AD_____
(Leave blank)

Award Number:
W81XWH-06-1-0502

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

PRINCIPAL INVESTIGATOR:
Arthur Gutierrez-Hartmann, M.D.

CONTRACTING ORGANIZATION:
University of Colorado Health Sciences Center
Aurora, Colorado 80045

REPORT DATE:
April 2009

TYPE OF REPORT:
Final

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

DISTRIBUTION STATEMENT: (Check one)

☑ Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only; report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-04-2009	Final	29-03-2006 - 28-03-2009

4. TITLE AND SUBTITLE

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

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0502

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Arthur Gutierrez-Hartmann, MD
Email: a.gutierrez-hartmann@uchsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Colorado Health Sciences Center *
Aurora, CO 80045-0508

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research
And Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

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.

15. SUBJECT TERMS
ETS, Ese-1, Esx, MCF-12A, transformation, mammary, breast cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	64	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

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 cytoplamic 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 ($2.5x10^6$ 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 specifally 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-bidning 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 trifluorolethanol.

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

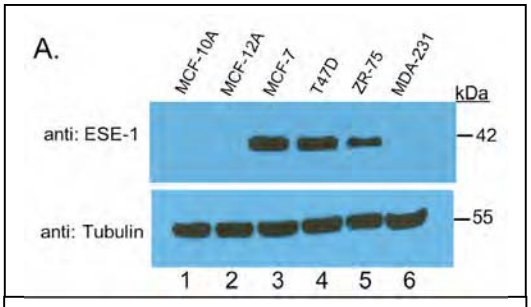


Fig. 1: Western blot of mammary cell whole cell lysates. Anti-Ese-1 (top) and anti-tubulin (bottom).

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.

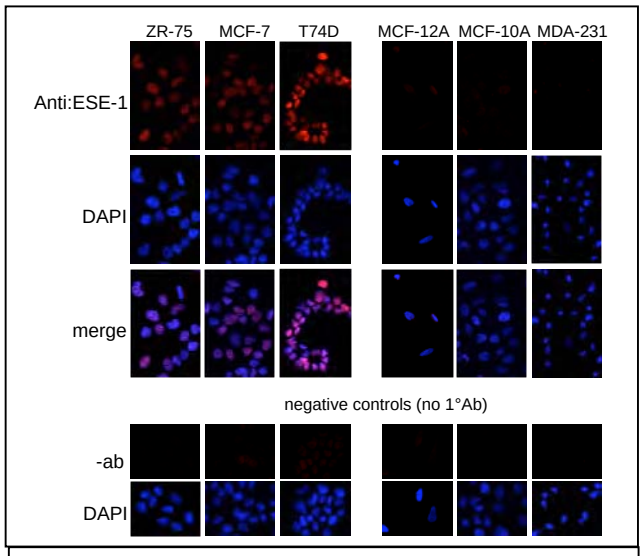


Fig. 2: ICC analysis of ESE-1 subcellular localization in breast cancer cells. Anti-Ese-1 (red), DAPI and Merge (purple). Negative controls shown in bottom panel.

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

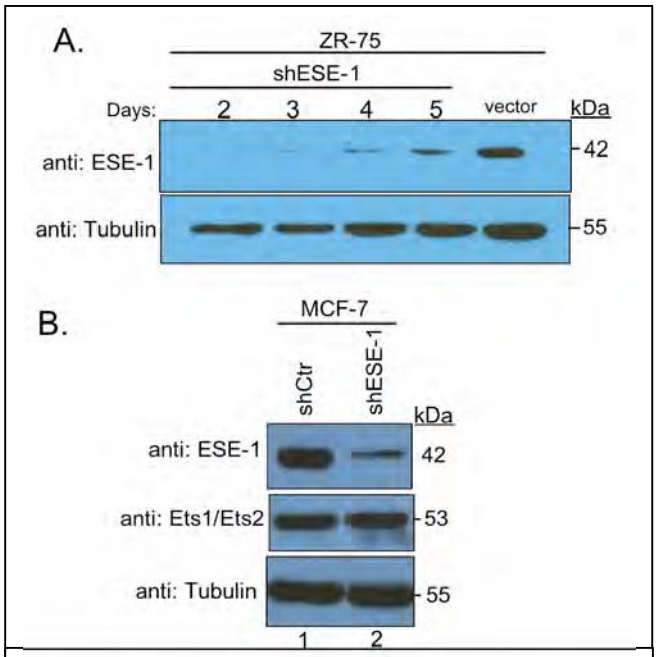


Fig. 3: Time-course of shEse-1 knock-down in ZR-75 and specificity of knockdown in MCF-7 breast cancer cells. Cell lysates prepared after 2-5 days post shESE-1 transfection were analyzed for ESE-1 and tubulin in ZR-75 cells (A); or 2-days post-transfection and blotted for ESE-1, Ets-1/2 and tubulin in MCF-7 cells (B).

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.

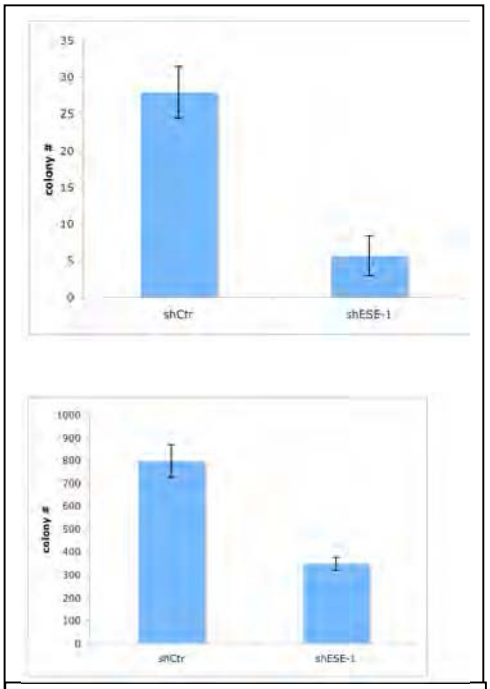


Fig. 4: Clonal cell growth (A) and soft agar colony formation (B) in MCF-7 cells post shCtr and shESE-1 transfection.

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

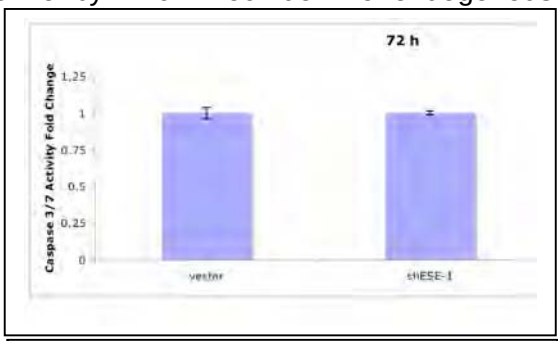


Fig. 5: Caspase 3/7 activity in MCF-7 cells 72-hr post shCtr and shESE-1 transfection.

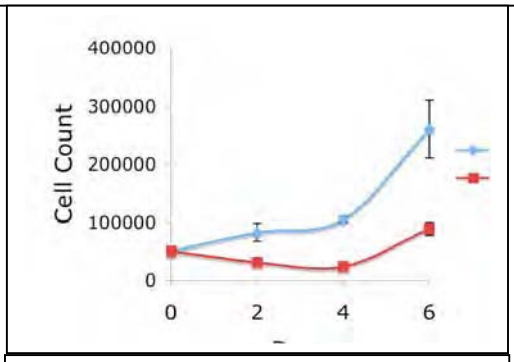


Fig. 6: Cell growth of MCF-7 cells (50000) 2, 4 and 6 days post-transfection with shCtr (blue) or shESE-1 (red).

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.

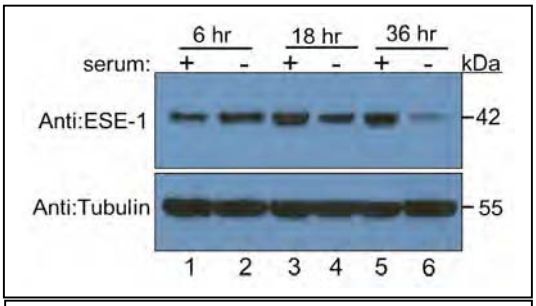


Fig. 7: Extinction of ESE-1 expression in MCF-7 cells upon serum withdrawal. Cells plated in (+) or (-) serum, and lysates prepared 6,18 and 36 hrs later and analyzed by WB for ESE-1 (top) and tubulin (bottom).

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 micro arrays.*

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.

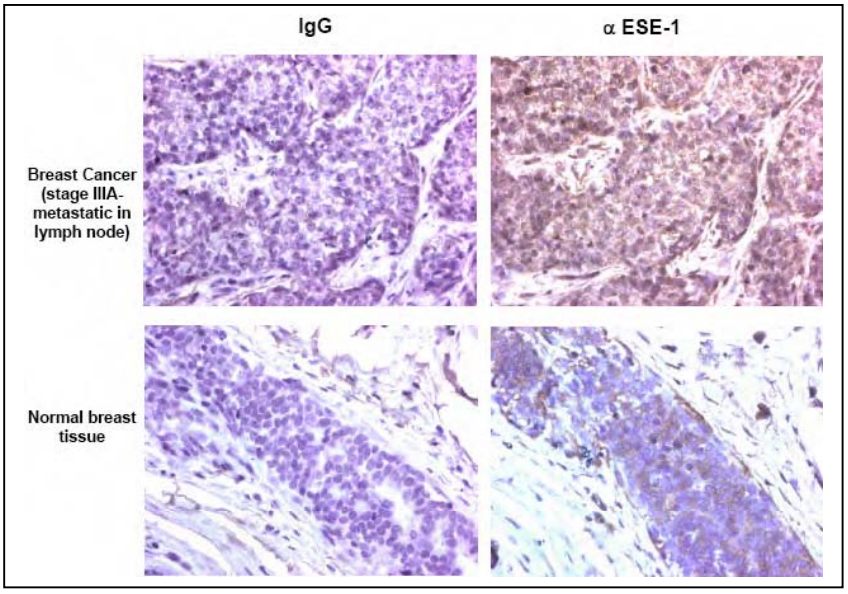


Fig. 8: **IHC analysis of ESE-1**. Stage IIIA breast cancer mets to LN (top panels) and normal breast tissue (bottom panels), probed with either IgG negative control (left panels) or anti-ESE-1 mAb405 (right panels) are shown. The 1^{o} Ab was used at 1:1000, and visualization was with DAB.

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:

1. Walker D, Prescott JD, **Gutierrez-Hartmann A**. Identification of ESE1 SAR-Domain Interacting Proteins that Mediate a Novel Cytoplasmic Transformation Mechanism. 97th Annual Meeting of the AACR, Washinton DC, April, 2006.
2. Prescott JD, Walker D, Tentler JJ, Poczobutt J, Schedin P, **Gutierrez-Hartmann A**. Ese-1 Transforms Human Mammary Epithelial Cells via a Unique 40-AA Domain Acting Through a Novel Cytoplasmic Mechanism. Mammary Gland Gordon Research Conference, Il Ciocco, Italy, June, 2006.
3. **Gutierrez-Hartmann A**. ETS transcription factors in epithelial morphogenesis and tumorigenesis. Pew Scholars Program in the Biomedical Sciences, 20th Anniversary Meeting, Cancun, Mexico, January, 2007.
4. Walker DM, Pozcobutt J, Gonzales MS, Horita H, **Gutierrez-Hartmann A**. ESE-1 is required to maintain the transformed phenotype of MCF-7 and ZR-75 human mammary epithelial cells. Era of Hope Meeting, Baltimore, MD, June, 2008.

Manuscripts

1. **Gutierrez-Hartmann A**, Duval DL, Bradford AP. ETS transcription factors in endocrine systems. *Trends Endocrinol Metab*, 18(4): 150-158, 2007.
2. Jedlicka P, **Gutierrez-Hartmann A**. ETS transcription factors in intestinal morphogenesis and disease. *Histology and Histopathology* 23:1417-24, 2008.
3. Jedlicka P, Sui X, Sussel L, **Gutierrez-Hartmann A**. Epithelial ETS transcription factors regulate enterocyte maturation, epithelial migration and architectural organization of the small intestinal crypt-villus unit in the mouse. *Am J Path*, 172:1280-190, 2009.
4. Jedlicka P, Sui X, **Gutierrez-Hartmann A**. The Ets dominant repressor En/Erm enhances intestinal epithelial tumorigenesis in ApcMin mice. *BMC Cancer* 9:197, 2009.
5. Walker DM, Pozcobutt J, Gonzales MS, Horita H, **Gutierrez-Hartmann A**. ESE-1 is required to maintain the transformed phenotype of MCF-7 and ZR-75 human mammary epithelial cells. *Intl Cancer J*, in press, 2010.
6. Prescott JD, Pozcobutt J, Gonzales M, **Gutierrez-Hartmann A**. Nuclear export and phosphorylation target sequences in ESE-1 mediate transformation of MCF-12A human mammary epithelial cells. *In preparation*.

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.
b. Komen Foundation (August, 2008): Reviewed but not funded.
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 nontransfromed, 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-7and 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

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

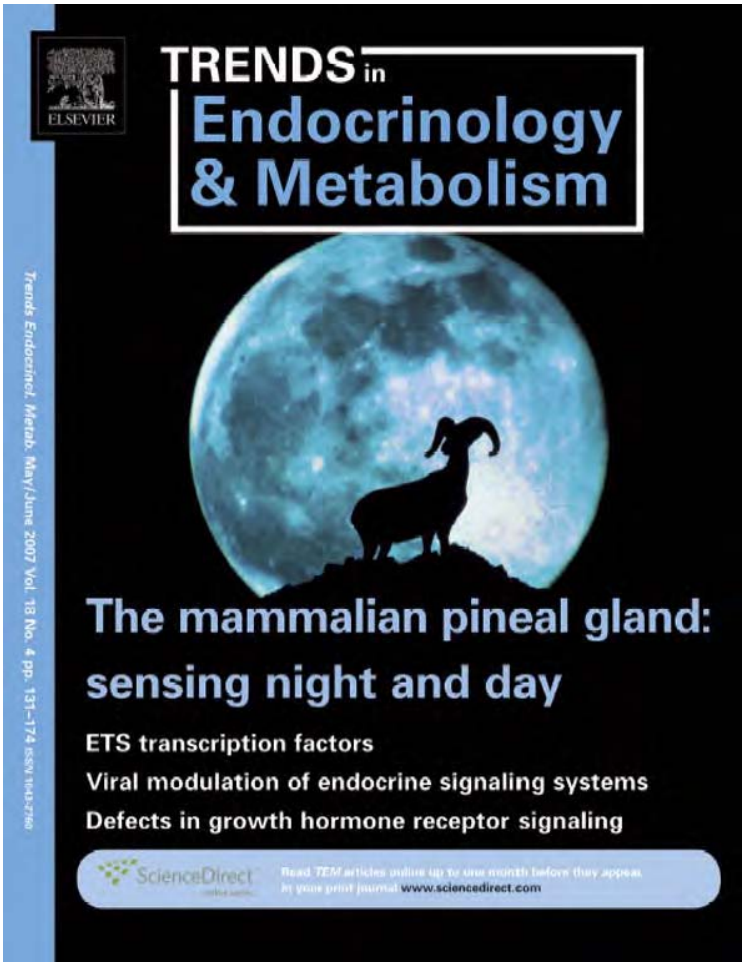
1. Wasylyk, B, and Nordheim, A**.** 1997. Ets transcription factors: Partners in the integration of signal responses., p. 251-284. *In* A. Papavassiliou (ed.), Transcription Factors in Eukaryotes. Landes Bioscience.
2. Wasylyk, B, Hagman, J, and Gutierrez-Hartmann, A**.** 1998. Ets transcription factors: nuclear effectors of the Ras/MAP kinase signaling pathway. Trends Biochem. Sci. 23**:**213-216.
3. Sapi, E, Flick, M, Rodov, S, and Kacinski, B**.** 1998. Ets-2 transdominant mutant abolishes anchorage-independent growth and anchorage-stimulating factor-stimulated invasion by BT20 breast carcinoma cells. Cancer Res. 58**:**1027-1033.
4. Delannoy-Courdent, A, Mattot, V, Fafeur, V, Fauquette, W, Pollet, I, Calmels, T, Vercamer, C, Boilly, B, Vandenbunder, B, and Desbiens, X**.** 1998. The expression of Ets-1 transcription factor lacking its activation domain decreases uPA proteolytic activity and cell motility, and impairs normal tubulogenesis and cancerous scattering in mammary epithelial cells. J. Cell Sci. 111**:**1521-1534.
5. Galang, C, Garcia-Ramirez, J, Solski, P, Westwick, J, Der, C, Neznanov, N, Oshima, R, and Hauser, C**.** 1996. Oncogenic Neu/ErbB-2 increases, Ets, AP-1 and NF-kB-dependent gene expression, and inhibiting Ets activation blocks Neu-mediated cellular transformation. J. Biol. Chem. 271**:**7992-7998.
6. Janknecht, R, and Nordheim, A**.** 1993. Gene regulation by Ets proteins. Biochim. Biophys. Acta 1155**:**346-356.
7. Chang, C, Scott, G, Kuo, W, Xiong, X, Suzdaltseva, Y, Park, J, Sayre, P, Erny, K, Collins, C, Gray, J, and Benz, C**.** 1997. ESX: A structurally unique Ets overexpressed early during human breast tumorigenesis. Oncogene 14**:**1617-1622.
8. Oettgen, P, Alani, R, Barcinski, M, Brown, L, Akbarali, Y, Boltax, J, Kunsch, C, Munger, K, and Libermann, T**.** 1997. Isolation and characterization of a novel epithelium-specific transcription factor, ESE-1, a member of the *ets* family. Mol. Cell. Biol. 17**:**4419-4433.
9. Tymms, M, Ng, A, Thomas, R, Schutte, B, Zhou, J, Eyre, H, Sutherland, G, Seth, A, Rosenberg, M, Papas, T, Debouck, C, and Kola, I**.** 1997. A novel epithelial-expressed ETS gene, ELF3: Human and murine cDNA sequences, murine genomic organization, human mapping to 1q.32.2 and expression in tissues and cancer. Oncogene 15**:**2449-2462.
10. Eckel KL, J.T. Tentler, G.J. Cappetta, S.E. Diamond, Gutierrez-Hartmann A. The epithelial-specific ETS transcription factor ESX/ESE-1/Elf-3 modulates breast cancer-associated gene expression. *DNA & Cell Biol*. 22:79-94, 2003.
11. Schedin PJ, Eckel KL, McDaniel SM, Prescott JD, Brodsky KS, Tentler JJ, Gutierrez-Hartmann A. ESX induces transformation and functional epithelial to mesenchymal transition in MCF-12A mammary epithelial cells. *Oncogene* 23:1766-1779, 2004.
12. Prescott JD, Koto K, Singh M, Gutierrez-Hartmann A. The ETS transcription factor ESE-1 transforms MCF-12A mammary epithelial cells via a novel cytoplasmic mechanism. *Mol Cell Biol* 24:5548-5564, 2004.

13. Manavathi B, Rayala SK, Kumar R. Phosphorylation-dependent regulation of stability and transforming potential of ETS transcriptional factor ESE-1 by p21-activated kinase 1. J Biol Chem 282:19820-30, 2007.

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

1. **Gutierrez-Hartmann A**, Duval DL, Bradford AP. ETS transcription factors in endocrine systems. *Trends Endocrinol Metab*, 18(4): 150-158, 2007.
2. Jedlicka P, **Gutierrez-Hartmann A**. ETS transcription factors in intestinal morphogenesis and disease. *Histology and Histopathology* 23:1417-24, 2008.
3. Jedlicka P, Sui X, Sussel L, **Gutierrez-Hartmann A**. Epithelial ETS transcription factors regulate enterocyte maturation, epithelial migration and architectural organization of the small intestinal crypt-villus unit in the mouse. *Am J Path*, 172:1280-190, 2009.
4. Jedlicka P, Sui X, **Gutierrez-Hartmann A**. The Ets dominant repressor En/Erm enhances intestinal epithelial tumorigenesis in ApcMin mice. *BMC Cancer* 9:197, 2009.
5. Walker DM, Pozcobutt J, Gonzales MS, Horita H, **Gutierrez-Hartmann A**. ESE-1 is required to maintain the transformed phenotype of MCF-7 and ZR-75 human mammary epithelial cells. *Intl Cancer J*, in press, 2010.

Provided for non-commercial research and educational use only.
Not for reproduction or distribution or commercial use.



This article was originally published in a journal published by Elsevier, and the attached copy is provided by Elsevier for the author's benefit and for the benefit of the author's institution, for non-commercial research and educational use including without limitation use in instruction at your institution, sending it to specific colleagues that you know, and providing a copy to your institution's administrator.

All other uses, reproduction and distribution, including without limitation commercial reprints, selling or licensing copies or access, or posting on open internet sites, your personal or institution's website or repository, are prohibited. For exceptions, permission may be sought for such use through Elsevier's permissions site at:

http://www.elsevier.com/locate/permissionusematerial

Review TRENDS in Endocrinology and Metabolism Vol.18 No.4

Full text provided by www.sciencedirect.com
ScienceDirect

ETS transcription factors in endocrine systems

Arthur Gutierrez-Hartmann[1], Dawn L. Duval[2,3] and Andrew P. Bradford[1]

[1] University of Colorado HSC, P. O. Box 6511, Mail Stop 8106, Aurora, CO 80045, USA
[2] University of Colorado HSC, 4200 E. 9th Ave., Campus Box C-238, Denver, CO 80262, USA
[3] Present address: Department of Clinical Sciences, Animal Cancer Center, Colorado State University, 300 West Drake Road, Fort Collins, CO 80523, USA

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, 10 in *Caenorhabditis elegans* and 9 in *Drosophila* that can be structurally categorized into 11 subfamilies (ETS, ERG, ELG, ELF, ESE, 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 (GABP)α, 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

Corresponding author: Gutierrez-Hartmann, A. (a.gutierrez-hartmann@UCHSC.edu).
Available online 26 March 2007.

