Journal of Cellular Biochemistry 102:549–559 (2007)

Defining ETS Transcription Regulatory Networks and their Contribution to Breast Cancer Progression

David P. Turner,[1,3] Victoria J. Findlay,[1,3] Omar Moussa,[1,3] and Dennis K. Watson[1,2,3]*

[1]Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina 29425
[2]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425
[3]Hollings Cancer Center, Medical University of South Carolina, Charleston, South Carolina 29425

Abstract ETS factors are members of one of the largest families of evolutionarily conserved transcription factors, regulating critical functions in normal cell homeostasis, that when perturbed contribute to tumor progression. The well documented alterations in ETS factor expression and function during breast cancer progression result in pleiotropic effects manifested by the downstream effect on their target genes. Multiple ETS factors bind to the same regulatory sites present on target genes, suggesting redundant or competitive functions. Furthermore, additional events contribute to, or may be necessary for, target gene regulation. In order to advance our understanding of the ETS-dependent regulation of breast cancer progression and metastasis, this prospect article puts forward a model for examining the effects of simultaneous expression of multiple transcription factors on the transcriptome of non-metastatic and metastatic breast cancer. Compared to existing RNA profiles defined following expression of individual transcription factors, the anti- and pro-metastatic signatures obtained by examining specific ETS regulatory networks will significantly improve our ability to accurately predict tumor progression and advance our understanding of gene regulation in cancer. Coordination of multiple ETS gene functions also mediates interactions between tumor and stromal cells and thus contributes to the cancer phenotype. As such, these new insights may provide a novel view of the ETS gene family as well as a focal point for studying the complex biological control involved in tumor progression. J. Cell. Biochem. 102: 549–559, 2007.

Key words: ETS; transcriptional regulation; breast cancer; microenvironment; proteome

Breast cancer is the second leading cause of cancer mortality in women in the Western World. In the United States, over 180,000 cases are diagnosed annually [Jemal et al., 2007]. Despite an encouraging trend towards reduced mortality, about one fourth of diagnosed patients will die from their disease. Breast cancer mortality is almost invariably attributable to metastasis that is clinically untreatable despite aggressive chemical and radiation therapies [Debies and Welch, 2001]. Additional studies directed towards elucidation of the factors involved in its progression should facilitate the design of molecularly based diagnostic and therapeutic approaches.

Breast cancer is a heterogeneous disease and understanding this complexity remains a major challenge for physicians and biologists. The proposed molecular mechanisms underlying breast cancer initiation and progression include over-expression of oncogenes such as Her2/neu [Slamon et al., 1989], myc [Escot et al., 1986] and H-ras [Rochlitz et al., 1989] or loss of tumor suppressor genes such as p53 [Davidoff et al., 1991], Rb [Fung and T'Ang, 1992] and PTEN [Dillon et al., 2007]. Several potential metastasis modulators have also been identified [Debies and Welch, 2001], however, no integrated molecular and cellular mechanisms for metastasis initiation and progression have emerged. More recent analyses of global changes in the transcriptome [Fan et al., 2006; Ivshina et al., 2006; Loi et al., 2007] and

Grant sponsor: National Institutes of Health; Grant number: P01 CA78582.

*Correspondence to: Dennis K. Watson, Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425.
E-mail: watsondk@musc.edu

Received 16 June 2007; Accepted 19 June 2007
DOI 10.1002/jcb.21494

proteome [Bertucci et al., 2006] of breast cancer cells has yielded additional mechanistic insights. Furthermore, recent molecular classification of breast cancer patients by gene expression profiling has the potential to provide patient outcome stratification [Dressman et al., 2006; Ma et al., 2007]. Microarray analysis of cell populations isolated by laser capture microscopy has defined expression patterns that are correlated with progression from ductal carcinoma in situ (DCIS) to invasive breast cancer [Schuetz et al., 2006]. The transcriptome studies also indicate that many of the molecular changes may be mediated by altered functions of transcription factors, including those of the ETS gene family. After providing a brief overview of the ETS factor family, their role in breast cancer progression will be presented. This will be followed by a discussion of areas of ongoing and future research that will provide insights into the ETS regulatory network and progression to metastatic breast cancer.

THE ETS FAMILY OF TRANSCRIPTION FACTORS

The oncogene v-ets is part of the transforming fusion protein of an avian retrovirus, E26 (*E*26 *t*ransforming *s*equence, ets). Subsequent identification of v-ets related genes from metazoan species established the Ets family as one of the largest families of transcriptional regulators, with diverse functions and activities (for review, see Seth and Watson [2005] and references therein). To date, 27 human ETS family members have been identified. All ETS genes retain a conserved sequence (the ETS domain) of ~85 amino acids that forms the winged helix-turn-helix DNA binding domain that recognizes a core GGAA/T sequence (ETS binding site, EBS). Binding of ETS proteins to target genes is facilitated by the binding of other transacting factors to cis-elements in proximity to the EBS. The second conserved domain found in a subset of ETS genes is the pointed (PNT) domain. This 65–85 amino acid domain is found in 11 of 27 human ETS genes and has in some cases been shown to function in protein–protein interaction and oligomerization. ETS factors are known to act as positive or negative regulators of the expression of genes that are involved in various biological processes, including those that control cellular proliferation, differentiation, development, hematopoiesis, apoptosis, metastasis, tissue remodeling, angiogenesis, and transformation. ETS proteins functional activity is modulated by post-translational modification(s) and interaction with other nuclear factors.

ETS FACTORS AND CANCER

The hallmark features of a cancer cell consist of uncontrolled proliferation, loss of differentiation, sustained cell division, increased angiogenesis, loss of apoptosis and a capacity to migrate and invade to other tissues and organs. All of these processes are driven by transient and/or permanent changes in gene expression profiles conferred through the activation or repression of cancer-associated genes. It is therefore clear that the role of transcriptional gene regulation in cancer progression cannot be understated and many transcription factors including ETS family members have been assigned as candidate oncogenes (ETS1, ETS2, MYC) or tumor repressors (PDEF, p53). The importance of ETS genes in human carcinogenesis is supported by the observations that ETS genes have altered expression patterns, are chromosomally amplified or deleted, or are located at translocation breakpoints in leukemias and solid tumors. Correlation of ETS gene expression levels with tumor progression occurs in human neoplasias such as thyroid, pancreas, liver, prostate, colon, lung, and breast carcinomas and leukemias [Seth and Watson, 2005].

