Award Number:
W81XWH-06-1-0502

TITLE:
Identification of Cytoplasmic Proteins Interacting with the Mammary Cell Transforming Domain of Ese-1

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REPORT DATE:
April 2009

TYPE OF REPORT:
Final

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

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Identification of Cytoplasmic Proteins Interacting with the Mammary Cell Transforming Domain of Ese-1

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The Ets family of transcription factors contains several members that are important components of the cellular pathways leading to tumorigenesis. The Ese-1 gene is an Ets member that is particularly relevant to breast cancer. Ese-1 is located on chromosome 1q32.1, in a region that is amplified in 50% of early breast cancers. Ese-1 mRNA is over-expressed in human breast ductal carcinoma in situ (DCIS). We discovered that Ese-1 transforms MCF-12A cells via a novel cytoplasmic mechanism in which a unique 40-amino acid (AA), serine- and aspartic acid rich (SAR) domain is necessary and sufficient for transformation. While the original goal was to identify cytoplasmic partners of the Ese-1 SAR motif that mediate transformation, due to difficulties, we pursued a more productive avenue of investigation. To this end, we have demonstrated that Ese-1 is required to maintain the transformed state of T47D, MCF-7 and ZR-75-1 breast cancer cells, and that it does so via a nuclear mechanism, by regulating cell proliferation.

ETS, Ese-1, Esx, MCF-12A, transformation, mammary, breast cancer
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INTRODUCTION

The Ets family of transcription factors contains several members that are important components of the cellular pathways leading to tumorigenesis (1). For example, several Ets members are downstream targets of oncogenic Ras (2); dominant-negative Ets reverses the transformed phenotype (3,4); and, Ets proteins have been shown to regulate a repertoire of genes that govern cellular survival, proliferation and migration (1,6). Moreover, several Ets factors have been implicated in breast cancer (1,6). However, the ability of Ets factors to transform human breast cells, the identity of the precise Ets factor required for breast cell transformation, and the molecular mechanism by which such an Ets factor mediates breast cell transformation, all remain unknown.

The ESE-1/ESX gene is an Ets member that is particularly relevant to breast cancer. ESE-1 is located on chromosome 1q32.1, in a region that is amplified in 50% of early breast cancers. ESE-1 mRNA is over-expressed in human breast ductal carcinoma in situ (DCIS) (7-9). Also, there is a positive feedback loop between the HER2/neu proto-oncogene and ESE-1, in that HER2/neu activation induces ESE-1 expression, while ESE-1 activates the HER2/neu promoter via a putative ESE-1 DNA binding site (7-9). Finally, HER2/neu and ESE-1 expression levels are positively correlated in human breast cancer cell lines (7-9). ESE-1 is expressed in both nuclear and cytoplasmic compartments. We discovered that ESE-1 initiates transformation of MCF-12A cells via a novel cytoplasmic mechanism in which a unique 40-amino acid (AA), serine- and aspartic acid rich (SAR) domain is necessary and sufficient for transformation (10-12). Furthermore, we reported that ESE-1 protein is abundantly expressed in the cytoplasm of human ductal carcinoma in situ (DCIS) specimens (12). However, identification of the cytoplasmic partners of the ESE-1 SAR motif and the precise mechanism by which cytoplasmic signaling mediated by the ESE-1 SAR motif occurs remain unknown. Current data reveals that this 40-AA SAR domain functions autonomously, indicating that this motif is sufficient to recognize and activate cytoplasmic partner proteins, thus transforming MCF-12A human mammary epithelial cells (12). Based on these results our original hypothesis was that the ESE-1 SAR motif is a modular functional domain that is required for breast cell transformation and that such a domain functions by interacting with specific cytoplasmic proteins. We proposed three original Specific Aims: (1) To purify and identify SAR-binding cytoplasmic proteins (SBCPs); (2) To validate that the identified SBCP is a bona fide downstream effector of Ese-1-mediated cellular transformation; and, (3) To determine the molecular mechanism of Ese-1/SBCP-mediated mammary cell transformation. Our original goal was to apply innovative and powerful state-of-the-art molecular yeast two-hybrid and protein biophysical methods (MALDI-TOF and LC-mass spectrometry) to identify proteins that interact with the ESE-1 SAR transformation domain. Below, I will include a summary of the approaches and results we obtained addressing these three aims. However, despite spending a considerable amount of time and effort pursuing these studies, we were not able to unambiguously identify a cytoplasmic protein as the key functional ESE-1 interacting partner mediating the transformed phenotype. Thus, we submitted a revised Statement of Work to the DOD DAMD, which was approved, and we shifted our research to a more productive avenue of investigation. In these revised studies, we set out to test the new hypothesis that endogenous ESE-1 is required to maintain the breast cancer transformed phenotype. To this end, we established three new Specific Aims (Revised Aims 1-3), and the progress towards these aims is also summarized below. Importantly, this new avenue of investigation was much more productive. Specifically, we discovered that endogenous ESE-1 is required to maintain the transformed phenotype in two breast cancer cell lines, via a proliferation mechanism and that endogenous ESE-1 is expressed in both cytoplasmic and nuclear
compartments in breast cancer specimens. Below is a summary of the data generated by this new direction.

**BODY A** (In Body A, we will present a summary of our progress towards the original 3 aims. Then in Body B, we will present a detailed summary of the revised and DOD-approved 3 aims).

**Original Task 1:** To purify and identify SAR-binding cytoplasmic proteins (SBCPs).

a) **GST-SAR Affinity purification of SAR-binding cytoplasmic proteins (SBCPs).**

To this end, we prepared cytoplasmic extracts from both nontransformed MCF12A mammary epithelial cells and from fully transformed T47D breast cancer cells. These cytoplasmic extracts were then first chromatographed through GST-only beads, to remove any proteins binding nonspecifically to GST and/or agarose beads. Next, we chromatographed the GST-only flow-through onto GST-only and GST-SAR affinity beads, washed each carefully with 0.1M KCl, then boiled the beads with Laemmli sample buffer and separated bound proteins on a 10% SDS-PAGE gel. The same two candidate bands of 52 kD and 42 kD were identified in both cytoplasmic extracts as binding specifically to GST-SAR vs GST-only. In collaboration with Dr. Natalie Ahn’s group at CU-Boulder, we used MALDI-TOF mass spectrometry to identify the 52 kD and 42 kD protein bands as EF-1α and γ-actin. A repeat GST-SAR affinity chromatography analysis also identified E/R/M (ezrin/radixin/moesin) protein.

b) **Yeast two-hybrid screening for SAR-binding cytoplasmic proteins (SBCPs).**

As an alternative approach to identify the SBCPs, we used the yeast two-hybrid screening approach. First, we established that Gal4-DBD-SAR does not self-activate reporter genes in the AH109 yeast strain. Next, we screened a human fetal brain cDNA library fused to the Gal4-AD (2.5x10⁶ clones; 74% of the human fetal brain library) with the Gal4-DBD-SAR bait protein, by mating these two yeast strains and plating on –TDO (-His, -Lue, -Trp) media. Library-bait interactions were tested on the following nutrient deprived media: –TDO (-His, -Trp, -Leu), –QDO (-Ade and -TDO), 25 mM 3-AT/–TDO, and x-α-gal/–TDO plates. We archived 321 clones that grew on initial selection plates (-His, -Trp, -Leu), and 79 of the 321 clones grew on either –QDO and/or screened successfully on x-α-gal/–TDO plates. We next focused on yeast colonies that grew with a diameter of 3 mm on –QDO plates and/or showing α-galactosidase activity on –TDO media. Only 11 of the 76 clones met this final criterion, and DNA sequence analysis and BLAT searches identified three plausible candidates: Rho Guanine Dissociation Inhibitor (Rho GDI), Na/K ATPase β-1, and a proposed Breast Cancer Associated protein (BCA3).

**Original Task 2:** To validate that the identified SBCP is a bona fide downstream effector of ESE-1-mediated cellular transformation.

a) **Co-immunoprecipitation (co-IP) of putative SAR-binding cytoplasmic proteins (SBCPs) with endogenous ESE-1.**

To determine whether any of the putative SBCPs identified by either GST-SAR affinity chromatography or yeast two-hybrid screening might function as bona fide downstream effectors of ESE-1-mediated transformation, we first addressed whether these proteins interact with endogenous ESE-1 by using a co-IP approach. However, we could not demonstrate that any of these putative SBCPs (EF-1α, γ-Actin, E/R/M, Rho GDO, Na/K ATPase, or BCA3) could specifically bind to the SAR domain, since none were able to co-immunoprecipitate with endogenous ESE-1. Instead, we found that most of these proteins co-immunoprecipitated with the transfected nonspecific control, GFP-only. Finally, the MALDI literature repeatedly identified EF-1α and γ-Actin as common nonspecific contaminants.
Original Task 3: To determine the molecular mechanism of ESE-1/SBCP-mediated mammary cell transformation.

Given that we could not unambiguously identify bona fide SAR-binding cytoplasmic proteins (SBCPs) in the previous two aims, we were unable to progress to this aim. However, we did attempt to perform structural studies of the SAR domain, to better understand how it might serve as a protein interaction motif. To this end, we purified recombinant GST-SAR protein and cleaved GST off, purified the SAR peptide and then performed circular dichroism structural studies. We determined that the SAR domain does not have any detectable secondary structure, even in the presence of trifluoroethanol.

BODY B (Revised Aims)

ETS factors comprise a large transcription factor family known to play a significant role in cellular development, differentiation, and transformation. Emerging evidence reveals that increased mRNA expression of the human Ets factor, ESE-1, is associated with breast cancer. Stable expression of ESE-1 transforms MCF-12A immortalized human mammary epithelial cells. However, little is known about ESE-1 protein expression and its role in maintaining the transformed phenotype in human breast cancer cell lines. We used an anti-ESE-1 mouse monoclonal antibody in Western blot and immunofluorescent cell analyses to show that ESE-1 is expressed as a nuclear protein in MCF-7, T47D and ZR-75 transformed, tumorigenic mammary epithelial cell lines, and that it is not expressed in transformed MDA-MB-231 and nontransformed MCF-10A and MCF-12A cells. In addition, specific knockdown of endogenous ESE-1 in the human breast carcinoma ZR-75 and MCF-7 cell lines decreased colony formation and anchorage independent growth. Mechanistically, ESE-1 knockdown decreased cellular proliferation, but had no effect on apoptosis. Finally, serum withdrawal resulted in a time-dependent, ~90% reduction of ESE-1 protein production in MCF-7 cells. These results establish that ESE-1 plays a key role in maintaining the transformed phenotype in breast cancer, thus providing a novel single-point target for breast cancer therapy.

Revised Task 1: To characterize ESE-1 expression in human breast cancer cells and to identify shRNAs capable of knocking-down endogenous ESE-1.

b) Untransformed MCF-10A and MCF-12A cells fail to express endogenous ESE-1, whereas MCF-7, T47D and ZR-75 breast cancer cell lines express ESE-1.

Using a small-grant support mechanism provided by our Cancer Center, we developed several novel, high-affinity, high-specificity murine monoclonal antibodies targeting amino acids 128-259, spanning the transcription activation domain (TAD), SAR, and AT-hook domains of human ESE-1 protein. The manuscript describing the generation and characterization of these antibodies is currently in preparation. For the studies in this report, we used one of these antibodies: anti-ESE-1 mAB405. Western blot analysis of whole cell extracts probing for endogenous ESE-1 was performed on a series of human transformed and nontransformed mammary epithelial cell lines and compared with qRT-PCR. As shown in Fig. 1, ESE-1 protein was not detected by Western blot analysis in the nontransformed MCF-10A and MCF-12A mammary epithelial cells lines. In contrast, ESE-1 protein was
detected in the tumorigenic MCF-7, T47D and ZR-75 cell lines, with the levels in MCF-7 and T47D being equivalent and greater than that expressed in ZR-75. Noteworthy, ESE-1 protein was not detectable in the highly metastatic MDA-MB-231 cells (Fig. 1A). Quantitative RT-PCR mRNA analysis validated these protein data (data not shown). To further confirm ESE-1 protein expression levels and its subcellular localization, we performed indirect immunofluorescence cytochemistry (ICC) studies, with cell nuclei counterstained with DAPI to define each cell (Fig. 2). These data revealed that MCF-10A, MCF-12A and MDA-MB-231 cells fail to express any ESE-1 protein detectable by this ICC method, whereas endogenous ESE-1 protein was detected in transformed MCF-7, T47D and ZR-75, and in each case ESE-1 was localized to the nucleus (Fig. 2). In general, the ICC data further confirmed the Western blot data and revealed ESE-1 protein to be localized in the nucleus.

c) **shRNA targeting of ESE-1 knocks down endogenous ESE-1 protein expression.**

In initial optimization studies of several shESE-1 constructs, we identified an shESE-1 construct (shESE-1), which targeted the ETS DBD of ESE-1, that optimally knocked down endogenous ESE-1 (data not shown). Using shESE-1, we established the time course of ESE-1 knockdown by transiently transfecting ZR-75 cells with shESE-1 and preparing whole cell lysates 2, 3, 4 and 5 days post-transfection (Fig. 3A). As a control, we transfected cells with an shRNA empty vector control (vector) and prepared whole cell lysates 2 days post-transfection (Fig. 3A). The whole cell lysates were then probed for ESE-1 and tubulin by Western blot analysis (Fig. 3A). These results show that compared to vector control, essentially a complete knockdown of ESE-1 occurs by 2 days, and a significant reduction of ESE-1 persists up to 5 days post-transfection of shESE-1 (Fig. 3A). The tubulin control shows that a nearly equivalent amount of protein was loaded in each lane. A similar time course of shESE-1 knockdown was performed in MCF-7 cells and this study
showed the same complete reduction of ESE-1 by 2 days, but in MCF-7 cells, this strong level of inhibition persisted up to 5 days (data not shown). Next, we sought to establish the specificity of ESE-1 knockdown. As noted above, this shESE-1 targeted the ETS DBD, which is conserved amongst ETS proteins. Computational analysis of the shESE-1 target sequence revealed it to be unique to ESE-1, with our target sequence showing minimal similarity only to ETS-1/ETS-2 (with only 4 of 19 nt being identical for each). As a negative control (shCtr), we used an shRNA construct that also targeted the ESE-1 ETS DBD, but which in optimization studies failed to inhibit ESE-1 expression. We transiently transfected MCF-7 cells with shCtr and shESE-1 shRNA vectors, prepared whole cell lysates 2 days post-transfection, and probed for ESE-1, Ets-1/Ets-2, and tubulin by Western blot analysis (Fig. 3B). This study reveals that the shCtr failed to inhibit endogenous ESE-1, while the shESE-1 vector resulted in a robust knockdown of ESE-1 in MCF-7 cells. We quantitated this inhibition by normalizing ESE-1 expression to tubulin and found that ESE-1 expression is reduced ~4-fold in the shESE-1 cells compared to shCtr. Finally, to determine the specificity of shESE-1 knockdown, we performed Western blot analysis for both ETS-1 and ETS-2, using an antibody that recognizes both ETS factors. As shown in Fig. 3B, neither the shCtr nor shESE-1 affected the levels of ETS-1 plus ETS-2, affirming shESE-1’s specificity to knockdown endogenous ESE-1, and that the shCtr failed to inhibit ESE-1, ETS-1 and ETS-2.

Revised Task 2. To determine whether ESE-1 knockdown in human breast cancer cell lines reverses the transformed phenotype.

a) Knockdown of ESE-1 reduces the clonal cell growth and soft agar colony-forming ability of ZR-75 and MCF-7 cells

In order to determine if ESE-1 was necessary for colony formation, ZR-75 and MCF-7 cells were each co-transfected with pEGFP-C3, to confer G418 resistance, and shCtr or shESE-1. Transfected cells were selected with G-418 for 14 days, resultant colonies were stained with Crystal violet, and counted by direct visualization. Shown are the results of the MCF-7 transfection revealing ~27 colonies per shCtr plate and only ~6 colonies formed in the presence of shESE-1, resulting in a 5.5-fold reduction in MCF-7 colony formation (Fig. 4A). Transfection of ZR-75 cells yielded ~43 and 3 cells with shCtr and shESE-1, respectively, resulting in a 13-fold reduction in ZR-75 colony formation (data not shown). Of note, several attempts to generate stable ESE-1 knockdown cell lines resulted in few, small colonies that failed to grow, precluding clonal expansion. Importantly, the similar inhibitory effect of ESE-1 knockdown on colony formation in two distinct breast cancer cell lines supports the critical role of ESE-1 in the growth of transformed mammary cells.

To further investigate the functional role of ESE-1 in the tumorigenic phenotype, we performed similar shRNA knockdown studies of ESE-1 and performed soft agar experiments using MCF-7 cells. We chose to focus on
MCF-7 cells for these soft agar experiments, because MCF-7 cells yielded larger colonies transient transfection resulted in a more effective and prolonged knockdown of endogenous ESE-1 (data not shown). This more prolonged knockdown after MCF-7 transient transfection is important, since the soft agar assay is over 14 days and we could not generate stable shESE-1 knockdown cells to then plate in soft agar. Because in the transient transfection approach not all cells are transfected and ESE-1 expression is likely to re-appear at later time points, compared to G418 selection methods, the resulting colony number in the shESE-1 knockdown cells presented here is likely an overestimate. Thus, we transiently transfected MCF-7 cells with shCtr or shESE-1 vector DNAs, plated the cells in soft agar and after 14 days counted the colonies growing in an anchorage independent manner (Fig. 4B). MCF-7 cells transfected with shCtr generated ~800 colonies, whereas shESE-1-transfected cells generated ~350 colonies, a 56% reduction in colony formation (Fig. 4b, plated in sextuplicate). A separate study showed that shESE-1 mediated a 64% reduction in MCF-7 soft agar colony number, with MCF-7 cells transiently transfected with shCTR- and shESE-1 yielding 575 and 209 soft agar colonies, respectively (data not shown). Cloning efficiency was determined by dividing the number of cells seeded by the number of colonies formed times 100. Control cells yielded a cloning efficiency of 1.6%, while shESE-1 cells had a cloning efficiency of 0.7%. Thus, taken together, these assays show that ESE-1 is required to maintain the tumorigenic phenotype of MCF-7 cells.

Revised Task 3: To determine the molecular mechanism of by which knock-down of endogenous ESE-1 regulates the transformed phenotype.

a) Reversion of the Transformed Phenotype is not due to Apoptosis

Having demonstrated a reduction in colony formation and anchorage independent growth, we next sought to address the mechanism responsible for the reversion of the malignant phenotype. We first tested whether knockdown of ESE-1 in MCF-7 cells resulted in apoptosis. In order to address this point, MCF-7 cells were transiently transfected with shCtr or shESE-1, and harvested at 48 and 72 hours. DNA laddering assay failed to show any shESE-1 induced apoptosis (data not shown). To further investigate apoptosis as a biological response to ESE-1 knockdown, we analyzed caspase 3 and/or 7 activation, using a luminescent enzyme activity assay (CaspaseGlo 3/7 Assay, Promega). With the data set to 1 for untransfected controls, these results show that there is no change in caspase 3/7 activity in shESE-1 transfected cells, compared to empty vector and shCtr transfected cells, at either the 48 (data not shown) or 72
hour time points (Fig. 5). Having excluded apoptosis as the cellular mechanism responsible for the shESE-1-induced inhibition of colony formation, we next sought to determine whether shESE-1 affected MCF-7 cellular proliferation.

b) **Reversion of the Transformed Phenotype is due to shESE-1-mediated Inhibition of MCF-7 Cell Proliferation**

The control of cellular proliferation is a key mechanism in the prevention of tumorgenicity and malignancy. ESE-1’s ability to transcriptionally regulate the growth-promoting Her2/neu and TGF-βRII receptors genes suggests that it has an important role in controlling cellular proliferation. In order to confirm ESE-1’s role in maintaining cellular proliferation in MCF-7 transformed cells, MCF-7 cells were transiently transfected with shCtr or shESE-1, and cellular proliferation was determined at 2-, 4- and 6-days post-transfection by counting total viable cells (Fig. 6). A representative total cell proliferation study, performed in duplicate, shows that shESE-1 cells display significantly reduced proliferation at each time point (Fig. 6). At the start of the study, 50,000 cells were plated, and the results reveal a 62%, 78% and 66% reduction in cell proliferation at 2- 4- and 6-days, respectively, in the shESE-1 knockdown cells compared to shCtr cells (Fig. 6). Similar results were obtained using an MTS proliferation assay, which showed a 1.6 fold (or ~62%) reduction in MTS absorbance at 6-days post-transfection in the shESE-1 transiently transfected MCF-7 cells, compared to shCtr control cells (data not shown). These data demonstrate that ESE-1 is required for optimal MCF-7 cellular proliferation and reveal the mechanism by which ESE-1 contributes to the transformed phenotype.

c) **Serum starvation extinguishes ESE-1 protein expression in MCF-7 cells**

Having shown that MCF-7 cells express endogenous ESE-1 protein (Fig. 1) and that ESE-1 expression is required to maintain the MCF-7 transformed phenotype by controlling cellular proliferation (Figs. 4-6), we sought to determine whether ESE-1 protein production was growth factor dependent. This question is relevant because ESE-1 protein regulates HER2/Neu promoter activity (10) and HER2/Neu has been shown to activate the ESE-1 promoter, suggesting that growth factors present in serum may play a critical role in regulating ESE-1 gene expression and ESE-1-mediated mammary cell proliferation. Here, we grew MCF-7 cells in either complete (10% FBS) or in serum-depleted (0.1% FBS) media for 6, 18 and 36 hrs, and then probed for ESE-1 and tubulin protein expression by Western blot analysis of whole cell extracts. As shown in Fig. 7, ESE-1 protein appears to increase slightly at the 6 hr post-starvation time-point, only to decrease progressively at the 18 and 36 hr post-starvation time-points, compared to complete media controls. Quantitation of the 36 hr post-starvation time-point revealed a 90% reduction of ESE-1 protein expression, when normalized to tubulin and compared to the 36 hr complete media control. The Western blot for tubulin reveals that protein loading was equivalent in all lanes. These data raise the interesting possibility that reduction in MCF-7 cell proliferation due to serum starvation may be due, at least in part, to reduced ESE-1 protein expression.
**Revised Task 4:** To identify cytoplasmic and nuclear ESE-1-interacting proteins.

a) *Identification of SAR-interacting cytoplasmic protein(s) and ESE-1-binding nuclear factors*

This was the original goal of this project, as noted above. This study has been frustratingly difficult, since we’ve applied SAR-affinity purification, yeast 2-hybrid and co-IP approaches to no avail. However, if funding become available, we plan to return to these studies, particularly since we generated two new high-affinity, highly-specific monoclonal antibodies targeting distinct ESE-1 domains, both of which IP endogenous ESE-1.

b) *Identification of cytoplasmic and nuclear expression of ESE-1 in breast cancer tissue microarrays.*

Although we were not able to identify SBCPs, we did use the monoclonal antibodies against ESE-1 that we generated to assess levels and subcellular localization of ESE-1 expression in human breast cancer specimens. Specifically, we have used anti-ESE-1 mAb405 and mAb1534 in preliminary IHC studies, optimizing dilution and antigen retrieval methods. As shown in Fig. 8, mAb405 at 1:1000 dilution shows ESE-1 signal (brown DAB ppt) in the nuclei and cytoplasm of breast cancer cells metastatic to the lymph node (LN: top R panel). In contrast, only a minimal, scattered ESE-1 signal is noted in normal MECs (lower R panel). The ESE-1 signal is specific, since a control IgG primary fails to yield any DAB signal at all (left panels), and not all cells are positive in the anti-ESE-1 panels.

