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CHARACTERIZATION OF GAP JUNCTION GENE EXPRESSION IN NORMAL AND TUMORIGENIC HUMAN BREAST EPITHELIAL CELLS

By

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ABSTRACT

CHARACTERIZATION OF GAP JUNCTION GENE EXPRESSION IN NORMAL AND TUMORIGENIC HUMAN BREAST EPITHELIAL CELLS

by

Tyng-Tyng Yang

Recently in our laboratory, we have identified two types of normal human breast epithelial cells, one is deficient in gap junctional intercellular communication (GJIC) (Type-I) and the other is competent in GJIC (Type-II). Furthermore, Type-I cells were found to express luminal epithelial cell markers (i.e. epithelial membrane antigen, EMA, and cytokeratin 18) and to be induced to differentiate by cholera toxin into Type-II cells which express myoepithelial cell-specific antigen (i.e. cytokeratin 14). The primary objective of this thesis is to characterize the expression of different gap junction (GJ) genes in these two types of cells and three representative breast cancer cell lines.

The results indicate that Type-II cells express cx43 and cx26 but not cx32 GJ genes by Northern blot analysis. In contrast, Type-I cells and GJIC-deficient breast carcinoma cell lines, MCF-7 and T47D, did not express any of these GJ genes. The GJIC competent carcinoma-sarcoma cell line, HSO578T, expressed cx43 but not cx26 or cx32 genes. In Western blot analyses, all these three breast cancer cell lines appear to contain low levels of cx43 protein. Immunofluorescence studies, however, showed that only HSO578T cells displayed punctate fluorescence along borders of cell-cell contact.

Experiments were also carried out to restore GJIC in MCF-7 cells by transfection with an amplifiable plasmid carrying a mutated dihydrofolate reductase gene and the cloned cDNA for rat cx26, cx32 or cx43. The results indicate that GJIC can be restored by all of the three GJ genes. After extended growth, most of these transfectants, however, lost the ability to perform GJIC. Northern blot analysis showed that these transfectants express mRNA of transfected rat GJ genes.

To:

My Parents and Grandmothers

For Their Love and Support

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INTRODUCTION

Gap junction channels provide a hydrophilic pathway for exchange of ions, metabolites and second messenger molecules between coupled cells. This kind of junctional communication has been implicated in the regulation of tissue homeostasis, cell growth and differentiation and other functions such as synchrony of contraction (Vitkauskas et al.,1985). On the other hand, loss of junctional intercellular communication is suggested to play a role in carcinogenesis especially in promotion phases by facilitating the clonal amplification of initiated cells by releasing them from the suppressive control of surrounding normal cells (Yotti et al., 1979; Yamasaki, 1990).

Breast cancer is the leading malignancy of American women. It was estimated that there were 150,000 new cases and 44,000 deaths from female breast carcinoma in 1990 (Silberberg et al., 1990). Almost all of these cancers are adenocarcinomas and, of these tumors, the vast majority are ductal adenocarcinoma of luminal epithelial cell origin (Page and Anderson, 1987). Normal human breast epithelial cells are capable of performing gap-junctional intercellular communication (GJIC) (Fentiman, 1980; Eldridge et al.,1989) and have been reported to express gap junction genes for Cx26 and Cx43 (Tomesetto et al.,1991; Lee et al., 1992). In contrast, most breast carcinoma cell lines were found to be deficient in GJIC (Fentiman et al., 1979) with the exception of one report (Eldridge et al., 1989) which showed that non-immortal cells derived from breast tumors were proficient in GJIC whereas in vitro chemically immortalized cell lines were deficient in GJIC.

Recently in our laboratory we were able to culture two types of human breast epithelial cells derived from reduction mammoplasty: one is deficient in GJIC (Type-I), the other is capable of performing GJIC (Type-II). Furthermore, Type-I cells were found to express luminal epithelial cell specific antigens (i.e. epithelial membrane antigen, EMA, and cytokeratin 18) and to be induced to differentiate into Type-II cells which express

cytokeratin 14, a myoepithelial cell marker. The primary objective of this thesis research is to determine which gap junction genes are expressed in these two types of cells.

In addition, since MCF-7 breast carcinoma cells are deficient in GJIC (Fentiman and Taylor-Papadimitriou, 1977) and since transfection of GJIC-deficient human hepatoma cells with gap junction genes has been shown to retard tumor growth in vivo in conjunction with restoration of GJIC (Eghbali et al., 1990), experiments were carried out to determine if GJIC in MCF-7 can be restored by the transfection of three rat gap junction genes (i.e. Cx26, Cx32 and Cx43) cloned in mammalian expressible and amplifiable vectors in our laboratory. These results are also presented in this thesis.

LITERATURE REVIEW

General Properties of Gap junctions

Gap junctions are composed of a cluster of membrane proteins which form intercellular channels facilitating the transfer of molecules between coupled cells. This kind of cell-cell communication was first demonstrated in parallel structural and electro-physiological studies of synapse between neurons underlying the escape mechanism of crayfish (Furshpan & Potter, 1959). In addition to the passage of ions carrying electric impulse, physiological studies have shown that gap junctions also provide a pathway for the transfer of small molecules, such as nucleotides, nutrients, cAMP other regulatory molecules, between adjacent cells. Moreover, the net transfer of molecules and ions through junctional channels is a passive process, depending only on electrochemical gradients (Sheridan, 1987). Experiments with fluorescent tracer molecules suggested that the channel can accommodate molecules of up to 1.0-1.5 kDa (Alberts et al., 1989).

The first gap junctional protein cDNA was isolated from rat liver expression library by using an antibody against isolated gap junctions in 1986 (Paul,1986). To date, the complete cDNA corresponding to three major different gap junction proteins have been cloned: the cDNA codes for a 32 kD protein (connexin 32) from rat liver (Paul, 1986), a 26 kD protein (connexin 26) from rat liver (Zhang & Nicholson, 1989), and a cDNA codes for a 43 kD protein (connexin 43) from rat heart (Beyer et al.,1988). Furthermore, several corresponding connexins have been found in human, chicken, and the African frog, Xenopus (Bennett et al., 1991).

This gap junction-mediated intercellular communication (GJIC) is believed to play an important role in normal tissue growth and development (Loewenstein, 1981). The disruption of junctional communication at early stages was shown to result in specific developmental defects in Xenopus (Warner et al., 1984). GJIC may also be required for

synchronized contraction and metabolic coordination of cells in a tissue (Vitkauskas and Canellakis, 1985). In addition, GJIC has been proposed to be involved in the promotion phase of carcinogenesis. The evidence came from studies which showed 12-O-tetradecanoyl phorbol-13-acetate (TPA) and other tumor promoters inhibited GJIC (Yotti et al., 1979; Murray and Fitzgerald, 1979; Trosko and Chang, 1988). By blockage of GJIC, TPA and other tumor promoters might interdict the flow of growth-inhibitory signals from normal cells to an initiated neighbor, thus giving the latter an opportunity to proliferate (Yotti at al., 1979).

Structure of Gap Junction

The basic structural unit of the gap junction channel is the connexon, comprised of six protein subunits (connexins) which are organized as a regular hexagonal lattice. Two connexons of coupled cells form a junctional channel providing a hydrophilic pathway to connect the cytoplasms of these two neighboring cells. The gap junction hemichannel can open or close by a lateral circular movement of connexin proteins. The functional pore size of the GJ is 1.5 nm in diameter and the interacting plasma membranes of this junction are separated by a "gap" of 2 to 4 nm (Alberts et al., 1989).

From the information of gap junction protein sequence and studies using monoclonal antibodies, the connexin topology has been proposed. While amino termini and carboxy termini are located inside the cytoplasm, the protein traverses through the membrane four times to form an M shape with two extracellular loops and one cytoplasmic loop (Unwin and Zamphigi, 1980; Dermietzel et al., 1990). The cytoplasmic regions are more variable among different connexins within a species and may confer functional and regulational differences such as the presence or absence of phosphorylation sites for cAMP-dependent kinase, tyrosine kinase or protein kinase C (Hertzberg and Johnson, 1988). However, the sequences of comparable isoforms are extremely well conserved across species. The striking feature of the two extracellular loops is the

presence of three cysteines in each. These particular amino acids may form disulfide bonds with cysteines in adjacent hemichannels, contributing to the formation of the intercellular junction (Bennett, 1991).

The Role of GJIC in Normal Cells

I. Embryogenesis and Development

The role of gap junction-mediated intercellular communication in embryogenesis was first experimentally studied in embryos of lower vertebrates. Gap junctions were first observed at the 8-cell stage of newt (Ito and Hori, 1966) and Xenopus (Slack and Palmer, 1969) when electrical coupling was detected. Warner, et al. (1984) microinjected a polyclonal antibody against purified rat liver-derived gap junction protein into one identified cell of the 8-cell stage Xenopus embryo. This treatment selectively disrupts both dye transfer and electrical coupling between the progeny cells. Moreover, it led to specific developmental defects of the injected embryo, as indicated by the absence of eye and deformed shape of the head (Warner et al., 1984).

In mammals, GJIC has been monitored in pre- and postimplantation mouse embryos. The development of regional pattern was observed, which led to the formation of "communication compartments". Cells in a given compartment are well coupled with one another, whereas dye coupling is severely restricted between various compartments (Lo, 1980). This type of compartmentation was suggested to represent early tissue determination (Welsch, F., 1990).

II. Maintenance and Growth of Cells and Organs

Normal cells of most adult tissues exchange metabolites through gap junctions. Therefore, they share a very similar internal environment with other cells of that organ (Vitkauskas and Canellakis, 1985). GJIC was first proposed by Loewenstein (1979) to have an important role in growth control. An example of total metabolic dependence

upon neighboring cells is the lens of the eye. The eye lens do not contact with the blood system directly but they are nourished totally by junctional communication with the neighboring epithelial cells which are in contact with the blood supply (Rae, 1982). According to the metabolic cooperation experiments, Gilula et al. (1978) reported that the uridine which is used for RNA synthesis by the oocyte was derived from its neighboring cumulus cells.

By using communication-competent mutant cells (i.e. auxotrophs for a metabolite) in in vitro studies, it was demonstrated that auxotrophic mutants can survive in a medium lacking their essential metabolite if they are placed in contact with communication-competent normal cells (Subak-Sharp et al., 1969; Hertzberg and Gilula, 1981). Similarly, communication-competent cells that are sensitive to a drug do not die in the medium containing the drug, if they are cocultured with communication-competent drug-resistant cells (Ledbetter and Lubin, 1979).

III. Synchrony of Contraction

It is important and essential for some organs to function in synchrony. In the heart, individual myocytes are connected to each other by extensive arrays of gap junctions (De Mello, 1982). Recently, an enzymatic isolation of viable heart cells has been developed and it allowed electrophysiological study of cell pairs (Kameyame, 1983). By taking the advantage of that method, all published studies have confirmed that electrical transmission in the heart muscles is by way of low resistance couplings (Metzger and Weingart, 1985; White et al., 1985; Noma and Tsuboi, 1987). Therefore, GJIC provides an effective means for electrical synchronization of the cardiac fibers.

Synchrony of contraction is also characteristic of the uterus during parturition. Gap junctions in these tissues from nondelivering animals are small and rare. However, GJs in the myometrium increase in size and numbers one day before parturition and are at their highest peak during delivery (Garfield, 1984). In delivering tissues, each cell has

about 1000 GJs with an average size of about 0.2 μm in diameter (Garfield et al., 1980). Afterward, the junctions decline sharply 24 hr following delivery and disappear until nonpregnancy levels are again attained (Garfield, 1984). It was suggested that increased electrical coupling in the myometrium at term facilitates improved current flow, permitting synchronization and coordination of electrical and contractile activities in the individual muscle fibers and normal delivery of the fetus(es) (Garfield et al., 1989).

IV. Coordination of Response

In multicellular organs, cells can communicate with each other by various mechanisms, including the formation of low-resistance junctions. Intercellular communication via gap junctions allows some tissues to respond in a coordinated manner to hormones or growth factors. One example is the in vivo study of dye coupling among cells in prolactin stimulated rat pancreatic islets. B cells of the isle of Langerhans, which produce insulin, are interconnected by gap junctions with a limited number of their surrounding cells of different function. While prolactin elevated in serum, under the condition of hyperinsulinemia, the B cells increase their number of gap junctions by approximately 2-fold and the extent of intercellular communication with their neighboring cells by as much as 10 to 20-fold (Michaels, 1982).

In another study Lawrence et al. (1978) cocultured rat ovarian granulosa cells and mouse myocardial cells. Following exposure of the cocultures to epinephrine, a hormone specific for myocardial cells, not only did the heart beat become faster, but the ovarian granulosa cells also responded by releasing luteinizing hormone. On the other hand, upon stimulating the coculture with a hormone specific for ovarain granulosa cells, follicle-stimulating hormone, the myocardial cell also showed a response. These studies suggest that cross-stimulation might result from intercellular communication of a common mediator. It means that when a hormone stimulates a cell within a target tissue, a

coordinated response by other cells within the population might be achieved by the gap junctional transfer of low-molecular weight mediators (Lawrence et al. 1978).

Regulation of Intercellular Communication

The rate of gap junctional communication is proportional to junctional area, namely the number of connexon pairs (Sheridan,1973). However, gap junctions are dynamic structures, which can be modulated by many endogenous and exogenous factors.

I. Intracellular Modulation of Junctional Permeability

a. Calcium

Intracellular injection of calcium solutions into cells of the salivary gland of the insect *Chironomous* decreased both electrical and dye coupling. This condition can be reversed by lowering calcium concentration (Rose et al., 1977). By using the calcium-sensitive compound, aequorin, as a probe, Rose and Loewenstein showed that calcium levels need to become elevated only in the region of the junction of the *Chironomous* cells to decrease the transfer (Rose and Loewenstein, 1976).

