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Gap junctional intercellular communication in quiescent and heterocellular mammary populations

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degree of Master of Science

***Short Title**: GJIC in quiescent and mixed mammary cell populations*

Abstract:

Gap junctions are protein transmembrane channels formed between apposing cells whose primary role is in mediating intercellular communication. This study addressed 1) the role of gap junctions in mammary epithelial cells during the transition from proliferation to quiescence, and 2) the mechanism of gap junction formation between different mammary cell types (heterocellular gap junctions). In the first part, proliferating mammary epithelial cells were induced to enter quiescence by serum deprivation or contact inhibition. Using Western blotting, we observed a dramatic reduction (up to 20-fold) in the expression of the gap junction protein, connexin43 (Cx43), during quiescence. Microinjection of the dye Lucifer yellow, however, revealed that there was no corresponding loss in gap junctional communication. Immunofluorescent staining for Cx43 revealed that intracellular pools of Cx43 were redistributed to the cell surface, possibly to maintain intercellular coupling. In the second part of our studies, the role of the cell adhesion molecule, E-cadherin, was studied in order to determine its role in the formation of heterocellular gap junctions between mammary fibroblasts and epithelial cells. Transfection of E-cadherin into fibroblasts was insufficient to increase the incidence of intercellular communication between the two distinct cell types. Interestingly, an intermediate cell type with properties of both epithelial cells and fibroblasts was able to bridge the communication gap. These studies may lead to insight into the role of gap junctions in mammary gland growth, development, and neoplasia.

Résumé:

Les jonctions GAP ou communiquantes sont des protéines transmembranaires formant des canaux entre cellules voisines. Leur principal rôle est d'établir une communication intercellulaire. Cette étude porte sur: 1) le rôle des jonctions communiquantes dans les cellules épithéliales mammaires pendant la transition de la phase proliférative à la phase de quiescence 2) le mécanisme de formation des jonctions communiquantes entre différentes types de cellules mammaires (jonctions communiquantes hétérologues). Dans la première partie, des cellules épithéliales mammaires sont rendues quiescentes par déprivation de serum ou inhibition de contact. Par des expériences d'immunoblot, nous avons observé une réduction (supérieure à 20 fois) de l'expression de la protéine de jonction communiquante, connexin43 (Cx43) pendant la phase de quiescence. Des expériences de microinjection de colorant jaune Lucifer ont cependant révelé qu'il n'y avait pas de perte correspondante de la communication intercellulaire. Dans la deuxième partie de notre étude, le rôle de la molécule d'adhésion E-cadhérine a été analysé afin de déterminer sa fonction dans la formation de jonctions communiquantes entre des fibroblastes et des cellules épithéliales mammaires. La transfection de E-cadhérine dans des fibroblastes n'a pas été suffisante pour accroître la communication intercellulaire entre les deux types cellulaires. Cependant, un type cellulaire hybride entre les cellules épithéliales et les fibroblastes était capable de générer des ponts de communication. Ces études nous permettent de mettre en évidence un rôle des jonctions communiquantes dans la croissance, le développement et la néoplasie de la glande mammaire.

for my loved ones

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I would like to extend my thanks to Dr. Dale Laird for his support, guidance and teaching. Dale has been a model of hard-work, integrity, and fairness and I could not have asked for a better supervisor. Under his tutelage, I have matured as a scientist and person and I have acquired "insight" that will continue to shape my life.

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To my family and friends, I am always grateful for your presence, support and love.

AMDG.

Preface:

All experimental work was performed by Michael A. Sia, except for the following:

1) In the first study (Chapter 2):

a) [^{3}H]thymidine incorporation was performed by Terry L. Woodward.

b) Significant revisions to the manuscript were made by Terry L. Woodward and Dale W. Laird.

2) In the second study (Chapter 3):

a) E-cadherin transfection, and Western blotting using the bovine mammary gland and mouse heart (Fig. 3) were performed by Terry L. Woodward.

b) The data presented in Fig. 4 (Western blotting and microinjection of Lucifer Yellow in homocellular populations) was also performed by Terry L. Woodward.

c) An initial draft of the paper was written by Terry. L. Woodward. However, substantial additions/revisions were made by Michael A. Sia in the Materials and Methods and Results sections. Additional advice and guidance were provided by Dale W. Laird.

The cell lines were generated in the laboratory of Jeffrey D. Turner (Chapter 2 and 3) and Orest W. Blaschuk (Chapter 3) provided us with reagents.

Chapter 1 of the thesis is a literature review covering a general overview of gap junctions and the main topics relevent to the studies performed in the thesis. Chapters 2 and 3 are written in manuscript form. Chapter 2 has been submitted for publication, as will Chapter 3 in the next few months. Chapter 4 is a concluding summary of the results and the contributions made to original knowledge.

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Chapter 1

LITERATURE REVIEW

1.1 Introduction

Cell junctions play critical roles in providing cells with the structural framework to interact with each other and with the extracellular matrix, in providing resistance to mechanical or shearing forces, and in permitting communication between cells (Alberts et al., 1994). There are three main functional groups of junctions: 1) occluding junctions which form impermeable or semipermeable barriers between the plasma membranes of adjacent cells; 2) anchoring junctions which are responsible for cell-cell or cell-matrix attachment; and 3) communicating junctions which permit the passage of electrical or chemical signals between cells (Alberts et al., 1994).

Gap junctions are the predominant communicating junction found in animal cells. Gap junctions are specialized transmembrane structures which permit the passage of ions, secondary messengers, and other small molecules to pass between closely apposed cells (reviewed, Bruzzone et al., 1996) . The passage of small molecules plays a role in mediating electrical and chemical coupling between cells. Defects in the gap junction proteins, termed connexins, or defects in connexin expression have been implicated in several pathological conditions such as cardiac ischemia and hypertrophy, Charcot-Marie-Tooth disease and visceroatrial heterotaxia syndrome (reviewed, Kumar and Gilula, 1996).

Intercellular communication via gap junctions has also been demonstrated to be involved in growth regulation and development and the loss of gap junctional communication has been

correlated with tumorigenesis (reviewed, Kumar and Gilula, 1996) . With the growing evidence that gap junctions play a role in maintaining cellular homeostasis and that loss of normal connexin expression and gap junction intercellular communication result in abnormal conditions, it has become more apparent that a greater understanding of gap junctions may provide therapeutic benefits. In this thesis, the roles of gap junctions and gap junction intercellular communication in cell proliferation and development will be addressed in quiescent and heterocellular mammary gland populations.

1.2 The Biological Importance of Gap Junctions

1. The role of gap junctions in normal tissues:

Gap junctions are transmembrane channels which allow small molecules (less than 1000 daltons) and secondary messengers such as cAMP to pass between cells (Caspar et al., 1977; Makowski et al, 1977). The passage of small molecules allows the junctions to mediate electrical and chemical coupling. For example, the synchronization of cardiac myocytes (Rudy and Quan, 1987; Cole et al., 1988; Spray and Burns, 1990), the peristaltic movements of the intestine (Burns, 1992), and myometrial contractions during labour (Risek et al., 1990; Winterhager et al., 1991) are all mediated by gap junctions. Gap junction intercellular communication (GJIC) plays a role in growth regulation by tumor suppression and in embryogenesis by allowing differentiating cells which will follow a similar developmental

pathway to remain coupled even if they have uncoupled from their surrounding tissue. The roles of gap junctions in cell growth and development are discussed in greater detail below. The permeability of gap junctions is regulated by cytosolic concentrations of free Ca^{2+}, voltage, and cytosolic pH (Rose and Loewenstein, 1975; Spray et al., 1981; Arellano et al., 1990).

2. Gap junction involvement in disease:

Aberrations in gap junction formation have been associated with several pathological conditions. The involvement of gap junctions in cardiac pathologies is especially well characterized. Disturbances in connexin expression have been associated with abnormal impulse propagation in hypertrophic and ischemic hearts (Kumar and Gilula, 1996). In knockout mice where the Cx43 gene was disrupted, the mice developed to term, but died shortly after birth as a result of defects resulting in a condition resembling congenital pulmonary stenosis in humans (Reaume et al., 1995). Other evidence exists which indicates that Cx43 plays a role in the development of the heart; Cx43 mutations have been linked to visceroatrial heterotaxia, a congenital condition that arises from defects in the left-right patterning of the heart (Britz-Cunningham et al., 1995). It has also been shown that following myocardial infarction, remodelling of the myocardium occurs. The cardiac myocytes will dedifferentiate to more closely resemble the phenotype of newborns and the remaining myocardium (i.e. connective tissue) will proliferate (Holtz, 1993). It is suggested that unlike newborn myocytes with their high density of gap junctions, hypertrophied dedifferentiated

cardiac myocytes are more susceptible to arrhythmias due to reduced gap junctional communication. In addition to cardiac diseases, gap junctions have also been implicated in Charcot-Marie-Tooth (CMT) disease which affects motor and sensory nerve function (Bergoffen et al., 1993; Paul, 1995; Spray and Dermietzel, 1995). The X-linked form of CMT (CMTX) involves abnormalities in the coding region of Cx32 that affect channel activity and result in defective Schwann cell function. Finally, the loss of gap junctional communication has been shown to be involved in tumorigenesis, a topic that is later addressed when looking at the role of gap junctions in growth regulation.

1.3 The Structure of Gap Junctions

1. Gap junction morphology:

Gap junctions were originally characterized in the 1960's by electron microscopy as structures formed at the close apposition of plasma membranes separated by a distinct 2-4 nm gap (Robertson, 1963; Revel and Karnovsky, 1967). The structures were subsequently identified using freeze-fracture replicas as dense arrays of intramembrane particles on the P-fracture face with complimentary arrays of pits on the E-fracture face (Kreutziger, 1968; Goodenough and Revel, 1970). Using x-ray diffraction techniques, several studies led to the construction of a model for gap junctions whereby each cell contributes a hemichannel, a connexon, that pairs with its counterpart from a neighboring cell to form a complete transmembrane channel (Caspar et al., 1977; Makowski et al., 1977). Gap junctions are

characterized by a hierarchical organization. Each connexon is itself an oligomer of six connexins, a family of homologous transmembrane proteins. Once connexons have paired, complete gap junction channels cluster at the cell surface and form gap junction plaques, a process that is believed to increase channel stability (Loewenstein, 1981; Yamaski, 1990; reviewed, Bruzzone et al., 1996).

2. The connexin family:

The simplest subunit of gap junctions is termed the connexin (Beyer et al., 1987), a ubiquitous nonglycosylated integral membrane protein, which varies in molecular weight and tissue distribution. Most cells in normal tissue express connexins; the sole exceptions tend to be terminally differentiated cells (skeletal muscle, erythrocytes, and circulating lymphocytes). There are currently thirteen known members of the multigene connexin family in rodents (reviewed, Kumar and Gilula, 1992; Bruzzone et al., 1996). There are two nomenclature systems used to identify connexins; the more commonly used system is based on the predicted molecular weight of the connexin polypeptide (Beyer et al., 1987), and the second is based on evolutionary considerations (Kumar and Gilula, 1992). Connexin32 (Cx32) and connexin43 (Cx43) are two of the more commonly expressed forms in the family of homologous transmembrane proteins (reviewed, Bruzzone et al., 1996).

Connexins appear to have a characteristic topology as demonstrated by immunolocalization studies using site-directed antibodies combined with membrane protection

assays (Beyer et al., 1987; Milks et al., 1988; Hertzberg et al., 1988; Beyer et al., 1989, Yancey et al., 1989; Laird and Revel, 1990). The connexin polypeptide traverses the plasma membrane four times forming a cytoplasmic loop, carboxy and amino termini found in the cytoplasm, and two extracellular loops linked by at least a single disulfide bond (and potentially three in total). The extracellular loops and the transmembrane domains of connexins have the greatest homology, whereas the cytoplasmic loop and carboxy terminus have the greatest variability suggesting that these latter regions may be important for regulation (Kumar and Gilula, 1996). Moreover, gap junctions formed by different connexins have also been shown to have different permeabilities (Brissette et al., 1994; Elfgang et al., 1995), thus enabling cells expressing different connexins to regulate selective responsiveness to certain metabolites or secondary messengers.

3. Heteromeric and heterotypic gap junctions:

Typically, connexons are homomeric in that they are composed of a single type of connexin, but several studies suggest that heteromeric conexons may also form in cell types that express more than one connexin (reviewed, Bruzzone et al., 1996). The earliest studies which reported the formation of heteromeric connexons involved the infection of SF9 insect cells with baculoviruses coding for Cx32 and Cx26 (Stauffer et al., 1991; Buehler et al., 1995; Stauffer, 1995). Recent studies *in vivo* have found that heteromeric channels form in chick lens fiber cells (Jiang and Goodenough, 1996) and bovine lens fiber cells (Konig and Zampighi, 1995). The role of heteromeric connexons is undetermined; they may simply be the

result of fortuitous events or they may participate in the regulation of intercellular communication in a manner distinct from homomeric connexons.

In addition to heteromeric connexons, the composition of gap junctions is further complicated by the presence of heterotypic gap junctions. Heterotypic gap junctions are formed by two homomeric connexons which are each composed of a different connexin. The formation of heterotypic channels is dependent on the compatibility between connexins (reviewed, Bruzzone et al., 1996). For example: 1) Cx31 is only functional in homotypic channels; 2) Cx40 has been found to only functionally interact with Cx37; and 3) Cx46 has been found to associate with several connexins. The compatibility between connexins may represent an additional regulatory mechanism governing the interactions between different cells in the formation of selective communication compartments. The importance of selective interactions will be further addressed when looking at the role of heterocellular gap junctions, channels between two different cell types.

1.4 The Life Cycle of Connexins

1. Trafficking:

Connexins are co-translationally imported in the endoplasmic reticulum (ER) and subsequently follow the classical secretory pathway (Falk et al., 1994). One possible

exception to co-translational import in the ER is Cx26 which has additionally been shown to post-translationally insert into microsomal membranes with a native orientation (Zhang et al., 1996). The mechanism of connexin oligomerization and the compartment(s) within which this occurs is, however, unique to gap junctions (Musil and Goodenough, 1993). In the case of other oligomeric proteins such as the T Cell receptor, the acetyl choline receptor and influenza hemagluttinin trimers, oligomerization occurs in the ER (Hurtley and Helenius, 1989). There is no conclusive data supporting connexin oligomerization in the ER, and in fact, Musil and Goodenough (1993) suggest that oligomerization occurs in the *trans* Golgi Network (TGN). Musil and Goodenough provided evidence in normal rat kidney cells using sucrose gradient velocity sedimentation, secretory transport blocks, and chemical cross-linking that connexin43 oligomerization occurs in a late secretory compartment which they proposed to be the TGN.

The transport of connexons from the TGN to the plasma membrane remains poorly understood, but it is believed to occur via the classical vesicular transport mechanism. Once at the cell surface, connexons will rapidly dock and form gap junction channels, a process that may be modulated by cell adhesion molecules, such as the cadherins (Mege et al., 1988; Musil et al., 1990b; Jongen et al., 1991; Meyer et al., 1992; Fujimoto et al., 1997). The gap junction channels will then migrate laterally within the plasma membrane to cluster and form gap junction plaques. The mechanism of internalization and subsequent degradation of gap junctions also remains obscure. The current evidence, mainly provided by electron microscopy, suggests that the entire gap junction is taken up into one of the donor cells as a

double membrane structure, an annular junction (Dermietzel et al., 1991; Risley et al., 1992; Naus et al., 1993). The degradation of internalized gap junctions may involve several mechanisms. The presence of connexins in lysosomes has been shown in several studies (Ginzberg and Gilula, 1979; Murray et al., 1981; Naus et al., 1993) and more recently, it has been shown that proteasomes may also be involved in connexin degradation (Laing and Beyer, 1995). The mechanism by which lysosomes and and proteasomes interact with connexins and the sequence of events involved in degradation remain to be determined.

2. Turnover:

The turnover of gap junctions is a dynamic process with a reported half-life of 5 h in the mouse liver (Fallon and Goodenough, 1981) and half-lives from 1.5-3.5 h in *in vitro* cell lines (Traub et al., 1987; Laird et al., 1991; Musil et al., 1990 a,b; Laird et al., 1995). Consequently, as a result of the dynamic nature of connexin assembly and disassembly, cells can respond to external stimuli by altering the number of functional channels.

1.5 The Role of Cadherins in Gap Junction Assembly

Gap junction formation is expected to be precisely regulated. As previously suggested, one possible family of molecules involved in gap junction formation may be the cadherins, calcium-dependent cell adhesion receptors, which bind cells by means of homophilic

interactions (reviewed, Takeichi, 1991). One of the more commonly expressed members of the cadherin family, E-cadherin, is a 120 kD transmembrane glycoprotein involved in the regulation of epithelial junction formation, morphogenesis, and cell-sorting. E-cadherin is primarily found in the adherens junctions of epithelial cells and interacts with the cytoskeleton through cytoplasmic proteins known as catenins that are essential for proper E-cadherin function (Boller et al., 1985; Behrens et al., 1985, Nagafuchi and Takeichi, 1988; Ozawa et al., 1989, 1990; Ozawa and Kemler, 1992). The loss of E-cadherin expression has been correlated with phenotypic alterations in epithelial cells and increased invasiveness in poorly differentiated carcinomas (Behrens et al., 1989; Schipper et al., 1991, Frixen et al., 1991).

E-cadherin or other cadherins may be responsible for the initial adhesion event that allows cells to recognize each other as homologous partners (Jongen et al., 1991), thereby bringing the plasma membranes of neighboring cells in close apposition such that regions can be established where connexon docking can occur. These regions would themselves exclude the presence of the cadherins since the size of the extracellular domain of the cadherin molecule is incompatible with a gap of 2-4 nm. In cell populations of the same type, there appears to be a direct link between cadherin expression and gap junction formation. In regenerating hepatocytes following partial hepatectomy, there is an initial decrease in the number and size of gap junctions, but between 48 to 60 hours postoperatively, gap junctions will reappear. Fujimoto and colleagues (1997) have recently shown using double-immunogold electron microscopy that the reappearance of gap junctions co-localizes with E-cadherin or alpha-catenin at cell-cell contact sites, implying that the cadherin-catenin complexes act as foci for

gap junction formation.

Studies have shown that mouse sarcoma 180 (S180) cells which failed to assemble gap junctions despite the expression of Cx43 acquired the ability to form functional gap junctions after transfection with E-cadherin (Mege et al, 1988; Musil et al., 1990). These reports also demonstrated that Fab' fragments from anti-E-cadherin antibodies disrupt gap junctions. Another study (Jongen et al., 1991) showed that calcium-dependent regulation of gap junctional communication is controlled by E-cadherin in mouse epidermal cells. The data obtained by Jongen and co-workers supported this conclusion based on two main lines of evidence: firstly, both E-cadherin expression and GJIC were Ca^{2+}-dependent and increases in GJIC induced by Ca^{2+} were accompanied by increases in the expression of E-cadherin; secondly, transfection of E-cadherin into mouse epidermal cells which had very low E-cadherin expression and low GJIC (independent of the Ca^{2+} levels in the media), resulted in a stable Ca^{2+}-dependent expression of E-cadherin as well as Ca^{2+}-dependent regulation of GJIC. Evidence for the involvement of another member of the cadherin family, N-cadherin, in gap junction formation was provided by Meyer and colleagues (1992) who were able to prevent gap junction assembly in reaggregating Novikoff cells using Fab' fragments from antibodies against N-cadherin.

The role of cadherins has, however, been made more complex by the finding that poorly-adhesive mouse L cells transfected with exogenous cadherins acquire strong cell-cell adhesion, but GJIC is inhibited in a glycosylation-sensitive manner (Wang and Rose, 1997).

The researchers in accordance with other authors (Doherty and Walsh, 1994; Miner et al., 1995; Fujimoto et al., 1997) suggest that intracellular signaling cascades resulting from cadherin mediated interactions may affect gap junction function and communication. Interestingly, ß-catenin, typically thought of as a cadherin associated molecule, has also been shown to regulate GJIC in *Xenopus* laevis embryos in a manner which cannot be accounted for simply by adhesion (Guger and Gumbiner; 1995). Therefore, the researchers proposed that there is a possible signaling role for ß-catenin.

1.6 Heterocellular Gap Junctions

Interactions between cells of different lineages is an important, though relatively uncommon event. Organs of the body consist of several types of tissues and may require coordinated control of growth and function. During development, cells of different lineages or following different developmental pathways are in direct contact with one another. In both instances, i.e. organ function and development, the formation and activity of heterocellular gap junctions (junctions between two different cell types) are involved. The high sequence homology of the transmembrane and extracellular regions of connexins may explain the finding that different cell types in one tissue, or cells from different tissues can be coupled to each other through gap junctions (Rook et al., 1992; reviewed, Bruzzone et al., 1996).

Gap junctions appear *in vivo* and *in vitro* between sinus node myocytes and fibroblasts, the

two main cell populations of the heart, and may play a role in the mechanical stretching of the wall of the right atrium (Rook et al., 1992; Kohl et al., 1994). Two myocytes can be separated by fibroblasts and remain electrically coupled via the fibroblasts to produce synchronous beating. Coculture systems composed of a fat-storing cell clone (CFSC-2G) and fresh isolated hepatocytes have also been developed *in vitro* to mimic the *in vivo* relationships found in the liver (Rojkind et al., 1995). More specifically, functional gap junctions form between fat-storing cells and hepatocytes. One role for heterocellular communication is to permit one cell type to influence the behaviour of another cell type. For instance, follicle cells exposed to an external stimulus may play a nutritional role by transferring regulatory molecules to oocytes, thus also allowing the oocytes to respond to the stimulus despite the lack of direct exposure (Schultz, 1985). An elegant study by Simon and colleagues (1997) has recently shown that heterocellular communication between oocytes and granulosa cells is critical for normal oogenesis with loss resulting in female infertility.

In other biological tissues, there may be necessary restrictions to the formation and function of heterocellular gap junctions. Early studies by Fentiman and colleagues (1976) addressed the question of whether or not epithelial cells and fibroblasts from the same tissue, such as the mammary gland, can interact directly and whether such an interaction could play a role in the development of epithelial tumors such as carcinomas of the breast. In the normal adult breast, epithelial cells and fibroblasts are separated by a basement membrane, but lose this restriction during tumor invasiveness. The researchers showed that mixed cultures of human fibroblasts (HumF) in direct contact with epithelial cells (HumE) selectively failed to

establish heterocellular communication even in the absence of a basement membrane. The researchers proposed that during the development of neoplasia, the two cell types may lose the specificity governing their interactions, thus leading to abnormal growth. However, in contrast to this study, a later study using human normal mammary epithelial cells (NMEC) and normal mamary fibroblasts (NMF) found excellent GJIC between the two cell lines (Tomasetto et al., 1993). The third chapter of my thesis will also examine GJIC between epthelial cells and fibroblasts in the mammary gland.

One consequence of restrictions on heterocellular gap junction formation is the sorting of epithelial cells and fibroblasts into separate domains, thus establishing communication compartments (Pitts and Kam, 1985). These communication compartments result in the ability to form concentration gradients within populations of cells and sharp concentration differences at heterocellular boundaries. One important application of this ability is in creating positional patterning during development, i.e. in regulating morphogenetic movements in embryogenesis.

Similar to the presence of heterocellular gap junctions between differentiating cells during embryonic development, heterocellular junctions are also present during tumorigenesis when cells may dedifferentiate and alter their phenotypes. A further analogy can be drawn between development and tumorigenesis, in that the expression of E-cadherin during development is critical for the regulation of morphogenetic movements, whereas the loss of E-cadherin expression appears to be one of the hallmarks of metastasis.

In contrast, cell adhesion has also been shown to be important in the formation of heterocellular gap junctions between metastatic tumor cells and vascular endothelium (El-Sabban and Pauli, 1991; 1995). The primary step in the colonization of organs by blood-borne cancer cells appears to be adhesion to the vascular endothelium. Adhesion is subsequently followed by the formation of functional gap junctions. The extent of communication between cancer cells is mediated by two factors, the expression levels of the same connexin and of compatible adhesion molecules. As with homocellular gap junctions, it thus appears that cadherins allow cells to recognize each other for proper channel formation. Whether or not the presence of compatible adhesion molecules is the sole factor that allows or restricts the formation of gap junctions betwen different cell types within the same tissue remains to be elucidated.

1.7 The Role of Gap Junctions in Growth Regulation

1. Transfection of connexins in communication-deficient cell lines:

A series of *in vitro* transfection experiments increasing gap junction expression in communication-deficient C6 glioma cells was performed whereby dramatic effects on cell coupling, growth and differentiation were observed (Zhu et al., 1991). Following transfection with Cx43 cDNA, there was a reduction in the proliferation of C6 cells and a phenotypic transformation to more closely resemble their normal progenitors (Naus et al., 1992). The

results suggest that intercellular coupling plays a role in maintaining normal cellular phenotype and possibly in the secretion of soluble growth-regulatory factors (Zhu et al., 1991). A similar transfection experiment performed using communication-deficient transformed mouse 10 T½ cells also demonstrated a restoration of junctional communication and inhibition of cell growth after transfection with Cx43 cDNA (Mehta et al., 1991).

Transfection experiments have also been carried out in human hepatoma cells (SKHep1) with cDNA encoding for Cx32 (Eghbali et al., 1991). The growth rates of the SKHep1 cell line were tested in culture and *in vivo*, as tumors in nude mice. While the growth rates of parental cells and transfected clones were similar when grown in culture, the growth rates varied *in vivo*. Tumors in nude mice were significantly slower in growth between well-coupled cells transfected with Cx32 than in poorly coupled cells. The results also support the notion that strong coupling impedes tumor growth, and conversely that reduction of functional gap junctions may directly contribute to the growth of tumor cells *in vivo* (Eghbali et al., 1991). Similarly, transfection studies with Cx26 and Cx43 in a human mammary carcinoma cell line (MDA-MB-435) confirmed the growth regulatory properties of gap junctions, but also demonstrated an additional role throught the restoration of differentiation potential (Hirschi et al., 1996). Mammary carcinoma cells transfected with Cx26 or Cx43 regained the capacity to form three-dimensional structures in a matrigel matrix, an ability characteristic of normal differentiated mammary cells, indicating that GJIC is involved in the morphogenesis of mammary gland structures.

2. Identification of gap junctions as putative tumor-suppressors:

A positive selection system was designed using subtractive hybridization to identify candidate tumor-suppressor genes (Lee et al., 1991). These researchers were able to show that tumor-derived human mammary epithelial cells differ from their normal counterparts by the lack of expression of several genes including a gene encoding Cx26 (Lee et al., 1992). Several studies have also shown that gap junction intercellular communication is also inhibited in response to tumor promoting agents such as TPA and oncogenes that encode tyrosine kinases (Atkinson et al., 1981; Azarnia and Loewenstein, 1984a;b; Chang et al., 1985; Crow et al., 1990; Swenson et al., 1990; Oh et al., 1991).

The results of these studies, in addition to the transfection experiments, support the hypothesis that gap junction communication plays a role in growth control. However, details of how gap junction communication reduces cell proliferation are not well understood.

1.8 Gap Junctions during the Cell Cycle

The role of gap junctions in growth regulation may be critically linked to their activity during the cell cycle, the basic unit of cell growth. Several studies have demonstrated alterations in gap junctions and GJIC during the cell cycle. Early freeze-fracture studies by Yee and Revel (1978) revealed morphological changes in gap junction plaques in the rat liver

following partial hepatectomy which induced hepatocytes to enter a state of proliferation. Later studies by Dermietzel and colleagues (1987) using immunofluorescent staining against Cx32 demonstrated differences in gap junctions between quiescent and proliferating hepatocytes. In both partially hepatectomized rat liver and proliferating cultured hepatocytes, reductions in Cx32 immunostaining were observed relative to their quiescent counterparts. These researchers suggested that the quantitative changes in gap junction expression may play a role in controlling proliferation in the liver. An alternate study in rat tracheal epithelium also revealed changes in gap junctions during specific stages of the cell cycle (Gordon et al., 1982). Mitotically inactive tracheal epithelium was characterized by the lack of gap junctions. However, when the epithelium was stimulated to proliferate, gap junctions were formed by the end of the DNA synthetic (S) period and subsequently disappeared during mitosis. The appearance and disappearance of gap junctions during specific phases of the cell cycle was an initial indication that gap junctions may play a role in the control of mitotic activity.