**KEY RESEARCH ACCOMPLISHEMENTS**

- Generation of shESE-1 constructs that successfully knock-down endogenous human ESE-1.
- Identification of endogenous ESE-1 as being required to maintain the transformed phenotype of MCF-7 and ZR-75 human mammary epithelial cells.
- Demonstration that the mechanism by which ESE-1 maintains the transformed phenotype is due to its requirement for cell proliferation.
- ESE-1 appears to contribute to the serum-induced proliferative response in breast cancer cells.

**REPORTABLE OUTCOMES**

*Abstracts:*


Manuscripts


Reagents Developed
1. shEse-1 knock-down vectors.

2. We also generated a number of reagents relevant to our original aims, including a number of Flag epitope-tagged SAR and ESE-1 constructs.

Doctoral training
1. Darius Walker, an Under-Represented Minority graduate student working on this project, completed his PhD thesis studies in the Molecular Biology Graduate Program.

Awards
1. Arthur Gutierrez-Hartmann, PI, was selected for the prestigious 2008 AACR-Minorities in Cancer Research - Jane Cooke Wright Lectureship Award.

2. Arthur Gutierrez-Hartmann, PI, was selected for the prestigious 2009 Endocrine Society Distinguished Educator Award.

3. Arthur Gutierrez-Hartmann, PI, was selected for the prestigious 2009 Keynote Speaker, Annual Meeting of the Network of Minority Research Investigators, NIDDK/NIH, Bethesda, MD.

New Grants
1. I have used the data generated with this DOD support to write new grant applications for:
a. R01 to NIH/NCI (Fall, 2008): This application was reviewed and scored, and received a competitive but nonfundable score. I plan to re-submit this July, 2010.


c. DOD Idea (Spring, 2009 and 2010): The 2009 application was not funded and the 2010 application was recently submitted.

d. DOD Concept Award (Spring, 2010): Recently submitted.

CONCLUSIONS

The ETS transcription factor family is known to play a significant role in many cancers, with aberrant expression of ESE-1 being correlated in nearly 50% of early human breast tumors. Previously, we have shown that stably expressed HA-ESE-1 or GFP-ESE-1 imposes the transformed phenotype on nontransformed, ESE-1-negative MCF-10A and MCF-12A cells (10-12), and that cytoplasmic localization of a 40-AA SAR domain is necessary and sufficient to mediate this effect (12). Consistent with this conclusion, PAK-1-mediated phosphorylation of the SAR domain of exogenous ESE-1 in the cytoplasm modulates stability and transformation potency of ESE-1 in ZR-75 cells (13). Thus, it is clear that the SAR domain of ESE-1 acts via cytoplasmic components to initiate the transformed state. While the original goal of this DOD Idea Award was to apply innovative and powerful state-of-the-art mass spectrometry, yeast two-hybrid and co-IP methods to identify proteins that interact with the ESE-1 SAR transformation domain, it became evident that despite multiple attempts, none of the putative SAR-binding cytoplasmic proteins that we identified by these approaches were bona fide. This raises the very real possibility that the interaction between the SAR domain and the key cytoplasmic effector is very transient, possibly with a high off-rate, such as might occur between a kinase (or phosphatase) and its substrate. Thus, we now believe that the SAR domain may interact and activate a kinase, or inhibit a phosphatase, as its mechanism of action. Unfortunately, we were not able to prove this point.

With the approval of the DOD, we revised our aims to address the new hypothesis that ESE-1 is required to maintain the transformed phenotype in fully transformed human breast cancer cells. In this regard, we were totally successful. Here we show that ESE-1 knockdown in transformed MCF-7 and ZR-75 cells that express endogenous ESE-1, reverses their transformed properties. Moreover, we demonstrate, by ICC, that ESE-1 is localized to the nucleus in MCF-7, T47D and ZR-75 cells, indicating that the ability of ESE-1 to maintain the transformed phenotype requires its role as a nuclear transcription factor. Of note, we have previously demonstrated that GFP-ESE-1 targeted to the nucleus of nontransformed MCF-10A and MCF-12A cells induces apoptosis, whereas transformed T47D and Sk-BR-3 cells tolerate nuclear expression of exogenous ESE-1 without inducing apoptosis, possibly because anti-apoptotic pathways have been up-regulated in these transformed cell. Taken together, these data suggest that ESE-1 initiates transformation in ESE-1-negative mammary epithelial cells via a cytoplasmic- and PAK-1-dependent mechanism, but once mammary epithelial cells are fully transformed, then ESE-1 is localized to the nucleus and functions as a transcription factor to maintain the transformed state.

While dominant-negative ETS approaches, which interfere with multiple ETS factors, have reversed the transformed phenotype in several breast cancer cell lines (NmuMG, MMT and BT20), here we show that the knockdown of a single ETS factor, ESE-1, has the same effect in MCF-7 and ZR-75-1 breast cancer cells. Importantly, we show that ESE-1 is required to maintain the transformed phenotype in MCF-7 and ZR-75-1 breast cancer cells, since shRNA-mediated ablation of endogenous ESE-1 protein resulted in decreased colony formation and anchorage-independent growth. Similar results were obtained with ZR-75 cells, with shESE-1 resulting in decreased colony formation and anchorage-independent growth (data not shown). Furthermore, mechanistic studies, using two separate approaches to measure apoptosis and proliferation, revealed that ESE-1 does not modulate apoptosis, but rather is required for cancer cell proliferation. Finally, we also demonstrate that serum is required to maintain ESE-1 protein production, raising the interesting
The possibility that reduced ESE-1 protein expression occurring upon serum starvation likely contributes to reduced MCF-7 cell proliferation noted in these conditions. Taken together, our findings contribute novel insights to our understanding of the critical role of ESE-1 in maintaining cell transformation of mammary epithelial cells via regulation of cellular proliferation.

REFERENCES


APPENDICES: PDF reprints of the following published reports have been included:


ETS transcription factors in endocrine systems

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E26 transformation-specific (ETS) transcription factors have become increasingly recognized as key regulators of differentiation, hormone responses and tumorigenesis in endocrine organs and target tissues. The ETS family is highly diverse, consisting of both transcription activators and repressors that mediate growth factor signaling and regulate gene expression through combinatorial interactions with multiple protein partners on composite DNA elements. ETS proteins have a role in the endocrine system in establishing pituitary-specific gene expression, mammary gland development and cancers of the breast, prostate and reproductive organs.

Introduction: structure and function of ETS proteins

The E26 transformation-specific (ETS) family is restricted to metazoans and thus represents an evolutionarily recent class of transcription factors [1–3]. It is one of the largest transcription factor families, consisting of 27 ETS genes in humans, 26 in mice, and 9 in Caenorhabditis elegans and 9 in Drosophila that can be structurally categorized into 11 subfamilies (ETS, ERG, ELG, ERF, TEL, PEA3, SPI, TCF and PDEF) [4,5] (Figure 1). The ETS family is defined by the ETS domain, which is a highly conserved DNA-binding domain (DBD) comprising 85 amino acids (AAs) that is folded into a winged helix-turn-helix DNA-binding motif that binds to a 5′-GGA(A/T)-3′ DNA core motif [2]. All ETS proteins, with the exception of GA-binding protein (GABPα, bind to DNA as a monomer and are auto-inhibited by virtue of two inhibitory regions that flank the DBD. Disinhibition, resulting in enhancement of ETS DBD activity and of target gene specificity, is achieved by three mechanisms: (i) flanking DNA sequences; (ii) partner–protein interactions; and (iii) kinase-mediated phosphorylation. ETS protein activation of target genes typically involves specific protein–protein interactions and such ETS–protein partner combinations frequently bind to bipartite DNA-binding sites [1–3]. Thus, although target gene selectivity of ETS factors is influenced by specific protein partners, the precise mechanism for achieving DNA-binding specificity, given the high level of redundancy, remains unclear [4,5]. Many ETS subfamilies (ETS, ERG, ELG, ESE, TEL and PDEF) contain the Pointed domain, which serves as a protein–protein interaction motif, several (TEL, ERF and TCF) contain a repressor domain and the majority (ETS, ERG, ELG, PEA3, ESE, SPI and TCF) contain a transcription activation domain (TAD) [1–3]. The activity of certain ETS factors is further regulated by subcellular localization. For example, YAN and TEL (members of the TEL subfamily) NET (also called SAP2, a member of the TCF subfamily) and ERF also contain a nuclear export sequence regulated by mitogen-activated protein kinases (MAPKs) or small ubiquitin-like modifiers (SUMOs), which controls their transcriptional repression activity [2]. By contrast, a cytoplasmic localization is required for ESE1 to transform MCF-12A and MCF-10A human mammary epithelial cell lines [6].

ETS factors are trans-acting phosphoproteins that have important roles in epithelial, hematopoietic, neuronal, endothelial and endocrine systems with key roles in cell migration, proliferation, differentiation and oncogenic transformation [1–3,7]. The founding member is encoded by the v-ets oncogene in the E26 retrovirus, which causes hematopoietic malignancies in chickens [1–3]. In humans, ETS factors are also associated with several malignancies. For example, chromosomal translocations involving ETS genes are found in 95% of human Ewing sarcoma cases and in several human hematopoietic malignancies [1]. In addition, amplification or upregulation of one or more ETS genes, including ETS1, ETS2, ER81, ERM, PEA3, PDEF and ESE1, is associated with a variety of epithelial cancers (e.g. lung, breast, colon and prostate) [1,2,7]. In mammalian cells, ETS proteins are key nuclear targets of growth factor and Ras oncogene signaling pathways, typically acting through the activating protein-1–ETS bipartite Ras response element (RRE) to regulate a repertoire of genes that control cell survival (anti-apoptosis), proliferation and motility [1–3]. Here, we focus on ETS factors in endocrine systems (Figure 2), with particular emphasis on their role in the regulation of pituitary-specific gene expression and tumorigenesis.

ETS factors regulating pituitary function

ETS factors have crucial roles in pituitary gonadotrope and lactotrope biology. Gonadotropes and lactotropes are two of the five hormone-secreting cell types that populate the anterior pituitary gland. Gonadotropes synthesize and secrete the glycoprotein hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH), which regulate gonadal function. Lactotropes produce the protein...
hormone prolactin (PRL), which stimulates breast development and lactation. Hypothalamic gonadotropin-releasing hormone (GnRH) regulates the production and secretion of LH and FSH in pituitary gonadotropes. Stimulation by GnRH leads to MAP kinase activation and ETS factor-mediated induction of the α-subunit gene through a GnRH response element [8]. The α-subunit is a common heterodimeric component of the gonadotrope-specific LH and FSH glycoprotein hormones. Activation of ETS factors in response to GnRH stimulation also induces the expression of the immediate-early gene Egr-1 through serum response elements [9]. Egr-1, in turn, is a primary activator of the LHβ-subunit gene in response to GnRH stimulation [8]. Thus, ETS factors are crucial mediators of the signaling pathways that regulate the responses of pituitary gonadotropes to hypothalamic GnRH stimulation (Figure 3a).

Pituitary lactotropes exhibit even greater dependence on ETS transcription factors for cellular growth, differentiation and PRL gene expression. Lactotrope cell growth and PRL synthesis and secretion are under tonic inhibitory regulation through hypothalamic-secreted dopamine acting on D2 receptors. The ERF repressor factor is expressed in pituitary lactotrope cell lines and it might be a key contributor to the negative effects of dopamine on PRL gene transcription and lactotrope cell growth [10]. Specifically, dopamine-mediated inhibition of extracellular-signal-regulated kinase (ERK) 1 and ERK2 activity increases ERF repressor action on the rat (r) PRL promoter, whereas growth factor-stimulated ERK activity reverses the repressor function of ERF through ERF phosphorylation [10].

ETS factors also regulate lactotrope differentiation. Pit-1 is a POU homeodomain, pituitary-specific transcription factor that governs the ontogeny of somatotrope, lactotrope and thyrotrope cell types and regulates growth hormone (GH), PRL and thyroid-stimulating hormone β gene transcription [11,12]. In GHFT pituitary precursor cells, which express low levels of Pit-1 but fail to express endogenous PRL or GH, PRL gene expression and the lactotrope phenotype is induced only by fibroblast growth factor-2 (FGF-2), and in an ETS-dependent fashion [13]. Specifically, this FGF-2 differentiating response was inhibited by a dominant-negative ETS construct and was augmented by the expression of ETS1. In the GH4 rat somatolactotrope cell line, FGF-2- and FGF-4-mediated induction of rPRL promoter activity is ETS dependent [14], and GH4 cells stably expressing FGF-4 produce tumors that are faster growing and more invasive when injected into Wistar rats [15]. Interestingly, the cooperation of ETS factors with Ikaros, a zinc-finger transcription factor, also regulates expression of the FGF-R4 isoform of the FGF receptors in GH4 somatolactotrope cells [16]. In addition, stable expression of a dominant-negative ETS construct (which encodes only the ETS-2 DBD) in GH4 cells was found to decrease PRL expression but had no effect on...
GH expression [17]. Furthermore, targeted expression of this same dominant-negative ETS construct to pituitary lactotropes in transgenic mice resulted in diminished lactotrope cell numbers (J. Tentler and A.G.H., unpublished). Taken together, these data reveal that ETS factors might represent a crucial determinant in both dopamine-mediated inhibitory lactotrope regulation and in growth factor-induced ontogeny of pituitary lactotropes.

One of the most characterized systems that best exemplifies the roles of various ETS factors in endocrine gene regulation is the lactotrope-specific \( \text{PRL} \) gene (Figure 3b). The proximal \( \text{rPRL} \) promoter contains four regions that bind to nuclear proteins derived from GH4 or GH3 rat pituitary somatolactotrope cells, with three of these regions [footprint (FP) I, III and IV] binding to Pit-1 [11,12]. An additional site was identified in promoter mutagenesis studies as the basal transcriptional element (BTE) [11,12]. The BTE site contains an ETS-binding site that is crucial for both basal activity of the \( \text{PRL} \) promoter and for responses to growth factors, including FGF-2, FGF-4, insulin, insulin-like growth factor, epidermal growth factor (EGF) and thyrotropin-releasing hormone [11,12,18]. Although electrophoretic mobility shift analyses have shown that the BTE site can bind to a variety of ETS factors, including ELK1, SAP1 (also known as ELK4), ETS1 and GABP\( \alpha \) (together with its non-DNA-binding partner GABP\( \beta_1 \)), BTE DNA-affinity purification of nuclear proteins from GH3 pituitary tumor cells identified the heterodimeric ETS factor GABP\( \alpha \)–\( \beta_1 \) as the key functional ETS factor binding to the BTE site [18]. Subsequent downregulation of the GABP\( \alpha \) and GABP\( \beta_1 \) subunits using small interfering RNA strategies in GH3 cells reduced the expression of endogenous PRL and established the role of GABP as a crucial regulator at the BTE in the PRL promoter [18].

A second crucial ETS regulatory site in the proximal \( \text{rPRL} \) promoter is a composite RRE, which is constituted by an ETS-binding site adjacent to a Pit-1 binding site, FP IV [3,19]. The \( \text{rPRL} \) RRE binds to ETS1 and Pit-1 and primarily mediates Ras activation of the \( \text{rPRL} \) promoter, but it also contributes to basal activity. Oncogenic V12Ras activates the Raf–MAPK kinase–MAPK signaling cascade, with MAPK directly phosphorylating chicken ETS1 [3]. Site-specific mutation of the ETS1 Thr82 MAPK phosphorylation site to Ala results in the loss of ETS1 enhancement of the Ras response [3]. Mutation of either the ETS1 or Pit-1 site diminishes the Ras response of the \( \text{rPRL} \) promoter in transient transfections of GH4 cells [19]. Similarly, expression of a dominant-negative ETS construct or Pit-1\( \beta \) (an alternatively spliced isoform that functions as a dominant-negative effector in pituitary cells) represses Ras activation of the \( \text{rPRL} \) promoter [3]. Contributing to this ETS–Pit-1 combinatorial code is the physical interaction of the TAD of ETS1 with the Pit-1 homeodomain (AAs 199–291), as demonstrated using
NMR approaches [20] (Figure 4). In addition, Pit-1 phosphorylation at Thr220, within the homeodomain, regulates the binding of Pit-1 to both ETS1 and the monomeric Pit-1 binding site within the RRE [20]. However, Pit-1 is also a unique cell-specific target for Ras signaling, and mutation analyses of Pit-1 have localized Ras responsiveness to AAs 60–80, at the C-terminus of the TAD [21]. This Pit-1 TAD region contributes to the Ras response through the p160 steroid receptor coactivator-1 [21]. Thus, the binding of Pit-1 and ETS1 to the composite RRE in the proximal rPRL promoter seems to generate a unique binding platform for Ras-stimulated coactivator complexes.

**ETS proteins in mammary gland development and breast cancer**

During embryogenesis, ETS1, PEA3, ERM, ER81 and ESE1 are expressed in the mammary gland, and the PEA3 subfamily members PEA3, ERM and ER81 have been shown to be expressed throughout mammary gland development [7,22–24]. In situ hybridization approaches have revealed that ERM and PEA3 are expressed in epithelial cells of the developing terminal end buds, suggesting that they have a role in branching morphogenesis [24]. PEA3 knockout mice show an increased number of proliferating cells and increased terminal end buds, implying that PEA3 functions as a repressor of mammary epithelial cell growth [24].