Alterations in cell cycle control are a critical step in carcinogenesis. Cell cycle arrest at the G_1-S phase by upregulation of the cyclin dependent kinase inhibitor p21 has been reported in response to DNA damage or oncogenic insult. The elevated level of p21 is known to be mediated through p53 and we have demonstrated PDEF mediated regulation of p21 expression [Feldman et al., 2003]. Interestingly, the ETS binding site in the promoter of p21 is associated with the corresponding p53 binding motif. The increased expression of the p21-activated kinase (PAK1) has been shown to be correlated with more aggressive breast cancer [Salh et al., 2002]. Recent studies have shown that PAK1 regulates the activity of the transforming ETS family member ESE-1 by phosphorylation [Manavathi et al., 2007]. This novel finding raises the possibility that using a specific inhibitor to the upstream effector of ESE-1 (e.g., PAK1-specific inhibitor CEP-1347)

may represent a novel approach for targeting a transcription factor in breast cancer.

ETS REGULATORY NETWORKS

Evidence suggests that multiple ETS factors act in concert to positively and negatively regulate the pathways that control progression to metastatic breast cancer. Studies examining ETS factor expression profiles in normal and cancerous breast cells have demonstrated that a diverse combination of at least 25 of 27 ETS family members examined are expressed at any one time in these cells [Galang et al., 2004; Hollenhorst et al., 2004]. This indicates a possible "ETS conversion" mechanism of gene regulation which provides the cell with an integrated mechanism by which to respond to a variety of intra- and extra-cellular signals efficiently [Hsu et al., 2004]. Furthermore, in breast cancer, up-regulation of multiple ETS factors including, ETS1 [Buggy et al., 2004], ETS2 [Buggy et al., 2006], PEA3 [Benz et al., 1997], ERM [Yarden and Sliwkowski, 2001], and ER81 [Bosc et al., 2001], is associated with poor prognosis and metastasis. In contrast other ETS factors including PDEF [Feldman et al., 2003; Doane et al., 2006], ESE-2 [Zhou et al., 1998], and ESE-3 [Tugores et al., 2001], are down-regulated during breast cancer progression within the same context. Reciprocal functional studies demonstrate the impact of such altered expression on the regulation of genes associated with proliferation, transformation, migration, invasion, anti-apoptosis, and angiogenesis [Seth and Watson, 2005] and include but are not exclusive to Her2/neu, uPA, MMPs, TIMPs, MET, Bcl2, maspin, VEGFR [Sementchenko and Watson, 2000], and survivin [Ghadersohi et al., 2007].

Reciprocal ETS regulation of a metastasis-associated gene has been clearly demonstrated at the urokinase plasminogen activator (uPA) promoter. Up-regulation of uPA has a causal role in enhancing matrix degradation, cytoskeleton re-organization, cell growth, migration and invasion (a pro-metastatic phenotype) and high levels of uPA in primary breast cancer is independently associated with poor outcome [Duffy, 2002]. ETS regulation of uPA has both positive and negative effects on breast cancer progression depending on the specific ETS factor expressed. ETS1 is over-expressed in invasive breast cancer and associated with increased uPA expression. In non-invasive (ETS1-) breast cancer cells, re-expression of ETS1 increases uPA levels leading to a pro-metastatic phenotype. Published work from this laboratory has demonstrated that the expression of another ETS family member PDEF is present in non-invasive, but lost in invasive, breast cancer cells. In contrast to the effect of ETS1 in non-invasive cells, PDEF re-expression in invasive cells represses endogenous uPA transcription leading to an inhibition of cell growth, migration and invasion (an anti-metastatic phenotype) [Feldman et al., 2003; Turner et al., 2007]. Intriguingly, although several potential EBS are found in the uPA promoter, both ETS1 and PDEF have been demonstrated to bind at the same consensus EBS in vivo.

Taken as a whole, this evidence strongly suggests the existence of distinct ETS expression regulatory networks that act in concert to positively or negatively regulate cancer associated genes. Significantly, each ETS network would result in distinct patterns of target gene expression, the elucidation of which may identify pro-metastatic and anti-metastatic signatures of gene expression that may predict the aggressive behavior of breast cancer cells.

ETS MEDIATED ANTI-AND PRO-METASTATIC SIGNATURES

Gene expression signatures consist of sets of gene profiles that are known to be predictive of a disease state and/or patient response to treatment. The combined statistical analysis of multiple gene sets obtained from independent gene microarray studies has resulted in an increased number of putative and validated "metastatic signatures" that predict the outcome of disease in cancer. In addition, comparison of gene expression profiles from primary and metastatic tumors in multiple cancer types reveals highly specific signatures that allow discrimination between primary and metastatic tumors. Similarly, by elucidating the expression networks conferred by ETS family members that elicit a pro-metastatic response (ETS1, ETS2, PEA3, etc.) and an anti-metastatic response (PDEF, ESE2, ESE3, etc.), improved pro- and anti-metastatic signatures may be defined that predict the aggressive behavior of cancer cells. In addition,

a better definition of genes whose expression is functionally important for metastatic progression will highlight new therapeutic targets.

