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Differential Expression of Key Signaling Proteins in MCF10 Cell Lines, a Human Breast Cancer Progression Model

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Abstract

Breast cancer is a heterogeneous disease that develops through a multistep process whose molecular basis remains poorly understood. The molecular mechanisms of breast cancer progression have been extensively studied using the MCF10 model. We summarized recent results on differential expression of proteins in the MCF10 cell series – MCF10A, MCF10AT1, MCF10DCIS.com and MCF10CA1a – and compared the ability of the latter 3 lines to form tumors in immunodeficient mice. In addition, we also investigated expression of several key signaling proteins in the MCF10 cell series corresponding to different stages of breast cancer progression. MCF10DCIS.com and MCF10CA1a cells were highly tumorigenic; MCF10CA1a cells showed more aggressive tumor growth than MCF10DCIS.com cells. HRAS-driven cancer initiation stage was accompanied by the increased expression of c-Myc, cyclin D1 and IGF-IR. Tumorigenic cell lines expressed higher levels of pErk, pAkt, Stat3 and Pak4 compared to nontumorigenic cells. The expression of CD44v, CD44v3, CD44v6, ERBB2, Cox2 and Smad4 correlated with the increased tumorigenicity of the MCF10 cell lines. The differences in expression of signaling proteins involved in breast cancer progression may provide new insight into the mechanisms of tumorigenesis and useful information for development of targeted therapeutics.

Keywords

Breast cancer progression; MCF10A; MCF10AT1; MCF10DCIS.com; MCF10CA1a; CD44

Introduction

Human breast cancer is a heterogeneous disease which evolves through a multistep process of accumulating genetic changes such as gene mutations, rearrangements and copy number amplifications (1, 2), loss of heterozygosity, and epigenetic alterations (3). Gene expression profiling studies have identified five distinct breast cancer subsets – luminal A, luminal B, ERBB2, basal-like, and normal breast-like – with intrinsically different gene signatures (4,

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Conflicts of Interest

No potential conflicts of interest to disclose.

5). These findings have been used in the clinic to stratify patients to different, subtype-specific treatments (6–8).

Breast cancer originates as benign hyperplasia of mammary duct epithelial cells, and progresses sequentially to atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC), which eventually metastasizes to distant organs (9, 10). Various stages commonly coexist in a single tumor suggesting that progression of breast cancer is not a linear process. The biological diversity emerges at the early DCIS stage (11); the morphological and molecular aberrations remain remarkably similar from DCIS to IDC indicating that progression to invasive breast cancer is associated with quantitative rather than qualitative molecular alterations (9, 12, 13). Another level of complexity stems from the differences in genetic background, environmental exposures, and treatment choices among breast cancer patients. Overall, all these factors have contributed to the relatively poor understanding of the molecular basis of breast cancer progression (14, 15).

The MCF10 cell series is a unique model of breast cancer progression. In this review, we summarize and discuss the key changes that occur in signaling pathways during MCF10 cancer progression using microarray gene profiling and proteomic data.

MCF10 Cell Lines as a Unique Model of Breast Cancer Progression

The MCF10 model is a series of cell lines that originated from the human breast epithelial cells MCF10A (16). These cell lines share the same genetic background and offer a unique model to study breast cancer progression in a cell culture system. The MCF10A cells are spontaneously immortalized, non-malignant cells obtained from a patient with fibrocystic breast disease (16). The MCF10A cell line is considered normal because it does not show any characteristics of invasiveness and does not form tumors when transplanted into immunodeficient mice.

MCF10AT1 is a pre-malignant cell line produced by transfection of MCF10A with constitutively active *HRAS* (16, 17); it forms simple ducts and lesions resembling human ADH and DCIS when transplanted into immunodeficient mice (18). Approximately 25% of the MCF10AT1 cells transplanted into mice eventually produce IDC which indicates tumorigenic potential of MCF10AT1 with slow progression (16).

MCF10DCIS.com is a cell line cloned from cell culture of a MCF10AT1 xenograft lesion. The MCF10DCIS.com cells reproducibly form DCIS-like comedo lesions that spontaneously progress to IDC as xenografts in immunodeficient mice (19).