In postnatal mammary glands, ETS factors have been shown to have key roles in pregnancy-induced, PRL-mediated mammary gland lobuloalveolar development and milk production and in breast tumorigenesis. In the early phase of pregnancy, a proliferative phase of mammary alveolar morphogenesis mediated by PRL and signal transducer and activation of transcription (STAT)5 is initiated, and the PRL–STAT5 pathway is responsible for the development of lobuloalveoli and the induction of lactation. Studies using elf5 and prlr gene knockout mice and rescue by targeted re-expression of elf5 in prlr nullizygous mammary epithelium revealed that the ETS factor Elf5 (also known as Ese-2) is the crucial downstream
ETS factors have a particularly relevant role in breast cancer. Although many ETS factors have been shown to be overexpressed in breast cancer, most of these studies have focused on mRNA rather than protein expression, and few have examined compartment-specific expression [7,22–24]. A recent comprehensive quantitative PCR and subtractive tissue analyses of 25 different ETS factors in normal and malignant murine mammary gland tissues and cell lines revealed that multiple ETS factors are expressed in lymph, stromal and epithelial mammary compartments [4]. For example, ELK4, ELF1 and ETS2 are the most abundant ETS factors expressed in the normal mammary gland, whereas PDEF, PEA3, ESE1, ESE2, ESE3, TEL (also known as ETV6) and NERF (also known as ELF2) mRNAs displayed significantly elevated expression in the epithelial cell compartment of mammary tumors [4]. Important data revealing a direct role of ETS proteins in mammary tumorigenesis have been reported, showing that a dominant-negative ETS2 DBD can block the anchorage-independent growth and cellular invasiveness of the NmuMG, MMT and BT20 breast cancer cell lines [27,28]. However, some of the most convincing reports stem from transgenic mice studies, showing that compound ets2 knockout MMTV–polyoma middle T (PyMT) mice, in which one ets2 allele has been knocked out, are more resistant to PyMT-induced breast tumorigenesis than are their wild-type counterparts [29]. Similarly, the generation of female mice expressing a homozgyous targeted ets2 gene, ets2(A72/A72), which precludes ERK phosphorylation of Thr72, blocked mammary tumors caused by transgenic-targeted oncogenes and seemed to do this exclusively through a stromal location [30,31]. Additionally, using immunohistochemical and western blotting approaches, several studies have revealed that specific ETS proteins, including members of the ETS, PEA3 and ESE subfamilies, are upregulated in breast cancer tumors and cell lines [7,22–24]. Although increased PEA3 and PDEF (also called PSE) mRNAs have been associated with breast cancer [7,22–24], their precise role in mammary tumorigenesis has been controversial. Studies testing their ability to transform mammary epithelial cells reveal that PEA3 and PDEF actually inhibit breast cancer cell growth [32–35]. Thus, although most reports implicate ETS factors in breast cancer, only the ETS factor ESE1 has been shown to confer an epithelial-to-mesenchymal transition phenotype and actually to transform human mammary epithelial cells [6,36–38].

ESE1 (also known as ESX or ELF3) is an epithelial-specific ETS protein that is particularly relevant to breast cancer because the ESE1 gene maps to human chromosome 1q32.1, in a region that is overrepresented in 50% of early breast cancers [7]. ESE1 mRNA and protein is overexpressed in human breast ductal carcinoma in situ, an early cancer stage that also overexpresses HER2 (also known as neu) [7]. Furthermore, a positive feedback loop between the HER2 proto-oncogene and ESE1 seems to exist [7]. ESE1 contains several unique features among ETS factors. For example, its mRNA expression is restricted to the mammary ductal epithelia and terminal ductal–lobular units, which are the most terminally differentiated, epithelial-derived cells [7]. Unique to ESE1 among ETS proteins are a 40-AA serine- and aspartic rich (SAR) motif and an HMG-like AT-hook domain, in addition to the Pointed, TAD and ETS DBD domains found in most ETS factors [7] (Figure 5a). Recent work revealed that ESE1 is required for the transformed phenotype of HER2- T47D breast cancer cells [36], that it transforms the immortalized but nontransformed MCF-12A and MCF-10A human mammary epithelial cells and that it does so through an autonomously functioning, unique 40-AA SAR domain acting through a novel cytoplasmic mechanism [6,37] (Figure 5b). A subsequent study screening ~250 cDNAs implicated in breast cancer independently identified ESE1 as being able to confer a motile phenotype upon MCF-10A cells, resulting in disorganized organoids in 3D culture [38], as previously reported [37]. The discovery that ESE1 functions in the cytoplasm to transform mammary epithelial cells established novel paradigms for ETS factor function and mechanisms by which transcription factors induce cell transformation.

ETS factors in the prostate

The expression of several ETS factors in the normal and/or cancerous prostate has been reported, including ETS1, ETS2, ELF1, ESE2 (also called ELF5), ER81, ERG, PDEF and PEA3, with ETS fusions having become one of the most common genetic markers of prostate cancer [1,39]. The ETS factor PDEF/PSE was originally isolated as a transcriptional regulator of the prostate-specific antigen gene.
Subsequently, PDEF/PSE mRNA was found to be increased in breast and prostate cancer, yet PDEF/PSE protein expression was found to be decreased as the prostate malignancy grade progressed [7]. These data have been interpreted to indicate that PDEF might actually function as a tumor suppressor and that a translation control mechanism seems to regulate PDEF protein expression in both prostate and breast cancers [7,32,35].

The first clinically relevant candidates for dominant oncogenes in prostate cancer are ETS fusion genes resulting from chromosomal translocation of the 5′ untranslated region of a prostate-specific, androgen-responsive, transmembrane serine protease gene (TMPRSS2) to ERG, ER81 (also known as ETV1) and PEA3 (also known as ETV4) ETS transcription factor genes [39]. Deletion of genomic DNA between TMPRSS2 (21q21.3) and ERG (21q21.2) results in the TMPRSS2:ERG fusion protein in ~50% of prostate cancers and chromosomal rearrangements between TMPRSS2 (21q21.3) and ER81 (7p21.2) or PEA3 (17q21) occur in another ~30%, indicating that TMPRSS2-ETS gene fusions might be the most common genetic abnormality defined to date in human malignancies [39]. These fusions result in androgen-mediated, robust induction of these various ETS factors, which are then thought to activate a repertoire of ETS-responsive genes, leading to prostate cell transformation [39].

ETS factors in reproductive tissues

The ETS family of transcription factors has crucial roles in the regulation of reproduction and embryogenesis. In the male, ERM is required in testicular Sertoli cells for spermatogonial stem cell self-renewal and transcriptional regulation of the stem cell niche [40]. Testicular germ cell tumors show allelic imbalance in the chromosomal region encoding TEL [41], and increased expression of ETS1 was associated with metastasis and angiogenesis [42]. PEA3 expression was increased in testicular seminomas, correlating with enhanced matrix metalloprotease (MMP) 2 levels [43].
In the female, uterine expression of ETS1 and the PEA3 subfamily have been implicated in endometrial angiogenesis and implantation [44,45]. ETS1 is also a crucial regulator of the uterine decidua PRL promoter and is dramatically upregulated during decidualization of the endometrial stroma [46]. In early postimplantation development, ETS2 expression is restricted to placental trophoblasts and is essential for placental function. Deletion of the DNA-binding domain of ETS2 in transgenic mice resulted in embryonic death before day 8.5, owing to defects in extra-embryonic tissue, including deficient MMP9 activity and failure of trophoblast proliferation [47]. Subsequent analysis indicated that ETS2 is necessary for the expression of extra-embryonic ectoderm (EXE) markers and anteroposterior patterning mediated by EXE-derived signals [48]. Deletion of a second trophoblast-specific ETS-related gene, elf-5, also results in concepts lacking EXE [49]. Several ETS factors have been investigated as prognostic markers in gynecological tumors. In cervical cancer, overexpression of ETS1, ERM (also called ETV5), ERG or ETS2 was associated with higher grade, metastasis and poor prognosis and correlated with elevated levels of MMP-1 and increased angiogenesis [50,51]. A polymorphism of the MMP-1 promoter, which generates an additional ETS-binding site, was also linked to advanced clinical stage and reduced survival in cervical and endometrial cancer [52,53]. Loss of the ETS repressor protein NET (also called SAP2) resulted in enhanced c-Fos expression in cervical cancer cells, a key event in transformation [54]. By contrast, expression of PEA3 suppressed the invasiveness of cervical carcinoma cells [55].

In endometrial tumors, increased expression of ETS1 and ELF1 is associated with advanced surgical stage, enhanced invasion and angiogenesis, and poor prognosis linked to elevated levels of MMPs [56,57]. ERM was also upregulated in endometrial carcinoma in tumor stages associated with myometrial invasion [58]. Accordingly, treatment of progesterone receptor B-expressing Ishikawa endometrial cancer cells with progesterone resulted in inhibition of cell growth and invasion, concomitant with decreased levels of ETS1 and MMPs [59], whereas estrogen-stimulated growth and invasion was associated with increased ETS1 and MMP expression [60]. Overall, evidence indicates that ETS factors, by virtue of their transcriptional regulation of MMPs and angiogenic genes, are crucial mediators of angiogenesis, invasion and metastasis in the development and progression of gynecological malignancies.

Increases in ETS1 and ELF1 are also linked to the malignant potential of ovarian cancer, being associated with higher grade and poor prognosis [61,62]. Overexpression of ETS1 in ovarian cancer cells also conferred resistance to chemotherapy [63]. Similarly to cervical cancer, expression of ETS1 and PEA3 correlated with elevated MMPs 1, 2 and 9 and increased angiogenesis, suggesting a central role for these ETS factors in the progression of ovarian carcinoma [64,65]. However, consistent with its inhibition of cervical cancer cell invasion, PEA3 is also reported to downregulate Her2 gene expression and reverse the transformed phenotype of ovarian cancer cells in vitro [66].

ETS factors in thyroid and pancreatic malignancies

Evidence suggests a role for ETS1 and ETS2 in the pathogenesis of thyroid cancer. Expression of ETS1 is increased in papillary and follicular thyroid carcinomas relative to benign nodules or normal tissue [67]. ETS1 and ETS2 expression and transcriptional activity were also increased in thyroid cancer cells. A dominant-negative ETS construct suppressed anchorage-independent growth and induced apoptosis in thyroid carcinoma but not in normal thyroid cell lines, implying a requirement for ETS1 and ETS2 to maintain the transformed phenotype [68]. In pancreatic adenocarcinoma, particularly in late-stage invasive tumors, elevated ETS2 protein was detected and PEA3 was shown to activate the mucin 4 promoter, an epithelial marker of pancreatic ductal carcinoma not present in normal tissue, suggesting that ETS factors might also be important in pancreatic cancer [69,70]. Finally, ETS1 has been implicated in ectopic expression of the endocrine ligand parathyroid hormone-related peptide, which is associated with bone metastases and hypercalcemia in several cancers, including breast cancer [71].

Conclusion

In summary, ETS factors have been shown to have crucial roles in development, differentiation and tumorigenesis in several endocrine systems. Studies in the pituitary, mammary and prostate glands have provided compelling insights into the functional role of specific ETS factors in these endocrine tissues. Emerging data in many other endocrine organs suggest a broader role for ETS transcription factors in endocrine pathophysiology. Future studies, targeting select endocrine tissues, by generating tissue-specific knockouts of individual ETS genes or expressing a dominant-negative ETS transgene, will provide an even greater understanding of the role of ETS factors in endocrine biology.

Acknowledgements

The authors wish to acknowledge grant support from the NIH, including R01 DK 376676 and R01 DK 46608 to A.G.H.; R01 DK 02946 to D.L.D.; and, R01 CA 104875 to A.P.B. Also, A.G.H. acknowledges BCRP BC051589 grant support from the DOD.

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Ets transcription factors in intestinal morphogenesis, homeostasis and disease

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Summary. Ets transcription factors comprise a large family of sequence-specific regulators of gene expression with important and diverse roles in development and disease. Most Ets family members are expressed in the developing and/or mature intestine, frequently in a compartment-specific and temporally dynamic manner. However, with the exception of the highly expressed Elf3, involved in embryonic epithelial differentiation, little is known about Ets functions in intestinal development and homeostasis. Ets factors show altered expression in colon cancer, where they regulate pathways relevant to tumor progression. Ets factors also likely act as important modifiers of non-neoplastic intestinal disease by regulating pathways relevant to tissue injury and repair. Despite a large body of published work on Ets biology, much remains to be learned about the precise functions of this large and diverse gene family in intestinal morphogenesis, homeostasis, and both neoplastic and non-neoplastic pathology.

Key words: Ets, Transcription factor, Intestine, Morphogenesis, Homeostasis, Cancer

Ets factors – Introduction

Ets factors comprise a large family of transcription factors related to each other by a conserved DNA-binding domain (DBD), the Ets domain. Found in metazoans, the number of individual Ets factors increases with the complexity of the organism, up to a total of 27 in humans (Gutierrez-Hartmann et al., 2007). Ets factors frequently function as mediators of extracellular signaling pathways (Wasylyk et al., 1998; Sharrocks, 2001; Oikawa and Yamada, 2003). As sequence-specific DNA-binding proteins, Ets factors interact with a core GGA(A/T) DNA sequence via the conserved Ets DBD, a winged helix-turn-helix structural motif (Graves and Petersen, 1998; Sharrocks, 2001; Oikawa and Yamada, 2003). Individual Ets factors regulate promoter activity directly via intrinsic activation or, less commonly, repression domains, or indirectly through interactions with other transcription-modulating proteins (Graves and Petersen, 1998; Wasylyk et al., 1998; Sharrocks, 2001; Oikawa and Yamada, 2003). Occasionally, some Ets factors may either activate or repress transcription depending on the precise promoter context (Oikawa and Yamada, 2003). Ets factors modulate the expression of a variety of genes involved in diverse cellular processes, including cell proliferation, differentiation, apoptosis, and cell-cell/cell-matrix interactions (Sementchenko and Watson, 2000). Many aspects of Ets biology have been the subject of recent reviews (Sharrocks, 2001; Oikawa and Yamada, 2003; Hsu et al., 2004; Seth and Watson, 2005; Gutierrez-Hartmann et al., 2007). This review will focus on Ets factors in the morphogenesis, homeostasis and disease of the intestinal tract.

Ets factors in intestinal morphogenesis and homeostasis

Mouse genetic studies have revealed unique Ets functions in a variety of biological processes. Gene inactivation (“knock-out”) studies have shown Ets factors to perform essential functions in: hematopoiesis and immune function (Ets1, Elf4, Fli1, Tel, Spi1, SpiB, GABPα); lymph/angiogenesis (Tel, Elk3); neurogenesis and neuromuscular function (Pea3, Erm, Erβ1, GABPα); spermatogenesis (Erm); development of extraembryonic tissues and early embryonic development (Ets2, Ets1, GABPα) (Maroulakou and Bowe, 2000; Lacorazza et al., 2002; Livet et al., 2002; Oikawa and Yamada, 2003; Ristevski et al., 2004; Rosmarin et al., 2004; Chen et al., 2005; Donnison et al., 2005; Zhou et al., 2005; Georgiades and Rossant, 2006; Hippenmeyer et al., 2007; O’Leary et al., 2007). As multiple Ets factors tend...
to be expressed in the same cell (Galang et al., 2004; Hollenhorst et al., 2004) and there appears to be substantial overlap in Ets DNA-binding specificity (Graves and Petersen, 1998), gene inactivation experiments may not reveal all Ets functions in vivo, due to potential compensatory activity by other co-expressed Ets factors. Indeed, tissue-specific expression of genetically modified Ets factors designed to block such compensatory activity has uncovered a number of Ets functions (de Kerchove D’Exaerde et al., 2002; Paratore et al., 2002; Parkinson et al., 2002; Thenevieu et al., 2007), including pulmonary airway morphogenesis not seen in Ets knock-out mice (Liu et al., 2003). As noted above, Ets factors frequently act as mediators of cell-cell signaling pathways, and this is true in development, where Ets factors often function as nuclear effectors of fibroblast growth factor (FGF) signaling (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001; Kawachi et al., 2002; Bertrand et al., 2003; Liu et al., 2003; Brent and Tabin, 2004).

A number of approaches have revealed widespread expression of Ets family members in the developing and mature mammalian intestine (Table 1, Fig. 1). Global expression profiling approaches performed on whole tissue have shown the expression of most Ets factors in the developing small intestine, many with temporally dynamic patterns of expression (Lepourcelet et al., 2005; Choi et al., 2006). By RT-PCR analysis, approximately one third of Ets factors analyzed showed changes in expression levels from mouse embryonic day 11 to 17, a period of dynamic tissue remodeling in the intestine (Choi et al., 2006). Global gene expression profiling of fractionated tissue and in-situ hybridization analysis have further demonstrated tissue compartment-specific expression in the intestine for many Ets factors (Kola et al., 1993; Maroulakou et al., 1994; Chotteau-Lelievre et al., 1997; Li et al., 2007).

In the developing mouse small intestine, PEA3, Elf1, Elf3, Ets2 and Erg are predominantly epithelial; Er81, Elf3, Elf2, Ets1, Erg and Fli1 are predominantly non-epithelial; and Erm appears to be expressed in both

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**Table 1. Physiologic Ets transcription factor expression in mammalian intestine, and altered expression in colon cancer.**

<table>
<thead>
<tr>
<th>Ets factor</th>
<th>Developmental expression (mouse)1</th>
<th>Relative expression (human adult sm int; colon; HCT-116 cells)2</th>
<th>Altered expression in colon carcinoma3</th>
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<tbody>
<tr>
<td>ER81/ETV1*</td>
<td>WTE (SAGE); nE (ISH; ma, 10.3)</td>
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<tr>
<td>PEA3/ETV4/E1AF*</td>
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<td>2; &lt;1; 17</td>
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<td></td>
</tr>
<tr>
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1 WTE: expression analyzed in whole tissue extract (SAGE [serial analysis of gene expression] of mouse embryonic small intestine from (Lepourcelet et al., 2005); RT: reverse transcriptase polymerase chain reaction) analysis of mouse embryonic small intestine from (Choi et al., 2006). Preemminently epithelial (E) or non-epithelial (nE) expression as determined by in-situ hybridization analysis (ISH; see text for references) and/or microarray (ma) analysis of chemically fractionated E18.5 mouse small intestine ((Li et al., 2007); numerical value is fold-enrichment in epithelial or non-epithelial compartment). 2 Number of mRNA molecules per 2x10^6 molecules of 18S rRNA, as determined by quantitative RT-PCR analysis of adult human whole tissue extracts (Hollenhorst et al., 2004); sm int: small intestine; HCT-116 cells: colon cancer cell line. 3 WTE: expression analyzed in whole tissue extract; RT: reverse transcriptase polymerase chain reaction; IHC: immunohistochemical analysis; ma: microarray analysis (tumor vs normal data from Oncomine™ Research database, www.oncomine.org, searched at p<0.001; number in brackets: number of studies showing this change). See text for additional detail, including references. *genetically inactivated (knocked-out) in mouse; NA: not found in mouse.
compartments (Table 1, Fig. 1). In the limited data available from the adult intestine, Elf3 expression remains epithelial and Ets1 remains non-epithelial (Table 1, Fig. 1). The resolution of such studies has thus far been largely limited to epithelium versus non-epithelial tissue. Although the latter is frequently referred to as “mesenchyme” or “stroma”, it is a complex tissue compartment composed of multiple different cell types. Thus, higher resolution expression studies will be required to determine which Ets factors are truly “mesenchymal/stromal”, and which are expressed in smooth muscle, vascular, neural, and immune/inflammatory cells (both those resident to the intestine and those incidentally transiting intestinal vasculature). In the adult intestine, expression of essentially all Ets transcription factors can be demonstrated at the whole tissue level, but transcript levels vary widely (Table 1). Elf3 and Ehf, two epithelial-specific Ets factors are expressed at high levels. Also showing relatively high-level expression are Ets1 and Ets2. In contrast, some Ets factors, notably members of the developmentally expressed PEA3 subfamily (Pea3, Erm and Er81) are expressed at relatively low levels in the adult. It remains to be determined whether low-level expression reflects a lesser requirement or essential, but spatially restricted, function. Finally, also at the whole tissue level, there appear to be some differences in Ets expression levels between the small intestine and colon. Notably, in the adult, the epithelial-specific Ets factors (Elf3, Elf5, Ehf and PDEF), as well as members of the PEA3 subfamily (Pea3, Erm and Er81), appear to be expressed at higher levels in the colon (Chotteau-Lelievre et al., 1997; Hollenhorst et al., 2004). In sum, available expression data strongly suggest important and potentially specific functions for Ets factors in the developing and mature intestine.

Of the nearly two-thirds of Ets factors genetically inactivated in the mouse thus far (Table 1), only one, the epithelial-specific Ets factor Elf3, has been reported to have an intestinal phenotype (Ng et al., 2002). Elf3−/− embryos manifest delayed and impaired villus morphogenesis, impaired enterocyte differentiation and altered microvillus structure, and altered goblet cell differentiation. Elf3−/− newborn animals exhibit diminished weight gain, and adults develop a “wasting” phenotype characterized by weight loss and diarrhea. Interestingly, the Elf3−/− embryonic phenotype is associated with diminished epithelial expression of transforming growth factor β type II receptor (TGFβRII), and both the impaired enterocyte and goblet cell differentiation can be rescued by transgenic TGFβRII expression in the intestinal epithelium (Flentjar et al., 2007). Thus, the critical role of Elf3 in epithelial differentiation in the developing intestine appears to be the facilitation of TGFβ signaling (Fig. 2). The role of Elf3 in regulation of TGFβRII expression in intestinal epithelium likely involves direct stimulation of TGFβRII promoter activity (Choi et al., 1998). Other Ets factors have been shown to be capable of regulating the TGFβRII promoter, with different, and often context-dependent, effects (Kopp et al., 2004). It will be interesting to see if other co-expressed Ets factors have a role in modulating TGFβRII expression in intestinal epithelium, and thus its differentiation.

Fig. 1. Ets expression in the developing mouse intestine. Ets factors with demonstrated developmental expression in the epithelium, non-epithelial tissue (“stroma”), or in whole tissue (unknown sublocalization). Red: genetically inactivated (knocked out) in mouse; asterisk (*): intestinal phenotype reported in knock-out mice.
demonstrated to be required for cecal morphogenesis in the mouse (Burns et al., 2004; Zhang et al., 2006). It remains to be determined if Ets factors are involved in this process, as they are in other FGF-mediated morphogenetic processes.