 1043-2760/$ – see front matter © 2007 Elsevier Ltd. All rights reserved. doi:10.1016/j.tem.2007.03.002

Author's personal copy

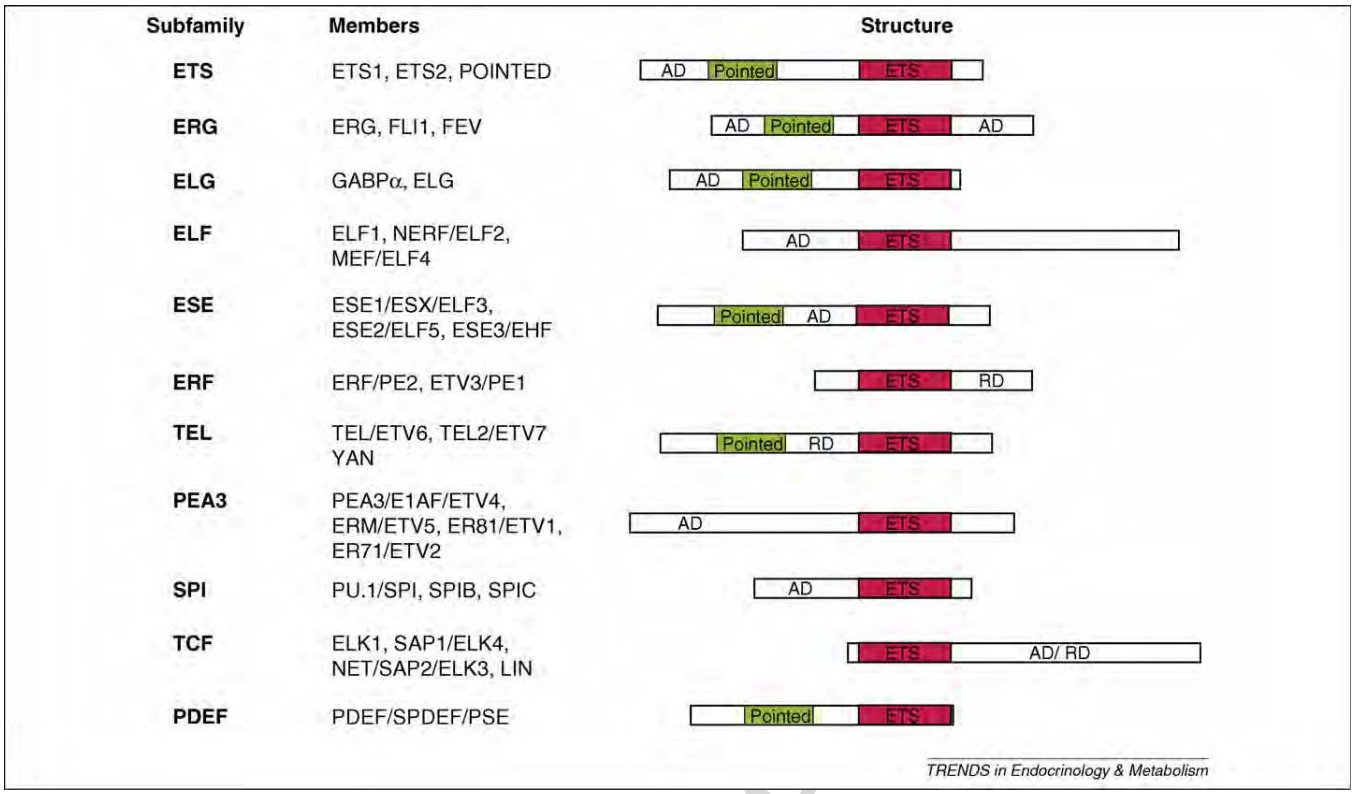


Figure 1. The ETS family of transcription factors. The main functional domains characteristic of members of each ETS sub family are depicted; alternative names for each member are given. Domains: AD, transcriptional activation domain; ETS, DNA binding domain; Pointed, basic helix–loop–helix pointed domain; RD, transcriptional repressor domain. Protein abbreviations: E1AF, E1A enhancer binding protein; EHF, ETS homologous factor; ELF, E74-like factor; ELG, ETS like gene; ER81, ETS related protein 81; ERF, ETS repressor factor; ERG, v-ets avian erythroblastosis virus E26 oncogene related; ERM, ETS related molecule; ESE, Epithelial specific ETS; ETS, v-ets erythroblastosis virus E26 oncogene homolog; ETV, ETS variant gene; FLI1, Friend leukemia virus integration 1; FEV, Fifth Ewing variant; GABP, GA repeat binding protein; LIN, abnormal cell lineage; MEF, myeloid ELF1-like factor; NERF, New ETS-related factor; PEA3, polyomavirus enhancer activator-3; PDEF, prostate derived ETS transcription factor; PSE, prostate epithelium-specific ETS; SAP, Serum response factor accessory protein; SPDEF, SAM pointed domain containing ETS transcription factor; SPI, spleen focus forming virus proviral integration oncogene; TEL, translocation, Ets, leukemia; TCF, Ternary complex factor.

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 gonatotropes. 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

Author's personal copy

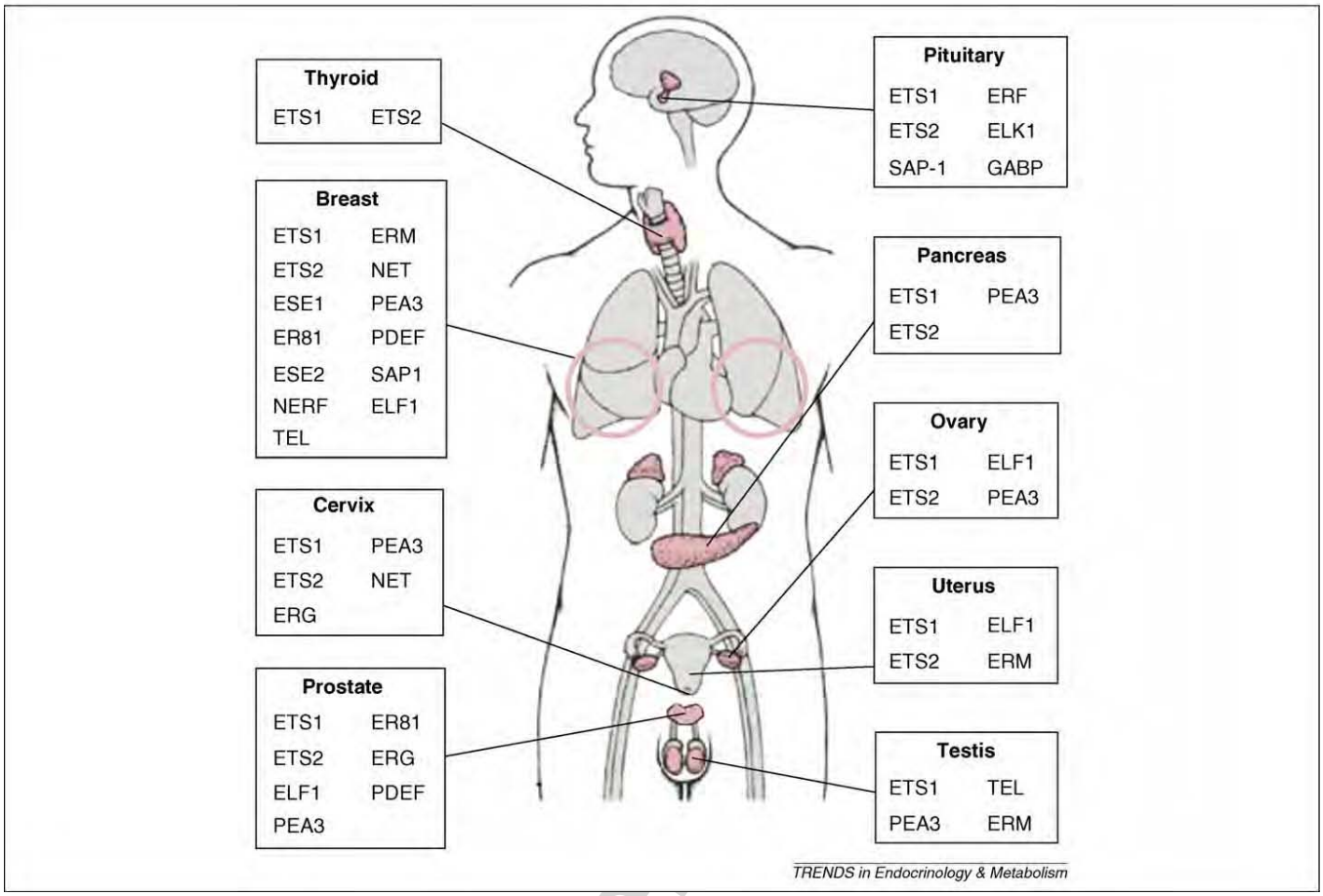


Figure 2. Mammalian ETS factor expression in endocrine organs and target tissues. A list of ETS factors implicated in normal function and/or tumorigenesis is shown for each human tissue. See text for discussion and corresponding references.

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 *PRL* gene (Figure 3b). The proximal 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 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 GABPα (together with its non-DNA-binding partner GABPβ1), BTE DNA-affinity purification of nuclear proteins from GH3 pituitary tumor cells identified the heterodimeric ETS factor GABPα–β1 as the key functional ETS factor binding to the BTE site [18]. Subsequent downregulation of the GABPα and GABPβ1 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 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 *rPRL* RRE binds to ETS1 and Pit-1 and primarily mediates Ras activation of the *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 *rPRL* promoter in transient transfections of GH4 cells [19]. Similarly, expression of a dominant-negative ETS construct or Pit-1β (an alternatively spliced isoform that functions as a dominant-negative effector in pituitary cells) represses Ras activation of the *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

Author's personal copy

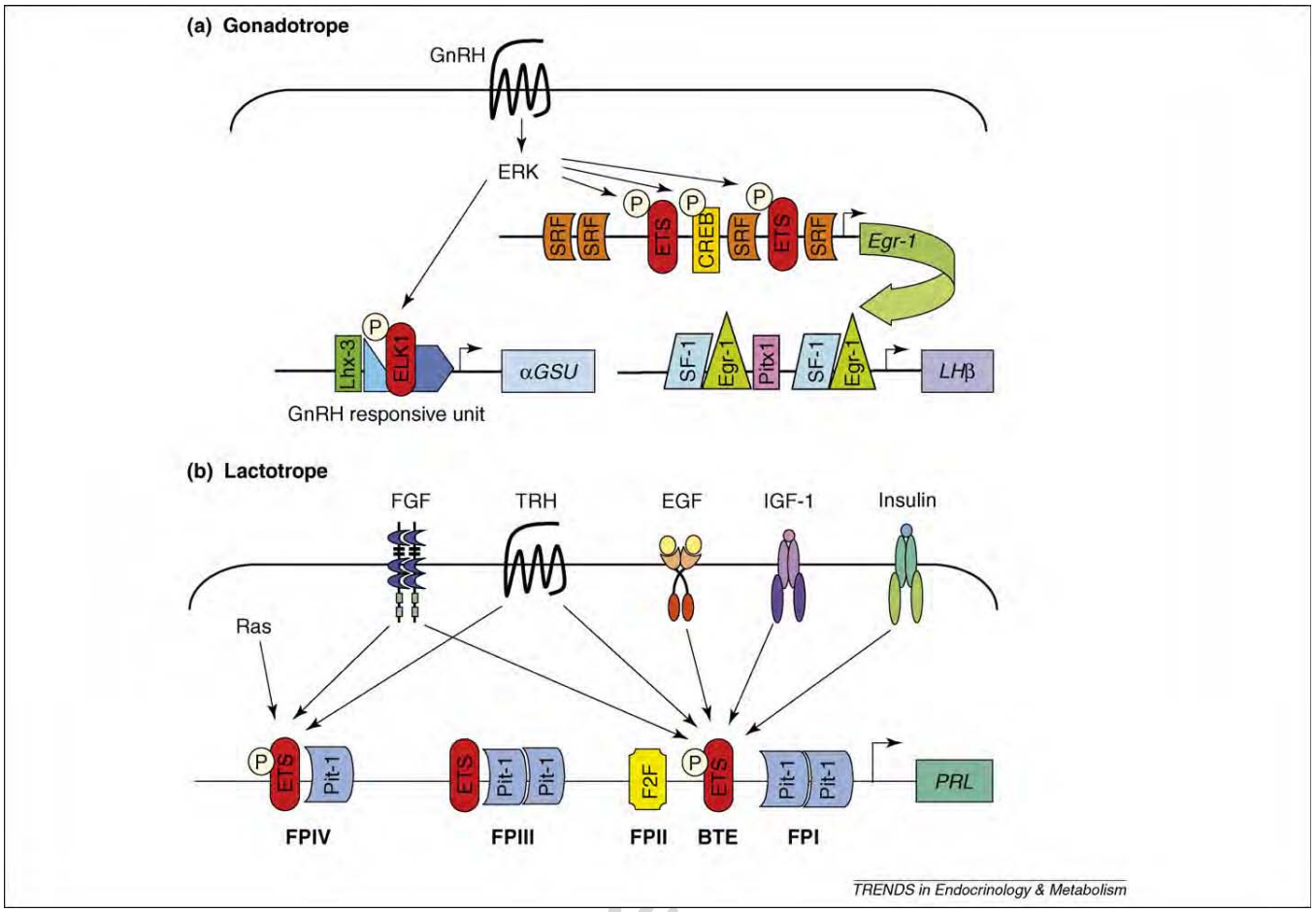


Figure 3. ETS factors as nuclear targets and integrators of signal transduction pathways regulating pituitary gonadotrope and lactotrope gene expression. **(a)** GnRH acting through the ERK pathway directly activates an ETS factor (ELK1) bound to the human α-glycoprotein subunit gene promoter (*αGSU*) within the GnRH responsive unit. Blue triangle, αBP1; hexagon, αBP2. GnRH also stimulates the expression of the human early growth response protein 1 gene (*Egr-1*) through ERK-dependent phosphorylation and activation of ETS factors (ETS) and the cyclic AMP response element binding protein (CREB). Upregulation of the transcription factor Egr-1 subsequently contributes to GnRH stimulation of the human *LHβ*-subunit gene promoter, through a composite response element consisting of binding sites for steroidogenic factor-1 (SF-1), the paired-like homeodomain transcription factor (Pitx1) and Egr-1. **(b)** ETS factors integrate multiple signaling pathways regulating *PRL* gene expression. Stimulation of the proximal –425 rat *PRL* gene promoter (*PRL*), in response to the indicated hormones, growth factors or oncogenic Ras, is mediated through ERK phosphorylation of distinct ETS factors (denoted by the circled P), described in the text, which bind to a composite ETS–Pit-1 binding site (FPIV) and/or an ETS binding site in the BTE. The binding of different ETS proteins to common response elements, and interactions with other transcription factors, provides a mechanism to confer highly specific responses to inductive signals and to coordinate and integrate hormonal and growth factor regulation of *PRL* gene expression. Abbreviations: FGF, fibroblast growth factors; IGF-1, Insulin like growth factor 1; Lhx-3, Lim-homeobox factor 3; SRF, serum response factor; TRH, thyroid-releasing hormone.

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

Author's Use

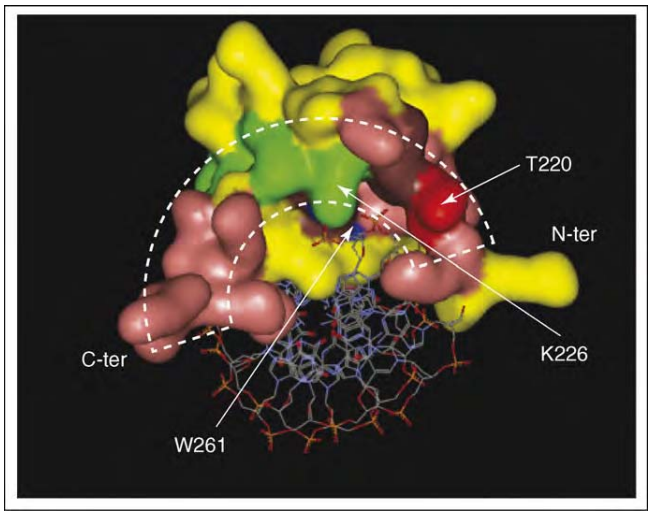


Figure 4. Chemical shift perturbations of the Pit-1 homeodomain induced by the binding of ETS1. A surface density representation of the Pit-1 homeodomain bound to DNA based on the crystal structure of the Pit-1 homeodomain. NMR shift perturbations of residues in the Pit-1 homeodomain (amino acids z199–291), in response to the binding of the Region III TAD (amino acids 190–257) of ETS1 [20], are indicated by color coding: red > purple > pink > green. Yellow residues are unaffected. The ETS interaction face on the Pit-1 homeodomain is indicated by a dashed white line. Amino acids highlighted in the ETS1 interaction face seem to affect the ETS1–Pit-1 interaction. W261 (blue) is in the hydrophobic DNA binding pocket, T220 (red) is a phosphorylation site and K226 (green) is a potential acetylation site. Adapted from Ref. [20]; copyright 2002 National Academy of Sciences USA.

effector of the PRL–STAT5 signaling pathway [25,26]. Thus, Elf5 (Ese-2) is necessary and sufficient for lactation-competent mammary gland development during pregnancy.

Several different lines of evidence suggest that 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 homozygous 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

Author's personal copy

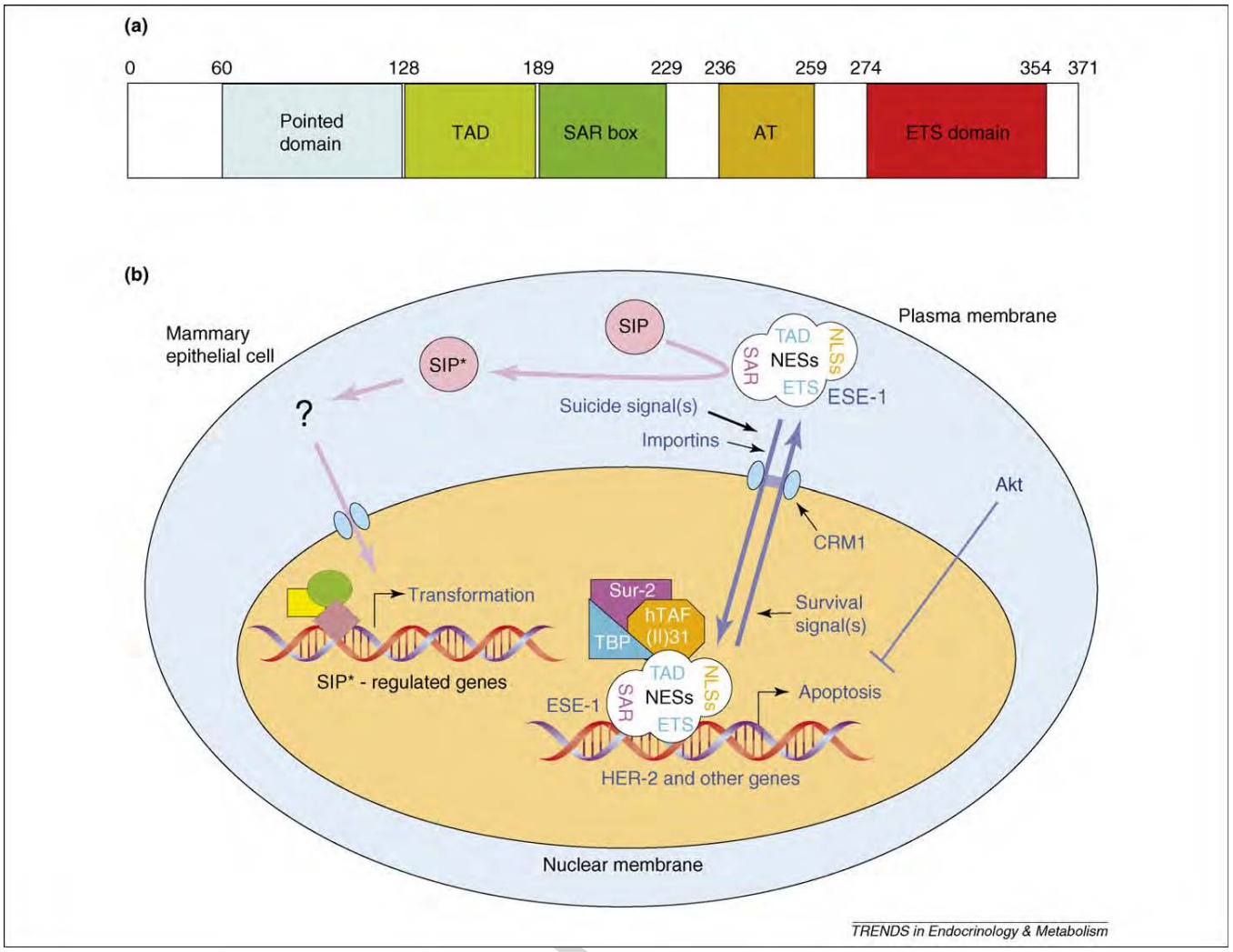


Figure 5. **(a)** Domain organization of ESE1. Shown is the 371 amino acid ESE1 protein, with the Pointed, TAD, SAR domain, AT-hook domain, and ETS DBD. Endpoints (residue numbers) are shown above each domain. **(b)** Model of ESE1 function in mammary gland biology. ESE1 is shown in the nucleus as binding to a target gene and activating an apoptotic pathway in normal epithelium, with nuclear localization governed by suicide signals. By contrast, survival signals are postulated to induce cytoplasmic localization of ESE1, through a Chromosomal region maintenance 1 (CRM1)-dependent mechanism, which is required for ESE1 to transform human mammary epithelial cells. The SAR domain is proposed to interact with SAR-interacting protein (SIP), converting it to the active form, SIP*, which then activates an unknown signal transduction pathway (shown as?), resulting in specific gene(s) transcription that establishes the transformed phenotype. Abbreviation: NES, nuclear export sequence; NLS, nuclear localization sequence; TAF, transcription activating factor; TBP, TATA binding protein.

[7]. 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].

Author

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 decidual 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 ectoplacental cone 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 conceptuses 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]. Over expression 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 46868 to A.G.H.; K01 DK 02946 to D.L.D.; and, RO1 CA 104875 to A.P.B. Also, A.G.H. acknowledges BCRP BC051589 grant support from the DOD.

