems to alter the levels of the endogenous myoep Cx43. We thus propose that loss of Cx43 impairs gap junctional communication and in the K5-Cx26
myoeeps which disrupts contractility possibly by preventing synchronization of the contractile pulse.
Chapter 2. Materials and Methods
2.1 Animals and Tissue Collection

Engineering of K5-Cx26 transgenic mice was described previously (131). Two K5-Cx26 mouse lines were used; line 73 and line 75, and both were maintained in the same FVB background. In all experiments the age- and stage-matched wild type littermates were used as control. K5-Cx26 males were crossed with wild-type (WT) females to generate transgenic positive females (Tg) and WT littermate controls. All animals were cared for in accordance with the Lawrence Berkeley National Laboratory Animal Welfare Regulatory Committee (AWRC# 0510, 0511). Mammary gland tissue was collected as previously described (132): the thoracic 2nd and 3rd mammary glands and inguinal 4th mammary glands were excised. The 2nd and third gland were used for RNA and protein extraction, while the 4th inguinal glands were either snap-frozen on dry ice for sectioning and immunohistochemistry or formalin fixed and paraffin embedded for sectioning and histologic analysis. For whole-mount analysis, one 4th inguinal mammary gland from each mouse was spread onto a glass slide, immersed in Carnoy’s fixative overnight, stained with carmine alum, and de-stained with acidic alcohol.

2.2 Primary Organoid Culture

The thoracic 2nd and 3rd mammary glands and 4th inguinal mammary glands were removed from mice. Minced tissue (4–8 glands) was gently shaken for 30 min at 37 °C in a 50-ml collagenase/trypsin mixture (0.2% trypsin, 0.2% collagenase type IV, 5% fetal bovine serum, 5 μg/ml insulin, 50 μg/ml gentamycin, in 50 ml of DMEM/F12). The collagenase solution was discarded after centrifugation at 1000 rpm and the pellet was re-suspended in 10 ml DMEM/F12. The suspension was centrifuged again at 1000 rpm for 10 min, re-suspended in 4 ml of DMEM/F12 + 40 μl of DNase (2 U/μl), and incubated for 5 min at ambient temperature with occasional shaking. The DNase solution was removed after centrifugation at 1000 rpm for 10 min. The DNase solution was discarded and the epithelial pieces were separated from the single cells (stromal cells including fibroblasts) through differential centrifugation; the pellet was re-suspended in 10 ml of DMEM/F12 and pulsed to 1500 rpm. The supernatant was then removed and the pellet was re-suspended in 10 ml DMEM/F12. Differential centrifugation was performed at least 4 times. The final pellet was re-suspended in the desired amount of basal medium (DMEM/F12 with 1% insulin, transferrin, selenium, and 1% penicillin/streptomycin) and seeded on top of a thin layer of Matrigel™ (Growth Factor Reduced Matrigel, BD Biosciences, San Jose, CA) for 24 h. After 24 h, the media was either replenished or changed to differentiation medium with lactogenic hormones: 1 μg/ml hydrocortisone (Sigma-Aldrich; GIH medium) and 1 μg/ml sheep Prl (Sigma-Aldrich) and dripped with 15% laminin-rich ECM (lrECM) (Matrigel™).
2.3 Fractionation of Mouse Mammary Epithelial Cell Types

To fractionate the epithelial subtypes from each other (i.e. the LEPs and myoeps from each other) isolated primary mammary organoids were re-suspended in Joklik’s Ca-free medium at 37°C for 15 min, then centrifuged at 1500 rpm for 10 min at room temperature and re-suspend them in 2 mL of Hank’s Balanced Salt Solution at 37°C for 5 min. The organoids were then incubated with 5 mL of serum-free DMEM/F12 with type-I DNase (50 µL of 2U/mL DNase I) to remove clumps and the reaction was stopped with 10% FBS in 5mL of L-15 medium or in calcium free DMEM/F12 with DNAase (50 µL, 2U/mL). The cells were then collected and washed and passed through a pre-separation filter and re-suspended in 500 µL MACS for magnetic sorting (MACS, Miltenyi Biotech) or 500µL FACS buffer for fluorescence activated cell sorting (FACS). Unconjugated primary antibodies were used for MACS separation EpCAM for LEPs or CD104 (β4-Integrin) for myoeps and AlexaFluor 647-anti mouse EpCAM, and FITC anti-mouse CD104 were used for FACS sorting.

2.4 Protein Extraction and Western Blot Analysis

Mammary glands were homogenized in 500 µL of extraction lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, phosphatase inhibitor cocktail of NaF (1 mM) and Na3VO4 (1.25 mM) and protease inhibitor cocktail from Calbiochem, EMD Biosciences, San Diego, CA). Protease inhibitors were added at a concentration of 40 µl/1 ml of lysis buffer, and phosphatase inhibitors cocktail at a concentration of 10 µl/1 ml each. The homogenate was centrifuged at 12,000 × g for 20 minutes at 4°C. For protein isolation from primary organoids, medium was removed from the well, and Matrigel + organoids were washed once with cold 1× PBS containing NaF (1 mM) and Na3VO4 (1.25 mM) and incubated with Matrigel™ extraction buffer: 1× PBS containing NaF (1 mM), Na3VO4 (1.25 mM), 5mM EDTA, on ice for 30 minutes with shaking to remove residual Matrigel™, then protein extraction from organoids proceeded as described above for whole mammary glands. The protein concentrations of supernatants were determined by Bio-Rad detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA).

Mammary protein (10 µg) was loaded onto 4-20% Tris-glycine gels for SDS-PAGE. Transfer efficiency was determined by Ponceau S staining and equal loading was confirmed by Laminin A/C levels (Santa Cruz Biotechnology, Inc.). Membranes were blocked for one hour in wash buffer (1xTBST: 100 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 0.1–0.3% Tween 20) with 5% milk for Cx proteins or 5% bovine serum albumin for milk proteins. Primary antibody was diluted in blocking buffer and the membranes were incubated overnight at 4°C in: polyclonal rabbit anti-mouse milk serum (Bissell laboratory) diluted 1:10,000 in blocking buffer, with polyclonal rabbit anti-Cx26 (Invitrogen Cat#51-2800) or monoclonal mouse anti-Cx26 (Invitrogen Cat#33-5800), and polyclonal rabbit
anti-Cx43 (Invitrogen Cat# 71-0700), polyclonal rabbit anti-catenin peptide (Santa Cruz biotechnology, inc. Cat# sc-7894 for α-catenin, Cat# sc-7199 for β-catenin) at a concentration of 0.5μg/ml in blocking buffer, and, with rabbit anti-myosin light chain 2 and anti-phospho-myosin light chain 2 (Cell signaling Car#3672, 3674) at a dilution of 1:200 on blocking buffer, rabbit anti-alpha smooth muscle actin (abcam, Cat#ab5694), rabbit anti-p63 (abcam, Cat#ab53039), rabbit anti-keratin 5 (Covance, Cat#PRB-160P) at a dilution of 1:500 in blocking buffer.

Membranes were then washed three times, for 10 min each and incubated with horse raddish peroxidase-conjugated secondary antibodies in 5% 1× TBST for 1 hour at room temperature. Chemiluminescent detection of bands was achieved using Pierce SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

2.5 RT-PCR

Total RNA was isolated with QIAGEN RNeasy Mini Kit (Valencia, CA). For cDNA synthesis, 20 ng of total cellular RNA was used to synthesize cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Primers for Cx26, Cx30, Cx32, Cx43, β-casein and whey acidic protein were used.