Several studies assaying for modifications in GJIC have also reported cell cycle correlated changes. Goodall and Maro (1986) assessed junctional coupling in mouse embryogenesis by dye injections and measurement of electrical continuity between cells. In early 8-cell mouse embryos, there is a loss of GJIC as the blastomeres enter mitosis which is only recovered during interphase of the 16-cell stage. Dye coupling has also been observed to be cell cycle modulated in early *Xenopus* laevis development (Su et al., 1990). In a clonal cell line of spontaneously immortalized rat granulosa cells, reduced GJIC was observed during mitosis using a fluorescence recovery after photobleaching assay (Stein et al., 1992). Resumption of

GJIC was established several hours after cytokinesis. Most recently, Xie and colleagues (1997) have shown a loss of GJIC in mitotic vascular endothelial cells. Moreover, these researchers have also identified and characterized a novel mitosis-specific form of Cx43 (modified by serine phosphorylation). This mitotic form has been suggested to be responsible for the closure and disassembly of gap junctions between mitotic cells and/or the internalization and degradation of junctional proteins from the cell surface. Alterations in gap junctions during specific stages of the cell cycle may permit cells to modulate their responsiveness to growth factors or regulatory molecules. For instance, quiescent, contact inhibited normal rat kidney cells (NRK) that were poorly communicating showed significant increases in GJIC upon treatment with TGF-ß relative to their proliferating counterparts (van Zoelen and Tertoolen, 1991). GJIC in quiescent NRK cells has also been shown to be inhibited by oncogenic ki-ras p21 and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator, while proliferating NRK cells were unresponsive (Paulson et al., 1994). The results of these experiments indicate that there is a cell stage dependent effect on GJIC responsiveness.

1.9 The Involvement of Gap Junctions in Development

Gap junction mediated cell coupling during development is first acquired in the 8-cell stage after compaction has begun in the mouse embryo (McLachlin and Kidder, 1986). The number of gap junctions and the rate of dye coupling progressively increase as development proceeds.

As previously discussed, GJIC between cells of the same type or between different types (heterocellular GJIC) is important in synchronizing cell behaviour and in coordinating the interactions between differentiating cells which are needed for establishing proper morphogenetic movements and organ architecture. An important experiment illustrating the role of gap junctions during development was carried out by Reaume and colleagues (1995) who created a null mutation in the murine gene encoding Cx43. Homozygous mouse embryos survived to term, but died shortly after birth. The most evident defects were found in the heart where the conus region, overlying the right ventricular outflow tract, was enlarged and filled with intraventricular septae that divided the outflow tract into blind-ended chambers. The defects in right ventricular outflow lead to neonatal cyanosis and perinatal death. Inhibition of gap junction activity by antibodies during embryogenesis has also been shown to lead to several defects. The injection of antibodies against connexins into mouse embryos at the 2-cell stage showed that the cells were communication incompetent and continued to divide, but were uncompacted (Lee et al., 1987). In the early amphibian embryo, injection of antibodies against connexins demonstrated blockage of cell coupling and patterning defects (Warner, 1987). Gap junctions have also been shown to be involved in establishing a communication gradient along the anterior-posterior axis of the developing chick limb bud (Coelho and Kosher, 1991). One possible role for gap junctions may thus be to establish gradient distributions of regulatory molecules which may be involved in specifying positional identities.

Originally, the expression of several gap junction genes (encoding Cx43, Cx32, and Cx26) had been shown to be regulated during early mouse embryogenesis and organogenesis (Nishi

et al., 1991). More recently, Davies and colleagues (1996) have found that at least six connexin mRNAs are present and translated during preimplantation development of the mouse, albeit at different stages of development. The differences in the expression patterns of these connexin genes may be related to differences in the communication needs required for proper fetal development as opposed to organ function in the neonatal or adult mouse. Changes in the expression patterns of connexin genes have been shown to be involved in the development of several organs, including the heart (Fishman et al., 1991) and the brain (Belliveau et al., 1991). The selective expression of gap junctions and selectivity in GJIC (via different connexins) thus play roles in development alongside cellular interactions mediated by growth factors. The selectivity of GJIC may be important in establishing and maintaining communication boundaries or gradients of regulatory molecules that are critical to normal embryonic patterning (Warner, 1992).

1.10 Gap Junctions in the Mammary Gland

The mammary gland consists of two epithelial cell types, secretory luminal cells and myoepithelial basal cells, separated from a fatty, collagenous stroma. In the mammary gland, gap junctions are important during lactation in synchronizing the responses of epithelial cells to mammogenic and lactogenic signals and in allowing the rapid passage of signals to synchronize the contractions of myoepithelial cells for the movement of milk into ducts (Pozzi et al., 1995). The role of gap junctions in nonlactating stages is still undetermined.

While the role of coupling has been studied for some time in cells of the mammary gland (Shen et al., 1976; McKay and Taylor-Papadimitriou, 1981; Berga; 1984), the identification of the connexins involved is still relatively new. There have been several conflicting reports as to the presence of connexins in the mammary gland and there appears to be species specific differences.

A study by Monaghan and colleagues (1994) identified Cx26 as the major connexin in the mouse mammary gland. Cx26 expression was absent in virgin mammary gland, appeared during pregnancy, and was most abundant during lactation before being greatly diminished during involution. These researchers were unable to detect Cx32, Cx40, and Cx43. This study is later contradicted by a report that Cx26, Cx32, and Cx43 were all expressed in the mouse mammary gland (Perez-Armendariz et al., 1995). The latter study found that Cx43 was constitutively expressed, but increased slightly during lactation, whereas Cx26 and Cx32 expression followed a pattern similar to that described by Monaghan and colleagues (1994). The expression of Cx26 and Cx43 was shown in a normal human mammary epithelial cell line (NMEC), but not in a mammary tumor cell line (TMEC) by Lee and co-workers (1992). Lee and co-workers also determined that Cx31.3, Cx32, Cx33, Cx37, and Cx40 were not expressed in either cell line. Monaghan and colleagues (1996) later confirmed the presence of Cx26 and Cx43 in human breast by immunostaining and polymerase chain reaction (PCR) analysis. A further study by Pozzi and colleagues (1995) looked at the expression and localization of Cx26, Cx32, and Cx43 in rodent and human mammary gland by PCR analysis and immunohistochemistry. Cx43 was found to be constitutively expressed in all three

species. Cx26 and Cx32 were found to be expressed only during lactation in the rodent mammary gland and neither Cx26 nor Cx32 was detected in human mammary gland. In summary, it appears that despite species differences, the major connexins expressed in the mammary gland are Cx26, Cx32, and Cx43. In contrast to humans and rodents, there does not appear to be any data concerning the connexins expressed in the mammary glands of bovines.

1.11 Objectives and Hypothesis

Intercellular communication between cells of different lineages during differentiation and development remains incompletely understood. This thesis will address the role of intercellular communication in quiescent and heterocellular mammary gland populations. Firstly, while it has been demonstrated that gap junctions play a role in growth control, the mechanism for this regulation has yet to be determined. One potential mechanism is cell cycle dependent modifications of connexins in order to regulate proliferation by altering the responsiveness and communicational needs of the cell. Secondly, in most mammalian tissues, gap junctions are the sole means of direct communication between cells of the same type, but it appears that cells of different types, such as epithelial cells and fibroblasts, can also communicate via gap junctions. However, the role of heterocellular communication in the mammary gland remains uncertain and the mechanism for this interaction and how it compares to that of homocellular junctions require further investigation.

In the first study, we examine the hypothesis that the expression, distribution, and coupling of Cx43 are altered during the transition between proliferation and quiescence (G_0). In our experiments, the model system utilized was a nontransformed bovine mammary epithelial cell line denoted MAC-T (Mammary Alveolar Cell-large T antigen) which could be brought into quiescence through contact inhibition or serum deprivation with little adverse effects. Our system allowed us to study factors, specifically the roles of gap junctions and GJIC during the cell cycle, in the control of cellular proliferation in mammary epithelium which are of significance in breast cancer research. Gap junctional expression and communication play critical roles in normal cellular function with loss possibly resulting in poor differentiation and acquisition of neoplastic phenotypes.

The second study specifically addresses the hypothesis that E-cadherin, a calcium dependent cell adhesion molecule, is essential and sufficient in establishing functional channels between heterotypic cells. Although it has been proposed that the high sequence homology in the extracellular regions of connexins is the main determinant in whether or not cells will form functional channels, results obtained in our laboratory suggest that other factors may be involved, such as the cadherin family. To this end, a model system was developed using mixed cultures of three distinct mammary cell lines which have been characterized as epithelial, fibroblast or intermediate cells based on morphological and immunocytochemical characteristics. Two of these lines were transfected with an E-cadherin plasmid. Functional assays were performed through micro-injection of the dye Lucifer yellow in mixed cultures to determine the extent of intercellular communication via gap junctions

between different cell types, prior and post E-cadherin transfection. Through our studies we plan to determine whether the expression or up-regulation of E-cadherin can establish and maintain intercellular communication in the mammary gland which may provide insight into the role of heterocellular communication and the possible implications that this may have in proper organ function, tumorigenesis, and development.

1.12 References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (1994). *Molecular Biology of the Cell.* 3rd edition. Garland Publishing Inc. New York.

Arellano, R. O., Rivers, A. and Ramson, F. (1990). Protein phosphorylation and hydrogen ions modulate calcium-induced closure of gap junction channels. Biophys J. **52,** 363-367.

Atkinson, M. M., Menko, A. S., Johnson, R. G., Sheppard, J. R. and Sheridan, J. D. (1981). Rapid and reversible reduction of junctional permeability in cells infected with a temperature-sensitive mutant of avian sarcoma virus. J. Cell Biol. **91,** 573-578.

Azarnia, R. and Loewenstein, W. R. (1984a). Intercellular communication and the control of growth: XII. Alteration of junctional permeability by simian virus 40. Roles of the large and small T antigens. J. Mem. Biol. **82,** 213-220.

Azarnia, R. and Loewenstein, W. R. (1984b). Intercellular communication and the control of growth: X. Alteration of Junctional permeability by the src gene. A study with temperature-sensitive mutant Rous sarcoma virus. J. Mem. Biol. **82,** 191-205.

Behrens, J., Birchmeier, W., Goodman, S. L. and Imhof, B. A. (1985). Dissociation of Madin-Darby canine kidney epithelial cells by the monoclonal antibody anti-Arc-1: mechanistic aspects and identification of the antigen as a component related to uvomorulin. J. Cell Biol. **101,** 1307-1315.

Behrens, J., Mareel, M. M., Van Roy, F. and Birchmeier, W. (1989). Dissecting tumor cell invasion: Epithelial cell acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. J. Cell Biol. **108,** 2435-2447.

Belliveau, D. J., Kidder, G. M. and Naus, C. C. G. (1991). Expression of gap junction genes during postnatal neural development. Developmental Genetics **12**, 308-317.

Berga, S. E. (1984). Electrical potentials and cell-cell dye movement in mouse mammary gland during lactation. Amer. J. Physiol. **247**, C20-C25.

Bergoffen, J., Scherrer, S. S., Wang, S., Scott, M. O., Bone, L. J., Paul, D. L., Chen, K., Lensch, M. W., Chance, P. F. and Fischbeck, K. H. (1993). Connexin mutations in X-linked Charcot-Marie-Tooth disease. Science **262**, 2039-2042.

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

Beyer, E. C., Kistler, J., Paul, D. L. and Goodenough, D. A. (1989). Antisera directed against connexin43 peptides react with a 43-kD protein localized to gap junctions in myocardium and other tissues. J. Cell Biol. **108**, 595-605.

Boller, K., Vestweber, D. and Kemler, R. (1985). Cell adhesion molecule uvomorulin is localized in the intermediate junctionsof adult intestinal epithelial cells. J. Cell Biol. **100**, 327-332.

Brisette, J. L., Kumar, N. M., Gilula, N. B. and Dotto, G. P. (1991). The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. Mol. Cell Biol. **11**, 5364-5371.

Brissette, J. L., Kumar, N. M., Gilula, N. B., Hall, J. E. and Dotto, G. P. (1994). Switch in gap junctionprotein expression is associated with selective changes in gap junctional permeability during keratinocyte differentiation. Proc. Natl. Acad. Sci. USA **91**, 1674-1678.

Britz-Cunningham, S. H., Shah, S. S., Zuppan, C. W. and Fletcher, W. H. (1995). Mutation of the connexin43 gap-junction gene in patients with heart malformations and defects of laterality. New Eng. J. Med. **322**, 1323-1329.

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

Buehler, L. K., Stauffer, K. A., Gilula, N. B. and Kumar, N. M. (1995). Single channel behavior of recombinant ß2 gap junction connexons reconstituted into planar lipid bilayers. Biophy. J. **68**, 1767-1775.

Burns, G. A. (1992). The tenia of the equinone intestinal tract. Cornell Veterinarian. **82**, 187-212.

Caspar, D., Goodenough, D., Makowski, L. and Phillips, W. (1977). Gap junction Structures I correlated electron microscopy and X-ray diffraction. J. Cell Biol. **74**, 605-628.

Chang, C. C., Trosko, J. E., Kung, H.-J., Bombrik, D. and Matsumura, F. (1985). Potential role of the src gene product in inhibition of gap junctional communication in NIH 3T3 cells. Proc. Natl. Acad. Sci. USA **82**, 5360.

Coelho, C. N. and Kosher, R. A. (1991). A gradient of gap junctional communication along the anterior-posterior axis of the developing chick limb bud. Developmental Biol. **148**, 529-535.

Cole, W. C., Picone, J. B. and Sperelakis, N. (1988). Gap junction uncoupling and discontinuous propogation in the heart. Biophys. J. **53**, 809-818.

Crow, D. S., Beyer, E. C., Paul, D. L., Kobe, S. S. and Lau, A. F. (1990). Phosphorylation

of connexin43 gap junction protein in uninfected and Rous sarcoma virus-transformed mammalian. Mol. Cell. Biol. **10**, 1754-1763.

Davies, T. C., Barr, K. J., Jones, D. H., Zhu, D. and Kidder, G. M. (1996). Multiple members of the connexin gene family participate in preimplantation development of the mouse. Developmental Genetics **18**, 234-243.

Dermietzel, R., Hertzberg, E. L., Kessler, J. A. and Spray, D. C. (1991). Gap junctions between cultured astrocytes: immunocytochemical, molecular, and electrophysiological analysis. J. Neurosci. **11**, 1421-1432.

Dermietzel, R., Yancey, S. B., Traub, O., Willecke, K. and Revel, J. P. (1987). Major loss of the 28-kD protein of gap junction in proliferating hepatocytes. J. Cell Biol. **105**, 1925-1934.

Doherty, P. and Walsh, F. S. (1994). Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. Curr. Opin. Neurobiol. **4**, 49-55.

Eghbali, B., Kessler, J. A. and Spray, D. C. (1991). Involvement of gap junctions in tumorigenesis: Transfection of tumor cells with connexin32 cDNA retards growth in vivo. Proc. Natl. Acad. Sci. USA **88**, 10701-10705.

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

El-Sabban, M. E., and Pauli, B. U. (1995). Adhesion-mediated gap junctional communication between lung-metastatic cancer cells and endothelium. Invasion and Metastasis **14**, 164-176.

Elfgang, C. Eckert, R., Lichtenberg-Frate, H., Butterweck, A., Traub, O., Klein, R. A., Hulser, D. F. and Willecke, K. (1995). Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. J. Cell Biol. **129**, 805-817.

Falk, M. M., Kumar, N. M. and Gilula, N. B. (1994). Membrane insertion of gap junction connexins: Polytopic channel forming membrane proteins. J. Cell Biol. **127**, 343-355.

Fallon, R. F. and Goodenough, D. A. (1981). Five-hour half-life of mouse liver gap-junction protein. J. Cell Biol. **90**, 521-526.

Fentiman, I., Taylor-Papadimitriou, J. and Stoker, M. (1976). Selective contact-dependent cell communication. Nature **264**, 760-762.

Fishman, G. I., Hertzberg, E. L., Spray, D. C. and Leinwand, L. A. (1991). Expression of connexin43 in the developing rat heart. Circ. Res. **68**, 782-787.

Frixen, U., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D. and Birchmeier, W. (1991). E-cadherin mediated cell-cell adhesion prevents invasiveness of human carcinoma cell lines. J. Cell Biol. **117**, 173-185.

Fujimoto, K., Nagafuchi, A., Tsukita, S., Kuraoka, A., Ohukuma, A. and Shibata, Y. (1997). Dynamics of connexins, E-cadherin and α-catenin on cell membranes during gap junction formation. J. Cell Sci. **110**, 311-322.
dc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. Cell **67**, 197-211.

Ginzberg, R. D. and Gilula, N. B. (1979). Modulation of cell junctions during differentiation and chicken otocyst sensory epithelium. Devel. Biol. **68**, 110-129.
646-653.

Goodall, H. and Maro, B. (1986). Major loss of junctional coupling during mitosis in early mouse embryos. J. Cell Biol. **102**, 568-575.

Goodenough, D. A. and Revel, J. -P. (1970). A fine structural analysis of intercellular junctions in mouse liver. J. Cell Biol. **45**, 272-290.

Gordon, R. E., Lane, B. P. and Marin, M. (1982). Regeneration of rat tracheal epithelium: changes in gap junctions during specific phases of the cell cycle. Exp. Lung Res. **3**, 47-56.

Guger, K. A. and Gumbiner, B. M. (1995). Beta-catenin has Wnt-like activity and mimics the Nieuwkoop signaling center in Xenopus dorsal-ventral patterning. Developmental Biol. **172**, 115-125.

Hirschi, K. H., Xu, C.-E., Tsukamoto, T. and Sager, R. (1996). Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. Cell Growth & Diff. **7**, 861-870.

Hertzberg, E. L., Disher, R. M., Tiller, A. A., Zhou, Y. and Cook, R. R. (1988). Topology of the M_r27,000 liver gap junction protein. J. Biol. Chem. **263**, 19105-19111.

Holtz, J. (1993). Myocardial hypertrophy after myocardial infarct: what is the significance of phenotype changes in cardiocytes? Herz. 18 Suppl. **1**, 387-394.

Hurtley, S. M. and Helenius, A. (1989). Protein oligomerization in the endoplasmic reticulum. Annu. Rev. Cell Biol. **5**, 277-307.

Jiang, J. X. and Goodenough, D. A. (1996). Heteromeric connexons in lens gap junction channels. Proc. Natl. Acad. Sci. USA **93**, 1287-1291.

Jongen, W. M. F., Fitgerald, D. J., Asamoto, M., Piccoli, C., Slaga, T. J., Gros, D., Takeichi, M. and Yamaski, H. (1991). Regulation of connexin43-mediated gap junctional intercellular communication Ca^{2+} in mouse epidermal cells is controlled by E-cadherin. J. Cell Biol. **114**, 545-555.

Kohl, P., Kamkin, A. G., Kiseleva, I. S. and Noble, D. (1994). Mechanosensitive fibroblasts in the sino-atrial node region of rat heart: Interactions with cardiomyocytes and possible role. Exp. Physiol. **79**, 943-956.

Konig, N. and Zampighi, G. (1995). Purification of bovine lens cell-to-cell channels composed of connexin44 and connexin50. J. Cell Sci. **108**, 3091-3098.

Kreutziger, G. O. (1968). Freeze-etching of intercellular junctions of mouse liver. In *Proc. 26th Electron Microsc. Soc. America*, pp 234-235. Claitor's Publishing, Baton Rouge.

Kumar, N. M. and Gilula, N. B. (1992). Molecular biology and genetics of gap junction channels. Semin. Cell Biol. **3**, 3-16.

Kumar, N. M. and Gilula, N. B. (1996). The gap junction communication channel. Cell **84**, 381-388.

Laing, J. G. and Beyer, E. C. (1995). The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. J. Biol. Chem. **270**, 26399-26403.

Laird, D. W. and Revel, J. P. (1990). Biochemical and immunochemical analysis of the arrangement of connexin43 in rat heart gap junction membranes. J. Cell Sci. **97**:109-117.

Laird, D. W., Castillo, M. and Kasprzak, L. (1995). Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor

cells. J. Cell Biol. **131**, 1193-1203.

Laird, D. W., Puranam, K. L. and Revel, J.-P. (1991). Turnover and phosphorylation of connexin43 gap junction protein in cultured cardiac myocytes. Biochem. J. **273**, 67-72.

Lee, S., Gilula, N. B. and Warner, A. E. (1987). Gap junctional communication and compaction during preimplantation stages of mouse development. Cell **51**, 851-860.

Lee, S. W., Tomasetto, C. and Sager, R. (1991). Positive selection of candidate tumor-suppressor genes by subtractive hybridization. Proc. Natl. Acad. Sci. USA **88**, 2825-2829.

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

Loewenstein, W. R. (1981). Junctional intercellular communication. The cell to cell membrane channel. Physiol. Rev. **61**, 829-913.

Makowski, L., Caspar, D., Phillips, W. and Goodenough, D. (1977). Gap junction structures II analysis of X-ray diffraction data. J. Cell Biol. **74**, 629-645.

McKay, I. A. and Taylor-Papadimitriou, J. (1981). Junctional communication pattern of cells cultured from human milk. Exp. Cell Res. **134**, 465-470.

McLachlin, J. R. and Kidder, G. M. (1986). Intercellular junctional coupling in preimplantation mouse embryos: effect of blocking transcription or translation. Developmental Biology. **117**, 146-155.

Mege, R. M., Matsuzaki, F., Gallin, W. J., Goldberg, J. I., Cunningham, B. A. and

Edelman, G. M. (1988). Construction of epitheloid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. Proc. Natl. Acad. Sci. USA **85**, 7274-7278.

Mehta, P. P., Hotz-Wagenblatt, A., Rose, B., Shalloway, D. and Loewenstein, W.R. (1991). Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. J. Membrane Biol. **124**, 207-225.

Meyer, R. A., Laird, D. W., Revel, J.-P. and Johnson, R. G. (1992) Inhibition of gap junction and adherens junction assembly by connexin and A-cam antibodies. J. Cell Biol. **119**, 179-189.

Milks, L. C., Kumar, N. M., Houghten, R., Unwin, N. and Gilula, N. B. (1988). Topology of 32-kD liver gap junction protein determined by site-directed antibody localizations. EMBO J. 7, 2967-2975.

Miner, P., Lampe, P., Atkinson, M. and Johnson, R. (1995). Extracellular calcium and cadherins regulate the process of gap junction assembly between cells in culture. In *Intercellular Communication through Gap Junctions* (ed. Y. Kanno, K. Kataoka, Y. Shiba, Y. Shibata and T. Shimazu), pp. 331-334. Elsevier, Amsterdam.

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

Monaghan, P., Clarke, C., Perusinghe, N. P., Moss, D. W., Chen, X.-Y. and Evans, W. H. (1996) Gap junction distribution and connexin expression in the human breast. Exp. Cell Res. **233**, 29-38.

Murray, S. A., Larsen, W. J., Trout, J., and Donata, S. T. (1981). Gap junction assembly and endocytosis correlated with patterns of growth in a cultured adrenocortical tumor cell (SW-13). Cancer Res. **41**, 4063-4069.

Musil, L. S., Beyer, E. C. and Goodenough, D. A. (1990a). Expression of the gap junction protein connexin43 in embryonic chick lens: Molecular cloning, ultrastructure localization, and post-translational phosphorylation. J. Membr. Biol. **116**, 163-175.

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

Musil, L. S. and Goodenough, D. A. (1993). Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. Cell **74**, 1065-1077.

Nagafuchi, A. and Takeichi, M. (1988). Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO 7, 3679-3684.

Naus, C. C. G., Zhu, D., Todd, S. D. L. and Kidder, G. M. (1992). Characteristics of C6 glioma cells overexpressing a gap junction protein. Cell. Molec. Neurobiol. **12**, 163-175.

Naus, C. C. G., Hearn, S., Zhu, D., Nicholson, B. J. and Shivers, R. R. (1993). Ultrastructural analysis of gap junctions in C6 glioma cells transfected with connexin43 cDNA. Exp. Cell Res. **206**, 72-84.

Nishi, M., Kumar, N. M. and Gilula, N. B. (1991). Developmental regulation of gap junction gene expression during mouse embryonic development. Developmental Biol. **146**, 117-130.

Oh, S. Y., Grupen, C. G. and Murray, A.W. (1991). Phorbol ester induces phosphorylation and down-regulation of connexin43 in WB cells. Biochem. Biophys. Acta **1094**, 243-245.

Ozawa, M. and Kemler, R. (1992). Molecular organization of the uvomorulin-catenin complex. J. Cell Biol. **116**, 989-996.

Ozawa, M., Baribault, H. and Kemler, R. (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO **8**, 1711-1717.

Ozawa, M., Ringwald, M. and Kemler, R. (1990). Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. Proc. Natl. Acad. Sci. USA **87**, 4246-4250.

Paul, D. L. (1995). New functions for gap junctions. Curr. Opin. Cell Biol. **7**, 665-672.

Paulson, A.F., Johnson, R. G. and Atkinson, M. M. (1994). Intercellular communication is reduced by TPA and Ki-ras p21 in quiescent, but not proliferating, NRK cells. Exp. Cell Res. **213**, 64-70.

Perez-Armendariz, E. M., Luna, J., Aceves, C. and Tapia, D.T. (1995). Connexins 26, 32 and 43 are expressed in virgin, pregnant and lactating mammary glands. Develop. Growth Diff. **37**, 421-431.

Pitts, J. D. and Kam, E. (1985). Communication compartments in mixed cell cultures. Exp. Cell Res. **156**, 439-449.

Pozzi, A., Rizek, B., Kiang, D. T., Gilula, N. B. and Kumar, N. M. (1995). Analysis of

multiple gap junction gene products in the rodent and human mammary gland. Exp. Cell Res. **220**, 212-219.

Reaume, A. G., de Sousa, P. A., Kulkarni, S., Langille, B. L., Zhu, D., Davies, T. C., Juneja, S. C., Kidder, G. M. and Rossant, J. (1995). Cardiac malformation in neonatal mice lacking connexin43. Science **267**, 1831-1834.

Revel. J.-P. and Karnovsky, M. J. (1967). Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. J. Cell Biol. **33**, C7-18.

Risek, B., Guthrie, S., Kumar, N. and Gilula, N. B. (1990). Modulation of gap junction transcription and protein expression during pregnancy in the rat. J. Cell Biol. **110**, 269-282.

Risley, M. S., Tan, I., Roy, C. and Saez, J. C. (1992). Cell-, age- and stage-dependent distribution of connexin43 gap junctions in testes. J. Cell Sci. **103**, 81-96.

Robertson, J. D. (1963). The occurence of a subunit pattern in the unit membranes of club endings in Mauthner cell synapse in the goldfish brain J. Cell Biol. **19**, 201-221.

Rojkind, M., Novikoff, P. M., Greenwel, P., Rubin, J., Rojas-Valencia, L., de Carvalho, A. C., Stockert, R., Spray, D., Hertzberg, E. L. and Wolkhoff, A. W. (1995). Characterization and functional studies on rat liver fat-storing cell line and freshly isolated hepatocyte coculture system. Amer. J. Pathology **146**, 1508-1520.

Rook, M. B., van Ginneken, A. C. G., de Jonge, B., El Aoumari, A., Gros, D. and Jongsma, H. J. (1992). Differences in gap junction channels between cardiac myocytes, fibroblasts, and heterologous pairs. Amer. J. Physiol. **263**, C959-977.