References

1 Seth, A. and Watson, D.K. (2005) ETS transcription factors and their emerging roles in human cancer. *Eur. J. Cancer* 41, 2462–2478
2 Sharrocks, A.D. (2001) The ETS-domain transcription factor family. *Nature* 2, 827–837
3 Wasylyk, B. *et al.* (1998) Ets transcription factors: nuclear effectors of the Ras/MAP kinase signaling pathway. *Trends Biochem. Sci.* 23, 213–216
4 Galang, C.K. *et al.* (2004) Changes in the expression of many Ets family transcription factors and of potential target genes in normal mammary tissue and tumors. *J. Biol. Chem.* 279, 11281–11292
5 Hollenhorst, P.C. *et al.* (2004) Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. *Nucleic Acids Res.* 32, 5693–5702
6 Prescott, J.D. *et al.* (2004) The ETS transcription factor ESE-1 transforms MCF-12A human mammary epithelial cells via a novel cytoplasmic mechanism. *Mol. Cell. Biol.* 24, 5548–5564
7 Feldman, R.J. *et al.* (2003) The epithelial-specific Ets factors occupy a unique position in defining epithelial proliferation, differentiation and carcinogenesis. *Anticancer Res.* 23, 2125–2131
8 Jorgensen, J.S. *et al.* (2004) Multiple and overlapping combinatorial codes orchestrate hormonal responsiveness and dictate cell-specific expression of the genes encoding luteinizing hormone. *Endocr. Rev.* 25, 521–542

Author's Personal Copy

9 Duan, W.R. *et al.* (2002) GnRH regulates early growth response protein 1 transcription through multiple promoter elements. *Mol. Endocrinol.* 16, 221–233
10 Liu, J.C. *et al.* (2002) Activation of Go-coupled dopamine D2 receptors inhibits ERK1/ERK2 in pituitary cells. A key step in the transcriptional suppression of the prolactin gene. *J. Biol. Chem.* 277, 35819–35825
11 Gourdji, D. and Laverriere, J-N. (1994) The rat prolactin gene: a target for tissue-specific and hormone-dependent transcription factors. *Mol. Cell. Endocrinol.* 100, 133–142
12 Dasen, J.S. and Rosenfeld, M.G. (2001) Signaling and transcriptional mechanisms in pituitary development. *Annu. Rev. Neurosci.* 24, 327–355
13 Lopez-Fernandez, J. *et al.* (2000) Differentiation of lactotrope precursor GHFT cells in response to fibroblast growth factor-2. *J. Biol. Chem.* 275, 21653–21660
14 Schweppe, R. *et al.* Functional components of fibroblast growth factor signal transduction in pituitary cells. *J. Biol. Chem.* 272, 30852–30859
15 Shimon, I. *et al.* (1996) Heparin-binding secretory transforming gene (hst) facilitates rat lactotrope cell tumorigenesis and induces prolactin gene transcription. *J. Clin. Invest.* 97, 187–195
16 Yu, S. *et al.* (2002) Fibroblast growth factor receptor 4 is a target for the zinc-finger transcription factor Ikaros in the pituitary. *Mol. Endocrinol.* 16, 1069–1078
17 Tentler, J.J. *et al.* (2003) Selective repression of rat prolactin gene by stable expression of dominant-negative Ets in GH4 pituitary cells. *Endocrine* 20, 3–12
18 Schweppe, R.E. *et al.* (2003) Purification and mass spectrometric identification of GA-binding protein (GABP) as the functional pituitary Ets factor binding to the basal transcription element of the prolactin promoter. *J. Biol. Chem.* 278, 16863–16872
19 Bradford, A.P. *et al.* (1996) GHF-1/Pit-1 functions as a cell-specific integrator of Ras signaling by targeting the Ras pathway to a composite Ets-1/GHF-1 response element. *J. Biol. Chem.* 271, 24639–24648
20 Augustijn, K.D. *et al.* (2002) Structural characterization of the Pit-1-Ets-1 interactions: phosphorylation regulates Pit-1/Ets-1 binding. *Proc. Natl. Acad. Sci. U. S. A.* 99, 12657–12662
21 Duval, D.L. *et al.* (2007) Differential utilization of transcription activation subdomains by distinct coactivators regulates Pit-1 basal and Ras responsiveness. *Mol. Endocrinol.* 21, 172–185
22 Coletta, R.D. *et al.* (2004) Transcriptional control of the cell cycle in mammary gland development and tumorigenesis. *J. Mammary. Gland Biol. Neoplasia.* 9, 39–53
23 Shepherd, T. and Hassell, J. (2001) Role of Ets transcription factors in mammary gland development and oncogenesis. *J. Mammary Gland Biol. Neoplasia* 6, 129–140
24 Kurpios, N.A. *et al.* (2003) Function of PEA3 Ets transcription factors in mammary gland development and oncogenesis. *J. Mammary Gland Biol. Neoplasia* 8, 177–190
25 Harris, J. *et al.* (2006) Socs2 and elf5 mediate prolactin-induced mammary gland development. *Mol. Endocrinol.* 20, 1177–1187
26 Zhou, J. *et al.* (2005) Elf5 is essential for early embryogenesis and mammary gland development during pregnancy and lactation. *EMBO J.* 24, 635–644
27 Sapi, E. *et al.* (1998) Ets-2 transdominant mutant abolishes anchorage-independent growth and macrophage-stimulating factor-stimulated invasion by BT20 breast carcinoma cells. *Cancer Res.* 58, 1027–1033
28 Delannoy-Courdent, A. *et al.* (1998) The expression of Ets-1 transcription factor lacking its activation domain decreases uPA proteolytic activity and cell motility, and impairs normal tubulogenesis and cancerous scattering in mammary epithelial cells. *J. Cell Sci.* 111, 1521–1534
29 Neznanov, N. *et al.* (1999) A single targeted Ets2 allele restricts development of mammary tumors in transgenic mice. *Cancer Res.* 59, 4242–4246
30 Man, A.K. *et al.* (2003) Ets2-dependent stromal regulation of mouse mammary tumors. *Mol. Cell. Biol.* 23, 8614–8625
31 Tynan, J.A. *et al.* (2005) Ets2-dependent microenvironmental support of mouse mammary tumors. *Oncogene* 24, 6870–6876
32 Ghadersohi, A. *et al.* (2007) Prostate-derived Ets transcription factor (PDEF) down regulates survivin expression and inhibits breast cancer cell growth *in vitro* and xenograft tumor formation *in vivo*. *Breast Cancer Res. Treat.* 102, 19–30
33 Xing, X. *et al.* (2000) The Ets protein PEA3 suppresses Her-2/neu overexpression and inhibits tumorigenesis. *Nat. Med.* 6, 189–195
34 Yu, Z. *et al.* (2006) Antitumor activity of an Ets protein, PEA3, in breast cancer cell lines MDA-MB-361DYT2 and BT474M1. *Mol. Carcinog.* 45, 667–675
35 Feldman, R.J. *et al.* (2003) Pdef expression in human breast cancer is correlated with invasive potential and altered gene expression. *Cancer Res.* 63, 4626–4631
36 Eckel, K.L. *et al.* (2003) The epithelial-specific ETS transcription factor ESX/ESE-1/Elf-3 modulates breast cancer-associated gene expression. *DNA Cell Biol.* 22, 79–94
37 Schedin, P.J. *et al.* (2004) ESX induces transformation and functional epithelial to mesenchymal transition in MCF-12A mammary epithelial cells. *Oncogene* 23, 1766–1779
38 Witt, A.E. *et al.* (2006) Functional proteomics approach to investigate the biological activities of cDNAs implicated in breast cancer. *J. Proteome Res.* 5, 599–610
39 Tomlins, S.A. *et al.* (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 310, 644–648
40 Chen, C. *et al.* (2005) ERM is required for transcriptional control of the spermatogonial stem cell niche. *Nature* 436, 1030–1034
41 Bergthorsson, J.T. *et al.* (2006) A genome-wide study of allelic imbalance in human testicular germ cell tumors using microsatellite markers. *Cancer Genet. Cytogenet.* 164, 1–9
42 Adam, M. *et al.* (2003) Angiogenetic protooncogene ets-1 induced neovascularization is involved in the metastatic process of testicular germ cell tumors. *Eur. Urol.* 44, 329–336
43 Gashaw, I. *et al.* (2005) Gene signatures of testicular seminoma with emphasis on expression of ets variant gene 4. *Cell. Mol. Life Sci.* 62, 2359–2368
44 Fujimoto, J. *et al.* (2003) Expression of ETS-1 related to angiogenesis in uterine endometrium during the menstrual cycle. *J. Biomed. Sci.* 10, 320–327
45 Koo, T.B. *et al.* (2005) Differential expression of the PEA3 subfamily of ETS transcription factors in the mouse ovary and peri-implantation uterus. *Reproduction* 129, 651–657
46 Brar, A.K. *et al.* (2002) An Ets motif in the proximal decidual prolactin promoter is essential for basal gene expression. *J. Mol. Endocrinol.* 29, 99–112
47 Yamamoto, H. *et al.* (1998) Defective trophoblast function in mice with a targeted mutation of Ets2. *Genes Dev.* 12, 1315–1326
48 Georgiades, P. and Rossant, J. (2006) Ets2 is necessary in trophoblast for normal embryonic anteroposterior axis development. *Development* 133, 1059–1068
49 Donnison, M. *et al.* (2005) Loss of the extraembryonic ectoderm in Elf5 mutants leads to defects in embryonic patterning. *Development* 132, 2299–2308
50 Fujimoto, J. *et al.* (2002) Clinical implications of expression of ETS-1 related to angiogenesis in uterine cervical cancers. *Ann. Oncol.* 13, 1598–1604
51 Simpson, S. *et al.* (1997) Altered expression of Erg and Ets-2 transcription factors is associated with genetic changes at 21q22.2-22.3 in immortal and cervical carcinoma cell lines. *Oncogene* 14, 2149–2157
52 Lai, H.C. *et al.* (2005) Matrix metalloproteinase 1 gene polymorphism as a prognostic predictor of invasive cervical cancer. *Gynecol. Oncol.* 96, 314–319
53 Nishioka, Y. *et al.* (2003) A relationship between matrix metalloproteinase-1 (MMP-1) promoter polymorphism and cervical cancer progression. *Cancer Lett.* 200, 49–55
54 van Riggelen, J. *et al.* (2005) Loss of net as repressor leads to constitutive increased c-fos transcription in cervical cancer cells. *J. Biol. Chem.* 280, 3286–3294
55 Iwasaki, M. *et al.* (2004) E1AF/PEA3 reduces the invasiveness of SiHa cervical cancer cells by activating serine proteinase inhibitor squamous cell carcinoma antigen. *Exp. Cell Res.* 299, 525–532
56 Fujimoto, J. *et al.* (2005) Sex steroid-dependent angiogenesis in uterine endometrial cancers. *J. Steroid Biochem. Mol. Biol.* 93, 161–165
57 Takai, N. *et al.* (2003) Clinical relevance of Elf-1 overexpression in endometrial carcinoma. *Gynecol. Oncol.* 89, 408–413
58 Planaguma, J. *et al.* (2005) Up-regulation of ERM/ETV5 correlates with the degree of myometrial infiltration in endometrioid endometrial carcinoma. *J. Pathol.* 207, 422–429

Author's personal copy

59 Saito, T. *et al.* (2004) Overexpressed progesterone receptor form B inhibit invasive activity suppressing matrix metalloproteinases in endometrial carcinoma cells. *Cancer Lett.* 209, 237–243
60 Mizumoto, H. *et al.* (2002) Acceleration of invasive activity via matrix metalloproteinases by transfection of the estrogen receptor-alpha gene in endometrial carcinoma cells. *Int. J. Cancer* 100, 401–406
61 Fujimoto, J. *et al.* (2004) Clinical implications of expression of ETS-1 related to angiogenesis in metastatic lesions of ovarian cancers. *Oncology* 66, 420–428
62 Takai, N. *et al.* (2003) The significance of Elf-1 expression in epithelial ovarian carcinoma. *Int. J. Mol. Med.* 12, 349–354
63 Wilson, L.A. *et al.* (2004) Role of the transcription factor Ets-1 in cisplatin resistance. *Mol. Cancer Ther.* 3, 823–832
64 Behrens, P. *et al.* (2001) The Ets-1 transcription factor is up-regulated together with MMP 1 and MMP 9 in the stroma of pre-invasive breast cancer. *J. Pathol.* 194, 43–50
65 Davidson, B. *et al.* (2003) Coordinated expression of integrin subunits, matrix metalloproteinases (MMP), angiogenic genes and Ets transcription factors in advanced-stage ovarian carcinoma: a possible activation pathway? *Cancer Metastasis Rev.* 22, 103–115
66 Hung, M.C. and Wang, S.C. (2000) Suppressing HER2/neu-mediated cell transformation by transcriptional repressors. *Breast Dis.* 11, 133–144
67 Fuhrer, D. *et al.* (2005) Evaluation of insulin-like growth factor II, cyclooxygenase-2, ets-1 and thyroid-specific thyroglobulin mRNA expression in benign and malignant thyroid tumours. *Eur. J. Endocrinol.* 152, 785–790
68 de Nigris, F. *et al.* (2001) Induction of ETS-1 and ETS-2 transcription factors is required for thyroid cell transformation. *Cancer Res.* 61, 2267–2275
69 Fauquette, V. *et al.* (2005) The antagonistic regulation of human MUC4 and ErbB-2 genes by the Ets protein PEA3 in pancreatic cancer cells: implications for the proliferation/differentiation balance in the cells. *Biochem. J.* 386, 35–45
70 Ito, Y. *et al.* (2002) Ets-2 overexpression contributes to progression of pancreatic adenocarcinoma. *Oncol. Rep.* 9, 853–857
71 Richard, V. *et al.* (2005) PTHrP gene expression in cancer: do all paths lead to Ets? *Crit. Rev. Eukaryot. Gene Expr.* 15, 115–132

Personal copy

Elsevier.com – linking scientists to new research and thinking

Designed for scientists' information needs, Elsevier.com is powered by the latest technology with customer-focused navigation and an intuitive architecture for an improved user experience and greater productivity.

The easy-to-use navigational tools and structure connect scientists with vital information – all from one entry point. Users can perform rapid and precise searches with our advanced search functionality, using the FAST technology of Scirus.com, the free science search engine. Users can define their searches by any number of criteria to pinpoint information and resources. Search by a specific author or editor, book publication date, subject area – life sciences, health sciences, physical sciences and social sciences – or by product type. Elsevier's portfolio includes more than 1800 Elsevier journals, 2200 new books every year and a range of innovative electronic products. In addition, tailored content for authors, editors and librarians provides timely news and updates on new products and services.

Elsevier is proud to be a partner with the scientific and medical community. Find out more about our mission and values at Elsevier.com. Discover how we support the scientific, technical and medical communities worldwide through partnerships with libraries and other publishers, and grant awards from The Elsevier Foundation.

As a world-leading publisher of scientific, technical and health information, Elsevier is dedicated to linking researchers and professionals to the best thinking in their fields. We offer the widest and deepest coverage in a range of media types to enhance cross-pollination of information, breakthroughs in research and discovery, and the sharing and preservation of knowledge.

Elsevier. Building insights. Breaking boundaries.
www.elsevier.com

Histol Histopathol (2008) 23: 1417-1424

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Ets transcription factors in intestinal morphogenesis, homeostasis and disease

Paul Jedlicka[1] and Arthur Gutierrez-Hartmann[2,3]

Departments of [1]Pathology, [2]Medicine, and [3]Biochemistry and Molecular Genetics, University of Colorado Denver, Aurora CO, USA

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.

Offprint requests to: Paul Jedlicka, Department of Pathology, University of Colorado Denver, Anschutz Medical Center, PO Box 6511, MS 8104, Aurora CO 80045, USA. e-mail: paul.jedlicka@uchsc.edu

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, Er81, GABPα); spermatogenesis (Erm); development of extraembryonic tissues and early embryonic development (Ets2, Elf5, 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; Theveneau 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; Oettgen et al., 1997; Maroulakou and Bowe, 2000; Vlaeminck-Guillem et al., 2000; Li et al., 2007). In the developing mouse small intestine, Pea3, Elf1, Elf3, Ehf, Ets2 and Erf are predominantly epithelial; Er81, Elk3, Elf2, Ets1, Erg and Fli1 are predominantly non-epithelial; and Erm appears to be expressed in both

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

Ets factor	Developmental expression (mouse)[1]	Relative expression (human adult sm int; colon; HCT-116 cells)[2]	Altered expression in colon carcinoma[3]
ER81/ETV1*	WTE (SAGE); nE (ISH; ma, 10.3)	2; 2; 3	Up (WTE: RT)
PEA3/ETV4/E1AF*	WTE (SAGE); E (ISH)	2; <1; 17	Up (WTE: RT, ma [2])
ERM/ETV5*	WTE (SAGE; RT); E/nE (ISH); nE (ma, 8.4)	1; 1; 32	Up (WTE: RT)
ELK1*	WTE (SAGE; RT)	5; 5; 19	
ELK3/NET/SAP2/ERP*	WTE (SAGE; RT); nE (ma, 53.0)	6; 3; 26	
ELK4/SAP1	WTE (RT)	14; 18; 16	
ELF1	WTE (SAGE; RT); E (ISH)	4; 4; 4	Up (WTE: ma [1])
ELF4/MEF/ELFR*	WTE (SAGE; RT)	7; 13; 9	
ELF2/NERF	WTE (SAGE); nE (ma, 2.2);	6; 7; 15	
ELF3/ESE1/ESX/ERT/JEN*	WTE (SAGE); E (ISH; ma, 5.7)	115; 449; 84	
ELF5/ESE2*	WTE (RT)	<1; <1; <1	
EHF/ESE3*	WTE (SAGE); E (ma, 11.1)	47; 183; 38	
SPI1/PU1*		16; 11; <1	
SPIB*		3; 3; <1	
SPIC	WTE (RT)	<1; <1; <1	
TEL/ETV6*	WTE (SAGE; RT)	11; 18; 10	Down (WTE: ma [1])
TEL2/TREF	NA	4; 5; 1	
PDEF/ESF/PSE	WTE (RT)	3; 17; <1	
GABPα/E4TF1*	WTE (SAGE)	12; 10; 38	
ETV2/ER71	WTE (RT)	2; 2; 4	
ETS1*	WTE (SAGE; RT); nE (ISH; ma, 104.3)	24; 18; 5	Up (IHC)
ETS2*	WTE (SAGE; RT); E (ISH; ma, 2.0)	68; 50; 25	Up (IHC; WTE: ma [1])
ERF	WTE (SAGE, RT); E (ma, 2.9)	3; 2; 3	
ETV3/PE1	WTE (SAGE; RT)	11; 7; 12	
ERG	nE (ISH; ma, 9.4)	3; 2; <1	
FLI1/ERGB*	WTE (SAGE; RT); nE (ma, 42.4)	4; 2; <1	Down (WTE: ma [1])
FEV	WTE (RT)	5; 3; <1	

[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)). Predominantly 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: semi-quantitative 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 ß type II receptor (TGFßRII), and both the impaired enterocyte and goblet cell differentiation can be rescued by transgenic TGFßRII 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 TGFß signaling (Fig. 2). The role of Elf3 in regulation of TGFßRII expression in intestinal epithelium likely involves direct stimulation of TGFßRII promoter activity (Choi et al., 1998). Other Ets factors have been shown to be capable of regulating the TGFßRII 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 TGFßRII expression in intestinal epithelium, and thus its differentiation.

Given the apparent ability of Ets factors to compensate for one another genetically, a possible reason for the paucity of intestinal phenotypes in other Ets knock-out animals is compensatory activity by Elf3. At the same time, the phenotype of Elf3 knock-out animals may not reflect the full spectrum of Elf3 function *in vivo* due to possible compensatory activity of other Ets factors, such as the closely related, and also highly expressed, Ehf. More precise analysis of the spatiotemporal patterns of Ets expression in the intestine, detailed analysis of intestinal development and homeostasis in individual, and potentially compound, Ets knock-out animals, as well as use of genetically modified Ets factors designed to overcome Ets compensation *in vivo* will be needed to fully characterize the functions of the many Ets factors expressed in the intestine. Of particular interest will be the other epithelial-specific Ets factors (Elf5, Ehf and PDEF), and other Ets factors implicated in morphogenic/homeostatic processes in other tissues, including members of the PEA3 subfamily (Pea3, Erm and Er81), Ets2 and GABPα. Interestingly, FGF signaling has been

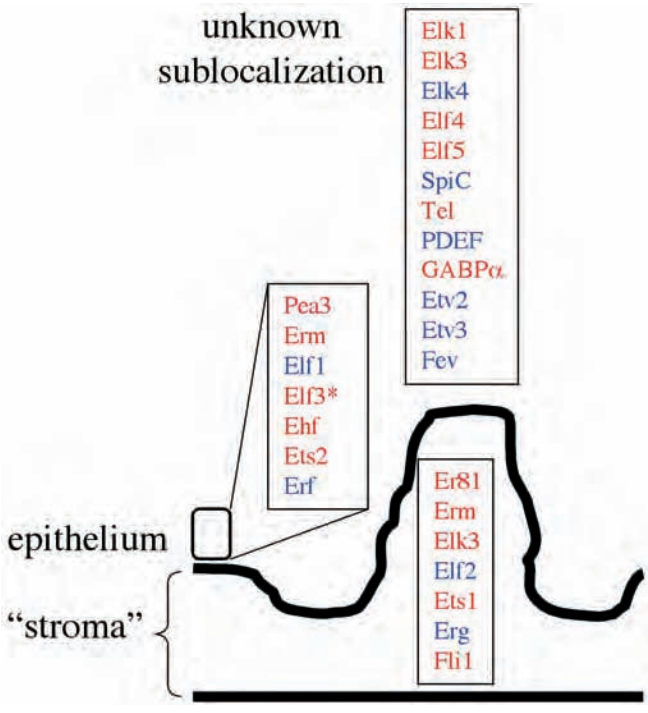


Fig. 1. Ets expression in the developing mouse intestine. Ets factors with demonstrated developmental expression in the epithelium, non-epithelial tissue ("stroma"), or in whole tissue (unknown sublocalization). Red: genetically inactivated (knocked out) in mouse; asterisk (*): intestinal phenotype reported 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 morphogenic 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; Gutierrez-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 ß3 (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 everexpression 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 ß-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 ß-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

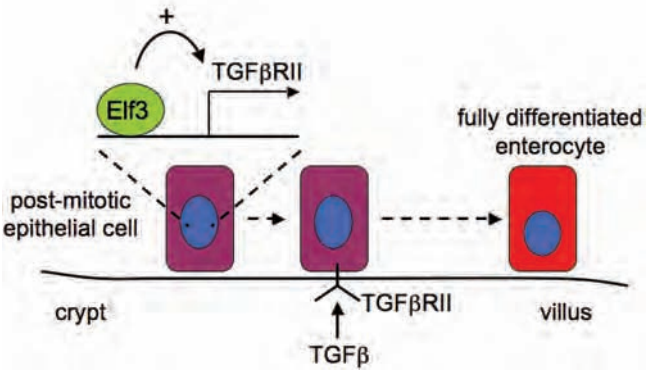


Fig. 2. Elf3 in developmental enterocyte differentiation. Elf3, expressed in developing intestinal epithelium, promotes epithelial expression of the TGFß type II receptor (TGFßRII), probably by directly stimulating TGFßRII promoter acivity. This presumably permits an epithelial response to the differentiating effects of TGFß, thus promoting epithelial differentiation along the enterocyte lineage. The same pathway also appears to regulate goblet cell terminal differentiation (see text).