MCF10CA1a is the most malignant and aggressive cell line from the MCF10A series; it was derived from the MCF10AT1 cells by multiple passages through immunodeficient mice. The MCF10CA1a cells rapidly generate large tumors without evidence of a precursor stage. In addition, intravenously injected MCF10CA1a cells readily produce tumors in the lungs of immunodeficient mice, indicating metastatic potential of the MCF10CA1a cells (20).

The MCF10 cell lines offer the opportunity to study genetic and molecular events during cancer progression from normal mammary epithelium to metastatic IDC (9, 10, 14, 15, 21, 22). Results from gene profiling studies of the MCF10 cell series (10, 14, 23, 24) are summarized in Table 1. Worsham and colleagues identified chromosomal aberrations associated with immortalization, transformation, and progression of the MCF10 cell series by using high-resolution mapping with selected gene probes (23). Using comparative microarray genomic hybridization and cDNA microarray, Rhee and Marella investigated gene expression changes associated with cancer progression in the MCF10 cell series (14,

24). Kadota and colleagues reported sequential mutation events correlated to the each MCF10 cancer progression stage in a genome-wide study combining a high density SNP analysis and a mutation analysis by sequencing (10).

Results from another group of studies investigating differential protein expression in the MCF10 cell series are also summarized in Table 1 (15, 21, 25–27). Kim and colleagues showed proteomic and phosphoproteomic alterations of PI3K, Akt, and mTOR signaling pathways – a direct target of HRAS transformation – and FOXO transcription factors in the MCF10 cell series (21). Choong and colleagues used proteome-wide profiling of the MCF10 cell series and identified novel proteins, AK1, ATOX1 and HIST1H2BM, associated with breast cancer progression (15). The differential expression of secreted proteins (25), tyrosine kinase substrates (26) or membrane glycoproteins (27) was also studied to identify biomarkers associated with breast cancer progression in the MCF10 cell series.

Tumorigenicity of MCF10 Cell Lines *in vivo*

Tumorigenicity and tumor growth rate of the MCF10AT1, MCF10DCIS.com and MCF10CA1a cells were examined *in vivo*. One million cells from each cell line except the MCF10A were injected into the mammary fat pad area of immunodeficient mice and the tumor volumes were measured. The MCF10AT1 cells did not form palpable tumors up to 60 days after the injection (Figure. 1). Compared to previous studies that reported sporadic incidences of IDC (16), we used fewer cells (10%) and a shorter experimental period which likely decreased the probability of producing IDC. In contrast, the MCF10DCIS.com cells formed tumors in all injected mice (n=9). The average tumor volume reached 1.03 ± 0.54 cm³ at 45 days after the injection (Figure 1). The MCF10CA1a cell also formed tumors in all mice (n=10), and the average tumor volume reached 1.79 ± 0.92 cm³ at 21 days after the injection (Fig. 1) indicating a faster growth of the MCF10CA1a tumors compared to that of the MCF10DCIS.com tumors. These data confirm the different tumorigenic potential of MCF10 cell lines *in vivo*.

Key Factors in the *HRAS*-driven Initiation Stage of Breast Cancer

Transformation of MCF10A cells with *HRAS* produced the MCF10AT1 cell line; the other two cell lines–MCF10DCIS.com and MCF10CA1a – were derived from the cell lines compared to MCF10A cells include c-Myc, cyclin D1 and insulin-like growth factor I receptor (IGF-IR) (Fig. 2A).

c-Myc, a transcription factor and key regulator of cell proliferation known to contribute to breast cancer development and progression, has been found overexpressed in 45% of breast tumors (28). Ras enhances the level of c-Myc by stabilizing the c-Myc protein (29). Furthermore, a study which crossed MMTV/*v* HA-ras and MMTV/*c-myc* transgenic mouse strains demonstrated a synergistic action of these two oncoproteins in accelerating mammary tumor formation (30). Amplification of c-Myc in the MCF10A cell line has been recently reported (10, 23), suggesting a collaborative nature of *HRAS* and c-Myc aberrant activity to initiate breast cancer.