ETS MEDIATED GENE REGULATION

Sparse information exists concerning the precise composition of the transcriptional complexes formed by ETS factors at their consensus-binding sites and no examination of the mechanism by which ETS family members compete for occupancy of the same promoter has been made. Transcription factor regulation is a highly complex process requiring an exact spatial and temporal coordination of multifaceted protein complexes in order to successfully regulate the 35,000–50,000 genes found in each human cell. Sequence specific transcription factors such as ETS factors play a crucial role in transcriptional regulation by initiating complex formation at their consensus binding motifs. Specificity is conferred through a complex series of protein-DNA and protein–protein interactions with a multitude of co-activator and/or co-repressor proteins (including chromatin remodeling and histone modifying enzymes). Correct complex formation regulates pre-initiation complex formation and in turn transcriptional activation. As mentioned above, all ETS factors recognize a common DNA binding motif (GGAA/T), and many are known to occupy the promoters of common genes [Hollenhorst et al., 2004]. In addition to some DNA sequence specificity, regulation is likely to be conferred by numerous intricate layers of control that may include intra- and extra-cellular signaling cascades, co-factor binding species, post-translational modifications, and alterations to protein/DNA conformation. Such layers of control will not only define the repertoire of ETS factors on specific target regulatory elements, but will also confer functional specificity and magnitude of transcriptional activation or repression.

To date, ETS factor research has mainly focused on the molecular mechanisms and functions of individual transcription factors and has produced a wealth of valuable insights into ETS factor function in both normal and cancerous cells. This research has identified a multitude (over 500) of direct ETS target genes, the precise expression pattern of which may drive many of the processes associated with cancer. How multiple ETS transcription factors compete for promoter occupancy and function as a regulatory network is unknown. It is now clear that to fully comprehend the dynamics of ETS-mediated regulation of cancer-associated genes they must be analyzed in the context of a dynamic transcriptional regulatory network and not as individual transcription factors. Recent advances in several experimental techniques are now allowing researchers to examine such networks on a global scale and allow a detailed examination of the multiprotein transcriptional complexes formed at the sites of gene promoters. These advances will now be discussed within the context of ETS mediated gene regulation.

WHOLE GENOME LOCATION ANALYSIS

Chromatin immunoprecipitation (ChIP) and DNA footprinting are well-established protocols for the analysis of protein-promoter interactions in vivo, but only allow for the examination of small experimentally isolated sections of individual promoter sequence. Sequential ChIP (SeqChIP) is an extension of the ChIP protocol, in which the immunoprecipitated chromatin is subjected to sequential immunoprecipitations with antibodies of different specificity. This provides a method of examining co-occupancy of defined promoters and can assign full occupancy, partial occupancy or no occupancy status. Furthermore, SeqChIP provides an experimental approach to simultaneously evaluate promoter occupancy and transcriptional status (e.g., histone H3 acetylation, phosphorylated RNAPII-CTD [Jackers et al., 2004]). However, this method is restricted to the analysis of small promoter regions, the boundaries defined by the sequences of the primers designed for the PCR amplification step. A second limitation of the ChIP or SeqChIP protocol is that it is dependent upon the quality and specificity of the antibodies used in the immunoprecipitation. However, ChIP has already been highly successful in identifying in vivo ETS occupancy at the promoters of numerous cancer related genes, including those regulating apoptosis (Bcl-2, Bcl-X_L), proliferation and senescence (Rb, p16), and migration and invasion (uPA, Slug).

To determine the global location of in vivo promoter binding sites of a specific protein, ChIP protocols have been combined with whole genome analysis methods to produce

"ChIP-on-chip" microarrays. ChIP products are hybridized to arrays consisting of promoter regions, CpG islands or whole genomes and are used to identify not only DNA binding sites, but also transcriptional co-factors and chromatin structure (Fig. 1).

In the context of an ETS transcription network, ChIP-on-chip analysis can potentially identify the full transcriptome for each individual ETS family member in any given scenario. For example, by defining the metastasis suppressing PDEF transcriptome in non-invasive cancer cells and metastasis activating ETS1 transcriptome in invasive cancer cells, specific ETS mediated transcriptional networks may be identified which confer an anti- or pro-metastatic phenotype. Furthermore, by comparing ChIP-on-chip data with microarray profiles obtained following ETS expression, direct and indirect targets for each ETS factor can be ascertained. It will also allow the identification of genes directly regulated by more than one specific ETS factor, such as that observed for uPA, which is directly regulated by both either ETS1 or PDEF, depending on cell context (Fig. 2A). In addition, the genes that are identified on expression microarrays and are not identified as direct targets on ChIP-on-chip microarrays will define indirect or downstream targets, providing further insight into the complex nature of ETS transcriptional regulatory networks. The identification of genome wide binding sites may also indicate the functions of various transcriptional regulators and help identify their target genes during development and disease progression. To date, ChIP-on-chip technology has already been used to determine binding hierarchies and co-factor requirements at promoters using time-course studies and protocols are currently being developed for organ and tissue examination.

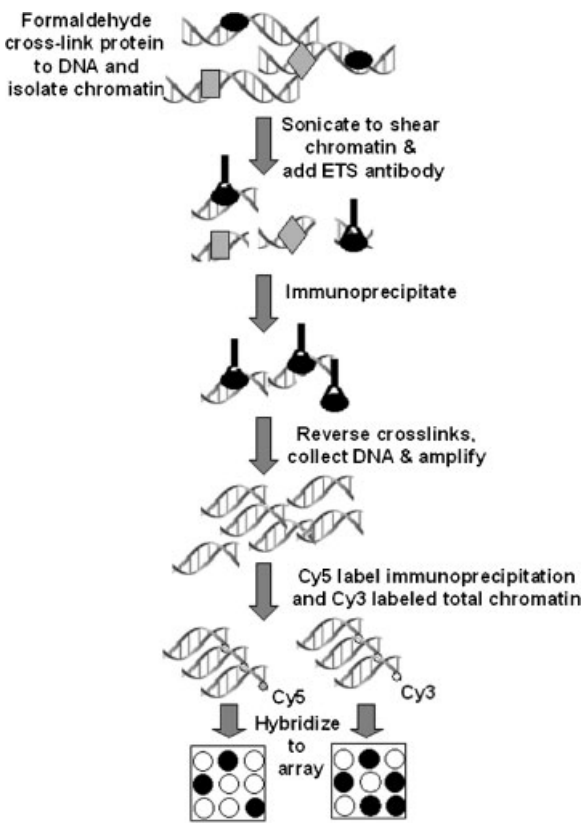

Fig. 1. Flow diagram of ChIP on chip analysis of promoter occupancy. Formaldehyde treatment is used to crosslink proteins to DNA. Chromatin is sheared into fragments by sonication. Chromatin bound complexes are isolated and an aliquot is removed to serve as a total chromatin control. Protein complexes are isolated by immunoprecipitation with an antibody directed against the protein of interest. The formaldehyde crosslinks are then reversed in both the immunoprecipitated and total chromatin samples and proteins are digested by Proteinase K. The chromatin fragments are then purified and subjected to whole genome amplification PCR. The amplified DNA samples are then labeled with unique fluorescent tags (e.g., Cy3 and Cy5). The two probes are competitively hybridized to a microarray.