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<th>Cx</th>
<th>Sense</th>
<th>Antisense</th>
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Real-time PCR was performed using LightCycler System (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Fast Start DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN) was used for PCR reaction. PCR data were analyzed with LightCycler Software ver.3 (Roche Diagnostics, Indianapolis, IN). Relative signals between Cxs and 18s rRNA were quantified.

2.6 Immunocytochemistry and Confocal Microscopy

Mammary gland cryosections (thickness, 5-15 μm) and cells were fixed in 10% buffered formalin and 80% methanol/20% acetone before immunolabeling with primary antibodies: rabbit anti-Cx26 (1 μg/ml), rabbit anti-keratin 5 antibody (2 μg/ml; Covance), FITC anti-alpha smooth muscle (Sigma), mouse anti-β-casein (Bissell Laborator). Cryosections and cells were then immunolabeled with secondary antibodies. DAPI staining was used to visualize nuclei before mounting.
with ProLong antifade (Invitrogen). Paraffin-embedded sections were subjected to rehydration with descending grades of ethanol baths to 1XTBS. Antigen retrieval was done by microwaving samples on high for 40 minutes in sodium citrate buffer (pH 6). Sections were then permeabilized and blocked with 0.1% Triton X-100 and 5% bovine serum albumin in TBS for 60 min for antibodies made in Rabbit and then incubated with antibodies. When using antibodies made in mouse, it was essential to block with the mouse-on-mouse (M.O.M™) blocking kit (Vector Labs), to block the endogenous mouse IgG which is abundant in tissue sections. Organoids cultured on top of Matrigel™ were smeared on glass slides and fixed on slides with 80% methanol/20% acetone. Primary single cells on 2D were cultured in chamber slides (Lab-Tek™, nunc) and fixed with 80% methanol/20% acetone. For all stained tissue and cells, primary antibody was visualized by incubating sections with Alexafluor dye-conjugated secondary antibodies. DAPI stain was used to visualize nuclei before mounting with ProLong antifade (Invitrogen). Immunolabeled cryosections, paraffin-embedded sections and cells and organoids were imaged on a Zeiss LSM 710 confocal microscope.

2.7 Mammary Gland Whole Mounts and Histology

To perform a general histological analysis of the mammary gland, paraffin-embedded sections of WT and K5-Cx26 mammary glands were stained with hematoxylin and eosin. Paraffin-embedded mammary gland sections were deparaffinized in xylene for 10 min, rehydrated in descending grades of ethanol baths, and stained with 1% hematoxylin (5 min) and 1% eosin (5 min). Sections were dehydrated in ascending grades of ethanol and xylene baths and mounted with Cytoseal (Richard-Allan Scientific). General histological analysis was performed by imaging several random areas with 63×, 40×, and 16× objective lenses mounted on a Zeiss Axioscope microscope workstation equipped with a Sony PowerHAD camera and Axiovision LE imaging software (Carl Zeiss Vision).

2.8 Oxytocin Assays

Ex vivo oxytocin assay was done as previously described by Plate et al., 2008. Pups were removed from the dam 1 h prior to the assay and the mouse was sacrificed on parturition day. Abdominal skin was cut and peeled off in order to expose the mammary gland. PBS or 0.5µg/ml–1 mg/ml oxytocin in PBS was applied directly on the mammary glands for 1 min and then removed. Milk entry into the ducts was monitored using a numeric camera. Photographs were taken before and after PBS or oxytocin exposure.

To visualize contraction in culture, primary organoids were seeded on top of a layer of Matrigel™ and supplied with lactogenic hormones (for 4 days to induce the production of milk proteins. After 4 days, organoids were subjected to oxytocin and tracked by live imaging for up to 120 minutes.
Quantification of contraction was done using imageJ software analysis tools. Briefly, acquired movies were collected as stacks of images with one image for every time point (1-5 minute intervals). Images were binarized such that organoids appeared dark in contrast with the background and the surface area of the organoid was calculated for each time point and plotted on a graph against time. Normalized area change was calculated as the change in surface area (\(\text{Area}_n - \text{Area}_i\))/\(\text{Area}_i\) (where \(\text{Area}_i\) represents the initial surface area) and the results were plotted against time.

### 2.9 Scrape Loading Assay

Primary cells were washed three times with warm PBS before addition of Lucifer yellow-CH (LY) at 0.1% dilution in PBS. Using a scalpel, cuts were made throughout the monolayer, followed by incubation with 5% LY in 150 mM LiCl for 10 min at 37°C. The cells were then washed with warm PBS and fixed with 4% formaldehyde. Slides were preserved by mounting in anti-fade and stored at 4 °C for later analysis and quantification.
Chapter 3. Results
**Rationale and Hypothesis**: The work in the Bissell laboratory is focused on understanding how signals in the organ microenvironment are intricately integrated to enable tissue-specific function and differentiation. Among microenvironmental cues, intercellular communication through GJs is essential to couple cells electrically and metabolically, and to regulate the selective passage of molecules of up to 1 KDa in size between adjacent cytoplasms. Identical or different Cxs can oligomerize to form homomeric or heteromeric connexons respectively and the docking connexons in turn make homotypic or heterotypic GJs. The identity and stoichiometry of Cx distribution within GJs determines the permeability and gating properties of the channel, thus, regulating the type and rate of molecules passing between cells. Unique channel types made of specific Cxs contribute to distinct functional states within the tissue, and in turn, the tissue context dictates Cx identity within GJs such that stage-specific function is achieved and maintained. We hypothesize that context-specific expression of Cx proteins is essential for proper Gap junctional communication and consequently is critical for tissue-specific function. Using the mammary gland as an “experimental organism” to investigate this hypothesis, I set out to characterize the mammary phenotype of the K5-Cx26 transgenic mouse where Cx26, a luminal mammary Cx, is ectopically expressed in the mammary myoeps otherwise lacking Cx26. K5-Cx26 female mice display a mammary gland impairment whereby transgenic dams are unable to feed their litters.