Cyclin D1 is another key regulator of cell cycle and one of the most frequently overexpressed oncoproteins in breast cancer (31). The expression of cyclin D1 is necessary for the transforming activity of *HRAS* (32, 33). Moreover, cyclin D1-null mice showed remarkable resistance to mammary tumorigenesis driven by the *NEU* or *RAS* oncogene (34). IGF-IR has critical roles in breast cancer growth, survival and transformation (35). A systematic review of results from clinical studies revealed an association between high concentrations of circulating IGF-I and an increased risk of breast cancer in pre-menopausal

women (36). Our data suggest that both cyclin D1 and IGF-IR contribute to HRAS-driven cancer initiation stage.

Key signaling proteins in malignant transformation of the MCF10 model by spontaneous mutagenesis

pErk, pAkt, signal transducer and activator of transcription 3 (Stat3), and Pak4 were highly expressed only in cell lines that form tumors quickly in immunodeficient mice—MCF10DCIS.com and MCF10CA1a. Importantly, their protein levels were markedly increased in the more aggressive MCF10DCIS.com and MCF10CA1a cells when compared with the MCF10AT1 cells (Fig. 2B).

Erk and Akt are central protein kinases that mediate cellular responses to a diverse range of extracellular stimuli, including growth factors and cytokines, to regulate cell cycle progression and cell motility (37). Although both the Erk pathway and the PI3-kinase activity can be stimulated by transfection of activated Ras (21), the high level of activated forms of Erk and Akt – pErk and pAkt – is found only in MCF10DCIS.com and MCF10CA1a cell lines indicating that overactivation of Erk and Akt might be critical for developing malignant breast cancer. Moreover, the most common activating PIK3CA mutation in human cancers (H1047R) (38, 39) has been detected in the MCF10CA1a cell line, but not in MCF10A and MCF10AT1 cells (10, 21), suggesting the PIK3CA activating mutation as a critical genetic alteration of malignant phenotype in both human breast cancer and the MCF10 cell model.

Activated Stat3 is found in approximately 70% of breast tumors and is often associated with invasive and metastatic cancers (40). Moreover, recent studies demonstrated that Stat3 has a crucial role in inducing and maintaining pro-carcinogenic inflammatory microenvironment during cancer progression (41).

Pak4, a serine/threonine kinase, has been associated with a signaling pathway leading to malignant transformation and found to be highly expressed in human breast cancer (42–44). A recent study demonstrated that overexpression of Pak4 in normal mammary epithelial cells disrupted the cell polarity and led to the formation of mammary tumors in immunodeficient mice (43).

Key proteins contributing to breast cancer progression in the MCF10 model

CD44v, CD44v3, CD44v6, ERBB2, Cox2 and Smad4 showed a gradual increase in protein expression from MCF10A to MCF10CA1a cells (Figure 2C). We hypothesized that this group of proteins could be associated with breast cancer progression since their increased expression corresponds to increased cellular malignancy in the MCF10 model.

CD44, encoded by a single gene, has multiple isoforms produced by alternative splicing of 10 variable exons (45). CD44 is a cell-surface glycoprotein involved in cell-cell and cell-extracellular matrix interactions, as well as in migration and invasion of cancer cells (46). CD44 also functions as a receptor for hyaluronan and other extracellular ligands such as osteopontin, collagens, and matrix metalloproteinases (MMPs) to mediate responses from the microenvironment, which lead to cancer cell survival and invasion (46). Several studies have examined the association of CD44s or CD44v expression with overall survival and metastasis in breast cancer but produced conflicting results (45, 47–49). Interestingly, CD44 has been used as a key cancer stem cell surface marker in various malignancies including breast cancer (46). Recent studies demonstrated that activation of CD44 by high molecular weight hyaluronan stabilizes multidrug-resistant (MDR) proteins in the cell membrane

suggesting a role of CD44 in drug resistance (50). CD44 also has a role as a co-receptor for multiple receptor tyrosine kinases such as ERBB2, IGF-IR, epidermal growth factor receptor (EGFR) and c-Met in cancer cells (51–53). Because of the wide range of functions linked to cancer progression and cancer stem cells, CD44 has become an attractive therapeutic target. Preclinical studies with CD44 siRNA, CD44-targeting antibodies, transcriptional inhibitors, and competitive antagonists have shown inhibition of cancer cell growth, tumorigenesis, and metastasis (54–58).

