Epithelial mesenchymal transition during the neoplastic transformation of human breast epithelial cells by estrogen

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Abstract. Epithelial-mesenchymal transition (EMT) in epithelial cells has been indicated as an important component of neoplastic transformation although, the genetic mechanism involved in this process has not been defined. The aim of this study was to evaluate the expression of different genes related to EMT such as E-cadherin, TGFß1, TGFß2, H-RAS, TWIST1, SNAIL2, SMAD5, FN1, CEACAM1 and JAG1 using the *in vitro-in vivo* model of the estrogen induced cell transformation developed in our laboratory. The E2-transformed MCF-10F (E2 70) cells and the tumorigenic cell line C5-A8-T8 (C5-T8) exhibit progressive loss of ductulogenesis as demonstrated by growth in collagen matrix. MCF-10F cells form ductal structures while E2 70 cells form solid spherical masses that in histological sections exhibit a pattern of growth resembling ductal hyperplasia or carcinoma *in situ*. The tumorigenic cells C5-T8 did not form structures on collagen acquiring an invasive pattern with spindle like features. We have observed a reduction in E-cadherin expression in E2 70 cells and a complete loss in C5-T8 cells. TGFß1, TGFß2, CEACAM1 and JAG1 were down-regulated in E2 70 and C5-T8 cells. SMAD5 and H-RAS were up-regulated in the tumorigenic C5-T8 cells whereas FN1, Twist1 and Snail2 were up-regulated in C5-T8 and down-regulated in E2 70. We conclude that the loss of expression of TGFß1, TGFß2, CEACAM1 and JAG1 are related to ductulogenesis and branching and the overexpression of H-RAS with loss of E-cadherin expression and up-modulation of TWIST1, SNAIL2 and SMAD5 expressions are involved in the EMT modulation.

Introduction

To infiltrate host tissue, malignant epithelial cells have to break their cell-to-cell adhesion and acquire motile properties (1). Such epithelial cell phenotype is reported as epithelial-mesenchymal transition (EMT) and was described during some physiological processes which are involved in embryogenesis and tissue repair (2). Current interest in this process stems from its developmental importance and its involvement in several adults diseases including the processes of breast cancer progression. In addition, EMT has been reported during duct morphogenesis in mammary gland (3), although, the genetic mechanism involved in this process has not been defined. Two particular changes are necessary for epithelial cells to acquire mesenchymal properties. The first is the lack of transcription in epithelial proteins such as E-cadherin and cytokeratin (4,5). E-cadherin is the adhesive component of adherens junctions and its expression has been described as dysfunctional or notably absent in most of advanced, undifferentiated and aggressive breast and other epithelial carcinomas (6). The second is an increased expression of specific mesenchymal proteins such as fibronectin and vimentin (7,8). The expression of such proteins is related to an abrupt change in cytoskeletal organization and loss of apico-basal polarity (9).

Some pathways have been described as a regulatory mechanism of EMT and to be involved in cancer progression and metastasis (4,8,9). Genes that are important in embryonic development such as *Twist, Smad, Snail* are frequently found to be culprits in cancer (4). The increased production of transcription factors such as TWIST and Snail2 (Slug), have been commonly used as molecular markers for EMT (5). Conversely, genes discovered for their oncogenetic role such as H-Ras and TGFß are often found to be key players in embryogenesis (10). The aim of this study was to evaluate the role of different genes related to EMT using the *in vitro-in vivo* model of the estrogen induced cell transformation developed in our laboratory (11).

Materials and methods

Ductulogenic assay. MCF-10F, E2 70 and C5-T8 were suspended at a final concentration of $7.5x10^3$ cells/ml in 89.3% (Vitrogen100) collagen matrix (Collagen Co., Palo Alto, CA, USA) and plated into 24-well chambers pre-coated with 89.3% of collagen base. They were fed with fresh high calcium media. The cells were examined under an inverted microscope for seven days. At the end of observation period the structures were photographed, fixed in 70% alcohol solution and processed for histological examination.