**3.1 Cx26 is Ectopically Expressed in the Mammary Myoeps of K5-Cx26 Mice**

To determine if Cx26 is indeed over- and ectopically expressed in the myoeps of K5-Cx26 mice, expression of Cx26 was studied in wild type (WT) and transgenic (Tg) tissues by quantitative RT-PCR (Q-PCR), western analysis and immunohistochemistry. Cx26 transcript levels were significantly higher in Tg tissue of virgin/nulliparous mice compared to WT littermates across all stages studied (Fig.1A). Immunostaining on frozen mammary tissue sections from glands of nulliparous WT and Tg littersmates established that Cx26 expression is only detectable in Tg tissue and that it is localized in the basal/myoep in the alveolar buds (Fig.1B). Western analysis confirmed higher expression of the protein in tissue of virgin/nulliparous, pregnant and lactating Tg mice (Fig.1C). To confirm the ectopic over-expression of Cx26 in the myoeps of Tg tissue, primary cells were isolated as previously described (133) and stained with antibodies against Cx26 and α-smooth muscle actin (α-sma), a myoep and smooth-muscle specific protein. Briefly, primary organoids from fresh mammary tissue of virgin or mid-pregnant mice were extracted by collagenase digestion; the isolated fragments containing epithelial and stromal components were purified to remove the contaminating fibroblasts and the remaining cells were then cultured for 2 hour on poly-L-lysine coated coverslips in medium containing serum to enhance attachment. The cells were later shifted to medium containing lactogenic hormones (prolactin, hydrocortisone, insulin, sodium selenite) and dripped with
15% lrECM. lrECM was shown previously to be required for expression of endogenous Cx proteins in mammary epithelial cell lines (75). After 3 days the cells were fixed and stained for Cx26 and α-sma, and indeed, Cx26 co-localized with α-sma only in cells derived from Tg but not WT tissue (Fig.1D). Thus, the expression of Cx26 was confirmed to be localized to the myoeps in the mammary glands of the Tg female mice. Interestingly, mRNA levels of all the other mammary Cxs, Cx30, Cx32 and Cx43 were comparable in the glands of lactating Wt and Tg littermates, establishing that ectopic expression of Cx26 in the mammary myoeps of K5-Cx26 Tg mice does not alter the mRNA expression of other mammary Cxs (Fig.1E).
Figure 1.

A-

![Graph showing expression of Cx26 in different conditions](image1.png)

B-

![Images showing WT and Tg conditions](image2.png)

C-

![Images of Cx26 and LAM A/C expression](image3.png)
Figure 1. Cx26 is ectopically expressed in mammary myoeps of K5-Cx26 mice. (A) Q-PCR analysis for the expression levels of Cx26 transcripts in nulliparous, mid-pregnant (day 14) and early lactating (day 2) WT and Tg tissues normalized to 18S rRNA (n=3). (B) Staining for Cx26 (green) in mammary tissue sections from WT and Tg nulliparous littermates. Cx26 is localized to the basal cells in the Tg tissue section. No significant staining is observed in sections from WT mice. Scale bar 20µm. (C) Western blot analysis of WT and Tg mammary gland homogenates at different time points of mammary development and corresponding quantification of Cx26 levels in comparison to Lamin A/C (LAM A/C) used as a loading control (n=3). (D) Primary cells from early pregnant mice cultured in differentiation medium (lactogenic hormones and 15% drip Matrigel) for 4 days and stained for Cx26 (Green; a,b,c,d) and α-sma (Red; c,d). Dual immunofluorescence shows co-localization of Cx26 with the myoep-specific protein α-sma. Cx26 co-localized with α-sma only in the transgenic cultures (d). DAPI staining (blue) marks nuclei (a,b,c,d). Scale bar 20µm.
3.2 Ectopic Expression of Cx26 Correlates with Impairment of Mammary Gland Function at Lactation

Most pups born to Tg dams die shortly after birth from starvation. Litter death starts from about day 2 of lactation until about day 10 when all the pups have died. Litter death is not due to the pups’ inability to suckle since the Tg dams exhibit normal maternal behavior including nest building, retrieval of displaced pups to the nest, and prolonged attempts at nursing. Moreover, the pups’ failure to thrive could be overcome when litters born to Tg mothers are nursed by WT dams (Fig. 2), suggesting that the starvation phenotype is due to a mammary defect during the lactation stage either caused by insufficient milk production or defects in milk release.

Figure 2.

Figure 2. Pups born to Tg dams die of starvation shortly after birth. Litters born to WT mothers have around 98-100% survival rate to weaning age (blue). Pups born to Tg mothers die from around day 4 to day 10 of lactation due to failure to thrive (red) but cross-fostering of pups born to Tg mothers by WT dams restores the survival rate to normal (blue and green) (n=3).
3.3 Lactation Failure is Due to Impaired Milk Delivery despite Normal Development of K5-Cx26 Mouse Mammary Glands

I asked whether the inability of the K5-Cx26 females to feed their litters at lactation is caused by defective development of the gland leading to reduced ductal outgrowth and incomplete alveologenesis, which may impair milk production. To assess whether the mammary glands of Tg female mice develop normally, mammary gland whole mounts and H&E-stained tissue sections from WT and Tg littermates at different stages of development were analyzed. No differences were observed in the extent of ductal and alveolar growth and size between the glands of WT and Tg mice (Fig.3A&B). Morphometric analysis on a large set of tissue samples from WT and Tg female mice where the ratios of epithelial to fat content in the mammary glands were calculated confirmed the absence of abnormalities in the extent of epithelial growth and alveolar development in the mammary glands of Tg female mice (Fig 4). Additionally, production of milk proteins was analyzed in mid-pregnancy (day 14), late-pregnancy (day 18) and early lactation (day 1) to investigate whether alveolar structures in the glands of Tg mice were capable of milk production. RT-Q-PCR analysis for β-casein and weigh acidic protein (WAP) mRNA revealed that the transcript levels for both genes were similar in the glands of WT and Tg littermates (Fig.5A). This was also confirmed at the protein level by Western analysis (Fig. 5B). Despite normal milk production by Tg dams, pups born to Tg mothers do not receive any milk as evident by lack of milk spots in their stomach in the first 2 days after birth (Fig.5C).
Figure 3.
Figure 3. Mammary glands of Tg mice develop normally. (A) Micrographs of stained whole mounts of mammary glands from WT and Tg mice at 14 week nulliparous mice, mid-pregnant (day 14), late pregnant (day 18) and early lactation (day 1). No difference is observed in gross size of mammary glands from WT and Tg littermates at all stages examined. (B) Higher magnification micrographs showing ductal and alveolar networks in the glands of WT and Tg mature nulliparous, late pregnant and early lactating littermates. No developmental defects are observed such as less ductal branching or reduced ducto-alveolar development.
Figure 4. Normal levels of epithelial to fat cells in the glands of Tg mice. H&E staining of tissue section from the mammary glands of mid-pregnant (day 14), early lactating (day 2) and mid-lactating (day 10) of WT and Tg littermates and quantification of number of epithelial (purple/blue cells) compared to fat (adipose) cells (white and light pink). Differences in epithelial/fat ratio in lactation day 10 are statistically significant (p<0.0001, n=12).
Figure 5.

A-

![Bar chart showing fold expression of β-casein and WAP in WT and Tg groups]

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C-

![Image of newborn mice with labels: WT mother and Tg mother]
Figure 5. Milk delivery but not production is impaired in Tg mice.
(A) Q-PCR analysis for β-casein and WAP mRNA revealed no significant difference in the transcript levels in WT and Tg tissue in mid-pregnancy (n=3).
(B) Western blot analysis with anti-β-casein antibodies revealed that the protein was expressed comparably between WT and Tg tissues in early lactation. Equal loading was assessed by Lamin A/C staining of membranes. (C) Photographs of pup born to WT mother with milk spot in stomach (left) and pup born to Tg mother.
3.4 Response to Oxytocin in the Mammary Glands of K5-Cx26 Mice is Impaired