Our results indicate that decreased expression of CD44s (the standard 85-kDa isoform) and increased expression of CD44v (the variants 100 to 250-kDa isoform) correlate with the increasing malignant potential of the MCF10 cell lines (Fig. 2C). A Western blot analysis with antibodies which specifically recognize CD44v3 and CD44v6 confirmed the increased expression of both of these isoforms in malignant MCF10 cells (Fig. 2C). In addition, overexpression of CD44s in the MCF10DCIS.com cells by transfection with a CD44s expression vector did not change the protein level of pErk and pAkt (data not shown). Our findings suggest that CD44v, but not CD44s, may be associated with aggressive and invasive breast cancer.

High expression of CD44v in malignant MCF10DCIS.com and MCF10CA1a cell lines can enhance the activities of multiple transmembrane receptor kinases (50); Fig. 3 summarizes membrane receptors known to interact with CD44 and their downstream signals. The CD44-hyaluronan interaction activates the ERBB signaling, induces the transcription of COX-2 (59), and also activates IGF-IR (52). CD44, particularly the CD44v6 isoform, acts as a co-receptor for Met through HGF binding, which in turn activates Stat3 and leads to tumor progression and invasion (60). The CD44-phosphorylated ERM (ezrin-radixin-moesin) proteins initiate the activation of TGF- β RII and downstream Smad signaling (61) (Fig. 3).

ERBB2 is overexpressed in approximately 25% of invasive breast cancer and is strongly associated with poor patient survival (62). A recent study demonstrated that overexpression of ERBB2 in the MCF10A cell line induced epithelial-mesenchymal transition and cell invasion (63). Two gene copies of ERBB2 are present in the MCF10AT1, MCF10DCIS.com and MCF10CA1 cell lines (23). Our results show gradually increased expression of ERBB2 proteins from MCF10AT1 to MCF10CA1a cells (Fig. 2C), suggesting a critical role of ERBB2 in breast cancer progression and invasion. COX-2 is one of the downstream targets of ERBB2 signaling pathway; a strong correlation between COX-2 and ERBB2 expression has been revealed in a large clinical investigation (64, 65). Moreover, up-regulated COX-2 expression has been associated with aggressive DCIS phenotype in both ER-positive and ER-negative breast cancers (66, 67). Our results also show a corresponding pattern between COX-2 and ERBB2 protein expression and its correlation to malignant potential in the MCF10 cell model (Fig. 2C).

Breast cancer invasion and metastasis

Metastasized tumor growth at distant sites is the main cause of death from breast cancer (68). Approximately 40% of early-stage breast cancer patients relapse and ultimately die of metastatic cancer, but accurate prediction of the risk of metastasis is still not possible (68). Comprehensive molecular profiling of the transition from DCIS and IDC to metastatic cancer has not yet identified tumor stage-specific signatures (22). Malignant precursor cells with metastatic capacity may already develop at early stages of tumorigenesis (69, 70). In addition, stromal cells in the environment surrounding the primary tumor are involved in facilitating metastasis (71). Therefore, both tumor microenvironment and epithelial cells have to be considered in tumor invasion and metastasis. Among the panel of MCF10 cell lines, the MCF10DCIS.com cells are particularly interesting because they can form

DCIS.com-like lesions which spontaneously progress into IDC in immunodeficient mice (72). Tumors from the MCF10DCIS.com xenograft showed increased expression of SDF-1 in stromal cells, which is known to be highly induced by tumor-associated fibroblasts, and increased expression of CXCR4, the receptor of SDF-1, in epithelial cancer cells during the DCIS to IDC transition (72). Although the MCF10DCIS.com xenograft model mimicked some aspects of the dynamic interaction between epithelial cancer cells and stromal cells, the utility of such model might be limited because of human-mouse differences in the epithelial-stromal interaction. In the present study, we did not attempt to identify molecules associated with cancer cell invasion and metastasis because the influence of microenvironment cannot be fully reproduced in the cell culture system we used for our study.