**Ets factors in intestinal epithelial neoplasia**

A number of different Ets factors have been shown to be misexpressed or overexpressed in human malignancies (Kurpios et al., 2003; Oikawa and Yamada, 2003; Seth and Watson, 2005; Gutiérrez-Hartmann et al., 2007). In hematologic malignancies, Ets overexpression appears to be driven at least in part by gene amplification (Rovigatti et al., 1986; Santoro et al., 1992; Baldus et al., 2004; Poppe et al., 2004). Gene amplification has also been demonstrated for Elf3 in breast cancer (Chang et al., 1997), and in prostate cancer a number of Ets factors become overexpressed by a chromosomal translocation-driven mechanism (Tomlins et al., 2005). The mechanisms governing Ets overexpression in other solid tumors remain to be defined. Much also remains to be learned about the precise downstream pathways mediating Ets tumor-modifying effects. Plausible candidate mechanisms, including effects on proliferation, survival, migration, invasion and angiogenesis, and targets, including extracellular matrix components and modifying enzymes, have been postulated based on tissue culture models and other studies, but largely remain to be demonstrated in vivo (Coletta et al., 2004; Hsu et al., 2004; Seth and Watson, 2005). In vivo, epithelial Pea3 and stromal Ets2 have been shown to promote mammary epithelial tumor growth in mouse models (Shepherd et al., 2001; Man et al., 2003). The stromal tumor-promoting effects of Ets2 act downstream of VEGF signaling, require MAP kinase-mediated phosphorylation of Ets2, and regulate downstream MMP expression (Man et al., 2003).

A number of Ets factors have been shown to be misexpressed/overexpressed in colon carcinoma (Table 1). Increased Ets1 and Ets2 expression in tumor cells correlates with adenoma to carcinoma progression, with Ets1 expression also correlating with carcinoma depth of invasion, lymphovascular invasion and metastasis (Nakayama et al., 2001; Ito et al., 2002). Ets1 is also expressed in tumor stroma, where it correlates with adenoma to carcinoma progression, lung metastasis, stromal expression of the matrix metalloproteases MMP-1 and MMP-9, and vascular expression of integrin β3 (Sato and Miwa, 2002; Behrens et al., 2003). Expression of Ets1, as well as possibly Ets2, in non-epithelial tissue compartments may explain the observed lower expression of these Ets factors in some colon cancer cell lines in comparison to unfractionated whole colon tissue (Hollenhorst et al., 2004). Ets2 is positively regulated by active Wnt signaling (van de Wetering et al., 2002), which may represent one mechanism for Ets overexpression in tumors.

Overexpression of Pea3, but not Erm, Er81, Ets1 or Ets2, predicts poor survival in colon carcinoma, and is associated with increased expression of MMP-1 and matrilysin/MMP-7 (Horiuchi et al., 2003). Pea3, Erm and Er81 expression levels are also increased in colon cancer cell lines relative to normal tissue (Crawford et al., 2001; Hollenhorst et al., 2004). In cultured colon cancer cells, Pea3 is required for MMP-1 and matrilysin/MMP-7 expression and invasive behavior (Horiuchi et al., 2003). Pea3, as well as the other members of the PEA3 Ets subfamily (Erm and Er81), cooperate with the β-catenin/TCF complex and c-Jun to stimulate matrilysin promoter activity (Crawford et al., 2001). Matrilysin functions as a tumor promoter in vivo (Witty et al., 1994; Wilson et al., 1997). Thus, stimulation of matrilysin expression likely represents an important mechanism of colon cancer promotion by Pea3 (Fig. 3). Other Ets-regulated tumor-promoting genes in colon cancer include Cox-2 and osteopontin (Liu et al., 2004; Wai et al., 2006). In addition, Ets factors may be involved in the regulation of cyclinD1 expression by β-catenin (Tetsu and McCormick, 1999).

Studies of other malignancies have suggested tumor suppressor functions for some Ets factors, including the epithelial-specific Ehf and PDEF (Gu et al., 2007; Turner et al., 2007a,b; Cangemi et al., 2008), and Elf4 (Seki et al., 2002). Expression of Elf3, Ehf and PDEF, three of the four epithelial-specific Ets factors, is several-fold lower in the HCT-116 colon cancer cell line compared to unfractionated whole colon tissue (Table 1), and the LoVo colon cancer cell line is also reported to lack detectable PDEF protein (Turner et al., 2007b). As these Ets factors are expressed predominantly, if not exclusively, in epithelia (Feldman et al., 2003), these differences are not likely to be accounted for by expression in the non-epithelial tissue compartment of

![Fig. 2. Elf3 in developmental enterocyte differentiation. Elf3, expressed in developing intestinal epithelium, promotes epithelial expression of the TGFβ type II receptor (TGFβRII), probably by directly stimulating TGFβRII promoter activity. This presumably permits an epithelial response to the differentiating effects of TGFβ, thus promoting epithelial differentiation along the enterocyte lineage. The same pathway also appears to regulate goblet cell terminal differentiation (see text).](image-url)
normal tissue. It remains to be determined whether the epithelial-specific Ets factors, as well as possibly others, have tumor suppressor functions in colon cancer, possibly in part through regulation of TGFβRII expression. Furthermore, while overexpressed Ets2 behaves as a tumor-promoter in multiple tumor types (Seth and Watson, 2005), physiologic or near-physiologic gene dosage of Ets2 has recently been shown to have a tumor "repressive" effect in a mouse model of colon cancer (Sussan et al., 2008). This suggests the interesting possibility that Ets factors may exert different, including opposing, tumor-modifying effects at normal/near-normal versus aberrantly high expression levels, due to differential binding and/or modulation of gene regulatory regions.

Finally, global gene expression profiling studies of unfractionated tumor and normal tissue confirm increased expression of Ets2 and Pea3 in colon cancer, and reveal additional alterations in Ets expression, including increased Elf1 and decreased Tel and Fli1 (Table 1). Furthermore, a study profiling tissue adjacent to sporadic colon carcinoma identified increased Erm, Er81 and Net expression, and decreased Eth, Elf1 and Elf4 expression, relative to tissue from individuals without cancer (Hong et al., 2007), suggesting possible functions for these Ets factors in colon cancer initiation and/or early progression. Taken together, the above data implicate Ets factors in many aspects of colon carcinoma initiation and/or progression, but much remains to be learned about their causal roles in vivo, as well as the precise mechanisms by which they effect these roles.

Ets factors in non-neoplastic disease of the intestine

Far less is known about Ets factor function(s) in non-neoplastic disease, but, as in neoplasia, Ets factors appear to be important disease modifiers. Ets1 exerts an anti-fibrogenic effect in fibroblasts, antagonizing the effects of TGFβ (Knittel et al., 1999; Czuwara-Ladkowski et al., 2002), and Ets1 knock-out mice exhibit features of autoimmune disease in multiple organs (Wang et al., 2005). Additionally, 3' polymorphisms of Ets1 are associated with different clinical manifestations of systemic lupus erythematosus (Sullivan et al., 2000). Ets2 and Fli1 overexpression has been observed in a number of inflammatory/autoimmune diseases (Trojanowska, 2000), and, interestingly, the normally epithelially restricted Elf3 becomes misexpressed in non-epithelial cells under conditions of inflammation (Rudders et al., 2001; Grall et al., 2003; Brown et al., 2004). Lastly, the Ets factor Net is required for VEGF-mediated angiogenesis in a mouse model of cutaneous wound healing (Zheng et al., 2003). Ets factors have been little studied in non-neoplastic intestinal disease, although quantitative alterations in Ets1 mRNA and protein have been observed in inflammatory bowel disease (Konno et al., 2004). Further, Elf3 has been shown to regulate the expression of the pro-inflammatory cytokine MIP-3α in enterocytes (Kwon et al., 2003). Global gene expression profiling of intestinal tissue from individuals with inflammatory bowel disease (IBD) compared to controls has not revealed significant alterations in the expression levels of Ets factors other than Ets1 at the whole tissue level (Hughes, 2005). However, given the complexity of tissue pathology in IBD, this does not exclude small and/or tissue subcompartment-restricted, but mechanistically significant, Ets expression changes. Similarly, it also does not exclude potentially important functions for physiologic levels of Ets during tissue injury and/or healing. Overall, given the important functions of Ets factors in epithelial, stromal, vascular and immune/inflammatory biology, it is likely that they have important roles in non-neoplastic intestinal disease, including IBD, but these roles largely remain to be identified and characterized.

Summary and perspectives

Ets transcription factors are widely expressed in the developing and mature intestine. Multiple approaches demonstrate tissue compartment and subcompartment-specific, and in some cases temporally dynamic, expression patterns for a number of Ets factors. This suggests specific functions for different Ets in intestinal morphogenesis and homeostasis, thus far demonstrated in vivo for only one Ets factor, Elf3. Elf3 controls intestinal epithelial differentiation during development by regulating the expression of TGFβRII in epithelial cells. Rigorous analysis of intestinal development, homeostasis and pathology in individual Ets knock-out animals, combined with creative genetic approaches, will be required to learn more about Ets functions in the
intestine in vivo. Expression and in vitro function studies suggest tumor-promoting roles for a number of Ets factors in colon cancer. It remains to be determined whether some Ets factors, including epithelial-specific Ets, function as tumor suppressors in colon cancer, as they appear to do in other malignancies. Given the important functions of Ets factors in epithelial, stromal, vascular and immune/inflammatory biology, it is likely that Ets factors have important roles in non-neoplastic diseases of the intestine, including inflammatory bowel disease, but, at present, these roles remain to be identified.

Acknowledgements. This work was supported by NIH K08 DK074557 (to PJ), and R01 DK37667 and DK46868 (to AGH).

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Ets transcription factors in intestinal biology


Expression analyses identify MLL as a prominent target of 11q23 amplification and support an etiologic role for MLL gain of function in myeloid malignancies. Blood 103, 229-235.


Ets Transcription Factors Control Epithelial Maturation and Transit and Crypt-Villus Morphogenesis in the Mammalian Intestine

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Members of the Ets transcription factor family are widely expressed in both the developing and mature mammalian intestine, but their biological functions remain primarily uncharacterized. We used a dominant repressor transgene approach to probe the function of epithelial Ets factors in the homeostasis of the crypt-villus unit, the functional unit of the small intestine. We show that targeted expression in small intestinal epithelium of a fusion protein composed of the Engrailed repressor domain and the Erm DNA-binding domain (En/Erm) results in marked disruption of normal crypt-villus homeostasis, including a cell-autonomous disturbance of epithelial matura-
tion, increased epithelial transit, severe villus dys-
morphogenesis, and crypt dysmorphogenesis. The epithelial maturation disturbance is independent of the regulation of TGFβRII levels, in contrast to Ets-mediated epithelial differentiation during development; rather, regulation of Cdx2 expression may play a role. The villus dysmorphogenesis is independent of alterations in the crypt-villus boundary and inappropriate β-catenin activation, and thus appears to represent a new mechanism controlling villus architectural organization. An Analysis of animals mosaic for En/Erm expression suggests that crypt nonauto-
nomous mechanisms underlie the crypt dysmorphogenesis phenotype. Our studies thus uncover novel Ets-regulated pathways of intestinal homeostasis in vivo. Interestingly, the overall En/Erm phenotype of disturbed crypt-villus homeostasis is consistent with recently identified Ets function(s) in the restriction of intestinal epithelial tumorigenesis. (Am J Pathol 2009, 174:1280–1290; DOI: 10.2353/ajpath.2009.080409)

Ets factors comprise a large family of transcription factors found in metazoa.1,2 Numbering as many as 27 in humans, Ets factors are related to each other by a con-
served DNA-binding domain (DBD), the Ets domain.1–3 The Ets domain is a winged helix-turn-helix structural motif, which binds to a core GGA(A/T) DNA sequence.1–4 On binding to DNA, Ets factors regulate gene promoter activity directly via intrinsic activation or, less commonly, repression domains, or indirectly through interactions with other transcription factors.1,2 Frequently acting as nuclear effectors of growth factor receptor-mediated sig-
naling pathways, Ets factors control the expression of genes involved in diverse cellular processes, including cell proliferation, apoptosis, differentiation, and cell-cell/ cell-matrix interactions.1,2,5,6

Ets factors are widely expressed in a variety of develop-
ing and adult mammalian tissues.7 Genetic inactiva-
tion of individual Ets factors in the mouse has revealed unique, essential Ets functions in diverse biological processes, including hematopoiesis, immune function, lymphangiogenesis, neurogenesis/neuromuscular func-
tion, spermatogenesis, early embryonic patterning, and development of extraembryonic tissues.1,7–16 Interest-
ingly, such studies have been remarkable for the ab-
sence of phenotypes in a number of tissues with Ets expression, especially those comprising solid organs. The relative paucity of Ets phenotypes in such tissues appears to be attributable to the multiplicity of expression of different Ets factors in the same cell, combined with the

Supported by the Cancer League of Colorado, The Children’s Hospital/ University of Colorado Denver Research Institute, and the National Institu-
tes of Health (K08 DK074557 to P.J. and R01 DK37667 and DK46868 to A.G.H.).

Accepted for publication December 9, 2008.

Supplemental material for this article can be found on http://ajp.
amipathol.org.

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ability of co-expressed Ets factors to compensate for each other’s function.4,17,18

Most Ets genes are expressed in the developing and/or mature mammalian intestine, frequently in a tissue compartment-specific and/or temporally dynamic manner.19 However, of the 16 (of 26 total) murine Ets genes inactivated to date, only 1, Elf3/ESE1/ESX/ERT/JEN, has been reported to have an intestinal phenotype.20 As in other systems,21 the absence of phenotypes in other Ets knockout mice is likely attributable to Ets genetic compensation in vivo. The use of genetically manipulated Ets factors with dominant activity has proven an effective way to overcome Ets genetic compensation. Such an approach has been used successfully to uncover and characterize Ets functions in a number of in vivo and in vitro systems, including lung morphogenesis, mammary tumorigenesis, and neuromuscular synapse function in the mouse.21–25 Neural crest differentiation,24,25 Schwann cell survival,26 and oncogenic cellular transformation.27,28 In the present study, we used the dominant Ets approach to probe the spectrum of functions of Ets transcription factors in the epithelial compartment of the mammalian intestinal crypt-villus unit. Specifically, we used an Ets-dominant repressor, composed of the repressor domain of the Drosophila Engrailed (En) protein fused to the DNA-binding domain of the Ets factor Erm/ Etv5, to block endogenous Ets activity in vivo. As shown herein, En/Erm expression in the small intestinal epithelium under control of the well-characterized villin promoter/enhancer reveals Ets functions in multiple aspects of crypt-villus homeostasis, including epithelial maturation, epithelial transit, and complex architectural organization of the crypt-villus unit.

Materials and Methods

DNA Constructs

The expression constructs pSG5-HA/En/Erm, pSG5-HA/ ErmDBD, and pSG5-HA/EnRD were derived from the construct pTRE-HEEN (generously provided by John Shannon, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH). pTRE-HEEN contains the murine Erm DNA-binding domain (DBD) (amino acids 364 to 449) and the Drosophila Engrailed repressor domain (EnRD; amino acids 2 to 298). pSG5-HA/ErmDBD and pSG5-HA/ EnRD were generated by polymerase chain reaction (PCR) amplification of the ErmDBD and EnRD, respectively, from pTRE-HEEN and subcloning into pTRE-HA (Clontech, Palo Alto, CA), followed by PCR amplification of the HA-tagged inserts and subcloning into pSG5.29 pSG5-HA/En/Erm was generated by PCR amplification of both the EnRD and ErmDBD from pTRE-HEEN and subcloning into pTRE-HA, followed by PCR amplification of the HA-tagged En/Erm fusion and subcloning into pSG5. A seven amino acid (GGSGGSG) spacer was added between the EnRD and ErmDBD of the En/Erm fusion during the first PCR cloning step. All constructs also contained a C-terminal nuclear localization sequence (NLS; PKKRRKV, from the SV40 large T antigen), added during the first PCR amplification step. pSG5-HA/Erm was generated by subcloning a full-length Erm cDNA, amplified from a mouse embryonic brain library by reverse transcriptase (RT)-PCR, into pTRE-HA (Clontech), and then subcloning of the HA-tagged insert into pSG5. pSG5-HA/Ets2 was generated by subcloning a full-length mouse Ets2 cDNA (generously provided by James Hagman, National Jewish Medical and Research Center, Denver, CO) into pCGN2-HA,30 and then subcloning the HA-tagged insert into pSG5. pSG5-HA/Ei331 was generated by subcloning HA-tagged full-length human Ei331 into pSG5. The reporter construct 8x(EBS)-TK-luciferase was generated by subcloning the 8xpal sequence (containing eight copies of the DNA-binding site GCAG-GAACGA from the rat stromelysin promoter) from 8xpal-pBLCAT31 into pA-TK-luciferase.32 The transgenic construct villin-En/Erm was generated by subcloning the HA-tagged En/Erm fusion (also containing the C-terminal NLS) from pTRE-HA/En/Erm into the p12.4-kb Vill plasmid (generously provided by Deborah Gumucio, University of Michigan, Ann Arbor, MI). All plasmid DNA constructs were confirmed by diagnostic restriction enzyme digestion and, when PCR was used in the cloning process, DNA sequencing.

Cell Culture, Transfection, Reporter Assays, and Immunoblotting

HeLa cells were grown as previously described.31 For assays of transcriptional activity, cells were plated in 96-well plates at a density of 4 × 10^4 cells per well, and were transfected 15 to 18 hours later with 100 ng of the 8xEBS-TK-luciferase reporter plasmid, 1 ng of Renilla-luciferase plasmid, and varying amounts of expression plasmid(s), with the total amount of DNA kept constant by the addition of empty pSG5 expression vector. The cells were harvested 18 to 24 hours later, and luciferase activity was measured as previously described.31 For protein expression analysis, HeLa cells (3 × 10^6 cells in 200 µl of medium) were mixed with varying amounts of expression plasmid(s), the total amount of DNA being kept constant at 10 µg by the addition of empty pSG5 expression vector. Cells were transfected by electroporation using a Bio-Rad (Hercules, CA) Gene Pulser set at 220 V and 960 µF. Electroporated cells were diluted into 3 ml of medium in 60-mm plates and incubated for 24 hours. Cells were harvested in 0.5 ml of phosphate-buffered saline (PBS)/ethylenediaminetetraacetic acid, pelleted, and lysed in 100 µl of hot (65°C) TEA lysis buffer (55 mM Tris-HCl, pH 7.5, 111 mM NaCl, 2.2 mM ethylenediaminetetraacetic acid, and 0.44% sodium dodecyl sulfate) with the complete protease inhibitor cocktail (Roche, Indianapolis, IN). The lysates were vortexed, placed on ice, boiled for 5 minutes, returned to ice, and passed 7 to 10 times through a 27-gauge needle using a 1-cc syringe. Lysate protein concentration was determined by the Bradford assay, using the Bio-Rad protein assay reagent. Equal amounts of total extract protein were resolved on a sodium dodecyl sulfate-polyacrylamide gel and transferred to Immobilon-P mem-
3% H2O2 for 10 minutes. For BrdU immunostaining, nonspecific peroxidase activity was blocked by incubation in Biocare (Walnut Creek, CA) medical decloaker. Endogenous mmol/L sodium citrate buffer, pH 6.0, for 1 hour in a retrieval was performed by incubating the slides in 10 sections were deparaffinized and rehydrated. Antigen chemical staining. For immunohistochemical staining, sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), or processed further for immunohistochemistry. Histology and Immunohistochemistry

For BrdU-labeling experiments, animals were injected intraperitoneally with 50 mg/kg body weight of BrdU (Sigma, St. Louis, MO) in PBS, 2 or 24 hours before euthanasia. All animals were euthanized using CO2. The small intestine was immediately harvested and cut into three segments of approximately equal length. Fecal contents were gently expelled, the lumen was injected with fixative (4% paraformaldehyde), and the intestine was rolled concentrically and placed in a histology cassette. Tissues were fixed for 24 hours in 4% paraformaldehyde at 4°C, after which the tissues were placed in 70% ethanol, processed further on a standard histology processor, and paraffin-embedded. Sections 4-μm-thick were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), or processed further for immunohistochemical staining. For immunohistochemical staining, sections were deparaffinized and rehydrated. Antigen retrieval was performed by incubating the slides in 10 mmol/L sodium citrate buffer, pH 6.0, for 1 hour in a Biocare (Walnut Creek, CA) medical decloaker. Endogenous peroxidase activity was blocked by incubation in 3% H2O2 for 10 minutes. For BrdU immunostaining, slides were incubated for 90 minutes in 2 N HCl, washed with ddH2O, incubated for 5 minutes in 0.1 mol/L sodium borate, and washed again with ddH2O, before blockade of peroxidase activity. Immunohistochemical staining was performed using the Vectastain ABC or ImmPRESS kit (Vector Laboratories, Burlingame, CA), and developed using diaminobenzidine (DAKO, Carpinteria, CA, or Sigma). Primary antibodies used were: horseradish peroxidase-conjugated goat anti-HA (1:25, Roche) rabbit anti-iAP (1:500; Abcam, Cambridge, MA), rabbit anti-i-Fabp (Jeffrey Gordon, Washington University. St. Louis, MO); goat anti-Mcm6 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Ki-67 (1:200; NeoMarkers, Fremont, CA), mouse anti-BrdU (1:40; BD Biosciences, San Jose, CA), rabbit anti-TGFβRII (1:100, Santa Cruz Biotechnology), mouse anti-Cdx2 (1:25, Abcam), and mouse anti-β-catenin (1:500; BD Biosciences/Transduction Laboratories). Biotinylated secondary antibodies used were: goat anti-rabbit IgG and goat anti-mouse IgG (Vector Laboratories), and donkey anti-goat IgG (Santa Cruz Biotechnology). All immunohistochemically stained slides were counterstained with hematoxylin, dehydrated, mounted, and coverslipped.