ETS PROTEOMICS

It has been estimated that as many as 10,000 different transcriptional regulators exist. It is likely that many of these regulators are integral components in a variety of transcriptional complexes resulting in the expression of a diverse array of target genes. Promoter binding specificity therefore will be driven by the exact composition and structural stoichiometry of the transcriptional complex. It is likely that four main classes of protein component will be identified in a specific transcriptional complex, (1) Co-factor proteins common to many transcriptional complexes; (2) co-factor proteins unique to specific, or specific sets of, transcriptional complex; (3) proteins associated with activation or repression, such as histone modification enzymes; (4) non-specific proteins not required for complex formation.

A common technical approach used for the analysis of protein complexes is to immunoprecipitate the target proteins from lysates prepared under mild conditions and identify the co-immunoprecipitated binding partners by mass spectrometry analysis on proteolytic fragments. A crucial aspect in the analysis of any protein complex is the need to isolate the complex

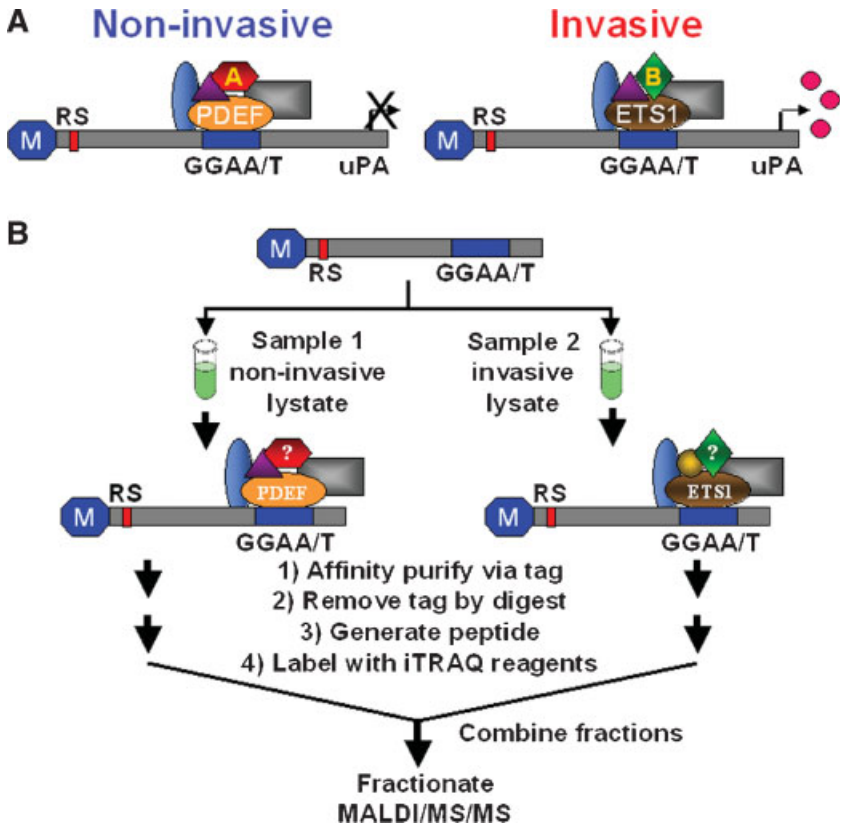

Fig. 2. Strategy for identifying unique protein components present as part of the multi-protein complex on ETS responsive promoters. **A**: Hypothetical model for the dual regulation of the uPA promoter by ETS family members. PDEF or ETS1 occupies the uPA promoter in non-invasive or invasive cells, respectively. Specificity for PDEF binding is conferred by the presence of the regulatory protein A. In invasive cells, specificity for ETS1 binding is conferred by the presence of the regulatory protein B. **B**: Single-step promoter DNA affinity purification and iTRAQ analysis of ETS transcriptional complexes. Nuclear extracts prepared from non-invasive and invasive cells are incubated with magnetically tagged (M) promoter constructs containing the EBS of choice. Complexes are isolated using the magnetic tag which is subsequently removed by restriction enzyme (RS) digestion. The isolated complexes are fragmented by proteolytic digestion and isotopically labeled with iTRAQ reagents and then combined. In order to identify and quantify the protein complex components, the combined sample is fractionated and analyzed by mass spectrometry and peptide database comparison.

under conditions that allow isolation of only true binding partners while eliminating contaminating proteins. Therefore, this methodological approach is very dependent upon the specificity of the antibody used in the immunoprecipitation step and on the ability to safely remove contaminating proteins, and crucially, not bona fida proteins by sequential washing steps.

Tandem affinity purification (TAP) is another method to purify and analyze protein complexes. Rather than rely on immunoprecipitation, this approach utilizes two affinity tags (e.g., calmodulin and streptavidin) fused to the N- or C-terminus of the protein of interest and separated by a protease cleavage site. The tagged protein is then expressed in a chosen cell type and isolated, together with its binding partners, via a tandem purification protocol directed against the affinity tags. After immobilization on the first affinity column, complexes are released using the protease cleavage site and immediately bound to a second affinity column directed against the second tag. The tagged protein, along with its binding partners is then released from the second column and analyzed by mass spectrometry to identify the unknown proteins. Both of these methods have been very successful in analyzing the proteome formed at the sites of transcriptional promoters. However, each of these methods does not allow an accurate assessment of the relative abundance of protein in any one complex under differing conditions.