Conclusions

Results of this study have identified differences in the expression level of several key signaling proteins among 4 cell lines of the MCF10 series, a model representing different stages of breast cancer. By linking the observed changes to capability of the cell lines to form xenograft tumors in immunodeficient mice – from immortalized but non-malignant to highly malignant and invasive – the analyzed proteins were grouped based on their potential roles in tumor development. We concluded that c-Myc, cyclin D1, and IGF-IR may have a role during the initiation stage of cancer development since their increased levels were found in all *HRAS*-transformed cells including those that did not form tumors in our experimental system. In contrast, high expression of pErk, pAkt, Stat3 and Pak4 was observed only in cell lines that form tumors in immunodeficient mice which suggests that these proteins are activated later and may be important for the maintenance of malignancy. CD44v, CD44v3, CD44v6, ERBB2, Cox2 and Smad4, which showed protein expression gradually increasing from non-malignant to highly malignant and invasive cells, may represent a group associated with breast cancer progression. CD44 is a particularly interesting protein whose multiple isoforms are likely involved in various stages of cancer development; our results showing an association between the increasing expression of CD44v and increasing malignant potential of the MCF10 cell lines suggest a benefit of high levels of CD44v for the cancer cell malignant progression possibly by activating multiple signaling pathways through receptor kinases.

Mapping of the complex network of molecular interactions leading to the selection of increasingly more aggressive cancer cells during progression of breast cancer requires experimental models that can be relatively easily studied, such as the MCF10. Although development of malignancy is a continuous process of cellular selection driven by an increasing capacity to proliferate and manage resources, and a decreasing capacity to die and interact with the environment, it may be mediated by a relatively small number of factors. Identification of these critical elements will ultimately lead to the design of more efficient therapeutics and better prognosis for cancer patients.

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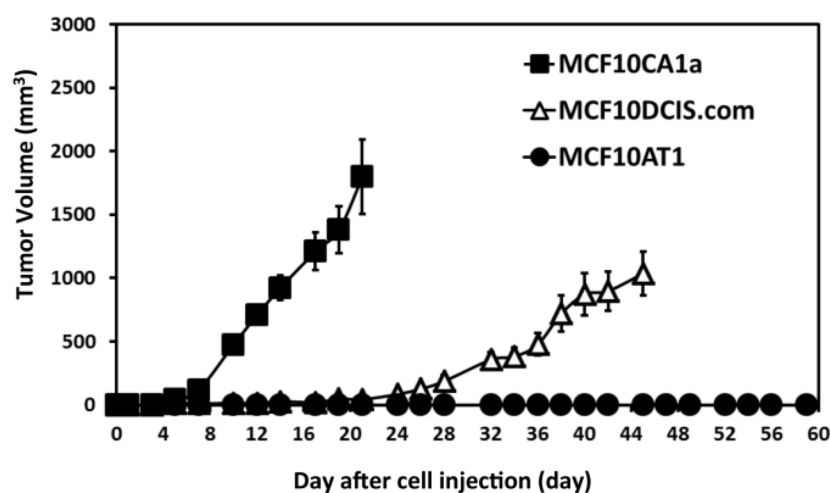


Figure 1. Tumor growth of MCF10 cell lines. MCF10AT1, MCF10DCIS.com and MCF10CA1a cell lines (1×10^6 cells per animal) were injected into mammary fat pad area of immunodeficient nu/nu mice as described previously (58)

Tumor volumes were measured to investigate the tumor growth rate for each cell line.