Rose, J. K. and Loewenstein, W. R. (1975). Permeability of cell junction depends on local

cytoplasmic calcium activity. Nature **254**, 250-282.

Rudy, Y. and Quan, W. (1987). A model study of the effects of the discreet cellular structure on electrical propogation in cardiac tissue. Circ. Res. **61**, 815-823.

Schipper, J. H., Frixen, U. H., Behrens, J., Unger, A., Jahnke, K. and Birchmeier, W. (1991). E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. Cancer Res. **51**, 6328-6337.

Schultz, R. M. (1985). Roles of cell-cell communication in development. Biol. Reproduction **32**, 27-42.

Simon, A. M., Goodenough, D. A., Li, E. and Paul, D. L. (1997). Female infertility in mice lacking connexin 37. Nature **385**, 525-529.

Shen, S. S., Hamamoto, S. T. and Pitelka, D. R. (1976). Electrophysiological study of coupling between cultured cells of the mouse mammary gland in five distinct physiological states. J. Membr. Biol. **29**, 373-382

Spray, D. C., Harris, A. L. and Bennet, M. V. L. (1981). Equilibrium properties of a voltage-dependent junctional conductance. J. Gen. Physiol. **77**, 75-94.

Spray, D. C. and Burt, J. M. (1990). Structure-activity relations of the cardiac gap junction channel. Am. J. Physiol. **258**, C195-C205.

Spray, D. C. and Dermietzel, R. (1995). X-linked dominant Charcot-Marie-Tooth disease and other potential gap junction disease of the nervous system. Trends Neurosci. **18**, 256-262.

Stauffer, K. A. (1995). The gap junction proteins ß1-connexin (connexin-32) and ß2-connexin (connexin-26) can form heteromeric hemichannels. J. Biol. Chem. **270**, 6768-6772.

Stauffer, K. A., Kumar, N. B., Gilula, N. B. and Unwin, N. (1991) Isolation and purification of gap junction channels. J. Cell Biol. **115**, 141-150.

Stein, L. S., Boonstra, J. and Burghardt, R. C. (1992). Reduced cell-cell communication between mitotic and nonmitotic coupled cells. Exp. Cell Res. **198**, 1-7.

Su, J. W., Tertoolen, L. G. J., de Laat, S. W., Hage, W. J. and Durston, A. J. (1990). Intercellular communication is cell cycle modulated during early Xenopus laevis development. J. Cell Biol. **110**, 115-121.

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

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. Science **251**, 1451-1455.

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

Traub, O., Look, J., Paul, D. L. and Willecke, K. (1987). Cyclic adeonosine monophosphate stimulates biosynthesis and phosphorylation of the 26 kDa gap junction protein in cultured mouse hepatocytes. Eur. J. Cell Biol. **108**, 1039-1051.

van Zoelen, E.J.J. and Tertoolen, L. G. J. (1991). Transforming growth factor-beta

enhances the extent of intercellular communication between normal rat kidney cells. J. Biol. Chem. **266**, 12075-12081.

Wang, Y. and Rose, B. (1997) An inhibition of gap-junctional communication by cadherins. J. Cell Sci. **110**, 301-309.

Warner, A. E. (1987). The use of antibodies to gap junction protein to explore the role of gap junctional communication during development. Ciba Foundation Symposium **125**, 154-167.

Warner, A. E. (1992). Gap junctions in development- a perspective. Seminars Cell Biol. **3**, 81-91.

Winterhager, E., Stutenkemper, R., Traub, O., Beyer, E. and Willecke, K. (1991). Expression of different connexin genes in rat uterus during decidualization and at term. Eur. J. Cell Biol. **56**, 1-7.

Xie, H.-Q., Laird, D. W., Chang, T.-H. and Hu, V. W. (1997). A mitosis-specific phosphorylation of the gap junction protein connexin43 in human vascular cells: biochemical characterization and localization. J. Cell Biol. (In press.).

Yamaski, H. (1990). Gap junctional intercellular communication and carcinogenesis. Carcinogenesis **11**, 1051-1058.

Yancey, S. B., John, S. A., Lal, R., Austin, B. J. and Revel, J.-P. (1989). The 43-kD polypeptide of heart gap junctions: Immunolocalization, topolgy and functional domains. J. Cell Biol. **108**, 2241-2254.

Yee, A. G. and Revel, J.-P. (1978). Loss and reappearance of gap junctions in regenerating

liver. J. Cell Biol. **78**, 554-564.

Zhang, J.-T., Chen, M., Foote, C. I. and Nicholson, B. J. (1996). Membrane integration of in vitro-translated gap junctional proteins: Co- and post-translational mechanisms. Molec. Biol. Cell **7**, 471-482.

Zhu, D., Caveney, S., Kidder, G. M. and Naus, C. C. G. (1991). Transfection of C6 glioma cells with connexin43 cDNA: Analysis of expression, intercellular coupling and cell proliferation. Proc. Natl. Acad. Sci. USA **88**, 1883-1887.

Chapter 2

Reduced Connexin43 Expression is not Correlated with a Loss of Gap Junction Communication in Quiescent Mammary Epithelium

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Running title: Connexin43 in quiescent mammary cells.

Key words: Quiescence, gap junctions, connexin43, intercellular communication

2.1 Abstract:

Gap junctions have been implicated in growth control, but at present it is unclear whether cells that enter a quiescent state continue to express functional gap junctions. To this end, MAC-T cells, a bovine mammary epithelial cell line, were serum starved for 48 h to induce a quiescent (G_0) state. [^{3}H]thymidine incorporation in quiescent cells was reduced by 97.3% from serum fed controls. Western blotting in conjunction with PhosphorImager analysis revealed up to a 20-fold decrease in the expression of the gap junction protein connexin43 (Cx43) and a shift towards the unphosphorylated form of Cx43 in quiescent cells. Dye coupling after microinjection, however, was not significantly altered in quiescent cells. In untreated cells, immunolabeling of Cx43 was predominantly perinuclear, though labeling was also present at sites of cell-cell apposition. In quiescent cells, intracellular labeling for Cx43 decreased, but no corresponding reductions were observed at the cell surface. Recovery from serum deprivation, resulted in increased thymidine incorporation that corresponded with an elevation in Cx43 protein expression and phosphorylation. In parallel, MAC-T cells were also induced to enter quiescence through contact inhibition. Despite a 20-fold reduction in 5-bromo-2'-deoxyuridine and substantial reductions in intracellular Cx43, contact inhibited MAC-T cells also maintained gap junction plaques and intercellular dye coupling. These experiments demonstrate that the maintenence of dye coupling in quiescent cells is correlated with a redistribution of intracellular stores of Cx43.

2.2 Introduction:

Gap junctions are transmembrane channels composed of two identical paired hemichannels, connexons, which allow the exchange of inorganic salts and small metabolites less than 1 kD between cells. Each connexon is an oligomer of integral membrane proteins, termed connexins, which vary in molecular weight and tissue distribution. Gap junctional communication permits the intercellular buffering of cytoplasmic ions, the synchronization of cellular behavior, and is involved in controlling cell proliferation (reviewed, Bruzzone et al., 1996). Gap junctional intercellular communication (GJIC) and/or connexin expression often increase when cells exit mitosis and subsequently enter a differentiated state, as demonstrated in the liver and mammary gland (Meyer and Overton, 1983; Ren et al., 1994; Neveu et al., 1995; Pozzi et al., 1995). Reciprocally, when highly proliferative cells are transfected with connexins to allow for or enhance GJIC, proliferation decreases and the cells frequently enter a differentiated state (Zhu et al., 1991; Mehta et al., 1991; Eghbali et al., 1991; Lin et al., 1995).

In adult vertebrates, most cells are maintained in a quiescent state; for example, hepatocytes and neurons are quiescent for extended periods of time (Pardee, 1989). Although these cells are in a quiescent state (G_0) within the cell cycle, they are still active in maintaining the metabolic, enzymatic, endocrine, and signal transduction events in the organ/system of which they reside (Pardee, 1989). The mammary gland is of particular

interest when examining entry and exit (G_0) from the cell cycle as the mammary gland undergoes cycles of proliferation, replicative dormancy and remodeling in the adult animal. Active mammary epithelial cell proliferation occurs during development, the late luteal phase, pregnancy and early lactation (Cowie et al., 1980; Potten et al., 1988; Anderson et al., 1989) and proliferative quiescence occurs during the follicular phase, mid and late lactation and involution (Cowie et al., 1980; Anderson et al., 1989). Although no reports have examined changes in GJIC during these periods, Pozzi and colleagues (1995) have found Cx32 and Cx26 were only expressed during lactation in the rat. Similarly, Monaghan and colleagues (1994) have reported changes in the expression of Cx26, Cx32, and Cx43 during different stages in the rodent adult life. While these studies have been performed *in vivo*, studies reporting *in vitro* models of mammary epithelial cell proliferation, quiescence and differentiation could more easily facilitate examination of GJIC (Hahm and Ip, 1990; Hahm et al., 1990; Huynh et al., 1991).

In vitro models have greatly extended our knowledge of the cell cycle under defined conditions (reviewed, Pines, 1994; Grana and Reddy, 1995; Meikrantz and Schlegel, 1995). Cells in culture can be stimulated to proliferate exponentially with serum, hormones or growth factors. Alternatively, cells can be maintained in a quiescent state by growth factor deprivation, growth inhibitors and/or contact inhibition. Connexin expression and gap junctional communication have been shown to increase or decrease following treatment of cells with exogenous growth inhibitors, depending on the growth inhibitor, concentration of growth inhibitor, cell type and culture conditions (Albright et

al., 1991; Chiba et al., 1994; Chandrass et al., 1995). Although several studies have used quiescent cells to examine alterations in GJIC (Shiba et al., 1989; 1990; Paulson et al., 1994; Chen et al., 1995), it is unclear whether the expression and distribution of connexin and function of gap junctions are altered during the induction into cellular quiescence and recovery back to a proliferative state.

We have chosen to use a well characterized bovine mammary epithelial cell line, MAC-T, to address the effects of cellular quiescence on connexin expression, distribution and GJIC. The MAC-T cell line is nontransformed, shows strong topo-inhibition and can be induced to functionally and morphologically differentiate in culture (Huynh et al., 1991). We demonstrate that Cx43 expression is downregulated up to 20-fold during cellular quiescence, but GJIC is not significantly decreased.

2.3 Materials and Methods:

1. Cell culture and drug-treatments:

The bovine mammary epithelial cell line, denoted MAC-T (Mammary Alveolar Cell-large T antigen) were used in all experiments. As previously described by Huynh et al. (1991), MAC-T cells were originally derived from mid-lactation bovine mammary tissue and were established in culture through transfection and subsequent low level expression of the SV40 large-T antigen. The MAC-Ts are nontransformed, clonal, non-tumorigenic

in nude mice, and show anchorage dependency as well as topo-inhibition. The cells were grown on 60 mm or 100 mm tissue culture dishes (with or without 12 mm glass coverslips). The dishes contained Dulbecco's Modified Eagles Media (DMEM) (Gibco-Life Technologies, Inc., N.Y.) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of streptomycin/ penicillin and 2mM glutamine. To induce quiescence, cells cultured for 2-3 days were serum starved for 48 h in unsupplemented DMEM (two media changes). Alternatively, cells were brought into a quiescent state through contact inhibition by long-term culture (7 days) in completely supplemented DMEM with one media change after 3 or 4 days. In some cases, MAC-T cells were treated with 5 μg/ml brefeldin A (BFA) (CedarLane Laboratories, Hornby, ON) for 30 min. Cells were maintained at 37 °C in an environment of 5% CO_2 and 95% air.

2. [^{3}H]thymidine incorporation:

[^{3}H]thymidine incorporation was based on the method used by Woodward et al. (1994). Cells were plated at a density of 2 x 10^4 cells/well in a 24 well plate (Nunclon, Denmark) in 1 ml DMEM supplemented with 10% FBS for 24 h. The cells were deprived of serum by cultivation in unsupplemented DMEM (two media changes) for 48 h. For 1 h, nonstarved cells and cells in the last hour of starvation were cultured in media supplemented with 1 μCi/ml methyl-[^{3}H]thymidine (ICN, Irvin, CA; s.a. = 64 Ci/mmol). The data were expressed as c.p.m./well.

3. BrdU incorporation and cell counts:

Labeling medium was prepared by diluting 10 mM 5-bromo-2'-deoxyuridine (BrdU) (Boehringer Mannheim Biochemica, Laval, QUE), a thousand-fold with complete tissue culture medium. Untreated cells or contact-inhibited cells grown on glass coverslips were incubated in labeling medium for 90 minutes at 37 °C. The cells were then washed with PBS and fixed in an acid-ethanol solution (95% ethanol and 5% acetic acid) for 30 minutes. The specimens were rehydrated by washing with PBS and incubated for 1 h at 37 °C with 2 M HCl in order to denature the DNA. The acid was then neutralized using several changes of 0.1 M borate buffer, pH 8.5. The coverslips were then washed with PBS and treated with mouse anti-BrdU-fluorescein antibodies (Boehringer Mannheim Biochemica, Laval, QUE) for 1 h. The coverslips were again washed with PBS, rinsed with distilled water and immunolabeled for Cx43 (as described below). The extent of BrdU incorporation was quantified by performing random cell counts (n=20) analyzing fields measuring 315 x 230 μm visualized under the 40X objective lens on the Zeiss LSM 410 inverted confocal microscope.

4. Immunofluorescent labeling:

Cells grown on coverslips were removed from growth media, rinsed with PBS and permeabilized using absolute ethanol for 10 min at room temperature. After washing with PBS, the cells were then incubated for 1 h with 2% bovine serum albumin (BSA) in order to block non-specific binding sites. Several washes with PBS were again administered, followed by a 1 h incubation with the primary antibody, 3-5 μg/ml of an affinity purified

anti-Cx43 antibody (CT-360). CT-360 is directed against the 360-382 peptide segment at the Cx43 carboxyl terminus (Laird and Revel, 1990) and can be blocked by the addition of 100 μg/ml of excess 360-382 peptide. Primary staining was followed by repeated washings with PBS and then a 1 h incubation with the secondary antibody, rhodamine conjugated goat anti-rabbit (250-fold dilution, Bio/Can Scientific, Mississauga, ON). The coverslips were then extensively washed with PBS and rinsed with distilled water prior to mounting on microscope slides.

In an attempt to identify the intracellular pool of Cx43, untreated and BFA treated cells were labeled for Cx43 and a *medial* Golgi apparatus protein. In this instance, cells grown on coverslips were first labeled for Cx43 using monoclonal anti-Cx43 antibody (Ingram & Bell, Montreal, QUE) (100-fold dilution) followed by rhodamine conjugated donkey anti-mouse (250-fold dilution). The cells were then labeled with 100-fold diluted rabbit anti-MG-160 serum (for a resident protein of *medial* Golgi cisterna) (Gonatas et al., 1989; Croul et al., 1990) followed by fluorescein conjugated goat anti-rabbit (250-fold dilution). The coverslips were mounted as previously described.

All fluorescent images were viewed using epifluorescent and/or confocal microscopy. For epifluorescence, cells were photographed with the Zeiss Axiophot fluorescence microscope using Kodak TMAX 400 film at 800 ASA. Confocal microscopy was used primarily for double-labeling experiments. Analysis of images was performed on a Zeiss LSM 410 confocal microscope as described by Laird et al. (1995).

5. Microinjection:

In order to assay for gap junctional communication, MAC-T cells grown on glass coverslips were pressure microinjected with 5% Lucifer yellow in 10 mM Hepes (pH 7.4, Molecular Probes, Eugene, OR). The cells were injected over a 20 min interval allowing Lucifer yellow to spread; dye ceased to spread 3-4 min after injection. Cells were then fixed (5 min after the last injection) with 3.7% formaldehyde in PBS for 5 min prior to mounting. Some coverslips were also permeabilzed for 30 min with 0.1% TX-100 after fixation and labelled for Cx43 as described above. The cells were viewed after injection and quantified for the extent (orders) of transfer using epifluorescent microscopy.

6. Western immunoblotting:

Untreated, serum-deprived or serum-recovered MAC-T cells were grown to 80-95% confluency in 100 mm dishes. The MAC-T cells were removed from the dishes with 0.25% trypsin with 2mM EDTA for 15 min. The cells were pelleted in media using a clinical centrifuge and resuspended in 5 ml of PBS on ice. An aliquot of cells was added to an equal volume of trypan blue and quantified using a hemocytometer. Samples were normalized for equal cell content. Dead cells stained with trypan blue were not counted and no significant difference in trypan blue staining between samples was observed. The cells suspended in PBS were repelleted and sonicated in 300-800 μl of modified Laemmli sample buffer containing 0.062 M Tris, pH 6.8, 10% glycerol, 4% SDS, 0.01% bromophenol blue, 10% 2-mercaptoethanol, 2mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 2mM EDTA, 50 mM NaF, and 500 μM NaVO4 (Laird et al., 1995). Cell

lysates were resolved on a 10% SDS-polyacrylamide gel with a bisacrylamide:acrylamide ratio of 0.4:30. Transfer of resolved proteins to nitrocellulose blot paper and immunostaining for Cx43 was performed as described by Laird et al. (1995). Blots were air dried and exposed to Amersham Hyperfilm-MP with an intensifying screen. In some instances, blots were quantified for Cx43 by overnight exposure in a PhosphorImager cassette and subsequent scanning for band intensity in a Molecular Dynamics PhosphorImager using ImageQuant Software.

2.4 Results:

1. Serum deprivation drives MAC-T cells into quiescence:

A clonal bovine mammary epithelial cell line, MAC-T, was induced to enter a quiescent state by depriving the cells of serum for 48 h. To confirm that the cells had entered quiescence, we assayed for the incorporation of [^{3}H]thymidine (Fig. 1). In serum starved cells, incorporation of [^{3}H]thymidine was drastically reduced by 97.3% from controls. Quiescent cells that were supplemented with serum for 24 and 48 h increased [^{3}H]thymidine incorporation to 72.4% and 66.9% of controls, respectively. The decrease after 48 h recovery may be accounted for by greater dissynchronization of cells during the cell cycle . The cells displayed no deleterious morphological change and survival was not compromised by starvation.

2. MAC-T cells express Cx43 and are dye coupled:

Immunofluorescence using an anti-Cx43 antibody (Laird and Revel, 1990), revealed distinct punctate distributions at areas of cell-cell apposition (as indicated by arrows in Fig. 2A) as well as at intracellular locations. However, the MAC-T cells express a high ratio of perinuclear intracellular staining relative to sparse cell surface staining. Microinjection of the dye Lucifer yellow was performed to determine the extent of coupling. In the same MAC-T cells which showed scattered plaques, there was extensive coupling (Fig. 2B, 2C).

3. Intracellular Cx43 resides in a BFA insensitive compartment:

We attempted to identify the intracellular pool of Cx43 through the use of Brefeldin A (BFA), an antibiotic that blocks protein trafficking within a fused ER/Golgi compartment (Misumi et al., 1986; Lippincott-Schwartz et al., 1989; Laird et al., 1995). Untreated cells and cells treated with BFA for 30 min were double immunofluorescently labeled for Cx43 and MG-160, a constituent of the *medial* Golgi (Fig. 3). In untreated MAC-T cells both Cx43 (Fig. 3A) and MG-160 (Fig. 3B) immunofluorescence were perinuclear with some areas of possible colocalization (as indicated by arrows in Fig. 3A-C). However, when MAC-T cells were treated with BFA, MG-160 was found dispersed throughout the cytoplasm (Fig. 3E, F-green), while most immunostainable Cx43 remains perinuclear (Fig. 3D, F-reddish-orange).

4. The intensity of Cx43 immunofluorescence decreases during quiescence:

Immunofluorescent labeling permitted the evaluation of changes in the distribution of

Cx43 during quiescence after two days of serum starvation (Fig. 4). In MAC-T cells that had entered quiescence (Fig. 4B), immunostaining of gap junctional plaques at the cell surface was still evident whereas the intracellular staining appeared more diffuse and less intense than in the controls (Fig. 4A). Immunofluorescent labeling of the cells after serum stimulation for 24 hours (Fig. 4C) revealed the presence of gap junction plaques at a level equal or greater than in either control or quiescent samples. Serum stimulated cells for 24 h also resulted in an incomplete restoration of perinuclear staining, but the intensity of immunostaining had increased relative to quiescent samples. After quiescent cells were stimulated for 48 hours (Fig. 4D), immunofluorescence was characterized by the presence of a high level of gap junctional plaques at areas of cell-cell apposition and perinuclear staining was restored.

5. Cx43 expression decreases and there is a shift towards the unphosphorylated species in quiescent cells:

To better understand the molecular changes in Cx43 during quiescence and recovery, biochemical analysis of the protein was performed using Western blotting (Fig. 5) in conjunction with PhosphorImager analysis (Table 1). The data in Table 1 represents the average of three separate blots using different sets of MAC-T cell lysates.

In untreated MAC-T cells (Fig. 5, lane a), three Cx43 species were detected: a species at 42 kDa corresponding to the unphosphorylated form of the protein, and species at 44 kDa and at 46 kDa with the latter two species corresponding to the phosphorylated forms

of the protein (Musil et al., 1990; Laird et al., 1991). In unsaturated exposures to a PhosphorImager, the phosphorylated forms of the protein accounted for 29.9% of the total Cx43 in the samples (Table 1). In quiescent samples (Fig. 5, lane b), the phosphorylated form was clearly evident while the two phosphorylated forms were slightly visible. Since the samples were standardized for equal cell numbers, a drastic 20-fold decrease (4.8% of control levels) in total Cx43 was apparent. The fraction of total Cx43 phosphorylated during quiescence also decreased to 10.9%. In samples of cells serum stimulated for 24 and 48 hours (Fig. 5, lanes c & d, respectively), the 44 and 46 kD species subsequently increased in intensity and protein expression recovered to 15.1% and 33.9% of controls, respectively. The fraction of phosphorylated Cx43 increased to 41.5% after a 24 hour recovery and to 45.2% after a 48 hour recovery.

With the knowledge that several connexins, namely Cx26, Cx32, and Cx43, have been reported to be expresed in the rodent mammary gland (Monaghan et al., 1994), we determined if Cx26 and/or Cx32 expression were endogenously present in proliferating MAC-T cells or up-regulated during quiescence. Using Western blot analysis with liver plasma membrane lysates as a positive control, we were unable to detect the presence of either Cx26 or Cx32 in proliferative, quiescent or serum-recovered MAC-T cell lysates (data not shown).

6. Extended quiescence results in a gradual depletion of intracellular and cell surface Cx43:

MAC-T cells were also brought into an extended quiescent state through serum starvation for three or four days (Fig. 6). Compared to control cells (Fig. 6A), quiescent cells after two days of starvation (Fig. 6B) possessed substantially less intracellular Cx43. After three days of starvation (Fig. 6C), little intracellular Cx43 was observed as were gap junction plaques. After four days (Fig. 6D), however, immunostaining for Cx43 was dispersed and gap junction plaques were rare. Western blot analysis for Cx43 (Fig. 7) confirms the progressive loss of Cx43 in cells that are maintained in a quiescenct state.

7. Quiescent cells maintain intercellular communication:

With the knowledge that Cx43 expression decreased during quiescence, microinjection of the dye Lucifer yellow was used to determine if there was a correlated decrease in functional coupling during quiescence (Table 2). In untreated cells (n=28), there was extensive dye coupling between MAC-T cells to greater than 3rd order transfer. Cells that had been brought into quiescence were also well coupled, but dye transfer was particularly reduced in >3rd order cells (n=34). Coupling appeared to increase slightly when cells were serum supplemented for 48 hours (n=26).

8. MAC-T cells can be brought into a quiescent state through contact inhibition:

As an alternative way to characterize MAC-T cells during quiescence, cells were cultured for 7 days to induce a state of contact inhibition. We assayed for the extent of

quiescence by immunostaining for the thymidine analog, 5-bromo-2'-deoxyuridine (BrdU) (Fig. 8).The percentage of cells incorporating BrdU dropped from 42 ± 2.7 (S.E.M.; n=20) in cells cultured at low density for 2-3 days (Fig. 8A) to 2 ± 0.4 (S.E.M.; n=20) in contact inhibited cells (Fig. 8B) as determined by randomized cell counts. The pattern of Cx43 immunostaining paralleled those of the cells brought into quiescence through serum starvation. Contact inhibited cells (Fig. 8D) also showed a decrease in perinuclear Cx43 staining compared to controls (Fig. 8C) while plaques at areas of cell-cell apposition were still apparent. Moreover, there was no significant change in gap junctional communication in contact inhibited cells as observed through quantification of dye transfer (Table 2).

2.5 Discussion:

In these trials we demonstrated that connexin43 (Cx43) protein was downregulated up to 20-fold during serum deprivation induced cellular quiescence (G_0). Interestingly, gap junctional intercellular communication (GJIC) was not substantially altered in quiescent cells, whether quiescence was induced by serum-deprivation or contact inhibition. Since rodent mammary glands contain Cx26, Cx32 and Cx43 (Monaghan et al., 1994), it was of interest to determine if Cx26 or Cx32 were compensating for the loss of Cx43. Although Cx32 or Cx26 were not detected in MAC-T cells, the possibility that these or other connexins are expressed to some level cannot be excluded. Immunocytochemical

analysis of Cx43 revealed a striking redistribution of this gap junction protein during cellular quiescence. Intracellular Cx43 stores were nearly depleted, while cell surface Cx43 immunostaining was equal to or greater than that seen in proliferating cells. Thus, at least in the early stages of quiesence, the redistribution of steady state pools of Cx43 to the cell surface may play a role in maintaining GJIC.

Cellular quiescence can be induced by 1) serum deprivation, 2) treatment with exogenous growth inhibitors, or 3) density-dependent contact inhibition. Treatment with growth inhibitors, such as retinoic acid and transforming growth factor betas have enhanced GJIC with induction of cellular quiescence in several cell lines, though results are variable (Albright et al., 1991; Chiba et al., 1994; Hossain and Bertram, 1994; Chandrass et al., 1995). Since these growth inhibitors can elicit a multitude of responses in addition to cellular quiescence, it is difficult to ascertain whether cellular quiescence is correlated with GJIC following treatment with these exogenous factors. For this reason, we chose to induce quiescence by serum deprivation or contact inhibition.

Serum deprivation is a common method to synchronize cells in G_0/G_1 by inducing proliferative cells to become quiescent, "proliferative quiescence". Reduced GJIC during serum deprivation in NRK cells has been reported (van Zoelen and Tertoolen, 1991; Paulson et al., 1994), though NRK cells infected with the Kirsten strain of the murine sarcoma virus (tsK-NRK) maintained GJIC during serum deprivation (Paulson et al., 1994). 3T3-L1 cells also remain well coupled in serum-deprived, quiescent conditions

(0.2% FBS) (Shiba et al., 1989). We have demonstrated that serum deprivation resulting in cellular quiescence substantially reduces Cx43 protein expression, but does not dramatically modify GJIC in MAC-T cells. Additionally, Cx43 protein and [^{3}H]thymidine incorporation increase toward prestarvation levels, when serum is added back to quiescent cultures. Importantly, MAC-T cells can be maintained in serum-free conditions for extended periods of time without increased cell death and can rapidly reenter the cell cycle upon serum addition. However, serum deprivation has been reported to induce apoptosis or cellular senescence in other cell lines including Balb/c 3T3 cells (Howard et al., 1993; Kulkarni and McCulloch, 1994; Lindenboim et al., 1995). Additionally, many cultured cell lines have reduced adherence to the substratum or to other cells when cultured in serum-free conditions, including HT1080 cells (Wang et al., 1995), MCF-7 and T47D cells (Woodward, unpublished observations).

