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An *in vitro*–*in vivo* model of epithelial mesenchymal transition in triple negative breast cancer

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Abstract

The loss of epithelial expression markers by neoplastic breast cancer cells in the primary tumor is believed to play a pivotal role during breast cancer metastasis. This phenomenon is the hallmark of the epithelial mesenchymal transition (EMT) process. Gene expression microarrays were performed to investigate key functional elements on an *in vitro* metastasis model derived from human breast epithelial cells (MCF10F) treated with 17 beta estradiol. We identified groups of SLUG associated genes modulated during EMT.

Introduction

Metastatic progression of breast cancer is a complex and clinically daunting process lacking fully identified mechanisms [1]. However, it is known that cumulative and sustained exposure to estrogens increases the risk of developing breast cancer [2–4]. Comparing five major breast cancer subtypes –basal-like, (ERBB2) – over expressing, normal breast tissue-like, subtype of luminal-like A and B [5–7] – basal-like breast cancer progresses toward malignancy with poor prognosis as measured in time to development of distal metastasis since these cells lose polarity and cell-to-cell junctions of epithelial differentiation and they acquire characteristics of mesenchymal cells lacking stable intercellular junctions [8–10]. In our laboratory, an *in vitro* basal-like tumor model of carcinogenesis was derived from immortalized ER α -negative human normal-like breast epithelial cell line MCF-10F [11,12] and gene expression analysis was performed on this model to examine the initiation phase of the cell transformation.

Experimental model of estrogen induced transformation of MCF-10F cells

To mimic the process involved in EMT, we used human breast epithelial cells (HBEC) derived from an *in vitro* model of carcinogenesis [13–15]. The model uses an immortalized ER α -negative human breast epithelial cell line, MCF-10F. As previously described, MCF-10F cells were cultured and treated with 70 nmol/L 17 β -estradiol. Transformed cells were collected 24 h after the last treatment and maintained for ten additional passages. These transformed ‘trMCF’ cells, progressively express phenotypes of *in vitro* cell transformation, colony formation in agar methocel, decreased ductulogenesis in collagen assay, and increased invasiveness in a Matrigel invasion system. For the following step, ‘trMCF’ cells were then seeded onto Matrigel invasion chambers, and cells that had degraded the reconstituted basal membrane and invaded were collected and identified as ‘bcMCF’ cells. The tumorigenic ability of ‘bcMCF’ cells was tested by injecting them into the mammary fat pad of 45-day-old female SCID mice. From the tumors formed by the ‘bcMCF’ cells, the

fourth cancer cell line ‘caMCF’ was isolated. The model is represented in Fig. 1. bcMCF is highly invasive and when injected in the tail of SCID mice induces metastatic lesions in the lung [14].

Gene expression changes during EMT

On the basis of the described model, we evaluated the changes in gene expression in the first step of EMT. Using Affymetrix chips (Human Genome U133 Plus 2.0 Array), we identified 2579 differentially expressed genes in trMCF cells when compared to MCF-10F cells (Fig. 2). Gene Ontology (GO) enrichment analyses were performed in the genes up and down-modulated in the trMCF cells. GO terms enriched by the downregulated genes in trMCF were mainly focused on extracellular matrix (ECM), adhesion, and immune response. The biological processes over-represented by the upregulated genes mainly belong to cell proliferation and RNA metabolism. By using the Ingenuity Pathway Analysis (IPA) software [16], we identified the most statistically significant canonical pathways over-represented by the genes modulated in the trMCF cells. Integrin signaling pathway showed to be an important pathway in which the malignancy starts to take place in the cell transformation of MCF-10F cells.

On the basis of the data, we have established a hypothetical pathway summarized in Fig. 3. Considering MCF10F is devoid of ER α and only expresses very small amount of ER β , the 17 β -estradiol could directly cause genomic aberrations on SLUG (phosphorylation, methylation, among others) without the mediation of ER α . SLUG was downregulated in trMCF, as a zinc finger transcriptional repressor [17,18], the downregulation of SLUG tends to disrupt the integrin signaling pathway and therefore alter the structure of ECM by suppressing the components like fibronectin1 and E-cadherin. This disrupted cross-talk between the epithelium and stroma could in turn initiate EMT [19–24]. Due to less integrin signal input derived from destructive ECM, the expression of SLUG is kept in suppressed status through the regulation of integrin-linked kinase (ILK) [23]. Thus the whole network forms a closed system under the control of SLUG expression. As the downstream of integrin pathways, NF κ B is suggested to be down regulated. As the response genes following NF κ B signaling, components related to IL8, CXC chemokine family and four TNF members all demonstrate suppressed status [25–28].