Calcium is considered an important cell "decoupler" under pathological conditions (De Mello, 1987). For instance, when heart fibers are damaged, a high resistance barrier is immediately established near the damaged area protecting the fibers from further damage. It is assumed that the movement of Ca^{++} through the damaged cells increases the resistance of the gap junctions in neighboring cells (De mello,1972). This is described as a "healing-over" phenomenon which appears to be a homeostatic mechanism by which undamaged cells are protected from the harmful effect (Vitkauskas and Canellakis, 1985).

The calcium down-regulated GJIC may be mediated by protein kinase C, a calcium-dependent protein kinase (Murray and Gainer, 1989).

b. pH

Decreases in intracellular pH have been associated with reversible uncoupling in a variety of cells. When embryonic cells of Xenopus were exposed to 100% CO2, the intracellular pH was reduced from 7.7 to 6.4, the membrane potential was decreased and the electrical coupling was abolished (Turin et al., 1977). In cardiac Purkinje fibers the intracellular injection of H^+ also causes cell decoupling (De Mello, 1980). Most of the time, lowering the pH causes a corresponding increase in the Ca^{++} concentration which is known to decrease electrical coupling (Rose and Rick, 1978).

c. Voltage

In experiments involving the rectifying synapse of crayfish, depolarization on the presynaptic side was found to increase junctional conductance (Furshpan et al, 1959). In amphibian blastomeres, transjunctional voltage applied from either side decrease the junctional conductance (Harris et al., 1981).

d. Cyclic adenosine 3',5'-monophosphate

The junctional permeability of various cultured mammalian cell types depends on the concentration of intracellular cyclic adenosine 3',5'-monophosphate (cAMP) (Wiener and Loewenstein, 1983). The permeability rises when the cells are exposed to exogenous cAMP or when they are stimulated to synthesize cAMP by choleragen or hormones. The permeability decreases when cAMP is lowered by the addition of serum or by the increase in cell density. For example, C1-1D cells, a mouse cancer cell type, are unable to perform GJIC in ordinary confluent culture but they became electrically coupled and capable of transferring fluorescent tracer molecules (carboxyfluorescein, 376 daltons) upon administration of cAMP, dibutyryl cAMP (db-cAMP), db-cAMP and caffeine or cholera toxin (Azarnia et al., 1981). It was hypothesized that junctional permeability is dependent on protein kinase A which is a cyclic AMP-activated enzyme (Wiener and Loewenstein, 1983).

II. Endogenous Modulation of Junctional Permeability

a. Hormones

Gap junctions are hormonally responsive in several types of tissue. In the female reproductive system, the granulosa cells of the ovarian follicle have gap junctions that increase with estrogen and decrease with ovulatory doses of human gonadotropin (Burghardt and Anderson, 1979; Larsen et al., 1981). Another example is the increase of GJIC in pancreatic B-cells when stimulated to secrete by insulin (Meda et al., 1979). It was suggested that these hormonal factors may exert their action through cAMP.

b. Growth Factors

Gap junctions are suspected to play a role in contact inhibition. Growth factors, by definition, are capable of inducing mitogenesis and overcome contact inhibition. Furthermore, many oncogenes are known to code for growth factor (e.g. sis) or growth factor receptor (e.g. erb B). As will be describe later, several oncogenes have been shown to block GJIC. Therefore, it is reasonable to suspect that GJIC might be blocked by certain growth factors. Indeed, EGF and TGF-β have been reported to block GJIC (Madhukar et al., 1989).

c. Oncogenes

A large body of evidence has been accumulated showing the important role of oncogenes in the process of malignant progression. In addition, many tumor cells are not able to perform GJIC. It is reasonable, therefore, to suspect the involvement of oncogene expression in modulation of gap junctions. To date, several oncogenes have been shown to modulate GJIC in various cell types. Rous sarcoma virus or v-src was first demonstrated to inhibit GJIC in different cell types (Atkinson et al., 1981, 1986; Azarnia and Loewenstein, 1984; Chang et al., 1985) Azarnia et al. (1988) reported that over expression of the cellular src gene in NIH/3T3 cells causes reduction of cell-to-cell

transmission of molecules in the 400- to 700-dalton range. By comparing the phosphorylation of connexin 43 in uninfected and Rous sarcoma virous (RSV) transformed mammalian fibroblasts, it was observed that connexin 43 protein in RSV transformed fibroblasts contained both phosphotyrosine and phosphoserine but in untransfected cells connexin 43 protein was phosphorylated only on serine residue. Thus, the presence of phosphotyrosine in connexin 43 was correlated with the loss of GJIC observed in RSV transformed fibroblasts (Crow et al., 1990). The ras oncogene was also found to down-regulate GJIC (El-Fouly et al., 1989; Vanhamme et al., 1989; De Feijter et al., 1990; De Feijter et al., 1992).

Even though numerous evidence shows that oncogene products decrease intercellular communication, not all studies published seem to obey this rule. The mutated T24 ras protooncogene transfected into NIH/3T3 cells does not alter the extent of metabolic cooperation of these cells from that of the parental NIH/3T3 cells (Vitkauskas and Canellakis, 1983). Myc and fos oncogenes, when introduced into NIH/3T3 cells, did not decrease the extent of metabolic cooperation between these cells (Vitkauskas et al., 1983). These results suggest that at least some proto-oncogenes may affect intercellular communication different from those of the corresponding viral oncogenes (Vitkauskas and Canellakis,1985), or that there might be a threshold level of the oncogene products needed to down regulate the GJIC (De Feijter et al., 1992).

III. Exogenous Modulation of Junctional Permeability

a. Inhibition of GJIC by Tumor Promoting agents

Blockage of GJIC was believed to play an important role in carcinogenesis since defective or selective GJIC was observed in many transformed or tumor cells (Loewenstein, 1979; Kanno, 1985; Yamasaki et al., 1987). In 1979, Yotti et al. reported that the mouse skin tumor promoter, TPA can eliminate the metabolic cooperation between 6-thiogunaine-resistant and wild type Chinese hamster V79 cells (Yotti et al.,

1979). Murray et al. (1979) working independently, also showed that tumor promoters, TPA and phorbol-12,13-didecanoate (PDD), inhibited the transfer of radioactively labeled chemicals from HEL/37 mouse epidermal cells to 3T3 cells during coculture. In addition, in vivo studies showed that the liver tumor promoters, phenolbarbital(PB) and p,p'-dichlorodiphenyltrichloroethane (DDT), reduced gap junctions of rat hepatocytes from freeze-fracture analysis (Sugie, et al., 1987). Anther group, using a GJ protein cDNA and northern blot analysis, demonstrated that the level of GJ protein mRNA in liver was markedly reduced after a few weeks of phenobarbital exposure (Mesnil, et al., 1988).

Actually, there are some confusing and contradictory observations in the literature. In culture, TPA did inhibit GJIC of mouse epidermal cells (Pasti et al., 1988), however, it did not inhibit GJIC of mouse epidermal cells when mice were painted with TPA (Kam and Pitts, 1988). However, Kalimi and Sirsat (1984) it reduce gap junction in TPA-treated mouse epidermis. In addition, transforming growth factor β did enhance transformation of BALB/c3T3 cells but failed to inhibit GJIC (Hamel et al., 1988). On the other hand, it has been shown to inhibit GJIC in vitro (Madhukar et al., 1989). Tumor promoters, such as okadaic acid or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), also failed to block GJIC (Boreiko et al., 1986, Abernethy et al., 1985).

This apparently contradictory evidence suggest that different tumor promoters may operate via different mechanisms. Alternatively, it is more likely that details of different protocols are the basis for the differences. There is also the possibility that some promoters might affect only initiated cells and not the normal cells. In the case of the negative effect of TCDD, it needs to be shown that the cells tested had TCDD receptors. Some have suggested that tumor promotion may involve multiple mechanism and GJIC inhibition may not be the sole mechanism of tumor promotion evoked by various chemicals (Yamasaki, 1990). For example, it was postulated that the tumor-promoting property of okadaic acid is the result of significant cytotoxicity to the mouse epidermis, with the resulting regenerative hyperplasia acting as the true promoter (Mordan, 1991).

However, this explanation does not rule out the role of gap junctions, since the dead cells (or removal of cells by surgery) would result in the lack of communication (Trosko et al., 1983)

b. Enhancement of GJIC by Anti-Tumor Promoters

Some studies show that certain anti-tumor promoting agents, such as cyclic AMP, retinoic acid, and glucocorticoids can antagonize the inhibitory effect of TPA on gap-junctional communication (Yamasaki and Enomoto, 1985). In addition, anti-tumor promoters can induce junctional communication between normal cells and chemically or virally transformed cells, resulting in the growth inhibition of the transformed cell types (Mehta et al, 1986). For example, retinoids are presently undergoing widespread clinical trials as cancer chemopreventive agents (Bertram et al., 1987). In the range of 10^{-8}-10^{-10} M, the retinoids are up-regulators of GJIC in both 10T1/2 cells and their carcinogen-initiated counterparts. This up-regulation was correlated strongly with inhibition of transformation (Hossain et al., 1989). When db-cAMP, fluocinolone acetonide, retinoic acid, or dexamethasone was added during the induction of BALB/c3T3 transformation by 3-methylcholanthrene, there was a 65-95% decrease in the number of transformed foci. These chemicals have been shown to reestablish intercellular communication between transformed and normal cells (Yamasaki et al., 1988).

GJIC and Expression of Gap Junction Genes in Normal and Tumorigenic Human Breast Epithelial Cells

Normal primary breast epithelial cells have been shown to communicate with homologous cells (Fentiman and Taylor-Papadimitrious, 1977; Fentiman, 1980; Eldridge et al., 1989). In contrast, most human breast cancer epithelial cell lines were found to be deficient in GJIC (Fentiman et al., 1979), including the MCF-7 (Fentiman and Taylor-Papadimitrous, 1979) used in this study. The few breast cancer cell lines competent in

cell-cell communication (i.e. MDA231 and HSO578T) were different from normal breast epithelial cells in showing non-selective communication (ability to communicate with calf lens cells) (Fentiman et al., 1979). Eldridge et al. (1989) compared GJICs in normal, immortal and non-immortal tumor-derived human breast epithelial cells. They reported that both normal and early passage tumor-derived cells were competent in GJIC in contrast to in vitro immortalized cell lines which were deficient in GJIC.

Thus far, many different gap junction genes have been identified: Cx26 (Zhang and Nicholson, 1989); Cx31 (Hoh et al., 1991); Cx32 (Paul, 1986); Cx43 (Beyer et al., 1987) and Cx46 (Beyer et al., 1988). Four of these genes have been mapped on human chromosomes; ch. 13 (Cx26), ch. x (Cx32), ch. 6 (Cx43) and ch. 13 (Cx46) (Wilecke et al., 1990; Hsieh et al., 1991). Using Northern blot analysis, mRNA species of Cx26 and Cx43 were found to be expressed in cultures of normal breast epithelial cells but not in tumor-derived breast epithelial cell lines including MCF-7 (Tomassetto et al., 1991; Lee et al., 1992). In contrast to normal breast epithelial cells, these breast carcinoma cell lines do not perform GJIC (Lee et al., 1992). Immunofluorescence studies using antibodies against different gap junction proteins have shown that normal breast epithelial cells express Cx43 but not Cx26 and Cx32 (Wilgenbus et al., 1992). None of these Cx genes were expressed in invasive ductal or lobular carcinoma (Wilgenbus et al., 1992). Based on the differential expression of the gap junction gene in normal and tumorigenic breast epithelial cells, Lee et al. (1991) proposed that Cx26 may be a tumor-suppressor gene of breast cancer. Similarly, transfection of GJIC-deficient mouse or human cells with Cx43 and Cx32 respectively have been shown to inhibit cell growth in vitro (Mehta et al., 1991) and to retard tumor growth in vivo (Eghbali et al., 1990) in conjunction with restoration of GJIC.

MATERIAL AND METHOD

Cell Culture Medium

In this study two different tissue culture media, a modified Eagle's minimal essential medium (MEM) and a modified MCDB 153, were used. The modified Eagle's MEM consisted of Earle's balanced salt solution, 50% increase of all vitamins and essential amino acids except glutamine, 100% increase of all non-essential amino acids and supplemented with 1 mM sodium pyruvate (referred to as D medium) and 5% fetal bovine serum (FBS) (Chang et al., 1981) was used to culture MCF-7, T47D and HSO578T cells. These tumor cell lines were available in our laboratory and were from America Type Culture Cell (ATCC) originally.

The MCDB 153 was modified by increasing the concentrations of calcium (0.15 mM) and six amino acids (Pittelkow and Scott, 1986) and adding recombinant human epidermal growth factor (EGF) (1 ng/ml), insulin (10 ug/ml), hydrocortisone (1 ug/ml), human transferin (10 ug/ml), ethanolamine (0.1 mM), and phosphoethanolamine (0.1 mM). A 1:1 mixture (v/v) of the modified MCDB 153 and the modified Eagle's MEM (without FBS) (referred to as MSU-1 medium) (developed by C.C. Chang) was used to develop the primary human breast epithelial cells from reduction mammoplasty and for culture of Type-II cells. Type-I cells can be cultured in the MSU-1 medium supplemented with 5% FBS.