Since the mammary glands of Tg females develop normally and are able to produce milk proteins, I hypothesized that the mammary functional impairment in K5-Cx26 Tg mice is due to a defect in milk delivery and ejection, possibly caused by defective contraction of the myoepithelial cells, and consistent with the targeting of the transgene to the myoepithelial compartment of K5-Cx26 mice. To determine if milk is being released to the ducts, an oxytocin-induced milk excretion assay was performed. WT and Tg littermates on the first day of lactation were separated from their pups to ensure that the milk would accumulate in the glands one hour prior to the addition of exogenous oxytocin. The mice were then sacrificed and their glands were immediately treated with exogenous oxytocin at different concentrations or with PBS as a control. In WT mice, low levels of milk were detected in ducts prior to the addition of oxytocin, however upon the addition of 100μg/ml of oxytocin to the mammary gland, ducts rapidly filled with milk. In K5-Cx26 mice, the glands were engorged with milk prior to oxytocin treatment. However, oxytocin addition did not induce a release of milk into the ducts even at higher doses (>100μg/ml) of oxytocin (Fig.6).
Figure 6.
Figure 6. Milk release in response to exogenous oxytocin is impaired in Tg mice. Mammary glands from WT or Tg littermates were treated with PBS or PBS containing 20µg/ml or 100µg/ml oxytocin and the expulsion of milk into the ducts was evaluated. \( n = 4 \) for WT, and \( n = 4 \) for Tg mice. Arrowheads indicate accumulation of milk within a duct after oxytocin exposure.
3.5 Primary Organoids from Tg Mammary Glands Fail to Contract in Response to Oxytocin Despite Normal Levels of Oxytocin Receptor

Since I observed that oxytocin-mediated milk release into the mammary ducts of Tg mice is impaired, I asked whether the defect in milk release is due to impaired myoep contractile function in the glands of Tg mice. To determine whether contraction of myoeps is impaired in Tg female mice, I used a culture assay not previously described whereby primary organoids from WT and Tg littermates were cultured in lactogenic medium and supplied with the contractile hormone, oxytocin and imaged live for >1 hr (134). Organoids from WT and Tg littermates are phenotypically similar when grown on 3D on top of lrECM, and they both express β-casein when treated with lactogenic hormones (Fig.7A). A previous study had revealed that addition of oxytocin to 3D cultures of rabbit mammary epithelial cells induces a contractile response after about 7 minutes of oxytocin addition (135). Whereas WT organoids contracted, pulsed in response to exogenous oxytocin in culture, Tg organoids failed to contract and pulse, indicating that K5-Cx26 acini are not capable of responding to oxytocin comparably to their WT counterparts (Fig.7B). Interestingly, the contraction defect is not due to decreased levels of oxytocin receptor in the mammary glands of K5-Cx26 dams compared to their wild type littermates. This was observed both in whole tissue homogenates from the mammary glands of WT and Tg mice and in primary organoids treated with oxytocin in culture. Contraction was quantified as the normalized change in surface area over time, and the quantification revealed that while the surface area of WT organoids fluctuated as a function of time after the addition of oxytocin, the surface area of Tg organoids did not vary with time after the addition of the hormone (Fig.7B).
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Graph showing normalized area change with minutes of OT addition.
**Figure 7. Primary mammary organoids from Tg mice do not contract in response to oxytocin.** (A) Primary organoids isolated from the glands of 6-week old virgin mice and cultured in differentiation medium that allow the production of β-casein stained for Cx26 and β-casein expression. β-casein was produced and secreted vectorially into the lumen in WT and Tg organoids by day 4 of culture. As expected, Cx26 localized with Keratin 5 in the transgenic organoids indicating myoep staining (f) but not in the WT organoids. DAPI staining (blue) marks nuclei. (B) Oxytocin (10^-6 i.u/ml) added on day 4 stimulated contraction (pulsing and decrease in size) in WT but not Tg organoid cultures. Quantification of the area change per minute was done using ImageJ software is shown on the right. (C) Oxytocin receptor (OTR) protein levels are not different in organoids isolated from the glands of WT and Tg mice. Lamin A/C was used for equal loading.
Summary of findings: mammary glands from WT and Tg lactating littermates respond differently to oxytocin treatment ex vivo; whereas oxytocin stimulates milk release into the mammary gland ducts of WT dams, oxytocin fails to induce milk release into the mammary ducts of K5-Cx26 dams. In addition, primary organoids isolated from transgenic glands display a different contractile activity in culture upon exogenous oxytocin addition compared to their wild type counterparts. This suggests that the problem is due to an inappropriate contractile response of myoeps despite the normal levels of oxytocin receptors in the mammary glands of K5-Cx26 dams. Thus, I asked whether signaling downstream of oxytocin addition in the transgenic dams is compromised. In addition, and since the endogenous myoep Cx43 has been shown to be essential for contractile activity of smooth muscle cells of the uterus, I investigated the expression of the endogenous Cx43 in the mammary tissue of K5-Cx26 female mice and the differentiation status of myoeps in Tg females.

3.6 The Mammary Glands of Tg Mice have Normal Levels of Differentiated Myoeps

I had proposed to characterize the mammary myoeps of K5-Cx26 mice and compare them to myoeps isolated from the glands of their WT littermates. The rationale for this experiment was to determine whether the mammary contraction defect observed in K5-Cx26 mice is caused by either lower numbers of myoeps, and subsequently lower ratio of myoep to epithelial cells in the glands of Tg mice, or, whether the myoeps are not fully differentiated. I performed a fractionation experiment to separate the myoeps from LEPs from the mammary glands of WT and Tg littermates. Briefly, EPCAM was used as a surface marker for LEPs and β4-integrin was used as myoepithelial surface marker. Isolated mammary epithelial cells from the glands of WT and Tg mice were incubated with FITC-conjugated EPCAM and Alexa-568-β4-integrin and sorted via FACS to separate the two fractions. No significant differences were observed in the ratios of mammary myoeps to LEPs between WT and Tg animals (Fig.8A). I then analyzed whether the myoeps from K5-Cx26 glands exhibited any differentiation defects by assessing levels of smooth muscle actin proteins, p63 and basal keratins. I noted no significant differences in the levels of myoep proteins in the cells isolated from WT and Tg animals and in whole tissue homogenates (Fig.8B).
Figure 8.

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Figure 8. Mammary myoeps of Tg mice differentiate normally. (A) Isolated primary luminal epithelial and myoepithelial cells from the glands of WT and Tg littermates were counted to reveal that there were no notable differences in the number and ratios of luminal epithelial to myoeps between WT and Tg animals. Primary cells were isolated from 3 pairs of WT and Tg littermates (n=3). (B) Analysis of myoep-specific proteins in the mammary glands of WT and Tg littermates did not reveal significant differences. (C) Analysis of myoep-specific proteins in the isolated myoeps also revealed not striking differences between WT and Tg cells.
3.7 Signaling to pMLC from Oxytocin is not Altered in the Mammary Glands and Primary Organoids of K5-Cx26 Mice

The oxytocin receptor (OTR) signals via a G-protein pathway to phosphorylate myosin-light-chain-2 which upon phosphorylation generates the force required to induce contraction (26). To test whether myosin-light chain 2 (MLC2) is phosphorylated upon addition of oxytocin in mammary glands and in primary organoids from the mammary glands of Tg mice, I collected protein from the glands of WT and Tg mid-pregnant and lactating littermates and from primary organoids isolated from WT and Tg littermates. No significant differences were observed in the ratios of pMLC2 to MLC2 in the tissue of mid-pregnant and early lactating WT and Tg littermates (Fig.9A). Similarly, organoids grown on top of lrECM in lactogenic medium for 2 days and treated with oxytocin (as previously described for contraction assay) were isolated and protein was collected from them at 20min, 40min, 80min and 120min intervals to assess the status of MLC-2 phosphorylation. No significant differences were observed in the ratios of pMLC2 to MLC2 across all time points examined between organoids from WT and Tg littermates, implying that the signaling pathway downstream from OTR stimulation is not impaired, but that the contractile defect is due to another problem in the mammary glands of K5-Cx26 mice (Fig.9B).
Figure 9.

