

THE TRANSCRIPTION FACTOR ETS-1 IN BREAST CANCER

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1. ABSTRACT

The proto-oncogene Ets-1 is a member of the Ets family of transcription factors which share a unique DNA binding domain, the Ets domain. Ets binding sites have been described on the promoters or enhancers of many proteinases and several Ets members transcriptionally regulate such promoters in transient cotransfection assays. Ets-1 is involved in both normal and pathological functions. Ets-1 is expressed in a variety of cells, including endothelial cells, vascular smooth muscle cells and epithelial cells. Ets-1 regulates the expression of several angiogenic and extracellular matrix remodeling factors promoting an invasive phenotype. The Ets family of transcription factors may play a role in the disease progression of breast cancer. In tumors, including breast neoplasia, Ets-1 expression is indicative of poorer prognosis. This review will summarize the role of Ets-1 in both the tumor cells, and the tumor endothelial cells as it relates to breast tumor growth and spread.

2. INTRODUCTION

The Ets family of transcription factors makes up a class of trans-acting phosphoproteins shown to have important roles in cell proliferation, differentiation, and oncogenic transformation (1). Ets proteins constitute a family of transcriptional factors that have now been implicated in the regulation of several proteinases, amongst other genes. The name "Ets" arises from an avian erythroblastosis virus, E26 sequence, and the name was later changed to E26 transformation specific sequence or Ets (2). Thirty members of the Ets family have been isolated which share a highly conserved DNA binding domain implicated in the recognition and binding to the PEA3 DNA sequence. Ets binding sites have been described on the promoters or enhancers of many proteinases and several Ets members transcriptionally regulate such promoters in transient cotransfection assays (3). The Ets transcription factor family is divided into subfamilies (Ets-1 and Ets-2, ERG, GABP, PEA3, ELK, ELF, and PU), based mainly on the sequence and location of the Ets domain, an 84-amino acid sequence present in all members of the family. Ets proteins bind to DNA

sequences having the core motif C/AGCAA/T (4). Ets transcription factors play a role in a variety of physiological and pathological processes, including embryogenesis, wound healing, and tumor progression. Their role in these processes is largely due to their ability to activate the transcription of several proteases, including urokinase plasminogen activator, collagenase I (MMP-1), stromelysin I (MMP-3), and gelatinase B (MMP-9) as well as integrin beta 3 (4). In addition to the functional Ets-1 binding sites found in proteases they have also been identified in other promoters including the promoters of genes encoding transcription factors such as JunB, growth factor receptors such as vascular endothelial growth factor (VEGF) receptor 1 (VEGFR-1), Tie-1 and Tie-2, matrix degrading proteases and their inhibitors and adhesion molecules such as beta 2 integrin, ICAM and VE-cadherin (5).

Ets-1 is usually localized in the nucleus, although it has been detected in the cytoplasm of quiescent endothelial cells and endometrial and ovarian cancer cells (6-8). The nuclear localization sequence for Ets-1 resides on its Ets domain as deletion of the C-terminal part of the Ets domain excludes Ets-1 from the nucleus (9).

Ets-1 is not ubiquitously expressed in embryonic and adult tissues. Rather, there is a restricted expression of Ets-1 during specific invasive processes, including the formation of new blood vessels during normal and pathological development (10). In embryos, Ets-1 is expressed in the blood islands of the yolk sac and in endothelial cells (EC). This embryonic expression is detected in ECs during both vasculogenesis and angiogenesis. In adults, Ets-1 is expressed in ECs during wound healing, tumor angiogenesis, and during re-endothelialization following denuding injury (11). In agreement with its expression during new blood vessel formation but not in mature vessels, *in vitro* studies found Ets-1 mRNA expression to be higher in proliferating but not confluent human EC (12). Ets-1 mRNA levels have been shown to be increased upon treatment with angiogenic factors such as tumor necrosis factor 1, phorbol myristate acetate, fibroblast growth factor (FGF) and VEGF (13).