To select for methotrexate-resistant transfectants of MCF-7 cells, the modified Eagle's MEM was supplemented with 10% dialyzed fetal bovine serum. The Amicon DC2 system with Diaflo hollow fiber cartridge H1P10 (10,000 MW cut-off) was used to prepare dialyzed FBS. Dialysate was circulated at a high rate around the fibers to remove salts and other small solute by diffusion. When the total volume of the serum was

reduced by 50%, PBS was added to restore the initial volume. After 5 cycles, about 97% of small molecules can be removed.

Cells

Human breast tissues were derived from reduction mammoplasty. They were minced to small fragments with scalpels and dissociated to single cells or cell aggregates with collagenase (1 mg/ml) in medium 199 (0.1 g tissue per ml) overnight at 37°C. Then, the cell suspensions were pelleted at 600 g for 10 min and incubated in MSU-1 medium supplemented with 5% FBS. To reduce fibroblasts in culture, the floating cell aggregates were transferred to new flasks after two hours. The next day, the medium was changed to serum-free MSU-1 medium. The cell culture developed in a week, with medium change once every 2 days, the cells could be dissociated with trypsin (0.01%)-EDTA (0.02%) in phosphate buffered saline (PBS) (calcium, magnesium-free) for subculture or storage in liquid nitrogen.

The differential rates of attachment on plastic surface by different types of cells were used to separate epithelial cells from fibroblasts. Cells taken from the freezer were plated in a 25 cm^2 flask containing 10 ml MSU-1 medium with 5% FBS and allowed to attach for 2 hours. The unattached cells in the supernatant were then transferred to a new flask which contained mostly epithelial cells. The original flask containing enriched fibroblasts was discarded. The next day, cells in the supernatant which contain mostly Type-I cells were transferred to a 100 mm culture plate. Ten ml MSU-1 medium (serum-free) was added into the flask which contain mostly type II cells.

Three human breast carcinoma cell lines were also used in this study, i.e. MCF-7,T47D and HSO578T. All these cell lines were cultured in the modified Eagle's MEM containing 5% FBS. All cultures were incubated in 37°C incubators supplied with humidified air and 5% CO_2.

Transfection and Selection of MCF-7 WITH CONNEXIN GENES

Plasmids (pSV(RI)-DHFR containing rat connexin 43, 32 or 26 cDNA insert) (Jou et al., 1993) were introduced into MCF-7 cells by using the cationic liposome-mediated transfection method. Lipofectin reagent (BRL cat no. 8292SA) is a 1:1 (v/v) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA), and dioleyl phosphatidyl-ethanolamine (DOPE) in filter sterilized water. Positive charged lipid, DOTMA, can form liposomes and interact spontaneously with DNA to form a liposome/polynucleotide complex with complete entrapment of the DNA. The fusion of the complex with cell membrane can deliver functional nucleic acid molecules to the tissue cultured cells.

MCF-7 cells were seeded in a 100-mm tissue culture dish in 10 ml of D medium with 5% fetal bovine serum. The cells were incubated until they were 40-50% confluent. For each 100-mm dish, 20 ug plasmid and 40 ug lipofectin reagent were diluted to 1.5 ml serum-free D medium separately and then were combined in a polystryene tube and mixed gently. The mixture was allowed to stand for 15 min at room temperature before it was added to the cultured cells which were washed twice with 5 ml serum-free medium prior to the addition. Cells were incubated for 16 hr before returning to D medium with 5% FBS (3 ml per plate). Twelve hours later, the cells were washed with serum-free medium again and exposed to selective medium (D medium supplemented with 10% dialyzed FBS and 400 ng/ml methotrexate-MTX).

The plasmids used contained mutant dihydrofolate reductase (DHFR) gene. DHFR catalyzes the NADPH-dependent reduction of folate to tetrahydrofolate which is an essential cofactor in the synthesis of glycine, purines and thymidine. The antineoplastic agent --MTX, a folate analogue, is a competitive inhibitor of DHFR, therefore, a deficiency of nucleotide pools and cell death may be induced by MTX. In transfectants that express the mutated DHFR gene, abundant mutant DHFR with reduced affinity for

mtx will be sufficient to neutralize MTX inhibition and to confer MTX-resistance to these cells.

Measurement of GJIC

The ability of cells to perform gap junctional intercellular communication was determined by the scrape loading and dye transfer method (El-Fouly et al., 1987). The dye used is Lucifer yellow (MW457.2, Sigma, cat. no. L0259) which is an intensely fluorescent 4-aminophthalimide and it can be detected with epifluorescence microscope at low, non-cytotoxic concentrations. Lucifer Yellow cannot diffuse through intact plasma membrane but its low MW permits it's transmission from one cell to another through gap junctions.

Confluent cultured cells grown in 35 mm culture plate for 2 days were washed several times with PBS. With 2 ml Lucifer Yellow solution (0.5 mg/ml) in the plate several scrape lines on the monolayer were made using a surgical blade. After 3 minutes incubation at room temperature the dye solution was decanted and the cell monolayers were rinsed several times with PBS. The cells in PBS or medium were examined under a Nikon epifluorescence microscope to determine the extent of dye distribution.

Isolation of Total RNA from Cells

Total RNA was extracted by using the modified single-step method of RNA isolation which was initially described by Chomczynski and Sacchi (1987). Under acidic condition, total RNA can be extracted in a single step, free of DNA, by using guanidinium thiocyanate, phenol and chloroform.

Confluent cell cultures in a 75 cm^2 flask were washed twice with PBS and then exposed to 1.5 ml of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) After making sure the cells were lysed, the flask was rocked for 5 min. The cell lysate was transferred to two 2.2 ml eppendorf tubes

equally and 75 µl of 2 M sodium acetate, pH 4.0, was added to each tube. The solution was vortexed and then 0.75 ml water-saturated phenol and 200 µl chloroform (chloroform: isoamyoalcohol=49:1) were added. The mixture was vortexed after addition of each solution and cooled on ice for 10 min. The sample was centrifuged at 10,000xg for 15 min at room temperature. The aqueous phase was transferred to a fresh tube, mixed with one volume of isopropanol and then placed at -20°C for at least 1 hr to precipitate RNA. The insolubilized RNA was pelleted at 10,000xg for 30 min. The pellet was resuspended in 200 ul of 4 M LiCl and gently vortexed for 10 min. Sedimentation at 10,000xg for 10 min was again performed and the resulting RNA pellet was dissolved in 200 ul of 0.5% SDS. 200 ul chloroform was then added and mixed to the aqueous phase by vortexing more than 1 min. After centrifugation at 10,000xg for 15 min, the upper aqueous phase was collected and precipitated by 2.5 volume of absolute ethanol and 1/10 volume of 3 M sodium acetate, pH 5.2. The pellet was washed with 75% ethanol, sedimented, air dried and dissolved in 30 ul of 0.5% SDS. The concentration and purity of RNAs was measured by reading at 260 nm and 280 nm.

Northern Blot Analysis

I. Electrophoresis of RNA in Gel Containing Formaldehyde

One gram of agarose was added to 100 ml of DEPC-treated ddH_2O and boiled in a microwave oven until about 30 ml of H_2O had evaporated. After cooling to 60°C, the gel solution was supplemented with 10 ml of 10 x MOPS (3-[N-Morpholino]propane-sulfonic acid) and 18 ml of formaldehyde to make a 1 x MOPS, 3% formaldehyde gel. After solidification, the gel was submerged in running buffer (1 x MOPS, 3% formaldehyde in DEPC-treated ddH_2O). Twenty micrograms of each RNA sample in 20 µl of sample buffer (50% formamide, 1 x MOPS, 6% formaldehyde, 1 µl of tracking dye and 1 µg/ml

of ethidium bromide) was heated at 68°C for 10 min and then chilled on ice. The samples were loaded into wells and electrophoresed at 90 volts for 4 hours.

II. Northern Blot

When the migration was complete, the gel was rinsed in excess DEPC-treated ddH_2O for 45 min to remove the formaldehyde. A picture of the gel along side with a transparent ruler was photographed above an ultraviolet illumination box. The gel was then soaked in 20 x SSC for 45 min with agitation. The RNAs were transferred from the gel to a nylon membrane (Hybond™-N, 0.45 micron; Amersham) by capillary transfer overnight. The membrane was rinsed with 2 x SSC and baked at 80°C in a vacuum oven for two hours. Furthermore, the side of the membrane carrying the RNA was exposed to a low dose of UV (4.5 min) on a UV transilluminator to crosslink RNA to the membrane.

III. Hybridization and Autoradiography

The whole length rat cDNAs for Cx43, Cx32 and Cx26, which were provided by Dr. Emmanuel Dupont, were used for making probes. Probes were made by using a random primed DNA labeling kit (Molecular Biology, Bochringer Mammheim)

Fifty nanograms of cDNA in 18 µl ddH_2O were denatured by heating at 95°C for 10 min and subsequently chilled on ice. The following components were added to the denatured cDNA: 6 ul of dATP/ dGTP/ dTTP (1:1:1), 4 ul of reaction buffer, 10 ul of [α-^{32}P]dCTP (50 uCi) and 2 ul of Klenow enzyme. The mixture was incubated at 37°C for 1 hour and the reaction was terminated by adding 10 ul of 0.2 M EDTA, pH 8.0. The reaction mixture was purified using a Bio-spin column (Bio-Rad, cat. no. 732-6006). The purified labeled-probe was denatured by heating at 95°C for 10 min and then chilled on ice just before being used.

During the time of making a probe, the membrane was pre-hybridized in a sealed bag containing 10 ml of hybridization buffer (5 x SSC, 5% Dextran Sulfate, 0.5% SDS, 5x Denhardt's and 250 ug/ml Herring sperm DNA) at 65°C for 1 hr. For hybridization, the

labeled probe was added to the bag and continuously incubated at the same temperature overnight. Next day, the membrane was taken out from the bag , rinsed with 2 x SSC , and washed twice with 0.5 x SSC at 65°C for 30 min. Autoradiographies were done at -70°C by using Kodak X-OMAT and regular screen.

Immunofluorescence staining of Gap Junctions

Cells were seeded on glass cover slips and cultured until they approached confluence. The cells were rinsed with 2 ml of Ca^{++} and Mg^{++} containing PBS for 3 times. After the last wash, the washing solution was removed as much as possible. The cells were fixed in 2 ml of 4% paraformaldehyde in 100 mM PIPES, pH 6.8, 1 mM $MgCl_2$ and 3% sucrose for 15 min. Fixation solution was freshly made by boiling paraformaldehyde in a solution of 100 mM PIPES and 1 mM $MgCl_2$ until dissolved, and then adding adequate sucrose. The solution was allowed to cool at room temperature. After fixation, the cultured cells on cover slips were rinsed thoroughly with 2 ml of 3% sucrose in PBS 2 times. Cells were permeabilized with 2 ml of -20°C methanol for 30 sec and washed subsequently with PBS twice. After these steps, the cells were incubated in 2 ml of 2 mg/ml sodium borohydride for 10 min, and followed by washing twice with PBS. Non-specific binding sites were blocked by incubating these cells in 10% normal goat serum (NGS) in PBS plus 0.05% Tween-20 at room temperature. One hour later, the solution was thoroughly removed by suction. The cells were incubated with 300 ul of primary antibody (1° Ab) (purified by Dr. Dupont) (1:50 dilution in 5% NGS, 0.3% gelatin and 0.05% Tween-20 in PBS) at room temperature for 2 hr. Diluted antibodies were centrifuged at least 15 min in a microcentrifuge before using. For the preimmune control, the diluted 1° antibody was incubated with corresponding peptide antigen (1:100 dilution) for 30 min before being used to incubate with cells. Afterward, the cover slip was rinsed twice, 5 min each, with 2 ml of 0.3% gelatin and 0.05% Tween-20 in PBS (washing buffer). The second incubation was carried out for 1 hr at room temperature in

the presence of Rhodamin-labeled conjugate afinipure Ducking anti-rabbit Ig G (ImmunoResearch laboratory, Inc. cat. no. 711-025-132) (1:200 dilution in the same Ab dilution buffer used for 1° Ab). After being rinsed with 2 ml washing buffer for 4 times, the cover slip was mounted onto a slide using Polysciences' Aqua/polymount. The cells were examined under an epifluorescence microscope using the G filter.

Protein Isolation and Quantification of Protein

Confluent cells culture in 25 cm^2 flask were rinsed twice with 5 ml of PBS then lysed in 0.5 ml of lysis buffer (20% SDS, 0.1 M Tris, pH 7.5 and 20 mM EDTA). The cell lysate was then transferred to a 2.2-ml eppendorf tube and sonicated for 30 sec. Subsequently, 100 µl of cell lysate was taken out for measuring the concentration of protein and 4 µl of β-mercaptoethanol was added to the remaining lysate.

A modified Bio-Rad DC protein assay was used for determining the protein concentration of the samples. First, samples were diluted 1:20, meanwhile, six protein standard solutions containing from 25 µg/ml to 800 µg/ml were prepared in the same buffer as the sample. 25 µl of standard or sample was pipeted into a 1.5-ml eppendorf tube, and mixed with 125 µl of reagent A' and 1 ml of reagent B (Bio-Rad cat no.500-0116). The tubes were vortexed after adding each reagent. After 15 min, the absorbance was read at 750 nm. The average absorbances of triplicate determination was used to calculate protein concentration.

Western Blot Analysis

I. SDS-Polyacrylamide Gel Electrophoresis of Protein

The Laemmli discontinuous buffer system (Laemmli, 1970) was used with 4.5% stacking gels and 12.5% separating gels. The apparatus was obtained from Bio-Rad Inc.