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![Graph showing pMLC2/MLC2 ratios for WT and Tg with 0' and 20' time points.](image)
C-
Figure 9. Signaling to the myosin-light-chain pathway is not altered in the primary mammary cells isolated from the glands of Tg mice. (A) Western blot analysis of oxytocin receptor (OTR), myosin light chain 2 (MLC2) and phospho-myosin light chain 2 (pMLC2) in WT and Tg tissue of mid-pregnant and mid-lactating mice show no significant differences in the levels of the protein between WT and Tg littermates. (B) Western blot analysis of PMLC2 and MLC2 before and after (20’) addition of oxytocin (OT). Quantification of band intensity levels from 3 different experiments (right) confirms that there are no significant differences in the ratios of PMLC2 to MLC2 between WT and Tg organoids before and after treatment with oxytocin. (C) Western analysis of downstream kinases from OT signaling shows no significant difference in protein levels between WT and Tg organoids before and after OT treatment.
3.8 Ectopic Expression of Cx26 Acts as a Trans-Dominant Negative for Cx43 Protein Levels

Cx proteins share a common structural topology including four transmembrane domains, two extracellular loops involved in mediating hemi-channel docking by non-covalent interactions, a cytoplasmic loop and N- and C- termini located in the cytoplasm. Cx26 differs from Cx43 in three aspects: it has a shorter C-terminal tail, lacks phosphorylation sites in its C-terminus, and while it is believed that Cx43 oligomerizes into a connexon in the trans-golgi network (TGN), Cx26 oligomerization occurs in the endoplasmic reticulum (52, 126). These two Cxs do not form heterotypic channels \textit{in vivo} or when co-expressed in Cx-deficient cell types in culture (126). Surprisingly, ectopic expression of Cx26 in the mammary myoeps of K5-Cx26 mice was associated with a down-regulation in the levels of Cx43 and its phosphorylation, suggesting that Cx26 in the myoeps acts as a trans-dominant negative to Cx43 (Fig.10A). However, Cx43 transcript levels did not vary significantly between the WT and Tg mice, arguing that ectopic expression of Cx26 might affect the post-translational modification and stability of the protein rather than influence the expression of the gene (Fig.10B). It has been shown previously that Cx43 protein expression and increased phosphorylation in late pregnancy and early lactation are independent of transcriptional control (106). This is an interesting observation because previous reports have established that loss of Cx43 function in another smooth muscle cell type causes a contractility defect (71). It would be interesting to show in future studies using cells isolated from the mammary glands of K5-Cx26 mice whether the loss of Cx43 protein is due to inhibition of protein translation, or increased Cx43 degradation. No differences were observed in the protein levels of the other mammary Cxs, Cx30 and Cx32 between WT and Tg littermates in late pregnancy and lactation (Fig.10C)
**Figure 10.**

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![Graph showing Cx43/18s rRNA expression levels for WT and Tg mice across different stages.]

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Figure 10. Cx26 acts as a trans-dominant negative to Cx43 in the glands of Tg mice. (A) Western analysis on mammary protein samples across different time points revealed that in late pregnant and early lactation, Cx43 expression is higher in wild type (WT) compared to Tg tissues. Lamin A/C is used for equal loading. (B) Q-PCR analysis of Cx43 transcript levels normalized to 18S rRNA (n=3): no significant difference in Cx43 mRNA levels is observed between WT and Tg at the gene expression level suggesting that loss of Cx43 in Tg tissue is at the post-transcriptional level. (C) Protein levels of Cx30 and C32 are similar in WT and Tg late pregnant and lactating littermates.
3.9 Gap Junctional Communication is Altered in Primary Mammary Cells Isolated from Tg Mice

One mechanism by which loss of Cx43 could cause a contractile defect is by inhibiting of the formation of competent gap junctional channels between myoeps. Cx43-GJs in the myoeps are thought to have the adequate gating and conductance characteristics permissive of electrical coupling to sustain contraction after initiation of the calcium transients. In the luminal epithelial cells Cx26 homotypic GJs are mostly present during mid-pregnancy to early lactation. At parturition and thereafter, Cx26 homotypic channels are replaced by Cx26-Cx32 or Cx26-Cx30 heterotypic GJs. This assembly is thought to be significant for the production and secretion of milk, since GJs made exclusively of Cx26 display increased sensitivity to the accumulation of taurine, which occurs during lactation (107, 112, 136). To test GJ intercellular communication (GJIC) in K5-Cx26 tissue, a scrape loading assay was performed on primary cells from the mammary tissue to assess functional coupling of cells by GJs. Briefly, cells cultured for 4 days were scraped by a sharp needle in the presence of a GJ permeating dye, Lucifer yellow. The dye, which cannot penetrate into the cells via membrane diffusion, can only enter to the injured cells via the introduced shear site and then diffuse to their neighbors via GJs. The cells were incubated with dye for 5 minutes and subsequently, washed extensively, fixed in 4% formaldehyde and stained for Cx26. Primary cells from the mammary glands of WT and Tg littermates allowed dye transfer across the monolayer as evident by dye spreading. In the cells derived from WT glands, Cx26 staining correlated with the presence of Lucifer Yellow in the cytoplasm. However, in cultures of cells from glands of Tg mice, cells expressing high amounts of Cx26 were dye-excluded, confirming that elevated and ectopic expression of Cx26 in the mammary myoeps of Tg mice correlates with loss of gap junctional coupling with neighboring cells (Fig.11).
Figure 11.
Figure 11. Gap junctional communication is altered in primary mammary cells isolated from the glands of Tg mice. Gap junctional communication assessed by a scrape loading assay. (A) Lucifer yellow dye (LY-Green) spreading after 10 min. (B) Staining for Cx26 (red) on the scratched cells. Dye spreading (green) is uniform across monolayers of primary mammary cells from WT mice, monolayers of primary Tg cells receive dye (green), except for cells highly expressing Cx26 (red) which are dye-excluded. (C) Quantification of number of cells expressing Cx26 and are dye excluded in scrape loaded primary cells isolated from the glands of WT and Tg littermates.
3.10 Inhibition of GJ Communication in WT Organoids Impairs Contraction in Response to Oxytocin