Ets-1 binding sites have been identified in numerous promoters of genes that are involved in angiogenesis, including the VEGF receptors, Flt-1 and Flk-1 (14,15). These results suggest that Ets-1 can be involved in angiogenesis associated with both normal development and tumor growth. Recently, Ets-1 deficient mice have been obtained by homologous recombination (16). The existence of viable ets-1 *-/-* mice suggests that Ets-1 is not essential for blood vessel formation in the embryo. Redundancy between the thirty Ets family members may account for this result, for example, the expression pattern of Ets-1 overlaps the patterns of Erg and Fli but not Ets-2, in endothelial cells during early embryonic development (5,17). Ets-1 over expression converts ECs from the quiescent to the invasive, angiogenic phenotype by inducing the expression of MMP-1, -3, and -9, and integrin beta 3 (18). Numerous studies support Ets having a role in regulating those genes involved in normal and pathological invasive processes.

3.1. ETS-1 and breast cancer cells

In addition to playing an important role in mammalian development, Ets family members have been directly implicated in the pathogenesis of a spectrum of human cancers. Ets-1 is produced by a variety of solid tumors, including epithelial, sarcomas and astrocytomas (for review 19). Dependent on tumor type, Ets-1 expression is either increased or exclusively found in invasive higher grade tumors: higher Ets-1 levels correlates with poor prognosis in cancer of the breast, ovary and cervix (20-22). Ets involvement in human malignancy is notable for its pleiotropic structural and functional contributions to the malignant phenotype (23). Given the crucial role of proteolytic enzymes in tumor invasion, *in vitro* studies suggest a possible role of Ets-mediated transcriptional regulation in metastatic progression (23). Supporting the relevance of Ets in this scheme *in vivo*, *in situ* hybridization studies on several types of cancer, including breast, lung, colon, pancreatic and thyroid, have demonstrated a clear association between tumor progression and Ets-1 expression, with higher expression in invasive cancers than in normal or benign tissues (19). Interestingly, in most cases, Ets-1 expression was predominantly found in the stromal component of the tumors, involving mainly peritumoral fibroblasts, but also endothelial cells and lymphocytes (3). The Ets-1 gene is amplified and overexpressed in myelodysplastic syndrome, an invasive proliferative disease. With regard to breast cancer, Ets-1 protein expression has been documented in both human breast cancer cells of established cell lines and of primary tumor isolates (24). As with the normal invasive processes like angiogenesis there is evidence that Ets proteins regulate genes involved in tumor invasion and metastasis (24). Ets-1 would seem to be responsible for transcriptional activation of those genes necessary for the increased mobility of cells.

The transcription factor Ets-1 regulates the expression of several angiogenic and extracellular matrix remodeling factors, and might be implicated in disease progression of breast cancer. The prognostic value of Ets-1 expression was assessed by RT-PCR in sporadic primary

breast cancer samples of patients with a median follow-up time of 62 months. Ets-1 expression levels correlated significantly with VEGF and PAI-1 in the same tissue. In both univariate and multivariate analyses Ets-1 expression showed significant prognostic value for relapse-free survival. Ets-1 is also a strong, independent predictor for poor prognosis in breast cancer. This seems, in part, to be attributable to its role in transcriptional regulation of specific factors involved in angiogenesis (VEGF) and extracellular matrix remodeling (PAI-1) (21). In order to demonstrate a topographical *in vivo* correlation between the expression of other important matrix proteins (MMP-1, MMP-9) and Ets-1, their expression was studied during breast cancer formation. All three genes were first expressed within both EC and stromal fibroblasts during the onset of stroma generation around intraductal and intralobular *in situ* carcinomas and they were significantly up-regulated in the stroma of invasive ductal and lobular cancers. The results of this study further supports the suggested *in vivo* role of Ets-1 in two coordinated invasive processes, angiogenesis and tumor invasion. Ets facilitates cancer progression by the sustained activation of matrix-degrading proteases which are initially expressed during the early stages of breast carcinogenesis (25). It could be speculated that extracellular matrix degradation products may in some way serve as inducers of Ets-1 and as such contribute to the facilitation of cancer progression.

We have successfully demonstrated that several breast cancer cell lines express Ets-1 mRNA and protein. These include the murine breast cancer cells 168FARN and 4T1 (26) and the human MCF-7 cells. E₂ induces an increase in expression of Ets-1 in all these cells lines in a time and dose dependent manner. Significant E₂-induced increases in both Ets-1 mRNA and protein are evident within 2 hours of treatment.

