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Deficient epithelial-fibroblast heterocellular gap junction communication can be overcome by co-culture with an intermediate cell type but not by E-cadherin transgene expression

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SUMMARY

Epithelial, fibroblast and intermediate cell lines were employed to examine the mechanism(s) essential for heterocellular gap junction intercellular communication in vitro. These cell lines were characterized extensively for cell type based on morphology, intermediate cytoskeletal proteins, cell adhesion molecules and their associated proteins, tight junction proteins as well as functional differentiation. All cell types expressed connexin43 and were dye-coupled in homocellular culture. Epithelial and intermediate cells or fibroblasts and intermediate cells readily assembled heterocellular connexin43-positive gap junction plaques when co-cultured, while gap junction plaques in mixed cultures of epithelial cells and fibroblasts were rare. Dye microinjection studies were used to show that there was little gap junction intercellular communication between epithelial cells and fibroblasts. However, intermediate cells were able to communicate with epithelial cells and, to a lesser extent, fibroblasts and could transfer dye to both epithelial cells and fibroblasts when all three cell types were cultured together. Fibroblasts that were stably transfected with a cDNA encoding E-cadherin had a greater tendency to aggregate and exhibited a more epithelial-like phenotype but heterocellular gap junction intercellular communication with epithelial cells, which endogenously express E-cadherin, was not enhanced. These results suggest that mutual expression of E-cadherin is insufficient to stimulate gap junction formation between epithelial cells and fibroblasts. Moreover, our results also demonstrate that communication gaps between epithelial cells and fibroblasts can be bridged by intermediate cells, a process that may be important in mammary gland development, growth, differentiation and cancer.

Key words: Gap junction, Connexin43, Cadherin, Heterocellular communication, Mammary cell

INTRODUCTION

Gap junctions are intercellular transmembrane channels that allow the exchange of amino acids, secondary messengers, calcium and other small molecules. The gap junction channel is composed of connexins, which oligomerize into hemichannels (connexons) that pair with connexons contributed from an adjacent cell (reviewed in Laird, 1996). The connexins (Cx) are a family of at least 14 proteins that are differentially expressed in nearly all cells (reviewed in Bruzzone et al., 1996). In many instances, homocellular gap junctions are assembled between cells of similar phenotype and gap junctions are formed by each cell contributing the same connexin constituent in a homotypic fashion (reviewed in Goodenough et al., 1996). However, in more recent years it has become clear that heterocellular gap junctions can form between cells of different types and by employing different members of the connexin family in a heterotypic arrangement (Bruzzone et al., 1996). In the current study we examine the essential mechanism for heterocellular gap junction formation in cells derived from the mammary gland.

The mechanisms of cellular selectivity regarding cell-cell communication have been under investigation since it was determined that heterocellular populations of fibroblasts and epithelial cells sort into communication compartments when co-cultured (Fentiman et al., 1976; Pitts and Burk, 1976). Homocellular populations of these same cells were well coupled through gap junctions, while heterocellular gap junctional intercellular communication (GJIC) was severely limited. Pitts and colleagues extended these early studies by discovering that the reduced coupling between different cell types is correlated with fewer junctions and that sparse heterocellular coupling can occur in vivo (Pitts and Kam, 1985; Kam et al., 1986). Other researchers have characterized

heterocellular gap junctions (GJ) in vivo and in vitro. For example, GJ between cardiac myocytes and surrounding fibroblasts (Rook et al., 1989; Laird and Revel, 1990); germ cells and Sertoli cells in the testis (Cyr et al., 1992); epithelial and fiber cells of the lens junctions (Goodenough et al., 1980; Rae and Kuszak, 1983; Bassnett et al., 1994); and between basal cells and principal cells in the epididymis (Cyr et al., 1996) have been documented. One of the best understood systems where heterocellular GJIC is essential occurs between the cumulus granulosa and the oocyte (Valdimarsson et al., 1993). In an elegant series of experiments, Simon and colleagues (1997) demonstrated that heterocellular Cx37 gap junctions between the oocyte and granulosa cells are critical for normal oogenesis as Cx37 knockout mice are infertile stemming from abnormalities in follicular growth and oocyte maturation. Using in vitro experiments, Fentiman and colleagues have found that it is possible that some cell types are not selective communicators and their promiscuity in GJIC may be related to their ability to adhere to different cell types (Fentiman et al., 1976).

Several reports have demonstrated that calcium-dependent cell adhesion is necessary for gap junction channel formation between cells that exhibit the same phenotype (Kanno et al., 1984; Jongen et al., 1991; Musil et al., 1990; Meyer et al., 1992; reviewed in Laird, 1996). In homocellular gap junction formation it has been proposed that cell adhesion is necessary to position apposing cell surface membranes for connexon docking and clustering of gap junction channels into plaques (Laird, 1996). However, it is not clear if cadherin-mediated cell adhesion is necessary for heterocellular gap junction assembly, especially since cell-specific cadherins have been shown to be responsible for homocellular cell-cell contact and clustering that occur in vivo and in vitro (Takeichi, 1991).

The molecular mechanisms that limit or facilitate the formation of heterocellular GJIC have not been investigated. To this end, we have generated and characterized an in vitro heterocellular system using three cell lines (fibroblasts, intermediate cells and epithelial cells). These well-characterized cell types were all obtained from the bovine mammary gland to ensure biological relevance and all were capable of extensive homocellular GJIC. Using this in vitro cell system we have determined that heterocellular GJ plaque assembly and GJIC between fibroblasts and epithelial cells is severely restricted. However, intermediate cells were found to be promiscuous and could act to bridge GJIC between fibroblasts and epithelial cells. Finally, our results showed that mutual expression of E-cadherin was insufficient in facilitating heterocellular GJIC between mammary fibroblasts and epithelial cells.

MATERIALS AND METHODS

Materials, cell lines and culture conditions

All media, sera and culture reagents were obtained from Gibco BRL (Burlington, ON), Becton Dickinson (St Laurent, QC) or Sigma Chemical Co. (St Louis, MO). Lipofectamine was obtained from Gibco BRL. All cell lines were grown in DMEM supplemented with 10% FBS, 100 units/ml of streptomycin/penicillin, and 2 mM glutamine. MAC-T cells were an established bovine mammary epithelial cell line that can be induced to morphologically and functionally differentiate in culture, similar to normal mammary alveolar epithelium in vivo (Huynh et al., 1991). FibE, FibC, 3hUnfil, primary fibroblasts and primary epithelial cells were isolated from bovine mammary tissue also, as previously described (Woodward et al., 1994, 1995). FibE cells were originally characterized as epithelial, but drifted in culture and represent a stable cell type with shared characteristics of both fibroblasts and epithelial cells and thus are referred to as an 'intermediate' or 'transitional' cell type. Cell morphology, phenotype and growth patterns of the other mammary cells did not drift in culture. FibC cells were characterized as being fibroblasts.