On the other hand, GJIC appears to play a role in establishing density dependent growth inhibition. Shiba and coworkers (1989; 1990) have demonstrated that nearly 100% of contact inhibited 3T3-L1 cells cultured in 2% calf serum communicated by GJIC, but only approximately 50% were coupled when cells escaped contact mediated growth inhibition by increasing sera concentration to 10%. Alternatively, Naus and colleagues (1992) demonstrated that contact inhibition could be induced when C6 glioma cells were transfected with a Cx43 cDNA. More evidence correlating GJIC and contact inhibition was documented by Ruch and coworkers (1995). In this study, inhibition of GJIC and reduction of Cx43 expression in Balb/c 3T3 cells by treatment with Cx43 antisense

oligonucleotides did not alter cellular growth rate, but saturation density was increased 3-fold, indicating a strong correlation between loss of GJIC and loss of contact inhibition (Ruch et al, 1995). Conversely, we have shown herein that MAC-T cells have a 20-fold lower incorporation of BrdU when maintained in confluent culture (density dependent contact inhibition), while maintaining GJIC. We have also demonstrated that cell surface localized Cx43 is maintained or enhanced in contact inhibited cells.

Extensive *in vivo* analysis of GJIC in the liver during cellular quiescence has previously been presented (Meyer et al., 1981; Dermietzel et al., 1987; Sugiyama et al., 1990; Hendrix et al., 1992). These studies have repeatedly demonstrated that GJIC is abundant during cellular quiescence and the expression of Cx32 and Cx26 are downregulated during DNA synthesis (Neveu et al., 1995). Recent reports in the mammary gland of rodents have indicated Cx26 expression increases during pregnancy and is maximal during lactation (Monaghan et al., 1994; Pozzi et al., 1995). Proliferation, on the other hand is maximal during pregnancy and cells enter a quiescent state as lactation progresses. Since cellular differentiation begins during pregnancy and culminates at lactation, connexin expression *in vivo* appears more closely correlated with cellular differentiation rather than cellular proliferation. However, connexin expression was not examined immunocytochemically in these *in vivo* studies to determine if the population of proliferating cells differed in connexin expression from the quiescent differentiated cells.

Our *in vitro* trials indicate that a predominantly BFA insensitive pool of connexin43,

likely localized in the *trans* Golgi Network or a later secretory compartment, is depleted in order to maintain GJIC in quiescent MAC-T cells during at least the early stages of MAC-T cell quiescence. This reorganization of connexin43 stores occurs whether MAC-T cells are hormone/growth factor deprived or hormone/growth factor supplemented but contact inhibited. In fact, after 4 days of serum deprivation, there is nearly a complete depletion of intracellular Cx43 stores. In MAC-T cells that were cultured in serum-containing media at confluence for 7 days, depletion of intracellular Cx43 staining and abundant cell surface Cx43 immunoflorescence was observed. Since cessation of cellular proliferation commonly preceeds functional differentiation, it is likely that maintenance of MAC-T cell GJIC during proliferative quiescence allows for enhanced responsiveness to differentiation factors (such as prolactin and laminin) or mitotic factors (in serum-starved cells).

Another factor involved in cell cycle control is dephosphorylation of key regulatory proteins by protein phosphatases and phosphorylation by kinases (Cyert and Thorner, 1989; Hunter; 1989; Nigg, 1993). In our studies, there was a slight shift to the non-phosphorylated form of Cx43 upon entry into G_0 and subsequent return to the phosphorylated forms upon re-entry into G_1. These findings suggest that cell cycle dependent or other protein kinases may be involved, the relevence of which lies in the fact that connexin phosphorylation has been demonstrated to alter cell coupling and may be important in regulating GJIC during normal development, differentiation, and growth (Saez et al., 1986; Somogyi et al., 1989; Brissette et al., 1991; Berthoud et al., 1992; 93;

Warn-Cramer et al., 1996). It is interesting that a consensus sequence for $p34^{cdc2}$ kinase (S/T-P-X-R/K) has been identified at the carboxy terminal of rat Cx43 (Kennelly and Krebs, 1991). In mammalian cells $p34^{cdc2}$, a component of the maturation-promoting factor required for progression through the cell cycle, undergoes a complex series of phosphorylation and dephosphorylation reactions when cells enter quiescence and are then subsequently induced to re-enter the mitotic cycle (Lee et al., 1988; Gaultier et al., 1991). Consensus sequences have also been determined for mitogen-activated protein kinase (MAP kinase) and purified MAP kinase has been shown to phosphorylate Cx43 on serine residues *in vitro* (Kanemitsu and Lau, 1993; Warn-Cramer et al., 1996). MAP kinase is known to play a role in the kinase cascade that results in G_0/G_1 transition (Tamemoto et al., 1992). The transition from G_0 to G_1 is involved in determining the post-embryonic cell proliferation rates and is defectively controlled in tumours (Pardee, 1974; 89). Moreover, the MAP kinase signal transduction pathway has been suggested to be directly relevent to the gap junctions by regulating Cx43 function through growth factors and oncogene products (Jou et al., 1995; Lau et al., 1996).The identification and role of protein kinases and phosphatases that may act on Cx43 at the G_0/G_1 transition remains to be investigated.

In summary, we have demonstrated that substantial downregulation of connexin expression does not necessarily translate into decreased GJIC. Instead, induction of cellular quiescence induces reorganization of Cx43 leading to the maintenence of cell surface plaques. These Cx43 reorganization events may act to enhance or expedite subsequent growth and differentiation responsiveness of quiescent mammary epithelial cells.

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2.7 References:

Albright, C. D., Grimley, P. M., Jones, R. T., Fontana, J. A., Keenan, K. D. and Resau, J. H. (1991). Cell-to-cell communication: a differential response to transforming growth factor-beta in normal and transformed (BEAS-2B) human bronchial epithelial cells. Carcinogenesis **12**, 1993-1999.

Anderson, T. J., Battersby, S., King, R. J. B., McPherson, K. and Going, J. J. (1989). Oral contraceptive use influences resting breast proliferation. Hum. Pathol. **20**, 1139-1144.

Berthoud, V. M., Ledbetter, M. L. S., Hertzberg, E. L. and Saez, J. C. (1992). Connexin43 in MDCK cells: regulation by a tumor-promoting phorbol and Ca^{2+}. Eur. J. Cell Biol. **57**, 40-50.

Berthoud, V. M., Rook, M., Hertzberg, E. L. and Saez, J. C. (1993). On the

mechanism of cell uncoupling induced by a tumor promoter phorbol ester in clone 9 cells, a rat liver epithelial cell line. Eur. J. Cell Biol. **62**, 384-396.

Brisette, J. L., Kumar, N. M., Gilula, N. B. and Dotto, G. P. (1991). The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. Mol. Cell Biol. **11**, 5364-5371.

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

Chandrass, K. J., Chanson, M., Spray, D. C. and Kessler, J. A. (1995). Transforming growth factor-beta-1 and forskolin modulate gap junctional communication and cellular phenotype of cultured Schwann cells. J. Neurosci. **15**, 262-273.

Chen, S., Pelletier, D. B., Ao, P. and Boynton, A. L. (1995). Connexin43 reverses the phenotype of transformed cells and alters their expression of cyclin/cyclin-dependent kinases. Cell Growth & Diff. **6**, 681-690.

Chiba, H., Sawada, N., Oyamada, M., Kojima, T., Iba, K., Ishii, S. and Mori, M. (1994). Hormonal regulation of connexin43 expression and gap junctional communication in human osteoblastic cells. Cell Struct. Funct. **19**, 173-177.

Cowie, A. T., Forsyth, I. A. and Hart, I. C. (1980). *Hormonal Control of Lactation.* (ed. F. Gross, M.M. Grumbach, A. Labhart, M.B. Lipsett, T. Mann, L.T. Samuels and J. Zander), Vol. 15. pp 1-145.S. Springer-Verlag. New York.

Croul, S., Mezitis, S. G. E., Stieber, A., Chen, Y., Gonatas, J. O., Goud, B. and Gonatas, N. K. (1990). Immunocytochemical visualization of the Golgi apparatus in

several species, including human, and tissues with an antiserum against MG-160, a sialoglycoprotein of rat Golgi apparatus. J. Histochem. Cytochem. **38**, 957-963.

Cyert, M. S. and Thorner, J. (1989). Putting it on and taking it off: Phosphoprotein phosphatase involvement in cell cycle regulation. Cell **57**, 891-893.

Dermietzel, R., Yancey, S. B., Traub, O., Willecke, K. and Revel, J. P. (1987). Major loss of the 28-kD protein of gap junction in proliferating hepatocytes. J. Cell Biol. **105**, 1925-1934.

Eghbali, B., Kessler, J. A. and Spray, D. C. (1991). Involvement of gap junctions in tumorigenesis: Transfection of tumor cells with connexin32 cDNA retards growth in vivo. Proc. Natl. Acad. Sci. USA **88**, 10701-10705.

Gaultier, J., Solomon, M. J., Booher, R. N., Bazan, J. F. and Kirshner, M. W. (1991). Cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. Cell **67**, 197-211.

Gonatas, J.O., Mezitis, S. G. E., Stieber, A., Fleischer, B. and Gonatas, N. K. (1989). MG-160: A novel sialoglycoprotein of the medial cisternae of the Golgi apparatus. J. Biol. Chem. **264**, 646-653.

Grana, X. and Reddy, E. P. (1995). Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene **11**, 211-219.

Hahm, H.A. and Ip, M. M. (1990). Primary culture of normal rat mammary epithelial cells within a basement matrix. I. Regulation of proliferation by hormones and growth

factors. In Vitro Cell Dev. Biol. **26**, 791-802.

Hahm, H.A., Ip, M. M., Darcy, K., Black, J. D., Shea, W. K., Forczek, S., Yoshimura, M. and Oka, T. (1990) Primary culture of normal rat mammary epithelial cells within a basement matrix. II. Functional differentiation under serum-free conditions. In Vitro Cell Dev. Biol. **26**, 803-814.

Hendrix, M. E., Skowron Lomneth, C., Wilfinger, W. W., Hertzberg, E. L., Mao, S. J. T., Chen, L. and Larsen, W. J. (1992). Quantitative immunoassay of total cellular gap junction protein connexin32 during liver regeneration using antibodies specific to the COOH-terminus. Tissue Cell **24**, 61-73.

Hossain, M.Z. and Bertram, J. S. (1994). Retinoids suppress proliferation, induce cell spreading, and up-regulate connexin43 expression only in postconfluent 10T1/2 cells: implications for the role of gap junctional communication. Cell Growth Diff. **5**, 1253-1261.

Howard, M.K., Burke, L. C., Mailhos, C., Pizzey, A., Gilbert, C. S., Lawson, W. D., Collins, M. K. and Latchman, N. S. (1993). Cell cycle arrest of proliferating neuronal cells by serum deprivation can result in either apoptosis or differentiation. J. Neurochem. **60**, 1783-1791.

Hunter, T. (1989). Protein-tyrosine phosphatases: The other side of the coin. Cell **58**, 1013-1016.

Huynh, H. T., Robitaille, G. and Turner, J. D. (1991). Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp. Cell Res. **197**, 191-199.

Jou, Y.-S., Layhe, B., Matesic, D. F., Chang, C.-C., de Feijter, A. W., Lockwood, L., Welsch, C. W., Klaunig, J. E. and Trosko, J. E. (1995). Inhibition of gap junctional intercellular communication and malignant transformation of rat liver epithelial cells by neu oncogene. Carcinogenesis **16**, 311-317.

Kanemitsu, M. Y. and Lau, A. L. (1993). Epidermal growth factor stimulates the diruption of gap junctional communication and connexin43 phosphorylation independent of 12-O-tetradecanoylphorbol 13-acetate-sensitive protein kinase C: The possible involvement of mitogen-activated protein kinase. Mol. Biol. Cell **4**, 837-848.

Kennelly, P. J. and Krebs, E. G. (1991). Consensus sequences as substrate-specificity determinants for protein kinases and protein phosphatases. J. Biol. Chem. **266**, 15555-15558.

Kulkarni, G. V. and McCulloch, C. A. (1994). Serum deprivation induces apoptotic cell death in a subset of Balb/c 3T3 fibroblasts. J. Cell Sci. **107**, 1169-1179.

Laird, D. W. and Revel, J. P. (1990). Biochemical and immunochemical analysis of the arrangement of connexin43 in rat heart gap junction membranes. J. Cell Sci. **97**:109-117.

Laird, D. W., Castillo, M. and Kasprzak, L. (1995). Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. J. Cell Biol. **131**, 1193-1203.

Laird, D. W., Puranam, K. L. and Revel, J.-P. (1991). Turnover and phosphorylation of connexin43 gap junction protein in cultured cardiac myocytes. Biochem. J. **273**, 67-72.

Lau, A. F., Kurata, W. E., Kanemitsu, M. Y., Loo, L. W. M., Warn-Cramer, B.

J., Eckhart, W. and Lampe, P. D. (1996). Regulation of connexin43 function by activated tyrosine protein kinases. J. Bioenergetics and Biomembranes. **28**, 359-368.

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

Lin, Z., Zhang, Z., Han, Y., Naus, C. C. G., Yu, K. R. and Holtzer, H. (1995). Functional expression of gap junction gene connexin43 and the myogenic differentiation of rhabdomyosarcoma cells. Sci. China B. **38**, 305-312.

Lindenboim, L., Diamond, R., Rothenberg, E. and Stein, R. (1995). Apoptosis induced by serum deprivation of PC12 cells is not preceded by growth arrest and can occur at each phase of the cell cycle. Canc. Res. **55**, 1242-1247.

Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S. and Klausner, R. D. (1989). Rapid redistribution of golgi proteins into the ER in cells treated with brefeldin A: Evidence for membrane recycling from Golgi to ER. Cell **56**, 801-813.

Mehta, P. P., Hotz-Wagenblatt, A., Rose, B., Shalloway, D. and Loewenstein, W.R. (1991). Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. J. Membrane Biol. **124**, 207-225.

Meikrantz, W. and Schlegel, R. (1995). Apoptosis and the cell cycle. J. Cell Bioch., **58**, 160-174.

Meyer, D. J., Yancey, S. B. and Revel, J.-P. (1981). Intercellular communication in normal and regenerating rat liver: a quantitative analysis. J. Cell Biol. **91**, 505-523.

Meyer, R. D. and Overton, J. (1983). Changes in intracellular junctions: I. Embryonic chick liver development. Dev. Biol. **99**, 172-180.

Misumi, Y., Misumi, Y., Miki, K., Takatsuki, A., Tamura, G. and Ikehara, Y. (1986). Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J. Biol. Chem. **261**, 11398-11403.

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

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

Naus, C. C. G., Zhu, D., Todd, S. D. L. and Kidder, G. M. (1992). Characteristics of C6 glioma cells overexpressing a gap junction protein. Cell. Molec. Neurobiol. **12**, 163-175.

Neveu, M. J., Hully, J. R., Babcock, K. L., Vaughan, J., Hertzberg, E. L., Nicholson, B. J., Paul, D. L. and Pitot, H. C. (1995). Proliferation-associated differences in the spatial and temporal expression of gap junction genes in rat liver. Hepatology **22**, 202-212.

Nigg, E. A. (1993). Targets of the cyclin-dependent protein kinases. Current Opinion in Cell Biol. **5**, 187-193.

Pardee, A.B. (1974). A restriction point for control of normal animal cell proliferation. Proc. Natl. Acad. Sci. USA **71**, 1286-1290.

Pardee, A.B. (1989). G_1 Events and Regulation of Cell Proliferation. Science **246**, 603-608.

Paulson, A.F., Johnson, R. G. and Atkinson, M. M. (1994). Intercellular communication is reduced by TPA and Ki-ras p21 in quiescent, but not proliferating, NRK cells. Exp. Cell Res. **213**, 64-70.

Pines, J. (1994). Protein kinases and cell cycle control. Semin. Cell Biol. **5**, 399-408.

Potten, C. S., Watson, R. J., Williams, G. T., Tickle, S., Roberts, S. A., Harris, M. and Howell, A. (1988). The effect of age and menstrual cycle upon proliferative activitiy of the normal human breast. Br. J. Cancer **58**, 163-170.

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

Ren, P., de-Feijter, A. W., Paul, D. L. and Ruch, R. L. (1994). Enhancement of liver cell gap junction protein expression by glucocorticoids. Carcinogenesis **15**, 1807-1813.

Ruch, R. J., Guan, X. and Sigler, K. (1995). Inhibition of gap junctional intercellular communication and enhancement of growth in BALB/c 3T3 cells treated with connexin43 antisense oligonucleotides. Mol. Carcinog. **14**, 269-274.

Saez, J. C., Spray, D. C., Nairn, A. C., Hertzberg, E. L., Greengard, P. and Bennett, M. V. L. (1986). cAMP increases junctional conductance and stimulates phosphorylation of the 27-Kda principal gap junction polypeptide. Proc. Natl. Acad. Sci. USA **83**, 2473-2477.

Shiba, Y., Sasaki, Y., Hirono, C. and Kanno, Y. (1989). Close relationship between modulation of serum-induced stimulation of DNA synthesis and changes in gap-junctional intercellular communication in quiescent 3T3-L1 cells caused by cyclic AMP and the tumor-promoting phorbol ester TPA. Exp. Cell Res. **185**, 535-540.

Shiba, Y., Sasaki, Y. and Kanno, Y. (1990). Inhibition of gap-junctional intercellular communication and enhanced binding of fibronectin-coated latex beads by stimulation of DNA synthesis in quiescent 3T3-L1 cells. J. Cell. Phys. **145**, 268-273.

Somogyi, R., Batzer, A. and Kolb, H.-A. (1989). Inhibition of electrical coupling in pairs of murine pancreatic acinar cells by OAG and isolated protein kinase C. J. Membr. Biol. **108**, 273-282.

Sugiyama, Y. and Ohta, H. (1990). Changes in density and distribution of gap junctions after partial hepatectomy: immunohistochemical and morphometric studies. Arch Histol Cytol. **53**, 71-80.

Tamemoto, H., Kadowaki, T., Tobe, K., Ueki, K., Izumi, T., Chatani, Y., Kohno, M., Kasuga, M., Yazaki, Y. and Akanuma, Y. (1992). Biphasic activation of two-mitogen activated protein kinases during the cell cycle in mammalian cells. J. Biol. Chem. **267**, 20293-20297.

van Zoelen, E.J.J. and Tertoolen, L. G. J. (1991). Transforming growth factor-beta

enhances the extent of intercellular communication between normal rat kidney cells. J. Biol. Chem. **266**, 12075-12081.

Wang, D., Birkenmeier, T. M., Yang, J., Venkateswarlu, S., Humphrey, L., Brattain, M. G. and Sun, L. (1995). Release from quiescence stimulates the expression of integrin alpha-5 beta-1, which regulates DNA synthesis in human fibrosarcoma HT1080 cells. J. Cell Phys. **164**, 499-508.

Warn-Cramer, B. J., Lampe, P. D., Kurata, W. E., Kanemitsu, M. Y., Loo, L. W. M., Eckhart, W. and Lau, A. F. (1996). Characterization of the mitogen-activated protein kinase phosphorylation sites on the connexin-43 gap junction protein. J. Biol. Chem. **271**, 3779-3786.

Woodward, T. L., Akers, R. M. and Turner, J. D. (1994). Lack of mitogenic response to EGF, pituitary and ovarian hormones in bovine mammary epithelial cells. Endocrine **2**, 529-535.

Zhu, D., Caveney, S., Kidder, G. M. and Naus, C. C. G. (1991). Transfection of C6 glioma cells with connexin43 cDNA: Analysis of expression, intercellular coupling and cell proliferation. Proc. Natl. Acad. Sci. USA **88**, 1883-1887.

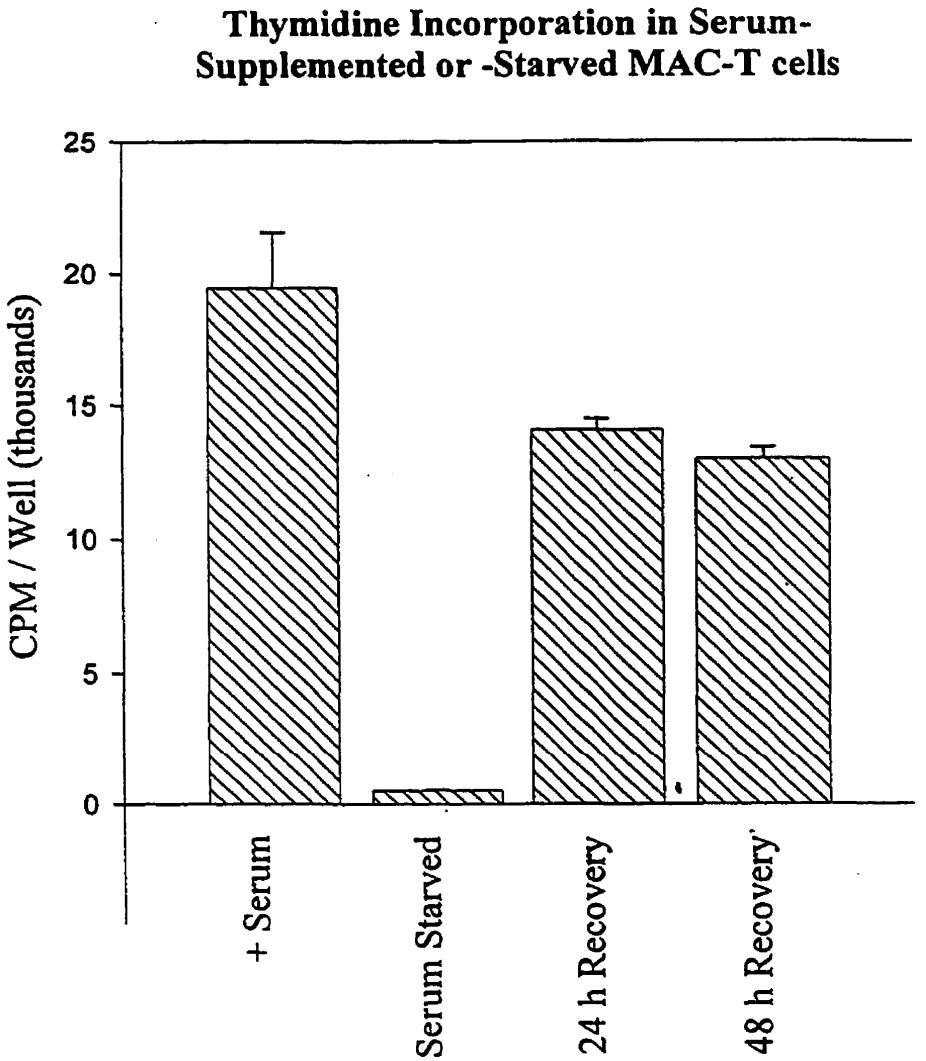

Figure 1. Serum starvation drives MAC-T cells into quiescence. In MAC-T cells that were serum starved for 48 hours, [^{3}H] thymidine incorporation was reduced by 97% from controls. Quiescent cells that were supplemented with serum for either 24 or 48 hours recovered from quiescence as [^{3}H] thymidine increased to approximately 70% of controls.

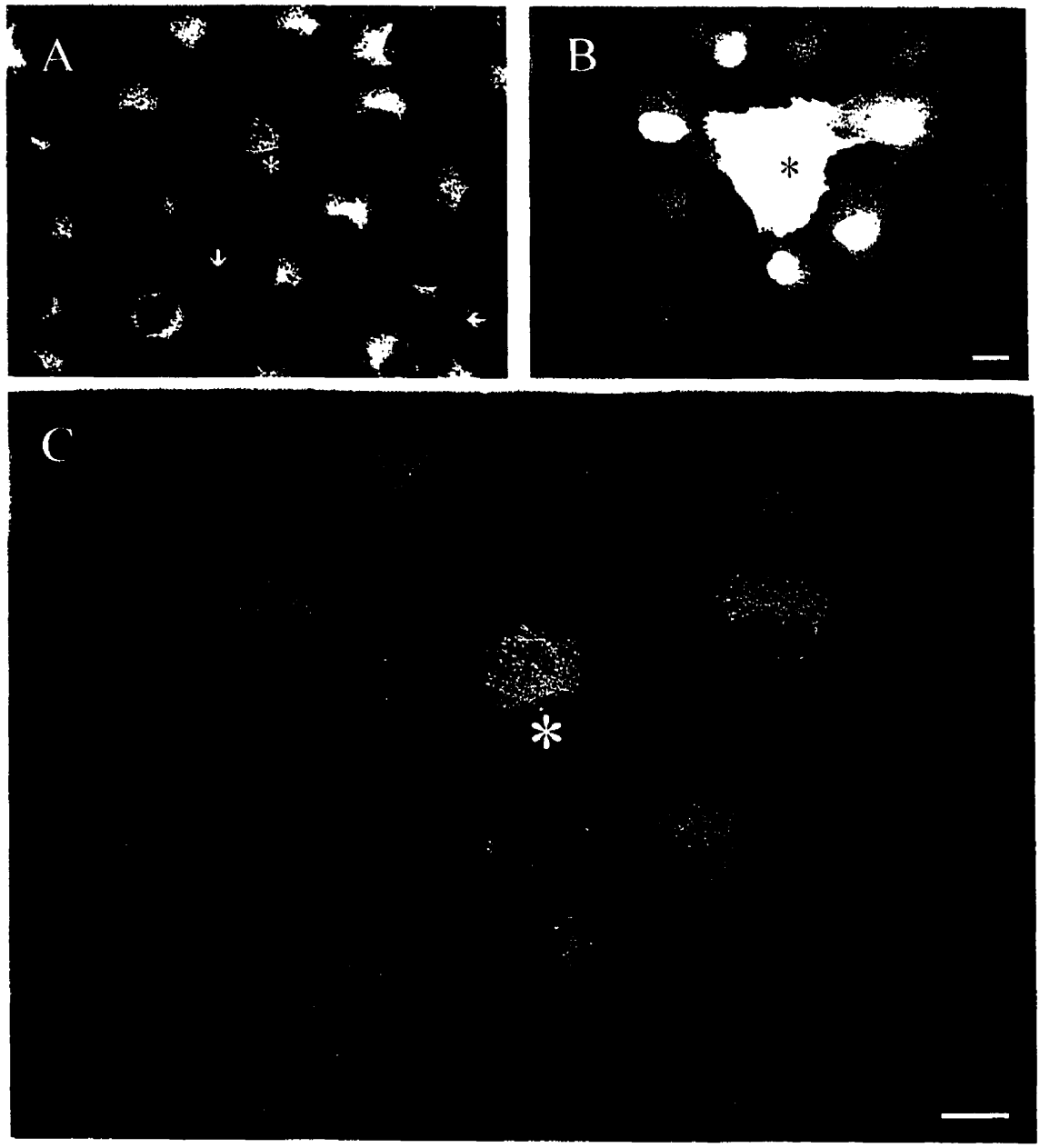

Figure 2. **MAC-T cells express Cx43 and are dye coupled.** Live MAC-T cells were microinjected with Lucifer yellow (injected cell indicated by asterisk), fixed and the same cells were labeled for Cx43. Confocal microscopic analysis revealed that Cx43 had a predominant perinuclear distribution (A) with scattered punctate distributions at areas of cell-cell apposition (arrows in A). Microinjection of Lucifer yellow demonstrated extensive cell coupling (B). Panel C represents the overlay of Cx43 immunostaining (red) and dye transfer (green) with areas of overlap appearing in yellow. Bar = 10 μm.