Breast tumor progression involves several other Ets-1 responsive genes. Among them is parathyroid-related peptide (PTHrP), which is expressed by many tumors and induces hypercalcaemia of malignancy (27). In addition to being a potent angiogenic factor, PTHrP also promotes the proliferative expansion of metastatic breast cancer cells in the bone by inducing bone degradation (28). This degradation leads to activation of TGF β which further stimulates PTHrP. The effect of TGF β on PTHrP involved a synergistic action between Ets-1 and Smad3 and is weakened by inhibitors that suppress Ets-1 expression (29). Several members of the ETS family of transcription factors contribute to tumorigenesis in many different tissues, including breast epithelium. The ESX gene is an epithelial-specific Ets member that is particularly relevant to breast cancer. ESX is amplified in early breast cancers, it is overexpressed in human breast ductal carcinoma *in situ* and there may be a positive feedback loop between the HER2/neu proto-oncogene and ESX. Recent studies have shown that ESX is able to differentially activate several malignancy-associated gene promoters which include ER81, HA-Ets-2, Ets-1, and EHF. ESX expression was also required for (*in vitro* or *in vivo*) cellular survival of non-transformed MCF-12A and transformed T47D human mammary cells (1).

Ets-1 and breast cancer

Ets-1 is upregulated both *in vivo* and *in vitro* in malignant melanoma, compared to benign melanocytic lesions and to primary melanocytes. Assessment of DNA-binding transactivation assays documented a strong Ets activity in melanoma cells. The invasive potential of the melanoma cells measured in a Boyden Chamber model was reduced up to 60 % by Ets-1 blockade. This can be attributed to the role of Ets-1 in transcriptional regulation of factors involved in invasion of melanoma cells (30).

Like ESX, PEA3, Ets-1, PDEF and ELF-3 transcripts have all been reported to be elevated in human breast tumors. Some of the Ets genes that are overexpressed in human breast tumors are also overexpressed in mouse models of this disease, notably *pea3*, as well as close paralogs ER81 and ERM. ER81 and ERM comprise the *pea3* subfamily of Ets genes and are coordinately overexpressed in mouse mammary tumors (31). Genetic analysis in mice reveal required roles for one or more of the *pea3* subfamily of Ets protein in the initiation and progression of mouse mammary tumors. The *pea3* subfamily genes are normally expressed in the primitive epithelium of mouse mammary buds during embryogenesis, and these three genes are expressed in epithelial progenitor cells during postnatal mammary gland development. Loss-of-function mutations in the mouse *pea3* gene results in increased numbers of terminal end buds and an increased fraction of proliferating cells in these structures, suggesting a role for *pea3* in progenitor cell renewal or terminal differentiation. Taken together these observations suggest that the *pea3* subfamily proteins play key regulatory roles in both mammary gland development and oncogenesis (31).

Ets-1 and AP-1 transcription factors have been shown to cooperate in a rat mammary cell line in stimulating transcription of osteopontin (OPN). Also, the independent presence of these transcription factors is associated with that of OPN in a group of human breast cancers. The presence of these transcription factors in human breast cancer is responsible in part, for the overexpression of OPN which in turn, is implicated in mammary neoplastic progression and metastasis (32). Urokinase plasminogen activator (uPA) has been associated with invasion and metastasis in breast cancer (33). Reporter transfection assays revealed that activation of uPA and MMP-9 collagenase promoters by EGF required the composite Ets and AP-1 transcription factor binding sites for an EGF response. Most notably, transfections with the Ets-1 and Ets-2 expression vectors potentiated uPA and MMP-9 promoter activation in response to EGF. These results suggest that transcription factors Ets-1 and Ets-2 provide the link, connecting EGF stimuli with activation of uPA and the 92 kD type IV collagenase promoters and may contribute to invasion phenotype (33).