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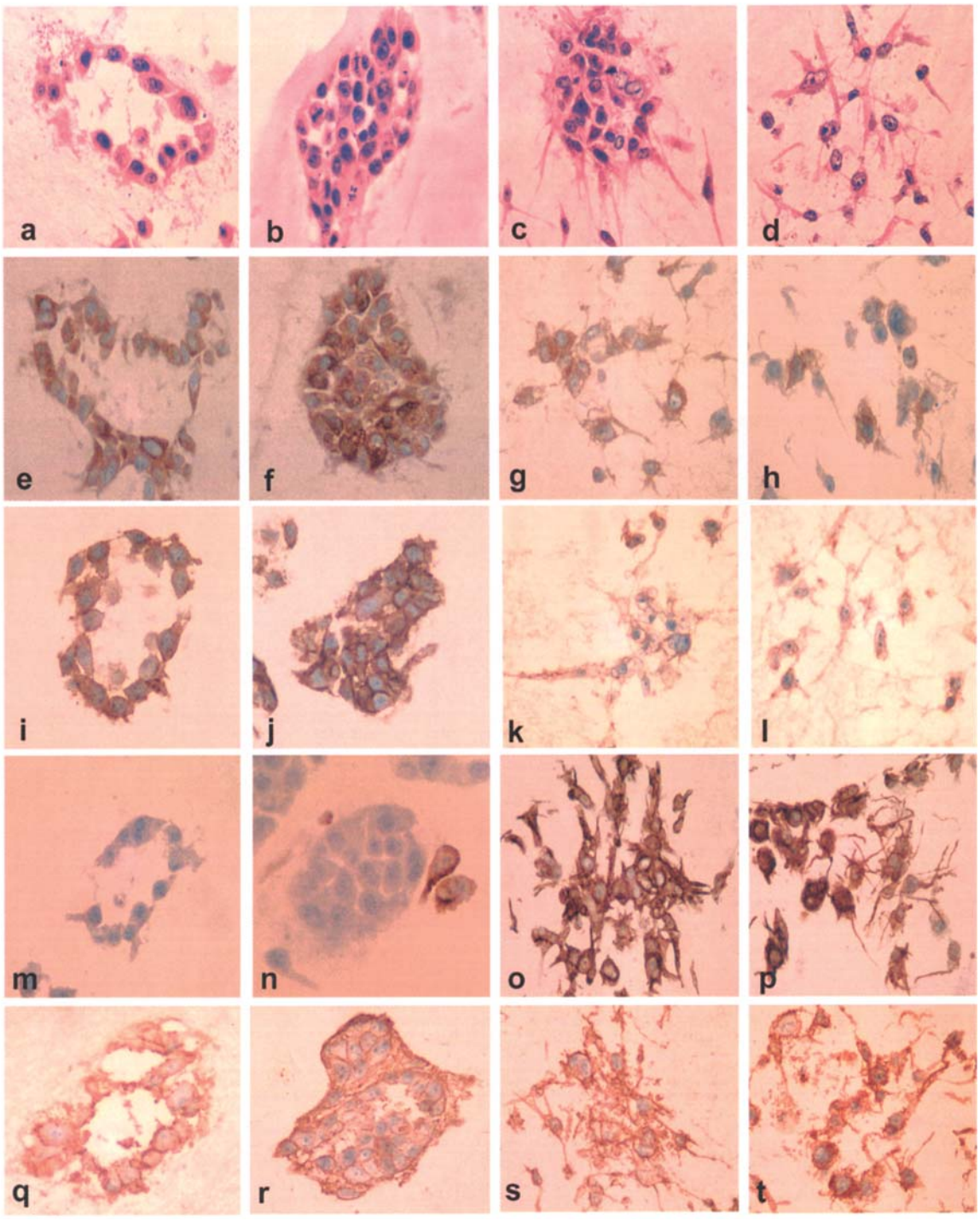

Figure 1. a, Histological section of MCF-10F cells growing in collagen matrix, H&E x40; b, E2 70 cells growing in collagen matrix, H&E x40; c and d, C5-T8 cells growing in collagen matrix, H&E x40; e, i, m and q, MCF-10F cells reacted with EMA, E-cadherin, vimentin, and fibronectin respectively (x40); f, j, n and r, E2 70 transformed cells reacted with EMA, E-cadherin, vimentin and fibronectin respectively (x40); g, h, k, l, o, p, and s, t, C5-T8 cells reacted with EMA, E-cadherin, vimentin and fibronectin respectively (x40).