Important advances in the field of quantitative proteomics now offer intriguing new ways to examine both the composition, and more importantly, the relative abundance of protein

occupancy at gene promoters. For example, single-step promoter DNA affinity purification coupled with iTRAQ (isotope tags for relative and absolute quantitation) is a proven, powerful technique of quantitative mass spectrometry used to compare the relative abundance of proteolytic peptides derived from sets of protein–protein and protein-DNA complexes [Ranish et al., 2003; Brand et al., 2004; Aggarwal et al., 2006]. Single-step promoter DNA affinity purification isolates transcriptional complexes by using immobilized promoter templates with a high affinity tag such as biotin, or covalently attached magnetic beads (Fig. 2B). Specific promoter templates are incubated with the nuclear lysates obtained from two experimental conditions and the complexes isolated using the affinity tag of choice. Once isolated, the tag is removed by digestion and protein peptides are generated by proteolysis. Each set of peptides is then labeled for mass spectrometry analysis using the iTRAQ system. iTRAQ consists of four (eight are in development) isobaric amine specific reagents that label the N-terminus of proteolytic peptides that are identical in mass but allow quantification via strong diagnostic signature ions when analyzed by MALDI-MS/MS. Labeled peptide samples are mixed and fractionated before analyzing by mass spectrometry. Database searching using the resultant peptide sequences identifies the protein composition within the isolated complex. Critically for the measurement of protein abundance levels, fragmentation of the isobaric tag produces low molecular mass reporter ions that are unique to the specific tag used to label each sample. The intensity of these reporter ions is directly relative to peptide levels and thus the relative abundance of protein within each complex can be assessed. As there are four isobaric tags currently available, four different conditions for complex formation can be examined in a single experiment or specificity can be increased by doubly labeling two experimental conditions.

The ETS regulatory network consists not only of the specific ETS factors present in a cell, but also a multitude of regulatory co-factors that

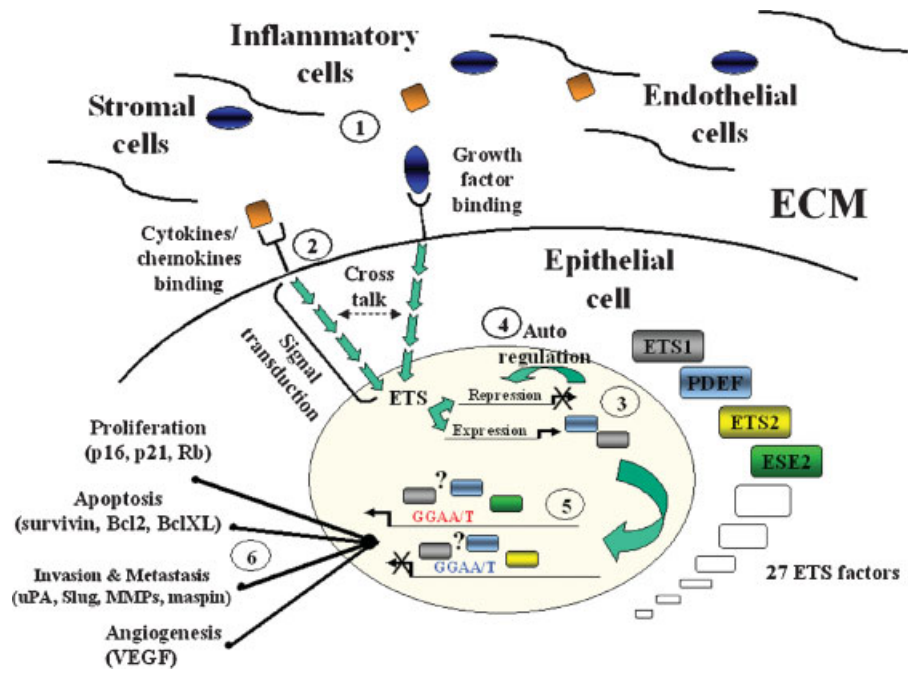

Fig. 3. Hypothetical model of the ETS regulatory network in breast cancer. Inflammatory cells are recruited by tumors through their secretion of chemokines, cytokines and growth factors (1). In response, the recruited inflammatory cells (and other cells of the microenvironment [e.g., stromal-derived fibroblasts and endothelial cells]) promote tumor proliferation and progression through additional secretion of biologically active molecules. This in turn results in the activation of intracellular signaling cascades via ligand binding at the cell surface of epithelial cells (2). The activated cascades directly or indirectly (through crosstalk) result in the expression and repression of varying combinations of the 27 ETS family members (3). ETS factors can regulate their own expression and/or that of other family members (4). The composition of ETS factors defines the transcriptional regulation of their target genes, many known to be involved in cancer progression (5). The altered expression of these genes has profound consequences on many cancer related pathways (6).

are essential for the ultimate definition of each ETS factors transcriptome (Fig. 3). By using quantitative proteomics to identify and quantify the regulatory co-factors required for ETS mediated gene regulation, it may be possible to identify the subtle mechanism by which a particular ETS family member can bind to its consensus sequence to the occlusion of other family members under different experimental conditions. For instance, by taking a snapshot of the transcriptional complex on the uPA promoter in its PDEF mediated repressed and ETS1 mediated activated state, key regulatory cofactors that confer ETS specificity will be identified and will provide valuable insight on the transcriptional control of metastasis-associated genes during breast cancer progression (Fig. 2A).

While the iTRAQ system has been demonstrated to greatly extend the utility of mass spectrometry for the analysis of macromolecular complexes [Ranish et al., 2003; Brand et al., 2004; Aggarwal et al., 2006], it is possible that this approach may generate false positives and/or false negatives. False positives may be due to the co-purification of non-specific proteins and false negatives from inaccuracies in mass spectrometric analysis due to low intensity ions [Ranish et al., 2003; Aggarwal et al., 2006]. Although a single step affinity purification protocol will help reduce both these factors, it is important to validate altered protein abundance levels using other in vitro and in vivo protocols. Such protocols could include Western blot examination of the lysates obtained from the single-step promoter DNA affinity purification and ChIP and SeqChIP coupled with quantitative real time PCR.