3.2 . ETS-1 and tumor endothelial cells

Ets-1 is transiently expressed in the two main blood vessel forming cell types, endothelial cells (ECs) and vascular smooth muscle cells (VSMC), when activated by the angiogenic factors, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiotensin

II, endothelin-1, tumor necrosis factor α or hydrogen peroxide (34-38). Activated ECs and VSMCs result in proliferation, migration and invasion of these cells. Inhibition of Ets-1 expression by transdominant negative Ets-1 mutant proteins or by anti-sense oligonucleotides directed against Ets-1 abrogated the ability of ECs to migrate, to adopt an invasive behavior or to form tubes in response to angiogenic growth factors. Constitutive expression of Ets-1, conversely, will mimic angiogenic stimulation and induced EC invasiveness. Tumor-induced angiogenesis involves increased Ets-1 in vascular stroma of cancerous lesions, and often correlates with tumor microvessel density (20,39,40).

Host endothelial cells are central to the process of tumor angiogenesis. The local EC/tumor microenvironment provides both diffusible (i.e., growth factors) and solid-state signals (i.e., extracellular matrix (ECM) proteins) that modulate the EC phenotype (quiescent vs. migratory). These molecular components also influence the process of EC sprouting, the first in a series of steps essential for new vessel formation.

The Ets-1 protein has oncogenic potential. Ets-1 is encoded by the *c-ets-1* proto-oncogene. Ets-1 is required for the transactivation of several genes encoding proteases involved in matrix degradation, such as MMP1, 2, 3, and 9 as well as uPA. All these proteases are necessary for matrix degradation which is required during both tumor invasion and the early phases of blood vessel formation. Ets-1 is transiently expressed within EC during tumor angiogenesis and is down regulated when vessel formation is completed. VEGF and bFGF, strong modulators of EC proliferation, induce Ets-1 in cultured EC (25).

The increased expression of the beta 3 integrin is important as $\alpha v \beta 3$ is associated with angiogenesis and is a ligand for PECAM-1 (CD-31), a glycoprotein involved in cell-cell junctions. Immunohistochemical staining demonstrated Ets-1 protein preferentially in the nucleus of those ECs with an epithelioid morphology consistent with an activated state, whereas quiescent flat-shaped ECs predominantly displayed cytosolic immunoreactivity (41).

On Matrigel, EC lines formed a cord-like network within 24h, with an increased ability of Ets1 transdominant negative mutant cells to spread on this substrate. In long-term studies, cells expressing Ets1 transdominant negative mutant showed a higher capacity to form branched structures; this effect was potentiated by FGF2. These results demonstrate a role of the Ets transcription factors in the regulation of the adhesive and morphogenetic properties of EC (5).

We employed an *in vitro* angiogenesis model that simulates the *in vivo* milieu for tumor capillary formation to study the direct effects of estrogen (26). 17 beta-estradiol (E_2) treatment significantly stimulated capillary sprouting within 8h in co-cultures of rat aortic endothelial cells (RAEC) and mouse mammary tumor cells. Co-cultures treated with either progesterone (P_4) or $E_2 + P_4$ showed

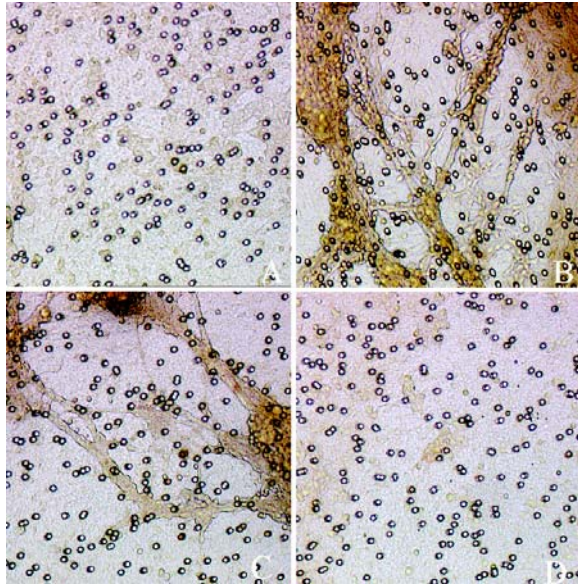


Figure 1. Inhibition of new vessel sprouting. 168FARN murine breast cancer cells were co-cultured with rat aortic ECs in either the absence (A) or presence (B,C,D) of 1×10^{-10} M E_2 for 24h. Cells were transfected with either Ets-1 missense (500nM, C) or Ets-1 antisense (500 nM, D). Note inhibition of complex capillary formation with antisense Ets-1 treatment (D) compared to E_2 -treated (B) or missense transfected (C) co-cultures. Small circles in all images represent membrane pores.(Reproduced with permission, 26).