Fifteen ml (for two gels) of 12.5% separating gel(4.2 ml acrylamide/Tris (30:0.8), 2.5 ml 1.5 M Tris, pH 8.8, 2.9 ml ddH_2O and 0.1 ml 10% SDS) was prepared and degassed for 15 min before being used. Then, 30 µl of ammonium persulfate (AP) and 270 µl of 1% N,N,N,N-Tetramethylethylenediamine (TEMED) were added to the gel solution to initiate polymerization. As soon as mixing well, the acrylamide solution was poured into the gap of glass plates. Isopropanol was spread over the gel to prevent oxygen from diffusing into the gels and inhibiting polymerization. After polymerization was complete (about 40 min), the overlay was poured off and the top of the gel was washed several times with deionized water to remove any unpolymerized acrylamide. While the separating gel was polymerizing, the stacking gel (1.5 ml Acrylamide/Tris (30:0.8), 2.5 ml 0.5M Tris, pH 8.3, 5 ml of ddH_2O and 0.1 ml 10% SDS) was prepared and degassed for around 10 min. The gel mixture was swirled rapidly when 150 µl of 10% AP and 750 µl of 1% TEMED were added. Without delay, the stacking gel solution was poured directly onto the surface of the polymerized resolving gel. Immediately, a clean Teflon comb was inserted into the stacking gel solution. More stacking gel solution was added to fill the space of the comb completely. The Teflon comb was removed carefully after polymerization was done (~ 20 min). The gels were then mounted in the electrophoresis apparatus. Moreover, Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. It was important to remove all bobbles which became trapped at the bottom of the gel between the glass plates. Finally, 20 µg of each of the samples which were prepared in 1 x SDS gel-loading buffer was loaded into the bottom of the wells. An equal volume of 1 x SDS gel-loading buffer was loaded into any wells that were unused. The gel was electrophoresed at 50 v until the dye front had moved into the resolving gel, the voltage was increased to 200 v. When the bromophenol blue reached the bottom of the resolving gel, the power was turned off.

II. Transfer of Protein from SDS-Polyacrylamide Gel to Solid Support

Four pieces of 3 MM paper and two pieces of polyvinylidene difluoride (PVDF) membrane were cut to the exact size of the SDS-polyacrylamide gel. One corner of the filters was marked with a soft-lead pencil.

These 3 MM papers were soaked in a shallow tray containing transfer buffer. The PVDF membranes were pre-wetted in methanol then soaked in transfer buffer. The transfer apparatus was set up in the following order: anode, porous pad, 3 MM paper, membrane, gel, 3 MM paper, porous pad and cathode. A glass pipette was used as a roller to squeeze out any air bubbles between layers. Twenty volts overnight were applied to transfer the protein.

Following transfer, the gels were stained with 0.1% Coomassie Brilliant Blue in methanol:H2O (1:1)/glacial acetic acid (9:1). Meanwhile, the membranes were rinsed with ddH_2O then stained with Ponceau S until the bands of protein were visible. The membranes were washed in several changes of deionized water. The standards were cut out from the membranes before immunoblotting.

III. Immunoblotting

Non-specific binding sites were blocked by soaking the PVDF membranes in 5% milk solution (40 mM Tris, pH7.5, 5% nonfat milk and 0.02% SDS) for at least one hour at room temperature with gentle agitation. The purified rabbit anti-rat connexin (Cx 43, Cx 32 or Cx 26) antibody, provided by Dr. Dupont, was diluted 1:25 with 5% milk solution. The control peptide was prepared in 1:100 dilution with Ab solution and preincubated for 30 min at room temperature. The membranes were incubated in a solution containing 1°Ab or 1°Ab and control peptide at room temperature for one hour. Afterward, these membranes were washed in 10 ml of 4% milk solution (40 mM Tris, 4% nonfat milk and 0.1% Tween 20) four times for 5 min each. The membranes were then incubated with 5 ml of a 1/500 dilution of biotinlayted anti-rabbit IgG (species-specific

$F(ab)_2$ fragment) (Amersham, cat. no. RPN 1064) in 4% milk solution for 1 hr and subsequently washed 4 times with the same solution. The membranes were incubated with a 1:5000 dilution of alkaline phosphatase-conjugated streptavidin (Amersham, cat. no. RPN 1234) in the solution mentioned above for one hour and sequentially washed four times with the solution of 20 mM Tris, 0.5 M NaCl and 0.1% Tween 20. Finally, these membranes were developed with the 5-bromo-4-chloro-3-inclonyl phosphate (BCIP)/ nitro blue tetrazolium (NBT) color development solution which was prepared by dissolving a 10 mg tablet of NBT in 30 ml of alkaline phosphatase buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl and 5 mM $MgCl_2$) and adding 100 µl of 5% BCIP.

Anchorage of Independent Growth (AIG)

Untransformed cells derived from solid tissues usually require a substrate to attach and to proliferate. In contrast, most tumor cells are capable of anchorage independent growth (AIG). The following procedure was used to test the ability of AIG.

A sufficient volume of 3.3% agarose (Sigma A-6013 Type-1 Low EEO) in PBS was prepared first and was kept at 45°C in a waterbath. Meanwhile, enough medium (D medium with 10% FBS or D medium with 10% dialyzed FBS and 400 ng/ml MTX) was warmed to 39°C in a waterbath. For the 0.5% hard agar layer, 0.45 ml of 3.3% agarose and 2.55 ml warm medium were mixed thoroughly by using a warm pipet and the mixture was immediately plated into a 60-mm dish. The hard agar layer was left at room temperature for 30 min to solidify. The 0.33% of soft agar was prepared by mixing 2.2 ml of medium, 0.3 ml of 3.3% agarose and $2x10^4$ cells in 0.5 ml of medium. This was then overlayed on the hard agar. Five milliliter of medium was added the second day and renewed every 4 days. Four weeks later, the culture was stained for 24 hours at 37°C with a solution containing 1 mg/ml of the tetrazolium salt, which is 2-(P-iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride (Sigma I-8377) in 0.9% of sodium chloride. The stained colonies in agar were dried (on a warming plate) and counted.

RESULTS

I. GJIC and Gap Junction Gene Expression in Normal Human Breast Epithelial Cells

a. Ability of Two Types of Normal Human Breast Epithelial Cells to Perform GJIC

Two primary human breast epithelial cell cultures (HME11 and HME14) derived from reduction mammoplasty from two different women were used in this study. The initial cultures were developed in MSU-1 medium. Both contain two morphologically distinguishable epithelial cell types. The first cell type is less reflective and less distinctive in cell boundaries (Fig. 1A and Fig. 2A for HME11 and HME14 respectively). This cell type (Type-I) is also characterized by its inability to perform GJIC based on studying using the scrape loading/dye transfer technique (Fig. 3A and Fig. 4A for HME11 and HME14 respectively). In contrast, the second cell type is cobblestone-shaped and is more reflective with distinctive cell boundaries (Fig. 1B and Fig. 2B for HME11 and HME14 respectively). More specifically, the second cell type (Type-II) is competent in GJIC (Fig. 3B and Fig. 4B for HME11 and HME14 respectively).

b. Expression of mRNA for Gap Junction Genes in Two Types of Normal Human Breast Epithelial Cells

The expression of gap junction genes was studied by Northern blot analysis using cloned cDNA for rat Cx26, Cx32 and Cx43 as probes. The results indicated that Type-I cells do not express gap junction genes for Cx43 (Fig. 6A, lane 3 and 5 for HME11 and HME14 respectively) and Cx26 (Fig. 7, lane 3 and 5 for HME11 and HME14 respectively). In contrast, Type-II cells were found to express genes for Cx43 (Fig. 6A, lane 4 and 6 for HME11 and HME14 respectively) and Cx26 (Fig. 7, lane 4 and 6 respectively). The breast stromal fibroblasts derived from the initial HME14 culture were

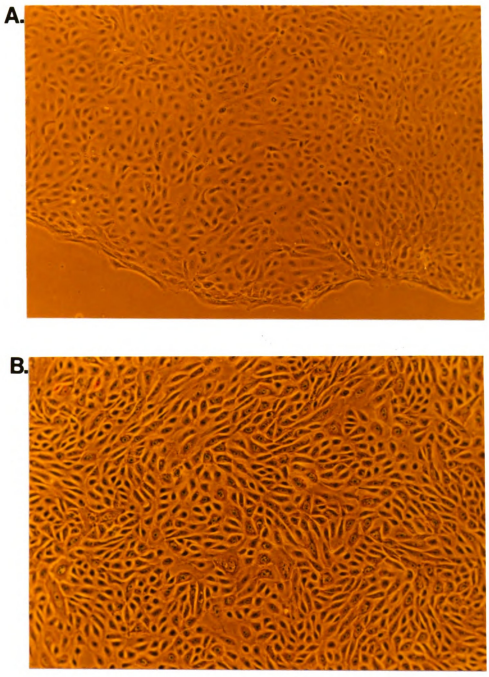

Figure 1. The morphology of two types of normal human breast epithelial cells. The photographs shown here are cells derived from HME 11: (A) Type-I cells (B) Type-II cells.

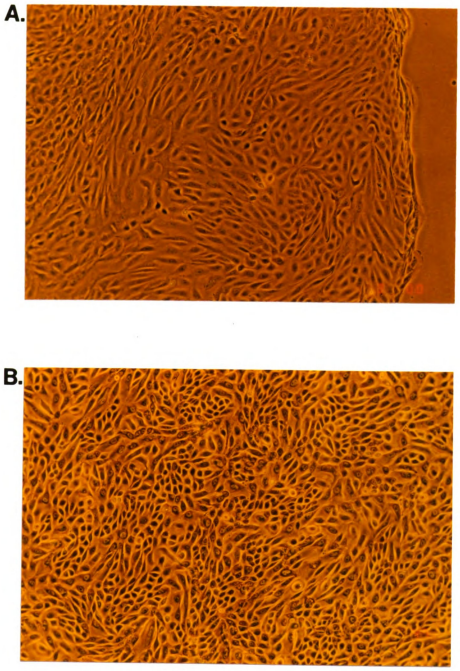

Figure 2. The morphology of two types of normal human breast epithelial cells from the primary culture, HME 14: (A) Type-I cells (B) Type-II cells.

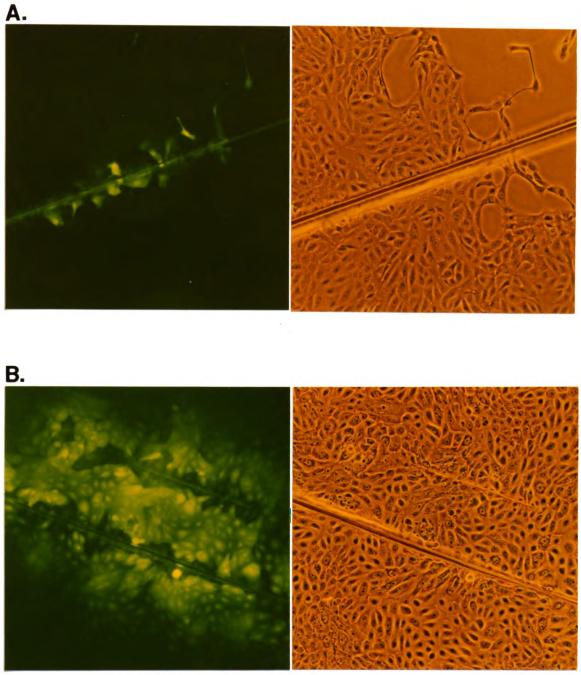

Figure 3. Gap junction mediated dye transfer was revealed by the scrape loading /dye transfer technique. (A) Type-I cells of HME 11 under a phase-contrast (right) or an epifluorescence (left) microscope. (B) Type-II cells of HME 11under a phase-contrast (right) or an epifluorescence (left) microscope.

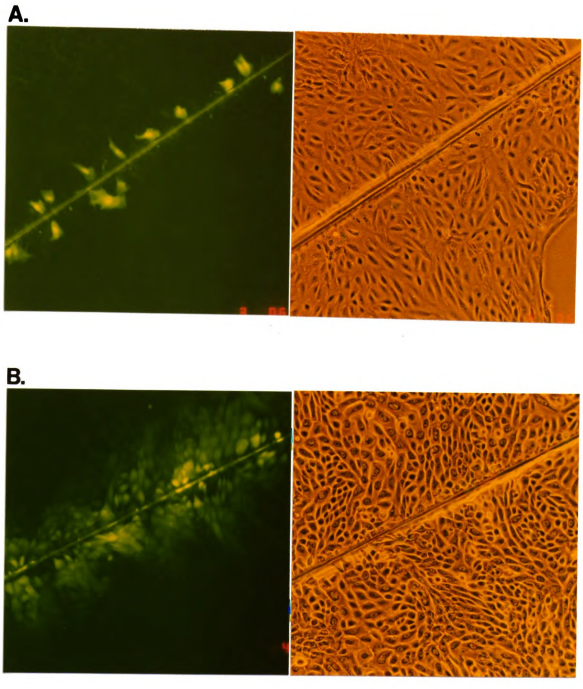

Figure 4. The ability of cells to perform GJIC was shown by the scrape loading /dye transfer technique. Type-I cells (A) and Type-II cells (B) of HME14 under a phase-contrast (right) or an epifluorescence (left) microscope.

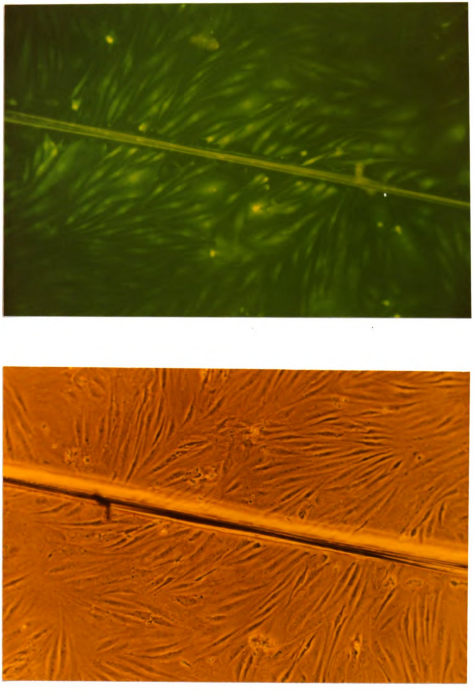

Figure 5. Gap junction mediated dye transfer of normal breast stromal fibroblasts derived from same culture as HME14. Same area of cells was observed under a phase-contrast (bottom) or an epifluorescence (top) microscope.