In order to determine if loss of gap junctional communication could be responsible for the contractile defect observed in the mammary glands of K5-Cx26 mice, primary organoids isolated from the glands of WT mice were treated with a GJ inhibitor (18-alpha-Glycyrrhetinic acid or 18αGA) or with DMSO (as the negative control). Oxytocin was then administered and primary organoids were tracked by live imaging. Whereas primary organoids treated with DMSO contracted as expected from former assays, organoids treated with the GJ inhibitor did not contract in culture when supplied with oxytocin in a dose-dependent manner, suggesting that loss of gap junctional communication is sufficient to inhibit contraction in WT organoids and implying that loss of contractility in the mammary myoeps of the K5-Cx26 mice could be due to loss of gap junctional coupling (Fig.12).
Figure 12. Inhibition of gap junctional communication impairs oxytocin-mediated contraction in primary mammary organoids from WT mice. a,b- Western analysis of PMLC2 and MLC2 levels in primary organoids treated with GJ inhibitor 18-alpha-Glycyrrhetinic acid (18αGA) at concentrations of 10µM and 50µM reveals no differences in the ratios of pMLC2 to MLC2. c- Normalized area change per minutes reveals that contraction is inhibited in a dose-dependent manner upon addition of GJ inhibitor.
3.11 Down-Regulation of Cx43 in WT Mammary Organoids Recapitulates the Contraction Defect of Tg Organoids

Previous studies have shown that expression of a mutant form of Cx43 protein in the mammary myoeps impairs contractility in the gland in response to oxytocin. Since K5-Cx26 mammary glands have lower levels of Cx43, I asked whether loss of Cx43 in the mammary myoeps is responsible for the mammary contractile defect. To establish whether loss of Cx43 recapitulates the contraction defect, Cx43 was knocked-down with shRNA in the mammary organoids of WT females. Organoids with lower Cx43 were cultured in lactogenic conditions, and the oxytocin assay was performed. I observed that down-regulation of Cx43 resulted in the loss of the contractile response to oxytocin, thus, proving that Cx43 loss alone is sufficient to impair contraction and suggesting that this is caused by a loss of gap junctional coupling between the myoeps that have lost Cx43 (Fig.13).
Figure 13.

a-

b-

![Graph](image)

![Graph](image)

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Figure 13. Down-regulation of Cx43 impairs oxytocin-mediated contraction in primary mammary organoids from WT mice. a- western blot showing lower of Cx43 in organoids treated with Cx43 shRNA. b- Lamin A/C was used as a loading marker and Cx43 levels in WT organoids treated with scrambled shRNA (Scrbl) and Cx43 shRNA were quantified. c- Representative quantification of contraction in organoids treated with Scrbl shRNA and with shRNA against Cx43 showing that loss of Cx43 inhibits contraction.
Chapter 4. Additional Experimental Results
4.1 Unexpected Phenotype of Primary Mammary Epithelial Cells from Tg Mice in Collagen I Gels

I had initially intended to quantify the magnitude of contraction of primary mammary epithelial cells isolated from WT and Tg littermates by assessing their ability to compress collagen I gels when they are embedded inside the gel. The Collagen I contraction assay requires that cells are embedded inside collagen I gels, and the gels are detached from the plate and allowed to float such that they are able to respond to the traction forces exerted by the cells upon contraction. Theoretically, contraction of the cells in collagen I will cause the fibers of collagen I to become compressed, consequently reducing the surface area of the gel. This change in surface area (or diameter) can be calculated and used as a correlative measure for the ‘magnitude’ of the contraction. Surprisingly, when I placed primary mammary epithelial cells from Tg female mice in collagen I, they clustered together and floated the collagen I gels prior to the addition of oxytocin. In comparison, primary mammary epithelial cells from WT female mice grew in collagen I and branched upon treatment with growth factors (Fig.14). It is not evident why primary cells isolated from the glands of Tg mice would cluster and detach the collagen-I gel from the plate surface, however, future studies should look at the expression of matrix metalloproteinases known to degrade collagen I.
Figure 14.
Figure 14. Mammary organoids from Tg mice aggregate inside collagen I gels and detach the gel from the plate. Micrographs of primary organoids from WT and Tg littermates embedded inside 3mg/ml Collagen I gels. While WT organoids grown and branch in the gel if stimulated with growth factors that induce branching, Tg organoids aggregate and detach the gel from the plate. This finding prevented the use of the collagen traction assay as a method to measure the difference in contractility between WT and Tg cells.
4.2 Ectopic Expression of Cx26 Alters the Levels of the Adhesion Proteins, E-Cadherin and β-Catenin

Cx43 has been shown to interact via its cytoplasmic domains with a number of adhesion proteins such as E-Cadherin and β-catenin (53). Concomitant with the decrease in Cx43 levels in the mammary glands of K5-Cx26 Tg mice, I observed very low levels of β-catenin, and a variation in the expression pattern of E-cadherin compared to age- and stage-matched WT littermates (Fig.15). The decrease in the levels of both proteins did not affect the development and differentiation of the gland. Further investigation established that when primary LEPs isolated from the mammary glands of Tg tissue were grown on top of lrECM, they expressed similar levels of both adhesion proteins implying that either the levels of E-Cadherin and β-catenin is only altered in the mammary myoeps of Tg mice, or that when the two cells types, LEPs and myoeps are in contact, down-regulation of E-Cadherin and β-catenin occurs by an unknown mechanism.
Figure 15. Lower levels of adherens junction proteins in the glands of Tg mice. (A) Western analysis for E-Cadherin levels and β-catenin in WT and Tg tissues showed differences in levels and expression pattern of both proteins. Two prominent bands for E-cadherin were seen at 120 KDa and 75 KDa in WT tissue that were down-regulated in the Tg glands. (B) Down-regulation of E-Cadherin protein in the glands of Tg mice compared to their WT littermates at mid-lactation (day 12).
4.3 Decreased Levels of E-Cadherin in the Mammary Glands of K5-Cx26 Mice Correlates with Increased Levels of N-Cadherin

The lower levels of Cx43, E-Cadherin and β-catenin could indicate that the epithelial cells in the mammary glands of Tg mice are undergoing a transformation. Analysis of the expression of other Cadherin isoforms showed that the decrease in E-Cadherin in the glands of Tg mice was accompanied by an increase in N-Cadherin expression, suggesting a possible Cadherin switch and a potential epithelial to mesenchymal transformation (EMT) occurring in the glands of Tg mice (Fig.16A). N-Cadherin expression is not observed in the mammary gland normally during the process of involution (Fig.16B).
Figure 16. N-Cadherin and Vimentin are expressed in the glands of lactating Tg mice. (A) The lower levels of E-cadherin were accompanied by increased levels of N-cadherin and vimentin in the glands of Tg mice at mid-lactation. (B) The expression of N-cadherin in WT tissue at different involution (Inv) time points was analyzed and no N-cadherin expression was observed from day 1 to day 7 of involution.
4.4 Phenotype of Chimeric WT/Tg Organoids

In order to determine if LEPs and myoeps from the glands of Tg mice form normal intercellular junctions and to pinpoint which cell type is responsible for the contractile defect, I fractionated the two cell types and recombined them in chimeric WT/Tg cultures on top of lrECM. Collected LEPs and myoeps were grown in basal medium (ITS-PS with fetal bovine serum) on top of non-adhesive substratum (polyHEMA). After 24 hours, luminal and myoepithelial fractions from the mammary glands of WT and Tg mice were co-cultured in 4 different permutations: WT LEPs with Tg myoeps, Tg LEPs with WT myoeps, WT LEPs with WT myoeps and Tg LEPs with Tg myoeps. In all conditions examined, co-cultured cells grew on top of lrECM and formed spheroid-like structures or aggregates. However, none of the spheroids contracted upon addition of oxytocin. Using chimeric co-cultures to determine whether LEPs or myoeps are responsible for the Tg mammary phenotype is not the optimal assay since recombining WT fractions together did not reproduce the contractile response observed in primary organoids. However, isolating the two cell types was an important step as it allowed the study of each cell type on its own and their comparison in more detail (Fig.17).
Figure 17. Chimeric co-cultures of WT and Tg mammary luminal epithelial and myoepithelial fractions form spheroid like structures on top of lrECM but do not contract upon oxytocin addition. Fractionated luminal eps and myoeps were used in chimeric WT/Tg cultures of the two cell types: WT luminal eps/WT myoeps (a), Tg luminal eps/Tg myoeps (b), Tg luminal eps/WT myoeps (c) and WT luminal eps/Tg myoeps (d). All chimeric co-cultures were stained for Cx26 (red) and α-SMA (green).
4.5 Phenotype of Primary Mammary Luminal Epithelial Cells from WT and Tg Littermates in Culture