E-cadherin expression

Fibroblasts (FibC) and intermediate (FibE) cells were subcultured for transfection and for continual passage. Cells were stably co-transfected with a plasmid expression vector containing E-cadherin, pBATEM2, and a neo plasmid, pBATneo, as a selectable marker (Nose et al., 1988) by lipofectamine as previously described (Woodward et al., 1995). Both plasmids were generous gifts from Dr Masatoshi Takeichi. Two days after transfection, cells were split 1:5 and subsequently selected in DMEM with 10-20% FBS, 2 mM glutamine and 1 mg/ml G418 sulfate. Selection was continued for 2 weeks. G418 sulfate at 1 mg/ml was previously determined to be 100% cytotoxic to all nontransfected cells within 5 days. Western blotting and immunofluorescent labeling revealed that the majority of cells in the selected cell line expressed E-cadherin at a level comparable to FibE cells and E-cadherin was localized to the plasma membrane.

Aggregation assay

In order to study cadherin-mediated cell aggregation, we employed the assay previously described by Nose et al. (1988) and modified by Wang and Rose (1997). Nearly confluent FibC (fibroblasts) and FibC cells transfected with cDNA encoding E-cadherin were treated with 0.25% trypsin in the presence of 1 mM EGTA at 37°C for 10-15 minutes. Trypsinized cells were washed with gentle pipeting in HCMF at 4°C in order to obtain a single cell suspension. Cells were quantified using a hemocytometer, spun, and subsequently resuspended ($2\text{-}6\times10^5$ cells) in 1 ml culture medium and placed in 35 mm dishes (Nunc, Denmark). The dishes were incubated on a rotary shaker (80 rpm) for 12 hours at 37°C in a 5% CO_2 atmosphere and then examined for cell aggregates under the microscope.

Immunocytochemistry

Fixed cells grown on coverslips were immunolabeled in the manner previously described by Laird et al. (1995). Antibodies against the following proteins were utilized: Cx43 (Laird and Revel, 1990), cytokeratin (Pan 1-8 anti-cytokeratin antibody; Boehringer-Mannheim Biochemica, QC), vimentin (Boehringer-Mannheim Biochemica, QC), E-cadherin (Transduction Laboratories, KY), β-catenin (Transduction Laboratories, KY), α-catenin (Sigma, MO), occludin (Zymed, CA) and ZO-1 (Developmental Studies Hybridoma Bank, IA). Briefly, cells were grown on glass coverslips and fixed with 100% ethanol, blocked with 10% horse serum or 2% BSA, rinsed in PBS and immunolabeled. Cells were labeled with 1-5 μg/ml anti-Cx43 antibody or 1:100 dilutions of cytoskeletal antibodies, and 1:250 for E-cadherin, β-catenin, α-catenin and occludin antibodies. Cells were subsequently rinsed 6× over 30 minutes in PBS and incubated for 1 hour with goat anti-mouse, goat anti-rat or donkey anti-rabbit antibodies conjugated to either rhodamine or FITC (Jackson ImmunoResearch Laboratories, Inc., PA). Coverslips were rinsed, mounted and analyzed on a Zeiss LSM 410 inverted confocal microscope as described previously (Laird et al., 1995). In some cases, cells were double-immunolabeled as described by Laird et al. (1995).

Microinjection

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

plated at a 3:1 ratio for FibC:FibE and FibC:MAC-T or a 1:1 ratio with MAC-T:FibE. Alternatively, FibC cells were permitted to reach 50% confluency before the addition of FibE and/or MAC-T cells, and utilized 2-3 days after the addition of the second population of cells. No detectable differences were observed between the two culturing procedures. The cells were assayed for the extent of GJIC through pressure microinjection of the dye, Lucifer Yellow (5%), in H_2O or 10 mM Hepes (pH 7.4, Molecular Probes, Eugene, OR). Typically, in heterocellular populations of cells, the microinjected cell was at least one order removed from contact with the second cell type. Cells were microinjected over an interval of 20 minutes and the coverslips were fixed with 3.7% formaldehyde in PBS for 5-10 minutes. In some cases, the coverslips were subsequently permeabilized with 0.1% Triton X-100 and immunolabeled. The cells were viewed using confocal microscopy (Zeiss LSM 410) to determine the extent and success of dye coupling. For tabulation of heterocellular populations (see Table 2), the following criteria were utilized: (1) microinjected cells had to successfully transfer dye to more than two cells within the same population of cells in order to be included and (2) microinjected cells were only considered to have successfully transferred dye to another cell type if more than one cell received dye.

Western immunoblotting

MAC-T cells, FibC cells, FibC cells transfected with E-cadherin (FibC TF), FibE cells, and FibE cells transfected with E-cadherin (FibE TF) were grown to 80-95% confluency in 100 mm dishes. The cells were then placed on ice, washed with PBS and pelleted in a clinical centrifuge. The pellet was resuspended with 600-800 μl of RIPA buffer (10 mM Na_2HPO_4, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholic acid, 1% SDS) to which inhibitors had been added (2 mM PMSF, 2 mM sodium orthovanadate, 1 mM sodium fluoride, 10 mM leupeptin, and 2 μg/ml aprotinin). The samples were sonicated, normalized for protein content using a bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) and then subjected to SDS-PAGE.

Lysates were resolved on a 10% SDS-polyacrylamide gel with a bisacrylamide:acrylamide ratio of 0.8:30. The resolved proteins were transferred to nitrocellulose blot paper and immunostained as described by Laird et al. (1995). The following antibody concentrations or dilutions were used: CT-360 (anti-Cx43) at 1μg/ml, anti-E-cadherin at 1:1000 and anti-β-catenin at 1:1000. The blots were air dried and exposed to Amersham Hyperfilm-MP with an intensifying screen.

RESULTS

Cell typing and characterization

Established mammary cells were characterized for cell type based on (1) morphology, (2) cytoskeletal intermediate filament proteins, (3) E-cadherin expression, (4) tight junction proteins and (5) mammary epithelial functional differentiation (β-casein expression/synthesis). The MAC-T cell line was chosen as an epithelial model since earlier reports had established that this cell line maintains its epithelial characteristics in culture (Huynh et al., 1991), unlike many other mammary epithelial cells (Huynh and Pollack, 1995). MAC-T cells stained positive for the intermediate filament protein cytokeratin (Fig. 1A,B; Table 1) and negative for the intermediate filament protein vimentin (Fig. 2A,B; Table 1). Additionally, MAC-T cells expressed the epithelial tight junction protein, occludin, the tight junction associated protein, ZO-1, and the epithelial adherens junction protein, E-cadherin (Table 1). This cell line has previously been shown to functionally and morphologically differentiate when cultured on an appropriate substratum in the