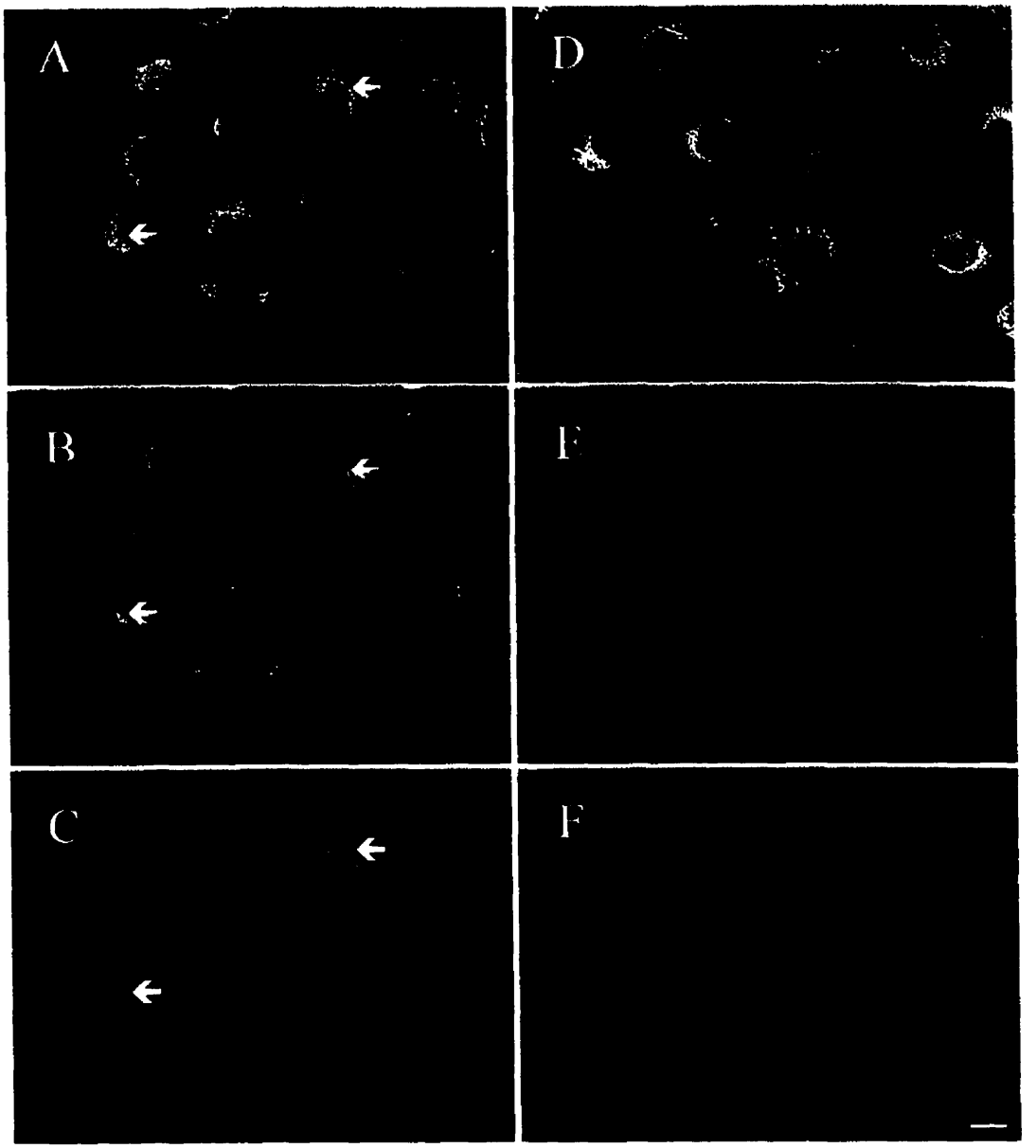

Figure 3. **Intracellular Cx43 resides in a BFA insensitive compartment.** In order to characterize the intracellular pool of Cx43, MAC-T cells were untreated or treated with 5 µg/ml brefeldin A for 30 min. Cells were subsequently double immunofluorescently labeled for Cx43 and a Golgi protein MG-160 and imaged by confocal microscopy. In untreated MAC-T cells both Cx43 (A) and MG-160 (B) immunostaining were perinuclear with some areas of possible colocalization as indicated in yellow by the overlay (C). However, when cells were treated with BFA, most Cx43 remained perinuclear (D) while MG-160 was found to be more dispersed throughout the cytoplasm (E). Panel F represents an overlay of the Cx43 (red) and MG-160 (green) immunostaining. Bar = 10 µm.

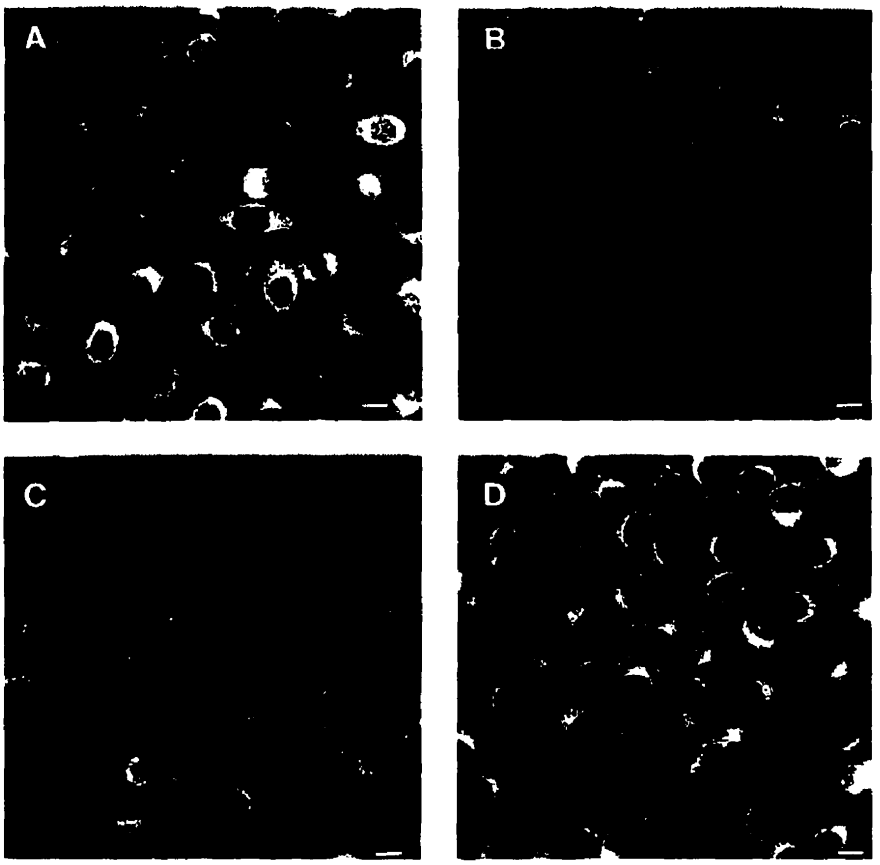

Figure 4. **Intracellular Cx43 immunostaining is reduced in quiescent cells.** Immunofluorescent labeling with an anti-Cx43 antibody was used to evaluate changes in the distribution of Cx43 during quiescence. In quiescent MAC-T cells (B), immunostaining of Cx43 at the cell surface was still evident though intracellular staining appeared more diffuse and of lower intensity than controls (A). Immunolabeling of cells after serum stimulation for 24 hours (C) and 48 hours (D) demonstrated a trend towards increased plaque formation and restoration of perinuclear staining. All images were taken at the same magnification and exposure using epifluorescent microscopy. Bar = 10 μm.

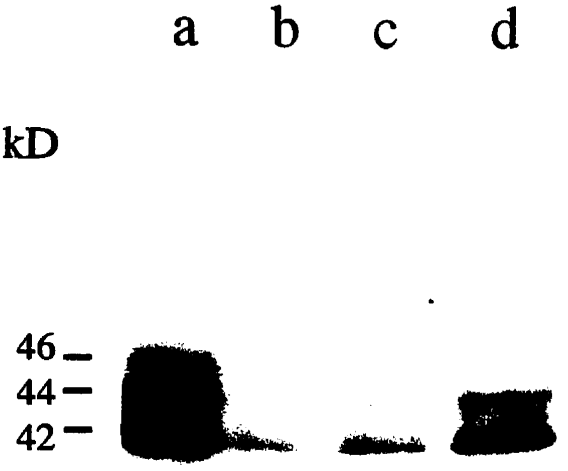

Figure 5. **Expression of Cx43 decreases in quiescent cells.** Western blots of cell lysates from control (lane a), quiescent (lane b), 24 hour serum supplemented after quiescence (lane c), and 48 hour serum supplemented after quiescence (lane d) MAC-T cells revealed that unphosphorylated Cx43 at 42 kD and phosphorylated Cx43 at 44 and 46 kD were present in all samples. PhosphorImager analysis revealed a 20 fold decrease in Cx43 expression in quiescent cells with a preferential decrease in phosphorylated species. Upon serum addition the expression of all Cx43 species increased, most notably the phosphorylated Cx43. All lanes were equalized for cell number.

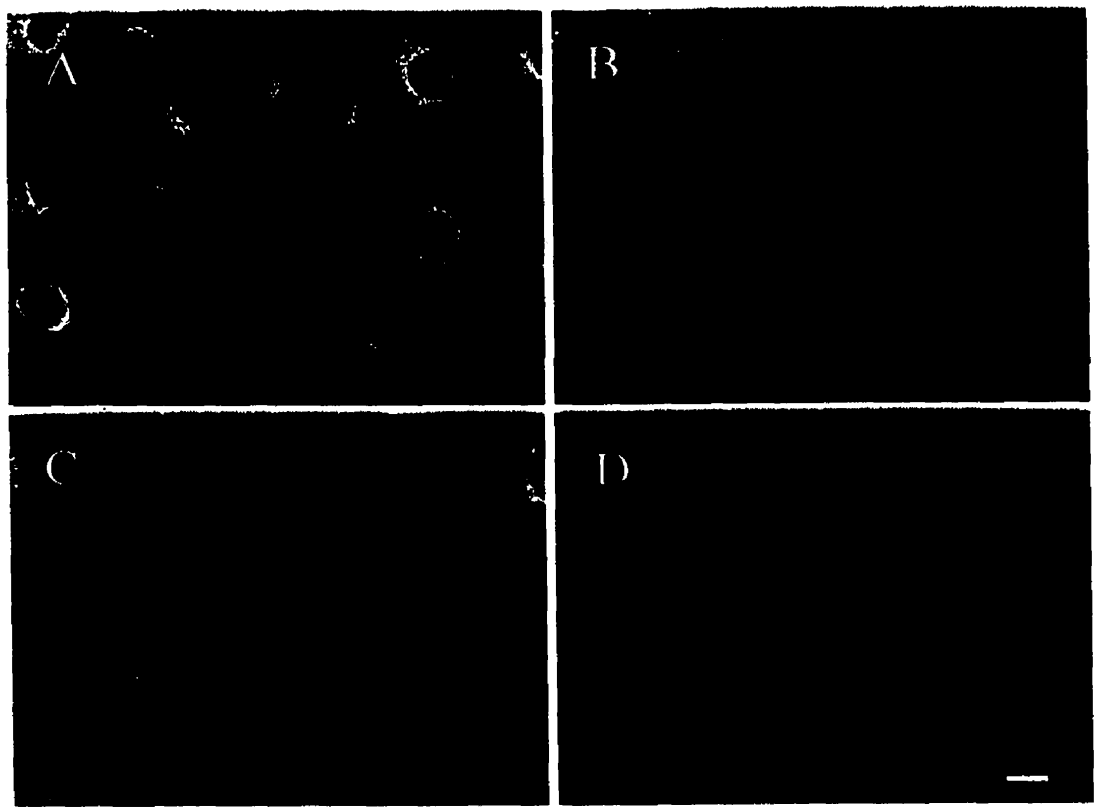

Figure 6. **Extended quiescence results in a depletion of cell surface Cx43.** MAC-T cells were serum starved for 2 days (B), 3 days (C) or 4 days (D) and analyzed for changes in the distribution of Cx43 immunofluorescence by confocal microscopy. In untreated cells (A), Cx43 displayed the typical pattern of abundant perinuclear staining and scattered plaques. Quiescent cells displayed an initial increase in cell surface plaques after 2 days of starvation which was depleted after a longer period of starvation. Intracellular Cx43 progressively decreased after induction of quiescence with little immunostaining evident after 4 days of starvation. All images were taken under the same parameters. Bar = 10 μm.

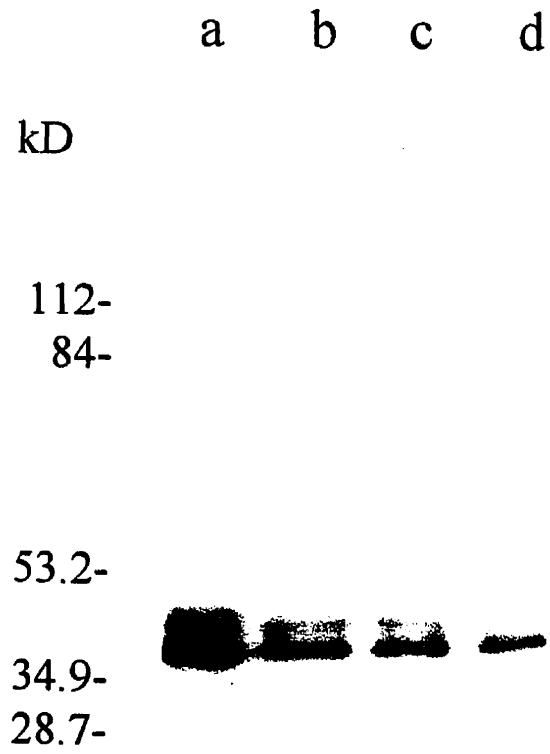

Figure 7. Cx43 expression progressively decreases during extended quiescence. Western blot analysis of control (lane a) MAC-T cells and cells serum starved for 2 days (lane b), 3 days (lane c), or 4 days (lane d) demonstrated a progressive decrease in all Cx43 species after entering quiescence. All lanes were equalized for cell number.

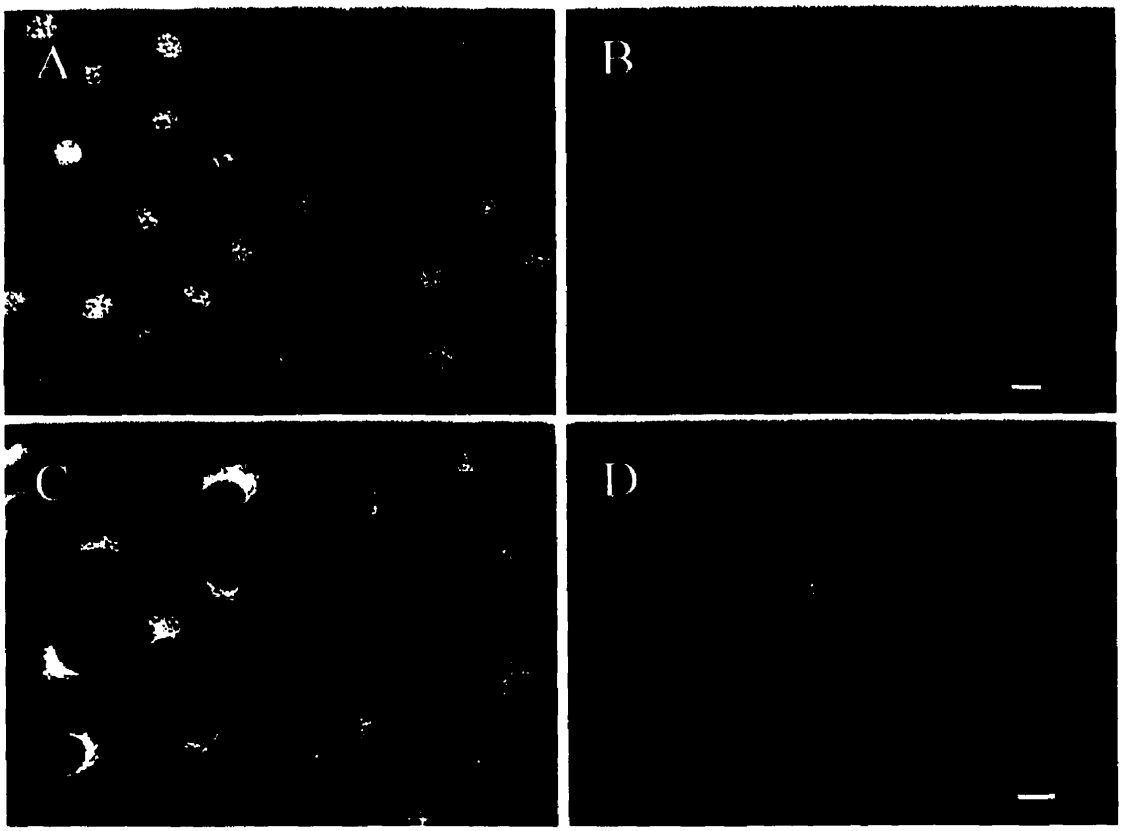

Figure 8. **Contact inhibition drives MAC-T cells into quiescence and results in redistribution of Cx43.** MAC-T cells were cultured for 7 days in the presence of serum growth factors to induce quiescence through contact inhibition. Cells were assayed for quiescence through the incorporation of the thymidine analog, 5-bromo-2'-deoxyuridine (BrdU) by immunofluorescent anti-BrdU counter-staining (A,B). In comparison to cells cultured for 2-3 days (A), anti-BrdU staining in cells cultured for 7 days showed a marked decrease (B). Intracellular immunostaining for Cx43 (C,D) decreased in contact inhibited cells (D). Panels A and B were taken at the same parameters, as were C and D, using confocal microscopy. Bar = 10 μm.

Table 1: PhosphorImager Analysis of Cx43 Protein in Quiescent and Serum-Recovered MAC-T Cells

	Control	Quiescence	24 h Rec.	48 h Rec.
44-46 kD	96.3	1.7	20.2	49.3
~42 kD	226.1	13.7	28.4	59.8
total volume	322.4	15.4	48.7	109.1
volume as a % of control	100.0	4.8	15.1	33.9
% of volume phosphorylated	29.9	10.9	41.5	45.2

The table presents the averages of PhosphorImager data obtained from three separate Western blots using different sets of untreated (control), quiescent and serum-recovered (24 or 48 hours after quiescence) MAC-T cell lysates. The numbers in the rows, 44-46 kD, ~42 kD, and total volume, represent actual numbers obtained using the PhosphorImager. The row, volume as a % of control, represents the total voume of Cx43 protein in each sample relative to the control. The % of volume phosphorylated is the fraction of the total volume of Cx43 in each sample that is accounted for by the 44-46 kD species.

Table 2: Quantification of Lucifer Yellow Transfer in Quiescent and Non-Quiescent MAC-T Cells

Dye Transfer	Control (n=28)	Quiescence (n=34)	24 h Rec. (n=25)	48 h Rec. (n=26)	Contact Inhibition (n=20)
1st order	7.2 ± 0.3	6.8 ± 0.2	6.8 ± 0.3	7.9 ± 0.3	8.3 ± 0.3
2nd order	11.8 ± 0.4	10.7 ± 0.5	10.7 ± 0.4	11.7 ± 0.6	12.5 ± 0.5
3rd order	13.5 ± 0.6	8.4 ± 0.8	9.6 ± 0.7	9.6 ± 1.1	12.7 ± 1.2
>3rd order	17.3 ± 2.6	5.7 ± 1.5	6.8 ± 1.1	9.4 ± 2.1	22.0 ± 3.6
Total	50.1 ± 3.3	32.2 ± 2.3	33.9 ± 2.0	39.0 ± 3.3	55.4 ± 4.7

Control, quiescent (serum-induced), serum-recovered (24 and 48 hours after serum-induced quiescence), and contact inhibited MAC-T cells were microinjected with Lucifer yellow to determine the extent of coupling. The table shows the average number of cells that received dye (along with the corresponding S. E. M. values) and the order of cell separation from the injected cell.

2.8 Summary:

Gap junctions play an important role in the proliferation, differentiation, and function of the mammary gland. In this part of the study, the role of gap junctional communication was addressed in proliferating mammary epithelial cells that were induced by serum deprivation or contact inhibition to enter a state of quiescence. We have shown that during quiescence, mammary epithelial cells will significantly alter expression levels of Cx43, but will maintain intercellular communication by redistributing intracellular pools of Cx43 to the cell surface. In addition, there is also an apparent modification in the phosphorylation state of the protein suggesting that cell cycle dependent kinases may be involved. The involvement of gap junctions during the cell cycle and in cellular proliferation are directly relevent to the study of tumorigenesis. In the next part of our study, we will further address the role of gap junctional communication in the mammary gland by looking at the mechanism of heterocellular gap junction formation. Heterocellular communication in the mammary gland may play a role during development and tumorigenesis when differentiating or dedifferentiating cells are in direct contact. In the adult mammary gland, however, there is a selective communication barrier between different cell types which may prevent inappropriate communication that leads to abnormal growth and function. We will address the possible involvement of cell adhesion molecules in establishing communication between mammary epithelial cells and fibroblasts. In our studies, we have also utilized a transitional cell type that expresses characteristics of fibroblasts and epithelial cells which allows us to study epithelial to mesenchyme transition, a process also associated with tumorigenesis.

Chapter 3

Heterocellular Gap Junctional Communication is Mediated by an Intermediate Cell Type but not by E-cadherin Transgene Expression

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Running title: Heterocellular GJIC is mediated by an intermediate cell type.

Key words: Gap junctions, connexin43, cadherins, heterocellular communication

3.1 Abstract:

Homocellular gap junctional intercellular communication (GJIC) occurs in nearly all cell types, though heterocellular GJIC has been identified far less frequently *in vivo*. We have developed a cell culture model to analyze homocellular and heterocellular GJIC using a mammary epithelial cell line (MAC-T), a fibroblast line (FibC) and an intermediate cell line (FibE). These cell lines were characterized extensively for cell type based on morphology, intermediate cytoskeletal filaments, cell adhesion molecules and their associated proteins, tight junction proteins as well as functional differentiation. All cell types expressed connexin43 and were dye coupled in homocellular culture. MAC-T cells and FibE cells had abundant intercellular plaques of Cx43 as observed by immunostaining. Gap junctional plaques were not observed between FibC and MAC-T cells. Similarly, there was little gap junctional intercellular communication between MAC-T cells and FibC cells. However, FibE cells, the intermediate cell type, were able to communicate with both cell types and could transfer dye between MAC-T cells and FibC cells when cultured together. Expression of an epithelial cadherin (E-cad), by stable transfection of FibC cells with an E-cad expressing plasmid vector, resulted in a more epithelial-like phenotype for the FibC cells, but did not enhance gap junctional communication with MAC-T cells, which endogenously express E-cad. GJIC was slightly enhanced, however, between MAC-T cells and FibE cells after FibE cells were transfected with E-cad. These results also demonstrate that communication gaps between epithelial cells and fibroblasts can be bridged by intermediate cells, a process that may be important in gland development, growth, differentiation and cancer.

3.2 Introduction:

Gap junctions are transmembrane channels between cells which allow the transfer of small molecules, amino acids, secondary messengers, and calcium. The gap junction channel is composed of two hemichannels, each from apposing cells. These hemichannels, or connexons, are formed by 6 polypeptides, the connexins (Beyer, 1993). The connexins are a family of at least 13 proteins that are differentially expressed in nearly all cells (reviewed in Bruzzone et al., 1996). Typically, gap junctions allow similar cells, homotypic cells, within a gland or organ to function coordinately, but independent of dissimilar cells, heterotypic cells. Therefore cells that are coupled by gap junctions can, potentially, 1) spread secondary messengers to induce proliferation or differentiation and 2) dilute harmful toxins; thereby causing the group of cells to function in concert (Holder et al., 1993; Wolburg and Rohlmann, 1995).

More than two decades ago, two independent research groups discovered that heterocellular populations (fibroblasts and epithelial cells) sort themselves, when co-cultured *in vitro*, into communication compartments (Fentiman et al., 1976; Pitts and Burk, 1976). Homocellular populations, either epithelial-epithelial or fibroblast-fibroblast, were well coupled through gap junctions, while heterocellular gap junctional intercellular communication (GJIC) was severely limited. Pitts and colleagues extended these early studies, indicating that the reduced coupling between different cell types is a result of fewer

junctions and that heterocellular coupling can occur *in vivo*, though this was an infrequent event (Pitts and Kam, 1985; Kam et al., 1986).

Other researchers have demonstrated that heterocellular GJIC does occur *in vivo* in defined systems. For example, GJIC between cardiac myocytes and surrounding fibroblasts (Rook et al., 1989; Laird and Revel, 1990), germ cells and Sertoli cells in the testis (Cyr et al., 1992), and between basal cells and principal cells in the epididymis (Cyr et al., 1996) has been documented. Other reports of *in vivo* heterocellular communication are less clear, as Bassnett and colleagues (1994) have presented compelling evidence that epithelial and fiber cells of the eye lens are not coupled by GJIC, contrary to previous reports (Goodenough et al., 1980; Rae and Kuszak, 1983; Miller and Goodenough; 1986). Cumulus-oocyte GJIC has been known to occur for many years (Valdimarsson et al., 1993). In an elegant series of experiments, Simon and colleagues (1997) have recently demonstrated that heterocellular Cx37 gap junctions between oocyte and granulosa cells is critical for normal oogenesis. Loss of Cx37 expression results in female infertility stemming from abnormalities in follicular growth, control of luteinization and oocyte maturation. It thus appears that although heterocellular gap junctions are not as prevalent as their homocellular counterparts, GJIC between different cell types serves an important biological role.

The mechanisms that limit or facilitate the formation of heterocellular GJIC have not been thoroughly investigated, despite many data that document heterocellular GJIC (or lack thereof) *in vitro*. Results concerning specificity of heterocellular GJIC *in vitro* have found

the absence (Fentiman et al., 1976) and presence (Tomasetto et al., 1993) of GJIC between epithelial cells and fibroblasts from the same gland. It has been suggested that some cell types are not selective communicators and their promiscuity in GJIC may be related to high numbers of gap junctions. We suggest, that discrepancies may result from poorly defined cell systems, or alternatively from drift of cells in culture. Cellular transdifferentiation in culture is frequent, and many cells contain properties of both epithelium and fibroblasts (Mork et al., 1990; Boyer and Thiery, 1993; Hay, 1995; Ronnov-Jessen et al., 1995). Furthermore, the extracellular matrix, growth factors in serum or exogenously added growth factors or hormones can induce transdifferentiation (Boyer et al., 1993; Miettinen et al., 1994).

Therefore, we have created a cell system using well defined fibroblasts and epithelial cells as well as an intermediate (or transitional) cell type. These well characterized cell types were all obtained from the mammary gland of a single species (bovine) to ensure species-species or gland-gland/organ differences were not an issue Using these cells we have determined that heterocellular GJIC between fibroblasts and epithelial cells is severely restricted. However, intermediate cells bridged GJIC between fibroblasts and epithelial cells. Homocellular cultures of either epithelial cells or fibroblasts had intercellular plaques of Cx43. However, these plaques were absent between the two cell types. Finally, our results indicate that cell adhesion molecules alone are insufficient in facilitating heterocellular GJIC, as stable transfection of fibroblasts with E-cadherin did not enhance heterocellular GJIC with epithelial cells, which endogenously express E-cadherin.

3.3 Materials and Methods:

1. Materials, cell lines and culture conditions:

All media, sera and culture reagents were obtained from GIBCO BRL (Burlington, Ont.), Becton Dickinson (St. Laurent, Que) or Sigma Chemical Co. (St. Louis, MO). Lipofectin was obtained from GIBCO BRL. All cell lines were grown in DMEM supplemented with 10% FBS, 100 units/ml of streptomycin/penicillin, and 2 mM glutamine. MAC-T cells were an established bovine mammary epithelial cell line that can be induced to morphologically and functionally differentiate in culture similar to normal mammary alveolar epithelium *in vivo* (Huynh et al., 1991). FibE and FibC cells were isolated from bovine mammary tissue also, as previously described (Woodward et al., 1994; 1995). Cell morphology, phenotype and growth patterns were not altered when the cells were cultured under the above conditions.