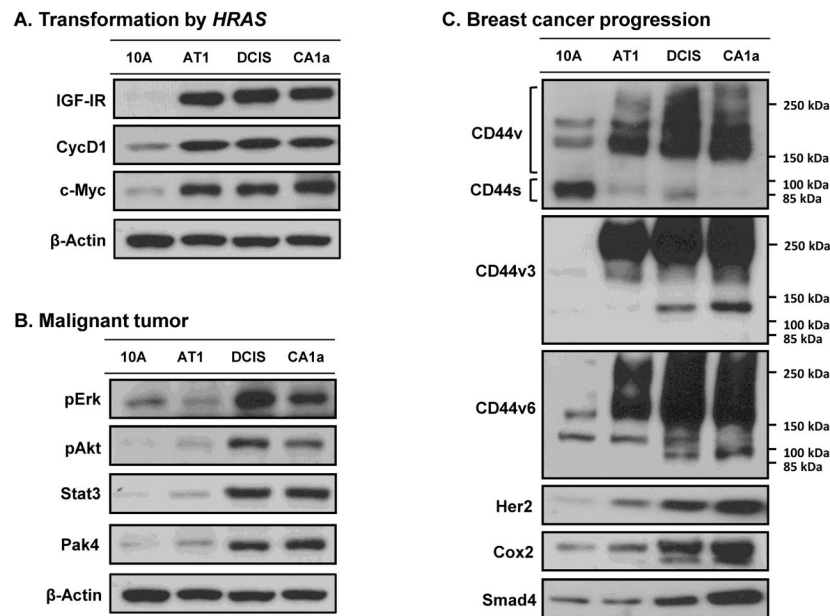


Figure 2. Comparison of the expression level for key signaling molecules among MCF10A, MCF10AT1, MCF10DCIS.com and MCF10CA1a cell lines

The four cell lines were incubated in the culture medium at 37°C, 5% CO₂ for 2 days (73). The expression level of key signaling proteins was analyzed by western blot as described previously (74). A. c-Myc, CycD1 and IGF-IR, whose protein expression levels are up-regulated in MCF10AT1, MCF10DCIS.com and MCF10CA1a cell lines, are categorized as a HRAS transformation-induced molecular event. B. pERK, pAKT, Stat3, Pak4, whose expression levels are up-regulated in MCF10DCIS.com and MCF10CA1a cell lines, are categorized as contributors of tumor malignancy. C. CD44v, CD44v3, CD44v6, Her2, COX2 and Smad4, whose expression level is gradually up-regulated from MCF10A to MCF10CA1a cell lines, are categorized as markers of breast cancer progression.

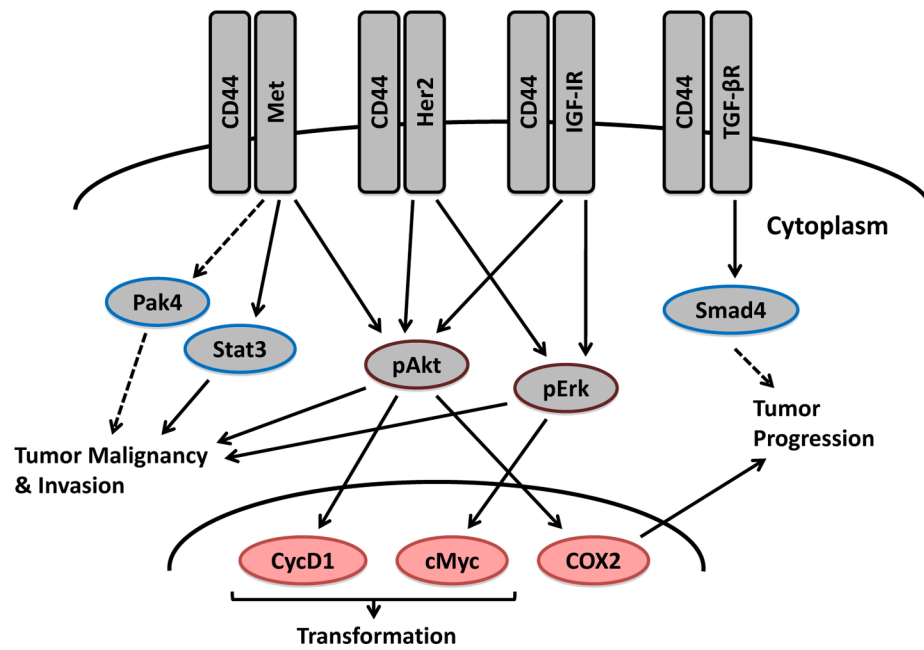


Figure 3. A schema of key signaling proteins in the MCF10 breast cancer model and their association with stages of cancer progression
Solid lines indicate the downstream signaling pathways whose involvement is supported by published data. Dotted lines represent the proposed pathways among the key signaling molecules supported by our findings.