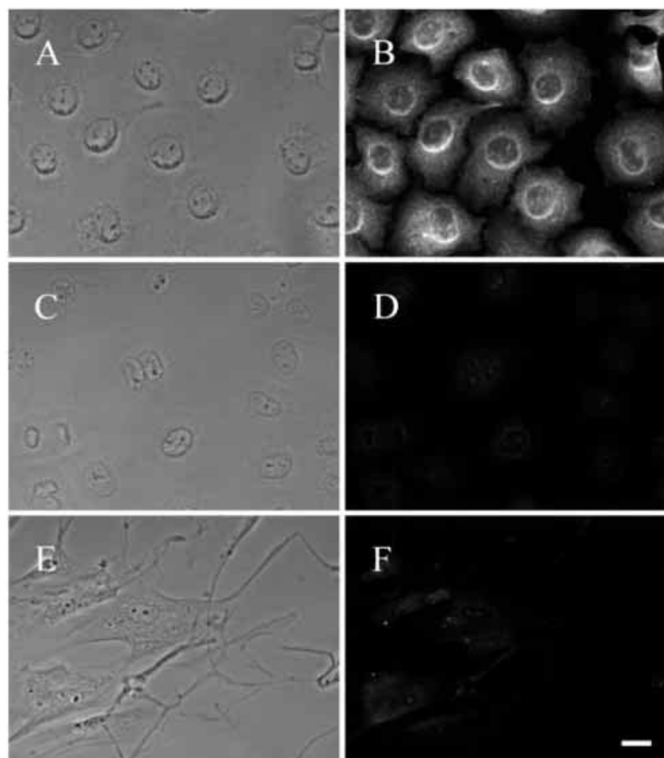

Fig. 1. Cytokeratin immunostaining in bovine mammary cell lines. MAC-T (A,B), FibE (C,D) and FibC (E,F) cells were immunostained with an anti-cytokeratin antibody and viewed under transmitted light for morphology (A,C,E) and fluorescence (B,D,F) for the distribution of the intermediate filament protein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

presence of prolactin (Huynh et al., 1991). MAC-T cells have been shown to synthesize and secrete large quantities of α and β-caseins in culture (Huynh et al., 1991). These characteristics are the hallmark of functionally differentiated mammary epithelial cells in vivo.

The bovine mammary fibroblast cell line, FibC, had a fibroblast morphology and, consistent with fibroblastic properties, the cell line was negative for cytokeratin (Fig. 1E,F) and positive for vimentin (Fig. 2E,F). FibC cells, as well, did not express epithelial tight junction or epithelial adherens junction proteins although they synthesized β-catenin (Table 1). The third cell type studied, FibE cells, had characteristics of both fibroblasts and epithelial cells. FibE cells expressed vimentin (Fig. 2C,D) and lacked cytokeratin (Fig. 1C,D). Additionally, this cell line did not express occludin (Table 1) and failed to express β-casein (T. L. Woodward, unpublished results). However, FibE cells have a cobblestone epithelial-like morphology, and express both E-cadherin and ZO-1 (Table 1). In summary, based on morphology, expression of hallmark proteins and functional differentiation, these three mammary cell lines, were characterized as epithelial cells (MAC-T), fibroblasts (FibC) and intermediate cells (FibE).

Cx43 expression and GJIC in homocellular cultures

Examination of connexin expression in the mammary gland of

Table 1. Characterization of bovine mammary cell lines

	Epithelial cells (MAC-T)	Intermediate cells (FibE)	Fibroblasts (FibC)
Morphology	Epithelial-like	Epithelial-like	Fibroblast-like
E-cadherin	Positive	Positive	Negative
β-catenin	Positive	Positive	Positive
ZO-1	Positive	Positive	Negative
Occludin	Positive	Negative	Negative
Vimentin	Negative	Positive	Positive
Cytokeratin	Positive	Negative	Negative
Actin	Positive	Positive	Positive

Bovine mammary epithelial cell lines were characterized by morphology and by immunofluorescence for the presence or absence of junctional proteins (E-cadherin, β-catenin, occludin, ZO-1) or cytoskeletal markers (vimentin, cytokeratin, actin).

humans and murine species has identified either Cx26 or Cx43 only (Monaghan et al., 1994; Pozzi et al., 1995), or both Cx26 and Cx43 (Lee et al., 1992), or developmentally regulated expression of Cx26, Cx32 and Cx43 (Pozzi et al., 1995). All three bovine mammary cell lines used in this study expressed several species of Cx43, a lower molecular mass (42 kDa) nonphosphorylated species and two phosphorylated species (44-46 kDa) (Fig. 3, insets). Cx26 and Cx32 were not immunodetected in any of the bovine mammary cell lines used in these studies, nor were these connexins found in lactating bovine mammary tissue (data not shown). When homocellular cultures of epithelial cells, intermediate cells and fibroblasts were microinjected with Lucifer Yellow to analyze GJIC, all three cell cultures were found to be well coupled (Fig. 3).

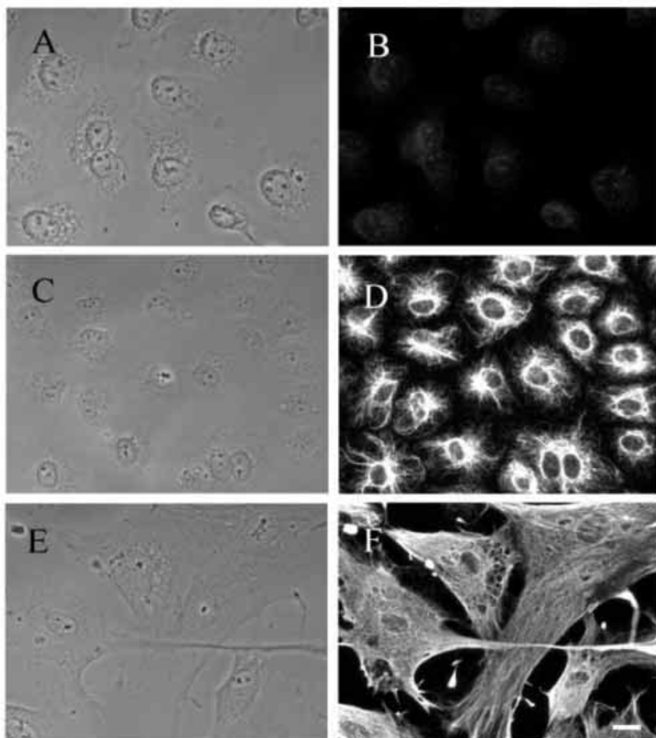

Fig. 2. Vimentin immunostaining in bovine mammary cell lines. MAC-T (A,B), FibE (C,D) and FibC (E,F) cells were characterized by immunofluorescent confocal microscopy for morphology under transmitted light (A,C,E) and for the distribution of the intermediate filament protein vimentin (B,D,F). The lack of vimentin expression in MAC-T cells suggests they are epithelial in nature. Although FibE cells exhibit an epithelial-like morphology, they express vimentin, a resident cell-specific protein in fibroblastic cells such as the FibC. All fluorescent images were taken using the same imaging conditions. Bar, 10 μm.