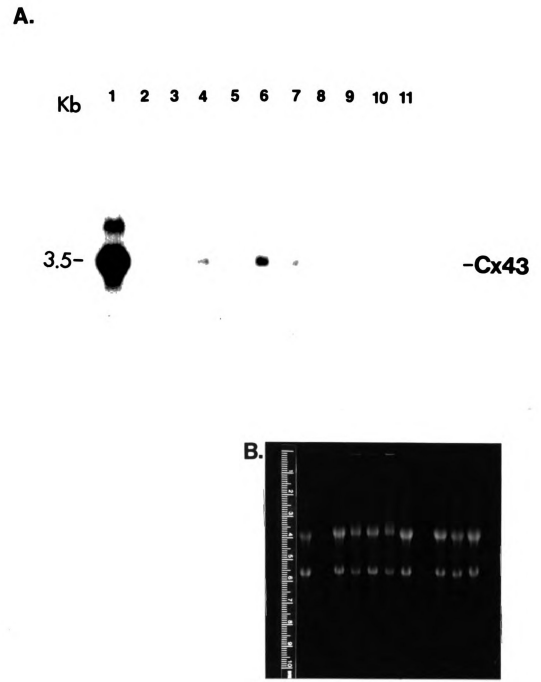

Figure 6. (A) Northern blot analysis of the expression of Cx43 gap junction gene in various cells. 1. Rat liver WB cells (positive control), 2. Blank, 3. HME11 type-I cells, 4. HME11 type-II cells, 5. HME14 type-I cells, 6. HME14 type-II cells, 7. HME14 stromal fibroblasts, 8. Blank, 9. HSO578T cells, 10. MCF-7 cells, 11. T47D cells. The amount of RNA loaded in each cell type or cell line was shown (B).

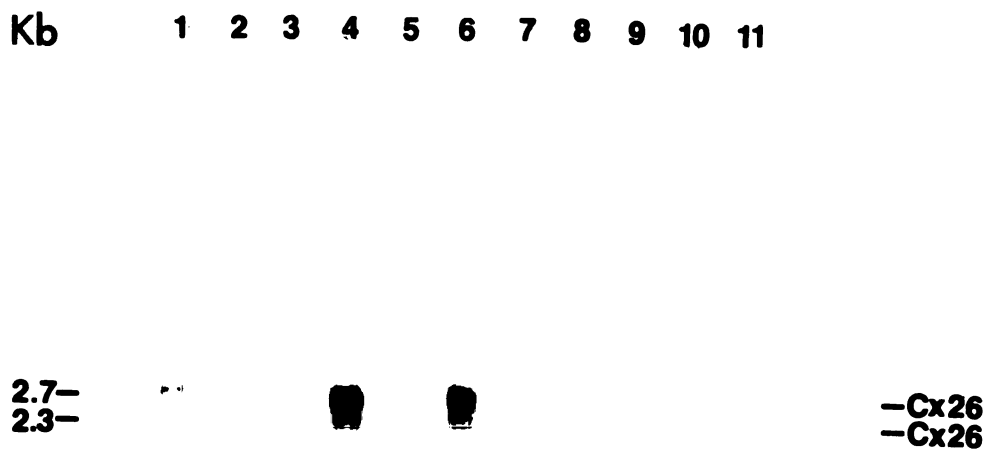

Figure 7. Northern blot analysis of Cx26 mRNA expression in various cell types and cell lines: 1. Rat liver WB cells, 2.Blank, 3. ME11 type-I cells, 4. HME11 type-II cells, 5. HME14 type-I cells, 6. HME14 type-II cells, 7. HME14 stromal fibroblasts, 8. Blank, 9. HSO578T, 10. MCF-7, 11. T47D.

competent in GJIC (Fig. 5) and were found to express Cx43 gene (Fig. 6A, lane 7) but not Cx26 gene (Fig. 7, lane 7). All these breast epithelial cells (HME11 and HME14) and stromal fibroblasts did not express the Cx32 gene (data not shown). As a control, the rat liver WB cell line expressed both Cx43 (Fig. 6A, lanel) and Cx26 (Fig. 7, lane 1)genes but not the Cx32 gene was used. The differential expression of gap junction mRNA in different cell types was studied under identical conditions using equal amounts of RNA (Fig. 6B).

c. Immunofluorescence Staining of Gap Junction

The presence of gap junctions on contiguous cell membranes was studied by immunofluorescence staining using specific antibodies against Cx26 or Cx43. No specific labeling for either connexin was observed for Type-I cells of HME11 (data not shown). The Type-II cells of HME14, however, displayed punctated fluorescence along borders of cell-cell contact after staining with either Cx43 (Fig. 8A) or Cx26 (Fig. 8B). The preimmune controls did not show any observable fluorescence staining (data not shown).

II. GJIC and Gap Junction Gene Expression in Three Breast Cancer Cell Lines

a. Ability to Perform GJIC

Two breast carcinoma cell lines, MCF-7 and T47D, and a breast carcinoma-sarcoma cell line, HSO578T, were used in this study. The two breast carcinoma cell lines were deficient in GJIC as studied by the scrape loading /dye transfer technique (Fig. 9B and 9C for MCF-7 and T47D respectively). In contrast, the breast carcinoma-sarcoma cell line HSO578T was competent in GJIC (Fig. 9A).

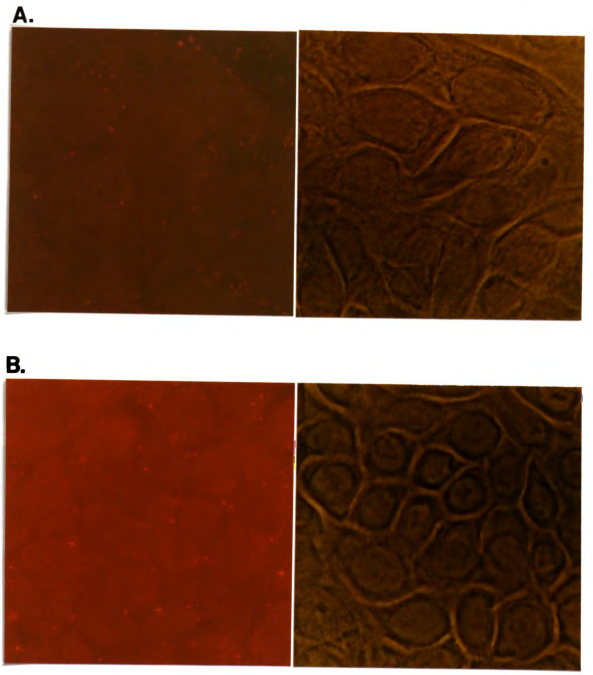

Figure 8. Immunocytochemical analysis of the presence of gap junction protein in cultured cells. (A) HME 14 type-II cells stained with antibodies against Cx43. (B) HME 14 type-II cells stained with anti-Cx26 antibodies.

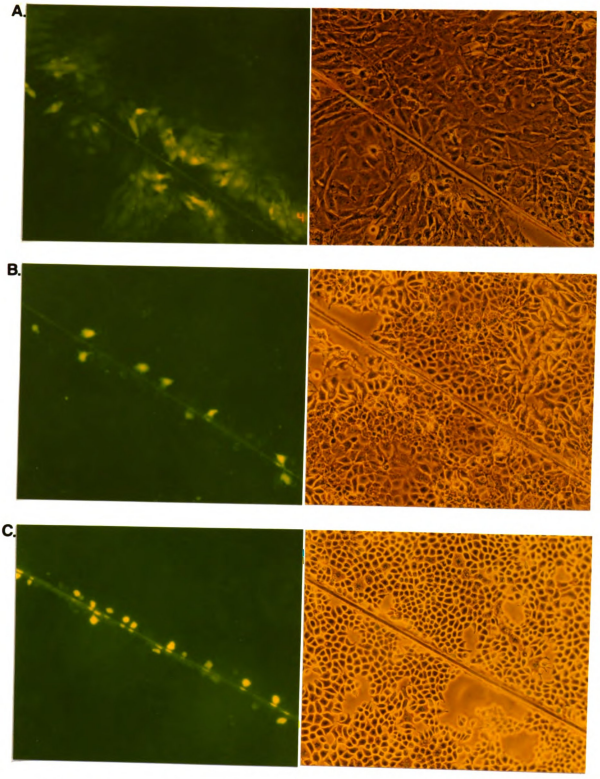

Figure 9. Gap junction mediated dye transfer of breast tumor cell lines. (A) HSO578T cells. (B) MCF-7 cells. (C) T47D cells. Same area of cells was observed under a phase-contrast (right) or an epifluorescence (left) microscope.

b. Expression of mRNA for Gap Junction Genes

Under the same experimental conditions as those used to study normal human breast epithelial cells, the MCF-7 and T47D did not express mRNA for Cx43 (Fig. 6, lane 10 and 11 respectively), Cx26 (Fig. 7, lane 10 and 11 respectively) and Cx32 (data not shown). The GJIC-competent HSO578T cells were found to express Cx43 mRNA (Fig. 6, lane 9) but not Cx26 mRNA (Fig. 7, lane 9) and Cx32 mRNA (data not shown).

c. Expression of Connexin Proteins

Total cellular proteins of the three breast cancer cell lines were used to detect the presence of connexin protein by Western blot analysis. The results showed that the three cell lines do not express Cx26 and Cx32 proteins (data not shown). A very weak expression of Cx43 protein, however, was observed for all the three cell lines (Fig. 10, lane 2, 3, 4 for MCF-7, T47D and HSO578T respectively). The HSO578T cells are different from MCF-7 and T47D cells by the presence of an additional Cx protein of lower molecular weight.

d. Immunofluorescence Staining o f Gap Junction

Since the three breast cancer cell lines were found to express low levels of Cx43 protein, immunofluorescence labeling was done to determine if Cx43 proteins are detectable on the membranes of these 3 cell lines. The results indicate that Cx43 protein was present in HSO578T cells as shown by the presence of punctated fluorescence along the borders of cell-cell contact (Fig. 11A). No Cx43 proteins were detected for MCF-7 and T47D by the immunofluorescence staining (Fig. 11B and 11C respectively). The presence of Cx43 appears to correspond to the gap junction function of these three cell lines.

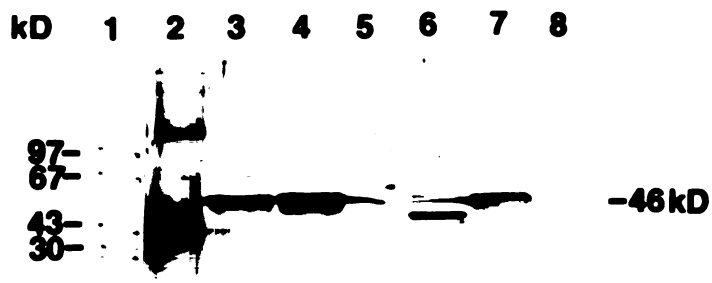

Figure 10. Western blot analysis of the expression of Cx43 protein in breast tumor cell lines. The membrane was incubated with antibodies against Cx43 protein.

1. Molecular weight marker. 2. Molecular weight marker. 3. MCF-7. 4. T47D.

5. HSO578T. 6. HME14 Type-II cells. 7. M13SV-2. 8. Rat heart.

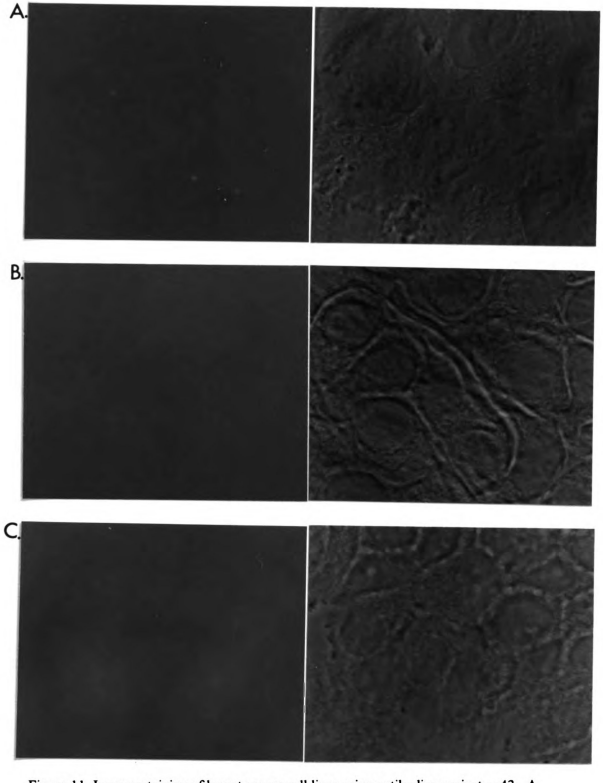

Figure 11. Immunostaining of breast cancer cell lines using antibodies against cx43. A. HSO578T. B. MCF-7. C. T47D.