only minimal endothelial cell (EC) sprouting when compared to E_2 treated cultures. Treatment with the E_2 agonist ICI 182,780 dramatically inhibited capillary formation in a statistically significant manner, demonstrating E_2 -specificity. Within hours of E_2 treatment ECs isolated from tumor cell/EC co-cultures demonstrated a statistically significant increase in both mRNA and protein levels of the transcription factor Ets-1. We observed increased matrix metalloproteinase (MMP) and decreased tissue inhibitor of metalloproteinase (TIMP) mRNA levels in these ECs following E_2 treatment. Ets-1 upregulates expression of the vascular endothelial growth factor (VEGF) receptor, Flt-1 and we detected increased Flt-1 mRNA levels in ECs co-cultured with tumor cells following E_2 treatment. Expression of Ets-1 contributes to destabilization of a quiescent EC phenotype in favor of an invasive angiogenic one, in part, by increasing expression of MMPs and integrin molecules that favor migration and invasion. Transfection of ECs with Ets-1 antisense prior to co-culture with E_2 resulted in a 95% inhibition in capillary formation (figure 1). We demonstrated, for the first time that physiological concentrations of E_2 directly and rapidly induced new capillary formation in a mammary tumor/EC co-culture system and suggest that this response may be mediated, in part, by an E_2 -induced increase in Ets-1 expression.

Other examples of steroid regulated Ets-1 expression are evident during the menstrual cycle, when EC dependent Ets-1 expression increases in the

proliferative phase and drops in the secretory phase, following changes in VEGF expression (42). Also, pregnancy stimulates the expression of Ets-1 in the ECs of the villous trophoblast, but not in maternal ECs (43).

Non steroidal stimulation, related to NO production, has been shown to influence Ets-1 expression. Bovine EC were cultured in type I collagen, with the addition of S-nitroso-N-acetylpenicillamine (SNAP), an NO donor, and stimulated the formation of tube-like structures (44). SNAP increased the expression of Ets-1 in a concentration dependent manner – reaching maximal levels 2h after treatment. The SNAP induced *in vitro* angiogenesis and increased Ets-1 mRNA levels were strongly reduced by the treatment of Ets-1 antisense. These results suggest that NO stimulated *in vitro* angiogenesis through the induction of Ets-1 expression. NO appears to stimulate EC differentiation to the angiogenic phenotype via the induction of Ets-1 transcription factor (44).

4. PERSPECTIVE

Ets-1 is involved in the regulation of invasive behavior of many normal and tumor cells. The ability to instigate an invasive phenotype is an important step for endothelial cells to convert to an angiogenic phenotype. Additionally, Ets-1 is overexpressed in pathological conditions requiring new blood vessel development. The relationship of Ets-1 and invasiveness is not limited to ECs however, VSMCs, epithelial and fibroblastic cells require Ets-1 to become invasive. For epithelial cancers, Ets-1 may serve a dual function: providing the cancer cells with nutrients and oxygen by inducing tumor vascularization and it may promote tumor invasion by activating ECM-degrading proteases in the cancer and/or in stromal cells. As such, high levels of Ets-1 in tumors often correlated with poorer prognosis. It would therefore, be desirable to develop therapies that target the Ets-1 gene.

The regulation of Ets-1 gene activity is rather complex. The current understanding of the function of Ets-1 is based on data partially obtained by methods which may not clearly allow distinction between the involvement of Ets-1 or that of another Ets proteins. Future studies will need to employ techniques such as post-transcriptional silencing by RNA interference, and new DNA binding assays, such as chromatin immunoprecipitation assays to demonstrate direct binding of Ets-1 to the gene of interest *in vivo* (19).

5. ACKNOWLEDGEMENTS

The authors are supported by a VA Merit Review Award (KB), and would like to thank the veterans of their facility who inspire them daily with their kindness and courage.

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Key Words: Ets-1, Breast, Cancer, Endothelial Cells, Gene, Review

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