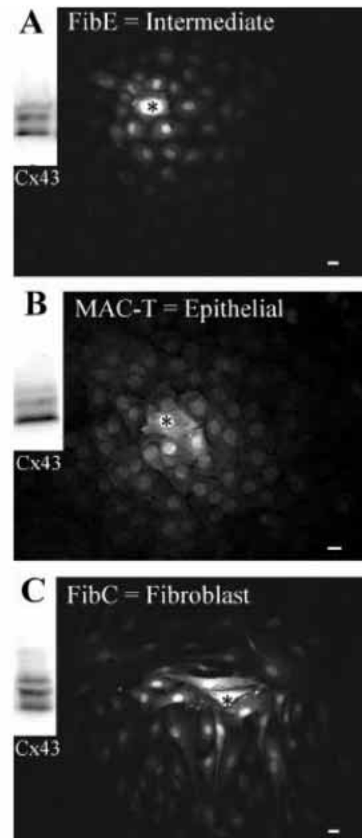

Fig. 3. Homocellular populations of intermediate cells, epithelial cells and fibroblasts express Cx43 and are well coupled. Live homocellular populations of intermediate FibE (A), epithelial MAC-T (B), and fibroblastic FibC (C) cells were microinjected with Lucifer Yellow (injected cells denoted by asterisks) and dye was observed to spread to neighboring cells in all cell types. Western blots were also performed using cell lysates of FibE (inset, A), MAC-T (inset, B) and FibC (inset, C). Both nonphosphorylated and phosphorylated species were observed in each cell type. Bars, 10 μm.

Heterocellular GJ and GJIC

Immunofluorescence studies performed in epithelial/ intermediate cell co-cultures revealed the formation of gap junctional plaques at heterocellular interfaces (Fig. 4A,C, arrows) as well as at homocellular interfaces (Fig. 4A,C, double arrows). Cells were distinguished on the basis of Cx43 (Fig. 4A) and vimentin (Fig. 4B) immunostaining. The presence of plaques suggested the possibility of intercellular communication between different cell types; thus, cell lines were co-cultured to examine heterocellular GJIC. Cx43 plaques were routinely (though less commonly) identified in intermediate/fibroblast co-cultures, whereas in epithelial/fibroblast co-cultures, heterocellular Cx43 plaques were rare (data not shown). Similarly, epithelial/intermediate cell (Fig. 5A,B) and fibroblast/intermediate cell (Fig. 5C,D) co-

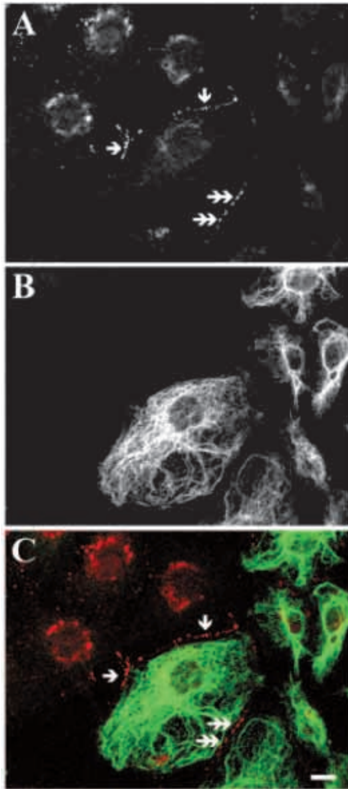

Fig. 4. Assembly of heterocellular gap junction plaques in co-cultures of epithelial and intermediate cell types. MAC-T and FibE cells were co-cultured and immunostained for Cx43 (A,C red) and vimentin (B,C green). Note the presence of Cx43 at locations where MAC-T cells are adjacent to FibE cells (arrows) as well as between neighboring FibE cells (double arrows). Bar, 10 μm.

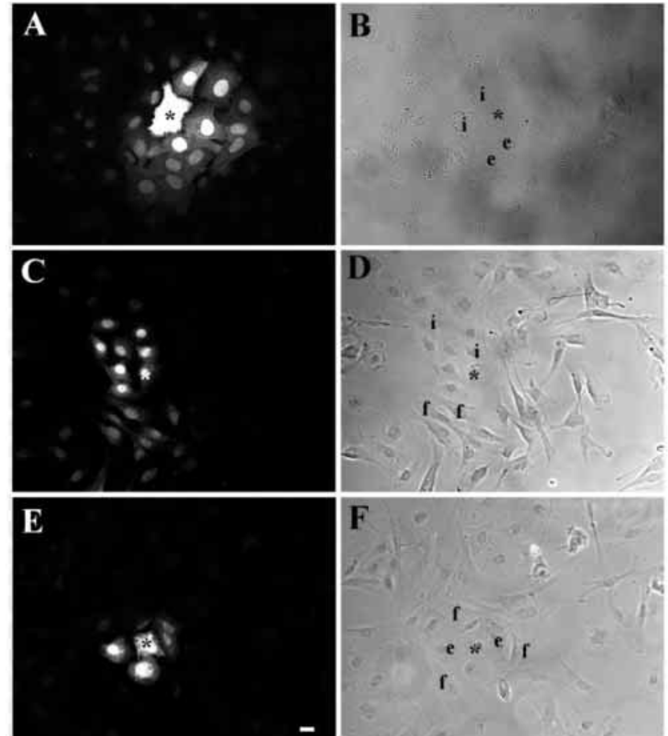

Fig. 5. Gap junction intercellular communication in heterocellular cultures. Heterocellular cultures of epithelial MAC-T and intermediate FibE cells (A,B), intermediate FibE cells and fibroblasts FibC (C,D) and epithelial MAC-T and fibroblasts FibC (E,F) were microinjected with Lucifer Yellow (A,C,E), and viewed under transmitted light (B,D,F). Mixed cell populations show dye coupling between epithelial cells (e) and intermediate cells (i) and between intermediate cells and fibroblasts (f), but poor coupling between epithelial cells and fibroblasts. The injected cell is indicated by an asterisk. Bar, 10 μm.

cultures commonly demonstrated heterocellular dye transfer of microinjected Lucifer Yellow, but dye transfer was rarely observed in fibroblast/epithelial co-cultures (Fig. 5E,F). Fibroblasts were identified by their spindle shaped morphology, while epithelial and intermediate cells, though similar in shape, could be separated by the presence of dark vesicle-like structures in intermediate cells when observed in transmitted light images and by the lack of perinuclear Cx43 immunostaining (data not shown). Primary cultures of fibroblasts and another bovine mammary fibroblast cell line (3hUnfil) also failed to assemble heterocellular Cx43 plaques when co-cultured with epithelial cells, while both populations of fibroblasts did form plaques with intermediate cells (data not shown).

Characterization of E-cadherin in stably transfected fibroblasts

In order to address the hypothesis that coexpression of the same connexin and a common cadherin would upregulate gap junctional communication between distinctly different cell types, fibroblasts and intermediate cells (which endogenously express E-cadherin) were transfected with a plasmid encoding E-cadherin. Fibroblasts transfected with a cDNA encoding E-cadherin expressed E-cadherin by western blot analysis (Fig. 6, lane b) and by immunocytochemistry (Fig. 7G,I) while untransfected fibroblasts lacked E-cadherin (Figs 6, lane c, 7E). The level of E-cadherin expression in these fibroblasts was comparable to that of endogenously expressed E-cadherin in intermediate cells (Fig. 6, lane e). No substantial change in the level of E-cadherin expression, as measured by western blot, was observed in intermediate cells after transfection with cDNA encoding E-cadherin (Fig. 6, lane d). Since E-cadherin expression was already abundant in intermediate cells, it is possible that endogenous E-cadherin may have been downregulated to compensate for transgene expressed E-cadherin. In comparison to wild-type fibroblasts (Fig. 6A), fibroblasts that were transfected with cDNA encoding E-cadherin aggregated more extensively into large cell clusters (Fig. 6B) suggesting that these cells expressed functional E-cadherin. Similar to epithelial and intermediate cells (Fig. 7A,C), E-cadherin was immunofluorescently localized to the plasma membrane of transfected fibroblasts, though the staining pattern appeared more punctate (Fig. 7G). Additionally, E-cadherin-expressing fibroblasts (Fig. 7H) were distinguishable from nontransfected fibroblasts (Fig. 7F) by their more epithelial-like morphology, although they were still distinct from the epithelial (Fig. 7B) and intermediate (Fig. 7D) cell lines. Double immunofluorescent labeling studies revealed that E-cadherin (Fig. 7I) and α-catenin (Fig. 7J) were colocalized in fibroblasts transfected with cDNA encoding E-