III. Transfection of GJIC-Deficient MCF-7 Cells with Cloned cDNA for Rat Connexin Genes

a. Selection of Transfectants

The transfectants were selected in D medium containing 10% dialyzed fetal calf serum and 0.4 ug/ml methotrexate since the plasmid carries not only the connexin gene but also the DHFR gene. When surviving colonies reach 5 mm in diameter, colonies were randomly selected to test their ability to perform GJIC by the scrape loading /dye transfer technique. Among 15 rat Cx43 gene transfectants tested, 7 were found to acquire the ability to perform GJIC. Five out of 21 Cx32 cDNA transfectants tested were competent in GJIC. For Cx26 cDNA transfectants, 3 out of 14 have restored their ability for GJIC. For plasmid controls similarly selected by methotrexate, 6 colonies were examined and none of them were capable of GJIC. All the positive clones showing GJIC and plasmid controls were isolated and propagated for further studies.

b. Gap-Junction Mediated Dye Transfer

The ability of rat gap junction cDNA transfectants to perform GJIC was restored in confluent cultures of early passage cells as shown by studies using the scrape loading /dye transfer technique. All colonies that were tested positive in preliminary screening still retained their ability for gap junction mediated dye transfer. Three representative Cx43 transfectants are shown in Fig. 12 (A. MCF-7-43-10; B. MCF-7-43-16; C. MCF-7-43-3). Similarly, three representative Cx32 transfectants are shown in Fig. 13 (A. MCF-7-32-2; B. MCF-7-32-11; C. MCF-7-32-20) and three Cx26 transfectants are shown in Fig. 14 (A. MCF-7-26-4; B. MCF-7-26-6; C. MCF-7-26-8). A representative plasmid control deficient in GJIC is shown in Fig. 15. These results clearly show that GJIC can be restored by transfection with either of the three cloned cDNA for rat Cx26, Cx32 or Cx43.

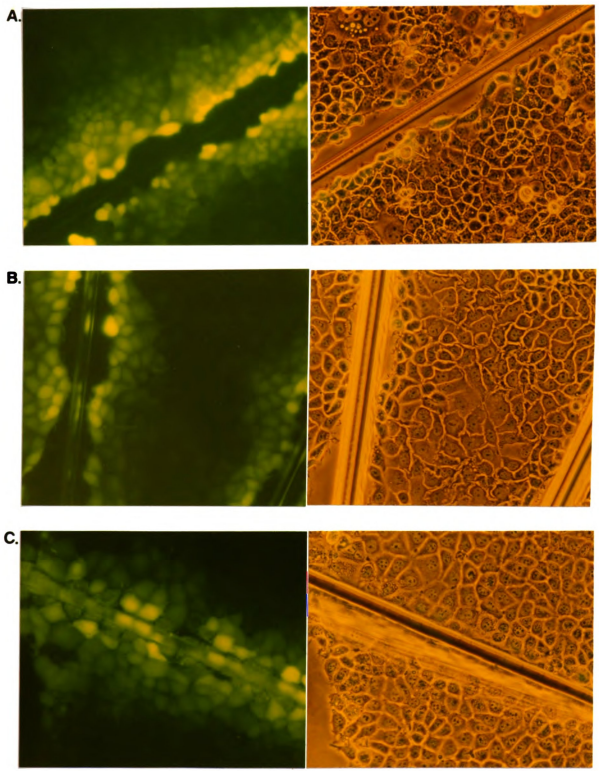

Figure 12. Gap junction-mediated dye transfer in three Cx43 transfectants: (A) MCF-7-43-10. (B) MCF-7-43-16. (C) MCF-7-43-3. Same area of cells was observed under a phase-contrast (right) or an epifluorescence (left) microscope.

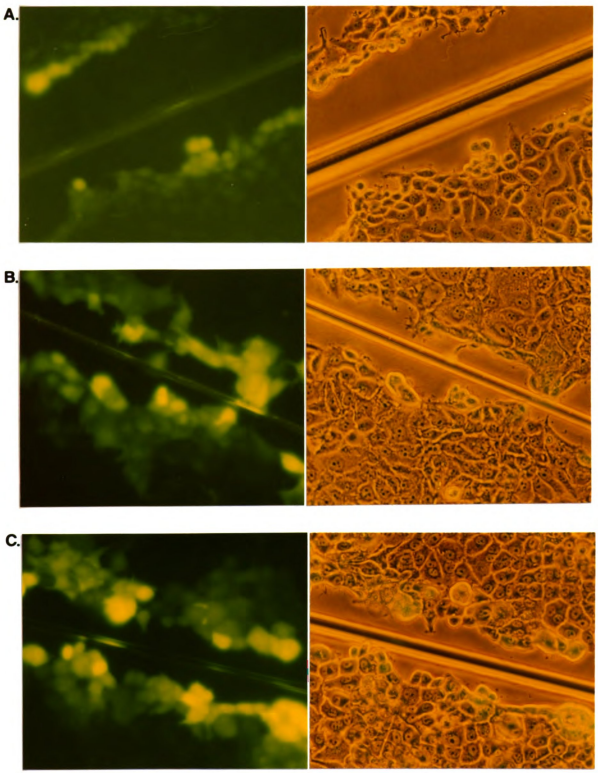

Figure 13. Gap junction-mediated dye transfer of three Cx 32 transfectants: (A) MCF-7-32-2. (B) MCF-7-32-11 (C) MCF-7-32-20. Same area of cells was observed under a phase-contrast (right) or an epifluorescence (left) microscope.

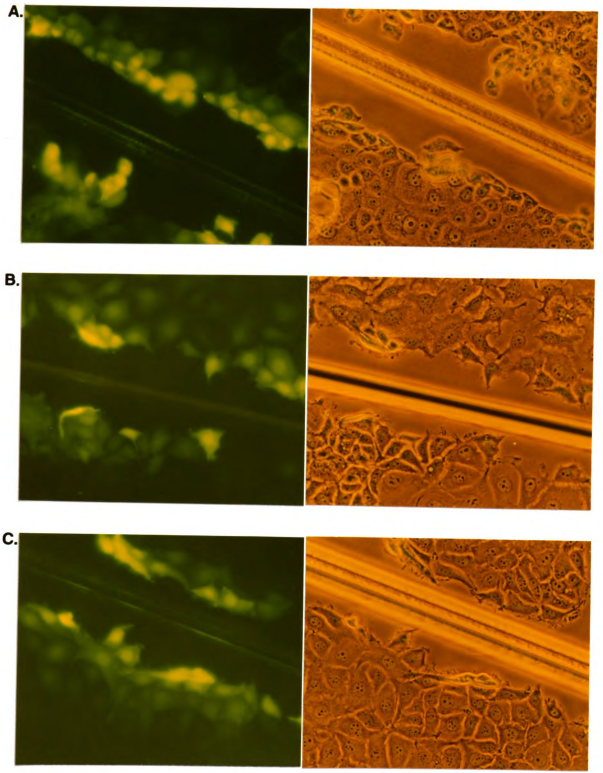

Figure 14. Gap junction-mediated dye transfer of three Cx26 transfectants:
(A) MCF-7-26-4. (B) MCF-7-26-6. (C) MCF-7-26-8. Same area was observed under a phase-contrast (right) or an epifluorescence (left) microscope.

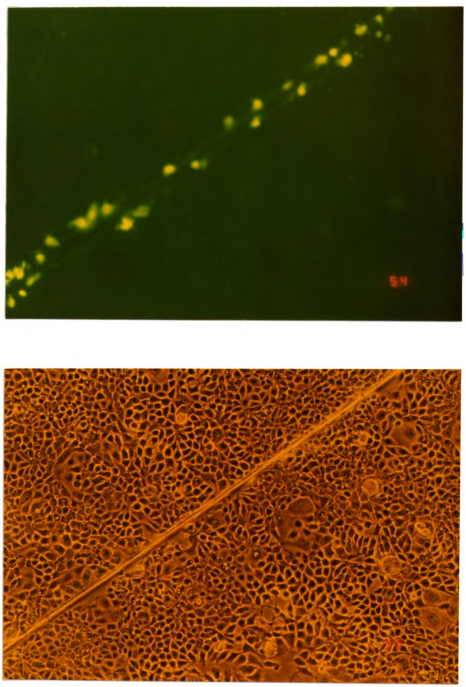

Figure 15. Gap junction-mediated dye transfer of a MCF-7 clone transfecting with plasmid pSV(RI)-DHFR (MCF-7-DHFR). Same area of cells was observed under an epifluorescence (top) or a phase-contrast (bottom) microscope.

The ability of these transfectants to perform GJIC, however, was found to decline after extended culture for all clones except one (MCF-7-43-10) which retained the ability after extended growth. Comparing the level of GJIC by the three different Cx gene transfectants at early passage, the Cx43 cDNA transfectants appear to have the highest level of GJIC.

c. Gap Junction Gene Expression

Five clones of Cx43 cDNA transfectants, three clones of Cx32 transfectants and three clones of Cx26 transfectants were analyzed by Northern blot for expression of transfected connexin genes. Total RNAs extracted from these cells were hybridized with radiolabeled rat Cx43, Cx32, Cx26 cDNA probes. The results show that all clones of transfectants proficient in GJIC expressed the respective mRNAs of the transfected connexin gene (Fig. 16, Cx43 cDNA transfectants; Fig. 17, Cx32 cDNA transfectants; fig. 18, Cx26 cDNA transfectants). The parental MCF-7 and plasmid control did not express any of these genes (Fig. 16-18, lane 1 for MCF-7; lane 2 for MCF-7-DHFR plasmid control). Two transcripts of different sizes were observed in these transfectants. The amount of the two transcripts was about equal for all Cx26 and Cx32 cDNA transfectants. For Cx43 cDNA transfectants, the low molecular weight transcript is negligible except for one clone (MCF-7-43-10). The presence of these two transcripts might have resulted from the differential processing of the mRNA transcribed from the connexin gene plasmid which carried an intron (about 0.61 Kb) at the 3' end of the connexin gene.

c. Anchorage Independent Growth (AIG)

To test if expression of gap junction genes might affect tumor cell phenotype, three clones of two different gap junction gene transfectants (i.e. Cx43 cDNA: MCF-7-43-3, MCF-7-43-10, MCF-7-43-16 and Cx26 cDNA: MCF-7-26-4, MCF-7-26-6, MCF-7-26-8) were examined for their ability of AIG. The plasmid control, MCF-7-DHFR, was included in the experiment. After 4 weeks of growth, the colonies formed in the agar were counted

under a microscope. The plasmid control had 39.5% colony-forming efficiency. For the Cx43 and Cx26 cDNA transfectants the average colony-forming efficiency was 28.5% and 38% respectively. Clearly, the expression of these two gap junction genes did not significantly affect the AIG of these cells.

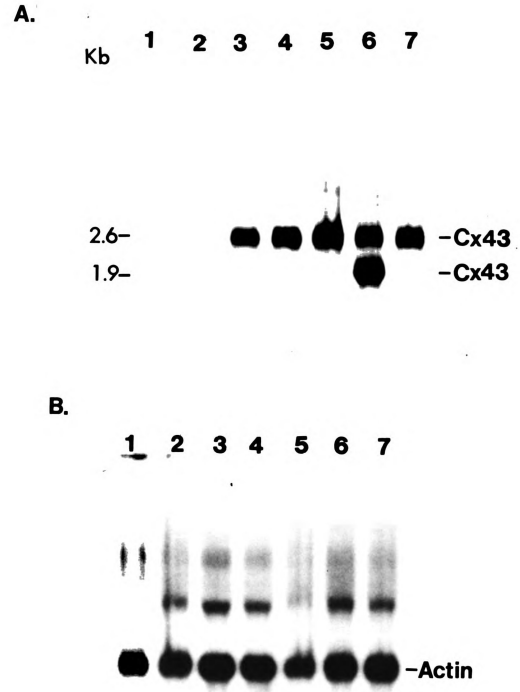

Figure 16. Northern blot analysis of Cx43 transfectants. (A) The membrane was hybridized with the rat Cx43 cDNA probe. (B) The same membrane was hybridized with actin DNA probe. 1. MCF-7. 2. MCF-7-DHFR. 3. MCF-7-43-2. 4. MCF-7-43-3. 5. MCF-7-43-4. 6. MCF-7-43-10. 7. MCF-7-43-16.

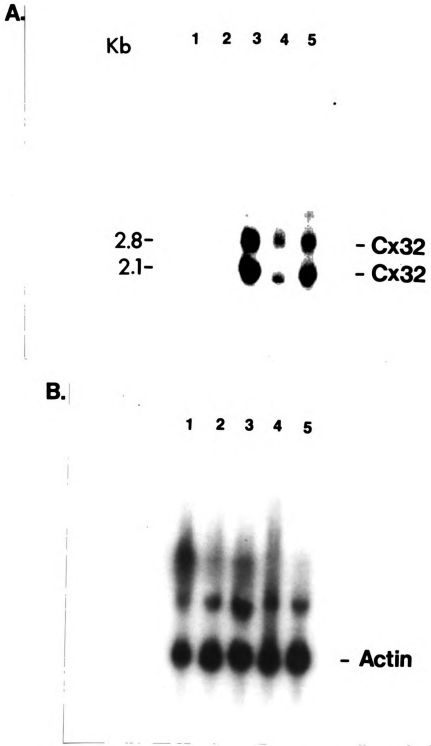

Figure 17. Northern blot analysis of Cx32 transfectants. (A) The membrane was hybridized with the rat Cx32 cDNA probe. (B) The same membranes was hybridized with actin DNA probe. 1. MCF-7. 2. MCF-7-DHFR. 3. MCF-7-32-2. 4. MCF-7-32-11. 5. MCF-7-32-20.