2. E-cadherin transfection:

FibC and FibE cells were split into two groups: one to be transfected and one to be passaged identicallly to the transfected. Cell were stably co-transfected with a plasmid expression vector containing E-cadherin, pBATEM2, and a neo plasmid, pBATneo, as a selectable marker (Nose et al., 1988) by lipofectamine previously described (Woodward et al., 1995). Both plasmids were generous gifts from Dr. Masatoshi Takeichi. Two days after transfection, cells were split 1:5 and subsequently selected in DMEM with 10-20% FBS, 2 mM glutamine, and 1 mg/ml G418 sulfate. Selection was continued for 2 weeks. G418 sulfate at 1 mg/ml was previously determined to be 100% cytotoxic to all nontransfected cells

within 5 days. Surviving cells were subsequently frozen or used directly in experiments. In addition to selection of positive transfectants, each cell line was screened for E-cadherin in transfected and nontransfected cells by Western blotting and immunocytochemistry.

3. Immunocytochemistry:

Fixed cells grown on coverslips were immunolabeled in the manner previously described by Laird et al. (1995). Antibodies against the following proteins were utilized: Cx43 (Laird and Revel, 1990), cytokeratin (Boehringer Mannheim Biochemica,QC), vimentin (Boehringer Mannheim Biochemica, QC), E-cadherin (Transduction Laboratories, KY), ß-catenin (Transduction Laboratories, KY), occludin (Zymed, CA), and ZO-1 (Developmental Studies Hybridoma Bank, IA). Briefly, cells were grown on glass coverslips and fixed with 100% ethanol, blocked with 10% horse sera or 2% BSA, rinsed in PBS and immunolabeled. Cells were labeled with 1-5 µg/ml anti-Cx43 antibody or 1:100 dilutions of cytoskeletal antibodies, and 1: 250 for E-cadherin, β-catenin and occludin antibodies. Cells were subsequently rinsed 6X over 30 min in PBS and incubated 1 h in secondary antibody, goat anti-mouse, goat anti-rat or donkey anti-rabbit rhodamine and FITC conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc., PA). Coverslips were rinsed, mounted and analyzed on a Zeiss LSM 410 inverted confocal microscope as described previously (Laird et al., 1995).

4. Microinjection:

Homocellular or heterocellular populations of FibE, FibC and/or MAC-T cells were grown on glass coverslips for 2-3 days prior to use in experimental protocols. Mixed cell

populations were simultaneously plated at a 3:1 ratio for FibC:FibE and FibC:MAC-T or 1:1 as with MAC-T:FibE. Alternatively, FibC cells were permitted to reach 50% confluency before the addition of FibE and/or MAC-T cells, and utilized 2-3 days after the addition of the second population of cells. No detectable differences were observed between the two culturing procedures. The cells were assayed for the extent of GJIC through pressure microinjection of the dye, Lucifer yellow (5%), in H_2O or 10 mM Hepes (pH 7.4, Molecular Probes, Eugene, OR). In the case of heterocellular populations of cells, an attempt was made to inject cells that were not directly in contact with a cell of a different type, but at least one order removed so that the possibility of two different cell types receiving dye from the same injection was minimized. Cells were microinjected over an interval of 20 minutes and the coverslips were fixed with 3.7% formaldehyde in PBS for 5-10 min. In some cases, the coverslips were subsequently permeabilized with 0.1% TX-100 and immunolabeled. The cells were viewed using confocal microscopy to determine the extent and success of dye coupling. For tabulation of heterocellular populations, the following criteria was utilized: 1) injections, at the very least, had to successfully transfer dye within the same population of cells in order to be included; and 2) injections were only considered as successful heterocellular dye transfers if the injected homocellular population of cells transferred dye to more than one cell of a different type.

5. Western immunoblotting:

MAC-T, FibC, FibC transfected with E-cadherin (FibC TF), FibE, and FibE transfected with E-cadherin (FibE TF) were grown to 80-95% confluency in 100 mm dishes. The cells

were then placed on ice, washed with PBS and pelleted in a clinical centrifuge. The pellet was resuspended with 600-800 µl of RIPA buffer (10mM Na_2HPO_4 pH 7.2, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 1% sodium deoxycholic acid, 1% SDS) to which inhibitors had been added (2mM PMSF, 2mM sodium orthovanadate, 1mM sodium fluoride, 10mM leupeptin, and 2 µg/ml aprotinin). The samples were sonicated, normalized for protein content using a bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) and then subjected to SDS-PAGE.

Alternately, bovine mammary gland lysates were prepared from lactating Holstein cows immediately following sacrifice. Parenchymal tissue, excluding major fibrous ducts, was removed and homogenized on ice in homogenization buffer (RIPA consisting of 150mM NaCl, 25mM Tris-HCl, 5mM EDTA, 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholic acid) to which inhibitors had been added (2mM PMSF, 0.5mM sodium orthovanadate, 5mM sodium fluoride, 1 µg/ml leupeptin, and 10 µg/ml aprotinin). The samples were homogenized using a Polytron tissue homogenizer and then centrifuged at 2400Xg at 4 °C for 5 min. Fat was removed and the supernantant was sonicated. The samples were recentrifuged twice at 13 000Xg at 4 °C for 15 min. The supernantant was removed and subjected to protein analysis using a Bio-Rad DC protein assay kit. Mouse heart samples were similarly prepared.

Lysates were resolved on a 10% SDS-polyacrylamide gel with a bisacrylamide:acrylamide ratio of 0.8:30. The resolved proteins were transferred to nitrocellulose blot paper and

immunostained as described by Laird et al. (1995). The following antibody concentrations/ dilutions were used: CT-360 (anti-Cx43) at 1µg/ml, anti-E-cadherin at 1:1000 and anti-ß-catenin at 1:1000. The blots were air dried and exposed to Amersham Hyperfilm-MP with an intensifying screen.

3.4 Results:

1. Cell typing and characterization:

Established mammary cells were characterized for cell type based on 1) morphology, 2) cytoskeletal intermediate filament proteins, 3) E-cadherin expression, 4) tight junction proteins, and 5) mammary epithelial functional differentiation (beta-casein expression/synthesis). The MAC-T cell line was chosen as an epithelial model, since earlier reports have established that this cell line maintains its epithelial characteristics in culture (Huynh et al., 1991), unlike many other mammary epithelial cells (Huynh and Pollack, 1995).

MAC-T cells stained positive for the intermediate filament protein cytokeratin (Fig. 1A, B; Table 1) and negative for the intermediate filament protein vimentin (Fig. 2A, B; Table 1). Additionally, the MAC-T cells expressed the epithelial tight junction protein, occludin, the tight junction associated protein, ZO-1, and the epithelial adherens junction protein, E-cadherin (Table 1). This cell line has previously been shown to functionally and morphologically differentiate when cultured on an appropriate substratum in the presence of

prolactin (Huynh et al., 1991). MAC-T cells have been shown to synthesize and secrete alpha and beta caseins, as well as lactose in culture. These characteristics are the hallmark of a functional mammary epithelial cell *in vivo*.

The bovine mammary fibroblast cell line, FibC, had a fibroblast morphology and consistent with fibroblastic properties, the cell line was negative for cytokeratin (Fig. 1E, F) and positive for vimentin (2E, F). FibC cells, as well, did not express epithelial tight junction or epithelial adherens junction proteins (Table 1). Finally, the FibE cell line had characteristics of both fibroblasts and epithelial cells. FibE cells expressed vimentin (Fig. 2C, D), the fibroblast intermediate filament, and lacked cytokeratin (Fig. 1C, D), the epithelial intermediate filament. Additionally, this cell line did not express the epithelial tight junction integral membrane protein occludin (Table 1) and did not functionally differentiate (Woodward, T.L., unpublished results). However, FibE cells have a cobblestone epithelial-like morphology, and express both E-cadherin and ZO-1 (Table 1).

Therefore based on morphology, marker proteins and functional differentiation, these 3 mammary cell lines, were characterized as epithelial cells (MAC-T), intermediate cells (FibE), and fibroblasts (FibC) throughout their use in these trials.

2. Connexin43 expression and GJIC in homotypic cultures:

Connexin43 expression was examined in lactating bovine mammary gland and the bovine mammary epithelial cell lines described above using Western blotting. The lactating mammary

tissue was positive for Cx43 indicating its *in vivo* presence in the bovine mammary gland (Fig. 3, lane b), although in lower quantities than in mouse heart (Fig. 3, lane a). All three cell lines expressed several forms of Cx43, a lower molecular weight (42 kDa) nonphosphorylated species and two phosphorylated species (44-46 kDa) (Fig. 4, insets). Examination of connexin expression in the mammary gland of humans or murine species have found expression of only Cx26 or Cx43 (Monaghan et al., 1994, Pozzi et al., 1995), or both Cx26 and Cx43 (Lee et al., 1992) or developmentally regulated expression of Cx26, Cx32 and Cx43 (Pozzi et al., 1995). Cx26 and Cx32 were not immuno-detected in any of the bovine mammary cell lines used in these studies, nor were these connexins found in lactating bovine mammary tissue (data not shown).

Immunostaining for Cx43 confirmed the presence of gap junctions in homocellular cultures of MAC-T cells, FibE cells and FibC cells. Although intracellular staining was particularly strong in MAC-T cells, all homocellular cultures had punctate immmmmostaining between cells and intracellular perinuclear staining (data not shown). Homocellular cultures of MAC-T, FibE and FibC cells were microinjected with Lucifer yellow to analyze GJIC. All cell types were well coupled by GJIC (Fig. 4).

3. Heterocellular GJIC:

Cell lines were cocultured to examine heterocellular GJIC. MAC-T/FibE (Fig. 5A) and FibC/FibE (Fig. 5D) cocultures commonly demonstrated heterocellular dye transfer of microinjected Lucifer yellow, but dye transfer was rarely observed in MAC-T/FibC cocultures

(Fig. 5G). The cell lines were distinguished based on Cx43 staining patterns characteristic of each cell line (Fig. 5B, E, H) and based on their morphological appearance (Fig. 5C, F, I). FibC cells were most readily identified by their spindle shaped morphology. FibE and MAC-T cells, though similar in shape, could be separated by the additional presence of dark vesicle-like structures in FibE cells when observed in transmitted light images and by the lack of perinuclear Cx43 immunostaining.

4. Characterization of E-cadherin in stably transfected fibroblasts:

In order to address the hypothesis that coexpression of the same connexin and a common cadherin upregulates gap junctional communication between distinctly different cell types, FibC cells (and FibE cells which endogenously express E-cadherin) were transfected with a plasmid encoding E-cadherin. E-cadherin transfected fibroblasts (FibC TF) expressed E-cadherin by Western blot analysis (Fig. 6) and by immunocytochemistry (Fig. 8). The level of E-cadherin expression in FibC TF was comparable to that of endogenously expressed E-cadherin in FibE cells and no significant change in E-cadherin expression was observed in FibE cells after transfection. Similar to MAC-T cells and FibE cells (Fig. 7B, D), E-cadherin was localized to the plasma membrane in FibC cells by immunofluorescence after transfection, though the staining pattern appeared more punctate (Fig. 8B, D). Additionally, E-cadherin expressing fibroblasts (Fig. 8C) were distinguishable from nontransfected fibroblasts (Fig. 8A) in culture by altered morphology. FibC TF cells were less elongated, enlarged and generally lacked lamellopodia that were present in nontransfected FibC cells. The cells appeared more

epithelial in morphology, though were still distinguishable from the MAC-T (Fig. 7A) and FibE (Fig. 7C) cell lines. The morphology of FibE cells transfected with E-cadherin (FibE TF) was not altered from nontransfected cells.

With the knowledge that the transfection of cadherins into cells can lead to changes in the expression of connexins (Mege et al., 1988; Musil et al., 1990), Western blotting was performed to examine the levels of Cx43 in FibC and FibE cells after transfection (Fig. 9). No detectable alterations in Cx43 levels were observed in either FibC or FibE cells. Using Western blot analysis, we also tested for the presence of ß-catenin, a protein associated with adherens junctions, which is required for proper E-cadherin function (Fig. 10). ß-catenin was expressed in all cell lines including FibC cells prior to transfection. Immunofluorescence studies localized ß-catenin in close proximity to the cell surface of all cell lines (Fig. 11; Fig. 12), as well as intracellularly in MAC-T cells (Fig. 11B). The pattern of staining mimicked that of E-cadherin in MAC-T (Fig. 11A, B), FibE (Fig. 11C, D), and FibC TF cells (Fig. 12 C, D). The additional presence of ß-catenin in nontransfected FibC cells (Fig. 12A, B) suggests that cadherins other than E-cadherin may be present. To date, however, we have been unable to detect other members of the cadherin family.

5. Quantification of heterocellular dye transfer:

Heterocellular GJIC was measured by Lucifer yellow dye transfer between different cell types in contact in coculture (Table 2). Directional transfer of dye was also assessed. MAC-T cells and the intermediate cell type, FibE, had the highest GJIC with 70-82% of cells

transferring dye to its heterocellular neighbors. Dye transfer between fibroblasts and the intermediate cell type was significantly lower at approximately 28%. Epithelial to fibroblast GJIC was very low at 12%. Fibroblasts transferred dye slightly more often to intermediate cells than intermediate cells to fibroblasts. This directional transfer was maintained following transfection of fibroblasts with E-cadherin.

After transfection with E-cadherin, FibE had slightly enhanced GJIC in both directions with MAC-T cells, FibE dye transfer to MAC-T cells increased slightly from 82% to 100% following transfection, while MAC-T to FibE dye transfer increased from 70% to 100%. Unexpectedly, GJIC in FibC TF was not substantially different than nontransfected FibC cells. Only slight differences in GJIC following transfection were noted between FibC TF and FibE or between FibC TF cells and FibE TF cells when compared to nontransfected FibC cells. MAC-T cells did not have greater GJIC with FibC TF than FibC cells. Therefore, despite constitutive expression of functional E-cadherin that altered the phenotype of FibC cells, GJIC between these fibroblasts and epithelial or intermediate cells was not substantially increased.

6. The role of intermediate cells in bridging communication gaps:

One interesting scenario that became more evident is the possibility that the intermediate cell type which communicates relatively well with the two distinct cell types may act to bridge the communication gap between epithelial cells and fibroblasts. In mixed cultures containing all three cell lines, micoinjected Lucifer yellow was observed to pass from fibroblasts to

epithelial cells via the intermediate cell type (Fig. 13A, D). Conversely, dye could also be transferred from epithelial cells to intermediate cells and then to fibroblasts (Fig. 14A, D) indicating that there is no directional selectivity. The three cell lines were distinguished by the presence of the cytoskeletal marker protein vimentin (Fig. 13B, 14B), which is found only in FibE and FibC cells, and by morphology (Fig. 13C, 14 C). Intermediate or transitional cells which have not fully differentiated or have dedifferentiated may thus be able to serve a role in passing molecular signals between two poorly communicating cell types during processes such as development and tumorigenesis.

3.5 Discussion:

We have analyzed Cx43 expression and GJIC between mammary epithelial cells, fibroblasts and an intermediate cell type. These studies have also investigated the function of E-cadherin in heterocellular GJIC. Our data demonstrates that cell surface Cx43, localized by immunocytochemical analysis, was abundant in homocellular cultures of fibroblasts, intermediate cells or epithelial cells. However, Cx43 plaques were not detected between epithelial cells and fibroblasts. Similarly, most cells in homocellular culture were able to transfer Lucifer yellow dye to their neighbors, and most intermediate cells and MAC-T cells were coupled by gap junctions. Fibroblast epithelial GJIC, on the other hand, was severely restricted. Plasmid directed expression of E-cadherin in intermediate cells increased GJIC between epithelial cells and intermediate cells to 100%. Despite E-cadherin transfected

fibroblasts having a more epithelial-like morphology following transfection, there was no change in GJIC between fibroblasts and epithelial cells when E-cadherin was localized at the cell surface of fibroblasts.

The mechanisms that limit or facilitate GJIC between heterotypic cell types is not well understood. The pioneering studies of Pitts and his colleagues revealed that GJIC between homotypic cells can be abundant, but is severely restricted between heterotypic cells (Pitts and Burk, 1976; Pitts and Kam, 1985). Unfortunately, these early studies were unable to determine if the different cell types expressed the same connexin and/or the same cell adhesion molecules, now known to be important in cell-cell adhesion and GJIC (Meyer et al., 1992). The first connexin was not cloned until 1986 (Goodenough et al., 1996) and connexin antibodies were not commerciably available. Fentiman and colleagues (1976) also found that heterocellular GJIC was limited in mammary cells. However, these researchers revealed that some cells in culture, calf lens epithelial cells for example, are non-selective communicators. Later studies by Tomasetto and colleagues (1993) found excellent communication between normal human mammary epithelial cells (TMEC) and normal human fibroblasts (TMEC). In our studies, epithelial cells and fibroblasts have very limited GJIC, but an intermediate cell type can communicate more efficiently with either cell type. In fact, GJIC between fibroblasts and epithelial cells can occur through this intermediate cell type.

Cells used in our studies were all obtained from the same gland (mammary gland) of the same species (bovine) as well as the same breed (Holstein-Friesian), thereby eliminating any

species or organ differences that may complicate measuring heterocellular communication. Furthermore, we have carefully characterized morphology, intermediate filaments, cadherins, catenins, tight junction (and associated) proteins as well as assessing epithelial differentiation to ensure cell typing. Since substantial drift frequently occurs during culture, it was important to type the cell lines before and during these studies. Many mammary epithelial cell lines have drifted significantly in culture, having altered morphology, differentiation, tumorigenicity, and even cell type (Mork et al., 1990; Huynh and Pollak, 1995; Ronnov-Jessen et al., 1995).

In vivo, heterocellular GJIC has been demonstrated in several instances (Kam et al., 1986; Laird and Revel, 1990; Cyr et al., 1992; Simon et al., 1997). In the adult mammary gland fibroblast and epithelial cells are most often separated by a basement membrane. Furthermore, during and immediately proceeding lactation, myoepithelial cells may completely "seal" off epithelial cells from the surrounding stroma. However, GJIC has been shown to occur between mammary myoepithelial cells and epithelial cells *in vitro* (Pitelka et al., 1973) and *in vivo* (Berga, 1984). Additionally, the mammary gland undergoes extensive postnatal development during puberty, pregnancy and lactation. At the onset of puberty, during the luteal phase of the menstrual cycle, and during pregnancy and early lactation, active cell proliferation occurs. During periods of ductal elongation and side branching, the basement membrane is degraded and epithelium invades the stromal tissue, thus facilitating direct epithelial-stromal contact (Howlett and Bissell, 1993). Additionally, metalloproteinase expression, basement membrane discontinuity and stromal tissue invasion are common characteristics of mammary epithelial tumors (Birkedal-Hansen et al., 1993; Guelstein et al.,

1993); these characteristics are magnified in metastatic tumors. Thus, the potential for fibroblast-epithelial contact may occur frequently in the normal and cancerous adult mammary gland.

Cell sorting, *in vitro* and *in vivo*, occurs by homotypic cells adhering to each other through cell type specific cell adhesion molecules (Takeichi, 1991). We have previously found in vitro cell sorting when MAC-T cells are cultured with fibroblasts for extended periods. The E-cadherin expressing MAC-T cells form islands separated by fibroblasts (that are E-cadherin negative). Cadherins have also been shown to be important in homotypic GJIC. For example, several groups have observed a positive correlation between cell adhesion molecule expression and GJIC (Kojima et al., 1994; El-Sabban and Pauli, 1994-1995; Jansen et al., 1996). It has been shown that when N-cadherin assembly is prevented by N-cadherin antibodies in reaggregating Novikoff cells, GJIC is prevented (Meyer et al., 1992). Likewise, others have demonstrated decreased or abolished GJIC in cells treated with anti-cell adhesion antibodies (Musil et al., 1990) or increased GJIC in cells transfected with cadherins (Jongen et al., 1991). Therefore, it seemed likely that heterocellular GJIC may not occur between mammary fibroblasts and epithelial cells, since their different cadherins would only allow them to form adherens junctions within a cell type and not between the two cell types. GJIC did, however, occur between fibroblasts and epithelial cells, albeit infrequently. Despite this infrequent heterocellular GJIC, we hypothesized that frequency of heterocellular GJIC may increase if fibroblasts expressed the epithelial specific E-cadherin. Morphology of the fibroblasts that stably expressed the E-cadherin transgene were markedly more epithelial-like.

However, heterocellular GJIC between E-cadherin transfected fibroblasts and epithelial cells was not more frequent than GJIC between non-transfected fibroblasts and epithelial cells (MAC-T and FibC 12.1% versus MAC-T and FibC TF 14.3%). Surprisingly, the intermediate cells, which endogenously express E-cadherin, when transfected with E-cadherin showed a slight increase in GJIC (FibE to MAC-T 81.8% versus FibE TF to MAC-T 100% and MAC-T to FibE 69.6% versus MAC-T to FibE TF 100%). Perhaps this should not have been unexpected since El-Sabban and Pauli (1994-1995) have previously found a rate limiting correlation between the expression level of cell adhesion molecules and GJIC between lung-metastatic cancer cells and endothelial cells.

The lack of correlation between cell adhesion molecules and GJIC has, however, been noted previously. Mesnil and colleagues (1993) determined that gene expression of an N-CAM homologue (DCC gene) was not correlated with GJIC. Whereas, Guger and Gumbiner (1995) found that enhanced expression of some cadherins does not alter gap junctional communication. Similarly, E-cadherin expression was correlated with the expressional patterns of connexin26 in hepatocytes, but connexin32 distribution or expression was not altered by E-cadherin expression (Kojima et al., 1994). Therefore, there appears to be a correlation between cell adhesion molecule expression and GJIC only in specific combinations of cell type, connexin expression and cadherin expression. Our results support this conclusion, but further extends the hypothesis that the expression of cell adhesion molecules may be more influential in modulating GJIC between similar cell types than distinct heterotypic cells.

The use of a 'transitional' cell type that expresses both epithelial and fibroblast characteristics allowed us to determine whether epithelial to mesenchyme transition (EMT) may provide a pathway for heterocellular GJIC. EMT has been shown to naturally occur during the development of several organs/glands (Duband et al., 1995; Hay, 1995). EMT, also occurs during tumorigenesis and may be a key element controlling the first steps of invasion and metastasis in epithelial derived tumors (Boyer and Thiery, 1993). EMT can be induced by 1) growth factors, 2) extracellular matrices and expression of their cognate cellular receptors (integrins), 3) and expression or repression of cell adhesion molecules. In fact, recent studies have demonstrated that established lines of normal mammary epithelial cells undergo EMT simply by treatment with TGF-β (Miettinen et al., 1994). Others have found extensive changes in the phenotype of low passage mammary cells by serum and growth factors (Ronnov-Jessen et al., 1995). The intermediate cell type used in our experiments (FibE, originally isolated from mammary epithelial cells) maintains its 'transitional' characteristics under our defined culture conditions. Interestingly, EMT is most likely to occur when epithelial-to-stromal cell contact may be greatest, during development and tumorigenesis. Our results support the hypothesis that transitional cells present during EMT could facilitate epithelial to stromal cell GJIC.

This study has demonstrated that true mammary epithelial cells rarely establish GJIC with fibroblasts *in vitro*. Intermediate cell types, however, form gap junctions with either fibroblasts or epithelial cells and communicate relatively well with either cell type. Intermediate cells may actually facilitate GJIC between the two distinct cell types, epithelial

cells and fibroblasts. Although cadherin expression has previously been shown to facilitate GJIC between some homotypic cells, expression of the same cadherin is not sufficient for heterocellular GJIC between fibroblasts and epithelial cells. Thus, the mechanism of heterocellular gap junction formation in the mammary gland may require more than an adhesion event. We hypothesize that GJIC is normally limited to homotypic cells in the adult mammary gland, though, during development, pregnancy, involution, or tumorigenesis, intermediate cells may catalyze heterocellular GJIC. These results are particularly compelling in light of substantial alterations in stromal cell behavior during breast epithelial cell carcinogenesis (desmoplasia) that have been suggested to alter tumor cell steroidal responsiveness, metalloproteinase expression, cell proliferation and tumorigenic potential.

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3.7 References:

Bassnett, S., Kuszak, J. R., Reinisch, L., Brown, H. G. and Beebe, D. C. (1994). Intercellular communication between epithelial and fiber cells of the eye lens. J. Cell Sci. **107**, 799-811.

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

Beyer, E.C. (1993). Gap junctions. Int. Rev. Cytol. **137C**, 1-37.

Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A. and Egler, J. A. (1993) Matrix metalloproteinases: a review. Crit. Rev. Oral. Biol. Med. 4, 197-250.

Boyer, B. and Thiery, J. P. (1993). Epithelium-mesenchyme interconversion as an example of epithelial plasticity. AMPMIS **101**, 257-268.

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

Casico, M., Kumar, N. M., Safarik, R. and Gilula, N. B. (1995). Physical characterization of gap junction membrane connexons (hemi-channels) isolated from rat liver. J. Biol. Chem. **270**, 18643-18648.

Cyr, D. G., Blaschuk, O. W. and Robaire, B. (1992). Identification and developmental regulation of cadherin messenger ribonucleic acids in the rat testis. Endocrinology **131**, 139-145.

Cyr, D. G., Hermo, L. and Laird, D. W. (1996). Immunocytochemical localization and regulation of connexin43 in the adult rat epididymis. Endocrinology **137**, 1474-1484.

Duband, J. L., Monier, F., Delannet, M. and Newgreen, D. (1995). Epithelium-mesenchyme transition during neural crest development. Acta Anat. Basel **154**, 63-78.

El-Sabban, M. E. and Pauli, B. U. (1994-1995). Adhesion-mediated gap junctional communication between lung-metastatatic cancer cells and endothelium. Inv. Metast. **14**, 164-176.

Fentiman, I., Taylor-Papadimitriou, J. and Stoker, M. (1976). Selective contact-dependent cell communication. Nature **264**, 760-762.

Goodenough, D. A., Dick II, J. S.B. and Lyons, J. E. (1980). Lens metabolic cooperation: A study of mouse lens transport and permeability visualized with freeze-substitution autoradiography and electron microscopy. J Cell Biol. **86**, 576-589.

Goodenough, D. A., Goliger, J. A. and Paul, D. L. (1996). Connexins, connexons, and intercellular communication. Annu. Rev. Biochem. 65, 475-502.

Guelstein, V. I., Tchypysheva, T. A., Ermilova, V. D. and Ljubimov, A. V. (1993). Myoepithelial and basement antigens in benign and malignant human breast tumors. Int. J. Cancer 53, 269-277.

Guger, K. A. and Gumbiner, B. M. (1995). Beta-catenin has Wnt-like activity and mimics the Nieuwkoop signaling center in Xenopus dorsal-ventral patterning. Dev. Biol. 172, 115-125.

Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. Acta Anat. Basel 154, 8-20.

Holder, J. W., Elmore, E. and Barrett, J. C. (1993). Gap junction function and cancer. Cancer Res. 53, 3475-3485.

Howlett, A. R. and Bissell, M. J. (1993). The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. Epith. Cell Biol. 31, 25-29.

Huynh, H. T., Robitaille, G. and Turner, J. D. (1991). Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp. Cell Res. 197, 191-199.

Huynh, H. T. and Pollak, M. (1995). HH2A, an immortalized bovine mammary epithelial cell line, expresses the gene encoding mammary derived growth inhibitor (MDGI). In Vitro Cell. Dev. Biol. 31, 25-29.

Jansen, L. A., Mesnil, M. and Jongen, W.M. (1996). Inhibition of gap junctional intercellular communication and delocalization of the cell adhesion molecule E-cadherin by tumor promoters. Carcinogenesis **17**, 1527-1531.

Jongen, W. M. F., Fitzgerald, D. J., Asamoto, M., Piccoli, C., Slaga, T. J., Gros, D., Takeichi,M. and Yamasaki, H. (1991). Regulation of connexin43-mediated gap junctional intercellular communication by Ca2+ in mouse epidermal cells is controlled by E-cadherin. J. Cell Biol. **114**, 545-555.

Kam, E., Melville, L. and Pitts, J. D. (1986). Patterns of junctional communication in skin. J. Invest. Dermatol. **87**, 748-753.