THE IMPORTANCE OF THE MICROENVIRONMENT

Breast tumors are complex tissues composed not only of neoplastic epithelial cells in which genetic and epigenetic events affect their changing phenotypes, but also a complex microenvironment composed of matrix proteins that provide biologically active molecules, and a cellular stromal component. It is now well documented that reciprocal interactions between neoplastic cells and the microenvironment promote growth, angiogenesis and metastasis [Hsu et al., 2004]. One component of the microenvironment is the inflammatory cells that are recruited by the release of cytokines and growth factors (e.g., monocyte chemoattractant protein-1, MCP-1; colony-stimulating factor, CSF-1) from tumor cells (Fig. 3). Resultant inflammatory responses regulate tumor development to a large extent by providing mediators of tissue homeostasis (e.g., soluble growth and survival factors, angiogenic factors, matrix remodeling enzymes, reactive oxygen species and other bioactive molecules) [van Kempen et al., 2006]. Relevant to this discussion, ETS family members regulate tumor expression of cytokines (e.g., MCB-1 [Zhan et al., 2005]) as well as response to specific growth factors (e.g., CSF-1) and chemokines (SDF-1 [Luo et al., 2005]). A reciprocal relationship exists between inflammatory cells (macrophages) expressing CSF-1 receptor and EGF with tumor cells expressing EGF receptor and CSF-1 that together promotes tumor metastasis [Wyckoff et al., 2004]. Similarly, stromal-derived fibroblasts secrete SDF-1 which binds to CXCR4 receptor on the surface of breast cancer cells to promote tumor growth and invasion through ETS1 and NFkB [Maroni et al., 2007]. The matrix metalloproteinases (MMPs) and uPA/plasmin system represent major classes of primarily stromal-derived ECM-degrading proteases, many of which are ETS target genes, with extensively documented roles in breast cancer invasiveness. Breast cancer cells can specifically activate the surrounding fibroblasts, macrophages and endothelial cells to produce uPA and MMPs [Toole, 2003]. Stromal derived MMP-3 expression promotes mammary carcinogenesis and MMP-1 stimulates growth and invasion pathways by cleaving protease activated receptor 1 (PAR1) on the tumor cell surface [Boire et al., 2005]. Evidence also exists to suggest the involvement of the uPA system and collagenase (MMP-13) in the transition from DCIS to invasive ductal carcinoma [Nielsen et al., 2007]. The important role for the stroma in cancer progression has led to efforts to target the tumor microenvironment and its components both for cancer therapy and for chemoprevention.

SIGNALING PATHWAYS IN 3D CULTURE

Another component of the microenvironment, the extracellular matrix (ECM), is also a key regulator of normal homeostasis and tissue phenotype. Important signals are lost when cells are cultured ex vivo on two-dimensional (2D)

plastic and restored using three-dimensional (3D) cultures, thus providing a model system to study genes involved in processes associated with tumor progression, and to investigate the mechanisms responsible for the associated phenotypic changes [Bissell et al., 2005].

Non-malignant mammary cells grown in a 3D context form polarized, growth-arrested acinus-like colonies [O'Brien et al., 2002]. Early studies revealed that this intact, well-ordered architecture is disrupted during the pathogenesis of epithelial tumors using human breast tumor cell lines grown in 3D cultures. These seminal experiments illustrated the dramatic contrast between normal cells and tumor cells grown in 3D culture when compared to 2D. These assays allow phenotypic discrimination between nonmalignant and malignant mammary cells, the latter of which form disorganized, proliferative, and nonpolar colonies [Petersen et al., 1992].

The formation of polarized, growth arrested multicellular structures that resemble acini is the reverse process of what occurs during the early stages of breast tumorigenesis. This phenotypic architecture provided a rationale to utilize microarray analyses to monitor gene expression changes as cells form acinar structures in 3D cultures [Fournier et al., 2006]. When compared to a previously published panel of microarray data for 295 breast cancer samples, the expression profile obtained allowed identification of a signature correlated with good outcome in breast cancer patients. This novel approach illustrates the power of combining multiple technologies in breast cancer research towards defining molecular markers of potential clinical utility.

Studies in 3D culture also highlighted the importance of signaling cascades and growth factor receptor signaling pathways in breast cancer progression by demonstrating that inhibition of the ECM receptor β1-integrin, present on the surface of malignant cells, results in a normal phenotype. Examination of the mechanisms responsible for this "phenotypic" reversion revealed that inhibition of β1-integrin function caused down regulation of both β1-integrin expression and the EGF receptor (EGFR). More importantly, these findings were only observed in 3D cultures. Later studies also showed a similar reversion through manipulation of the ERK and PI3K signaling pathways [Wang et al., 2002].

3D CULTURE SYSTEMS

The 3D in vitro system is an invaluable tool for cancer biologists. These models can reproduce the in vivo behavior of tumor cells and can mimic cell stromal interactions, thus providing a system in which to investigate the many regulatory feedback mechanisms that exist between cellular components in a well defined environment. 3D culture models exist for a number of different tissues. There are currently two methods available for 3D systems using the breast as a model [Lee et al., 2007]. This includes the 3D embedded method in which cells are cultured in an ECM. One commonly used mixture is that derived from the Engelbreth–Holm–Swarm (EHS) murine tumor, commercially available as Matrigel™. In this method the epithelial cells are completely embedded within the ECM and grown in the presence of culture media containing growth factors and hormones that are necessary for proliferation and survival. The second method, the 3D on-top assay, involves seeding the cells on top of the formed ECM gelled bed in a dilute solution of ECM. This method requires a shorter amount of time, a decreased amount of EHS, and facilitates imaging (as colonies are in a single plane). This method is ideal for time-lapse imaging and in situ immunostaining of cells that form invasive stellate structures. It is also more cost-effective. However, using the 3D embedded assay, colonies can be (a) fully extracted for immunostaining, DNA, RNA and protein extraction, (b) partially (in-well) extracted for immunostaining or (c) fixed in gel for whole cell immunostaining. Frozen sections for subsequent immunostaining may also be obtained after embedding of whole cultures in Optimal Cutting Temperature (OCT) compound.