cadherin (Fig. 7I,J, arrows). With the knowledge that cadherin expression can lead to changes in the expression and distribution of connexins (Mege et al., 1988; Musil et al., 1990), western blotting was performed to examine the levels of Cx43 in fibroblasts and intermediate cells after transfection (Fig. 8). The expression of E-cadherin did not influence the already high levels of Cx43 in fibroblasts or the previously low levels of Cx43 in intermediate cells (Fig. 8). Using western blot analysis, we also tested for the presence of β-catenin, a protein associated with adherens junctions, which is required for proper E-cadherin function (Fig. 9). β-catenin was expressed in all cell lines including fibroblasts prior to transfection (Fig. 9). Immunofluorescence studies localized β-catenin at the cell surface of all cell lines (Fig. 10A,C,E,G), as well as intracellularly in epithelial cells (Fig. 10A). The presence of β-catenin in untransfected fibroblasts (Fig. 10E) suggests that cadherins other than E-cadherin may be present. To date, however, we have been unable to detect other members of the cadherin family.

Quantification of heterocellular dye transfer

A dye transfer assay was used to measure GJIC between heterocellular populations of cells (Table 2). Epithelial cells and the intermediate cell type had the highest GJIC with 73.5% of microinjected cells transferring dye to heterocellular neighbors. Dye transfer between fibroblasts and the intermediate cell type was significantly lower at approximately 28.3%. Epithelial cell to fibroblast GJIC was low at 12.1%. Fibroblasts transferred dye more effectively to intermediate cells than intermediate cells to fibroblasts. After transfection with cDNA encoding E-cadherin, the instance of intermediate cells communicating with epithelial cells increased to 100% (Table 2). Since epithelial-to-intermediate cell GJIC was already high before transfection (73.5%), we did not investigate why E-cadherin expression slightly increased GJIC. Unexpectedly, heterocellular GJIC between fibroblasts, which expressed E-cadherin, and epithelial cells remained low at 14.3%. Furthermore, E-cadherin expression only induced

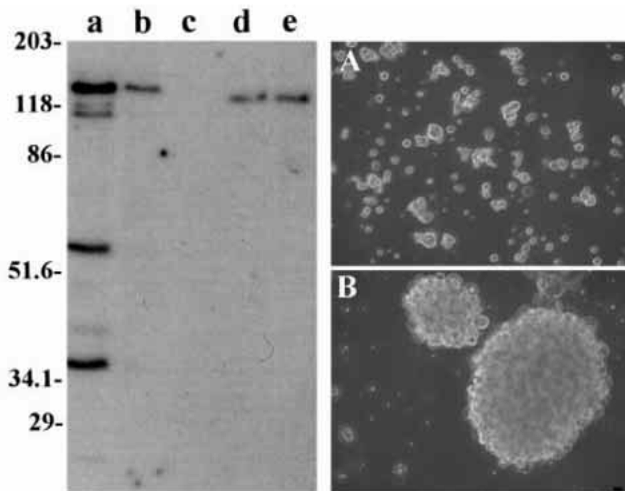

Fig. 6. Western blot and aggregation analysis of E-cadherin in bovine mammary cells. Western blots of lysates from epithelial cells (lane a), fibroblast transfected with cDNA encoding E-cadherin (lane b), fibroblasts (lane c), intermediate cells transfected with cDNA encoding E-cadherin (lane d) and intermediate cells (lane e) revealed that all cell lines, with the exception of the fibroblasts (lane c), contained full-length E-cadherin at 120-130 kDa. The level of E-cadherin expression in fibroblasts after transfection with cDNA encoding E-cadherin was comparable to that of intermediate cells. Equal amounts of total protein were loaded in each lane and molecular masses are expressed as kDa. Fibroblasts (A) and fibroblasts transfected with cDNA encoding E-cadherin (B) were examined for their ability to aggregate, as described in the Materials and methods. Fibroblasts remained as single cells or small aggregates (A) while fibroblasts that expressed E-cadherin aggregated into large colonies (B). Bar, 25 μm.

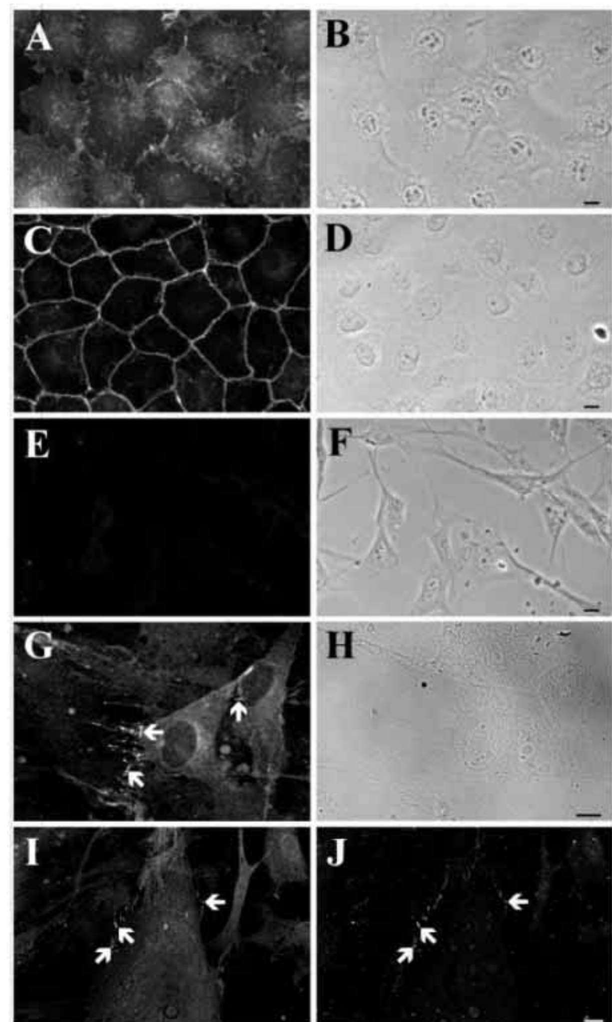

Fig. 7. Characterization of E-cadherin distribution in mammary cell lines and colocalization with α-catenin. Distribution of E-cadherin in epithelial cells (A,B), intermediate cells (C,D), fibroblasts (E,F) and fibroblasts after transfection with cDNA encoding E-cadherin (G,H). E-cadherin staining was endogenously present in epithelial cells and intermediate cells at the cell surface (A,C), with additional intracellular staining present in the epithelial cells. Cell surface immunostaining for E-cadherin was only observed in fibroblasts after transfection (G, arrows). The corresponding transmitted light images are shown (B,D,F,H). Fibroblasts that were transfected with cDNA encoding E-cadherin (I) were double immunofluorescently labeled with anti-E-cadherin and anti-α-catenin antibodies. As expected E-cadherin and α-catenin colocalized (I,J, arrows). Bars, 10 μm.