Histological and immunohistochemical analyses. Tissues fixed in alcohol 70%, dehydrated and embedded in paraffin were cut at 5-μm thickness and stained with hematoxylin and eosin for histological analysis. For immunohistochemical analysis, tissue sections were mounted on aminoalkyl-silane-coated or positively charged slides, deparaffinized, rehydrated and incubated in 2% hydrogen peroxide at room temperature for 15 min for quenching endogenous peroxidase activity. The sections were sequentially incubated in two changes of Target Retrieval Solution at 98˚C for 5 min each. All tissue sections were incubated in diluted normal blocking serum for 20 min. Excess serum was blotted from the slides and sections were incubated with the following antibodies: HHF35 a mouse anti-human muscle actin primary antibody, epithelial membrane antigen (EMA) clone E29, AE1, anti-human low molecular weight cytokeratin (Biogenex, San Ramon, CA), E-Cadherin, (Becton Dickinson Biosciences), Vimentin monoclonal mouse anti-human

Table I. Immunohistochemical expression profile of human breast epithelial cells transformed with estradiol.

Antibody	MCF-10F cells	E2 70 cells	C5-T8 cells
EMA	++++	++++	++
HHF35	-	-	-
AE1 cytokeratin	++++	++	-
E-cadherin	++++	++	-
Vimentin	+	-	++++
Fibronectin	+++	++	++++

Negative (-), weak (+), moderate (++), marked (+++) and strong (++++).

antibody (Dako Cytomation Colorado Inc.), and Fibronectin P1H11 mouse monoclonal raised against a cell binding domain of fibronectin of human origin (Santa Cruz Biotechnology, Inc., CA). After incubation in a humidity chamber at 4˚C overnight, sections were washed in buffer and incubated with horse biotinylated secondary antibody (Vector Laboratories. Inc., Burlingame, CA, USA) at room temperature for 30 min followed by a 30-min incubation with Vectastain Elite avidin-biotin complex kit (Vector Laboratories), washed in PBS buffer, and incubated in peroxidase substrate solution containing hydrogen peroxide and 3, 3'-diaminobenzidine-HCl for 2 min. Sections incubated with no immune serum were used as negative controls. All sections were lightly counterstained with hematoxylin. Immunostaining was evaluated by examination of slides under a bright field microscope, and graded according to the intensity of the brown staining.

Real-time RT-PCR. Total RNA was isolated from growing cells at 70-80% confluence using TRIzol (InVitrogen) according to manufacturer's instructions. The RNA was treated with DNase I (Invitrogen) and cleaned using RNeasy kit (Qiagen). The concentration and quality of RNA were determined spectrophotometrically and by capillary gel eletrophoresis (Agilent 2100 Bioanalyser, Palo Alto, CA). Real-time reverse transcriptase PCR (Real-time RT-PCR) was used to quantify the expression of E-cadherin, TGFß1, TGFß2, H-RAS, TWIST1, SNAIL2, SMAD5, FN1, CEACAM1 and JAG1. The TaqMan One Step RT-PCR kit (Applied Biosystems) was used and the assays ran using Applied Biosystems 7900 HT instrument. The TATA box-binding protein (TBP) was used as endogenous RNA control and each sample was normalized on the basis of its TBP content. The Ct was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe pass a fixed threshold above baseline. The SDS 2.1 software based on the comparative Ct method was used for data analysis. The comparative method calculates the relative gene expression using the following equation: relative quantity = $2^{-\Delta\Delta Ct}$ (User Bulletin 2, Applied Biosystems). For each gene, the expression level was compared to expression in the parental cell line MCF-10F.