Results

The En/Erm Dominant Repressor Potently Blocks Promoter Activity by Ets Transcription Factors

Most Ets transcription factors are expressed in the developing and/or mature mammalian intestine. Although 16 (of 26 total) Ets genes have been genetically inactivated in the mouse to date, only 1 (Eii3) has been reported to have an intestinal phenotype. The absence of intestinal phenotypes in the other Ets knockout animals is likely attributable to genetic compensation by co-expressed Ets factors. Ets genetic compensation can be overcome by the use of genetically manipulated Ets factors with dominant activity. We adopted this approach to probe the spectrum of Ets functions in the homeostasis of the intestinal crypt-villus axis in vivo. One possible dominant Ets approach, previously used by some, is the use of the Ets DNA-binding domain alone as a competitive inhibitor. We considered such an approach, but were concerned that it would rely too heavily on high expression levels, which can be difficult to achieve in transgenic systems. Because most Ets factors function predominantly as transcriptional activators, an alternative approach, used by others, is the use of an Ets dominant repressor, composed of the Ets DNA-binding domain (DBD) and the Droso phila Engrailed repressor domain. The Engrailed repressor domain functions by an active repression mechanism, and should thus effect more potent blockade of endogenous Ets promoter activity than the Ets DBD alone at similar expression levels. To test this, we generated the HA epitope-tagged construct En/Erm, composed of the Engrailed repressor domain (EnRD) fused to the amino terminus of the DNA-binding domain of the Ets factor Erm (ErmDBD). As shown in Figure 1A, when tested in a transient co-transfection assay, En/Erm was able to fully block transcriptional activation of an Ets-responsive reporter construct by Ets1. In comparison, an equivalent amount of the Erm DBD alone had a much weaker effect (Figure 1A). Importantly, EnRD alone had little effect on Ets activation (Figure 1A), indicating that the blocking effect of the En/Erm construct requires DNA binding, and...
The 12.4-kbp villin promoter-enhancer fragment, a HA epitope tag, the villin-En/Erm dominant repressor transgene. The transgene consists of B expression (plasmid pSG5, and all except Ets1 have an N-terminal HA epitope tag. Protein and SD of triplicate transfections. All constructs were expressed from the co-transfected Renilla-luciferase construct; results are expressed as mean determined by quantitative luminometry, was normalized to the activity of Ets, and Elf3, in a transient co-transfection assay. As shown in Figure 1B, En/Erm was able to block activation of an Ets-responsive reporter by all three Ets factors, at equivalent (for Ets2) or lower (for Erm and Elf3) relative protein expression levels. Thus, En/Erm has the ability to block the activity of multiple different Ets factors, and should therefore be a good reagent for probing the spectrum of Ets functions in vivo.

Characterization of Villin-En/Erm Transgenic Animals
En/Erm expression was targeted to intestinal epithelium using the well-characterized 12.4-kbp villin promoter. This promoter drives gene expression in small intestinal epithelium, and to a lesser extent colonic epithelium, from approximately embryonic day 12.5 on through adulthood, with expression greater in the villi than crypts. The modular organization of the villin-En/Erm transgene is shown in Figure 1C. Multiple transgenic lines were analyzed. Transgene expression was assayed by RT-PCR (not shown) and immunohistochemical staining against the HA epitope tag (Figure 2, A and B). Animals from one stable transgenic line and two independent transgene-positive mosaic founders manifested robust transgene expression detectable by immunohistochemistry (Figure 2B) and similar phenotypes in the small intestine under physiological conditions. These animals were thus further analyzed in detail. The similarity of the phenotypes in animals arising from three independent transgene insertion events confirms that the phenotypes are attributable to En/Erm expression, and not integration site effects. In agreement with previous studies, transgene expression was higher in the villi than the crypts (Figure 2B), and greater in the small intestine than the colon (data not shown). The transgene was expressed specifically in the nuclei of epithelial cells (Figure 2B), as expected and required for its dominant repressor effect on Ets-regulated gene expression.

En/Erm Expression in Small Intestinal Epithelium Reveals Novel Ets Functions in Villus Epithelial Maturation and Transit
In animals stably expressing immunohistochemistry-detectable En/Erm transgenic protein, the morphology of enterocytes (absorptive epithelial cells) along the length of the small intestine appeared abnormal on H&E-stained histological sections. In enterocytes from nontransgenic animals, a gradual histomorphological change could be seen as the cells progressed from the villus base to the villus tip, characterized by increasing cytoplasmic eosinophilia, and rounding and more basal position of the nucleus (Figure 2, C, E, and F). In contrast, in En/Erm-expressing animals from the stable transgenic line, en-
enterocytes along the length of the villus maintained a morphology that resembled the cells at the base, characterized by darker, more amphophilic cytoplasm, and more elongated and centrally positioned nuclei (Figure 2, D, G, and H). By H&E histomorphology, this phenotype thus suggested impaired enterocyte maturation.

To further characterize this phenotype, we examined the expression of markers of enterocyte differentiation and maturation. The transgenic enterocytes manifested a well-formed PAS-positive glycocalyx, ultrastructurally well-formed microvilli, and expression of intestinal alkaline phosphatase (iAP) and intestinal fatty acid binding protein (iFabp) (Figure 3, A–J; and data not shown); indeed, as in control animals, there appeared to be an appropriate gradient of increased iFabp expression from villus base to tip in the transgenic intestines. Thus, commitment to the enterocyte lineage and some aspects of enterocyte maturation were intact in the transgenic animals. However, in striking contrast to controls, transgenic intestines showed inappropriately persistent expression of Mcm6, a marker of villus epithelial immaturity, along the entire villus axis (Figure 3, K–P); furthermore, in the setting of high transgene levels, we observed residual Ki-67 expression in more superficial villus epithelium, not seen in controls (Figure 3, Q and R). Because the expression of these proteins is normally restricted to the less mature cells in the crypt and more basal villus epithelium (Figure 3, K–M, and Q), these findings support the presence of a disturbance of maturation in the transgenic enterocytes. Interestingly, the overall findings of histomorphological immaturity and misexpression of some (eg, Mcm6), but not other (eg, iFabp), enterocyte

Figure 2. Transgene expression and impaired enterocyte maturation in En/Ern-expressing small intestine. HA immunostaining of small intestine from control (A) and transgenic (B) animals shows the expression of En/Ern protein specifically in epithelial nuclei of transgenic animals. En/Ern expression was strong in the villus epithelium (B, open arrowhead), and was also observed in the superficial aspects of crypts (B, filled arrowhead), but not the deep aspects of crypts (B, arrow); no immunostaining was observed in control animals (A). H&E-stained sections of small intestine from control (C, detailed views of villus in E and F) and transgenic (D, detailed view of villus in G and H) animals, note morphological resemblance of transgenic enterocytes at the villus tip to enterocytes at the villus base (filled arrowheads, enterocytes at villus base; open arrowheads, enterocytes at villus tip).

Figure 3. Characterization of enterocyte maturation disturbance in En/Ern transgenic animals. Histochemical (PAS) and immunohistochemical (iAP, iFabp, Mcm6, and Ki-67) staining of small intestine from control (Tg−; A, C, E–G, K–M, and Q) and transgenic (Tg+; B, D, H–J, N–P, and R) animals. Arrowheads in A and B: PAS-positive glycocalyx on enterocytes; arrowheads in C and D: iAP expression in superficial aspect of enterocytes (insets: detailed views of villus tips); arrowheads in E–J: iFABP expression in enterocyte cytoplasm (F and H detailed views of villus tips in E and H, respectively, G and J; detailed views of villus bases in E and H, respectively); arrowheads in K–P: Mcm6 expression in enterocyte nuclei at villus base (filled arrowheads) and villus tip (open arrowheads); filled arrowheads in Q and R: upper limit of residual Ki-67 immunopositivity in villus enterocytes; open arrowheads in Q and R: solitary ectopic Ki-67 immunopositivity in transgenic superficial villus enterocytes (inset detailed view).
maturation markers suggest that En/Erm expression results in enterocyte maturation dys-synchrony.

The disturbed maturation of the En/Erm-expressing enterocytes prompted us to examine the intestinal epithelium for proliferative activity, which is normally restricted to the crypt but can inappropriately spread to villi in the context of some genetic manipulations. By H&E examination, no mitotic figures were detected in the villus epithelium, and brief (2-hour) in vivo BrdU labeling revealed epithelial proliferative activity appropriately restricted to the crypt compartment, as in controls (Figure 4, A and B). Thus, the immature-appearing En/Erm-expressing enterocytes are postmitotic cells, and the transgenic intestine maintains a normal crypt-villus boundary. We next examined the rate of epithelial transit along the crypt-villus axis because this could in principle account for altered epithelial maturation in the transgenic animals. After cell division in the crypt, intestinal epithelial cells normally migrate in an orderly manner out of the crypt, and from villus base to tip, where they are ultimately shed into the lumen. The rate of epithelial transit can be assayed in vivo by a BrdU pulse followed by an extended chase period (eg, 24 hours), which allows postmitotic, BrdU-labeled cells to migrate up the axis. As shown in Figure 4, C–F, En/Erm-expressing animals showed much more rapid epithelial migration compared with nontransgenic animals. En/Erm expression thus results in more rapid epithelial transit along the crypt-villus axis.

The identical disturbance of enterocyte maturation was observed in En/Erm-expressing foci of the independent transgenic founders with mosaic expression of the transgene. This was evident by both histomorphological examination (Figure 5, A–D) and Mcm6 immunostaining (Figure 5, E–H). Interestingly, individual mosaic villus in these animals showed an abrupt transition between immature En/Erm-positive cells and directly adjacent En/Erm-negative cells, with the latter consistently exhibiting maturation appropriate for their position along the villus axis (Figure 5, I–J). This indicates that the En/Erm-induced disturbance of enterocyte maturation is cell-autonomous, and thus unlikely to be attributable to global acceleration of epithelial transit alone.

Enterocytes represent one epithelial lineage in the small intestine, the others being the secretory lineages, composed of goblet cells, Paneth cells, and neuroendocrine cells. To determine whether En/Erm expression affected these lineages, we performed quantitative morphometric analysis of secretory epithelial cells. The overall number and localization of goblet cells, neuroendocrine cells, and Paneth cells were not significantly different from nontransgenic controls (data not shown). Thus, En/Erm expression does not affect secretory epithelial cell specification.

Ets-regulated epithelial expression of transforming growth factor-β type II receptor (TGFβRII) has recently been shown to play an important role in small intestinal epithelial differentiation during embryogenesis. We thus examined TGFβRII expression in En/Erm-expressing small intestines with disturbed maturation. In contrast to the dysmature embryonic small intestine of Elf3 knockout mice, we did not see loss of TGFβRII expression in
adult En/Erm-expressing small intestine (Figure 6, A and B). Thus, additional, TGFβRII-independent, Ets-mediated pathways appear to regulate epithelial differentiation in the adult small intestine. Interestingly, in the setting of high-level En/Erm expression, we observed inappropriate persistence of villus Cdx2 expression in the proximal small intestine (Figure 6, C and D), suggesting a possible role for this important regulator of intestinal epithelial differentiation in the disturbed maturation phenotype.

**En/Erm Expression in Small Intestinal Epithelium**

Results in Villus and Crypt Architectural Disorder

Small intestinal villi maintain an exquisitely ordered architecture under normal homeostasis, characterized by even spacing, unidirectional (radial) growth, relatively constant height for a given intestinal segment, and absence of branching. Compared with villi from nontransgenic animals (Figure 7A), villi in En/Erm-expressing animals showed marked architectural disorganization (Figure 7, B and C), including branch formation (arrows), mid-villus changes in the direction of growth (arrowheads), and villus bridging. The degree of villus dysmorphogenesis correlated with the level of En/Erm expression in individual animals. On average, as many as 14.5% of villi showed branching in a given intestinal segment (Figure 7D). Villus branching was also observed in the independent transgenic animals mosaic for En/Erm expression (Figure 7D). Recently, ectopic activation of β-catenin in villus epithelium, either directly or secondary to blockade of Hedgehog signaling, has been shown to result in marked disruption of villus morphogenesis during intestinal development.39,40 However, we did not observe ectopic nuclear translocation, indicative of activation, of β-catenin in the dysmorphic En/Erm-expressing intestine (Figure 7, E–J; in control experiments, the same antibody was able to robustly detect inappropriate activation of β-catenin in the setting of epithelial neoplasia (Supplemental Figure S1 available at

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**Figure 6.** Analysis of potential mediators of the disturbed epithelial maturation phenotype. TGFβRII (A and B)- and Cdx2 (C and D)-immunostained small intestine from control (Tg−) and transgenic (Tg+) animals. Note similar level of expression of TGFβRII in control and transgenic animals (arrowheads in A and B), and inappropriate persistence of Cdx2 expression throughout villi of transgenic animals compared with controls (arrowheads in C and D). TGFβRII is cytoplasmic, whereas Cdx2 is nuclear. Insets: Magnified views of villus epithelium.

**Figure 7.** Villus architectural dysmorphogenesis in En/Erm-expressing small intestine. A–C: H&E-stained sections of small intestine from control (Tg−) and transgenic (Tg+) animals (arrow: villus branching; arrowheads: villus turns; asterisk: villus bridging). D: Quantiﬁcation of villus branching in control (Tg−, n = 80) and transgenic (Tg+, n = 53) animals, and transgene-expressing foci in mosaic animals (Tg+tm, n = 2), in proximal (PSI), mid (MSI), and distal (DSI) small intestine. β-Catenin immunostaining in control (E–G) and transgenic (H–J) animals. Note nuclear staining (ﬁlled arrowheads, G and J) limited to epithelial cells at bases of crypts, and membranous (ﬁlled arrowheads, F and D) but no nuclear (open arrowheads, F and D) staining in villi, in both transgenic and control animals.
was little effect of En/Erm on individual crypt size and morphology (Figure 8C). However, in larger foci of En/Erm protein expression, alterations in the crypt compartment were observed, including crypt branching and disorder of orientation (Figure 8, D and E). Morphometric analysis of crypt epithelial proliferation and apoptosis in the mosaic animals did not reveal statistically significant differences between En/Erm expressing and nonexpressing foci (Figure 8, H and I). Thus, the alterations in crypt homeostasis in areas of En/Erm expression appear to involve crypt nonautonomous mechanisms, independent of significant effects on crypt epithelial proliferation or apoptosis.

Discussion

**Ets Factors and Intestinal Epithelial Maturation and Transit**

Our studies using targeted expression of the Ets dominant repressor En/Erm in the intestine reveal multiple novel roles for epithelial Ets transcription factors in small intestinal crypt-villus homeostasis. First, En/Erm expression leads to disturbed maturation of the enterocyte epithelial lineage. This phenotype is interesting, and to our knowledge unique, in that it shows features of dysynchrony, whereby some maturation steps, namely accumulation of cytoplasmic iFabp, proceed normally, whereas others, namely extinction of nuclear Mcm6, do not. Indeed, this phenotype suggests that enterocyte maturation in the adult intestine is controlled by multiple parallel and independent pathways.

The Ets transcription factor Elf3 has recently been shown to be an important regulator of intestinal epithelial differentiation during embryonic development, raising the possibility that the En/Erm phenotype is attributable to blockade of Elf3 in the adult intestine. However, although superficially similar (impaired epithelial differentiation/maturation), the En/Erm phenotype differs from the Elf3−/− phenotype in several respects. We do not observe the microvillus dysmorphogenesis or decreased goblet cell numbers seen in Elf3−/− embryonic intestine. Further, in contrast to Elf3−/− embryonic intestine, we do not observe loss of TGFβRII expression in association with the villin-En/Erm disturbed epithelial maturation phenotype. Interestingly, epithelial-specific deletion of TGFβRII in the mouse small intestine is reported to be free of phenotypic changes under conditions of homeostasis. Together, these findings suggest that signaling via TGFβRII, although apparently important in the developing intestine, may have a less important role in epithelial differentiation in the adult intestine. Interestingly, our studies suggest a possible role for Cdx2 in Ets-mediated epithelial maturation in the adult intestine. Cdx2 is known to be able to promote intestinal epithelial differentiation. However, in the proximal adult small intestine, Cdx2 is expressed in a diminishing gradient along the villus axis (Figure 6C), opposite to the gradient of epithelial maturation. Moreover, we observed inappropriately persistent Cdx2 expression in the setting of
En/Erm-induced epithelial immaturity. Together, these findings suggest that Cdx2 may function as a negative regulator of intestinal epithelial maturation, and that the role of Ets factors in adult intestinal epithelial maturation may involve down-regulation of Cdx2 expression. Whether the En/Erm epithelial dysmaturation phenotype uncovers TGFβRII-independent activity of Elf3, and/or the activity of other Ets factor(s) remains to be determined.

The increased intestinal epithelial transit in villin-En/Erm animals is the first demonstration of a role for Ets factors in epithelial movement along the crypt-villus axis, an important parameter of intestinal homeostasis. Given the villus-predominant pattern of En/Erm expression and the lack of a crypt-autonomous En/Erm phenotype in the transgenic animals, we think that the increased transit phenotype uncovers a specific function of Ets factors in the control of epithelial movement along the crypt-villus axis, rather than a nonspecific effect of increased crypt epithelial production. Little is currently known about mechanisms specifically controlling epithelial transit in the intestine. E-cadherin has been shown to be one important regulator of this process. However, by immunohistochemical staining, we did not observe alterations in E-cadherin expression or localization in villin-En/Erm animals with increased transit (data not shown). Thus, Ets factors appear to regulate intestinal epithelial transit independently, or alternatively downstream, of E-cadherin. The apparent absence of a compensatory increase in epithelial production in response to the accelerated transit in the transgenic animals is somewhat surprising. We suspect that this reflects our inability to detect small changes in proliferation and/or apoptosis in our analyses; indeed, although not statistically significant, there appeared to be a trend toward increased crypt epithelial proliferation in the mid-small intestine of transgenic animals, where phenotypes were also generally most pronounced (Figure 8F). Interestingly, it is worthy of mention that alterations in villus epithelial transit, apparently unaccompanied by changes in crypt epithelial production, have been reported in the setting of other genetic manipulations of the intestine.

**Ets Factors and Architectural Organization of theCrypt-Villus Axis**

The crypt-villus unit of the small intestine forms relatively late in development, and from here on maintains an exquisitely organized architecture: individual units are precisely spaced, and the separate identity, size (depth of crypts and height of villi), and orientation (perpendicular to the length of the intestine) of the crypt and villus components are precisely maintained. Opposing activities of Wnt and Hh signaling, as well as Ephrin signaling, itself regulated by the Wnt pathway, normally restrict epithelial proliferation to the crypt compartment, and differentiation to the villus compartment. Disruption of the Wnt and Hh pathways, directly or indirectly resulting in ectopic activation of β-catenin, can give rise to marked alterations in villus architecture and loss of the normal crypt-villus boundary. Interestingly, En/Erm expres-
effects via lower affinity sites) at supraphysiological expression levels. Finally, the Ern/Erm phenotype of disturbed crypt-villus homeostasis also suggests possible roles for Ets factors in the control of epithelial regeneration/repair after injury.

Acknowledgments

We thank Melissa Gonzalez for generating the 8x(EBSS)-TK-luciferase reporter construct; the University of Colorado Cancer Center Transgenic/Knockout Core Facility for generation of villin-En/Erm transgenic mice; Jana Polzer, Janet Lieber, Michael Medina, and Sara Garza-Williams for assistance with mouse histology; Janet Lieber and Gary Mierau for assistance with electron microscopy; Heide Ford and Erica McCoy for assistance with HA immunostaining; John Shannon (Cincinnati Children's Hospital Medical Center), Deborah Gumucio (University of Michigan), Jeffrey Gordon (Washington University), and James Hagman (National Jewish Medical and Research Center) for reagents; and Heide Ford and Sean Colgan for critical reading of the manuscript.

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The Ets dominant repressor En/Erm enhances intestinal epithelial tumorigenesis in Apc\textsuperscript{Min} mice

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Published: 22 June 2009


Accepted: 22 June 2009

This article is available from: http://www.biomedcentral.com/1471-2407/9/197

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Abstract

Background: Ets transcription factors have been widely implicated in the control of tumorigenesis, with most studies suggesting tumor-promoting roles. However, few studies have examined Ets tumorigenesis-modifying functions in vivo using model genetic systems.