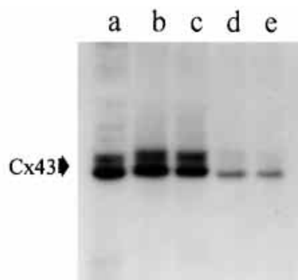

Fig. 8. Western blot analysis of Cx43 in bovine mammary cells. Western blot studies of lysates obtained from epithelial cells (lane a), E-cadherin expressing fibroblasts (lane b), fibroblasts (lane c), intermediate cells after transfection with cDNA encoding E-cadherin (lane d) and intermediate cells (lane e) revealed that there were no significant changes in the expression levels of Cx43 in either intermediate cells or fibroblasts following transfection. Equal amounts of total protein were loaded in each lane.

slight improvements in GJIC between fibroblasts and intermediate cell types (Table 2) even though both cell types expressed similar levels of E-cadherin. Therefore, despite constitutive expression of functional E-cadherin that altered the phenotype and the ability of fibroblasts to aggregate, GJIC between fibroblasts and epithelial cells or between fibroblasts and intermediate cells did not substantially increase.

The role of intermediate cells in bridging communication gaps

We investigated the possibility that the intermediate cell type, which communicates with both epithelial cells and fibroblasts, may act to bridge the communication gap between epithelial cells and fibroblasts. In mixed cultures containing all three cell lines, microinjected Lucifer Yellow was observed to pass from fibroblasts to epithelial cells via the intermediate cell type (Fig. 11A,D). Conversely, dye could also be transferred from epithelial cells to intermediate cells and then to fibroblasts (Fig. 12A,D) indicating that there is no directional selectivity. The three cell lines were distinguished by the presence of the cytoskeletal marker protein vimentin (Figs 11B, 12B), which is found only in intermediate cells and fibroblasts, and by morphology (Figs 11C, 12C). Intermediate or 'transitional' cells that have not fully differentiated or have dedifferentiated may thus be able to serve a role in passing molecular signals between two poorly communicating cell types. Triple culture injections were not quantified because of the difficulty in identifying appropriate cell clusters where all three cell phenotypes were represented and fibroblasts were clearly separated from epithelial cells by several intermediate cells.

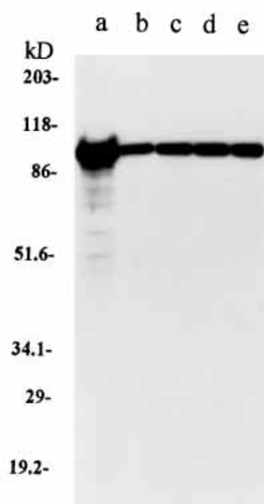

Fig. 9. Western blot analysis of β-catenin expression in bovine mammary cells. Western blots using an anti-β-catenin revealed that epithelial cells (lane a), E-cadherin expressing fibroblasts (lane b), fibroblasts (lane c), intermediate cells transfected with cDNA encoding E-cadherin (lane d) and intermediate cells (lane e) all expressed β-catenin at approx. 92 kDa. An equal amount of total protein was loaded in each lane.

Table 2. Quantification of heterocellular dye transfer

Co-culture Cell types	Direction and success of dye transfer		Overall success of dye transfer
	a to b	b to a	
a) Epithelial b) Intermediate	16/23=69.6%	9/11=81.8%	25/34=73.5%
a) Epithelial b) Intermediate+E-cad	27/27=100%	15/15=100%	42/42=100%
a) Epithelial b) Fibroblasts	6/34=17.6%	2/32=6.3%	8/66=12.1%
a) Epithelial b) Fibroblasts+E-cad	3/35=8.6%	6/28=21.4%	9/63=14.3%
a) Fibroblasts b) Intermediate	10/22=45.5%	5/31=16.1%	15/53=28.3%
a) Fibroblasts+E-cad b) Intermediate	15/28=53.6%	4/27=14.8%	19/55=34.5%
a) Fibroblasts b) Intermediate+E-cad	14/26=53.8%	7/18=38.9%	21/44=47.7%
a) Fibroblasts+E-cad b) Intermediate+E-cad	10/16=62.5%	5/22=22.7%	15/38=39.5%

Co-cultures of mammary cells were microinjected before and after transfection with cDNA encoding E-cadherin (E-cad). The direction of dye transfer is noted.

DISCUSSION

Substantial evidence exists that calcium-dependent cell adhesion molecules (cadherins) play a critical role in gap junction assembly and intercellular communication between most homocellular populations of cells (see Laird, 1996, for a review). Heterocellular GJIC has been reported to occur in vitro (Tomasetto et al., 1993), but there are no reports where strict cell typing and connexin analysis of heterotypic cell populations were conducted before analyzing GJIC. It is also unclear if E-cadherin-mediated cell adhesion is essential and/or sufficient to stimulate the molecular events necessary for the establishment of GJIC between cells that possess distinct phenotypes. In order to explore these questions we have analyzed Cx43 expression and GJIC between mammary epithelial cells, fibroblasts and an intermediate cell type and determined whether expression of E-cadherin could overcome heterotypic GJIC barriers. Our data demonstrated that GJIC was abundant in homocellular cultures of fibroblasts,