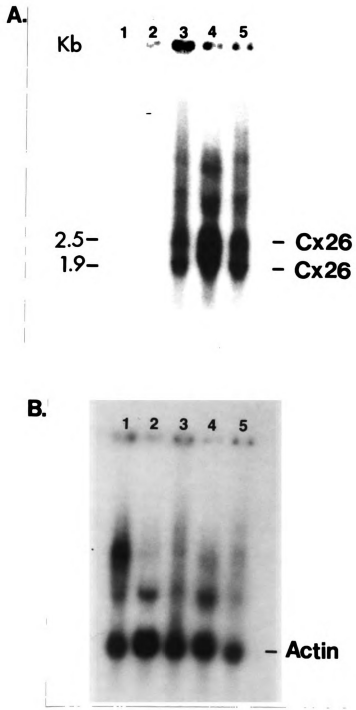

Figure 18. Northern blot analysis of Cx26 transfectants. (A) The membrane was hybridized with the rat Cx26 cDNA probe. (B) The same membrane was hybridized with actin DNA probe. 1. MCF-7. 2. MCF-7-DHFR. 3. MCF-7-26-4. 4. MCF-7-26-6. 5. MCF-7-26-8.

DISCUSSION

The objectives of this thesis research were: 1) to determine what gap junction genes are expressed in normal human breast epithelial cells and 2) to determine if GJIC can be restored in MCF-7 cells by transfection with cloned cDNA for different rat gap junction genes. The major results obtained from this study may be summarized as follows: 1) The two primary human breast epithelial cell cultures derived from reduction mammoplasty from two women (i.e. HME11 and HME14), like other normal breast epithelial cell cultures developed and studied by this laboratory, contain two morphologically distinctive cell types: one is deficient in GJIC (Type-I) and the other is competent in GJIC (Type-II); 2) The Type-I cells from both cultures did not express gap junction genes for Cx26, Cx32 or Cx43 as revealed by Northern analysis; 3) The Type-II cells from both cultures expressed the transcript for Cx26 and Cx43 but not Cx32; 4) The human breast carcinoma cell lines, MCF-7 and T47D, are deficient in GJIC and do not express detectable amount of mRNA for Cx26, Cx32 and Cx43, whereas, the breast carcinoma-sarcoma cell line HSO578T is competent in GJIC and expressed mRNA for Cx43 but not Cx26 and Cx32 genes similar to normal human breast stromal fibroblasts; 5) The GJIC can be restored in MCF-7 cells by transfection with cloned cDNA for rat Cx26, Cx32 and Cx43; 6) Most MCF-7 transfectants lost the acquired GJIC gradually after extended culture and 7) These MCF-7 transfectants expressing gap junction genes do not seem to significantly alter their growth rate in vitro and ability of AIG.

Previous studies have shown that normal human breast epithelial cells are competent in GJIC (Fentiman and Taylor-Papadimitriou, 1977; Fetiman, 1980;Eldridge et al., 1989) and expressed Cx26 and Cx43 genes (Lee et al., 1992). These phenotypes are similar to those exhibited by Type-II cells of our normal breast epithelial cell culture. There is good reason to believe that those cultures (e.g. Eldridge et al., 1989; Lee et al., 1992) are Type-II cells since the media (i.e. MCDB 170, DFCI-1) used in their studies are

known to promote the growth of Type-II cells (personnel observation). The Type-I cells were first identified and reported by our laboratory (Chang et al., 1990) and apparently have not been studied by other laboratory. Furthermore, the Type-I cells can be induced by cholera toxin (present in the DFCI-1 medium) to differentiate into Type-II cells (Chang et al., 1991) and can be converted to Type-II cells by culturing in the MCDB 170 medium (Clonetics MEGM medium) (personnel observation).

Type-I cells were deficient in GJIC and did not express gap junction genes for Cx26, Cx32 and Cx43. These phenotypes are also shared by the breast carcinoma cell lines, MCF-7 and T47D and other cell lines studied by Lee et al. (1992). The in vivo study also indicates that the Cx43 gene was not expressed in invasive ductal carcinoma (Wilgenbus et al., 1992). This correlation might indicate that most human breast carcinomas are derived from Type-I cells rather than from Type-II cells. Similarly, the breast sarcoma cell line, HSO578T, and normal human breast stromal fibroblasts were competent in GJIC and expressed Cx43 but not Cx32 and Cx26 genes. This might indicate that the HSO578T cell line is of fibroblast origin instead of epithelial cell origin. With further substantiation, the above observation might be used as a marker to distinguish breast sarcoma from breast carcinoma.

Based on differential expression of the Cx26 gene in normal and tumorigenic breast epithelial cells, Lee et al. (1991) proposed that the Cx26 gene may be a tumor-suppressor gene of breast cancer. The major characteristics of a tumor suppressor gene is its expression in normal cells and the absence of its function in tumor cells. By this criterion and in light of the fact that Cx26 and Cx43 gap unction genes are not expressed in Type-I normal human breast epithelial cells which express luminal epithelial cell specific antigens (i.e. EMA and cytokeratin 18) (Chang et al., in preparation), the hypothesis is clearly in doubt. We have observed that the differentiation of Type-I to Type-II cells involves a wholesale transition of gene expression, the most notable among them are morphological change, growth requirements, keratin gene and gap junction gene

expression. Therefore the gap junction gene expression might be correlated with cell growth and differentiation at the transcriptional level. The blockage of this differentiation in Type-I cells might have implications in carcinogenesis. Furthermore, since blockage of GJIC might be a mechanism for tumor promotion, one reason that breast carcinoma is of ductal luminal origin might be the deficiency in GJIC of Type-I cells which are suspected as the putative breast epithelial stem cells.

The ability to perform GJIC in communication-deficient MCF-7 cells can be restored by transfection with cloned cDNA for rat Cx26, Cx32 or Cx43 gap junction genes. Since MCF-7 cells do not express any of these genes, the results indicated that functional cell-cell communication can be carried out by any one of the 3 gap junction genes. Different from the rat liver WB cells GJIC-deficient mutants transfected with the same Cx43 gene and plasmid (Jou et al., 1993), the restoration of GJIC in MCF-7 cells did not require amplification of the transfected gap junction gene. The discrepancy might be due to the difference in mechanisms of GJIC deficiency between the two types of cells. Unlike the MCF-7 cells, the WB cell mutants are defective in post-translational phosphorylation of Cx43. Most MCF-7 transfectants, however, were unable to sustain the initial level of GJIC after extended growth. The loss of GJIC in MCF-7 transfectants could be at transcriptional or translational level. Further studies involving DNA methylation and Western blot analysis should be able to discern the possible underlying mechanisms. In addition, since the transfected gap junction genes are carried by an amplifiable vector, it is possible to study whether the loss of GJIC in MCF-7 transfectants might be prevented by amplification of the transfected gap junction genes.

The MCF-7 gap junction gene transfectants do not seem to change their ability of AIG. Since most of these transfectants were unable to sustain the restored GJIC, the effect of GJIC on these phenotypes may not be accurately studied. Although tumorigenic cells are usually AIG competent, the AIG competent cells are not necessarily tumorigenic. The MCF-43-10 clone was found to maintain GJIC after extended growth. This cell line

should be useful to study whether restoration of GJIC might affect the tumorigenicity of MCF-7 cells in immune-deficient mice.

REFERENCES

Abernethy, D.J., W.F. Greenlee, J.C. Huband and C.J. Boreiko. 1985. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) promotes the transformation of C3H/10T1/2 cells. Carcinogenesis 6:651-653.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson. 1989. Molecular Biology of the cell, p 798-802. Garland Publishing, Inc. New York & London.

Atkinson, M.M., A.S. Memko, R.G. Johnson, J.R. Sheppand, and J.D. Shenidan. 1981. Rapid and reversible redunction of junctional permeability in cells infected with a temperature-sensitive mutant of avian sarcoma virus. J. Cell Biol. 91:573-578.

Atkinson, M.M., S.K. Anderson, J.D. Sheridan. 1986. Modification of gap junctions in cells transfected by a temperature-sensitive mutant of rous sarcoma virus. J. membr. Biol. 9:53-64.

Azarinia, R., G. Dahl, and W.R. Loewenstein. 1981. Cell junction and cyclic AMP: III. promotion of junctional membrane permeability and junctional membrane particles in a junction-deficient cell type. J. Membrane Biol. 63:133-146.

Azarnia, R., and W.R. Loewenstein. 1984. Intercellular communication and the control of growth: X. alternation of junctional permeability by the src gene. A study with temperature-sensitive mutant rous sarcoma virus. J. Membr. Biol. 82:191-205.

Azarnia, R., S. Reddy, T.E. Kmiecik, D. Shalloway and W.R. Loewenstein. 1988. The cellular src gene product regulates junctional cell-to-cell communication. Science 239: 398-400.

Bennett, M.V.L., L.C. Barrio, T.A. Bargiello, D.C. Spray, E. Hertzberg, and J.C. Saez. 1991. Gap junctions: new tools, new answers, new qutestions. Neuron 6:305-320.

Bertram J.S., N.L. Kolonel, and F.L. Jr Meyskens. 1987. Rationale and strategies for chemoprevention of cancer in humans. Cancer Res. 47:3012-3031.

Beyer, E.C., D.C. Paul, and D.A. Goodemough. 1987. Connexin 43: a protein from rat heart homologous to a gap junction protein from liver. J. Cell Biol. 105:2621-2629.

Beyer, E.C., D.A. Goodenough, D.C. Paul. 1988. The connexins, a family of related gap junction proteins. In Gap Junctions (ed., E.C. Hertzberg and R.G. Johson), p 167-175. Alan Liss. New York.

Boreiko, C.J., D.J. Abernethy, J.H. Sanchez and B.H. Dorman. 1986. Effect of mouse skin tumor promoters upon [^{3}H]uridine exchange and focus formation in cultures of C3H/10T1/2 mouse fibroblasts. Carcinogenesis 7:1095-1099.

Burghardt, R.C. and E. Anderson. 1979. Hormonal modulation of ovarian interstitial cells with particular reference to gap junctions. J. Cell Biol. 81:104.

Chang, C.C., J.A. Boezi, S.T. Warren, C.L.K. Sabourin, P.K. Liu, L. Glatzer and J.E. Trosko. 1981. Isolation and characterization of a UV-sensitive hypermutable aphidicolin-resistant Chinese hamster cell line. Somat. Cell Genetics 7:235-253.

Chang, C.C., J.E. Trosko, H.J. Kung, D. Bombick and F. Matsumura. 1985. Potential role of the src gene product in inhibition of gap junctional communcation in NIH/3T3 cells. Proc. Natl. Acad. Sci. USA 82:5360-5364.

Chang, C.C., S. Nakatsaka, G. Kalimi, J.E. Trosko and C.W. Welsch. 1990. Characterization of two types of normal human breast epithelial cells that are either deficient or proficient in gap-junctional intercellular communication. J. Cellular Biochem. Supplement 14B p.331.

Chang, C.C., C.Y. Kao, S. Nakatsuka, J.E. Trosko and C.W. Welsch. 1991. Identification of a subpopulation of normal human breast epithelial cells with stem cell characteristics. Proc. Am. Assoc. Cancer Res. 32:40

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochem. 162:156-159.

Crow, D.S., E.C. Beyer, D.L. Paul, S.S. Kobe and A.F. Lau. 1990. Phosphorylation of connexin43 gap junction protein in uninfected and Rous sarcoma virus-transformed mammalian fibroblasts. Mol. Cell. Biol. 10:1754-1763.

De Feijter, A.W., J.S. Ray, C.M. Weghorst, J.E. Klaunig, J.I. Goodman, C.C. Chang, R.J. Ruch, and J.E. Trosko. 1990. Infection of rat liver epithelial cells with v-Ha-ras: Correlation between oncogene expression, gap junctional communication, and tumonigenicity. Mol. Carcinogenesis 3:54-67.

De Feijter, A.W., J.E. Trosko, D.B. Krizman, R.M. Lebovitz, and M.W. Lieberman. 1992. Correlation of increased levels of Ha-ras T24 protein with extent of loss of gap junction function in rat liver epithelial cells. Mol. Carinogenesis 5:205-212.

De Mello, W.C. 1972. The healing-over process in cardiac and other muscle fibers. In electrical phenomena in the heart (ed. W.C. De Mello), p 323. Academic Press Inc, New York.

De Mello, W.C. 1980. Influence of intracellular injection of H^+ on the electrical coupling in cardiac Purkinjier fibers. Cell Biol. Int. Rep. 4:51-57.

De Mello, W.C. 1982. Cell-to-cell communication in heart and other tissue. Prog. Biophys. Mol. Biol. 39:147-182.

De Mello, W.C. 1987. Modulation of junctional permeability. In cell-to-cell communication (ed., W.C. De Mello), p 43. Plenum Press, Inc. New York, New York.

Dermietzel, R., T.K. Hwang, and D.S. Spray. 1990. The gap junction family: structure, function, and chemistry. Anal. Embryol. 182:517-528.

Doolittle, R.F., M.W. Hunkarpiller and K.C. Robbins. 1983. Simian sarcoma virus oncogene v-sis, is derived from the gene(s) encoding a platelet derived growth factor. Science 221:275-277.

Downward, J., Y. Yasden and E. Mays. 1984. Close similarity of epidermal growth factor receptor and v-erb B oncogene protein sequences. Nature 307:521-527.

Eghbali, B., J.A. Kessler, and D.C. Spray. 1990. Expression of gap junction channels in communication incompetent cells after stable transfection with cDNA encoding connexin 32. Proc. Natl. Acd. Sci. USA 87:1328-1331.

El-Fouly, M.H., J.E. Trosko and C.C. Chang. 1987. Scrape-loading and dye transfer, a rapid and simple technique to study gap junctional intercellular communication. Exp. Cell Res. 168:422-430.