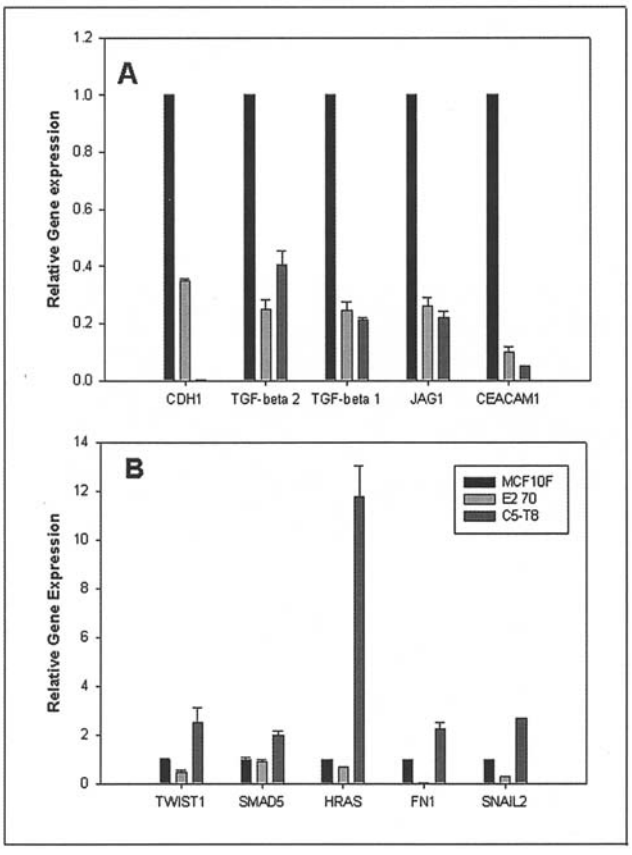

Figure 2. Comparative expression of genes related to EMT process during the neoplastic transformation of breast epithelial cells by estrogen.

Results

Ductulogenic assay. MCF-10F, E2 70 and C5-T8 cell lines presented with different phenotypes when growing in collagen matrix. MCF-10F exhibits a duct-like growth pattern (Fig. 1a) whereas E2 70 cell line forms spherical masses that in the histological sections resemble a ductal hyperplasia or carcinoma *in situ* (Fig. 1b). The cell line C5-T8 grows in an invasive spread pattern with no structure formation (Fig. 1c) or as single spindle cells resembling an invasive ductal carcinoma (Fig. 1d).

In order to further characterize the cell phenotype we studied the expression of muscle actin protein using HHF35 primary antibody and the expression of epithelial membrane antigen using EMA Mc-5 primary antibody (Table I). There was no HHF35 staining in MCF-10F, E2 70 and C5-T8 cells (Table I) indicating that they are not myoepithelial cells, otherwise, all cell lines were positive for EMA (Fig. 1e-h) revealing their breast epithelial nature. Keratin was significantly reduced from the MCF-10F to the C5-T8 cells (Table I). E-cadherin was strongly positive in MCF-10F cells and started decreasing the reactivity in the E2 70 cells (Fig. 1i and j), being almost negligible in C5-T8 cells (Fig. 1k and l). Analyzing vimentin expression, MCF-10F and E2 70 cell lines are negative (Fig. 1m and n), whereas the C5-T8 cells have a strong dark brown staining (Fig. 1o and p). The staining for fibronectin also shows an increase in the intensity for the C5-T8 (Table I).

Gene expression study. We have determined by RT-PCR the expression of E-cadherin, TGFß1, TGFß2, H-RAS, TWIST1, SNAIL2, SMAD5, FN1, CEACAM1 and JAG1 genes (Fig. 2A). We observed a reduction in E-cadherin expression in E2 70 cells and a complete loss in C5-A8-T8 cells. TGFß1, TGFß2, CEACAM1 and JAG1 were down-regulated in E2 70 and C5-A8-T8 cells. SMAD5 and H-RAS were up-regulated in the tumorigenic C5-A8-T8 cells whereas FN1, TWIST1 and SNAIL2 were up-regulated in C5-A8-T8 and down-regulated in E2 70 (Fig. 2B).

Discussion

Recent evidence regarding the role of cell plasticity in mammary gland development and breast cancer progression has been reported (2,3). Originally, differentiated epithelial cells grow linked together and move as epithelial sheets (12). Alternatively, as occurs during embryogenesis, epithelial cells can dissociate and migrate as individual cells. One property of such cells is the ability to detach from the epithelium and express locomotory ability. The manifestation of this property has been described as an intense change in cell differentiation. Epithelial cells lose their own epithelial characteristics and acquire a mesenchymal phenotype. This phenotype change is followed by the loss in apico-basal polarity and cytoskeleton reorganization. Migrating cells no longer express cytokeratin filaments and express vimentin filaments associated to a loss of cell-to-cell adhesion (13). This process is the base of the epithelial to mensenchymal transition (EMT) concept.