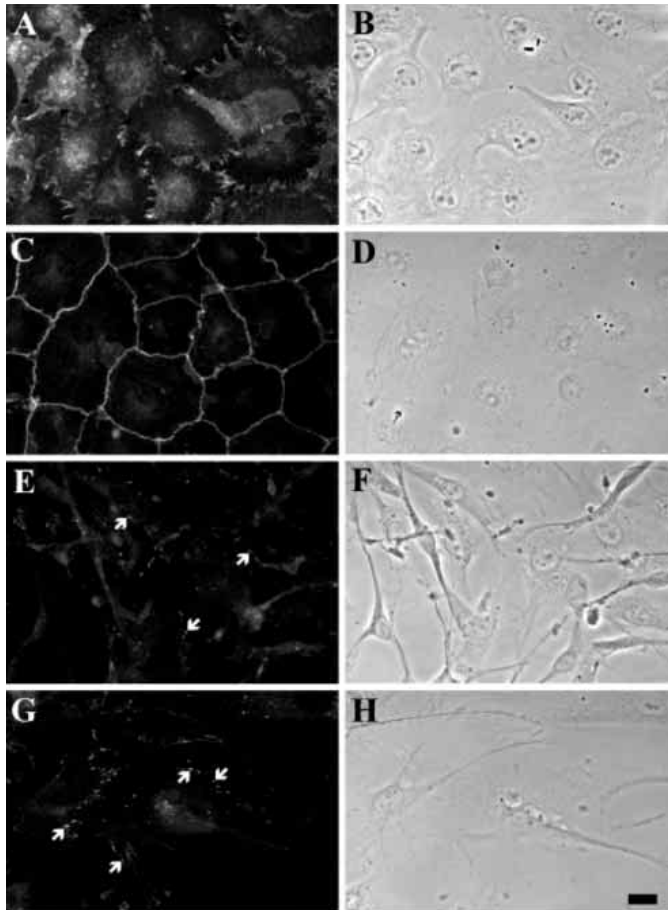

Fig. 10. Characterization of β-catenin immunofluorescence in mammary cell lines. Epithelial cells (A,B), intermediate cells (C,D), fibroblasts (E,F) and E-cadherin expressing fibroblasts (G,H) were immunostained with an anti-β-catenin antibody. β-catenin was distributed in a rim-like pattern at the periphery of intermediate cells while epithelial cells had both a cell surface distribution as well as an intracellular pool. β-catenin was distributed in a punctate pattern at the cell surface of fibroblasts cells before (E, arrows) and after (G, arrows) transfection with cDNA encoding E-cadherin. Cell morphology is shown in the corresponding transmitted light images (B,D,F,H). Bar, 10 μm.

intermediate cells or epithelial cells. In heterocellular co-cultures, intercellular Cx43 plaques and GJIC were established between the epithelial and intermediate cells and, to a more limited extent, between intermediate cells and fibroblasts. In contrast, epithelial cells established few Cx43 plaques and communicated poorly with fibroblasts, demonstrating that heterocellular GJIC is severely limited when compared to homocellular GJIC. The cell surface expression of exogenous E-cadherin in fibroblasts (1) colocalized with functionally required α-catenin and co-distributed with β-catenin, (2) changed the morphology of the cells to a more epithelial-like phenotype and (3) promoted cell aggregation, but failed to increase heterocellular GJIC with epithelial or intermediate cells that express high levels of endogenous E-cadherin. These studies suggest that E-cadherin expression alone in two cell types with distinct phenotypes is insufficient to upregulate heterocellular GJIC. Furthermore, our results with triple co-cultures suggest that intermediate or transitional cell types are more promiscuous and can serve as bridges to metabolically couple cell types that have distinct phenotypes.

In vivo, heterocellular GJ formation or GJIC have been demonstrated in several instances (Kam et al., 1986; Cyr et al., 1992; Simon et al., 1997). In the adult mammary gland, fibroblast and epithelial cells are most often separated by a basement membrane. Furthermore, during and immediately after lactation, myoepithelial cells may completely ‘seal’ off epithelial cells from the surrounding stroma. However, GJIC has been shown to occur between mammary myoepithelial cells and epithelial cells in vitro (Pitelka et al., 1973) and in vivo (Berga, 1984). Additionally, the mammary gland undergoes extensive postnatal development during puberty, pregnancy and lactation. At the onset of puberty, during the luteal phase of the menstrual cycle, and during pregnancy and early lactation, active cell proliferation occurs. During periods of ductal elongation and side branching, the basement membrane is degraded and epithelium invades the stromal tissue, thus facilitating direct epithelial-stromal contact. Studies have reported that the basal lamina becomes ‘indistinct’ where proliferating end-bud epithelium invades the stroma (Sakakura, 1991). Electron microscopic examination of tissues from several organs (i.e. intestine, salivary glands, kidney, lung, and eye) have revealed cytoplasmic processes that extend through the basement membrane resulting in direct epithelium-fibroblast contact (reviewed in Sakakura, 1991). Additionally, metalloproteinase expression, basement membrane discontinuity and stromal tissue invasion are common characteristics of mammary epithelial tumors (Birkedal-Hansen et al., 1993; Guelstein et al., 1993). These characteristics are magnified in metastatic tumors. Thus, the potential for fibroblast-epithelial contact directly or through transitional cell types may occur often in both normal and cancerous adult mammary gland.

Although Fentiman and colleagues (1976) had previously reported the lack of epithelial-fibroblast GJIC in human mammary cells in culture, Tomasetto and colleagues (1993) found excellent communication between normal human mammary epithelial cells and normal human fibroblasts in vitro. We have determined that bovine mammary epithelial cells and fibroblasts were found to have very limited GJIC, but a well-characterized intermediate cell type could communicate with both cell types. The discrepancy with these results may be linked to the transdifferentiation that may occur in culture. In our study, we have carefully characterized morphology, intermediate filaments, cadherins, catenins, tight junction (and associated) proteins, as well as assessing epithelial differentiation, to ensure cell typing. Since substantial drift frequently occurs during culture, it was important to fully characterize the cell lines before and during these studies. Many mammary epithelial cell lines have been reported to drift significantly and rapidly in culture resulting in altered morphology, differentiation, tumorigenicity and even cell type (Mork et al., 1990; Huynh and Pollak, 1995; Ronnov-Jessen et al., 1995).

Cell sorting of true epithelial and true fibroblasts occurs in vitro and in vivo, by cells of similar phenotype adhering to each other through cell type-specific cell adhesion molecules (Takeichi, 1991). We have found that MAC-T cells (E-cadherin positive) form islands separate from fibroblasts (E-cadherin

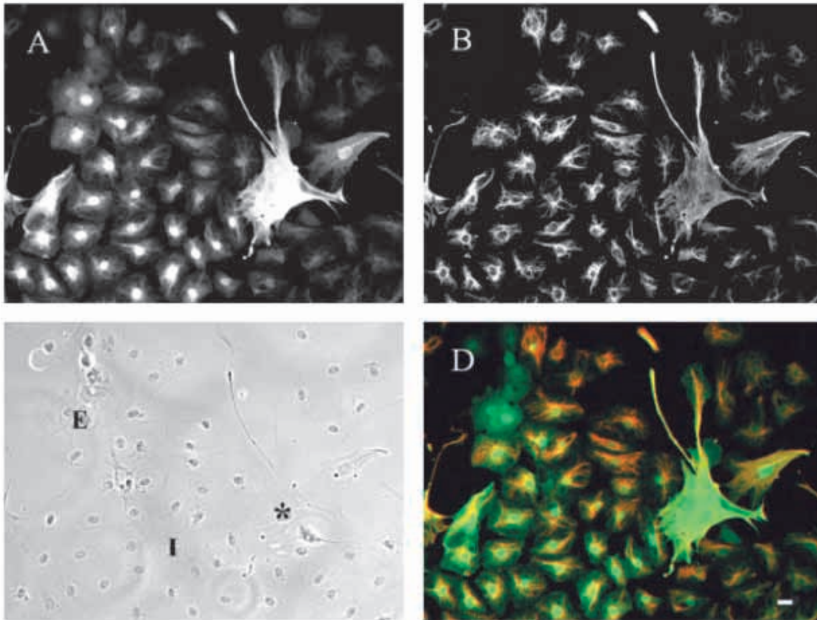

Fig. 11. Dye transfer from fibroblasts to epithelial cells via intermediate cells in triple cell co-cultures. When Lucifer Yellow was microinjected into a fibroblast (asterisk in C), dye transferred to epithelial 'E' cells via a group of intermediate 'I' cells (A,C,D). The intermediate cells and fibroblasts can be distinguished from epithelial cells by the presence of anti-vimentin immunostaining (B,D). The morphological appearances of intermediate cells and fibroblasts were used to distinguish between these two cell types. D represents an overlay of Lucifer Yellow dye transfer (green) and anti-vimentin staining (red) with overlapping areas appearing in yellow. Bar, 10 μm.