Mechanisms

Estrogen is the dominant trigger for triple negative breast cancer initiation

Mainly via two receptors, estrogen receptors α and β (ER α and ER β), estrogens exert their functions which contribute to ductal elongation and branching [29,30]. The most widely acknowledged mechanism of estrogen induced breast cancer is through binding of the hormone to nuclear receptors [31], initiating potent mitogenic effects; also, estrogens are metabolized to quinones, and these in turn act as direct mutagenic agents [32]. Although ER α is well defined as prognostic marker and therapeutic target with many well defined mechanisms [33], the genesis mechanism of malignant triple-negative breast cancer remains to be elucidated due to their weak, or lacking, expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [34].

The malignant transformation established on ER α negative human immortalized breast epithelial cell line supports the non-ER α -mediated mechanism [35]. In response to 17 β -estradiol, or the metabolites 2- and 4-hydroxyestradiol, genomic abnormalities were detected in transformed cell lines on p53 mutation, loss of heterozygosity in chromosomes 11 and 13 [13]. In line with the *in vitro* system, breast cancer can also be introduced on ER α knockout mice expressing oncogene Wnt-1 or ovariectomized rats treated with estradiol [36]. Once the dominant mammary carcinogenesis directed by ER α is absent, multiple elements can

take the position, or collaborate with each other, to conduct breast cancer initiation, since the binding sites for estrogen are not limited to ER α and ER β . The potential candidates vary from the plasma membrane-anchored ER α spliced variants to G protein-coupled receptors, like GPR30 [37].

ECM alteration is a dynamic niche accompanied with metastasis progression

The change of cell motility and proliferation metabolism are always accompanied by ECM reorganization, and the remodeling process is tightly regulated by the balance between matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) [38]. Corresponding MMPs are capable of degrading specific ECM components and their potent activities can induce extravasations of transformed cell. From ECM constructing perspective, a certain expression pattern of ECM is able to represent the invasive status for transformed cells which is a promising direction for predicting tumor development. For example, the relative density of ECM holding the invasive cells is usually diluted due to the down regulation of ECM components, like fibronectin1, which will contribute to the raised cell motility through cell skeleton alterations brought by decreased expression of vimentin. Since the ECM-cell attachment and cell-cell attachment are both required by mesenchymal cell proliferation [39], the new synthesized cell-cell attachment components, such as keratin and E-cadherin, are adjusted to balance the diminished the ECM-cell attachment. The distinct polarity is another important characteristic of epithelial cells, which is the missing feature in cancer [39]. ECM is believed to be in charge of the establishment and maintenance of tissue polarity and structure, for example, integrin β 1 maintains tissue polarity in mammary gland.

ECM remodeling is also involved in tumor associated inflammation with the initial function to suppress tumor growth [40]. Some ECM components may act as chemoattractants for immune cell recruitment, such as acetylated tripeptide Pro-Gly-Pro, the proteolysis product from collagen I degradation by MMP8 or 9, can functionally mimic CXCL8 as the chemoattractant for neutrophils in a lung inflammation model. Furthermore, type I collagen can block the polarization of macrophage and inhibit its activation which causes fewer cancer cells to be removed by the immune system [41].

Appropriate contacts with ECM are required by cancer stem cells for expansion and differentiation. Theoretically, the formation of lineage-specific ECM can be derived by the deregulation of deregulated ECM dynamics. The potent signaling receptors from altered ECM may allow cancer stem cells to anchor to a specific environment for polarity and maintain asymmetric cell division properties. The destruction of ECM would cause the balance disruption of expansion and differentiation on stem cells and lead to cancer stem cell release as potential metastasis mechanism.

Conclusion

This study integrates functional genomic data analyses to elucidate the progressive molecular events in the E2-mediated malignant transformation of ER (–) HBECs. Genomic aberrations progressively accumulated as the cells expressed more aggressive phenotypes (*i.e.*, in the tumorigenic bcMCF and caMCF) in comparison with the non-tumorigenic trMCF cells. These findings revealed an intrinsic and independent network within E2-induced gene expression alterations, and tumorigenesis in ER α (–) HBECs.