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 TGFßRII 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 Ehf, 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 TGFß (Knittel et al., 1999; Czuwara-Ladykowska 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 TGFßRII 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

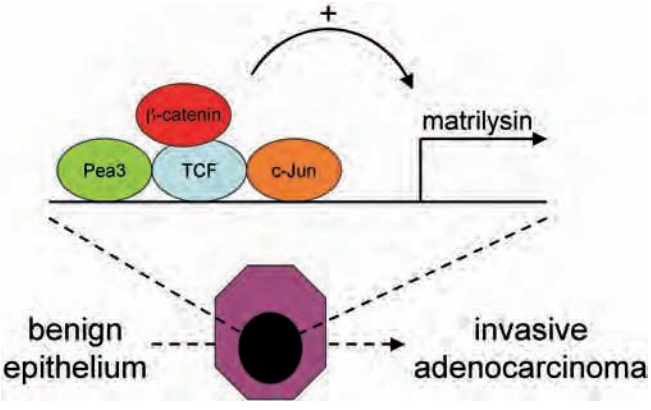


Fig. 3. Pea3 in colon cancer. Pea3 is overexpressed in tumor cells where it cooperates with the ß-catenin/TCF complex and c-Jun to stimulate the expression of the tumor promoter matrilysin. Matrilysin is expressed from early on in the adenoma-carcinoma sequence and may have multiple tumorigenesis-modifying functions (Witty et al., 1994; Takeuchi et al., 1997; Wilson et al., 1997). Other Ets-regulated tumor-promoting targets in colon cancer include Cox-2 and osteopontin, and Ets factors in the stromal, vascular and other surrounding tissue compartments likely also have tumor-modifying functions (see text).

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).

References

Baldus C.D., Liyanarachchi S., Mrozek K., Auer H., Tanner S.M., Guimond M., Ruppert A.S., Mohamed N., Davuluri R.V., Caligiuri M.A., Bloomfield C.D. and de la Chapelle A. (2004). Acute myeloid leukemia with complex karyotypes and abnormal chromosome 21: Amplification discloses overexpression of APP, ETS2, and ERG genes. Proc. Natl. Acad. Sci. USA 101, 3915-3920.

Behrens P., Mathiak M., Mangold E., Kirdorf S., Wellmann A., Fogt F., Rothe M., Florin A. and Wernert N. (2003). Stromal expression of invasion-promoting, matrix-degrading proteases MMP-1 and -9 and the Ets 1 transcription factor in HNPCC carcinomas and sporadic colorectal cancers. Int. J .Cancer 107, 183-188.

Bertrand V., Hudson C., Caillol D., Popovici C. and Lemaire P. (2003). Neural tissue in ascidian embryos is induced by FGF9/16/20, acting via a combination of maternal GATA and Ets transcription factors. Cell 115, 615-627.

Brent A.E. and Tabin C.J. (2004). FGF acts directly on the somitic tendon progenitors through the Ets transcription factors Pea3 and Erm to regulate scleraxis expression. Development 131, 3885-3896.

Brown C., Gaspar J., Pettit A., Lee R., Gu X., Wang H., Manning C., Voland C., Goldring S.R., Goldring M.B., Libermann T.A., Gravallese E.M. and Oettgen P. (2004). ESE-1 is a novel transcriptional mediator of angiopoietin-1 expression in the setting of inflammation. J. Biol. Chem. 279, 12794-12803.

Burns R.C., Fairbanks T.J., Sala F., De Langhe S., Mailleux A., Thiery J.P., Dickson C., Itoh N., Warburton D., Anderson K.D. and Bellusci S. (2004). Requirement for fibroblast growth factor 10 or fibroblast growth factor receptor 2-IIIb signaling for cecal development in mouse. Dev. Biol. 265, 61-74.

Cangemi R., Mensah A., Albertini V., Jain A., Mello-Grand M., Chiorino G., Catapano C.V. and Carbone G.M. (2008). Reduced expression and tumor suppressor function of the ETS transcription factor ESE-3 in prostate cancer. Oncogene 27, 2877-2885.

Chang C.H., Scott G.K., Kuo W.L., Xiong X., Suzdaltseva Y., Park J.W., Sayre P., Erny K., Collins C., Gray J.W. and Benz C.C. (1997). ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. Oncogene 14, 1617-1622.

Chen C., Ouyang W., Grigura V., Zhou Q., Carnes K., Lim H., Zhao G.Q., Arber S., Kurpios N., Murphy T.L., Cheng A.M., Hassell J.A., Chandrashekar V., Hofmann M.C., Hess R.A. and Murphy K.M. (2005). ERM is required for transcriptional control of the spermatogonial stem cell niche. Nature 436, 1030-1034.

Choi M.Y., Romer A.I., Hu M., Lepourcelet M., Mechoor A., Yesilaltay A., Krieger M., Gray P.A. and Shivdasani R.A. (2006). A dynamic expression survey identifies transcription factors relevant in mouse digestive tract development. Development 133, 4119-4129.

Choi S.G., Yi Y., Kim Y.S., Kato M., Chang J., Chung H.W., Hahm K.B., Yang H.K., Rhee H.H., Bang Y.J. and Kim S.J. (1998). A novel ets-related transcription factor, ERT/ESX/ESE-1, regulates expression of the transforming growth factor-beta type II receptor. J. Biol. Chem. 273, 110-117.

Chotteau-Lelievre A., Desbiens X., Pelczar H., Defossez P.A. and de Launoit Y. (1997). Differential expression patterns of the PEA3 group transcription factors through murine embryonic development. Oncogene 15, 937-952.

Coletta R.D., Jedlicka P., Gutierrez-Hartmann A. and Ford H.L. (2004). Transcriptional control of the cell cycle in mammary gland development and tumorigenesis. J. Mammary Gland Biol. Neoplasia 9, 39-53.

Crawford H.C., Fingleton B., Gustavson M.D., Kurpios N., Wagenaar R.A., Hassell J.A. and Matrisian L.M. (2001). The PEA3 subfamily of Ets transcription factors synergizes with beta-catenin-LEF-1 to activate matrilysin transcription in intestinal tumors. Mol. Cell Biol. 21, 1370-1383.

Czuwara-Ladykowska J., Sementchenko V.I., Watson D.K. and Trojanowska M. (2002). Ets1 is an effector of the transforming growth factor beta (TGF-beta) signaling pathway and an antagonist of the profibrotic effects of TGF-beta. J. Biol. Chem. 277, 20399-20408.

de Kerchove D'Exaerde A., Cartaud J., Ravel-Chapuis A., Seroz T., Pasteau F., Angus L.M., Jasmin B.J., Changeux J.P. and Schaeffer L. (2002). Expression of mutant Ets protein at the neuromuscular synapse causes alterations in morphology and gene expression. EMBO Rep. 3, 1075-1081.

Donnison M., Beaton A., Davey H.W., Broadhurst R., L'Huillier P. and Pfeffer P.L. (2005). Loss of the extraembryonic ectoderm in Elf5 mutants leads to defects in embryonic patterning. Development 132, 2299-2308.

Feldman R.J., Sementchenko V.I. and Watson D.K. (2003). The epithelial-specific Ets factors occupy a unique position in defining epithelial proliferation, differentiation and carcinogenesis. Anticancer Res. 23, 2125-2131.

Flentjar N., Chu P.Y., Ng A.Y., Johnstone C.N., Heath J.K., Ernst M., Hertzog P.J. and Pritchard M.A. (2007). TGF-betaRII rescues development of small intestinal epithelial cells in Elf3-deficient mice. Gastroenterology 132, 1410-1419.

Galang C.K., Muller W.J., Foos G., Oshima R.G. and Hauser C.A. (2004). Changes in the expression of many Ets family transcription factors and of potential target genes in normal mammary tissue and tumors. J. Biol. Chem. 279, 11281-11292.

Georgiades P. and Rossant J. (2006). Ets2 is necessary in trophoblast for normal embryonic anteroposterior axis development. Development 133, 1059-1068.

Grall F., Gu X., Tan L., Cho J.Y., Inan M.S., Pettit A.R., Thamrongsak U., Choy B.K., Manning C., Akbarali Y., Zerbini L., Rudders S., Goldring S.R., Gravallese E.M., Oettgen P., Goldring M.B. and Libermann T.A. (2003). Responses to the proinflammatory cytokines interleukin-1 and tumor necrosis factor alpha in cells derived from rheumatoid synovium and other joint tissues involve nuclear factor kappaB-mediated induction of the Ets transcription factor ESE-1. Arthritis Rheum. 48, 1249-1260.

Graves B.J. and Petersen J.M. (1998). Specificity within the ets family of

transcription factors. Adv. Cancer Res. 75, 1-55.

Gu X., Zerbini L.F., Otu H.H., Bhasin M., Yang Q., Joseph M.G., Grall F., Onatunde T., Correa R.G. and Libermann T.A. (2007). Reduced PDEF expression increases invasion and expression of mesenchymal genes in prostate cancer cells. Cancer Res. 67, 4219-4226.

Gutierrez-Hartmann A., Duval D.L. and Bradford A.P. (2007). ETS transcription factors in endocrine systems. Trends Endocrinol. Metab. 18, 150-158.

Hippenmeyer S., Huber R.M., Ladle D.R., Murphy K. and Arber S. (2007). ETS transcription factor Erm controls subsynaptic gene expression in skeletal muscles. Neuron 55, 726-740.

Hollenhorst P.C., Jones D.A. and Graves B.J. (2004). Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. Nucleic Acids Res. 32, 5693-5702.

Hong Y., Ho K.S., Eu K.W. and Cheah P.Y. (2007). A susceptibility gene set for early onset colorectal cancer that integrates diverse signaling pathways: implication for tumorigenesis. Clin. Cancer Res. 13, 1107-1114.

Horiuchi S., Yamamoto H., Min Y., Adachi Y., Itoh F. and Imai K. (2003). Association of ets-related transcriptional factor E1AF expression with tumour progression and overexpression of MMP-1 and matrilysin in human colorectal cancer. J. Pathol. 200, 568-576.

Hsu T., Trojanowska M. and Watson D.K. (2004). Ets proteins in biological control and cancer. J. Cell Biochem. 91, 896-903.

Hughes A.L. (2005). Consistent across-tissue signatures of differential gene expression in Crohn's disease. Immunogenetics 57, 709-716.

Ito Y., Takeda T., Okada M. and Matsuura N. (2002). Expression of ets-1 and ets-2 in colonic neoplasms. Anticancer Res. 22, 1581-1584.

Kawachi K., Masuyama N. and Nishida E. (2002). Essential role of the transcription factor Ets-2 in *Xenopus* early development. J. Biol. Chem. 4, 4.

Knittel T., Kobold D., Dudas J., Saile B. and Ramadori G. (1999). Role of the Ets-1 transcription factor during activation of rat hepatic stellate cells in culture. Am. J. Pathol. 155, 1841-1848.

Kola I., Brookes S., Green A.R., Garber R., Tymms M., Papas T.S. and Seth A. (1993). The Ets1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation. Proc. Natl. Acad. Sci. USA 90, 7588-7592.

Konno S., Iizuka M., Yukawa M., Sasaki K., Sato A., Horie Y., Nanjo H., Fukushima T. and Watanabe S. (2004). Altered expression of angiogenic factors in the VEGF-Ets-1 cascades in inflammatory bowel disease. J . Gastroenterol. 39, 931-939.

Kopp J.L., Wilder P.J., Desler M., Kim J.H., Hou J., Nowling T. and Rizzino A. (2004). Unique and selective effects of five Ets family members, Elf3, Ets1, Ets2, PEA3, and PU.1, on the promoter of the type II transforming growth factor-beta receptor gene. J. Biol. Chem. 279, 19407-19420.

Kurpios N.A., Sabolic N.A., Shepherd T.G., Fidalgo G.M. and Hassell J.A. (2003). Function of PEA3 Ets transcription factors in mammary gland development and oncogenesis. J. Mammary Gland Biol. Neoplasia 8, 177-190.

Kwon J.H., Keates S., Simeonidis S., Grall F., Libermann T.A. and Keates A.C. (2003). ESE-1, an enterocyte-specific Ets transcription factor, regulates MIP-3alpha gene expression in Caco-2 human colonic epithelial cells. J. Biol. Chem. 278, 875-884.

Lacorazza H.D., Miyazaki Y., Di Cristofano A., Deblasio A., Hedvat C., Zhang J., Cordon-Cardo C., Mao S., Pandolfi P.P. and Nimer S.D. (2002). The ETS protein MEF plays a critical role in perforin gene expression and the development of natural killer and NK-T cells. Immunity 17, 437-449.

Lepourcelet M., Tou L., Cai L., Sawada J., Lazar A.J., Glickman J.N., Williamson J.A., Everett A.D., Redston M., Fox E.A., Nakatani Y. and Shivdasani R.A. (2005). Insights into developmental mechanisms and cancers in the mammalian intestine derived from serial analysis of gene expression and study of the hepatoma-derived growth factor (HDGF). Development 132, 415-427.

Li X., Madison B.B., Zacharias W., Kolterud A., States D. and Gumucio D.L. (2007). Deconvoluting the intestine: molecular evidence for a major role of the mesenchyme in the modulation of signaling cross talk. Physiol. Genomics. 29, 290-301.

Liu Y., Borchert G.L. and Phang J.M. (2004). Polyoma enhancer activator 3, an ets transcription factor, mediates the induction of cyclooxygenase-2 by nitric oxide in colorectal cancer cells. J. Biol. Chem. 279, 18694-18700.

Liu Y., Jiang H., Crawford H.C. and Hogan B.L. (2003). Role for ETS domain transcription factors Pea3/Erm in mouse lung development. Dev. Biol. 261, 10-24.

Livet J., Sigrist M., Stroebel S., De Paola V., Price S.R., Henderson C.E., Jessell T.M. and Arber S. (2002). ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools. Neuron 35, 877-892.

Man A.K., Young L.J., Tynan J.A., Lesperance J., Egeblad M., Werb Z., Hauser C.A., Muller W.J., Cardiff R.D. and Oshima R.G. (2003). Ets2-dependent stromal regulation of mouse mammary tumors. Mol. Cell. Biol. 23, 8614-8625.

Maroulakou I.G. and Bowe D.B. (2000). Expression and function of Ets transcription factors in mammalian development: a regulatory network. Oncogene 19, 6432-6442.

Maroulakou I.G., Papas T.S. and Green J.E. (1994). Differential expression of ets-1 and ets-2 proto-oncogenes during murine embryogenesis. Oncogene 9, 1551-1565.

Nakayama T., Ito M., Ohtsuru A., Naito S. and Sekine I. (2001). Expression of the ets-1 proto-oncogene in human colorectal carcinoma. Mod. Pathol. 14, 415-422.

Ng A.Y., Waring P., Ristevski S., Wang C., Wilson T., Pritchard M., Hertzog P. and Kola I. (2002). Inactivation of the transcription factor Elf3 in mice results in dysmorphogenesis and altered differentiation of intestinal epithelium. Gastroenterology 122, 1455-1466.

O'Leary D.A., Noakes P.G., Lavidis N.A., Kola I., Hertzog P.J. and Ristevski S. (2007). Targeting of the ETS factor GABPalpha disrupts neuromuscular junction synaptic function. Mol. Cell Biol. 27, 3470-3480.

Oettgen P., Alani R.M., Barcinski M.A., Brown L., Akbarali Y., Boltax J., Kunsch C., Munger K. and Libermann T.A. (1997). Isolation and characterization of a novel epithelium-specific transcription factor, ESE-1, a member of the ets family. Mol. Cell Biol. 17, 4419-4433.

Oikawa T. and Yamada T. (2003). Molecular biology of the Ets family of transcription factors. Gene 303, 11-34.

Paratore C., Brugnoli G., Lee H.Y., Suter U. and Sommer L. (2002). The role of the Ets domain transcription factor Erm in modulating differentiation of neural crest stem cells. Dev. Biol. 250, 168-180.

Parkinson D.B., Langner K., Namini S.S., Jessen K.R. and Mirsky R. (2002). beta-Neuregulin and autocrine mediated survival of Schwann cells requires activity of Ets family transcription factors. Mol. Cell Neurosci. 20, 154-167.

Poppe B., Vandesompele J., Schoch C., Lindvall C., Mrozek K., Bloomfield C.D., Beverloo H.B., Michaux L., Dastugue N., Herens C., Yigit N., De Paepe A., Hagemeijer A. and Speleman F. (2004).

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.

Raible F. and Brand M. (2001). Tight transcriptional control of the ETS domain factors Erm and Pea3 by Fgf signaling during early zebrafish development. Mech. Dev. 107, 105-117.

Ristevski S., O'Leary D.A., Thornell A.P., Owen M.J., Kola, I. and Hertzog P.J. (2004). The ETS transcription factor GABPalpha is essential for early embryogenesis. Mol. Cell. Biol. 24, 5844-5849.

Roehl H. and Nusslein-Volhard C. (2001). Zebrafish pea3 and erm are general targets of FGF8 signaling. Curr. Biol. 11, 503-507.

Rosmarin A.G., Resendes K.K., Yang Z., McMillan J.N. and Fleming S.L. (2004). GA-binding protein transcription factor: a review of GABP as an integrator of intracellular signaling and protein-protein interactions. Blood Cells Mol. Dis. 32, 143-154.

Rovigatti U., Watson D.K. and Yunis J.J. (1986). Amplification and rearrangement of Hu-ets-1 in leukemia and lymphoma with involvement of 11q23. Science 232, 398-400.

Rudders S., Gaspar J., Madore R., Voland C., Grall F., Patel A., Pellacani A., Perrella M.A., Libermann T.A. and Oettgen P. (2001). ESE-1 is a novel transcriptional mediator of inflammation that interacts with NF-kappa B to regulate the inducible nitric-oxide synthase gene. J. Biol. Chem. 276, 3302-3309.

Santoro A., Maggio A., Carbone P., Mirto S., Caronia F. and Acuto S. (1992). Amplification of ETS2 oncogene in acute nonlymphoblastic leukemia with t(6;21;18). Cancer Genet. Cytogenet. 58, 71-75.

Sato T. and Miwa A. (2002). Ets-1 and integrin beta3 for lung metastasis from colorectal cancer. Apmis 110, 347-353.

Seki Y., Suico M.A., Uto A., Hisatsune A., Shuto T., Isohama Y. and Kai H. (2002). The ETS transcription factor MEF is a candidate tumor suppressor gene on the X chromosome. Cancer Res. 62, 6579-6586.

Sementchenko V.I. and Watson D.K. (2000). Ets target genes: past, present and future. Oncogene 19, 6533-6548.

Seth A. and Watson D.K. (2005). ETS transcription factors and their emerging roles in human cancer. Eur. J. Cancer 41, 2462-2478.

Sharrocks A.D. (2001). The ETS-domain transcription factor family. Nat. Rev. Mol. Cell Biol. 2, 827-837.

Shepherd T.G., Kockeritz L., Szrajber M.R., Muller W.J. and Hassell J.A. (2001). The pea3 subfamily ets genes are required for HER2/Neu-mediated mammary oncogenesis. Curr. Biol. 11, 1739-1748.

Sullivan K.E., Piliero L.M., Dharia T., Goldman D. and Petri M.A. (2000). 3' polymorphisms of ETS1 are associated with different clinical phenotypes in SLE. Hum. Mutat. 16, 49-53.

Sussan T.E., Yang A., Li F., Ostrowski M.C. and Reeves R.H. (2008). Trisomy represses Apc(Min)-mediated tumours in mouse models of Down's syndrome. Nature 451, 73-75.

Takeuchi N., Ichikawa Y., Ishikawa T., Momiyama N., Hasegawa S., Nagashima Y., Miyazaki K., Koshikawa N., Mitsuhashi M. and Shimada H. (1997). Matrilysin gene expression in sporadic and familial colorectal adenomas. Mol. Carcinog. 19, 225-229.

Tetsu O. and McCormick F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398, 422-426.

Theveneau E., Duband J.L. and Altabef M. (2007). Ets-1 confers cranial features on neural crest delamination. PLoS ONE 2, e1142.

Tomlins S.A., Rhodes D.R., Perner S., Dhanasekaran S.M., Mehra R., Sun X.W., Varambally S., Cao X., Tchinda J., Kuefer R., Lee C., Montie J.E., Shah R.B., Pienta K.J., Rubin M.A. and Chinnaiyan A.M. (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310, 644-648.

Trojanowska M. (2000). Ets factors and regulation of the extracellular matrix. Oncogene 19, 6464-6471.

Turner D.P., Findlay V.J., Moussa O. and Watson D.K. (2007a). Defining ETS transcription regulatory networks and their contribution to breast cancer progression. J. Cell. Biochem. 102, 549-559.

Turner D.P., Moussa O., Sauane M., Fisher P.B. and Watson D.K. (2007b). Prostate-derived ETS factor is a mediator of metastatic potential through the inhibition of migration and invasion in breast cancer. Cancer Res. 67, 1618-1625.

van de Wetering M., Sancho E., Verweij C., de Lau W., Oving I., Hurlstone A., van der Horn K., Batlle E., Coudreuse D., Haramis A.P., Tjon-Pon-Fong M., Moerer P., van den Born M., Soete G., Pals S., Eilers M., Medema R. and Clevers H. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111, 241-250.

Vlaeminck-Guillem V., Carrere S., Dewitte F., Stehelin D., Desbiens X. and Duterque-Coquillaud M. (2000). The Ets family member Erg gene is expressed in mesodermal tissues and neural crests at fundamental steps during mouse embryogenesis. Mech. Dev. 91, 331-335.

Wai P.Y., Mi Z., Gao C., Guo H., Marroquin C. and Kuo P.C. (2006). Ets-1 and runx2 regulate transcription of a metastatic gene, osteopontin, in murine colorectal cancer cells. J. Biol. Chem. 281, 18973-18982.

Wang D., John S.A., Clements J.L., Percy D.H., Barton K.P. and Garrett-Sinha L.A. (2005). Ets-1 deficiency leads to altered B cell differentiation, hyperresponsiveness to TLR9 and autoimmune disease. Int. Immunol. 17, 1179-1191.