Since I observed a down-regulation of intercellular adhesion proteins E-cadherin and β-catenin in the mammary glands of K5-Cx26 mice, I wanted to test whether the loss of these adhesion proteins affect the formation of 3D acinar-structures inside 3D IrECM and affect the architectural integrity of LEPs. Isolated WT and Tg LEPs were grown as single cells inside 3D IrECM and treated with lactogenic hormones which allow them to differentiate. After 7 days, single cells isolated from the mammary glands of WT and Tg littermates formed acinar-like structures, expressed milk proteins and comparable levels of E-Cadherin and β-catenin. This suggests that functional and structural differentiation in culture could be recovered in the absence of the K5-Cx26 myoeps and further proves that in the context of the K5-Cx26 mammary glands, the interaction between the myoeps and LEPs causes the observed differences in the adhesion protein levels (Fig.18).
Figure 18. Fractionated luminal epithelial cells from WT and Tg littermates do not have altered adhesion proteins. (A) Fractionated primary mammary LEPs from WT and Tg littermates are grown on top of 3D lrECM and treated with lactogenic hormones form acinar structures and have similar membranous intercellular E-cadherin staining. Cx26 expression in the isolated LEPs is similar in the WT and Tg cells. (B) Western analysis for E-cadherin and β-catenin in WT and Tg primary LEPs reveals that levels of both proteins are comparable in WT and Tg cells.
4.6 Wnt 5a Levels in the Mammary Glands of Tg Mice

A recent study has shown that in a transgenic mouse model where WNT5A is overexpressed in the mammary epithelium driven by the mouse mammary tumor virus promoter (M5a mice), lactation was impaired due to a failure of the epithelium to respond to oxytocin, and subsequently failure of milk ejection. The milk ejection problem was shown to correlate with impaired gap junctional communication between cells of the M5a mouse by specifically regulating Cx43 phosphorylation at Serine 368 (137). Given the phenotypic similarity between this mouse and the K5-Cx26 mouse model, I investigated the expression of Wnt5a in the mammary glands of WT and Tg littermates in mid-pregnancy. Wnt5a is known to be upregulated in mi-pregnancy and my results shot that Wnt5a expression is lower in the glands of K5-Cx26 mice in mid-pregnancy compared to their WT littermates. Wnt5a overexpression in the M5a mouse correlated with hyperphosphorylation of Cx43 at Serin 368 and loss of gap junctional communication. In the case of the K5-Cx26 mouse, the opposite pattern was observed: Cx43 levels and phosphorylation status were lower in the Tg mice compared to the WT littermates and Wnt5a levels were also lower (Fig.19). It is not clear what is the relationship between Cx43 and Wnt5a, Whether Cx43 regulates Wnt5a or the opposite can not be determined, however, it seems that there is a correlation in the expression patterns of the two proteins which has implications on gap junctional communication and is relevant for mammary gland function.
**Figure 19.**

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**Figure 19. Wnt5a levels are lower in the mammary glands of Tg mice.** Western Analysis and quantification of Wnt5a levels in mid-pregnant WT and Tg littermates revealed that Wnt5a levels are lower in the glands of Tg mice compared to their WT littermates. β-actin was used to determine equal loading.
4.7 Integrin Expression in the Mammary Glands of Tg Mice

Previous studies have shown that the expression of α3β1 integrin is essential for the contractile activity of myoeps, both in cultured primary cells and in a mouse model lacking expression of the α3 subunit. I tested whether the expression of integrins is altered in the mammary glands of K5-Cx26 mice compared to their WT littermates and found that while β1 integrin levels are unchanged, α3 integrin levels are lower in the glands of K5-Cx26 mice in lactation. The former study identified the FAK/Pak/rac pathway in mediating the effect of α3 loss in the myoeps (26). Investigation of the levels of signals downstream from α3 integrin in cultured primary organoids from WT and Tg animals did not show any significant differences in the levels of these proteins upon treatment with oxytocin. It is possible that α3 levels are regulated in response to the loss of contractile activity in the K5-Cx26 myoeps and that the down-regulation of α3 occurs as a feedback mechanism due to the lack of engagement of the contractile apparatus in the Tg myoeps (Fig.20).
Figure 20. Integrin levels in the mammary glands of Tg mice. Western analysis of β1-integrin levels and α3-integrin levels revealed no difference in the levels of β1-integrin between the glands of WT and Tg nulliparous, mid-pregnant (P.14) and early lactating (L.2) littermates. However, the levels of α3-integrin were lower in the glands of Tg mid-pregnant and early lactating mice. Ponceau staining indicates equal loading.
4.8 MMP-7 Expression in the Mammary Glands of Tg Mice

A previous study found that Cx43 levels were correlated to presence of matrix metalloproteinase-7, matrilysin. In fact the study found that MMP-7 affects the levels of Cx43 and electrical conduction between myocardial cells after myocardial infarction. MMP-7/- mice had higher levels of Cx43 after myocardial infarction and better survival (138). I tested the levels of MMP-7 in the mammary glands of WT and Tg littermates and found that MMP-7 are significantly higher in the glands of K5-Cx26 mice. This is interesting because MMP-7 was found to regulate Cx43 protein levels and not expression of the gene. Thus, the increased levels of MMP-7 could account for the observed down-regulation in Cx43 protein (Fig.21).
Figure 21. MMP-7/ Matrilysin levels are lower in the glands of K5-Cx26 mice. Q-PCR analysis of MMP-7 mRNA levels in the glands of WT and Tg nulliparous littermates revealed significantly higher levels of MMP-7 mRNA in the glands of Tg mice compared to their WT littermates. 18s rRNA was used as an internal control for mRNA levels.
Chapter 5. Discussion
Intercellular interaction and communication via GJs is essential for normal mammary function \((108, 109)\). In the mammary gland, Cx distribution is asymmetric, with Cx26, Cx30, Cx32 expressed in the luminal epithelium and Cx43 in the myoeps \((105, 107)\). Despite the fact that many tissues express two or more members of the Cx family and that two Cxs can be co-expressed in the same cell, there is little functional overlap between Cx subtypes. The need for molecular heterogeneity of GJ channel proteins in vivo is not surprising: Cx-pairing specificity dictates which cell types are able to communicate via GJs, and, modulates the type and rate of molecules passing through GJs \((54, 127)\).