The hypothesis originates from the SLUG transcriptional change under the effects of E2 treatment, since SLUG can be regulated by estrogen through many pathways, such as MTA gene and E-box [42,43]. SLUG and SNAI1 belong to the Snail family of proteins; both contain an NH₂-terminal repression domain and a COOH-terminal zinc-finger DNA-binding

domain [42]. The downregulation of SLUG exerts its role of transcriptional regulator through binding E-box and alters the expression of many ECM components such as fibronectin1, E-cadherin, keratins and vimentin. In our model, the ECM component is represented by the expression variance of fibronectin1 which is involved in cell adhesion, growth, migration, differentiation and wound healing processes. The altered mesenchymal gene expression is believed to be the hallmark of EMT which involves differentiation of polarized epithelial cells to a migratory fibroblastoid phenotype, a phenomenon that is increasingly considered to be an important event during cancer progression and metastasis.

Of interest, SLUG was downregulated in trMCF and the downregulation of SLUG at the transformation phase was first examined by us and this sharp turn in expression may indicate a trigger mechanism of EMT in E2 induced basal-like breast cancer progression. The expression of SLUG is altered at a relative early phase during tumorigenesis, thus the mechanism can still exert its function through other oncogenes, like p53, which is usually malfunctioned at later phase. The downstream components of SLUG are mainly ECM molecules, and many proved metastasis markers, such as vimentin and E-cadherin, are linked together by this hypothesis. Additionally, the ECM alteration triggered by SLUG can connect with the ERK system and control TGF β expression through integrin signaling, so that, the connections of both cell proliferation and invasiveness events are established to the hypothesis [44,45].

Integrins function as heterodimeric receptors for extracellular matrix proteins, mediating cell anchorage [46]. Due to the ECM destruction, the integrin signaling pathway was the most significantly altered pathway in the progression of the neoplastic transformation. In Table 1, the integrin compositions for fibronectin, collagen and laminin are altered to incorporate with the change of ECM components, thus, the signal starting with integrin will be decreased. The integrin signaling pathway was enriched with dysregulated genes in trMCF, indicating that this pathway was affected in early stages of cell transformation. In addition, GO analysis revealed enrichment of upregulated genes in the cell proliferation process in tumorigenic cells which can support the dysfunctional integrin signaling in same direction. Our results support the concept that E2-induced breast cancer is a polygenic disease with a large range of genomic instabilities. E2 and/or its metabolites can directly cause genomic aberrations and transcriptome alterations without the mediation of ER α . As a consequence, changes in gene expression result in disrupted integrin signaling and epithelial to mesenchymal transition.

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Figure 1.
In vitro cell model of basal breast cancer. The figure depicts the different stages of cancer progression.

Figure 2.
Heatmap of differentially expressed genes in E2 cells compared to the parental cell line MCF10F. Red squares represent higher expression values and green squares represent lower expression values.

Figure 3. Proposed pathway of progressive molecular events in the 17 β -estrodiole mediated malignant transformation in the MCF10F model of cancer progression.

Table 1

Dysregulated genes involved in integrin signaling pathway

Symbol	Gene name	Log ₂ fold change trMCF vs MCF10F
CADM3	Cell adhesion molecule 3	-2.46
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	7.55
CDH16	Cadherin 16, KSP-cadherin	1.59
CDH3	Cadherin 3, type 1, P-cadherin (placental)	3.98
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-2.59
CXCL2	Chemokine (C-X-C motif) ligand 2	-2.19
CXCL3	Chemokine (C-X-C motif) ligand 3	-2.92
CXCL5	Chemokine (C-X-C motif) ligand 5	-3.3
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte Chemotactic protein 2)	-5.48
CXCR7	Chemokine (C-X-C motif) receptor 7	-5.41
DSP	Desmoplakin	2.22
FN1	Fibronectin 1	-9.28
IL8	Interleukin 8	-4.33
ITGA6	Integrin, alpha 6	-1.1
ITGA6	Integrin, alpha 6	-1.1
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	-1.24
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	-1.77
ITGB6	Integrin, beta 6	6.73
ITGB8	Integrin, beta 8	1.24
KRT15	Keratin 15	8.18
KRT16	Keratin 16	3.75
KRT6A	Keratin 6A	3.38
KRT6B	Keratin 6B	6.79
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	-1.44
SNAI2(SLUG)	Snail homolog 2 (Drosophila)	-2.61
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	-1.47
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	-2.92
TNFRSF10D	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	-1.74
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	-1.52
VIM	Vimentin	-3.11