Table 1

Analysis, characterization and gene profiling studies using the MCF10 breast cancer progression model

Reference	Analysis	Differential expression of genes or proteins in the MCF10 breast cancer progression
Worsham et al. (2006) (23)	Multiplex ligationdependent probe amplification assay (MLPA)	- MCF10A: Loss of one copy of CCND2 and IGF1R/ Homozygous loss of ERBB2, CDKN2A and CDKN2B gene/ Gain of MYC - MCF10AT1: Restoration of ERBB2 and CCND2 / Gain of IL13, VEGF, HRAS, TRAF2 - MCF10CA1a: Gain of BCAS2, IL12A and MME/ Restoration of IGF1R
Rhee et al. (2008) (14)	Comparative microarray analysis	- Down-regulation of TNFSF7, S100A4, S100A7, S100A8 and S100A9, and KLK5 and THBS1 were associated with transformation and progression of breast cancer in MCF10AT model - Down-regulation of genes in malignant cell lines can be epigenetically reversed
Marella et al. (2009) (24)	Spectral karyotyping, Array comparative genomic hybridization (aCGH) and cDNA microarray	- Up-regulated genes in MCF10CA1a: SEPP1, DCN, FBN1, PTGER2, AOX1, MUC1, MMP2, FN1, RB1, CDKNB1, CCND3, IL7 and IL18 - Down-regulated genes in MCF10CA1a: CDH1, IL1B, S100A14, BDKRB2, VEGF, BRAF, ERBB2, EGFR, HRAS, MYC, PTEN, IL1A and IL1B
Kadota et al. (2010) (10)	Combination of high density SNP analysis and mutation analysis by sequencing	- MCF10A: CDKN2A deletion and MYC amplification - MCF10AT1: HRAS activation - MCF10CA1h: activating PIK3CA mutation/LRP1B, FHIT and CDH13 deletion - MCF10CA1a: activating PIK3CA mutation/LRP1B and RUNX1 deletion
Kim et al. (2009) (21)	Immunohistochemistry and western blot analysis	- Increased Ras, Rac, Rho and active forms of PDK1, eIF4E and 4EBP1 protein level in malignant cells of MCF10AT model - Western blot of cell lines and immunohistochemistry of xenograft tumor demonstrated elevated expression of phospho- AKT and phospho-FOXO 1,3a and 4
Choong et al. (2010) (15)	Proteome-wide analysis	- The cancer progression of MCF10AT1 model is associated with a major-reprogramming in metabolism - MCF10CA cell lines: increased expression of AK1 and ATOX1 / decreased expression of HIST1H2BM
Mbeunkui et al. (2007) (25)	Analysis of conditioned medium proteome using LC-MS/MS	- Differential expression of secreted proteins in MCF10AT model - High secretion level of alpha-1-antichymotrypsin and galectin-3-binding protein in MCF10DCIS.com and MCF10CA cell lines - Galectin-3-binding protein has been associated with aggressiveness of other types of cancers
Chen et al. (2007) (26)	Combination of phosphotyrosyl affinity enrichment, iTRAQ and LC-MS/MS analysis	TOLLIP, WBP2, NSFL1C, SLC4A7, CYFIP1 and RPS2 were detected as novel proteins that underwent differential phosphorylation during breast cancer progression in MCF10AT model
Wang et al. (2008) (27)	Combination of membrane extraction and lectin affinity methods with LCMS/ MS analysis	Differential expression of membrane glycoprotein in MCF10AT model

Reference	Analysis	Differential expression of genes or proteins in the MCF10 breast cancer progression
		CD44, Gamma-glutamyl hydrolase, Galectin-3-binding protein and Syndecan-1 were associated with malignant breast cancer cell lines