Methods: Using mice expressing a previously characterized Ets dominant repressor transgene in the intestinal epithelium (Villin-En/Erm), we examined the consequences of blocking endogenous Ets-mediated transcriptional activation on tumorigenesis in the Apc\textsuperscript{Min} model of intestinal carcinoma.

Results: En/Erm expression in the intestine, at levels not associated with overt crypt-villus dysmorphogenesis, results in a marked increase in tumor number in Apc\textsuperscript{Min} animals. Moreover, when examined histologically, tumors from En/Erm-expressing animals show a trend toward greater stromal invasiveness. Detailed analysis of crypt-villus homeostasis in these En/Erm transgenic animals suggests increased epithelial turnover as one possible mechanism for the enhanced tumorigenesis.

Conclusion: Our findings provide in vivo evidence for a tumor-restricting function of endogenous Ets factors in the intestinal epithelium.

Background

Members of the Ets transcription family, numbering up to 27 in humans, are widely expressed in developing and mature tissues, and regulate diverse cellular processes [1,2]. Ets factors are also frequently misexpressed in the setting of neoplasia. Many Ets factors become overexpressed in tumors and appear to play tumor-promoting roles, while a limited number, notably the epithelial specific Ets, may perform tumor suppressor functions [1,3-5]. However, to date, most information about Ets functions in tumorigenesis has come from cell culture and animal xenograft models. Indeed, very few studies have examined Ets functions in tumorigenesis in vivo using model genetic systems [6-8]. Ets factors are widely expressed in the intestine, and often misexpressed in carcinoma of the colon, but their tumor-modifying roles in intestinal epithelial neoplasia in vivo largely remain to be defined [9].

We have previously generated and characterized transgenic mice expressing an Ets dominant repressor (En/
Erm) with broad Ets-blocking activity in the small intestinal epithelium [10]. Nearly all members of the Ets transcription factor family are expressed in the mature mammalian intestine, but expression levels vary widely [9,11]. Our previous study characterized the phenotypic consequences of transgene expression at immunohistochemically detectable levels, which resulted in marked disturbance of crypt-villus homeostasis [10]. Additional transgenic lines, expressing En/Erm at levels detectable by RT-PCR but not immunohistochemistry (“low expressors”), did not manifest an overt dysmorphogenic phenotype under normal physiologic conditions, despite the fact that En/Erm is able to block Ets activity at substochiometric levels in vitro [10]. To determine whether this low-level En/Erm expression has phenotypic consequences under pathologic conditions, we tested its effect on intestinal epithelial tumorigenesis in the ApcMin mouse, a well-established model of multiple intestinal neoplasia [12-14].

We find that animals with low-level En/Erm expression develop more than twice as many tumors in the small intestine as non-transgenic ApcMin controls. Interestingly, while these animals do not manifest the overt crypt-villus dysmorphogenesis phenotype under conditions of homeostasis previously described for high-level En/Erm expressors [10], they do show a mild increase in epithelial transit. Thus, the increase in tumor number in the En/Erm animals may in part be due to increased crypt-villus epithelial turnover. Moreover, on histologic analysis, tumors from En/Erm-expressing animals show a trend toward greater stromal invasion. Our studies in a genetic tumor model thus uncover an unexpected role for epithelially expressed Ets factors in the restriction of tumorigenesis in the intestine.

Methods

Animals

Villin-En/Erm transgenic animals were generated as previously described [10], and were maintained in an FVB/N background. ApcMin/+ animals were obtained from Jackson Laboratories and were maintained in a C57BL/6j genetic background. Experimental and control animals for the tumor study were both derived from a cross between Villin-En/Erm animals and ApcMin/+ animals. The studies were thus carried out in a hybrid (C57BL/6j × FVB/N) background, as done by others [15]. In order to control for possible confounding effects of the modifier-of-Min locus (Mom1), the major modifier of tumor multiplicity in the ApcMin strain [16], the Mom1 genotype (resistant versus sensitive) was determined, as previously described [15,17], and such analyses indicated that all animals in the study were heterozygous (Mom1<sup>SR</sup>) for the Mom1 locus. The presence of the Villin-En/Erm transgene and ApcMin mutation were determined by PCR genotyping of tail-biopsy DNA, as previously described ([10]; http://jaxmice.jax.org). Tumor number, size and histology in Apc<sup>Min</sup> and Apc<sup>Min</sup>;Villin-En/Erm animals were evaluated in H+E-stained sections of the complete length of the small intestine by a pathologist (PJ) blinded to the genotype. All animal work was carried out under protocols approved by the Institutional Animal Care and Use Committee.

Histology and immunohistochemistry

Animals were euthanized using CO<sub>2</sub> followed by cervical dislocation. The small intestine was immediately harvested and cut into two to three segments of approximately equal length. Fecal contents were gently expelled, the lumen was injected with fixative (4% paraformaldehyde), and the intestine was rolled concentrically and placed in a histology cassette. Fixation was for 24 hours in 4% paraformaldehyde at 4°C, after which the tissues were placed in 70% ethanol, processed further on a standard histology processor and paraffin-embedded. Sections 4 µm thick were stained with hematoxylin and eosin (H+E) or processed further for immunohistochemical staining. For immunohistochemical staining, sections were deparaffinized and rehydrated. Antigen retrieval was performed by incubating the slides in 10 mM sodium citrate buffer, pH 6.0, for 1 hour in a Biocare Medical Decloaker. Endogenous peroxidase activity was blocked by incubation in 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes. Immunohistochemical staining was performed using the M.O.M. (mouse on mouse) kit (Vector Laboratories), and developed using DAB (Dako or Sigma). Primary antibodies used were mouse anti-smooth muscle actin (Dako, 1:100) and mouse anti-E-cadherin (BD Biosciences, 1:100). Mmc6 and -catenin immunohistochemical staining, and BrdU labeling and immunohistochemical staining were performed as described previously [10]. All immunohistochemically stained slides were counterstained with hematoxylin, dehydrated, mounted and coverslipped.

RT-PCR

Following euthanasia, the small intestine was removed, cut into multiple segments and fecal contents were gently expelled. The intestinal segments were opened lengthwise and stored in RNAlater (Ambion) at 4°C overnight. The tissue was removed from RNAlater and laid mucosal surface up onto Petri dish lids. The mucosa was gently scraped off with a razor blade and collected in a 1.5 ml tube. Trizol (1 ml; Invitrogen) was added, the tissue was homogenized with a disposable pestle (Fisher), and RNA was isolated per manufacturer protocol (Invitrogen) and stored at -80°C. Ten (10) µg of RNA were treated with DNase using a DNA-free kit (Ambion), and 1.6 µg of treated RNA were reverse transcribed using Superscript III reverse transcriptase (Invitrogen), per manufacturer protocol, using random primers in a 20 µl reaction. In paral-
Intestinal En/Erm expression increases tumor number in Apc<sup>Min</sup> mice

We have previously generated and characterized transgenic animals expressing the Ets dominant repressor En/Erm, composed of an HA epitope tag, the Engrailed repressor domain and the DNA-binding domain of the Ets factor Erm, in the intestinal epithelium under control of the Villin promoter. En/Erm can potently block transcriptional activation by multiple members of the Ets family and, when expressed at immunohistochemically detectable levels in the intestinal epithelium, causes a severe disturbance of crypt-villus homeostasis [10]. We established additional lines, which stably integrated the Villin-En/Erm transgene, but did not express En/Erm at immunohistochemically detectable levels. Animals from such lines did not manifest an overt disturbance of crypt-villus homeostasis (Figure 1A), indicating a threshold level of transgene expression for the previously described dramatic dysmorphogenesis phenotype [10]. Such animals did, however, express the transgene at levels detectable by RT-PCR (Figure 1B). Expression levels of different Ets factors in the intestine vary widely [9]. We thus wondered whether such low transgene expressors, free of phenotype under conditions of normal homeostasis, might manifest a phenotype under pathologic conditions, by uncovering a differential requirement for endogenous Ets factor(s) expressed at lower relative levels.

We thus examined the effect of low-level En/Erm expression on intestinal epithelial tumorigenesis. Our prior studies showed that the Villin-En/Erm transgene is expressed primarily in the small intestine [10]. We therefore chose the Apc<sup>Min</sup> mouse as the tumor model, as these animals develop multiple epithelial tumors predominantly in the small intestine [13]. The tumor studies were carried out in a hybrid (C57BL/6J × FVB/N) genetic background, since the Villin-En/Erm transgenic lines were generated in the FVB/N strain while the background of the Apc<sup>Min</sup> animals was C57BL/6J. As others have done in similar studies [15], we determined the genotype of the major modifier of tumor multiplicity in the Apc<sup>Min</sup> strain, Mm1, in order to control for possible genetic background differences between control (Apc<sup>Min</sup>) and experimental (Apc<sup>Min</sup>;Villin-En/Erm) groups. Such analyses indicated that all animals in both control and experimental groups were heterozygous for the Mm1 locus, and thus similarly susceptible to Apc<sup>Min</sup>-driven tumorigenesis.

Figure 1
Intestinal histology and En/Erm expression in transgenic animals in the tumor study. (A) Representative histology (H+E-stained sections) of mid small intestine from non-transgenic (Tg<sup>-</sup>) and Villin-En/Erm transgenic (Tg<sup>+</sup>) animals under conditions of normal homeostasis. The transgenic animals show crypt-villus morphology indistinguishable from control non-transgenic animals. (B) Levels of Actin and En/Erm transgene RNA in non-transgenic (Tg<sup>-</sup>) and transgenic (Tg<sup>+</sup>) animals, as determined by RT-PCR (cycle number: 25).

As shown in Table 1, Apc<sup>Min</sup>;Villin-En/Erm animals developed 2.4 times as many total small intestinal tumors as Apc<sup>Min</sup> controls (16.5 vs. 6.8, p = 0.03). The Apc<sup>Min</sup>;Villin-En/Erm group included approximately equal numbers of animals from three independent Villin-En/Erm transgenic lines, each of which developed on average more tumors than the Apc<sup>Min</sup> control group (data not shown), indicating that the differences in tumor number were due to En/Erm expression rather than transgene integration site effects. Apc<sup>Min</sup>;Villin-En/Erm mice developed more tumors in each intestinal segment (proximal, mid and distal) compared to the Apc<sup>Min</sup> controls, although the differences were greatest and most statistically significant in the mid small intestine (8.4 vs. 2.8, p = 0.03; Table 1 and Figure 2). Interestingly, this is the segment where we previously observed the most severe crypt-villus dysmorphogenesis phenotypes in high En/Erm expressor
Table 1: Summary of animal tumor data

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<td>DSI</td>
<td>2.9 (0.3)</td>
<td>4.8 (0.4)</td>
<td>0.37</td>
</tr>
<tr>
<td>Tumor size (total; cm)*</td>
<td>0.31 (0.03)</td>
<td>0.31 (0.02)</td>
<td>0.89</td>
</tr>
<tr>
<td>PSI</td>
<td>0.33 (0.07)</td>
<td>0.25 (0.04)</td>
<td>0.33</td>
</tr>
<tr>
<td>MSI</td>
<td>0.20 (0.02)</td>
<td>0.27 (0.01)</td>
<td>0.001</td>
</tr>
<tr>
<td>DSI</td>
<td>0.32 (0.04)</td>
<td>0.32 (0.02)</td>
<td>0.82</td>
</tr>
<tr>
<td>% tumors with stromal invasion</td>
<td>10.9 (4.6)</td>
<td>19.3 (4.6)</td>
<td>0.17</td>
</tr>
<tr>
<td>% histologically aggressive tumors</td>
<td>3.7 (2.8)</td>
<td>4.7 (2.4)</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* largest dimension from slide
*** from two-tailed student t-test with unequal variance
SEM: standard error of the mean
PSI, MSI and DSI: proximal, mid and distal small intestine, respectively

As expected, tumor numbers in the colon were lower, and not significantly different between the ApcMin;Villin-En/Erm and ApcMin groups (0.64 vs. 0.75, p = 0.80), consistent with the lower levels of transgene expression in this part of the intestinal tract [10,18]. There was no significant difference in average overall small intestinal tumor size between the ApcMin;Villin-En/Erm and ApcMin groups (Table 1), suggesting that the En/Erm tumor-promoting effect acts predominantly at an early stage of tumorigenesis. One exception was the mid small intestine, where a modest, but statistically significant, increase in tumor size was observed in the ApcMin;Villin-En/Erm group (Table 1); hence, locally, En/Erm may also promote later (post-initiation) stages of tumorigenesis. Thus, low-level expression of the Ets dominant repressor En/Erm enhances intestinal tumorigenesis in ApcMin mice.

As discussed, the low expressor transgenic animals in the tumor studies did not manifest an overt intestinal dysmorphogenesis phenotype, as judged by H&E histomorphology, in contrast to the previously described high expressor animals [10]. To determine whether more subtle phenotypes might be present under conditions of homeostasis, we performed additional analyses. As shown in Fig. 3, Mcm6 immunohistochemical staining confirmed the presence of normal epithelial maturation in the Villin-En/Erm animals, indistinguishable from non-transgenic controls. Interestingly, by in vivo BrdU labeling, the low expressor En/Erm animals did show a modest increase in crypt-villus epithelial transit, smaller than previously described in high expressors [10], but clearly different from non-transgenic controls (Figure 3). Thus, the tumor-promoting effect of En/Erm may act in part by increasing epithelial turnover.

Effect of En/Erm expression on tumor invasiveness in ApcMin mice

By histologic analysis, tumors in both control (ApcMin) and experimental (ApcMin;Villin-En/Erm) groups included adenomas and adenocarcinomas with similar morphologic features. We found evidence of stromal invasion (of the lamina propria and beyond; Figure 4A–C) in 70% of the animals. There was a trend toward greater invasiveness in the ApcMin;Villin-En/Erm group (19.3% vs. 10.9% of tumors), although this difference did not reach statistical significance (p = 0.17) (Table 1). Further, nearly one third of the tumors showing stromal invasion manifested highly aggressive histology, characterized by
small, poorly differentiated glands and clusters of malignant epithelial cells in a densely collagenized, desmoplastic stroma (Figure 4D and 4E). Such tumors expressed high levels of both cytoplasmic and nuclear (activated) -catenin, but retained epithelial characteristics, including membranous E-cadherin expression (Figure 4F and 4G). Adenomas associated with such tumors tended to contain regions with cytologic features of high-grade dysplasia, including large nuclei with prominent nucleoli and brisk mitotic activity (data not shown). The incidence of these aggressive tumors was not significantly different between the experimental (ApcMin;Villin-En/Erm) and control (ApcMin) groups (4.7% vs. 3.7%, p = 0.78). Thus, a relatively high proportion of ApcMin-induced neoplastic lesions gives rise to invasive and histologically aggressive adenocarcinomas in a C57BL/6J x FVB/N hybrid genetic background, and En/Erm-expressing tumors show a trend toward greater stromal invasiveness.

**Discussion**

Members of the Ets transcription factor family are frequently misexpressed or otherwise dysregulated in diverse human malignancies [1,4,5]. Although Ets factors have been shown to regulate a variety of cellular processes relevant to tumorigenesis [1,4,5], relatively little is known about their effects on tumor initiation and/or progression in vivo. Indeed, to date, few studies have examined Ets functions in tumorigenesis using animal genetic models (Table 2). In mouse models of breast cancer, PEA3 and Ets2 have been shown to exert tumor-promoting effects, the former in the epithelium and the latter in the stroma [6-8]. In the present study, we examined the effect of expression of the Ets dominant repressor transgene En/Erm on ApcMin-driven tumorigenesis in the intestine. Suprisingly, we find that low-level expression of En/Erm in the intestinal epithelium more than doubles the tumor number in ApcMin mice. This effect appears to act at an early stage in tumor formation, as En/Erm seems to have little effect on tumor size. Interestingly, while lacking the overt crypt-villus dysmorphogenesis phenotype of high-expressor animals, the low-expressor mice analyzed in this tumor study do retain a degree of increased crypt-vil-
Invasive adenocarcinomas in animals in tumor study. (A-C) H+E-stained (A: low-power view; B: high-power view) and SMA immunostained (C) histologic sections of a representative invasive tumor. Note neoplastic epithelium invading through the muscularis propria (mp) of the intestinal wall (solid arrowheads) and into the outermost serosa layer (s and open arrowheads; m: mucosa; SMA immunostain highlights well-oriented muscularis propria layer). (D-G) Histologically aggressive lesions. H+E-stained (D: low-power view; E: high-power view) histologic sections showing poorly formed glands and clusters of neoplastic epithelial cells (asterisk in D and arrow in E) in a densely collagenized desmoplastic stroma. These aggressive lesions express high-levels of cytoplasmic and nuclear beta-catenin (arrowhead in F; inset: detailed view), but retain membranous E-cadherin expression (arrowheads in G; inset: detailed view).
lus epithelial transit. This suggests that the En/Ern tumor-promoting effect may act in part through increased epithelial turnover. As an interesting corollary, it also implies that epithelial maturation and transit, found to be coordinately disrupted in the high-expressor En/Ern animals [10], are regulated by different mechanisms, as the phenotypes are genetically separable in the low-expressor mice.

Most Ets factors function as transcriptional activators [1,2]. Since En/Ern exerts its effect by blocking Ets-mediated transcriptional activation [10], our findings imply that the endogenous Ets factors blocked by En/Ern normally function to restrict tumorigenesis in the intestinal epithelium. Due to the high conservation of the Ets domain, the En/Ern protein is able to block transcriptional activation by multiple different Ets factors [10], making it difficult to determine which individual Ets is/are responsible for this tumorigenesis-modifying effect. We have previously shown that higher (immunohistochemically detectable) levels En/Ern expression result in small intestinal crypt-villus dysmorphogenesis, probably by interfering with the activity of relatively abundant Ets factors in the intestine, such as Elf3, Ehf and/or Ets2 [10]. In contrast, possible candidate Ets factors responsible for the tumor phenotype include those with normally lower relative expression levels in the intestine, such as PEA3, ERM and/or ELF1 [9]. Alternatively, the tumor phenotype may be uncovering a differential requirement for a more highly expressed Ets. Interestingly, similar to our findings, Sasson et al recently observed an inverse relationship between Ets2 gene copy number and tumor number in the ApcMin model of intestinal carcinoma. Tumor size is relatively unaffected, indicating that this effect acts predominantly at the level of tumor initiation or/and early promotion. Histologic examination of the tumors suggests that En/Ern expression may also promote stromal invasion. Together, these findings from an animal genetic model provide in vivo evidence for an unexpected role for endogenous Ets factors in the restriction of epithelial tumorigenesis in the intestine. Moreover, our studies suggest that the ApcMin mutation may provide a good model for invasive human intestinal carcinoma in the C57BL/6J x FVB/N hybrid genetic background.

**Conclusion**

Expression of the Ets dominant repressor En/Ern in the small intestine, at levels that do not cause crypt-villus dysmorphogenesis, results in a marked increase in tumor number in the ApcMin model of intestinal carcinoma. Tumor size is relatively unaffected, indicating that this effect acts predominantly at the level of tumor initiation or/and early promotion. Histologic examination of the tumors suggests that En/Ern expression may also promote stromal invasion. Together, these findings from an animal genetic model provide in vivo evidence for an unexpected role for endogenous Ets factors in the restriction of epithelial tumorigenesis in the intestine. Moreover, our studies suggest that the ApcMin mutation may provide a good model for invasive human intestinal carcinoma in the C57BL/6J x FVB/N hybrid genetic background.

**Competing interests**

The authors declare that they have no competing interests.
Authors’ contributions
PJ designed the study and analyzed the data. PJ and XS carried out the experiments. PJ and AGH wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We wish to thank Jana Polzer, Janet Lieber, Michael Medina and Sara Garza-Williams for assistance with mouse histology, and Heide Ford for critical reading of the manuscript. This work was supported by the Cancer League of Colorado, The Children’s Hospital/University of Colorado Denver Research Institute, NIH K08 DK74557 (to PJ), and NIH R01 DK37667 and DK46868 (to AGH).

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Pre-publication history
The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/9/197/prepub

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ESE-1 is Required to Maintain the Transformed Phenotype of MCF-7 and ZR-75-1 Human Breast Cancer Cells

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Abstract: Background: ETS factors comprise a large transcription factor family known to play a significant role in cellular development, differentiation, and transformation. The human Epithelial Specific Ets factor-1, ESE-1, is particularly relevant in breast cancer. Specifically, increased mRNA expression of ESE-1 and the Her2/neu proto-oncogene are correlated in breast cancer, and activation of the Her2/Neu receptor induces ESE-1 gene transcription. Stable expression of ESE-1 initiated transformation of ESE-1-negative MCF-12A immortalized human mammary epithelial cells, leading to increased migration, invasion and anchorage independent growth. However, little is known about ESE-1 protein expression and its role in maintaining the transformed phenotype in human breast cancer cell lines. Results: Here, we used an anti-ESE-1 mouse monoclonal antibody in Western blot and immunofluorescent cell analyses to show that ESE-1 is expressed as a nuclear protein in MCF-7, T47D and ZR-75-1 transformed, tumorigenic mammary epithelial cell lines, and that it is not expressed in transformed MDA-MB-231 and nontransformed MCF-10A and MCF-12A cells. In addition, specific knockdown of endogenous ESE-1 in the human breast carcinoma ZR-75-1 and MCF-7 cell lines decreased colony formation and anchorage independent growth. Mechanistically, ESE-1 knockdown decreased cellular proliferation, but had no effect on apoptosis. Conclusions: These results establish that the knockdown of a single ETS factor, ESE-1, is sufficient to reverse the transformed phenotype in breast cancer and demonstrate that ESE-1 is required for cellular proliferation. Thus, ESE-1 plays a key role in maintaining the transformed phenotype in breast cancer, providing a novel single-point target for therapy.