PERSPECTIVES

Understanding the molecular crosstalk between diverse cell populations is now easier than ever, which is the power of the tools that are now available to us. Biological events that are associated with epithelial cancers, such as the filling of the luminal space, loss of polarization, escape from proliferative suppression, invasive behavior and loss of cell adhesion can now be studied, thus providing a more physiologically relevant approach to the

analysis of gene function and cell phenotype ex vivo.

Important advances in transciptome and proteome analysis have started to allow detailed investigation into the global regulation of genes by transcription factors. By combining the data obtained from techniques such as ChIP on chip and iTRAQ, it will be possible to examine gene regulation in the context of a large and complex network of interactions mediated by multiple transcription factors and their regulatory co-factors (Fig. 3). In the context of the transcriptional regulation of cancer, such insight will allow a greater understanding of the mechanism of tumor suppressor repression and oncogene activation, such as that observed in the ETS regulation of uPA during metastasis. Furthermore, it has become apparent that future studies will move forward from a reductionist view with primary focus on epithelial cell towards models that also consider the contribution of the tumor cell microenvironment to the cancer phenotype. For example, efforts should focus on the use of 3D culture systems that more faithfully represent the histological complexity of epithelial tissues in vivo. Collectively, application of these technological advances will provide significant mechanistic insight into breast cancer progression, leading to improved diagnostic and prognostic markers and novel therapeutic opportunities.

ACKNOWLEDGMENTS

We apologize to those researchers whose work could not be cited because of space limitations or was only cited indirectly by referring to reviews or more recent publications.

REFERENCES

Aggarwal K, Choe LH, Lee KH. 2006. Shotgun proteomics using the iTRAQ isobaric tags. Brief Funct Genomic Proteomic 5:112–120.

Benz CC, O'Hagan RC, Richter B, Scott GK, Chang CH, Xiong X, Chew K, Ljung BM, Edgerton S, Thor A, Hassell JA. 1997. HER2/Neu and the Ets transcription activator PEA3 are coordinately upregulated in human breast cancer. Oncogene 15:1513–1525.

Bertucci F, Birnbaum D, Goncalves A. 2006. Proteomics of breast cancer: Principles and potential clinical applications. Mol Cell Proteomics 5:1772–1786.

Bissell MJ, Kenny PA, Radisky DC. 2005. Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: The role of extracellular matrix and its degrading enzymes. Cold Spring Harb Symp Quant Biol 70:343–356.

Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A. 2005. PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell 120:303–313.

Bosc DG, Goueli BS, Janknecht R. 2001. HER2/Neu-mediated activation of the ETS transcription factor ER81 and its target gene MMP-1. Oncogene 20:6215–6224.

Brand M, Ranish JA, Kummer NT, Hamilton J, Igarashi K, Francastel C, Chi TH, Crabtree GR, Aebersold R, Groudine M. 2004. Dynamic changes in transcription factor complexes during erythroid differentiation revealed by quantitative proteomics. Nat Struct Mol Biol 11:73–80.

Buggy Y, Maguire TM, McGreal G, McDermott E, Hill AD, O'Higgins N, Duffy MJ. 2004. Overexpression of the Ets-1 transcription factor in human breast cancer. Br J Cancer 91:1308–1315.

Buggy Y, Maguire TM, McDermott E, Hill AD, O'Higgins N, Duffy MJ. 2006. Ets2 transcription factor in normal and neoplastic human breast tissue. Eur J Cancer 42: 485–491.

Davidoff AM, Kerns BJ, Pence JC, Marks JR, Iglehart JD. 1991. p53 alterations in all stages of breast cancer. J Surg Oncol 48:260–267.

Debies MT, Welch DR. 2001. Genetic basis of human breast cancer metastasis. J Mammary Gland Biol Neoplasia 6: 441–451.

Dillon RL, White DE, Muller WJ. 2007. The phosphatidyl inositol 3-kinase signaling network: Implications for human breast cancer. Oncogene 26:1338–1345.

Doane AS, Danso M, Lal P, Donaton M, Zhang L, Hudis C, Gerald WL. 2006. An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen. Oncogene 25:3994–4008.

Dressman HK, Hans C, Bild A, Olson JA, Rosen E, Marcom PK, Liotcheva VB, Jones EL, Vujaskovic Z, Marks J, Dewhirst MW, West M, Nevins JR, Blackwell K. 2006. Gene expression profiles of multiple breast cancer phenotypes and response to neoadjuvant chemotherapy. Clin Cancer Res 12:819–826.

Duffy MJ. 2002. Urokinase-type plasminogen activator: A potent marker of metastatic potential in human cancers. Biochem Soc Trans 30:207–210.

Escot C, Theillet C, Lidereau R, Spyratos F, Champeme MH, Gest J, Callahan R. 1986. Genetic alteration of the c-myc protooncogene (MYC) in human primary breast carcinomas. Proc Natl Acad Sci USA 83:4834–4838.

Fan C, Oh DS, Wessels L, Weigelt B, Nuyten DS, Nobel AB, van't Veer LJ, Perou CM. 2006. Concordance among gene-expression-based predictors for breast cancer. N Engl J Med 355:560–569.

Feldman RJ, Sementchenko VI, Gayed M, Fraig MM, Watson DK. 2003. Pdef expression in human breast cancer is correlated with invasive potential and altered gene expression. Cancer Res 63:4626–4631.

Fournier MV, Martin KJ, Kenny PA, Xhaja K, Bosch I, Yaswen P, Bissell MJ. 2006. Gene expression signature in organized and growth-arrested mammary acini predicts good outcome in breast cancer. Cancer Res 66: 7095–7102.

Fung YK, T'Ang A. 1992. The role of the retinoblastoma gene in breast cancer development. Cancer Treat Res 61: 59–68.

Galang CK, Muller WJ, Foos G, Oshima RG, Hauser CA. 2004. Changes in the expression of many Ets family transcription factors and of potential target genes in normal mammary tissue and tumors. J Biol Chem 279: 11281–11292.