Analyzing the morphology and the immunohistochemical pattern in MCF-10F in collagen gel culture, we were able to demonstrate that during the neoplastic transformation the mammary epithelial cell is able to change its phenotype, acquiring a mesenchymal property infiltrating the surrounding extra-cellular matrix. Two important observations in our study support the idea that both the loss and the uncontrolled activation of EMT process could be involved in estrogen-induced carcinogenesis of the breast epithelia. Such phenotype changes are responsible for blocking the cell's ability to form duct-like structures in collagen gel culture and induce the cells to grow in a pattern mimicking an *in situ* ductal carcinoma in human breast. Analyzing the immunohistochemical pattern in such structures, we observed that the cells do not have a mesenchymal differentiation disabling them from invading the extra-cellular collagen matrix. However, to acquire an invasive phenotype (C5-T8), E2 transformed cells must be submitted to a selection process through the invasion Boyden chamber as described previously (11). E2 70 cell line grow forming spherical structures in collagen gel, however those cells that invade the membrane when plated inside the Boyden chamber are those that have a spread pattern of growth throughout the extra-cellular collagen matrix and the one that will form tumors in the heterologous hosts. The phenotypical changes could be due to particular modulation in genes involved in EMT control. E2 70 cells have reduced E-cadherin transcription, an epithelial specific protein, however, fibronectin 1, a mesenchymal specific gene associated to cytoskeleton reorganization and cell motility, are down-regulated. This genotype is consistent to an *in situ* neoplasia (2). The tumorigenic cell line C5-T8, shows a complete loss of E-cadherin transcription associated with an up-regulation in FN1 as is also demonstrated by more intense cytoplasmic staining in these cells. Such genotype is highly associated to mesenchymal differentiation that enables C5-T8 cells to invade the extra-cellular collagen matrix (2).

TGFß and H-RAS are described as EMT trigger processes (10,14,15). In our model we observed that TGBß1 and TGFß2 are down modulated in transformed and tumorigenic cell lines. On the other hand, the C5-T8 cell line has a significant increase in H-RAS transduction. Such an observation supports the hypothesis that the EMT process is triggered by the H-RAS pathway rather than the TGFß pathway at least in our model. The oncogene Ras activates the MAPK pathway that is essential for EMT and metastasis processes in an *in vivo* system (2). Genes related to EMT during embryogenesis, also associated to this change, are differently expressed among MCF-10F, E2 70 and C5-T8 cell lines.

The Smad group of intracellular proteins transduces signal by members of the TGFß family from the cell surface to the nucleus. Binding to receptors for the TGFß family members leads to the phosphorylation and activation of the receptor regulated Smads, which include Smads 1, 2, 3, 5, and 8 (16,17). The Smad-dependent signaling pathway appears to be necessary for TGFß-induced EMT and Smad5 is activated by members of the bone morphogenetic protein (BMP) branch of the TGFß superfamily (17-19). SMAD5 increased expression was observed in colorectal cancer by immunohistochemistry (20,21).

TWIST was recently reported as an E-cadherin protein repressor and is able to induce EMT (1) and, it has been found to be correlated with metastasis in various cancers including breast, prostate and hepatic cancers (22,23). Knocking-down Twist by RNAi prevents metastasis and overexpression of Twist in two human epithelial cell lines which causes both complete EMT and E-cadherin repression (1).

SNAIL2 (Slug) is a zinc finger transcriptional repressor closely related to the Snail family. It has been found in vertebrate and is involved in the control of gastrulation (24,25). SNAIL2 can down-regulate E-cadherin expression. Its expression is related to undifferentiated breast carcinomas and all ductal invasive carcinomas with lymph nodes positive to SNAIL2 expression (26).

TWIST1, SMAD5 and SNAIL2 are up-regulated in tumorigenic cells and are normal or down-regulated in the E2 70 cell line. We believe that such genes are involved in the process of carcinogenesis in our model and are associated to the induction in mesenchymal phenotype in tumorigenic cells.

We also studied the expression of two genes *JAG1* and *CEACAM1* that are related to the process of duct morphogenesis (19,27). Analyzing the phenotype in our model, there is a progressive loss in duct morphogenesis and we observed a down-regulation in both genes, in the E2 70 and in the C5-T8 cell lines. Supporting that such genes are involved in breast cancer progression and the loss of duct morphogenesis is an important step in breast carcinogenesis.

Altogether our data allow us to postulate that the loss of expression of TGFß1, TGFß2, CEACAM1 and JAG1 is

related to the loss of duct morphogenesis, and that the over-expression of H-RAS with loss of E-cadherin expression and up-modulation of FN1, TWIST1, SNAIL2 and SMAD5 expressions are involved in the EMT modulation.

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