Kojima, T., Sawada, N., Oyamada, M., Chiba, H., Isomura, H. and Mori, M. (1994). Rapid appearance of connexin26-positive gap junctions in centrilobular hepatocytes without induction of mRNA and protein synthesis in isolated perfused liver of female rat. J. Cell Sci. **107**, 3579-3590.

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

Laird, D. W. and Revel, J.-P. (1990). Biochemical and immunochemical analysis of the arrangement of connexin43 in rat heart gap junction membranes. J. Cell Sci. **97**, 109-117.

Laird, D. W., Castillo, M. and Kasprzak, L. (1995). Gap junction turnover, intracellular trafficking and phosphorylation of connexin43 in brefeldin-A treated rat mammary tumor cells. J. Cell Biol. **131**, 1193-1203.

Mege, R. M., Matsuzaki, F., Gallin, W. J., Goldberg, J. L, Cunningham, B. A. and Edelman, G. M. (1988). Construction of epitheloid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. Proc. Natl. Acad. Sci. USA **85**, 7274-7278.

Mesnil, M., Piccoli, C., Klein, J. L., Morand, I. and Yamasaki, H. (1993). Lack of correlation between the gap junctional communication capacity of human colon cancer cell lines and expression of the DCC gene, a homologue of a cell adhesion molecule (N-CAM). Jpn. J. Cancer Res. **84**, 742-747.

Meyer, R. A., Laird, D. W., Revel, J.-P. and Johnson, R. G. (1992). Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. J. Cell Biol. **119**, 179-189.

Miettinen, P.J., Ebner, R., Lopez, A. R. and Derynck, R. (1994). TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J. Cell Biol. **127**, 2021-2036.

Miller, T. M. and Goodenough, D. A. (1986). Evidence for two physiologically distinct gap junctions expressed by the chick lens epithelial cell. J. Cell Biol. **102**, 194-199.

Monaghan, P., Perusinghe, N., Carlile, G. and Evans, W. H. (1994). Rapid modulation of gap junction expression in mouse mammary gland during pregnancy, lactation, and involution. J. Histoch. Cytoch. **42**, 931-938.

Mork, C., van Deurs, B. and Petersen, O. (1990). Regulation of vimentin expression in cultured human mammary epithelial cells. Differentiation **43**, 146-156.

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

Nose, A., Nagafuchi, A. and Takeichi, M. (1988). Expressed recombinant cadherins mediate cell sorting in model systems. Cell **54**, 993-1001.

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

Pitts, J. D. and Burk, R. R. (1976). Specificity of junctional communication between animal cells. Nature **264**, 762-764.

Pitts, J. D. and Kam, E. (1985). Communication compartments in mixed cell cultures. Exp. Cell Res. **156**, 439-449.

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

Rae, J. L. and Kuszak, J. R. (1983). The electrical coupling of epithelium and fibers in the frog lens. Exp. Eye Res. **36**, 317-326.

Ronnov-Jessen, L., Petersen, O. W., Koteliansky, V. E. and Bissell, M. J. (1995). The origin of the myofibroblasts in breast cancer. J. Clin. Invest. **95**, 859-873.

Rook, M. B., Jongsma, H. J. and de Jonge, B. (1989). Single channel currents of homo- and heterologous gap junctions between cardiac fibroblasts and myocytes. Pfluegers Arch. **414**, 95-98.

Simon, A. M., Goodenough, D. A., Li, E. and Paul, D. L. (1997). Female infertility in mice lacking connexin 37. Nature **385**, 525-529.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. Science **251**, 1451-1455.

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

Valdimarsson, G., De-Sousa, P. A. and Kidder, G. M. (1993). Coexpression of gap junction proteins in the cumulus-oocyte complex. Mol. Reprod. Dev. **36**, 7-15.

Wolburg, H. and Rohlmann, A. (1995). Structure-function relationships in gap junctions. Int. Rev. Cytol. **157**, 315-373.

Woodward, T. L., Akers, R. M. and Turner, J. D. (1994). Lack of mitogenic response to EGF, pituitary and ovarian hormones in bovine mammary epithelial cells. Endocrine **2**, 529-535.

Woodward, T. L., DuMont, N., O'Connor-McCourt, M., Turner, J. D. and Philip, A. (1995). Characterization of transforming growth factor-β growth regulatory effects and receptors on bovine mammary cells. J. Cell. Phys. **165**, 339-348.

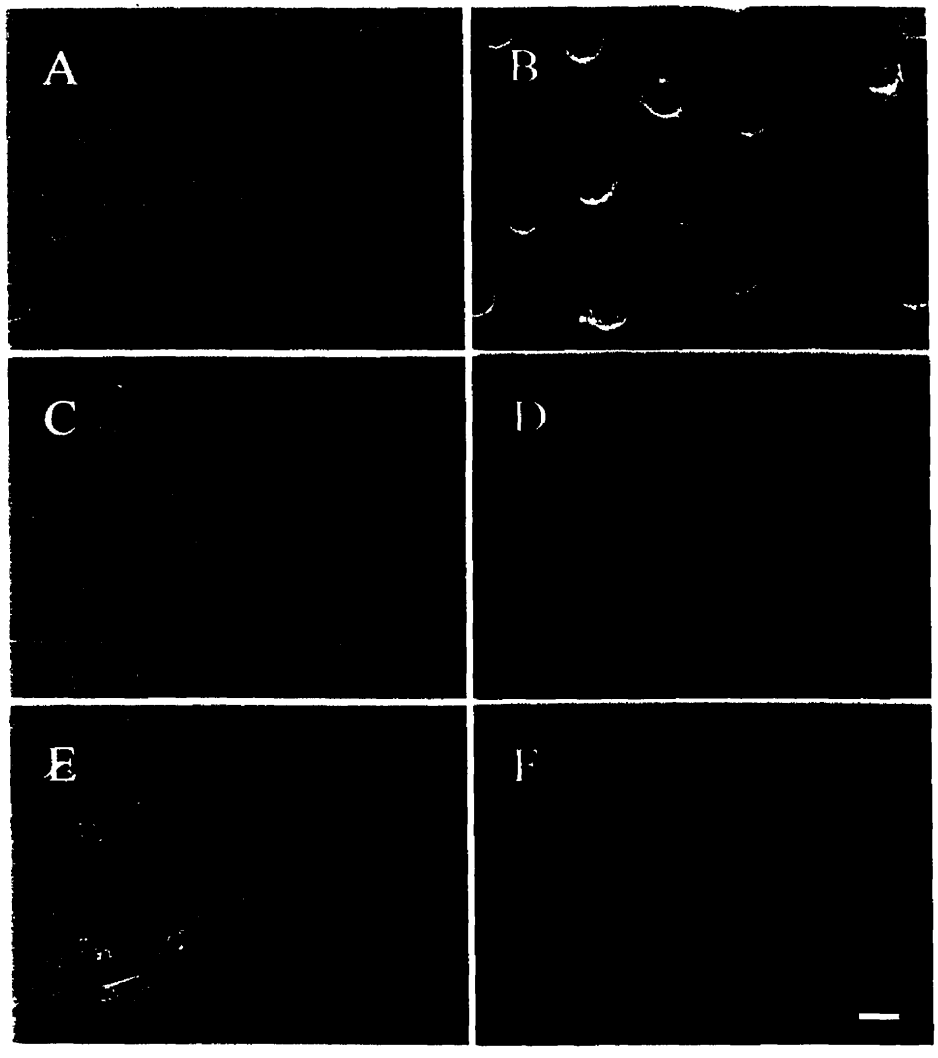


Figure 1. Cytokeratin immunostaining in bovine mammary cell lines. MAC-T (A, B), FibE (C, D), and FibC (E, F) cells were immunostained with an anti-cytokeratin antibody (B, D, F) and viewed under transmitted light for morphology (A, C, E) and fluorescence for the distribution of the intermediate filament protein cytokeratin. Consistent with the characterization of MAC-T cells as being epithelial in nature, these cells express cytokeratin which is absent in FibE and FibC cells. All fluorescent images were taken at the same parameters. Bar = 10 μm.

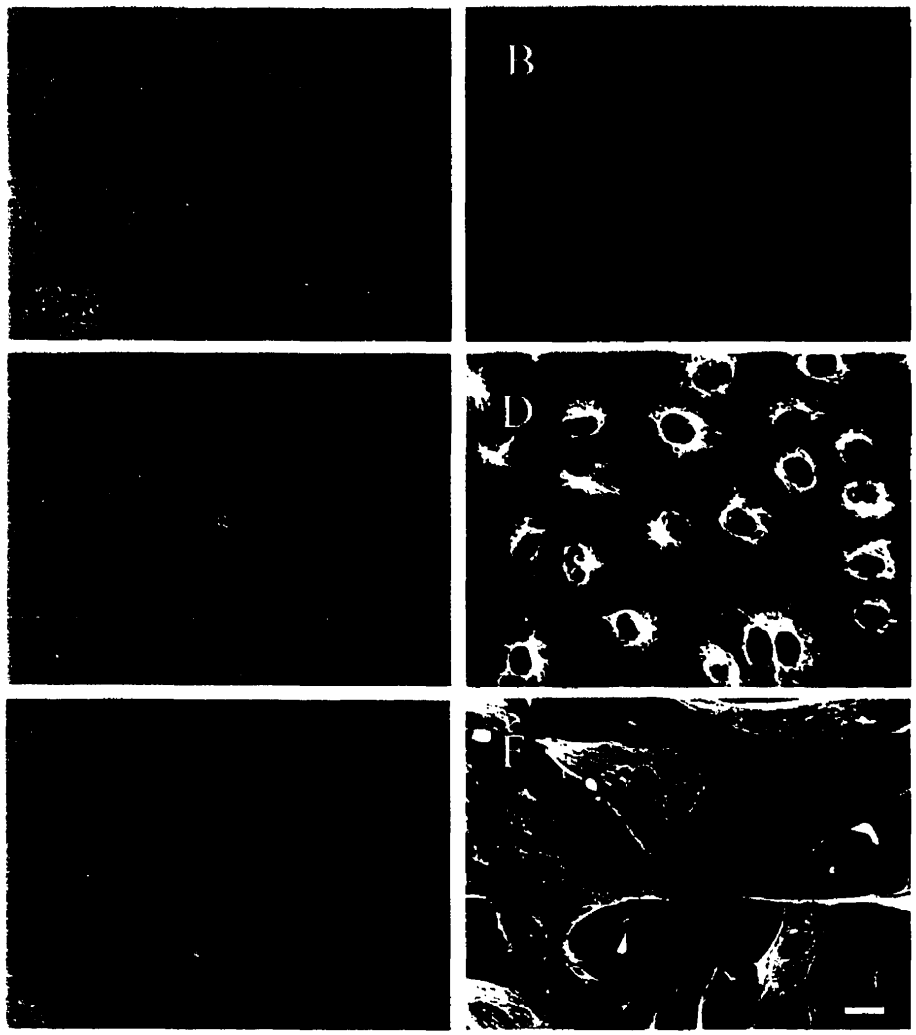


Figure 2. Vimentin immunostaining in bovine mammary cell lines. MAC-T (A, B), FibE (C, D), and FibC (E, F) cells were characterized by confocal microscopy for morphology under transmitted light (A, C, E) and for the distribution of the intermediate filament protein vimentin after immunofluorescent staining using an anti-vimentin antibody (B, D, F). The lack of vimentin expression in MAC-T cells suggests it is epithelial in nature. Although FibE cells exhibit an epithelial-like morphology, they express vimentin, typical of fibroblastic cells such as the FibC. All fluorescent images were taken at the same parameters. Bar = 10 μm.

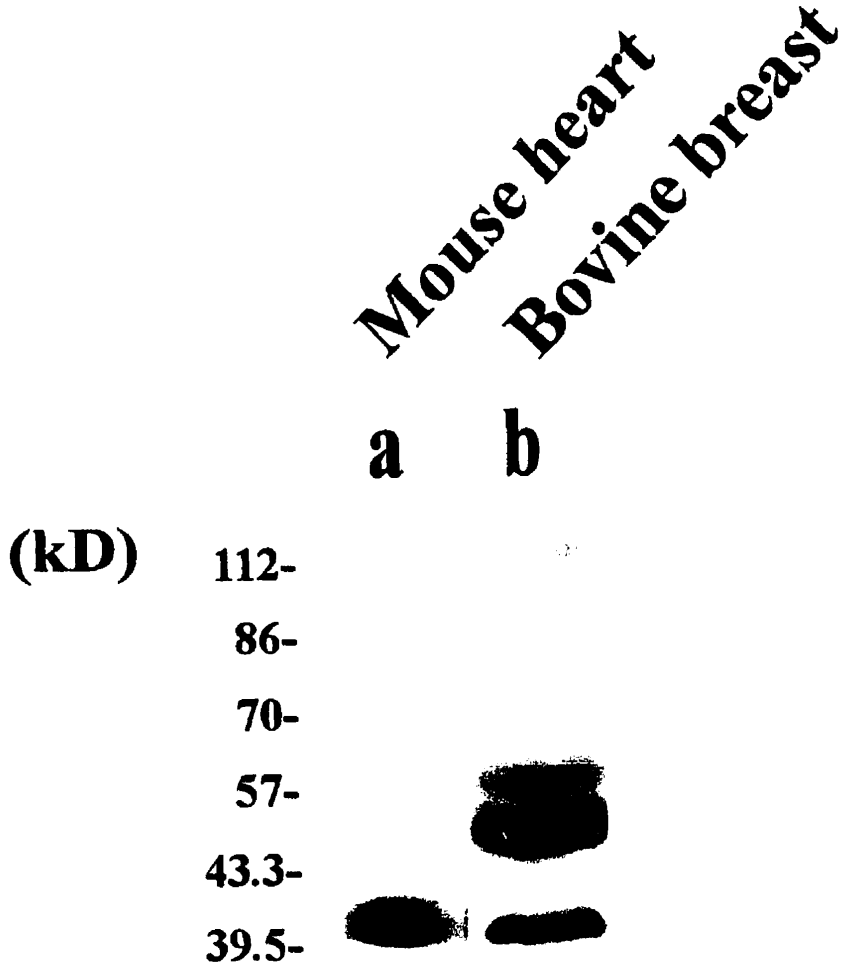


Figure 3.**Cells from the bovine breast express Cx43 *in vivo*.** Bovine mammary gland (15.6 μg) (lane b) and mouse heart (0.72 μg) (lane a) were Western blotted for Cx43. Note that the bovine mammary gland expresses Cx43 at ≈ 42 kD.

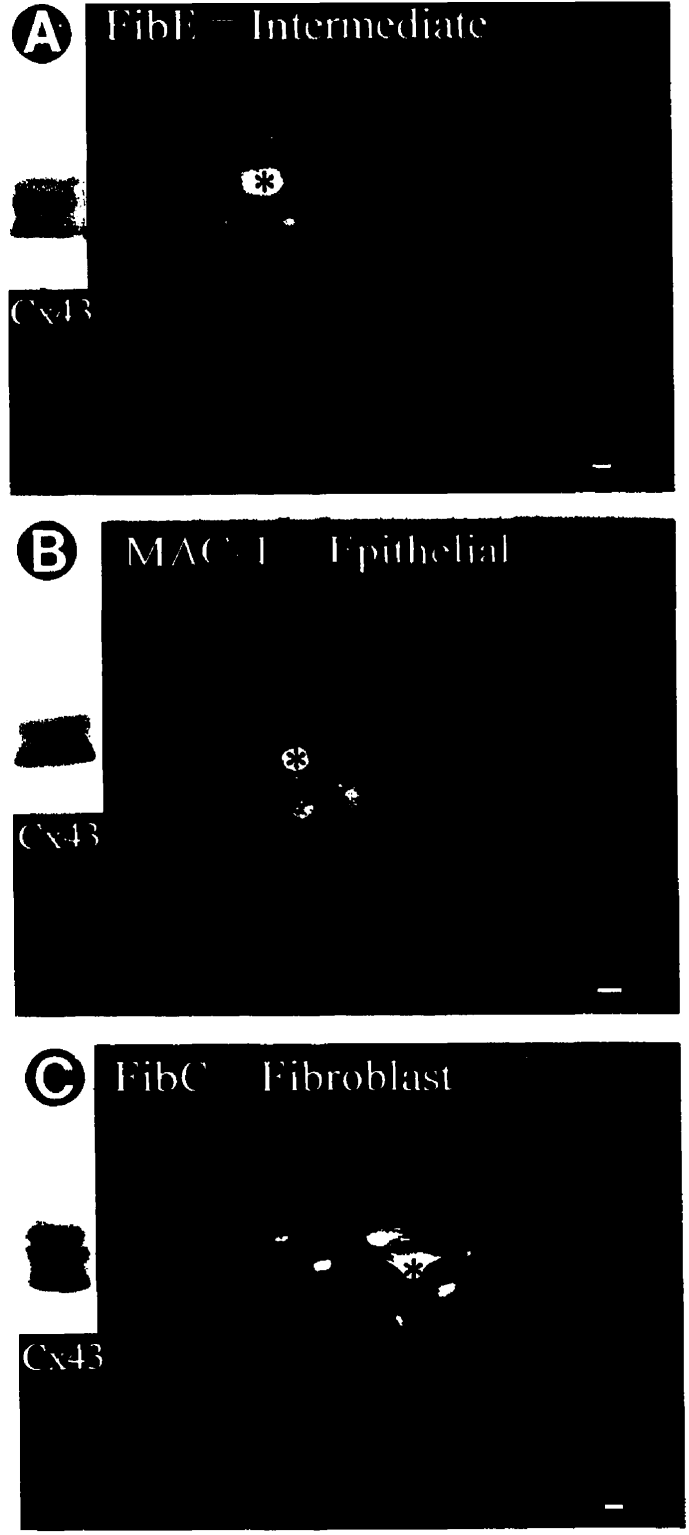


Figure 4. **Homocellular populations of intermediate cells, epithelial cells and fibroblasts express Cx43 and are well couple.** Live homocellular populations of intermediate FibE (A), epithelial MAC-T (B), and fibroblastic FibC (C) cells were microinjected with Lucifer yellow (injected cells denoted by asterisks) and dye was observed to spread to neighbouring cells in all cell types. Western blots were also performed using cell lysates of FibE (inset, A), MAC-T (inset, B), and FibC (inset, C). Several species of Cx43 were present in each cell type, a nonphosphorylated species and phosphorylated species. Bar = 10 μm.

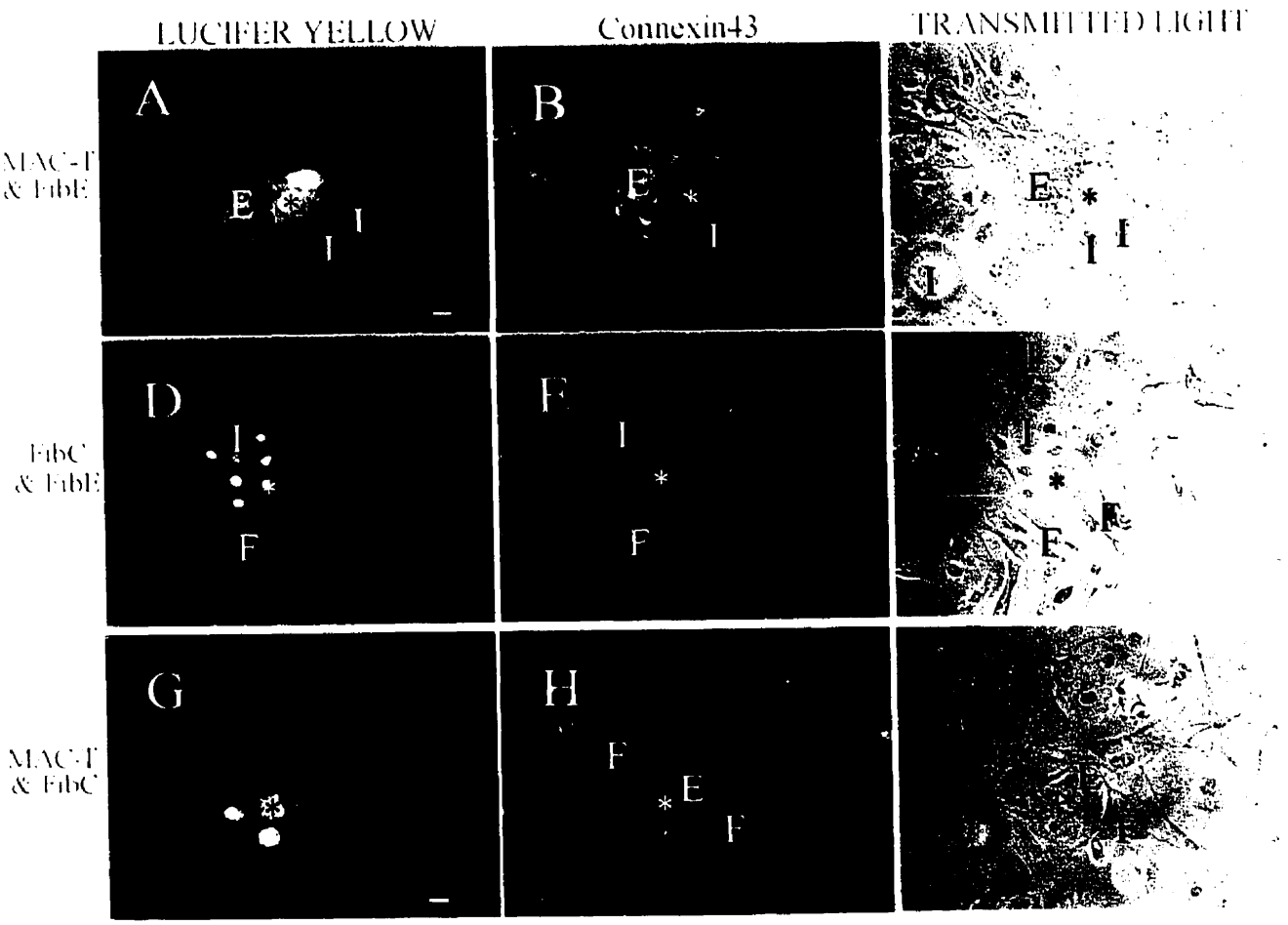


Figure 5. **Intercellular communication and connexin43 immunostaining in heterocellular cultures.** Heterocellular cultures of MAC-T/FibE (A-C), Fib C/FibE (D-F), and MAC-T/FibC (G-I) were microinjected with Lucifer yellow (A, D, G), immunostained for Cx43 (B, E, H), and viewed under transmitted light (C, F, I). Mixed cell populations show coupling between MAC-T cells (denoted by E= epithelial) and FibE cells (denoted I= intermediate) and between FibE cells and FibC cells (denoted F= fibroblasts), but poor coupling between epithelial cells and fibroblasts. The injected cell is indicated by an asterisk and the corresponding fields shown for Cx43 immunostaining and transmitted light images aid in distinguishing each cell line. Bar = 10 μm.

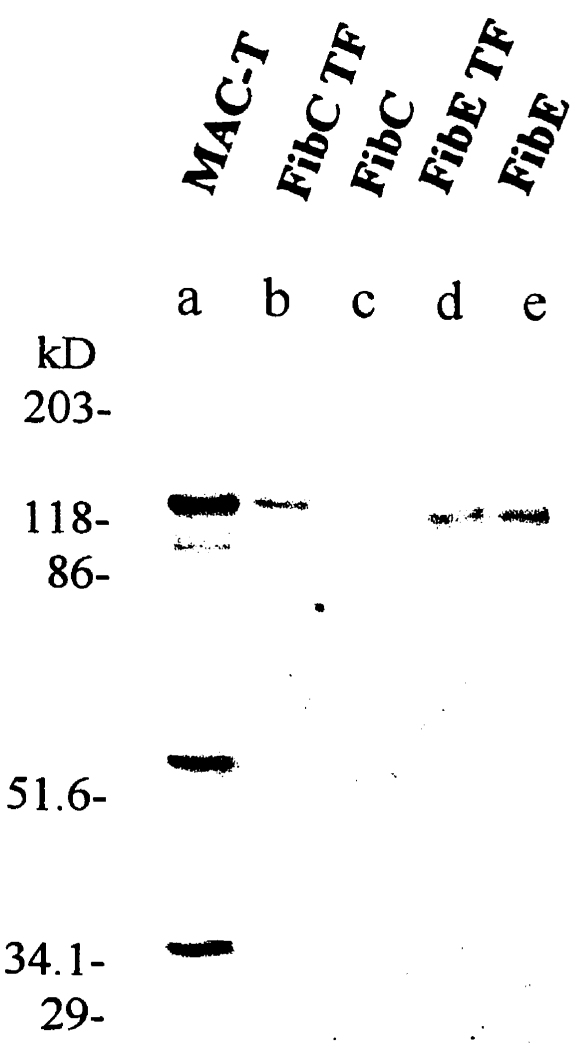


Figure 6. Western blot analysis of E-cadherin in bovine mammary cells. Western blots of lysates from MAC-T (lane a), FibC TF (lane b), FibC (lane c), FibE TF (lane d), and FibE (lane e) cells revealed that all cell lines, with the exception of the non-transfected FibC cells (lane c), contained full-length E-cadherin at $M_r \approx 120\text{-}130$ kD. E-cadherin expression was not upregulated in FibE cells after transfection with E-cadherin, but the level of E-cadherin expression in FibC cells was comparable to that of FibE after transfection. The cell lysates contain equal amounts of protein.

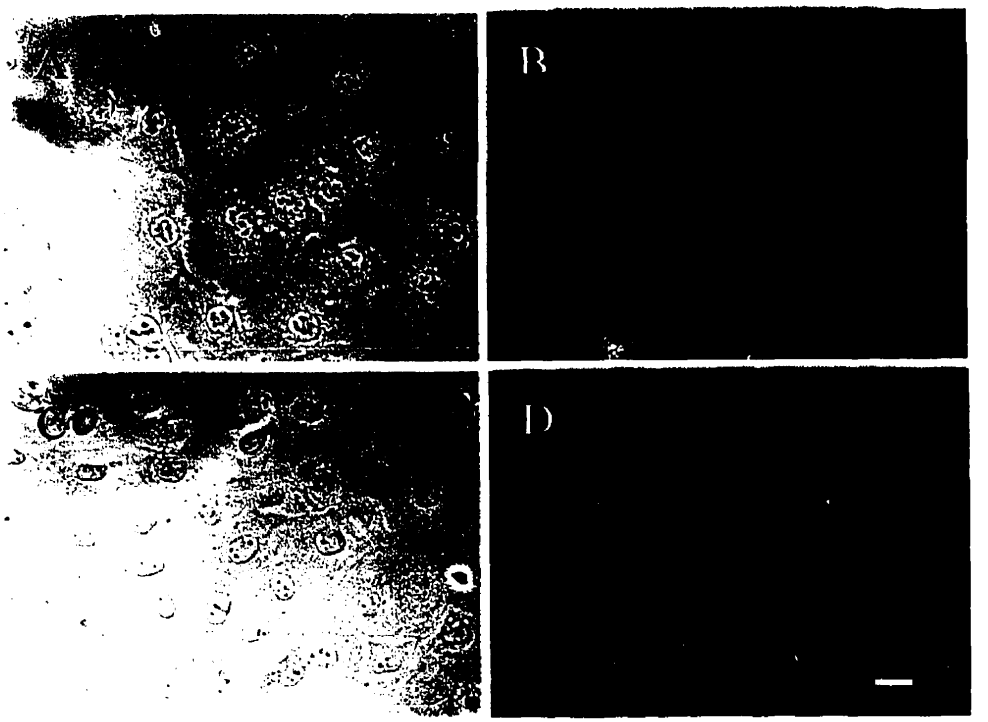


Figure 7. **Characterization of E-cadherin distribution in MAC-T and FibE cells.** The MAC-T (A, B) and FibE (C, D) cell lines were immunofluorescently stained for E-cadherin. E-cadherin staining was endogenously present in MAC-T and FibE cells at the cell surface (B, D), with additional intracellular staining present in MAC-T cells. The corresponding transmitted light images are shown (A, C). Bar =10 μm.