Ghadersohi A, Pan D, Fayazi Z, Hicks DG, Winston JS, Li F. 2007. Prostate-derived Ets transcription factor (PDEF) downregulates survivin expression and inhibits breast cancer cell growth in vitro and xenograft tumor formation in vivo. Breast Cancer Res Treat 102:19–30.

Hollenhorst PC, Jones DA, Graves BJ. 2004. Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. Nucleic Acids Res 32:5693–5702.

Hsu T, Trojanowska M, Watson DK. 2004. Ets proteins in biological control and cancer. J Cell Biochem 91:896–903.

Ivshina AV, George J, Senko O, Mow B, Putti TC, Smeds J, Lindahl T, Pawitan Y, Hall P, Nordgren H, Wong JE, Liu ET, Bergh J, Kuznetsov VA, Miller LD. 2006. Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer. Cancer Res 66:10292–10301.

Jackers P, Szalai G, Moussa O, Watson DK. 2004. Ets-dependent regulation of target gene expression during megakaryopoiesis. J Biol Chem 279:52183–52190.

Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. 2007. Cancer statistics, 2007. CA Cancer J Clin 57:43–66.

Lee GY, Kenny PA, Lee EH, Bissell MJ. 2007. Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods 4:359–365.

Loi S, Piccart M, Sotiriou C. 2007. The use of gene-expression profiling to better understand the clinical heterogeneity of estrogen receptor positive breast cancers and tamoxifen response. Crit Rev Oncol Hematol 61:187–194.

Luo Y, Cai J, Xue H, Miura T, Rao MS. 2005. Functional SDF1 alpha/CXCR4 signaling in the developing spinal cord. J Neurochem 93:452–462.

Ma Y, Qian Y, Wei L, Abraham J, Shi X, Castranova V, Harner EJ, Flynn DC, Guo L. 2007. Population-based molecular prognosis of breast cancer by transcriptional profiling. Clin Cancer Res 13:2014–2022.

Manavathi B, Rayala SK, Kumar R. 2007. Phosphorylation-dependent regulation of stability and transforming potential of ETS transcriptional factor ESE-1 by p21-activated kinase 1. J Biol Chem 282:19820–19830.

Maroni P, Bendinelli P, Matteucci E, Desiderio MA. 2007. HGF induces CXCR4 and CXCL12-mediated tumor invasion through Ets1 and NF-kappaB. Carcinogenesis 28:267–279.

Nielsen BS, Rank F, Illemann M, Lund LR, Dano K. 2007. Stromal cells associated with early invasive foci in human mammary ductal carcinoma in situ coexpress urokinase and urokinase receptor. Int J Cancer 120: 2086–2095.

O'Brien LE, Zegers MM, Mostov KE. 2002. Opinion: Building epithelial architecture: Insights from three-dimensional culture models. Nat Rev Mol Cell Biol 3:531–537.

Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ. 1992. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc Natl Acad Sci USA 89:9064–9068.

Ranish JA, Yi EC, Leslie DM, Purvine SO, Goodlett DR, Eng J, Aebersold R. 2003. The study of macromolecular complexes by quantitative proteomics. Nat Genet 33: 349–355.

Rochlitz CF, Scott GK, Dodson JM, Liu E, Dollbaum C, Smith HS, Benz CC. 1989. Incidence of activating ras oncogene mutations associated with primary and metastatic human breast cancer. Cancer Res 49:357–360.

Salh B, Marotta A, Wagey R, Sayed M, Pelech S. 2002. Dysregulation of phosphatidylinositol 3-kinase and downstream effectors in human breast cancer. Int J Cancer 98:148–154.

Schuetz CS, Bonin M, Clare SE, Nieselt K, Sotlar K, Walter M, Fehm T, Solomayer E, Riess O, Wallwiener D, Kurek R, Neubauer HJ. 2006. Progression-specific genes identified by expression profiling of matched ductal carcinomas in situ and invasive breast tumors, combining laser capture microdissection and oligonucleotide microarray analysis. Cancer Res 66:5278–5286.

Sementchenko VI, Watson DK. 2000. Ets target genes: Past, present and future. Oncogene 19:6533–6548.

Seth A, Watson DK. 2005. ETS transcription factors and their emerging roles in human cancer. Eur J Cancer 41:2462–2478.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A., et al. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707–712.

Toole BP. 2003. Emmprin (CD147), a cell surface regulator of matrix metalloproteinase production and function. Curr Top Dev Biol 54:371–389.

Tugores A, Le J, Sorokina I, Snijders AJ, Duyao M, Reddy PS, Carlee L, Ronshaugen M, Mushegian A, Watanaskul T, Chu S, Buckler A, Emtage S, McCormick MK. 2001. The epithelium-specific ETS protein EHF/ESE-3 is a context-dependent transcriptional repressor downstream of MAPK signaling cascades. J Biol Chem 276: 20397–20406.

Turner DP, Moussa O, Sauane M, Fisher PB, Watson DK. 2007. Prostate-derived ETS factor is a mediator of metastatic potential through the inhibition of migration and invasion in breast cancer. Cancer Res 67:1618–1625.

van Kempen LC, de Visser KE, Coussens LM. 2006. Inflammation, proteases and cancer. Eur J Cancer 42:728–734.

Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, Turley EA, Bissell MJ. 2002. Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. J Natl Cancer Inst 94:1494–1503.

Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J. 2004. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 64:7022–7029.

Yarden Y, Sliwkowski MX. 2001. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2:127–137.

Zhan Y, Brown C, Maynard E, Anshelevich A, Ni W, Ho IC, Oettgen P. 2005. Ets-1 is a critical regulator of Ang II-mediated vascular inflammation and remodeling. J Clin Invest 115:2508–2516.

Zhou J, Ng AY, Tymms MJ, Jermiin LS, Seth AK, Thomas RS, Kola I. 1998. A novel transcription factor, ELF5, belongs to the ELF subfamily of ETS genes and maps to human chromosome 11p13-15, a region subject to LOH and rearrangement in human carcinoma cell lines. Oncogene 17:2719–2732.