Deciphering the unique functions of Cx subtypes has been possible with mouse knock-in studies that enabled the exchange of Cx subtypes with each other. In the mammary gland, knock-in of Cx26, Cx32 and Cx40 in place of Cx43 revealed that only Cx40, but not Cx26 or Cx32, can functionally substitute for Cx43 loss without altering mammary function \((115)\). While the lethality of Cx43 deficient mice was rescued in these knock-in mouse models, Cx43KICx40, Cx43KICx32 (Cx4332/32) and Cx43KICx26 (Cx4343/26) differed functionally and morphologically from each other and from wild-type mice \((115, 116)\). Moreover, the inter-Cx substitutions exhibited different and tissue-specific phenotypes. For example, while Cx40 was able to substitute for Cx43 in the mammary gland, Cx40KICx43 mice presented with a variety of defects, including male infertility due to incomplete spermatogenesis and hypotrophic testis. Cx32 however, was unable to functionally replace Cx43 in both tissues. Interestingly the mammary phenotypes of Cx4343/26 and Cx4332/32 mice also differed from each other, suggesting that the effects of Cx-substitutions are not only tissue-specific, but that within the same tissue they are also Cx-dependent \((116)\).

In this study I revealed the consequences of mis-expressing a luminal Cx in the myoepithelium. Using a K5-Cx26 transgenic mouse model, I showed that mammary glands of K5-Cx26 female mice develop normally but the transgenic females are unable to feed their pups, leading to litter starvation and demise in early to mid-lactation. Furthermore I showed that the mammary impairment is not caused by deficient milk protein expression but by a defect in milk delivery to the pups. Surprisingly, I found that ectopic expression of Cx26 to the myoeps alters the expression of endogenous Cx43 and inhibits GJ-mediated dye coupling in myoeps expressing high levels of Cx26. However, ectopic expression of Cx26 in the myoeps of K5-Cx26 did not interfere with mammary gland development from birth until parturition, despite Cx26 acting as a dominant negative to Cx43 protein during these stages. This is in contrast to the Cx4343/26 mouse whereby the replacement of one allele of Cx43 by Cx26 leads to defective alveolar expansion preventing Cx4343/26 dams from making sufficient amounts of milk for feeding the pups \((116)\). Interestingly, the K5-Cx26 mammary phenotype is strikingly similar to the Cx4332/32 phenotype where both copies of Cx43 are replaced by Cx32. In both cases, the mammary gland develops normally but the mice are unable to deliver the milk to their pups. These findings together suggest that Cx43 has a complex relationship during mammary gland development, but
that it has a very specific role after parturition, in particular for milk delivery. Cx43 seems to be required for myoepithelial contraction and other Cxs are not able compensate for this function. Loss of Cx43 in other contractile tissues, such as in the uterine myometrium has been implicated in decreased contraction in response to oxytocin. In particular, expression of a mutant Cx43 in the uterine myometrium of the Gja1(Jrt)/+ mouse model, caused delayed parturition the mouse and the birth of suffocated pups (117). The delayed and defective parturition was attributed to decreased contractions in myometrial smooth muscle cells, and the contractile impairment correlated with lower gap junctional communication between myoepithelial cells to about 15% relative to their wild type counterparts. Loss of gap junctional coupling was caused by a dominant-negative interference of the mutant Cx43 with the endogenous protein (117).

This is the first report of a dominant-negative interaction between WT Cx26 and Cx43 in vivo. I showed that Cx43 mRNA expression is not affected by the ectopic expression of Cx26, but that the protein levels of Cx43 are significantly lower compared to WT littermates, and Cx43 is not phosphorylated. Interestingly, Cx26 and Cx43 are co-expressed in many tissues and in cultures cells, but to date they have not been shown to assemble into heteromeric hemi-channels. Cx26 and Cx43 proteins are structurally distinct, and whereas Cx43 has been shown to have multiple phosphorylation sites, Cx26 remains unphosphorylated and has a significantly shorter carboxy-tail compared to other Cx isotypes, and particularly relative to Cx43 (62). Different studies have revealed variable routes of shuttling of Cx26 and Cx43 to the membrane. One hypothesis suggests that Cx26 is spatially segregated from Cx43 and cannot oligomerize with it en route to the membrane(47, 48). A study using Cos and HeLa cells showed that targeting of Cx43-GFP to GJs was inhibited in cells treated with Brefeldin A, a drug that disassembles the Golgi network. However, GJs constructed of Cx26-GFP were only minimally affected by Brefeldin A, but were sensitive instead to Nocodazole treatment, suggesting that Cx26 shuttles to the membrane via microtubules rather than through the secretory pathway and confirming the proposed hypothesis of alternate routing (46). However, another study using HEK and HeLa cells found that pharmacological treatment with Brefeldin A or nocodazole affected both Cx26 and Cx43 similarly suggesting that both isotypes followed similar routes of cellular trafficking and assembly into GJs (126). In contrast, yet another study using NRK cells revealed that disruption of microtubules with nocodazole inhibited the recruitment of Cx43-GFP into GJs but had limited effect on the transport and clustering of Cx26-YFP into GJs within the photobleached regions of cell-cell contact suggesting that Cx43 shuttles using the MT network, which is plausible given that Cx43 has a tubulin binding site (50-52). The conflicting findings from these studies point to a more complex regulation of these two proteins in the different cell systems and culture conditions examined, and suggest that there are other factors involved in the trafficking control of these two proteins. Another striking feature of these two Cx isotypes is that their co-expression in the same cell was found to reduce the total intercellular junctional
conductance to a little more than 10% of that in cells expressing only a single Cx (either Cx26 alone or Cx43 alone) (126). The presence of incompatible connexons might be behind the observed reduction of GJIC by preventing functional docking of apposing hemi-channels and causing the accumulation of uncoupled hemi-channels at the intermembrane junction. Alternatively, the lowered conductance might be caused by negative interference between the adjacent connexons which recruit gating regulators away from the nearby channels resulting in uncooperativity between the distinct channels at the GJ plaque region. The second hypothesis possibly accounts for the phenotypes observed in K5-Cx26 myoeps. Alternatively, it is possible that Cx26 is able to reach the membrane in K5-Cx26 myoeps but that channels remain closed, or that they are impermeable for specific molecules that are important for electrical conductance and the spread of the depolarization wave during oxytocin-mediated contraction. I have not determined how Cx26 causes down-regulation of Cx43 protein in the mammary myoeps of K5-Cx26 mice, but the findings emphasize the importance of segregated distribution of GJ proteins in the mammary gland, and further highlight the important role of Cx43 and gap junctional communication in contractile function of mammary myoeps.

The most striking feature of the K5-Cx26 mouse mammary gland is its ability to develop normally and attain its functional milk-synthesizing capacity despite the altered levels of a number of molecules including E-cadherin, β-catenin and Wnt5a and α3-integrin. Although further studies will be needed to pin-point how the differences in levels of these proteins affect the mammary gland of the K5-Cx26 mouse, it is obvious from this study and others that signaling networks are integrated intricately in the mammary gland to control lactation and milk delivery to the pups. This work ties with the general hypothesis in the Bissell laboratory that signaling pathways in the tissue are intricately regulated and integrated in a dynamic and reciprocal fashion to achieve tissue-specific function. How are signals relayed between all these molecules? This is yet to be investigated, but this study has identified potential novel cross-talks between pathways that control mammary gland contractile function (Model proposed in scheme below).
Model of known and potential interactions regulating contractile function in the mammary gland.
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