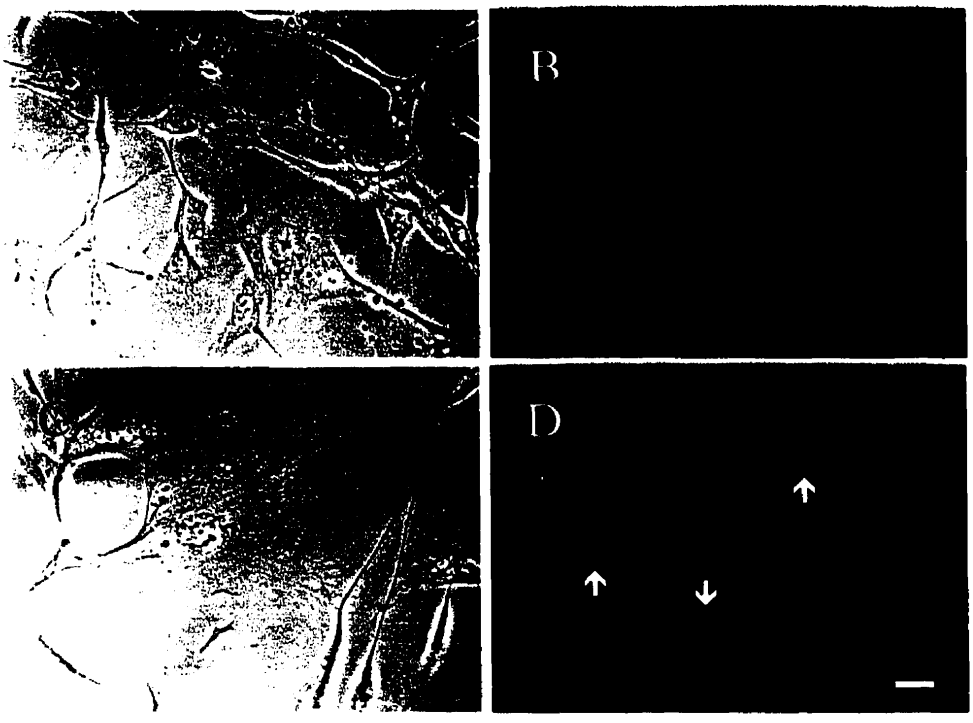


Figure 8. Characterization of E-cadherin distribution in FibC, and FibC TF. The bovine mammary fibroblasts, FibC, were immunofluorescently stained for E-cadherin prior to (B) and post-transfection (D) with a plasmid encoding E-cadherin. Cell surface immunostaining (see arrows) for E-cadherin was only observed in FibC cells after transfection (FibC TF cells). The corresponding transmitted light images are shown for FibC, prior to (A) and post-transfection (C). Bar =10 μm.

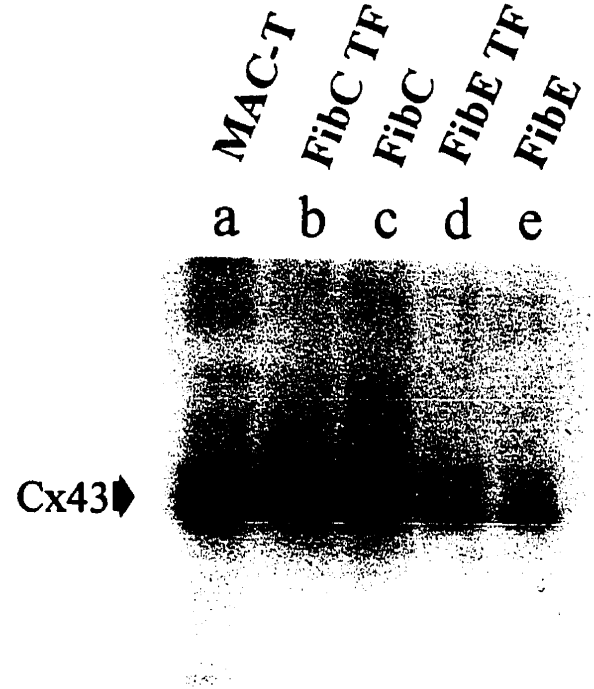


Figure 9. **Western blot analysis of Cx43 in bovine mammary cells.** Western blot studies of MAC-T (lane a), FibC TF (lane b), FibC (lane c), FibE TF (lane d), and FibE (lane e) cell lysates revealed that there were no alterations in the expression levels of Cx43 in either FibE or FibC following transfection. The expression levels of Cx43 in FibC and MAC-T cells were comparable; expression of Cx43 was substantially lower in FibE cells. The cell lysates have been standardized for protein content.

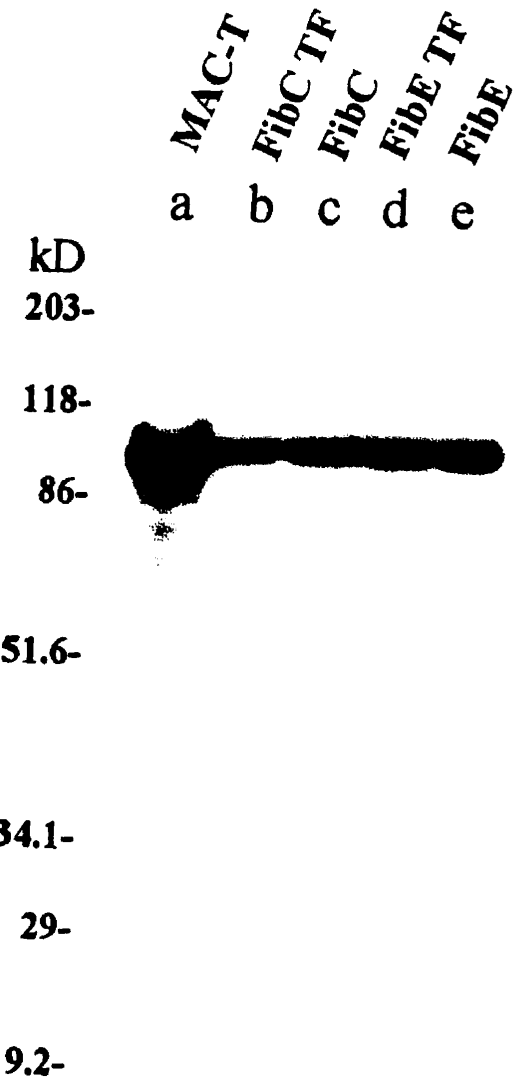


Figure 10. **Western blot analysis of ß-catenin expression in bovine mammary cells.** Western blots using an anti-ß-catenin revealed that MAC-T (lane a), FibC TF (lane b), FibC (lane c), FibE TF (lane d), and FibE (lane e) cells all expressed ß-catenin at ≈ 92 kD. The lysates have been standardized for protein content.

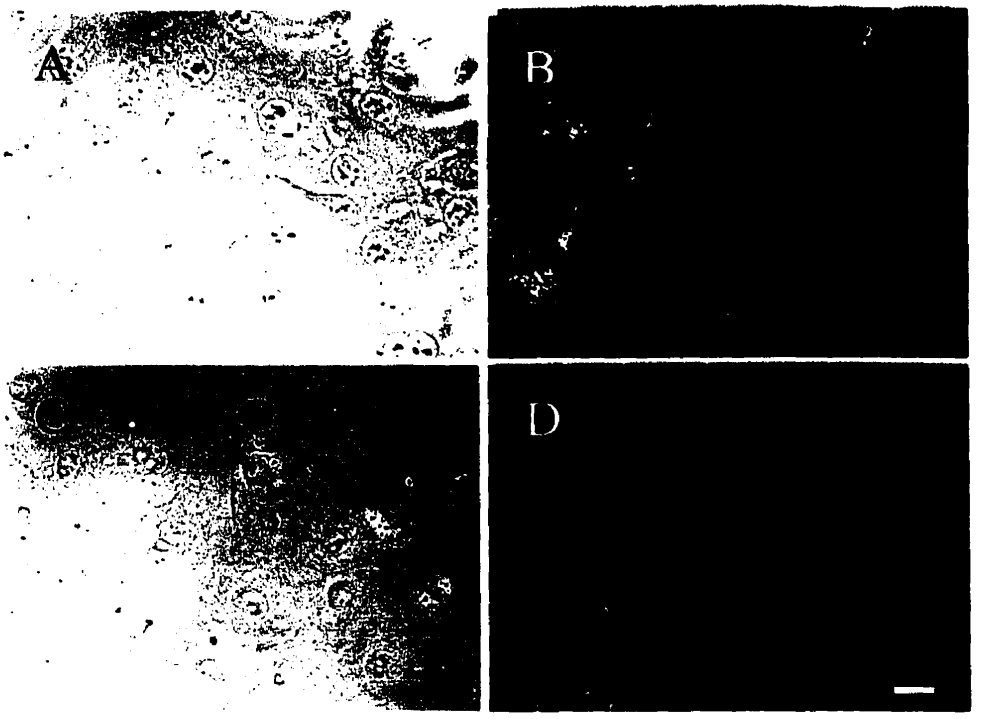


Figure 11. **Characterization of ß-catenin immunofluorescence in MAC-T and FibE cells.** Immunostaining with an anti-ß-catenin antibody revealed distinct staining patterns in bovine mammary cells. ß-catenin was distributed in a rim-like pattern at the periphery of FibE (D) and MAC-T cells (B) with additional intracellular pools in MAC-T cells. Cell morphology is shown in the corresponding transmitted light images of MAC-T (A) and FibE (C) cells. Bar = 10 μm.

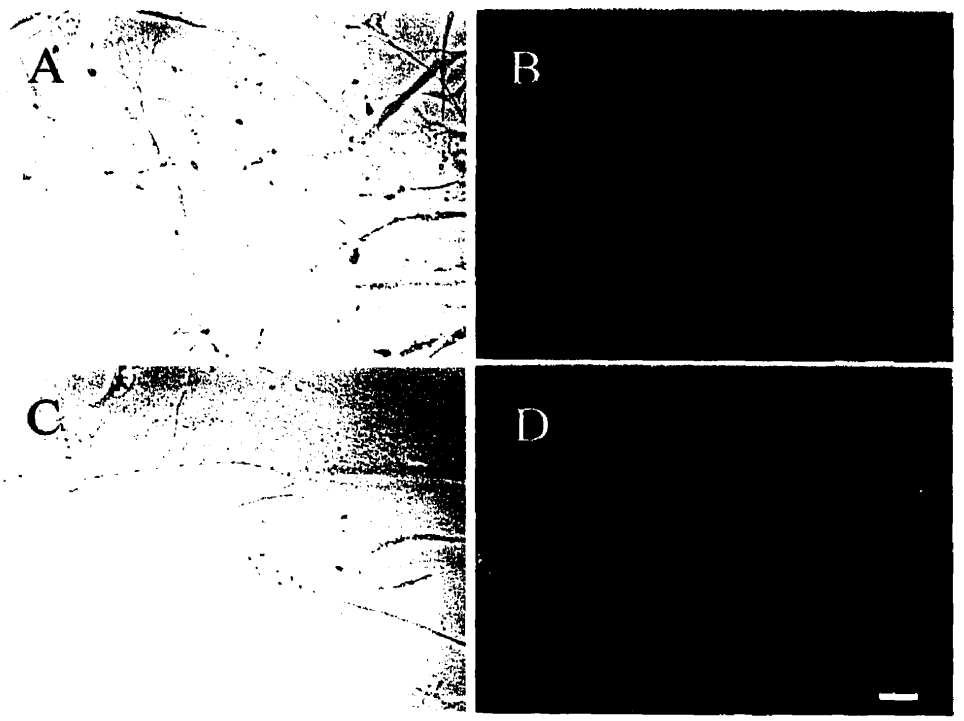


Figure 12. **Characterization of ß-catenin immunofluorescence in FibC and FibC TF cells.** Immunostaining with an anti-ß-catenin antibody revealed that ß-catenin was distributed in a punctate pattern at the cell surface of FibC cells prior to (B) and post-transfection (D). The respective transmitted light images, A and C, are presented. Bar = 10 µm.

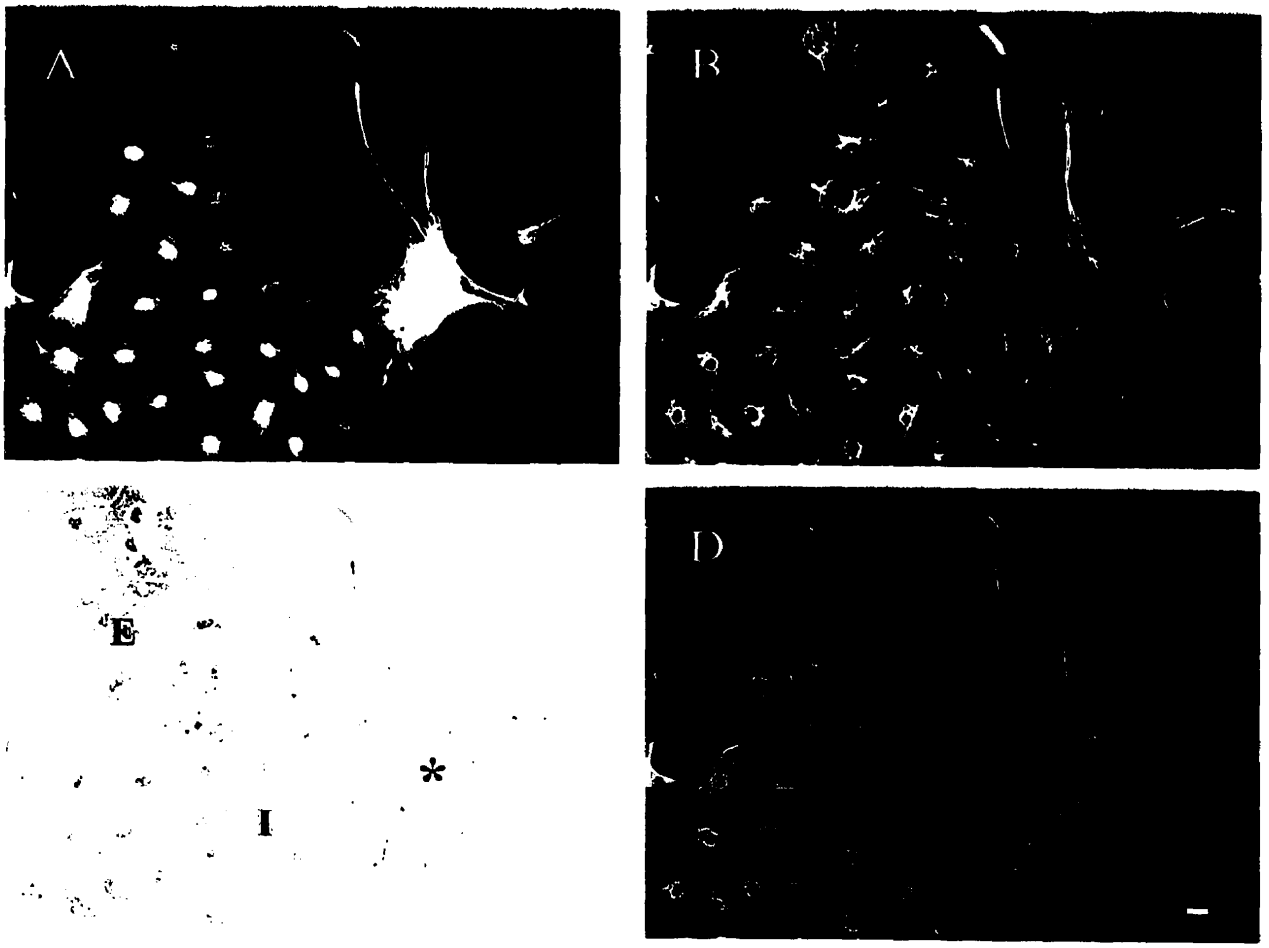


Figure 13. **Dye transfer from fibroblasts to epithelial cells via intermediate cells in triple cell cocultures.** When Lucifer yellow was microinjected into a fibroblast (A, D), dye transferred to epithelial "E" cells via a group of intermediate "I" cells. The injected cell is denoted by an asterisk in the transmitted light image (C). The intermediate cells and fibroblasts can be distinguished from epithelial cells by the presence of anti-vimentin (B, D) immunostaining. The morphological appearances of intermediate cells and fibroblasts is used to distinguish between these two cell types. Panel D represents an overlay of Lucifer yellow dye transfer (green) and anti-vimentin staining (red) with overlapping areas appearing in yellow. Bar = 10 μm.

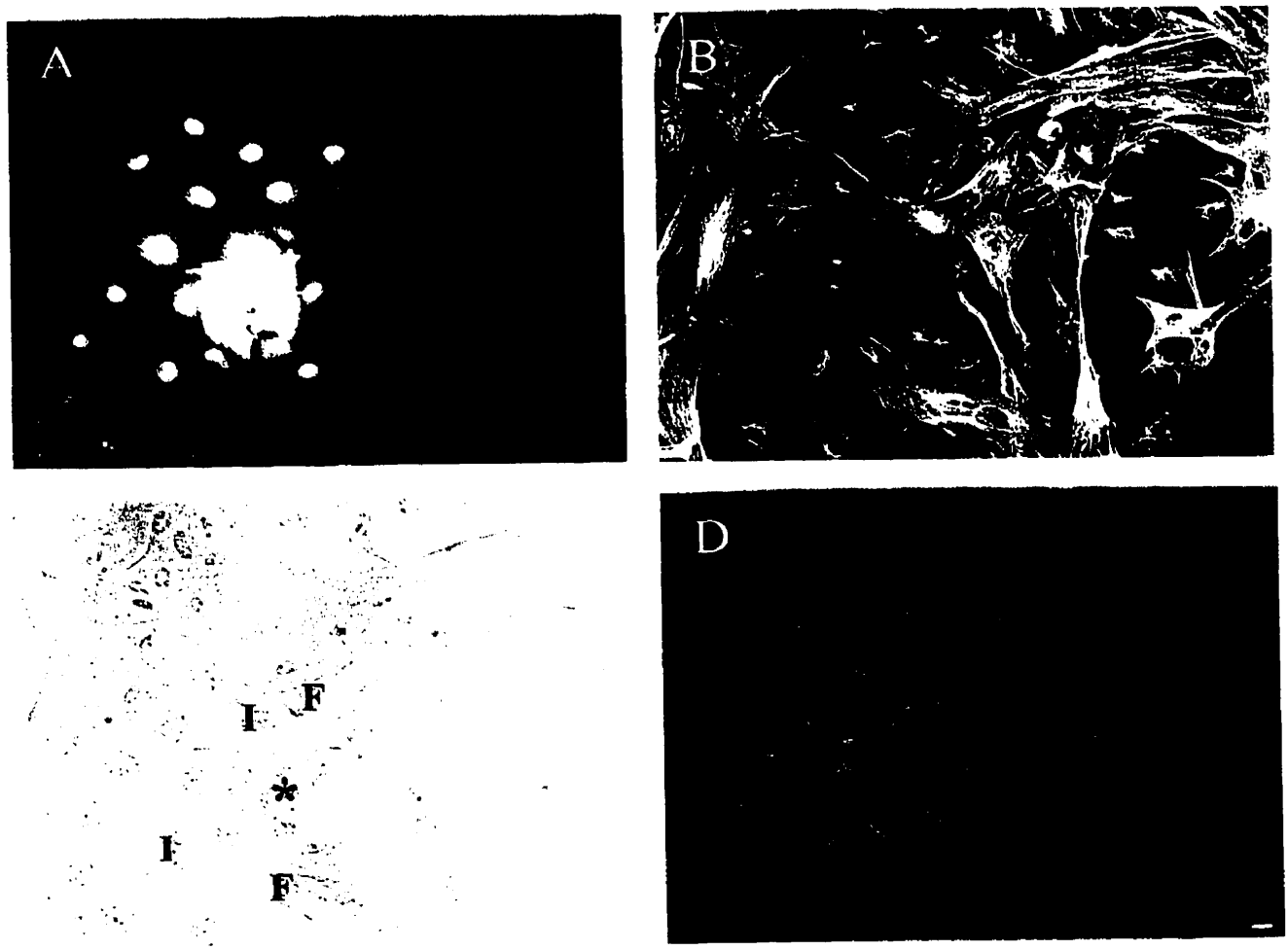


Figure 14. **Dye transfer from <u>epithelial cells to fibroblasts</u> via intermediate cells in triple cell cocultures.** When Lucifer yellow was microinjected into an epithelial cell (A, D), dye transferred to nearby fibroblasts "F" via adjacent intermediate "I" cells. The injected cell is denoted by an asterisk in panel C. The cell types are distinguished based on anti-vimentin immunofluorescent staining (B, D) and morphology under transmitted light (C). Panel D represents an overlay of dye transfer (green) and anti-vimentin immunofluorescence (red) with areas of overlap in yellow. Bar = 10 μm.

Table 1: Characterization of Bovine Mammary Cell Lines

	MAC-T	FibE	FibC
Morphology	Epithelial-like	Epithelial-like	Fibroblast-like
E-cadherin	+	+	-
Occludin	+	-	-
ZO-1	+	+	-
Vimentin	-	+	+
Cytokeratin	+	-	-

Bovine mammary cell lines, MAC-T, FibC and FibE, were characterized for morphology and by immunofluorescence for the presence/absence of marker proteins, cytoskeletal (vimentin, cytokeratin) or junctional (E-cadherin, occludin, ZO-1) proteins. These marker proteins enabled classification of MAC-T cells as epithelial cells, FibC cells as fibroblasts, and FibE as an intermediate cell type with properties of both epithelial cells and fibroblasts.

Table 2: Quantification of Heterocellular Dye Transfer

MAC-T & FibE			
Heterocellular Dye Transfer	Total 25/34= 73.5%	MAC-T--> FibE 16/23= 69.6%	FibE--> MAC-T 9/11= 81.8 %

MAC-T & FibE TF			
Heterocellular Dye Transfer	Total 42/42= 100%	MAC-T--> FibE TF 27/27= 100%	FibE TF--> MAC-T 15/15= 100%

MAC-T & FibC			
Heterocellular Dye Transfer	Total 8/66= 12.1%	MAC-T--> FibC 6/34= 17.6%	FibC--> MAC-T 2/32= 6.3%

MAC-T & FibC TF			
Heterocellular Dye Transfer	Total 9/63= 14.3%	MAC-T--> FibC TF 3/35= 8.6%	FibC TF--> MAC-T 6/28= 21.4%

FibC & FibE			
Heterocellular Dye Transfer	Total 15/53= 28.3%	FibC-->FibE 10/22= 45.5%	FibE-->FibC 5/31= 16.1%

FibC TF & FibE			
Heterocellular Dye Transfer	Total 19/55= 34.5%	FibC TF--> FibE 15/28= 53.6%	FibE--> FibC TF 4/27= 14.8%

FibC & FibE TF			
Heterocellular Dye Transfer	Total 21/44= 47.7%	FibC --> FibE TF 14/26= 53.8%	FibE TF--> FibC 7/18= 38.9%

FibC TF & FibE TF			
Heterocellular Dye Transfer	Total 15/38= 39.5%	FibC TF--> FibE TF 10/16= 62.5%	FibE TF--> FibC TF 5/22= 22.7%

The following table represents the quantification of heterocellular dye transfer. Cell lines (MAC-T, FibC, FibC TF, FibE, and FibE TF) were co-cultured in various combinations. Lucifer yellow was injected into one population of cells and observed for the frequency of successful transfer into cells of a different type. In all cases, injections had to fulfill two criteria: firstly, all injections, at the very least, had to transfer dye within the same population as the injected cell; and secondly, in the case of successful heterocellular dye transfer, more than one cell of a type other than the injected cell had to receive dye. In all scenarios of coculture, the total number of successful injections are presented and the success of dye transfer in either direction is presented.

Chapter 4

CONCLUSIONS AND ORIGINAL CONTRIBUTIONS

4.1 Conclusion:

We have shown that decreased Cx43 expression is not correlated with changes in gap junctional intercellular communication (GJIC) in quiescent mammary epithelial cells. GJIC appears to be maintained by the redistribution of intracellular Cx43 to the cell surface. In addition to alterations in the expression levels of Cx43, phosphorylation of Cx43 undergoes modest modifications at the G_0/G_1 transition suggesting the possible involvement of cell cycle dependent kinases. With the knowledge that loss of gap junctions has been shown to be involved in tumorigenesis, modifications in Cx43 and GJIC during the cell cycle may be possible mechanisms by which cells regulate proliferation. Gap junctional communication may permit cells to maintain responsiveness to regulatory or differentiation factors.

In the case of GJIC between different cell lineages, a process proposed to be involved in tumorigenesis and proper development, we have examined the role of E-cadherin expression as a critical mechanism for gap junction formation. We have shown that expression of the same cadherin, coexpressed with the same connexin, is insufficient in establishing heterocellular gap junction formation between distinct cell lines of the mammary gland. Transitional or intermediate cells may, however, act as mediators of GJIC between poorly coupled cell types. These studies underlie the importance of selective communication between different cell lineages in the mammary gland and support the proposal that GJIC between mammary fibroblasts and epithelial cells is uncommon and can be associated with abnormal growth, i.e. tumorigenesis, which is also marked by the presence of intermediate cell types.

4.2 Contributions to Original Knowledge:

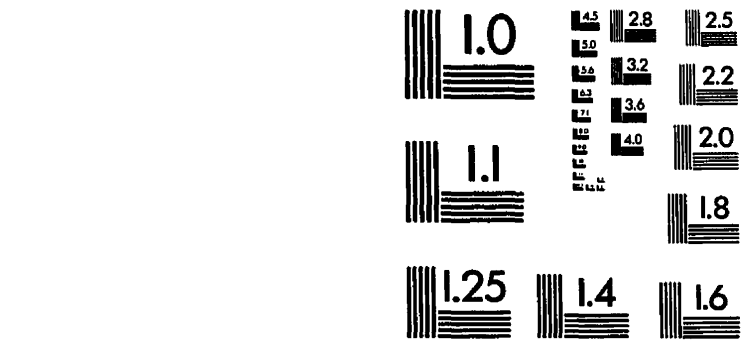
Chapter 2:

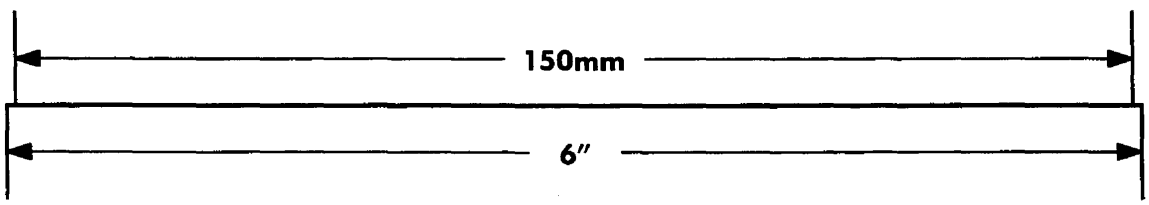
In this study, we have demonstrated that there are drastic reductions in Cx43 expression in the bovine mammary gland during quiescence and a modest change in Cx43 phosphorylation. However, these changes do not correlate with a significant down-regulation in GJIC.

Chapter 3:

We have shown that E-cadherin which has been reported to be necessary for gap junction formation is, itself, insufficient in establishing heterocellular communication between well characterized epithelial cells and fibroblasts of the mammary gland. Our studies also support the low incidence of heterocellular communication between these two cell lines. In addition, we have provided evidence suggesting that intermediate (transitional) cell types can bridge and regulate communication between mammary fibroblasts and epithelial cells.

In contrast to human and murine mammary glands, there is also a dearth of information concerning the role of gap junctions in the bovine mammary gland. Our studies represent, to our knowledge, the only experiments directly addressing the role of gap junctions in the bovine mammary gland.





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