Keywords: ETS, transformation, breast cancer, shRNA.

INTRODUCTION

The ETS transcription factor family is composed of 27 members in humans, and ETS proteins appear to have important roles in cellular proliferation, differentiation and transformation [1-3]. This large family of transcription factors is characterized by a conserved winged helix-turn-helix DNA binding domain (DBD), the ETS domain, which mediates binding to target DNA sequences [3]. ETS proteins function as transcriptional activators or repressors, and are regulated by protein-protein interactions and mitogen-activated protein kinase (MAPK) phosphorylation [1-3]. In particular, activation of the Ras proto-oncogene has been shown to mediate phosphorylation of several ETS factors [3, 4]. This is significant because the Ras pathway is critical in regulating cell cycle and proliferation [3, 4]. This, in conjunction with ETS chromosomal translocations and over-expression, suggests that this family has a major role in oncogenesis [1, 5, 6].

Epithelial specific ETS factor-1, ESE-1 (also known as ESX, Jen and ERT, and Elf3 in mice) is a ~42 kDa protein, and it is the defining member of the epithelial-restricted, ESE subfamily of ETS transcription factors [7-10].

ESE-1 cDNA was first isolated while screening cDNA from human keratinocytes and pancreatic cancer tissue for novel transcripts containing the ETS domain [7, 9]. Since ESE-1’s initial isolation, its mRNA expression has been documented in several human and rodent epithelial tissues, including placenta, lung, kidney, prostate, intestine, breast, skin, retina and other epithelia [7-10]. During mouse embryo development, Elf3 mRNA expression levels increase progressively, from embryonic day 7 to day 17, and in post-embryonic mammary gland development, Elf3 is found in virgin, pregnant and involuting mammary glands [11]. The ESE-1 locus, 1q31.1, is located in a region that is commonly amplified in human breast cancer [10, 12]. Furthermore, over-expression of ESE-1 mRNA has been demonstrated in lung cancer and in Her2/neu-positive ductal carcinomas in situ (DCIS) [10, 12]. This increased ESE-1 mRNA expression has been shown to correlate with increased expression of the Her2/Neu proto-oncogene in breast cancer cell lines and in 45 primary ductal breast cancer samples [12, 13]. In addition, activation of the Her2/Neu receptor induces ESE-1 gene transcription [12, 13]. Therefore, a positive-feedback-loop is predicted based on increased ESE-1 expression in response to Her2/neu receptor activation and ESE-1 protein then binding and activating the Her2/neu promoter [13-16].

An increasingly large body of evidence reveals that ETS factors are particularly important in breast cancer [1, 5]. A thorough mRNA expression analysis of 25 ETS factor members in normal and cancerous mouse mammary glands sho-
pered that expression of Pdef, Pea3, Elf3/ESE-1, Elf5/ESE-2, Ehf/ESE-3, ETV6/TEL, and Elf2/NERF mRNAs was elevated in the epithelial cell compartment of mammary tumors [17]. Moreover, expression of a dominant-negative ETS factor, to overcome the redundancy of ETS factor expression, reversed the transformed phenotype in NmuMG, MMT and BT20 breast cancer cell lines [18, 19]. To determine whether a single ETS factor could impose the transformed phenotype, we stably expressed HA-ESE-1 or GFP-ESE-1 fusions in the ESE-1-negative, non-transformed MCF-10A and MCF-12A human mammary cell lines, and demonstrated that ectopic ESE-1 increased cellular proliferation, migration, invasion and colony number in soft agar [20, 21]. Moreover, ESE-1 was identified as a factor enhancing cell migration and altered morphogenesis in 3D assays in a separate and unbiased analysis of a collection of 1000 cDNAs relevant to breast cancer, in which each of 1000 cDNAs were ectopically expressed in MCF-10A cells [22]. Finally, we have reported that ESE-1 initiates transformation of MCF-12A mammary epithelial cells via an autonomously functioning, unique 40-amino acid serine and aspartic rich (SAR) domain acting via a novel cytoplasmic mechanism [20]. While dominant-negative and gain-of-function experiments demonstrate that ETS factors, in particular ESE-1, mediate the transformed state in breast cancer, no study to date has demonstrated that ESE-1 alone is required to maintain the transformed phenotype.

To investigate the potential role of ESE-1 in maintaining a malignant phenotype, we used shRNA targeting ESE-1 and a highly-specific ESE-1 mouse monoclonal antibody (Walker et al., in preparation), in order to characterize and monitor ESE-1 protein expression in several nontransformed and transformed mammary epithelial cell lines. Here we show that shRNA targeting ESE-1 specifically ablates endogenous ESE-1 expression in ZR-75-1 and MCF-7 cells, leading to an inhibition of clonogenicity and anchorage independent colony growth in these cells. Moreover, we show that ESE-1 knockdown did not induce apoptosis, but rather diminished MCF-7 cellular proliferation. These results establish that ESE-1 plays a critical role in maintaining the transformed state and that it does so by controlling cell proliferation. Thus, ESE-1 provides a potential single-point target for future breast cancer therapy.

MATERIALS AND METHODOLOGY

Cell Lines

MCF-7, T47D, MDA-MB-231, MCF-10A, and MCF-12A cells were maintained as described previously [15, 21]. ZR-75-1 cells were cultured in minimum essential medium (MEM) supplemented with 5% FBS, 1x non-essential amino acids, and 1 μM insulin. For soft agar experiments, ZR-75-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS.

shRNA Constructs and Transfection

ESE-1 shRNA and shGFP oligonucleotides were designed using Oligoengine (Seattle, WA). The oligonucleotide targeting ESE-1 (shESE-1) is as follows: forward oligo 5’ ACAGCAACATGACCTACG AACATGACCTACGATTCAAGAGATCGTAGGTCATGGTTGCTGT-reverse oligo 5’ ACAGCAACATGACCTACG ATCTCTTGAAATCGTAGTATGGCACGTGT. The ESE-1 negative control shRNA oligonucleotide is as follows: forward oligo 5’ GCTCAACGAGGGCCTCATGTTCAAG AGACATGAGGCCCCCCCTCGTGGAGC reverse oligo 5’ GCTC AACGAGGGCCTCATGTTCAAGACATGAGGCCCCCTC GTTGGAGC. This negative control (shCtrl) was originally designed to target ESE-1 expression, but since qRT-PCR and Western blot studies showed it failed to inhibit ESE-1 expression, we used it as a negative control. The ESE-1 shRNA and shCtrl were cloned into pSuper at the BgII and HindIII restriction sites. Each pSuper shRNA vector was co-transfected along with pEGFP-C3 at a 10 to 1 ratio (pSuper: pEGFP) into MCF-7 or ZR-75-1 cells using Qiagen Effectene, with a 12 to 1 ratio of DNA to Effectene. Cell lysates were generated two days post-transfection, unless otherwise noted. The shESE-1/ob4 construct was identified as capable of reducing endogenous ESE-1 protein, after testing an additional five shESE-1 constructs in the pLKO.1 lentiviral plasmid backbone, which contains a puro-mycin expression cassette. A single shGFP in the pLKO.1 vector served as a negative control. All of these pLKO.1 shRNAs were purchased from Open Biosystems (Huntsville, AL) and transfected as plasmid DNAs, not as packaged lenti-viruses. The oligonucleotide targeting ESE-1 (shESE-1/ob4) sequence targets the TAD and is as follows: 5’-GCTCTTCTGATGAGCTCAGTTG. The GFP negative control shRNA oligonucleotide is as follows 5’-TACACAGCCCAACAGGCTTACAT.

Western Blotting

Western blot analysis was performed essentially as described previously [15, 20], but cells lysis was completed by re-suspending them and incubating them on ice for 25 min in 0.1% NP-40, 50 mM Hepes pH 7.2, 250 mM, 2 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 1 mM benzamidine, 1 mM DTT, 25 μg/ml aprotinin, 25 μg/ml trypsin inhibitor, 25 μg/ml leupeptin, and 25 μg/ml β-glycerophosphate. Cells were then vortexed in four 30 second intervals, followed by a 15 min centrifugation at 13 000 g at 4°C, and the supernatant was collected. Protein concentrations were determined using a Bio-Rad detergent-compatible protein assay.

Quantitative RT-PCR

The qRT-PCR study was performed as described [23]. Plasmid pEGFP-ESE-1 [20] was used to generate sense-strand ESE-1 RNA, which was then used as an absolute standard for qRT-PCR. Primers and probes for ESE-1 were designed using the Prism 7700 sequence detection software (Primer Express, Perkin-Elmer Corp./Applied Biosystems (PE ABI), Foster City, CA), resulting in the following oligos: Forward ESE-1 Primer: (1109)AGCGGGGCACTAGTGCTGTT; Reverse Primer: (1173)ACGAGCTCGGCCCC GTAAGGATGGATCCCGCATGCTG; and TaqMan Probe: (1131)AACAACTGGGAG ATTCCTGGAACGGG(1154). Total RNA was prepared from cells using RNA Stat-60 (Tel-Test “B”, Inc.) and qRT-PCR was performed using an ABI PRISM 7700 Sequence detector.
(PE ABI), with PCR reactions monitored in real time. Reverse transcription, PCR conditions, real-time data acquisition and analyses were performed as described [23]. ESE-1 mRNA was normalized to the amount of 18s rRNA (PE ABI, P/N 4308310) in each sample.

Immunocytochemistry (ICC)

Cells (50 000) were plated directly onto glass cover slip in a 12 well tissue culture plate. Two days post-plating, cells were fixed with 2% paraformaldehyde (PFA) in 1x PBS for 20-25 min at room temperature (RT), followed by three 5 min washes in 1x PBS. Cells were permeabilized at RT with 0.5% Triton X-100 in 1x PBS for 10 minutes, followed by three 10 min washes in 100 mM glycine in 1x PBS. Permeabilized cells were blocked in a 1x PBS, 0.5% Tween-20, 10% goat serum, 0.05% bovine serum albumin (BSA) blocking buffer at RT within a moisture chamber for 1-2 h. Cells probed for ESE-1 were incubated in 1:500 antibody-blocking buffer overnight at 4°C in a moisture chamber. To measure auto-fluorescence, cells were incubated overnight at 4°C with blocking buffer alone, shRNA transfected cells underwent the same procedure at 48 h post-transfection.

Colony Formation Assays

MCF-7 and ZR-75-1 cells were transfected in suspension with Effectene, as described above, using 250 000 cells and 800:80 ng of shRNA:pEGFP-C3 in sterile 1.5 ml microfuge tube per transfection. Transfected cells (500 000 MCF-7 and 250 000 ZR-75-1) were then seeded on 60 mm plates, and 48 h post-transfection, cells were treated with 50 µg/ml G418 (Gibco) for 14 days. On day fourteen, cells were fixed with 2% PFA for 30 min, washed twice in PBS, and then stained with 30 seconds with crystal violet and washed for 1 min in ddH2O. Colonies were counted by direct visual analysis.

Colony Formation in Soft Agar

Anchorage independent growth was determined by assaying colony formation in soft agar. MCF-7 or ZR-75-1 cells were transfected with shESE-1 or shCtr plasmids and 50 000 cells were seeded by re-suspending cells in 1.5 ml per well of 0.3% agar noble (BD Scientific Difco) mixed with growth medium, and plated in 6 well plates previously covered with 1.5 ml of 0.6% agar noble base layer per well. Optimal colony formation by ZR-75-1 cells required additional modifications, including switching to RPMI 1640 medium supplemented with 10% serum and concentrating the medium, so that the concentration of nutrients was 1x after mixing with the agar. MCF-7 and ZR-75-1 cells grown in soft agar cultures were fed every three days up to 14 days. Resulting colonies were stained overnight at 37°C in PBS with 150 µl of 1M nitroblue tetrazolium chloride (Amresco) in PBS and quantitated using Metamorph or ImageJ imaging software, set to a colony threshold size of 150 microns.

Apoptosis Assay

MCF-7 cells were transiently transfected with empty vector, shCtr or shESE-1 DNA (~1x10⁶ cells per DNA) and used for both the DNA laddering and caspase 3/7 assays. For the DNA laddering, transfected cells were harvested 24 h post-transfection, cells were counted and genomic DNA was isolated from 100,000 cells. DNA was analyzed on a 1% agarose gel stained with ethidium bromide. Positive control cells were treated with 75 µg/ml of TRAIL plus 1 µg/ml of cycloheximide. For the Caspase-Glo 3/7 assay transfected MCF-7 cells were collected 24 h post-transfection with 1x PBS/EDTA, counted and 5 000 transfected cells were plated in a 96-well plate. Caspase 3/7 reagent was added to cells at 48 h and 72 h time points, and assays were performed as described by the manufacture (CaspaseGlo 3/7 Assay, Promega).

Proliferation Assays

Total cell count proliferation assays were conducted by plating 50 000 cells/well in a 12-well plate, and transfecting these cells with pEGFP-C3 and the indicated shRNA. Cells were harvested with 1x PBS/EDTA on days 2, 4, and 6 post-transfection, and viable cells were counted using the Vi-Cell counter (Beckman Coulter). MTS proliferation assays were conducted by transfecting MCF-7 cells with pEGFP-C3 and the indicated shRNA, and harvested 24 h later by suspension into 1x PBS-EDTA. Viable cells were counted by staining cells with Trypan blue and using a Vi-Cell-cell viability analyzer (Beckman Coulter), with 5 000 cells/well plated in a 96-well plate. Six days later, cells were counted using the MTS proliferation assay, as described by manufacturer (Promega).

RESULTS

MCF-7, T47D and ZR-75-1 Human Breast Cancer Cells Express ESE-1 Protein, whereas Transformed MDA-MB-231 and Nontransformed MCF-10A and MCF-12A Cells do not

In order to characterize and monitor ESE-1 protein expression in basal and shRNA knockdown conditions in various human mammary epithelial cell lines, we generated several highly specific mouse monoclonal antibodies against an ESE-1 peptide spanning amino acids 128-259. This region includes the transcription activation domain (TAD), SAR, and AT-hook domains. Details describing the generation and characterization of these antibodies will be presented elsewhere (Walker DM, in preparation), but in this report we limited our studies to the use of one of these, anti-ESE-1 mAb405. Western blot analysis of whole cell extracts probing for endogenous ESE-1 was performed on a series of human transformed and nontransformed mammary epithelial cell lines and compared with qRT-PCR. As shown in Fig. (1A), ESE-1 protein was not detected by Western blot analysis in the nontransformed MCF-10A and MCF-12A mammary epithelial cells lines. In contrast, ESE-1 protein was detected in the tumorigenic MCF-7, T47D and ZR-75-1 cell lines, with the levels in MCF-7 and T47D being equivalent and greater than that expressed in ZR-75-1. Noteworthy, ESE-1 protein was not detectable in the highly metastatic MDA-MB-231 cells (Fig. 1A). In order to obtain a direct comparison of ESE-1 protein and mRNA levels in this same panel of mammary epithelial cell lines, we next performed a quantitative RT-PCR analysis. This qRT-PCR study revealed that MCF-10A and MCF-12A nontransformed cells do not express any detectable ESE-1 mRNA, whereas the transformed MCF-7, T47D, ZR-75-1 and MDA-
MB-231 cells all express ESE-1 mRNA, but to varying degrees (Fig. 1B). The MCF-10A, MCF-12A and MCF-7 mRNA and protein data do correlate with each other (Fig. 1).

Fig. (1). ESE-1 protein is expressed in several human breast cancer cell lines. A. Western blot of whole cell extracts (100 μg) generated from MCF-10A (lane 1), MCF-12A (lane 2), MCF-7 (lane 3), T47D (lane 4), ZR-75-1 (lane 5) and MDA-MB-231 (lane 6) cells, probed with anti-ESE-1 mAb405 antibody (1:1000 dilution) and anti-tubulin mouse monoclonal antibody (1:10 000 dilution, CP06, Calbiochem). B. Quantitative RT-PCR analysis of endogenous ESE-1 in MCF-10A, MCF-12A, MCF-7, T47D, ZR-75-1 and MDA-MB-231 human mammary cell lines. Total RNA (1 μg) was generated from each cell line and used for qRT-PCR analysis (ABI PRISM 7700, PE ABI). ESE-1 mRNA in each sample calculated relative to absolute ESE-1 values that were derived from a standard curve, using a known amount of sense-strand ESE-1 RNA, and then normalized to total input RNA, using 18S rRNA measured from 1 ng total RNA.

However, for each given amount of ESE-1 protein expressed, the T47D mRNA level is lower than expected, whereas the ZR-75-1 and MDA-MB-231 mRNA levels are higher than expected (Fig. 1). To further confirm ESE-1 protein expression levels and its subcellular localization, we performed indirect immunofluorescence cytochemistry (ICC) studies, with cell nuclei counterstained with DAPI to define each cell (Fig. 2). These data revealed that MCF-10A, MCF-12A and MDA-MB-231 cells fail to express any ESE-1 protein detectable by this ICC method, whereas endogenous ESE-1 protein was detected in transformed MCF-7, T47D and ZR-75-1, and in each case ESE-1 was localized to the nucleus (Fig. 2). However, the ICC and Western blot data did not strictly correlate, since T47D cells displayed the strongest ICC signal, yet in the Western blot, the T47D signal was equivalent to the MCF-7 lane, but more than the ZR-75-1 lane (Figs. 1 and 2). As a negative control, primary anti-ESE-1 monoclonal antibody was omitted for each cell line, and this study revealed that the ICC signal is dependent on the primary anti-ESE-1 antibody (Fig. 2, lower panel). In general, the ICC data further confirmed the Western blot data and revealed ESE-1 protein to be primarily localized in the nucleus.

Fig. (2). ESE-1 is localized to the nucleus in human breast cancer cell lines. The top row are confocal images of ZR-75-1, MCF-7, T47D, MCF-12A, MCF-10A and MDA-MB-231 mammary epithelial cells probed with the anti-ESE-1 mAB405 antibody (1:500 dilution), followed by a Cy3-conjugated goat anti-mouse secondary antibody (1:200 dilution). ZR-75-1, MCF-7, T47D show a positive (red) signal and are grouped in the left panel, whereas MCF-12A, MCF-10A and MDA-MB-231 are negative and are grouped in the right panel. The second row from the top shows confocal images of the same cells stained with DAPI (100 ng/ml; blue nuclei), and the third row shows the merge of the top two rows. Shown at the bottom are the negative controls, depicting confocal images of these same cells probed with blocking solution and the Cy3-conjugated goat anti-mouse secondary antibody (1:200 dilution), but omitting the anti-ESE-1 mAb. The bottom row shows confocal images of DAPI-stained, negative control cells.

shRNA Targeting of ESE-1 Knocks Down Endogenous ESE-1 Protein Expression

ZR-75-1 and MCF-7 are estrogen receptor (ER)-positive human breast cancer cell lines, with the latter being a classic model system used to study estrogen-dependent tumorigenesis. The detection of endogenous ESE-1 in ZR-75-1 and MCF-7 cells makes these ideal cell lines to study the role of ESE-1 in maintaining tumorigenesis, and whether ESE-1 contributes to cellular survival, apoptosis and/or proliferation. To address these points, we used an shRNA approach to knockdown endogenous ESE-1 in ZR-75-1 and MCF-7 cells. In initial optimization studies of several shESE-1 constructs, we identified an shESE-1 construct (shESE-1), which targeted the ETS DBD of ESE-1, that optimally knocked down endogenous ESE-1 (data not shown). Using shESE-1, we first sought to establish the time course of ESE-1 knockdown by transiently transfecting ZR-75-1 cells with shESE-1 and
preparing whole cell lysates 2, 3, 4 and 5 days post-transfection (Fig. 3A). As a control, we transfected cells with an

**Knockdown of ESE-1 Reduces the Colony-Forming Ability of MCF-7 and ZR-75-1 Cells**

In order to determine if ESE-1 was necessary for colony formation, MCF-7 and ZR-75-1 cells were each co-transfected with pEGFP-C3, to confer G418 resistance, and shCtr or shESE-1. Transfected cells were selected with G-418 for 14 days, resultant colonies were stained with Crystal violet, and counted by direct visualization (Figs. 4A and 4B). The MCF-7 and ZR-75-1 cells transfected with shCtr yielded ~27 and ~43 colonies per plate, respectively, with ZR-75-1 colonies being larger than the MCF-7 colonies (Fig. 4A). Quantitation of triplicate colony formation assays indicated that only ~six MCF-7 colonies formed in the presence of shESE-1, resulting in a 5.5-fold reduction in MCF-7 colony formation and only ~three ZR-75-1 colonies formed in the presence of shESE-1, resulting in a 13-fold reduction in ZR-75-1 colony formation (Fig. 4B). Of note, we show a 14-day selection with G418, since several attempts to generate stable ESE-1 knockdown cell lines resulted in very few, small colonies that failed to grow, thus making clonal expansion unsuccessful. Importantly, the similar inhibitory effect of ESE-1

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**Fig. (3).** shESE-1 knocks down endogenous ESE-1 protein in ZR-75-1 and MCF-7 cells in a specific and prolonged manner. A. Time-course of ESE-1 knockdown after transient shESE-1 transfection in ZR-75-1 cells. ZR-75-1 cells (~60% confluent, 10 cm plate) were transiently transfected with shESE-1 (6.5:0.65 μg; shESE1:pEGFP-C3), and lysates were harvested on days 2, 3, 4 and 5 post-transfection. The negative control lysate was generated 2 days after transfecting ZR-75-1 cells with pSuper/empty vector. Western blot of whole cell lysates (100 μg) using the anti-ESE-1 mAB405 (1:1000) is shown in the top panel, with the same blot stripped and re-probed with tubulin antibody (1:10 000) and shown in the bottom panel. B. Specificity of shESE-1 knockdown in MCF-7 cells. MCF-7 cells were transiently transfected with shCtr or shESE-1, harvested 48 h post-transfection and Western blot of 100 μg of whole cell extract with anti-ESE-1 mAB405 (1:1000) shown in the top panel. The middle panel shows a Western blot of 35 μg of the same MCF-7 whole cell extracts, probed with anti-Ets1/Ets2 rabbit polyclonal antibody (1:10 000 dilution, Santa Cruz, c-275). The PVDF membrane shown in the top panel was washed and re-probed with anti-tubulin antibody (1:10 000), and the resultant Western blot is shown in the bottom panel. C. Arbitrary densitometry units (ADU) measuring the ESE-1 densitometry signal normalized against tubulin, with the shCtr signal set to 1.
Knockdown on colony formation in two distinct breast cancer cell lines supports the critical role of ESE-1 in the growth of transformed mammary cells.