Wasylyk B., Hagman J. and Gutierrez-Hartmann A. (1998). Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem. Sci. 23, 213-216.

Wilson C.L., Heppner K.J., Labosky P.A., Hogan B.L. and Matrisian L.M. (1997). Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc. Natl. Acad. Sci. USA 94, 1402-1407.

Witty J.P., McDonnell S., Newell K.J., Cannon P., Navre M., Tressler R.J. and Matrisian L.M. (1994). Modulation of matrilysin levels in colon carcinoma cell lines affects tumorigenicity in vivo. Cancer Res. 54, 4805-4812.

Zhang X., Stappenbeck T.S., White A.C., Lavine K.J., Gordon J.I. and Ornitz D.M. (2006). Reciprocal epithelial-mesenchymal FGF signaling is required for cecal development. Development 133, 173-180.

Zheng H., Wasylyk C., Ayadi A., Abecassis J., Schalken J.A., Rogatsch H., Wernert N., Maira S.M., Multon M.C. and Wasylyk B. (2003). The transcription factor Net regulates the angiogenic switch. Genes Dev. 17, 2283-2297.

Zhou J., Chehab R., Tkalcevic J., Naylor M.J., Harris J., Wilson T.J., Tsao S., Tellis I., Zavarsek S., Xu D., Lapinskas E.J., Visvader J., Lindeman G.J., Thomas R., Ormand C.J., Hertzog P.J., Kola I. and Pritchard M.A. (2005). Elf5 is essential for early embryogenesis and mammary gland development during pregnancy and lactation. EMBO J. 24, 635-644.

Accepted May 7, 2008

The American Journal of Pathology, Vol. 174, No. 4, April 2009
Copyright © American Society for Investigative Pathology
DOI: 10.2353/ajpath.2009.080409

Epithelial and Mesenchymal Cell Biology

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

Paul Jedlicka,* Xiaomei Sui,*† Lori Sussel,‡ and Arthur Gutierrez-Hartmann†‡

From the Departments of Pathology, Medicine,† and Biochemistry and Molecular Genetics,‡ University of Colorado Denver, Aurora, Colorado*

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 maturation, increased epithelial transit, severe villus dysmorphogenesis, 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 nonautonomous 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 metazoans.[1,2] Numbering as many as 27 in humans, Ets factors are related to each other by a conserved 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 signaling 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 developing and adult mammalian tissues.[7] Genetic inactivation of individual Ets factors in the mouse has revealed unique, essential Ets functions in diverse biological processes, including hematopoiesis, immune function, lymph/angiogenesis, neurogenesis/neuromuscular function, spermatogenesis, early embryonic patterning, and development of extraembryonic tissues.[1,7–16] Interestingly, such studies have been remarkable for the absence 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 Institutes 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.amjpathol.org.*

Present address of L.S.: Department of Genetics and Development, Columbia University, New York, NY.

Address reprint requests to Paul Jedlicka, Department of Pathology, University of Colorado Denver, Anschutz Medical Center, PO Box 6511, MS 8104, Aurora CO 80045. E-mail: paul.jedlicka@ucdenver.edu.

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,[21–23] 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 (GGGSGGG) 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; PKKKRKV, 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/Elf3 was generated by subcloning HA-tagged full-length human Elf3[31] into pSG5. The reporter construct 8x(EBS)-TK-luciferase was generated by subcloning the 8xpal sequence (containing eight copies of the DNA-binding site GCAGGAAGCA from the rat stromelysin promoter) from 8xpal-pBLCAT[31] into pA_3-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 500 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 mmol/L triethanolamine, pH 7.5, 111 mmol/L NaCl, 2.2 mmol/L 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-

branes (Millipore, Bedford, MA). Immunoblotting was performed as previously described.[31] Primary antibodies used were anti-HA mouse monoclonal (1:1000; Covance, Princeton, NJ) and anti-tubulin mouse monoclonal (1:1000; Oncogene, Cambridge, MA); secondary antibody used was horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000, Bio-Rad). Detection was performed using the SuperSignal West Pico chemiluminescent substrate kit (Pierce, Rockford, IL).

Transgenic Animals

The villin-En/Erm transgenic construct, excised from the vector backbone by digestion with *Pme*I, was injected into the pronuclei of fertilized ova from FVB/N mice by the University of Colorado Cancer Center Transgenic/Knockout Core Facility. Transgenic animals were identified by PCR genotyping of tail-biopsy DNA using primers to the Engrailed repressor domain (5′-TGGAGTTTAGCCGGCAACAG-3′ and 5′-TGGCATCGCTCATCTTGGAG-3′); PCR of mouse actin DNA (primers 5′-TATCCTGACCCTGAAGTACC-3′ and 5′-GGTCAGGATCTTCATGAGGT-3′), performed in the same reaction, served as a control. Transgenic animals were maintained in an FVB/N background. Adult transgenic animals were subjected to phenotypic analysis, with littermates or age-matched non-transgenic animals serving as controls.

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 CO_2. 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% H_2O_2 for 10 minutes. For BrdU immunostaining, slides were incubated for 90 minutes in 2 N HCl, washed with ddH_2O, incubated for 5 minutes in 0.1 mol/L sodium borate, and washed again with ddH_2O, 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 rat anti-HA (1:25, Roche) rabbit anti-iAP (1:500; Abcam, Cambridge, MA), rabbit anti-iFabp (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 Activation by Ets Transcription Factors

Most Ets transcription factors are expressed in the developing and/or mature mammalian intestine.[19] Although 16 (of 26 total) Ets genes have been genetically inactivated in the mouse to date, only 1 (Elf3) has been reported to have an intestinal phenotype.[20] 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.[21–28] 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.[23,25–28] 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,[1,2,4] an alternative approach, used by others, is the use of an Ets dominant repressor, composed of the Ets DNA-binding domain (DBD) and the *Drosophila* Engrailed repressor domain.[21,22,24] The Engrailed repressor domain functions by an active repression mechanism,[33] 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

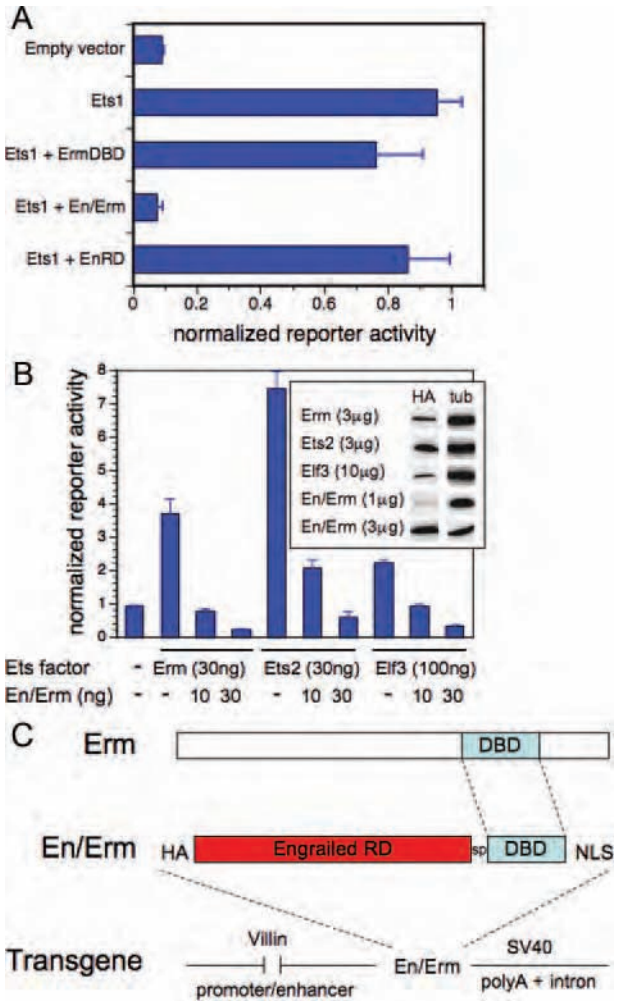


Figure 1. Transcriptional blocking activity of the En/Erm dominant repressor *in vitro*. HeLa cells were transfected with the reporter plasmid 8x(EBS)-TK-luciferase and the indicated plasmid DNA expression constructs. **A:** Transfected DNA amounts were: Ets1, 50 ng; ErmDBD, 250 ng; En/Erm, 150 ng; EnRD, 50 ng (chosen to normalize for differences in construct expression levels). **B:** Transfected DNA amounts were as shown. Reporter activity, determined by quantitative luminometry, was normalized to the activity of the co-transfected Renilla-luciferase construct; results are expressed as mean and SD of triplicate transfections. All constructs were expressed from the plasmid pSG5, and all except Ets1 have an N-terminal HA epitope tag. Protein expression (**B**, **inset**) was determined by immunoblotting with antibody against HA, and tubulin (tub) as loading control. **C:** Modular organization of the villin-En/Erm dominant repressor transgene. The transgene consists of the 12.4-kbp villin promoter-enhancer fragment, a HA epitope tag, the *Drosophila Engrailed* repressor domain (RD), a 7-amino acid (GGGSGGG) spacer (sp), the Erm DNA-binding domain (DBD), a 7-amino acid (PKKKRKV) SV40 large T-antigen nuclear localization sequence (NLS), and a SV40 intron and polyA tail.

is not attributable to nonspecific activity of the En repressor domain alone. These findings are similar to those of Liu and colleagues,[21] who, of note, uncovered an Ets pulmonary dysmorphogenesis phenotype using a transgene expressing an Engrailed-ErmDBD fusion, but not the Erm DBD alone. Based on these analyses, we selected En/Erm for blocking Ets-dependent gene expression *in vivo*.

Because of the high conservation of the DBD throughout the Ets family,[4] we expected that En/Erm would be capable of blocking the activity of a number of different Ets factors. To test this, we examined En/Erm blocking activity against Erm, Ets2, and Elf3, representative members of different subfamilies expressed in the intestinal epithelium.[19] Blockade by En/Erm was tested at both low- and high-protein expression levels, relative to Erm, Ets2, 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.[34] 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,[34] 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-

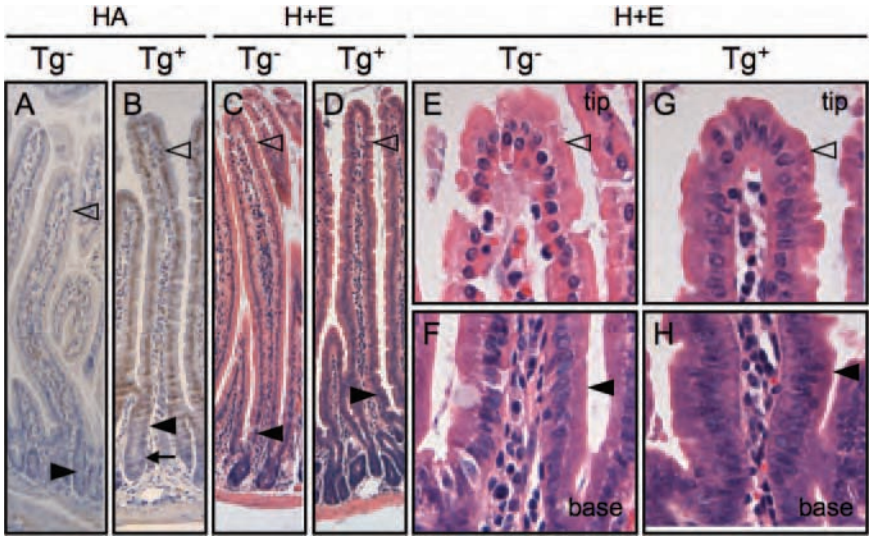


Figure 2. Transgene expression and impaired enterocyte maturation in En/Erm-expressing small intestine. HA immunostaining of small intestine from control (**A**) and transgenic (**B**) animals shows the expression of En/Erm protein specifically in epithelial nuclei of transgenic animals. En/Erm expression 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).

terocytes 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,[35] 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

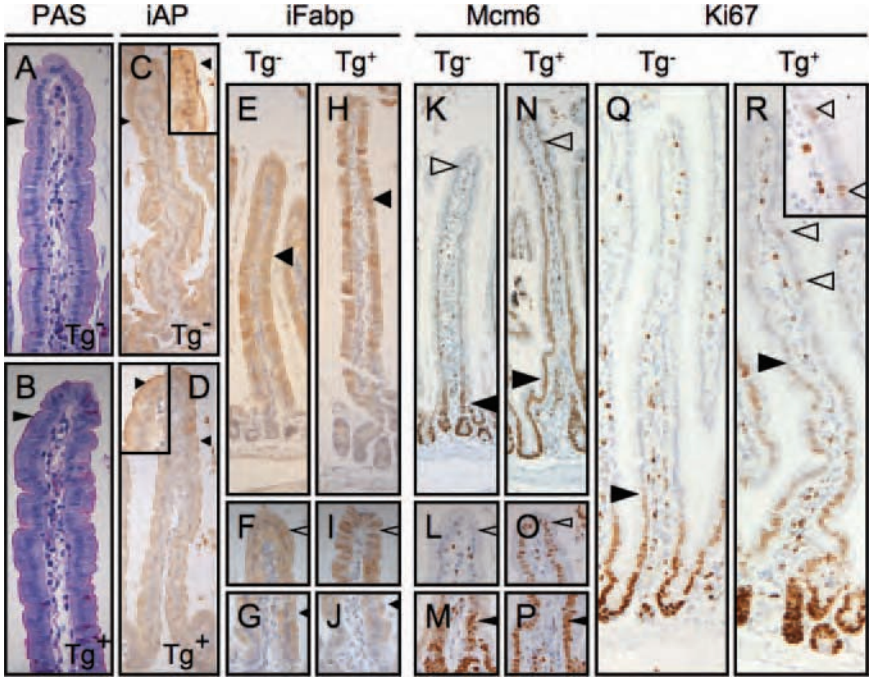


Figure 3. Characterization of enterocyte maturation disturbance in En/Erm transgenic animals. Histochemical (PAS) and immunohistochemical (iAP, iFabp, Mcm6, and Ki-67) staining of small intestine from control (Tg^-: **A**, **C**, **E--G**, **K--M**, and **Q**) and transgenic (Tg^+: **B**, **D**, **H--J**, **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 **I:** 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**; **L** and **O:** detailed views of villus tips in **K** and **N**, respectively; **M** and **P:** detailed views of villus bases in **K** and **N**, respectively; **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).

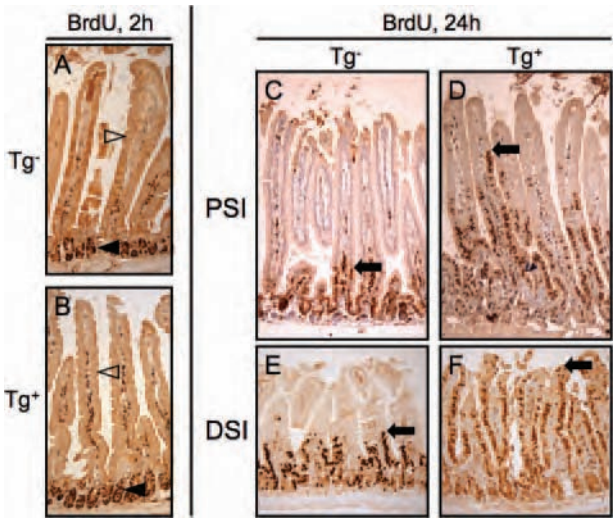


Figure 4. BrdU immunostaining of small intestine from control (Tg^-: **A**, **C**, and **E**) and transgenic (Tg^+: **B**, **D**, and **F**) animals pulsed *in vivo* with BrdU and analyzed 2 hours (**A** and **B**) or 24 hours (**C--F**) later (**filled arrowheads:** crypt epithelial cells; **open arrowheads:** villus epithelial cells; **arrows:** upper limit of epithelial cell transit after BrdU incorporation in the crypt; PSI: proximal small intestine; DSI: distal small intestine).

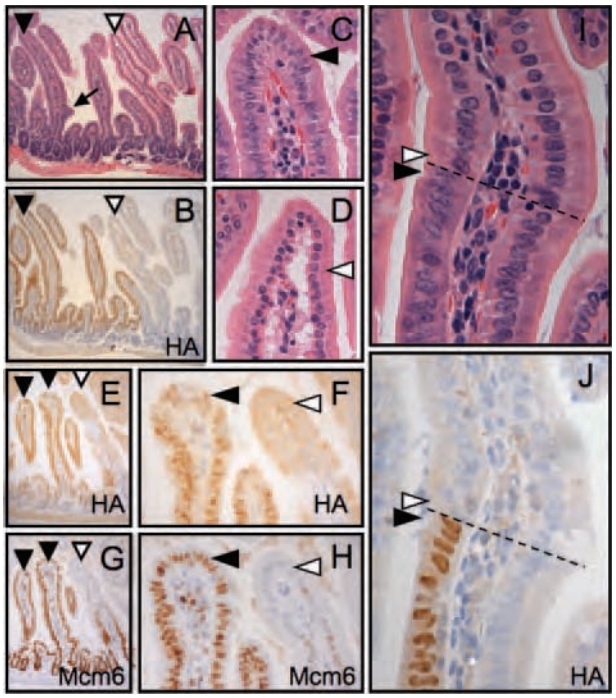


Figure 5. Characterization of enterocyte maturation disturbance in En/Erm mosaic animals. H&E-stained (**A**) and HA-immunostained (**B**) small intestinal focus mosaic for En/Erm expression (**filled arrowhead:** En/Erm expressing villus; **open arrowhead:** nonexpressing villus; **arrow:** incipient villus branch). Detailed views of the tips of the En/Erm expressing (**C**) and nonexpressing (**D**) from **A** (**arrowheads:** enterocytes). HA (**E** and **F**) and Mcm6 (**G** and **H**) immunostaining of a small intestinal focus mosaic for En/Erm expression (**arrowhead** designations are as in **A--D**). As above, En/Erm expression appears as immunopositivity in epithelial nuclei (**filled arrowhead**; contrast with absence of nuclear immunopositivity, shown by **open arrowheads**, in adjacent villus). Detailed view of H&E-stained (**I**) and HA-immunostained (**J**) individual villus mosaic for En/Erm expression. Note immature morphology of En/Erm-expressing enterocyte (**filled arrowhead**), but maturation appropriate for position along villus axis (**dashed line**) of adjacent nonexpressing enterocyte (**open arrowhead**).

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.[36] 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.[36] 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.[37] 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 villi 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.[36] 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.[20,38] 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,[20] we did not see loss of TGFβRII expression in

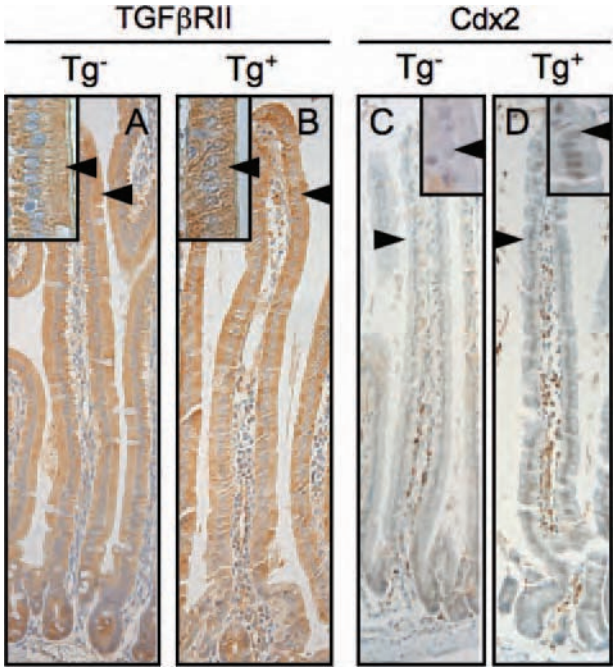


Figure 6. Analysis of potential mediators of the disturbed epithelial maturation phenotype. TGFβRII (**A** and **B**)- and Cdx2 (**C** and **D**)-immunostained small intestine from control (Tg^-) and transgenic (Tg^+) animals. Note similar level of expression of TGFβRII in control and transgenic animals (**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.

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 an 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 dysmorphogenic 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

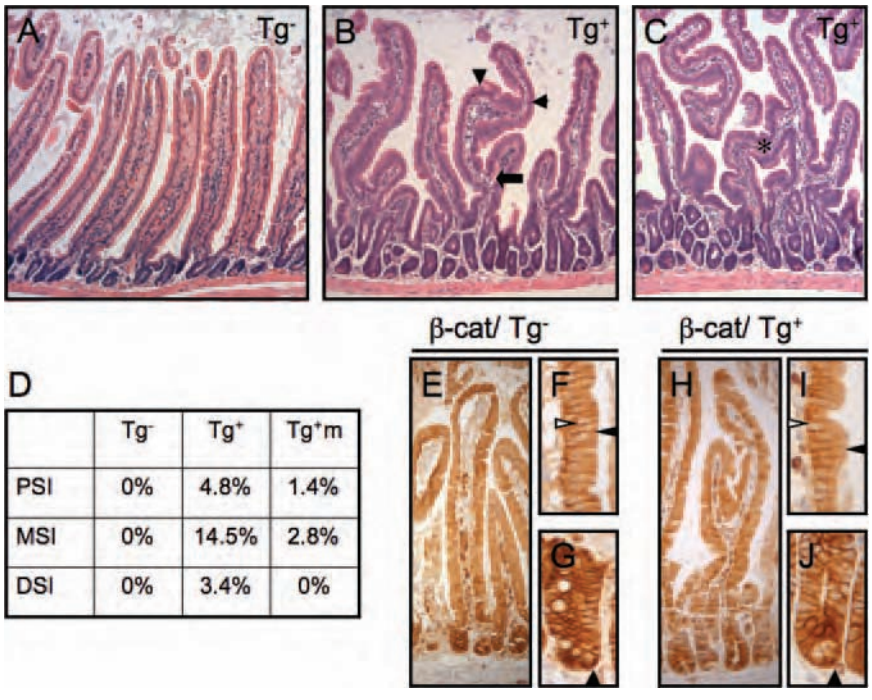


Figure 7. Villus architectural dysmorphogenesis in En/Erm-expressing small intestine. **A–C:** H&E-stained sections of small intestine from control (Tg^-) and transgenic (Tg^+) animals (**arrow:** villus branching; **arrowheads:** villus turns; **asterisk:** villus bridging). **D:** Quantitation of villus branching in control (Tg^-, $n = 8$) and transgenic (Tg^+, $n = 3$) animals, and transgene-expressing foci in mosaic animals (Tg^+m, $n = 2$), 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 **I**) but no nuclear (**open arrowheads**, **F** and **I**) staining in villi, in both transgenic and control animals.