El-Fouly, M.H., J.E. Trosko, C.C. Chang, S.T. Warren. 1989. Potential role of the human Ha-ras oncogene in the inhibition of gap junctional intercellular communication. Mol. Carcinogenesis 2:131-135.

Eldridge, S.R., T.W. Martens, C.A. Sattler, and M.N. Gould. 1989. Association of decreased intercellular communication with the immortal but not the tumorigenic phenotype in human mammary epithelial cells. Cancer Res. 49:4326-4331.

Fentiman, I.S., and J. Taylor-Papadimitriou. 1977. Cultured human breast cancer cells lose selectivity in direct intercellular communication. Nature (London) 269:156-158.

Fentiman, I.S., J. Hurst, R.L. Ceriani, and J. Taylor-Papadimitriou. 1979. Junctional intercellular communication pattern of cultured human breast cancer cells. Cancer Res. 39:4739-4743.

Fentiman, I.S. 1980. Cell communication in breast cancer. Ann. R. Coll. Surg. Eng., 62:280-286.

Furshpan, E.J., and D.D. Potter. 1959. Transmission at the giant moter synapses of the crayfish. J. Physiol. (London) 145:289-325.

Garfield, R.E., D. Merrett, and A.K. Grover. 1980. Gap junction formation and regulation in myometrium. Am. J. Physiol. 239:c217.

Garfield, R.E. 1984. Control of myometrial function in preterm versus term labor. Clin. Obstet. Gynecol. 27:572-591.

Garfield, R.E., W.C. Cole, and M.G. Blennerhassett. 1989. Gap junctions in uterine smooth muscle. In cellinteractions and gap junctions volume II. (ed. N. Specelakis and W.C. Cole), p 247. CRC Press, Inc. Boca Raton, Florida.

Gilula, N.B., M.L. Epstein, and W.H. Beers. 1978. Cell-to-cell communication and ovulation, a study of the cumulus-oocyte complex. J. Cell Biol. 78:58-75.

Hamel, E., F. Katoh, G. Mueller, W. Birchmeier and H. Yamasaki. 1988. Transformation growth factor β as a potent promoter in two-stage BABL/c3T3 cell transformation. Cancer Res. 48:2832-2836.

Herris, A.L., D.C. Spray and M.V.L. Bennett. 1981. Kinetic properties of a voltage-dependent junctional conductance. J. Gen. Physiol. 77:95-117.

Hertzberg, E.L. and N.B. Gilula. 1981. Gap junction communication. Ann. Rev. Physiol. 43:479-491.

Hertzberg, E.L., and R.G. Johnson. 1988. Gap junctions. Alan R. Liss, Inc., New York.

Hoh, J.H., S.A. John, and J.P. Revel. 1991. Molecular cloning and characterization of a new member of the gap junction gene family, connexin 31. J. Biol. Chem. 266: 6524-6531.

Hossain, M.Z., L.R. Wilkens, P.P. Mehta, W. Loewenstein, and J.S. Bertram. 1989. Enhancement of gap junctional communication by retinoids correlates with their ability to inhibit neoplastic transformation. Carcinogenesis 10:1743-1748.

Hsieh, C.L., N.M. Kumar, N.B. Gilula, and U. Francke. 1991. Distribution of genes for gap junction membrane channel proteins on human and mouse chromosomes Somatic Cell. Mol. Genetics 17:191-200.

Ito, S., and N. Hori. 1966. Electrical characteristics of Triturus eggs during cleavage. J. Gen. Physiol. 49:1019.

Jou, Y.S., E. Dupont, S.C. Lu, B.V. Madhukar, S.Y. Oh, J.E. Trosko and C.C. Chang. 1993. Restoration of gap junction intercellular communication in a communication deficient rat liver cell mutant by transfection with connexin43 cDNA. Mol. Carcinogenesis (in press).

Kalimi, G.and S.M. Sirsat. 1984. The relevance of gap junctions to stage I tumor promotion in mouse epidermis. Carcinogenesis 5:1671-1677.

Kalimi, G., C.C. Chang, P. Edwards, E. Dupont, B.V. Madhukar, E. Standbridge and J.E. Trosko. 1990. Re-establishment of gap junctional communication in a non-tumorigenic Hela-normal human fibroblasts hybrid. Proc. Am. Assoc. Cancer res. 31:318.

Kam, E. and J.D. Pitts. 1988. Effects of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate on junctional communication in intact mouse skin: persistence of homologous communication and increase of epidermal-dermal coupling. Carcinogenesis 9:1389-1394.

Kameyame, M. 1983. Electrical coupling between ventricular paired cells isolated from guinea-pig heart. J. Physiol. (London) 336:345.

Kanno, Y. 1985. Modulation of cell communication and carcinogensis. J. Physiol. 35:693-707.

Larsen, W.J., H.N. Tung and C. Polking. 1981. Response of granulosa cell gap junctions to human chorionic gonadotropin (hCG) at ovulation. Biol. Reproduct. 25:1119.

Lawrence, T.S., W.H. Beers and N.B. Gilula. 1978. Transmission of hormonal stimulation by cell-to-cell communication. Nature 272:501-506.

Ledbetter, M.L.S. and M. Lubin. 1979. Transfer of potassium, a new method of cell-cell coupling. J. Cell Biol. 80:150-159.

Lee, S.W., C. Tomasetto and R. Sager. 1991. Positive selection of candidate tumor-suppressor genes by subtractive hybridization. Proc. Nat. Accd. Sci. USA 88:2825-2829.

Lee, S.W., C. Tomasetto, D. Paul, K. Kejomarsi, and R. Sager. 1992. Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. J. Cell Biol. 118:1213-1221.

Lo, C.W. 1980. Gap junctions and development. In "development of mammals" (ed., M.H. Johnson) Vol. 4, p 39-80. Elserier, New York.

Loewenstein, W.R. 1979. Junctional intercellular communication and the control of growth. Biochim. Biophy. Acta 560:1-65.

Loewenstein, W.R. 1981. Junctional intercellular communication: the cell-to-cell membrane channel. Physiol. Reviews 61:829-913.

Meda, P., A. Perrelet and L. Orci. 1979. Increase of gap junctions between pancreatic B-cells during stimulation of insulin secretion. J. Cell Biol. 82:441.

Madhukar, B.V., S.Y. Oh, C.C. Chang, M. Wade and J.E. Trosko. 1989. Altered regulation of intercellular communication by epidermal growth factor, transforming growth factor-β, and peptide hormones in normal keratinocyte. Carcinogenesis 10:13.

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

Mehta, P.P., J.S. Bertramand W.R. Loewenstein. 1986. Growth inhibition of transformed cells correlates with their junctional communication with normal cells. Cell 44:187-196.

Mesnil, M., D.J. Fitzgerald, and H. Yamasaki. 1988. Phenobarbital specifically reduces gap junction protein mRNA level in rat liver. Mol. Carcinog. 1:79-81.

Metzger, P., and R. Weingart. 1985. Electric current flow in cell pairs isolated from adult rat hearts. J. Physiol. (London) 336:177.

Michaels, R.L. 1982. Increased dye coupling among cells in prolactin stimulated pancreatic islets. J. Cell Biol. 95:94a

Mordan, L.J. 1991. Carcinogenesis models. Science 251:142-143.

Murray, A.W., and D.J. Fitzgerald. 1979. Tumor promoters inhibit metabolic cooperation in cocultures of epidermal and 3T3 cells. Biochem. Biophys. Res. Commun. 91:395-401.

Murray, A.W., and H.S.C. Gainer. 1989. Regulation of gap junctional communication by protein kinase. In cell interactions and gap junction (ed., N. Sperelakis and W.C. Cole), p 98. CRC Press, Inc. Boca Raton, Florida.

Noma, A., and N. Tsuboi. 1987. Dependence of junctional conductance on proton, calcium and magresium ions in cardiac paired cells of guinea-pig. J. Physiol. (London) 382:193.

Page, D.L., and T.L. Anderson. 1987. Diagnostic Histopathology of the breast. Edinburgh, Churchill Livingstone.

Pasti, G., E. Rivedal, S.H. Yuspa, C.L. Herald, G.R. Pettitand P.M. Blumberg. 1988. Contrasting duration of inhibition of cell-cell communication in primary mouse epidermal cells by phorbol 12,13-dibutyrate and by bryostatin 1. Cancer Res. 48:447-451.

Paul, D.L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. J. Cell Biol. 103:123-134.

Pittelkow, M.R., and R.E. Scott. 1986. New techniques for the *in vitro* culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. Mayo Clin. Proc. 61:771-777.

Rae, J.I. 1982. Curr. Top. Eye Res. 35:598-609.

Rose, B. and W.R. Loewenstein. 1976. Permeability of a cell junction and the local cytoplasmic free ionized calcium concentration: a study with aequorin. J. Membrane Biol. 28:87

Rose, B., I. Simpson and W.R. Loewenstein. 1977. Calcium ion produces graded changes in permeability of membrane channels in cell junction. Nature 267:625.

Rose, B. and R. Rick. 1978. Intracellular pH, intracellular free Ca and junctional cell-cell coupling. J. Membrane Biol. 44:377-415.

Sheridan, J.D. 1973. Functional evaluation of low resistance junctions: influence of cell shape and size. Am. Zool. 13:1119-1128.

Sheridan, J.D. 1987. Cell communication and growth. In cell-to-cell communication (ed., W.C. De Mello), p 188. Plenum Press, Inc. New York, New York.

Silberberg, E., C.C. Boring, T.S. Suives. 1990. Cancer Statistics, 1990. CA 40:8-26.

Slack, C., and J.F. Palmer. 1969. The permeability of intercellular junctions in the early embryo of xenopus laevis, studies with a fluorescent tracer. Exp. Cell Res. 55:416.

Subak-Sharpe, J.H., R.R. Burk and J.D. Pitts. 1969. Metabolic cooperation between biochemically marked mammalian cells in tissue culture. Cell Sci. 4:353-367.

Sugie, S., H. Mori, and M. Takahashi. 1987. Effect of *in vivo* exposure to the liver tumor promoters phenobarbital or DDT on the gap junctions of rat hepatocytes: a quantitative freeze-fracture analysis. Carcinogrenesis 8:45-51.

Tomasetto, C., S.W. Lee, D. Paul, and R. Sager. 1991. Gap junction communication in normal and tumoral mammary epithelial cells. J. Cell Biol. 115,190a.

Trosko, J.E., and C.C. Change. 1988. Non-genotoxic mechanism in carcinogenesis: role of inhibited intercellular communication. In: Banbury report 31: carinogen risk assessment: new directions in qualitative and quantitative aspects, p 139-170. Cold Spring Harbor Laboratory, New York.

Turin, L., and A.E. Warner. 1977. Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryos. Nature 270:56-57.

Unwin, P.N.T., and G. Zamphigi. 1980. Structure of the junction between communicating cells. Nature 283:545-549.

Vanhamme, L., S. Rolin and C. Szpirer. 1989. Inhibition of gap-junctional intercellular communication between epithelial cells transformed by the activated H-ras-1 oncogene. Exp. Cell Res. 180:297-301.

Vitkauskas, G.V. and E.S. Canellakis. 1983. J. Cell Biol. 97:80a

Vitkauskas, G.V., and E.S. Canellakis. 1985. Intercellular communication and cancer chemotherapy. Biochim. Biophy. Acta 833:19-34.

Warner, A.E., S.C. Guthrie, and N.B. Gilula. 1984. Antibodies to gap-junctional protein selectively discrupt junctional communication in the early amphibian embryo. Nature 311:127-131.

Welsch, F. 1990. Teratogens and cell-to-cell communication. In "cell Intercommunication" (ed., W.C. De Mello), p137. CRC Press Inc. Boca Raton, Florida.

White, R.L., D.C. Spray, A.C. Campos de Carvalho, B.A. Wittenberg, and M.V.L. Bennett. 1985. Some electrical and pharmacological properties of gap junctions between adult remtricular myocytes. Am. J. Physiol. 249:c447.

Wiener, E.C., and W.R. Loewenstein. 1983. Correction of cell-to-cell communication defect by introduction of a protein kinase into mutant cells. Nature 305:433-435.

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

Willecke, K., S. Jungbluth, E. Dahl, H. Hennemann, R. Heynkes, and K.H. Grzeschik. 1990. Six genes of the human connexin gene family coding for gap junctional proteins are assigned to four different human chromosomes. Eur. J. Cell Biol. 53:275-280.

Yamasaki, H. and T. Enomoto. 1985. Role of intercellular communication in BALB/c3T3 cell transformation. In Carcinogenesis vol. 9, a comprehensive survey-mammalian cell transformation : mechanisms of carcinogenesis and assays for carcinogens (ed. J.C. Barrett and R.W. Tennant) p.179-194. Raven press,New York.

Yamasaki, H., M. Hollstein, M. Mesnil, N. Martel, and A.M. Aguelon. 1987. Selective lack of intercellular communication between transformed and nontransformed cells as a common property of chemical and oncogene transformation of BALB/C3T3 cells. Cancer Res. 47:5658-5664.

Yamasaki H. and F. Katoh. 1988. Further Evidence for the Involvement of gap-junctional intercellular communication in induction and maintenence of transformed foci in BALB/C3T3 cells. Cancer Res. 48:3490-3495.

Yamasaki, H. 1990. Gap junctional intercellular communication and carcinogenesis. Carcinogenesis 11:1051-1058.

Yotti, L.P., C.C. Change, and J.E. Trosko. 1979. Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. Science 206: 1089-1091.

Zhang, J.T., and B.J. Nicholson. 1989. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. J. Cell Biol. 109:3391-3401.