**Knockdown of ESE-1 Inhibits Anchorage Independent Growth of MCF-7 and ZR-75-1 Cells**

To further investigate the functional role of ESE-1 in the maintenance of tumorigenic phenotype, we evaluated the effects of ESE-1 knockdown on anchorage independent growth of MCF-7 and ZR-75-1 breast cancer cells using soft agar assays. To this end, we transiently transfected MCF-7 and ZR-75-1 cells with shCtr or shESE-1 vector DNAs, plated the cells in soft agar and after 14 days counted the colonies growing in an anchorage independent manner (Fig. 4C and 4D). Because in the transient transfection approach not all cells are transfected and ESE-1 expression is likely to re-appear at later time points, compared to G418 selection methods, the resulting colony number in the shESE-1 knockdown cells presented here is likely an overestimate. The graph in Fig. (4C) shows quantification of MCF-7 soft agar colonies derived from 5 independent experiments. MCF-7 cells transfected with shCtr generated on average ~780 colonies, whereas MCF-7 cells transfected with shESE-1 generated ~470, a 40% reduction in colony formation. The cloning efficiency, determined by dividing the number of colonies growing in an anchorage independent manner by the number of colonies plated, was used to calculate the growth advantage of shESE-1 transfected cells compared to shCtr transfected cells.

Fig. (4). ESE-1 knockdown abrogates MCF-7 and ZR-75-1 colony formation and diminishes soft agar colony formation. A. MCF-7 and ZR-75-1 colonies stained with Crystal violet. MCF-7 cells (top panels) or ZR-75-1 cells (bottom panels) were transfected with shCtr or shESE-1 (using different DNA preparations for the ZR-75-1 and MCF-7 cells), selected for 14 days with 500 μg/ml of G418, and resultant colonies were stained with Crystal violet. B. Direct visual quantitation of MCF-7 (grey bars) and ZR-75-1 (dark bars) colonies. Cells were transfected in triplicate and selected with G418, as above, and the resultant colonies were counted by visual inspection. The shESE-1-mediated colony reduction in MCF-7 and ZR-75-1 cells is statistically significant to p=0.008 and p=0.0006, respectively, using the Student’s t-test. C. Quantitation of MCF-7 soft agar colonies. MCF-7 cells were transiently transfected with shCtr or shESE-1, plated in soft agar and resulting colonies were counted after 21 days using imaging software. Data shown are average of 5 independent assays. D. Quantitation of ZR-75 soft agar colonies. Data shown are an average of 5 independent assays. Results from each experiment were normalized to shCtr and expressed as % control.
colonies by the number of cells seeded, was on average 1.6% for shCtr-transfected MCF-7 cells and 0.9% for shESE-1 transfected MCF-7 cells. Colony formation in soft agar by ZR-75-1 cells was less efficient and less reproducible than by MCF-7 cells. Despite further optimization, the number of soft agar colonies formed by ZR-75-1 cells fluctuated from assay to assay, possibly because these cells were sensitive to slight changes in assay conditions. Nevertheless, we consistently observed that the shESE-1-transfected ZR-75-1 cells formed less colonies than the cells transfected with shCtr. For quantification, we normalized the colony number in each assay to shCtr (100%). The graph in Fig. (4D) shows quantification of ZR-75-1 soft agar colonies, derived from 5 independent experiments. ZR-75-1 cells transfected with shESE-1 formed on average 22% fewer colonies than the cells transfected with shCtr.

To address the possibility that the observed growth-inhibitory effects of shESE-1 on MCF-7 cells are due to a nonspecific, off-target knockdown, we used another shESE-1, labeled as shESE-1/ob4. As previously noted, in our initial studies testing of seven shESE-1 constructs, only one resulted in knockdown of ESE-1. Thus, to identify another shRNA construct, we tested an additional five constructs in a distinct vector backbone (pLKO.1), and identified shESE-1/ob4 as one that resulted in measurable ESE-1 knockdown (Fig. 5). MCF-7 cells were transiently transfected with shGFP control or shESE-1/ob4, both in the pLKO.1 background, and whole cell lysates were analyzed by Western blotting. The results show that the level of endogenous ESE-1 protein was partially inhibited by shESE-1/ob4 relative to control, and that the amount of protein loaded was equivalent in each lane (Fig. 5A). Quantitation of ESE-1 protein expressed, normalized to tubulin, shows that shESE-1/ob4 mediates a 27% knockdown (Fig. 5B). MCF-7 cells transfected with shGFP generated ~1343 colonies, whereas shESE-1/ob4-transfected cells generated ~874 colonies, a 35% reduction in colony formation (Fig. 5C). While shESE-1/ob4 mediated only a partial inhibition of soft agar colony formation, the effect essentially matched the knockdown level of ESE-1. Taken together, these functional assays indicate that the effects of shESE-1 and shESE-1/ob4 are unlikely to be due to nonspecific off-target responses, and demonstrate that ESE-1 is required to maintain the transformed phenotype of MCF-7 cells.

Reversion of the Transformed Phenotype is not due to Apoptosis

Having demonstrated a reduction in colony formation and anchorage independent growth, we next sought to address the mechanism responsible for the reversion of the malignant phenotype. We first tested whether knockdown of ESE-1 in MCF-7 cells resulted in apoptosis. In order to address this point, MCF-7 cells were transiently transfected with empty vector, shCtr or shESE-1, and harvested at 48 and 72 hours. We tested for apoptosis by using two separate assays, DNA laddering (Fig. 6A) and caspase 3/7 analyses (Fig. 6B and 6C). The DNA laddering assay showed no DNA fragmentation at the 48-hour time point for vector control, indicating that the transfection method alone did not induce apoptosis (Fig. 6A, lane 1). Similarly, there was no DNA fragmentation at the 48-hour time point in the shESE-1 or shCtr knockdown lanes (Fig. 6A, lanes 2-3). At the 72-hour time point there is minimal DNA fragmentation in the empty vector (Fig. 6A, lane 4) and shCtr (Fig. 6A, lane 5) controls, and no detectable fragmentation in the shESE-1 treated cells (Fig. 6A, lane 5). As a positive control, MCF-7 cells were treated with Trail plus cycloheximide, and these
cells display robust DNA fragmentation evincing apoptosis (Fig. 6A, lane 7). To further investigate apoptosis as a biological response to ESE-1 knockdown, we analyzed caspase 3 and/or 7 activation, using a luminescent enzyme activity assay (CaspaseGlo 3/7 Assay, Promega). With the data set to 1 for untransfected controls, these results show that there is no change in caspase 3/7 activity in shESE-1 transfected cells, compared to empty vector and shCtr transfected cells, at either the 48 or 72 hour time points (Figs. 6B and 6C). Having excluded apoptosis as the cellular mechanism responsible for the shESE-1-induced inhibition of colony formation, we next sought to determine whether shESE-1 affected MCF-7 cellular proliferation.

**Fig. (6).** Knockdown of ESE-1 does not induce DNA laddering or Caspase 3/7 activity. MCF-7 cells were transiently transfected with empty vector (lanes 1 & 4), shESE-1 (lanes 2 & 5) and shCtr (lanes 3 & 6), and genomic DNA was isolated at 48 and 72 h post-transfection. Isolated DNA (5 μg) was separated on a 1% agarose gel and stained with ethidium bromide. Positive control MCF-7 cells (lane 7) were treated with 75 μg/ml of trail plus 1 μg/ml of cycloheximide for 24 h, and DNA (5 μg) was isolated and analyzed as above. B. and C. For the caspase assays MCF-7 cells were transiently transfected with empty, shESE-1 and shCtr DNAs in triplicate, and caspase 3/7 activity was measured at 48 h (B) and 72 h (C) post-transfection. The caspase 3/7 activity of untransfected controls at 48 and 72 h was set to 1, and the caspase 3/7 activity of transfected cells was normalized to the untransfected value and expressed as fold-change.
Reversion of the Transformed Phenotype is due to shESE-1-Mediated Inhibition of MCF-7 Cell Proliferation

The control of cellular proliferation is a key mechanism in the prevention of tumorigenicity and malignancy. ESE-1 has been shown to regulate promoter activity of the Her2/neu and TGF-βRII genes [13, 24-27]. These two plasma membrane receptors contribute to the regulation of breast cancer cell growth and proliferation. ESE-1’s transcriptional regulation of these receptors suggests that it has an important role in controlling cellular proliferation. In order to confirm ESE-1’s role in maintaining cellular proliferation in MCF-7 transformed cells, MCF-7 cells were transiently transfected with shCtr or shESE-1, and cellular proliferation was determined at 2-, 4- and 6-days post-transfection by counting total viable cells (Fig. 7A) and 6-days post-transfection using an MTS assay (Fig. 7B). A representative total cell proliferation study, performed in duplicate, shows that shESE-1 cells display significantly reduced proliferation at each time point (Fig. 7A). At the start of the study, 50,000 cells were plated, with shCtr cells showing 82,500, 105,000 and 260,000 at 2-, 4- and 6-days post-transfection and the shESE-1 cells showing 31,000, 23,500 and 89,000 at the same time points. These results reveal a 62%, 78% and 66% reduction in cell proliferation at 2-4- and 6-days, respectively, in the shESE-1 knockdown cells compared to shCtr cells (Fig. 7A). Similar results were obtained using an MTS proliferation assay, which allowed us to perform 8 replicates in a 96-well format, and showed a 1.6 fold (or ~62%) reduction in MTS absorbance at 6-days post-transfection in the shESE-1 transiently transfected MCF-7 cells, compared to shCtr control cells (Fig. 7B). These data further demonstrate that ESE-1 is required for optimal MCF-7 cellular proliferation and reveal the mechanism by which ESE-1 contributes to the transformed phenotype.

DISCUSSION

The ETS transcription factor family is known to play a significant role in many cancers, with aberrant expression of ESE-1 being detected in nearly 50% of early human breast tumors. In addition, ectopically expressed ESE-1 has been shown to impart the transformed phenotype on ESE-1-negative MCF-12A and MCF-10A nontransformed mammary epithelial cell lines [15, 20-22, 28]. While dominant-negative ETS approaches, which interfere with multiple ETS factors, have reversed the transformed phenotype in several breast cancer cell lines (NmuMG, MMT and BT20) [18, 19], here we show that the knockdown of a single ETS factor, ESE-1, has the same effect in MCF-7 and ZR-75-1 breast cancer cells. Importantly, we show that ESE-1 is required to maintain the transformed phenotype in MCF-7 and ZR-75-1 breast cancer cells, since shRNA-mediated ablation of endogenous ESE-1 protein resulted in decreased colony formation and anchorage-independent growth (Fig. 4-6). Furthermore, mechanistic studies, using two separate approaches to measure apoptosis and proliferation, revealed that ESE-1 does not modulate apoptosis in MCF-7 cells, but rather is required for their proliferation (Figs. 6 and 7). Taken together, this paper contributes novel insights to our understanding of the critical role of ESE-1 in maintaining cell transformation of mammary epithelial cells via regulation of cellular proliferation.

Protein expression and characterization studies of ESE-1 in breast cancer cell lines and tissues have been limited, in large part due to the lack of highly-specific anti-ESE-1 antibodies. Similar to our results shown here, most studies using Western blot analysis of breast cancer cell lines have reported that MCF-10A, MCF-12A and MDA-MB-231 cells typically do not express ESE-1, whereas MCF-7, ZR-75-1 and T47D cells do express it [13, 28-30]. However, unlike our results, one group reported ESE-1 protein expression in MCF-12A cells [13], and another group reported that T47D cells fail to express ESE-1 protein [30]. We have consis-
tently not been able to detect ESE-1 in MCF-12A cells, using PCR to detect mRNA (Fig. 1B) [21] and Western blot, IHC or ICC analyses to detect protein (Figs. 1A and 2) [20]. Thus, one possibility to explain this discrepancy is that MCF-12A cells expressing ESE-1 have undergone spontaneous transformation, suggesting that MCF-12A cells may be poised to be easily transformed, requiring the use of low passage cells grown in defined media to avoid transformation and possible activation of ESE-1 expression [21, 31]. Another discrepancy is that we show the level of ESE-1 protein to be MCF-7 = T47D > ZR-75-1, whereas previous reports show MCF-7 > ZR-75-1 >> T47D [13, 29, 30]. This disparity could be explained by slight differences in growth conditions or subclone characteristics. Indeed, we have found ESE-1 expression to vary in certain T47D sublines (data not shown). While many more cell lines would have to be analyzed to reach a rigorous conclusion, it is noteworthy that MCF-10A, MCF-12A and MDA-MB-231 cells, which fail to express Her2/neu, also fail to express ESE-1; whereas ZR-75-1, T47D and MCF-7, which express detectable to low to minimal levels of Her2/neu, respectively, do express ESE-1. Finally, we show that ESE-1 mRNA levels generally correlate with protein data in the mammary cell lines studied here, with T47D and MDA-MB-231 showing a slight discordance between the mRNA and protein levels (Fig. 1).

ESE-1 contains several functional NLS and NES signals [20, 32], suggesting that ESE-1 shuttles between the nuclear and cytoplasmic compartments. We and others have demonstrated that transient transfection and adenoviral transduction studies consistently show nuclear localization of ESE-1/Elf-3 and we have used such transient ESE-1 expression assays to map the transcriptional properties and sites of co-factor interactions of ESE-1/Elf-3 as a nuclear effector [15, 20, 29, 32-35]. Here, using MAb405 in ICC studies, we show that endogenous ESE-1 is detected in the nucleus in MCF-7, T47D and ZR-75-1 human mammary epithelial cancer cells (Fig. 2). However, several IHC studies of endogenous ESE-1 show cytoplasmic ± nuclear localization in T47D and ZR-75-1 cell lines [28, 36], and mammary, retinal pigment and synovial epithelial cells [13, 20, 29, 37-39]. Thus, the differences in subcellular localization of ESE-1 noted in these various studies may be due to the different experimental conditions, different antibodies used for detection, and different types of assays: e.g., IHC vs. ICC, transient vs. stable, ectopic vs. endogenous, in vitro vs. in vivo, and non-transformed vs. transformed. Nevertheless, in combination, these studies reveal that ESE-1 can be detected in the nuclear and/or cytoplasmic compartments.

Previously, we have shown that stably expressed HA-ESE-1 or GFP-ESE-1 initiates and imposes the transformed phenotype upon nontransformed, ESE-1-negative MCF-10A and MCF-12A cells [20, 21], and that cytoplasmic localization of a 40-AA SAR domain is necessary and sufficient to mediate this effect [20]. Consistent with this conclusion, PAK-1-mediated phosphorylation of the SAR domain of exogenous ESE-1 in the cytoplasm modulates the stability and transformation potency of ESE-1 in ZR-75-1 cells [28]. Here we show that ESE-1 knockdown, in transformed MCF-7 and ZR-75-1 cells that express endogenous ESE-1, reverses their transformed properties. We used an additional, distinct shESE-1 construct (shESE-1/ob4) in MCF-7 cells to address any nonspecific effects using shESE-1 to knock-down ESE-1. Although the ESE-1 knockdown and colony inhibition was less with the shESE-1/ob4 construct compared to shESE-1 (~30% vs. ~40%), the reduction in soft agar colony formation was correlated with the level of endogenous ESE-1 protein (Figs. 4 and 5). Thus together, these two knockdown studies confirm that the reversal of the transformed phenotype is due to reduction of ESE-1, rather than any nonspecific effects of shRNA expression.

Moreover, we demonstrate, by ICC, that ESE-1 is localized to the nucleus in MCF-7, T47D and ZR-75-1 cells, indicating that the ability of ESE-1 to maintain the transformed phenotype requires its role as a nuclear transcription factor. Of note, we have previously demonstrated that GFP-ESE-1 targeted to the nucleus of nontransformed MCF-10A and MCF-12A cells induces apoptosis, whereas transformed T47D and Sk-Br-3 cells tolerate nuclear expression of exogenous ESE-1 without inducing apoptosis, possibly because anti-apoptotic pathways have been up-regulated in these transformed cells [20]. Taken together, these data suggest that ESE-1 initiates transformation in ESE-1-negative mammary epithelial cells via a cytoplasmic- and PAK-1-dependent mechanism, but once fully transformed, these cells require the nuclear transcription properties of ESE-1 to maintain the transformed phenotype.

ETS factors have been associated with a number of malignancies, particularly in the mammary gland and prostate [1]. The large redundancy of ETS factor genes in humans (~27) has required dominant-negative ETS approaches, whereby function of multiple ETS factors is blocked. This dominant-negative method has validated that ETS factors are required for mammary cell transformation [1]; however, such studies have failed to identify whether an individual ETS factor is sufficient to maintain the transformed phenotype. A key significance of this report is that we use shRNA technology to knockdown a single ETS factor, ESE-1, and this loss of function study supports that ESE-1 is necessary to maintain the transformed phenotypes in MCF-7 and ZR-75-1 cells. Our previous gain of function studies support that ESE-1 can be sufficient for transformation [21]. Thus, the clinical significance is that ESE-1 provides a novel single-molecule target for breast cancer therapy. The optimal ESE-1 19-nt target sequence (shESE-1) maps to the DBD. While BLAST searches with this 19-nt sequence only identified ESE-1, manual analyses of ETS DBD sequences revealed that ETS-1 and ETS-2 showed the closest similarity, with only 4/19 matches. While MCF-7 cells express low levels of ETS-2 and minimal amounts of ETS-1, this level of mismatches is very unlikely to allow the ESE-1 shRNA to target either ETS-1 or ETS-2 [40, 41]. Nevertheless, we performed Western blot analysis and demonstrated specificity for knockdown of ESE-1, since there was no change in the level of what is likely to be ETS-2 (Fig. 3B). Relevant to this report is the Ets2 knock-out study revealing that transgenic mice expressing mammary-targeted polyoma virus middle T oncogene crossed to heterozygous female mice carrying only one wild-type Ets2 allele, resulted in smaller tumors compared to crosses with wild-type controls [42]. Further analysis revealed that Ets2 functions in the stromal compartment to regulate mammary epithelial tumor growth [43]. The transgenic papers show a ~50% reduction in mouse mammary tumor size, due to the stromal effects of Ets2, whereas we show an ~80-90% reduction in
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The Open Cancer Journal, 2010, Volume 3

...members of the Gutierrez-Hartmann laboratory for their helpful comments and suggestions. We also thank Dr. Robert Winn for advice in optimizing the soft agar assay and Dr. Rafael Nemenoff for use of the Metamorph program. We used DNA Sequencing, Tissue Culture, Imaging and Quantitative PCR Core Facilities of the Colorado Comprehensive Cancer Center (supported by grant NIH P30 CA 46934). We acknowledge the following grant support, including: NIH F31 GM 72203 to DMW, DOD BCRP W81XWH-05-1-0352 to JP, UNCF-Pfizer and ACS Postdoctoral Fellowship Awards to MSG, and DOD BCRP W81XWH-06-1-0502 to AGH.

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