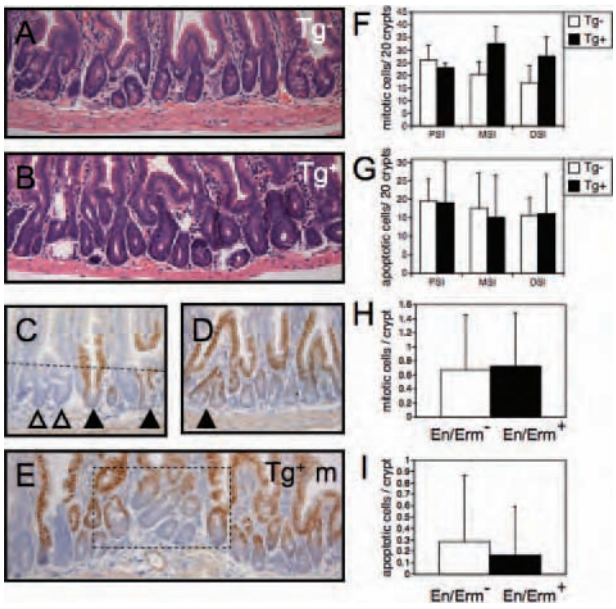


Figure 8. Crypt alterations in En/Erm-expressing small intestine. **A** and **B:** H&E-stained small intestine from control (Tg^-) and transgenic (Tg^+) animals. Note crypt disorder, including increased variation in crypt position and size in transgenic animals (**B**) relative to controls (**A**). **C–E:** HA immunostaining of small intestine in mosaic transgenic animals (**open arrowheads:** non-expressing crypts; **black arrowheads:** En/Erm-expressing crypts; dashed line in **E:** crypt-villus boundary; dashed box in **G:** crypt dysmorphogenesis in a large En/Erm-expressing mosaic focus. Crypt mitotic (**F**) and apoptotic (**G**) activity in proximal (PSI), mid (MSI), and distal (DSI) small intestine of control (Tg^-, $n = 7$ and 8, respectively) and transgenic (Tg^+, $n = 3$ for each) animals. Average mitotic (**H**) and apoptotic (**I**) cells per En/Erm nonexpressing (En/Erm^-, white bars) and expressing (En/Erm^+, black bars) crypts in mosaic animals (49 total expressing and nonexpressing crypts along the entire small intestine were scored for each; values are expressed as average and SD). Mitotic and apoptotic cells were scored by their characteristic morphology on H&E-stained sections, in well-oriented, fully-visualized crypts; none of the comparisons between experimental and control groups yielded statistically significant differences ($P < 0.05$).

http://ajp.amjpathol.org). These findings suggest that the En/Erm-induced villus dysmorphogenesis occurs independently of inappropriate β-catenin activation, and appears to uncover a novel pathway regulating normal villus architecture.

Like the villi, the small intestinal crypts maintain an orderly architecture, with relatively constant spacing, orientation, and size of crypts along the length of the intestine. In contrast to nontransgenic animals (Figure 8A), the crypts in En/Erm-expressing animals showed greater variation in number, size, and orientation (Figure 8B). As in the case of the villus dysmorphogenesis, the crypt phenotype correlated with the level of En/Erm expression in individual animals. By morphometric analysis, we did not observe statistically significant differences in crypt epithelial proliferation or apoptosis, possible mechanisms of altered crypt homeostasis (Figure 8, F and G). There appeared to be an overall trend toward increased crypt number and cellularity in En/Erm-expressing animals, but these differences did not reach statistical significance (data not shown). Interestingly, in the mosaic lines, similar crypt changes were observed, but were dependent on the size of the En/Erm-expressing focus. In areas with highly mosaic expression (ie, extensive intermixing of En/Erm-positive and -negative crypts), there 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 dyssynchrony, 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,[20,38] 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.[41] 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.[36] However, in the proximal adult small intestine, Cdx2 is expressed in a diminishing gradient along the villus axis (Figure 6C),[42] 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.[43] 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.[37,44]

Ets Factors and Architectural Organization of the Crypt-Villus Axis

The crypt-villus unit of the small intestine forms relatively late in development,[36] 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.[36,40] 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.[39,40] Interestingly, En/Erm expression causes villus dysmorphogenesis of similar severity, but independent of alterations in the crypt-villus boundary or inappropriate β-catenin activation. Little is currently known about mechanisms specifically regulating parameters of villus morphology, including villus height, direction, and absence of branching. Our studies identify Ets factors as novel regulators of villus morphology in the mature adult small intestine. Our analyses further suggest that Ets factors may regulate crypt number, position, and/or size, by crypt nonautonomous mechanisms possibly involving epithelial-stromal communication pathways.

Summary and Perspectives

Our studies using the Ets dominant repressor En/Erm identify Ets factors as important regulators of epithelial maturation, epithelial transit, and crypt-villus architecture in the adult small intestine. Little is currently known mechanistically about these important parameters of intestinal homeostasis. Examination of candidate mechanisms, namely TGFβRII expression, E-cadherin expression, and β-catenin localization, suggests that these do not contribute to the observed phenotypes. Thus, the mechanisms by which Ets factors regulate adult intestinal homeostasis appear to represent novel pathways. Unraveling of these pathways will in part require precise knowledge of both segmental and subcompartmental Ets expression in the adult intestine. Unfortunately, such information is not currently available, but existing data from whole tissue expression analysis[18] allow one to speculate as to the relevant Ets factor(s). Elf3 appears to be the most highly expressed Ets factor in the adult intestine,[18] and the En/Erm phenotype is dependent on robust transgene expression. Given the demonstrated role of Elf3 in epithelial differentiation in the developing intestine,[20,38] we suspect that the En/Erm phenotype may be attributable to blockade, probably incomplete, of Elf3 and simultaneous blockade of other highly expressed Ets factor(s) in the small intestine, such as Ehf/ESE-3 and/or Ets2.[18]

What does the En/Erm phenotype reveal about possible Ets roles in intestinal pathology? The overall phenotype—increased cell transit, impaired differentiation, and crypt-villus disorder—is that of a disturbed and overactive crypt-villus axis. Ets factors are generally overexpressed in intestinal epithelial neoplasms, and appear to be tumor promoting.[19] Surprisingly, however, in the only genetic study of Ets function in intestinal epithelial neoplasia published thus far, Ets2, at physiological or near-physiological gene dosage, was shown to restrict rather than enhance tumor multiplicity.[45] Thus, in contrast to the generally tumor-promoting effects of overexpressed Ets factors in established tumors, the maintenance of crypt-villus homeostasis by physiological levels of at least some epithelial Ets factors may have an overall effect of restricting tumor initiation and/or early promotion. Further studies will be required to determine to what extent this duality of function reflects inherent properties of different Ets factors, promoter context-dependent transcriptional activities (activation versus repression) of individual Ets, and/or gain-of-function type phenomena (ie, promoter

effects via lower affinity sites) at supraphysiological expression levels. Finally, the En/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(EBS)-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.

References

1. Oikawa T, Yamada T: Molecular biology of the Ets family of transcription factors. Gene 2003, 303:11–34
2. Sharrocks AD: The ET: S-domain transcription factor family. Nat Rev Mol Cell Biol 2001, 2:827–837
3. Gutierrez-Hartmann A, Duval DL, Bradford AP: ETS transcription factors in endocrine systems. Trends Endocrinol Metab 2007, 18:150–158
4. Graves BJ, Petersen JM: Specificity within the ets family of transcription factors. Adv Cancer Res 1998, 75:1–55
5. Sementchenko VI, Watson DK: Ets target genes: past, present and future. Oncogene 2000, 19:6533–6548
6. Wasylyk B, Hagman J, Gutierrez-Hartmann A: Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem Sci 1998, 23:213–216
7. Maroulakou IG, Bowe DB: Expression and function of Ets transcription factors in mammalian development: a regulatory network. Oncogene 2000, 19:6432–6442
8. Chen C, Ouyang W, Grigura V, Zhou Q, Carnes K, Lim H, Zhao GQ, Arber S, Kurpios N, Murphy TL, Cheng AM, Hassell JA, Chandrashekar V, Hofmann MC, Hess RA, Murphy KM: ERM is required for transcriptional control of the spermatogonial stem cell niche. Nature 2005, 436:1030–1034
9. Donnison M, Beaton A, Davey HW, Broadhurst R, L'Huillier P, Pfeffer PL: Loss of the extraembryonic ectoderm in Elf5 mutants leads to defects in embryonic patterning. Development 2005, 132:2299–2308
10. Georgiades P, Rossant J: Ets2 is necessary in trophoblast for normal embryonic anteroposterior axis development. Development 2006, 133:1059–1068
11. Hippenmeyer S, Huber RM, Ladle DR, Murphy K, Arber S: ETS transcription factor Erm controls subsynaptic gene expression in skeletal muscles. Neuron 2007, 55:726–740
12. Livet J, Sigrist M, Stroebel S, De Paola V, Price SR, Henderson CE, Jessell TM, Arber S: ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools. Neuron 2002, 35:877–892
13. O'Leary DA, Noakes PG, Lavidis NA, Kola I, Hertzog PJ, Ristevski S: Targeting of the ETS factor GABPalpha disrupts neuromuscular junction synaptic function. Mol Cell Biol 2007, 27:3470–3480
14. Ristevski S, O'Leary DA, Thornell AP, Owen MJ, Kola I, Hertzog PJ: The ETS transcription factor GABPalpha is essential for early embryogenesis. Mol Cell Biol 2004, 24:5844–5849
15. Rosmarin AG, Resendes KK, Yang Z, McMillan JN, Fleming SL: GA-binding protein transcription factor: a review of GABP as an integrator of intracellular signaling and protein-protein interactions. Blood Cells Mol Dis 2004, 32:143–154
16. Zhou J, Chehab R, Tkalcevic J, Naylor MJ, Harris J, Wilson TJ, Tsao S, Tellis I, Zavarsek S, Xu D, Lapinskas EJ, Visvader J, Lindeman GJ, Thomas R, Ormandy CJ, Hertzog PJ, Kola I, Pritchard MA: Elf5 is essential for early embryogenesis and mammary gland development during pregnancy and lactation. EMBO J 2005, 24:635–644
17. Galang CK, Muller WJ, Foos G, Oshima RG, Hauser CA: Changes in the expression of many Ets family transcription factors and of potential target genes in normal mammary tissue and tumors. J Biol Chem 2004, 279:11281–11292
18. Hollenhorst PC, Jones DA, Graves BJ: Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. Nucleic Acids Res 2004, 32:5693–5702
19. Jedlicka P, Gutierrez-Hartmann A: Ets transcription factors in intestinal morphogenesis, homeostasis and disease. Histol Histopathol 2008, 23:1417–1424
20. Ng AY, Waring P, Ristevski S, Wang C, Wilson T, Pritchard M, Hertzog P, Kola I: Inactivation of the transcription factor Elf3 in mice results in dysmorphogenesis and altered differentiation of intestinal epithelium. Gastroenterology 2002, 122:1455–1466
21. Liu Y, Jiang H, Crawford HC, Hogan BL: Role for ETS domain transcription factors Pea3/Erm in mouse lung development. Dev Biol 2003, 261:10–24
22. Shepherd TG, Kockeritz L, Szrajber MR, Muller WJ, Hassell JA: The pea3 subfamily ets genes are required for HER2/Neu-mediated mammary oncogenesis. Curr Biol 2001, 11:1739–1748
23. de Kerchove D'Exaerde A, Cartaud J, Ravel-Chapuis A, Seroz T, Pasteau F, Angus LM, Jasmin BJ, Changeux JP, Schaeffer L: Expression of mutant Ets protein at the neuromuscular synapse causes alterations in morphology and gene expression. EMBO Rep 2002, 3:1075–1081
24. Paratore C, Brugnoli G, Lee HY, Suter U, Sommer L: The role of the Ets domain transcription factor Erm in modulating differentiation of neural crest stem cells. Dev Biol 2002, 250:168–180
25. Théveneau E, Duband JL, Altabef M: Ets-1 confers cranial features on neural crest delamination. PLoS ONE 2007, 2:e1142
26. Parkinson DB, Langner K, Namini SS, Jessen KR, Mirsky R: Beta-neuregulin and autocrine mediated survival of Schwann cells requires activity of Ets family transcription factors. Mol Cell Neurosci 2002, 20:154–167
27. Langer SJ, Bortner DM, Roussel MF, Sherr CJ, Ostrowski MC: Mitogenic signaling by colony-stimulating factor 1 and ras is suppressed by the ets-2 DNA-binding domain and restored by myc overexpression. Mol Cell Biol 1992, 12:5355–5362
28. Wasylyk C, Maira SM, Sobieszczuk P, Wasylyk B: Reversion of Ras transformed cells by Ets transdominant mutants. Oncogene 1994, 9:3665–3673
29. Green S, Issemann I, Sheer E: A versatile in vivo and in vitro eukaryotic expression vector for protein engineering. Nucleic Acids Res 1988, 16:369
30. Gordon DF, Lewis SR, Haugen BR, James RA, McDermott MT, Wood WM, Ridgway EC: Pit-1 and GATA-2 interact and functionally cooperate to activate the thyrotropin beta-subunit promoter. J Biol Chem 1997, 272:24339–24347
31. Eckel KL, Tentler JJ, Cappetta GJ, Diamond SE, Gutierrez-Hartmann A: The epithelial-specific ETS transcription factor ESX/ESE-1/Elf-3 modulates breast cancer-associated gene expression. DNA Cell Biol 2003, 22:79–94
32. Ng L, Forrest D, Haugen BR, Wood WM, Curran T: N-terminal variants of thyroid hormone receptor beta: differential function and potential contribution to syndrome of resistance to thyroid hormone. Mol Endocrinol 1995, 9:1202–1213
33. Jaynes JB, O'Farrell PH: Active repression of transcription by the engrailed homeodomain protein. EMBO J 1991, 10:1427–1433
34. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL: Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J Biol Chem 2002, 277: 33275–33283
35. Haigis K, Sage J, Glickman J, Shafer S, Jacks T: The related retinoblastoma (pRb) and p130 proteins cooperate to regulate homeostasis in the intestinal epithelium. J Biol Chem 2006, 281:638–647
36. Hauck AL, Swanson KS, Kenis PJ, Leckband DE, Gaskins HR,

Schook LB: Twists and turns in the development and maintenance of the mammalian small intestine epithelium. Birth Defects Res C Embryo Today 2005, 75:58–71
37. Stappenbeck TS, Gordon JI: Rac1 mutations produce aberrant epithelial differentiation in the developing and adult mouse small intestine. Development 2000, 127:2629–2642
38. Flentjar N, Chu PY, Ng AY, Johnstone CN, Heath JK, Ernst M, Hertzog PJ, Pritchard MA: TGF-betaRII rescues development of small intestinal epithelial cells in Elf3-deficient mice. Gastroenterology 2007, 132:1410–1419
39. Kim BM, Mao J, Taketo MM, Shivdasani RA: Phases of canonical Wnt signaling during the development of mouse intestinal epithelium. Gastroenterology 2007, 133:529–538
40. Madison BB, Braunstein K, Kuizon E, Portman K, Qiao XT, Gumucio DL: Epithelial hedgehog signals pattern the intestinal crypt-villus axis. Development 2005, 132:279–289
41. Muñoz NM, Upton M, Rojas A, Washington MK, Lin L, Chytil A, Sozmen EG, Madison BB, Pozzi A, Moon RT, Moses HL, Grady WM: Transforming growth factor beta receptor type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation. Cancer Res 2006, 66:9837–9844
42. Silberg DG, Swain GP, Suh ER, Traber PG: Cdx1 and cdx2 expression during intestinal development. Gastroenterology 2000, 119:961–971
43. Hermiston ML, Wong MH, Gordon JI: Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautonomous regulation of cell fate in a self-renewing system. Genes Dev 1996, 10:985–996
44. Wong MH, Rubinfeld B, Gordon JI: Effects of forced expression of an NH2-terminal truncated beta-catenin on mouse intestinal epithelial homeostasis. J Cell Biol 1998, 141:765–777
45. Sussan TE, Yang A, Li F, Ostrowski MC, Reeves RH: Trisomy represses Apc(Min)-mediated tumours in mouse models of Down's syndrome. Nature 2008, 451:73–75

BMC Cancer

BioMed Central

Research article

Open Access

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

Paul Jedlicka*[1], Xiaomei Sui[1,2] and Arthur Gutierrez-Hartmann[2,3,4]

Address: [1]Department of Pathology, University of Colorado Denver, Aurora CO, USA, [2]Department of Medicine, University of Colorado Denver, Aurora CO, USA, [3]Department of Biochemistry, University of Colorado Denver, Aurora CO, USA and [4]Department of Molecular Genetics, University of Colorado Denver, Aurora CO, USA

Email: Paul Jedlicka* - paul.jedlicka@ucdenver.edu; Xiaomei Sui - xiaomei.sui@ucdenver.edu; Arthur Gutierrez-Hartmann - a.gutierrez-hartmann@ucdenver.edu

* Corresponding author

Published: 22 June 2009

BMC Cancer 2009, **9**:197 doi:10.1186/1471-2407-9-197

Received: 23 December 2008
Accepted: 22 June 2009

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

© 2009 Jedlicka et al; licensee BioMed Central Ltd.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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^{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^{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 substoichiometric 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 Apc^{Min} 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 Apc^{Min} 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. $Apc^{Min/+}$ 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 $Apc^{Min/+}$ 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 Apc^{Min} 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 ($Mom1^{S/R}$) for the Mom1 locus. The presence of the Villin-En/Erm transgene and Apc^{Min} 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^{Min} and Apc^{Min};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_2 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 um 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_2O_2 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). Mcm6 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) ug of RNA were treated with DNase using a DNA-free kit (Ambion), and 1.6 ug of treated RNA were reverse transcribed using Superscript III reverse transcriptase (Invitrogen), per manufacturer protocol, using random primers in a 20 ul reaction. In paral-

lel control reactions, reverse transcriptase was omitted. One ul of the reverse transcription reaction was then PCR-amplified using primers to Engrailed (for detection of the transgenic transcript) or Actin (control), as previously described [10], and the products were resolved on a 2% agarose gel stained with ethidium bromide.

Results

Intestinal En/Erm expression increases tumor number in Apc^{Min} 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^{Min} 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^{Min} 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^{Min} strain, Mom1, in order to control for possible genetic background differences between control (Apc^{Min}) and experimental (Apc^{Min};Villin-En/Erm) groups. Such analyses indicated that all animals in both control and experimental groups were heterozygous for the Mom1 locus, and thus similarly susceptible to Apc^{Min}-driven tumorigenesis.

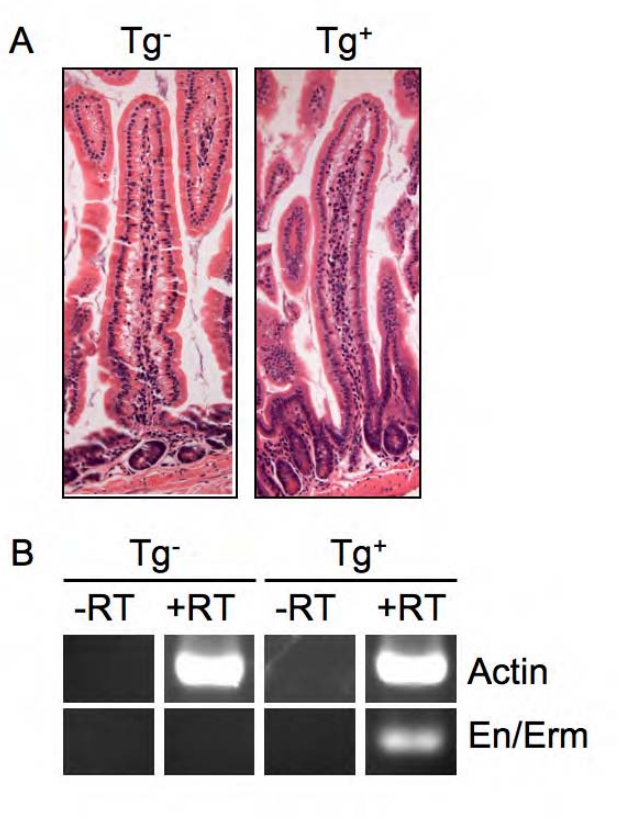


Figure 1
Intestinal histology and En/Erm expression in transgenic animals in the tumor study. (A) Representative histology (H+E-stained sections) of mid small intestine from non-transgenic (Tg^-) and Villin-En/Erm transgenic (Tg^+) animals under conditions of normal homeostasis. The transgenic animals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

As shown in Table 1, Apc^{Min};Villin-En/Erm animals developed 2.4 times as many total small intestinal tumors as Apc^{Min} controls (16.5 vs. 6.8, p = 0.03). The Apc^{Min};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^{Min} 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^{Min};Villin-En/Erm mice developed more tumors in each intestinal segment (proximal, mid and distal) compared to the Apc^{Min} 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

	Apc^{Min} (n = 12)	Apc^{Min}; Villin-En/Erm (n = 11)	
	Mean (SEM)	Mean (SEM)	p-value**
Animal age (months)	14.3 (1.3)	13.0 (1.1)	0.45
Tumor number (total)	6.8 (2.0)	16.5 (3.4)	0.03
PSI	1.3 (0.1)	3.4 (0.2)	0.07
MSI	2.8 (0.3)	8.4 (0.4)	0.03
DSI	2.9 (0.3)	4.8 (0.4)	0.37
Tumor size (total; cm)*	0.31 (0.03)	0.31 (0.02)	0.89
PSI	0.33 (0.07)	0.25 (0.04)	0.33
MSI	0.20 (0.02)	0.27 (0.01)	0.001
DSI	0.32 (0.04)	0.32 (0.02)	0.82
% tumors with stromal invasion	10.9 (4.6)	19.3 (4.6)	0.17
% histologically aggressive tumors	3.7 (2.8)	4.7 (2.4)	0.78

* 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

animals [10]. As expected, tumor numbers in the colon were lower, and not significantly different between the Apc^{Min};Villin-En/Erm and Apc^{Min} 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 Apc^{Min};Villin-En/Erm and Apc^{Min} 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 Apc^{Min};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 Apc^{Min} 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 Apc^{Min} mice

By histologic analysis, tumors in both control (Apc^{Min}) and experimental (Apc^{Min};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 Apc^{Min};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

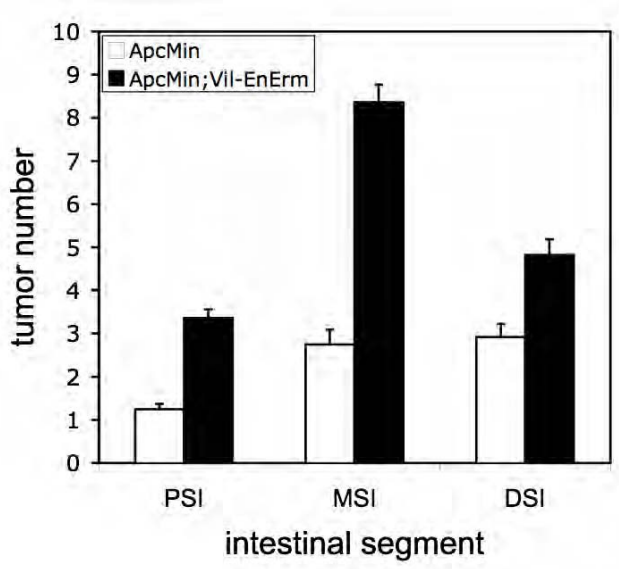


Figure 2
Tumor number in the small intestines of Apc^{Min} and Apc^{Min}; Villin-En/Erm mice, broken down by longitudinal segment (PSI, MSI and DSI denote proximal, mid and distal small intestine, respectively). Data are expressed as mean and standard error of the mean.