negative) when co-cultured (data not shown). A link between cadherin adhesion and GJIC was demonstrated when anti-N-cadherin antibodies were shown to prevent Novikoff cell aggregation and GJIC (Meyer et al., 1992). Likewise, others have demonstrated decreased or abolished GJIC in cells treated with anti-cell adhesion antibodies (Musil et al., 1990) or increased GJIC in cells transfected with cadherins (Jongen et al., 1991). Consequently, we proposed that heterocellular GJIC may not occur between mammary fibroblasts and epithelial cells, since their different cadherin expression would only allow them to form adherens junctions among cells of the same type. Our results showed that very few gap junctions formed between bovine mammary fibroblasts and epithelial cells.

We hypothesized that the frequency of heterocellular GJIC may increase if fibroblasts expressed the epithelial specific E-cadherin. The morphology of the fibroblasts that stably expressed the E-cadherin transgene was markedly more epithelial-like. In addition, the transfected fibroblasts expressed levels of E-cadherin that were equal to E-cadherin levels in intermediate cells but, unlike the intermediate cells, they established poor heterocellular GJIC with epithelial cells. The transfection of fibroblasts with cDNA encoding E-cadherin did not cause an increase in Cx43 expression, nor did it change the localization of Cx43. The lack of changes in GJIC could not be explained by lack of cell surface E-cadherin, since transfection caused fibroblasts to aggregate extensively, further demonstrating that E-cadherin was functional in the fibroblasts. While no other studies on the role of cadherins in heterocellular GJIC have been reported it has been shown that N-cadherin expression in mouse L cells (Wang and Rose, 1997) and

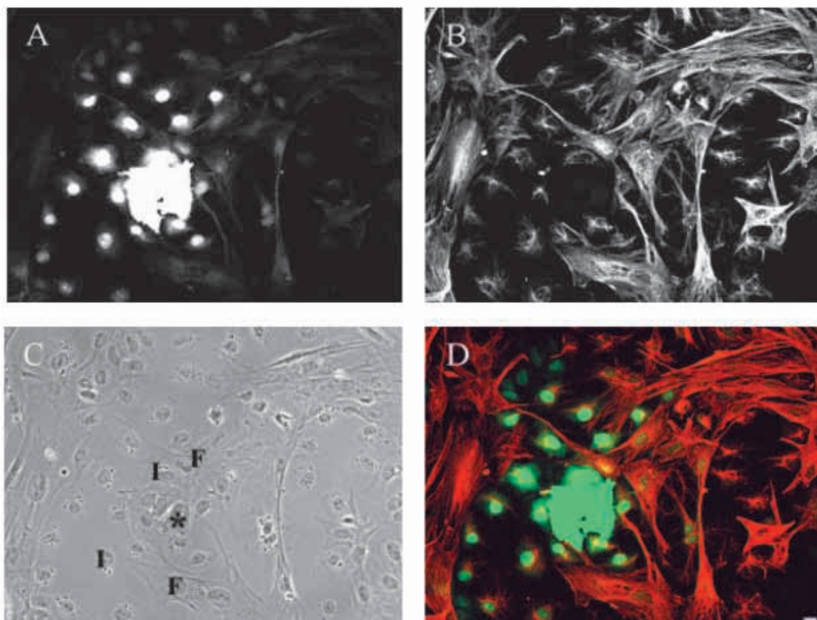

Fig. 12. Dye transfer from epithelial cells to fibroblasts via intermediate cells in triple cell co-cultures. When Lucifer Yellow was microinjected into an epithelial cell (asterisk in C), dye transferred to nearby fibroblasts 'F' via adjacent intermediate 'I' cells (A,C,D). The cell types are distinguished on the basis of anti-vimentin immunofluorescent staining (B,D) and morphology under transmitted light (C). D represents an overlay of dye transfer (green) and anti-vimentin immunofluorescence (red) with areas of overlap in yellow. Bar, 10 μm.

expression of the N-CAM homologue (DCC gene) (Mesnil et al., 1993) can inhibit or fail to enhance GJIC. We conclude that the functional expression of E-cadherin in two distinct cell types is insufficient to modulate heterocellular GJIC.

The use of an intermediate (transitional) cell type that shares both epithelial and fibroblast characteristics allowed us to determine whether epithelial to mesenchyme transition (EMT) may provide a pathway for heterocellular GJIC. EMT has been shown to occur during the development of several organs/glands (Duband et al., 1995; Hay, 1995). EMT also occurs during tumorigenesis and may be a key element controlling the first steps of invasion and metastasis in epithelial derived tumors (Boyer and Thiery, 1993). EMT can be induced by: (1) growth factors, (2) extracellular matrices and expression of their cognate cellular receptors (integrins), and (3) loss of E-cadherin, a known tumor suppressor. Recent studies have demonstrated that established lines of normal mammary epithelial cells rapidly undergo EMT simply by treatment with TGF-β (Miettinen et al., 1994). Others have found extensive changes in the phenotype of low passage mammary cells by altering serum and growth factors (Ronnov-Jessen et al., 1995). During EMT, the transitional cell type will often express proteins associated with both epithelial and mesenchymal cells, including cell adhesion molecules. We have been unable to demonstrate the expression of any cell adhesion molecules other than E-cadherin in the FibE line. This intermediate cell type (originally isolated from mammary epithelial cells) maintains its ‘transitional’ characteristics under our defined culture conditions. Interestingly, EMT is most likely to occur when epithelial-to-stromal cell contact may also be the highest, during development and tumorigenesis. Our results support the hypothesis that intermediate cells present during EMT could facilitate epithelial to stromal cell GJIC.

This study substantiated the observation of others concerning the inability of true mammary epithelial cells to establish GJIC with fibroblasts in vitro. Intermediate cell types, however, form gap junctions with either fibroblasts or epithelial cells and can communicate with either cell type. Intermediate cells may actually facilitate GJIC between the two distinct cell types, epithelial cells and fibroblasts. Although cadherin expression has previously been shown to facilitate GJIC between homotypic cells, expression of the same cadherin is not sufficient for heterocellular GJIC between bovine mammary fibroblasts and epithelial cells. Thus, the mechanism of heterocellular gap junction formation in the mammary gland may require more than an adhesion event. We propose that GJIC is normally attributed to homocellular cells in the adult mammary gland, though, during development, pregnancy, involution and especially tumorigenesis, transitional cells may catalyze heterocellular GJIC. These results are particularly compelling in light of substantial alterations in stromal cell behavior during breast epithelial cell carcinogenesis (desmoplasia) that have been suggested to alter tumor cell steroidal responsiveness, metalloproteinase expression, cell proliferation and tumorigenic potential.

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