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 (Apc^{Min};Villin-En/Erm) and control (Apc^{Min}) groups (4.7% vs. 3.7%, p = 0.78). Thus, a relatively high proportion of Apc^{Min}-induced neoplastic lesions gives rise to invasive and histologically aggressive adenocarcinomas in a C57BL/6J × 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,

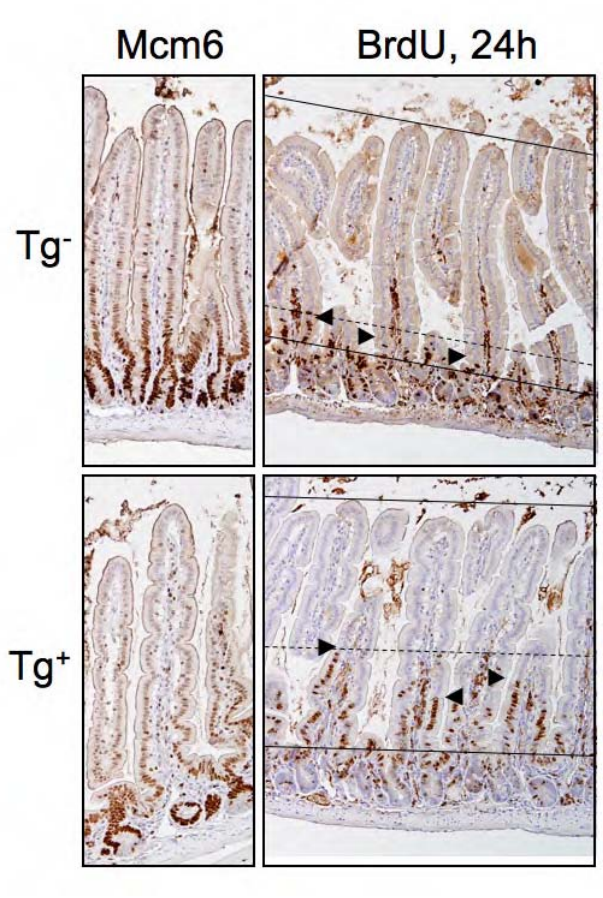


Figure 3
Analysis of epithelial maturation and transit in the small intestines of En/Erm low-expressor mice (Tg^+) and non-transgenic controls (Tg^-). Transgenic animals show appropriate loss of nuclear Mcm6 expression along the crypt-villus axis, indistinguishable from controls, reflecting normal epithelial maturation. In contrast, crypt-villus epithelial transit, as determined by in vivo BrdU labeling [10], is increased in transgenic animals compared to controls (top solid line: villus tips; bottom solid line: crypt-villus junction; dashed line: overall upper limit of epithelial transit; arrowheads: upper limit of epithelial transit in individual villi). Representative images of mid small intestine are shown for both groups.

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 Apc^{Min}-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 Apc^{Min} 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-

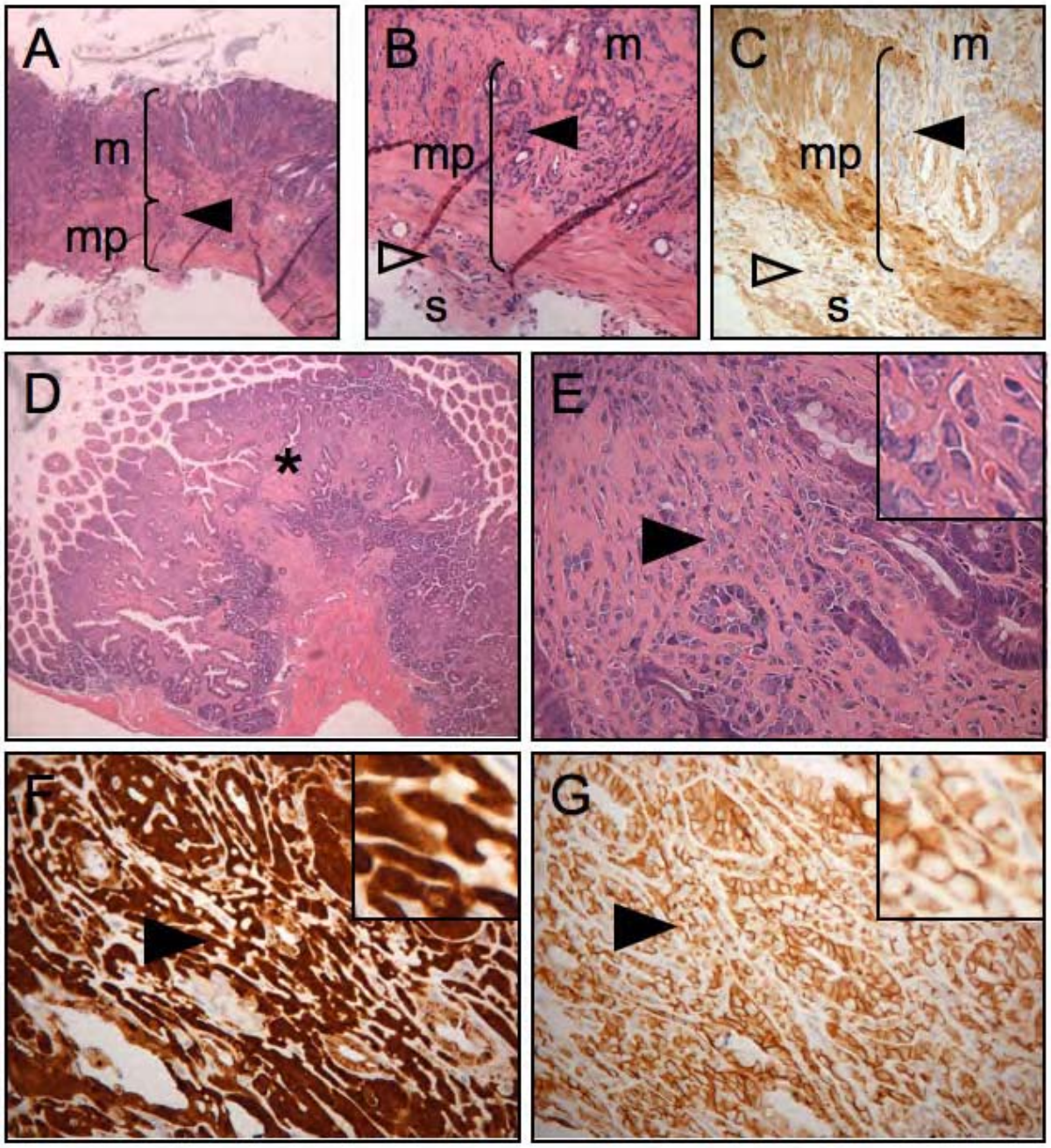


Figure 4

Invasive adenocarcinomas in animals in tumor study. (A-C) H+E-stained (A: low-power view; B: high-power view) and SMAimmunostained (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: lowpower 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).

Table 2: Summary of genetic studies examining Ets factor functions in tumorigenesis

Ets	Genetic manipulation	Tissue	Tumor model	Effect on tumorigenesis	Subcompartment with effect	Reference
PEA3 subfamily	Dominant negative	Mammary gland	MMTV-Neu	Inhibition (increased latency; decreased number and size)	Epithelium	[7]
Ets2	Hypomorphic mutant	Mammary gland	MMTV-PyMT MMTV-Neu	Inhibition (increased latency; decreased number and size)	Stroma	[6,8]
Ets2	One extra gene copy	Intestine	Apc^{Min}	Inhibition (decreased number)	Not determined	[18]
Ets family	Dominant repressor	Intestine	Apc^{Min}	Promotion (increased number)	Epithelium	This paper

lus epithelial transit. This suggests that the En/Erm 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/Erm 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/Erm exerts its effect by blocking Ets-mediated transcriptional activation [10], our findings imply that the endogenous Ets factors blocked by En/Erm normally function to restrict tumorigenesis in the intestinal epithelium. Due to the high conservation of the Ets domain, the En/Erm 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/Erm 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 Elf 1 [9]. Alternatively, the tumor phenotype may be uncovering a differential requirement for a more highly expressed Ets. Interestingly, similar to our findings, Sussan et al recently observed an inverse relationship between Ets2 gene copy number and tumor number in the Apc^{Min} model, suggesting that Ets2 normally functions to restrict intestinal tumor formation [19]. Thus, while frequently overexpressed in colon cancer, at least some Ets factors, including Ets2 and those blocked by the En/Erm transgene in our studies, appear to normally restrict, rather than promote, epithelial neoplasia in the intestine. Indeed, it may be that the same Ets factors manifest different functions in neoplasia at different expression levels due to differential promoter binding and regulation.

Secondly, our studies suggest that the Apc^{Min} mutation may provide a good model of human intestinal cancer in the C57BL/6J × FVB/N hybrid genetic background. In this background, the overall tumor burden is lower and the proportion of invasive lesions higher than in the pure C57BL/6J background, in which animals die relatively early from intestinal obstruction caused by numerous non-invasive adenomas. Thus, this mixed background model approximates the human disease, and could be useful for studying genetic parameters controlling carcinoma invasion and metastasis.

Conclusion

Expression of the Ets dominant repressor En/Erm in the small intestine, at levels that do not cause crypt-villus dysmorphogenesis, results in a marked increase in tumor number in the Apc^{Min} 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/Erm 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 Apc^{Min} mutation may provide a good model for invasive human intestinal carcinoma in the C57BL/6J × 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).

References

1. Oikawa T, Yamada T: **Molecular biology of the Ets family of transcription factors.** *Gene* 2003, **303:**11-34.
2. Sharrocks AD: **The ETS-domain transcription factor family.** *Nat Rev Mol Cell Biol* 2001, **2(11):**827-837.
3. Feldman RJ, Sementchenko VI, Watson DK: **The epithelial-specific Ets factors occupy a unique position in defining epithelial proliferation, differentiation and carcinogenesis.** *Anticancer Res* 2003, **23(3A):**2125-2131.
4. Hsu T, Trojanowska M, Watson DK: **Ets proteins in biological control and cancer.** *J Cell Biochem* 2004, **91(5):**896-903.
5. Seth A, Watson DK: **ETS transcription factors and their emerging roles in human cancer.** *Eur J Cancer* 2005, **41(16):**2462-2478.
6. Man AK, Young LJ, Tynan JA, Lesperance J, Egeblad M, Werb Z, Hauser CA, Muller WJ, Cardiff RD, Oshima RG: **Ets2-dependent stromal regulation of mouse mammary tumors.** *Mol Cell Biol* 2003, **23(23):**8614-8625.
7. Shepherd TG, Kockeritz L, Szrajber MR, Muller WJ, Hassell JA: **The pea3 subfamily ets genes are required for HER2/Neu-mediated mammary oncogenesis.** *Curr Biol* 2001, **11(22):**1739-1748.
8. Tynan JA, Wen F, Muller WJ, Oshima RG: **Ets2-dependent microenvironmental support of mouse mammary tumors.** *Oncogene* 2005, **24(46):**6870-6876.
9. Jedlicka P, Gutierrez-Hartmann A: **Ets transcription factors in intestinal morphogenesis, homeostasis and disease.** *Histol Histopathol* 2008, **23(11):**1417-1424.
10. Jedlicka P, Sui X, Sussel L, Gutierrez-Hartmann A: **Ets transcription factors control epithelial maturation and transit and crypt-villus morphogenesis in the mammalian intestine.** *Am J Pathol* 2009, **174(4):**1280-1290.
11. Hollenhorst PC, Jones DA, Graves BJ: **Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors.** *Nucleic Acids Res* 2004, **32(18):**5693-5702.
12. Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF: **Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene.** *Science* 1992, **256(5057):**668-670.
13. Heyer J, Yang K, Lipkin M, Edelmann W, Kucherlapati R: **Mouse models for colorectal cancer.** *Oncogene* 1999, **18(38):**5325-5333.
14. Boivin GP, Washington K, Yang K, Ward JM, Pretlow TP, Russell R, Besselsen DG, Godfrey VL, Doetschman T, Dove WF, *et al.*: **Pathology of mouse models of intestinal cancer: consensus report and recommendations.** *Gastroenterology* 2003, **124(3):**762-777.
15. Lawrance AK, Deng L, Brody LC, Finnell RH, Shane B, Rozen R: **Genetic and nutritional deficiencies in folate metabolism influence tumorigenicity in Apcmin/+ mice.** *J Nutr Biochem* 2007, **18(5):**305-312.
16. Dietrich WF, Lander ES, Smith JS, Moser AR, Gould KA, Luongo C, Borenstein N, Dove W: **Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse.** *Cell* 1993, **75(4):**631-639.
17. Gould KA, Luongo C, Moser AR, McNeley MK, Borenstein N, Shedlovsky A, Dove WF, Hong K, Dietrich WF, Lander ES: **Genetic evaluation of candidate genes for the Mom1 modifier of intestinal neoplasia in mice.** *Genetics* 1996, **144(4):**1777-1785.
18. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL: **Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine.** *J Biol Chem* 2002, **277(36):**33275-33283.
19. Sussan TE, Yang A, Li F, Ostrowski MC, Reeves RH: **Trisomy represses Apc(Min)-mediated tumours in mouse models of Down's syndrome.** *Nature* 2008, **451(7174):**73-75.

Pre-publication history

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

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

Publish with **Bio Med Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

BioMedcentral

Open Access

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

Darius M. Walker[1], Joanna M. Poczobutt[1], Melissa S. Gonzales[2,4], Henrick Horita[3] and Arthur Gutierrez-Hartmann[*,1,2]

Molecular Biology Program[1], and Departments of Medicine[2], Biochemistry and Molecular Genetics[3] and Pharmacology[4], University of Colorado Denver, Anschutz Medical Center, Aurora, CO, 80045, USA

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]. The 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-

*Address correspondence to this author at the Molecular Biology Program, and Departments of Medicine, University of Colorado Denver/AMC, P. O. Box 6511, Mail Stop 8106, Aurora, CO 80045, USA; Tel: 303-724-3921; Fax: 303-724-3920; E-mail: a.gutierrez-hartmann@UCHSC.edu

wed 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, 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 shCtr oligonucleotides were designed using Oligoengine (Seattle, WA). The oligonucleotide targeting ESE-1 (shESE-1) is follows: forward oligo 5' ACAGC AACATGACCTACGATTCAAGAGATCGTAGGTCATG TTGCTGT-reverse oligo 5' ACAGCAACATGACCTACG ATCTCTTGAATCGTAGGTCATGTTGCTGT. The ESE-1 negative control shRNA oligonucleotide is as follows: forward oligo 5' GCTCAACGAGGGCCTCATGTTCAAG AGACATGAGGCCCTCGTTGAGC reverse oligo 5' GCTC AACGAGGGCCTCATGTCTCTTGAACATGAGGCCCTC GTTGAGC. This negative control (shCtr) 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 shCtr were cloned into pSuper at the *Bgl*II and *Hind*III 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'-GCTCTTC TGATGA GCTCAGTTG. The GFP negative control shRNA oligonucleotide is as follows 5'-TACAACAGCCACAACG TCTAT. Each pLKO.1 shRNA vector was co-transfected along with pEGFP-C3 at a 10 to 1 ratio (pLKO.1:pEGFP) into MCF-7 cells using Qiagen Effectene, with a 12 to 1 ratio of DNA to Effectene. Cell lysates were generated two days post-transfection, as noted above.

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 Na_3VO_4, 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℃, 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)AGCCGGGCCATGA GGTAC(1126); Reverse Primer: (1173)ACGAGTCGCCGG CCAT(1158); and TaqMan Probe: (1131)ACAAACGGGAG ATCCTGGAACGGG(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 tranfected 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 500 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 for 30 seconds with crystal violet and washed for 1 min in ddH_2O. 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 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 (~$1x10^6$ 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 3 000 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**).

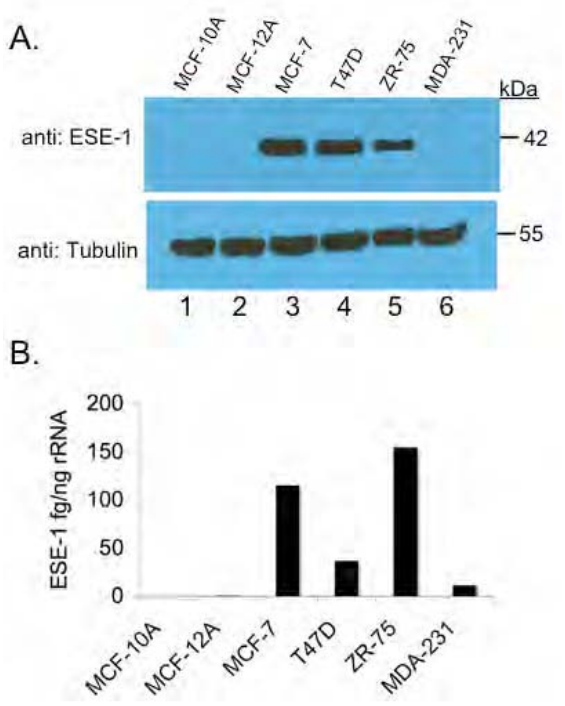


Fig. (1). ESE-1 protein is expressed in several human breast cancer cell lines. **A**. Western blot of whole cell extracts (100 µg) generated from MCF-10A (lane 1), MCF-12A (lane 2), MCF-7 (lane 3), T47D (lane 4), ZR-75-1 (lane 5) and MDA-MB-231 (lane 6) cells, probed with anti-ESE-1 mAB405 antibody (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.

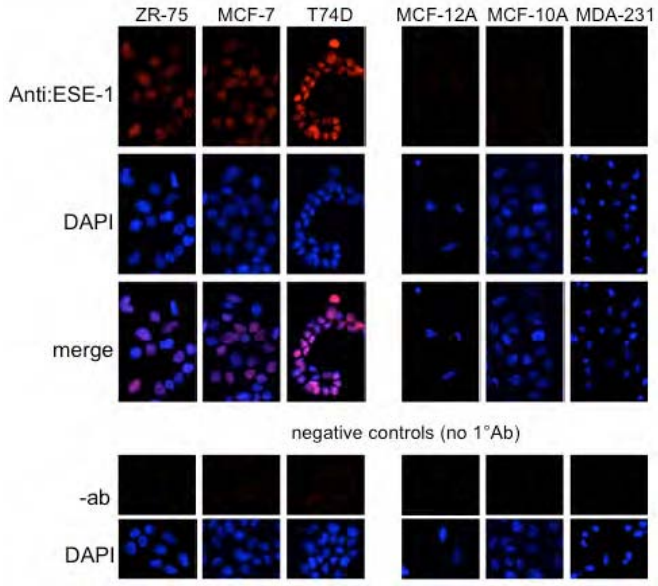


Fig. (2). ESE-1 is localized to the nucleus in human breast cancer cell lines. The top row are confocal images of ZR-75-1, MCF-7, T47D, MCF-12A, MCF-10A and MDA-MB-231 mammary epithelial cells probed with the anti-ESE-1 mAB405 antibody (1:500 dilution), followed by a Cy3-conjugated goat anti-mouse secondary 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 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, an almost complete knockdown of ESE-1 occurs by 2 days, with reduction of ESE-1 persisting 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, indicating that differences in protein loading fail to explain the significant reduction in ESE-1 detected. A similar time course of shESE-1 knockdown was performed in MCF-7 cells and this study showed the same strong reduction of ESE-1 by 2 days, and this level of inhibition persisted up to 5 days in MCF-7 (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 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 (Fig. **3C**). 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.

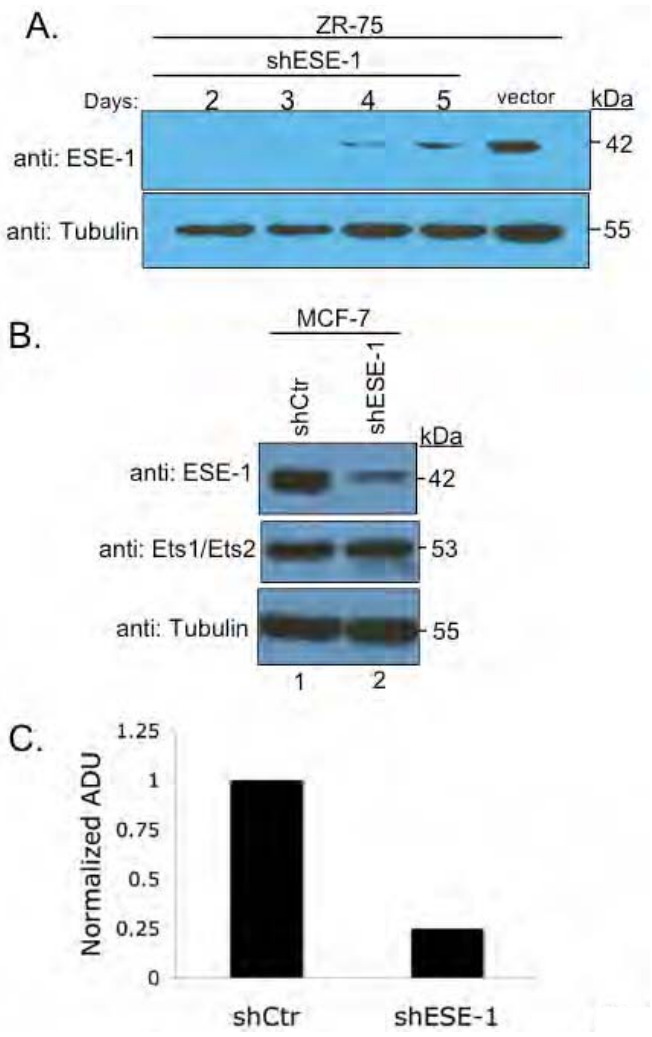


Fig. (3). shESE-1 knocks down endogenous ESE-1 protein in ZR-75-1 and MCF-7 cells in a specific and prolonged manner. **A**. Time-course of ESE-1 knockdown after transient shESE-1 transfection in ZR-75-1 cells. ZR-75-1 cells (~60% confluent, 10 cm plate) were transiently transfected with shESE-1 (6.5:0.65 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 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

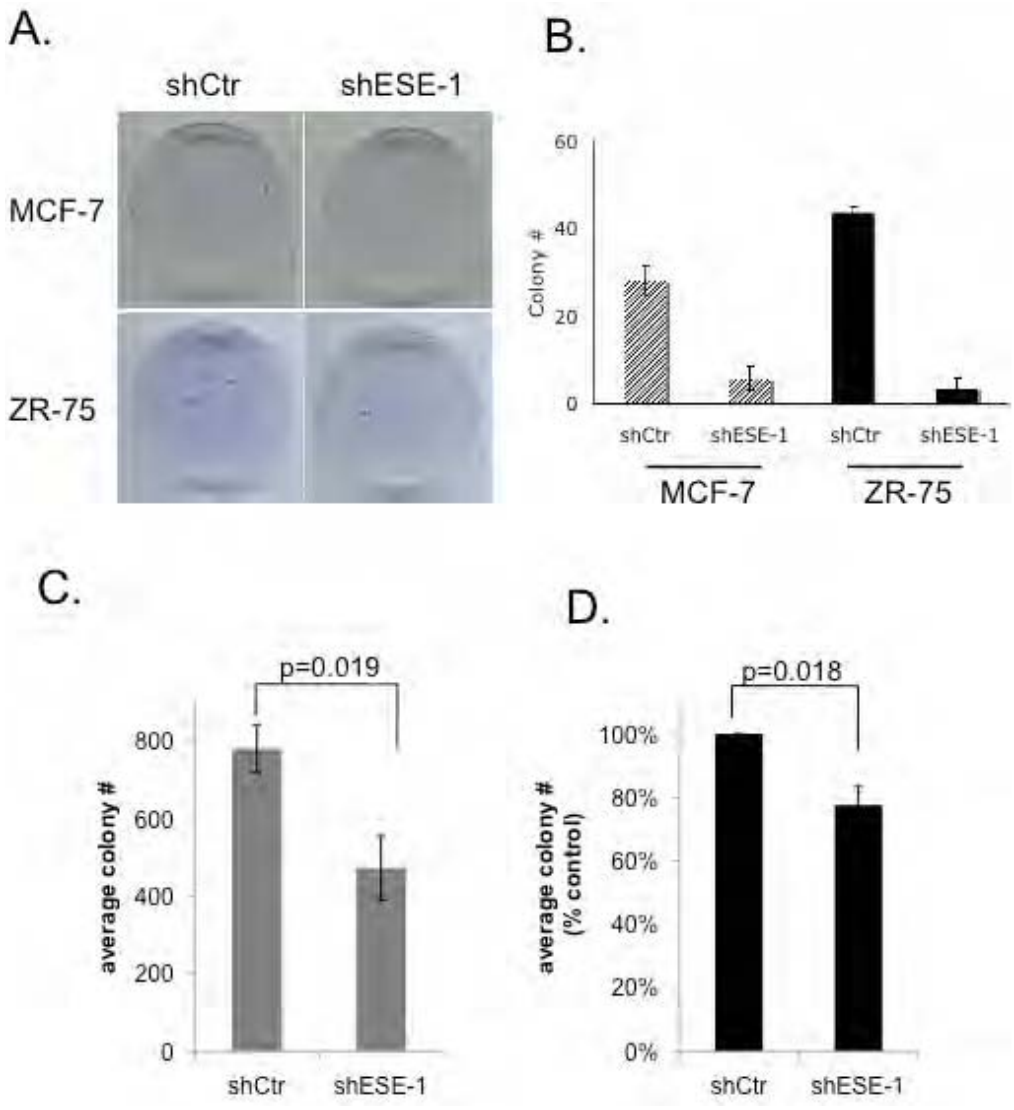


Fig. (4). ESE-1 knockdown abrogates MCF-7 and ZR-75-1 colony formation and diminishes soft agar colony formation. A. MCF-7 and ZR-75-1 colonies stained with Crystal violet. MCF-7 cells (top panels) or ZR-75-1cells (bottom panels) were transfected with shCtr or shESE-1 (using different DNA preparations for the ZR-75-1 and MCF-7 cells), selected for 14 days with 500 ug/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.

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 knock-down 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 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.

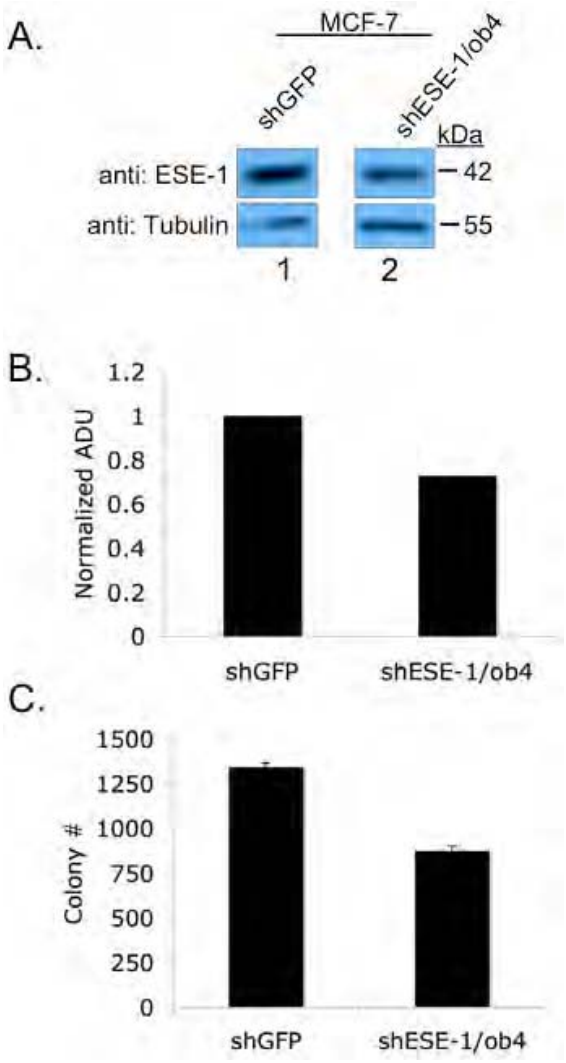


Fig. (5). shESE-1/ob4-mediated partial knockdown of endogenous ESE-1 protein correlates with the partial reduction in soft agar colony count in MCF-7 cells. A. ESE-1 Western blot. MCF-7 cells were transiently transfected with shGFP or shESE-1/ob4, harvested 48 h post-transfection and Western blot of 50 μg of whole cell extract with anti-ESE-1 mAB405 (1:1000) shown in the top panel. 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. **B**. Relative quantitation of ESE-1 expression. Arbitrary densitometry units (ADU) measuring the ESE-1 densitometry signal normalized against tubulin, with the shCtr signal set to 1. **C**. Quantitation of soft agar colonies. MCF-7 cells were transiently transfected with shGFP or shESE-1/ob4, plated in sextuplicate or triplicate and colonies were quantitated using the Metamorph imaging software (threshold 150-250 microns). shESE-1/ob4 mediated a 35% reduction in MCF-7 soft agar colony formation, which was significant to a $p=4.4x10^{-10}$ value, using the Student's t-test.

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

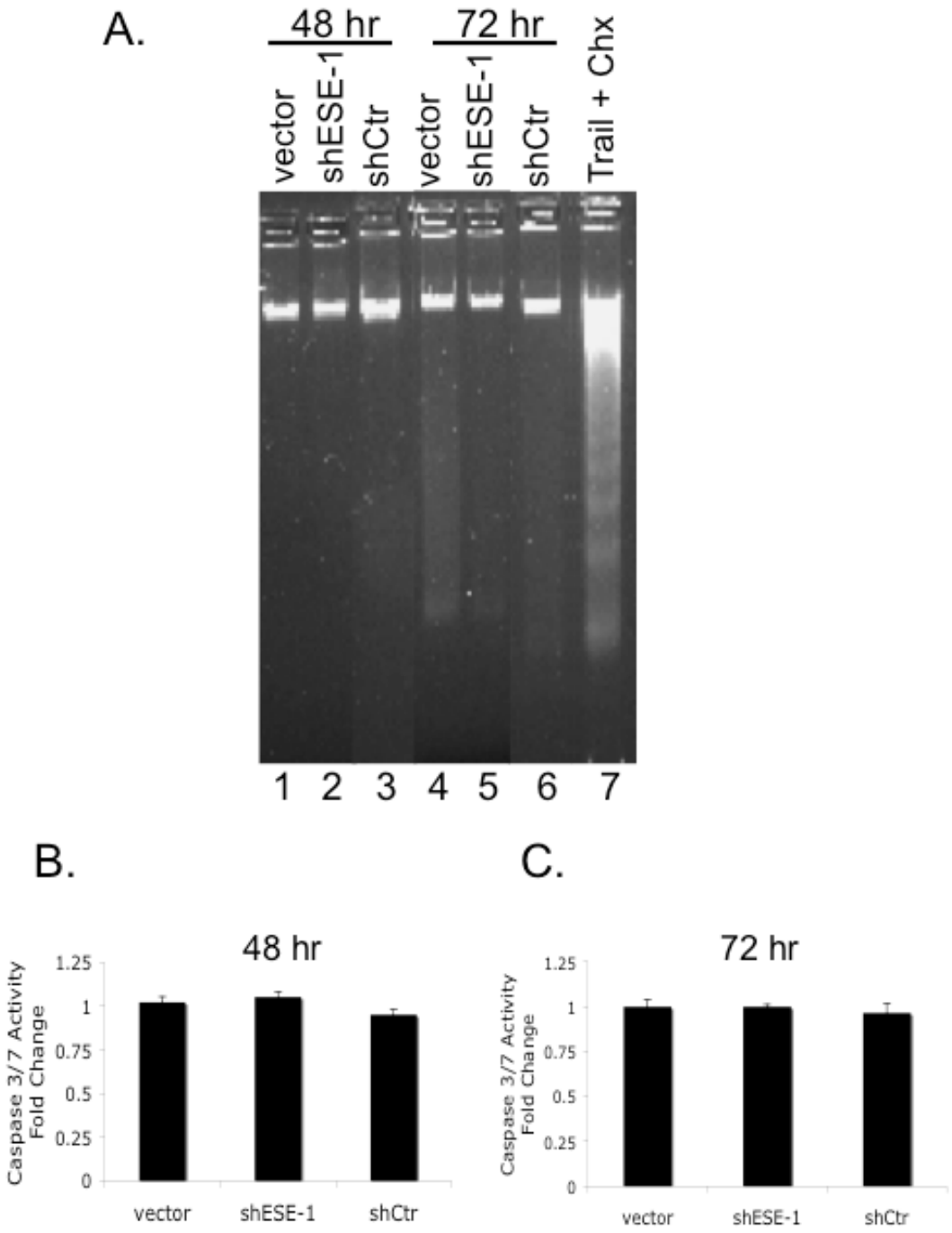


Fig. (6). Knockdown of ESE-1 does not induce DNA laddering or Caspase 3/7 activity. MCF-7 cells were transiently transfected with empty vector (lanes 1 & 4), shESE-1 (lanes 2 & 5) and shCtr (lanes 3 & 6), and genomic DNA was isolated at 48 and 72 h post-transfection. Isolated DNA (5 μg) was separated 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 capsase 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.

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.

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.

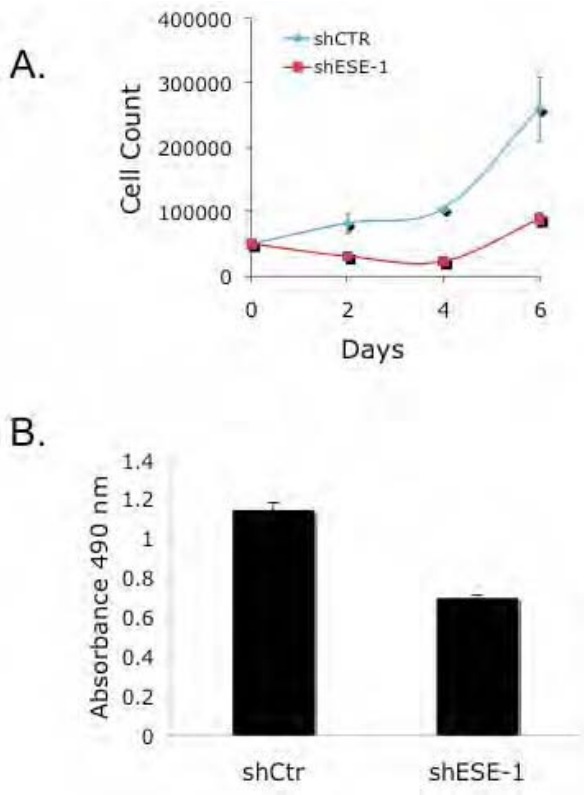


Fig. (7). Proliferation of MCF-7 cells is reduced with knockdown of ESE-1. Also, serum starvation of MCF-7 cells reduces endogenous ESE-1 protein expression. A. Total cell counts over 6 days. MCF-7 cells were transiently transfected with shCtr (diamonds) or shESE-1 (squares), and 24 h post-transfection cells were counted and 50 000 seeded at time 0. Cells were collected on days 2, 4, and 6 with 1 x PBS/EDTA and counted using the Beckman Coulter Vi-cell. **B**. MTS proliferation assay at 6 days. MCF-7 cells were transiently transfected with shCtr or shESE-1, replicated eight times, and 24 h post-transfection cells were counted and 5 000 cells were plated at time 0. Cells were grown for 6 days, harvested and MTS measured at 490 nm, as in Methods. The difference in shCtr vs shESE-1 was significant to p=1.67E-07, using the Student's t-test. C. MCF-7 cells were plated in either 10% serum (+) or 0.1 % serum (-) media and harvested at 6 h (lanes 1, 2), 18 h (lanes 3, 4) and 36 h (lanes 5, 6) later. The top panel shows a Western blot of MCF-7 whole cell extracts (100 μg) probed with anti-ESE-1 mAB405 antibody (1:1000 dilution). The bottom panel shows the same blot stripped and re-probed with anti-tubulin antibody (1:10 000).

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 nontransformed *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 knockdown 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

colony formation in ZR-75-1 and MCF-7 cells stably expressing shESE-1 (Fig. **4A** and **B**) and a ~20 and ~40% reduction in soft agar colony numbers in ZR-75-1 and MCF-7 cells, respectively, transiently expressing the shESE-1 (Fig. **4C** and **D**). Indeed, the colonies growing in G418-selected MCF-7 cells, with stable knockdown of ESE-1, consistently failed to expand, underscoring that ESE-1, when more fully ablated, has a dominant growth and/or survival inhibitory effect in mammary epithelial cells. In combination, these data reveal that specific, individual ETS factors have critical roles in mammary carcinogenesis, but in distinct tissue compartments, with Ets2 functioning in the stroma and ESE-1 functioning in the mammary epithelial cell.

Having demonstrated that ESE-1 is required to maintain the transformed phenotype of MCF-7 cells, we proceeded to mechanistic studies evaluating whether ESE-1 knockdown induced apoptosis or affected cell proliferation. Specifically, we used both DNA laddering and caspase 3/7 cleavage assays, in order to rigorously assess whether ESE-1 knockdown caused apoptosis, and the data show that ESE-1 knockdown does not induce MCF-7 apoptosis (Fig. **6**). Instead, two separate experimental approaches, total cell counts and MTS assay, revealed that ESE-1 knockdown has a key effect on MCF-7 cell proliferation (Fig. **7**). Again, these studies required transient transfection of shESE-1 in order to expand the cells over the 6-day assay period, and despite the fact that not all cells take up plasmid DNA, shESE-1 knockdown resulted in a 73% and 46% inhibition of proliferation in the total cell count and MTS assays, respectively, at 6 days (Fig. **7**). Certain ETS proteins control cell proliferation by inducing growth factors and/or their receptors, with ESE-1 shown to trans-activate HER2/neu [1, 5, 15, 35, 44, 45]. Indeed, heregulin/HER2, EGF and serum induce ESE-1 promoter activity in Sk-Br-3 human breast cancer cells. Furthermore, lactogenic competency induced in murine HC-11 mammary cells by dexamethasone, insulin and prolactin, also increased ESE-1 mRNA expression [13]. These findings suggest that a forward autocrine regulatory loop may exist between ESE-1 and growth factors, such as HER2/neu, and that such a regulatory loop contributes to the regulation of cell proliferation, and ultimately transformation, by ESE-1.

CONCLUSION

Although an increasing number of reports have underscored the important role of ETS factors in human breast cancer, the exact role of any single ETS factor in maintaining the transformed phenotype in human breast cancer cell lines remains undefined. This report establishes that the knockdown of a single ETS factor, ESE-1, is sufficient to reverse the transformed phenotype of MCF-7 and ZR-75-1 breast cancer cell lines and demonstrates that ESE-1 is required for cellular proliferation. Taken together, this paper contributes novel insights to our understanding of the critical role of ESE-1 in maintaining the transformed state in breast cancer, providing a novel single-point target for therapy.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. Heide Ford and Pepper Schedin for critical review of the manuscript. We thank 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.

REFERENCES

[1] Gutierrez-Hartmann A, Duval DL, Bradford AP. ETS transcription factors in endocrine systems. Trends Endocrinol Metab 2007; 18: 150-8.

[2] Oikawa T, Yamada T. Molecular biology of the Ets family of transcription factors. Gene 2003; 303: 11-34.

[3] Sharrocks AD. The ETS-domain transcription factor family. Nat Rev Mol Cell Biol 2001; 2: 827-37.

[4] Wasylyk B, Hagman J, Gutierrez-Hartmann A. Ets transcription factors: nuclear effectors of the Ras/MAP kinase signaling pathway. Trends Biochem Sci 1998; 23: 213-6.

[5] Seth A, Watson DK. ETS transcription factors and their emerging roles in human cancer. Eur J Cancer 2005; 41: 2462-78.

[6] Tomlins SA, Rhodes DR, Perner S, *et al.* Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 2005; 310: 644-8.

[7] Andreoli J, Jang S-I, Chung E, Coticchia C, Steinert P, Markova N. The expression of a novel, epithelium-specific ets transcription factor is restricted to the most differentiated layers in the epidermis. Nucleic Acids Res 1997; 25: 4287-95.

[8] Feldman RJ, Sementchenko VI, Watson DK. The epithelial-specific Ets factors occupy a unique position in defining epithelial proliferation, differentiation and carcinogenesis. Anticancer Res 2003; 23: 2125-31.

[9] Oettgen P, Alani R, Barcinski M, *et al.* Isolation and characterization of a novel epithelium-specific transcription factor, ESE-1, a member of the *ets* family. Mol Cell Biol 1997; 17: 4419-33.

[10] Tymms M, Ng A, Thomas R, *et al.* A novel epithelial-expressed ETS gene, ELF3: Human and murine cDNA sequences, murine genomic organization, human mapping to 1q.32.2 and expression in tissues and cancer. Oncogene 1997; 15: 2449-62.

[11] Neve R, Chang C-H, Scott G, *et al.* The epithelium-specific Ets transcription factor ESX is associated with mammary gland development and involution. FASEB J 1998; 12: 1541-50.

[12] Chang CH, Scott GK, Kuo WL, *et al.* ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. Oncogene 1997; 14: 1617-22.

[13] Neve RM, Ylstra B, Chang CH, Albertson DG, Benz CC. ErbB2 activation of ESX gene expression. Oncogene 2002; 21: 3934-8.

[14] Benz C, O'Hagan R, Richter B, *et al.* HER2/Neu and the Ets transcription activator PEA3 are coordinatley upregulated in human breast cancer. Oncogene 1997; 15: 1513-25.

[15] Eckel KL, Tentler JJ, Cappetta GJ, Diamond SE, Gutierrez-Hartmann A. The epithelial-specific ETS transcription factor ESX/ESE-1/Elf-3 modulates breast cancer-associated gene expression. DNA Cell Biol 2003; 22: 79-94.

[16] O'Hagan R, JA H. The PEA3 Ets transcription factor is a downstream target of the HER2/Neu receptor tyrosine kinase. Oncogene 1998; 16: 301-10.

[17] Galang CK, Muller WJ, Foos G, Oshima RG, Hauser CA. Changes in the expression of many Ets family transcription factors and of potential target genes in normal mammary tissue and tumors. J Biol Chem 2004; 279: 11281-92.

[18] Delannoy-Courdent A, Mattot V, Fafeur V, *et al.* The expression of Ets-1 transcription factor lacking its activation domain decreases uPA proteolytic activity and cell motility, and impairs normal tubulogenesis and cancerous scattering in mammary epithelial cells. J Cell Sci 1998; 111: 1521-34.

[19] Sapi E, Flick M, Rodov S, Kacinski B. Ets-2 transdominant mutant abolishes anchorage-independent growth and macropahge-stimulating factor-stimulated invasion by BT20 breast carcinoma cells. Cancer Res 1998; 58: 1027-33.

[20] Prescott J, Koto K, Singh M, Gutierrez-Hartmann A. The ETS transcription factor ESE-1 transforms MCF-12A human mammary epithelial cells via a novel cytoplasmic mechanism. Mol Cell Biol 2004; 24: 5548-64.

[21] Schedin P, Eckel-Mahan K, McDaniel S, *et al.* ESX induces transformation and functional epithelial to mesenchymal transition in MCF-12A mammary epithelial cells. Oncogene 2004; 23: 1766-79.

[22] Witt AE, Hines LM, Collins NL, *et al.* Functional proteomics approach to investigate the biological activities of cDNAs implicated in breast cancer. J Proteome Res 2006; 5: 599-610.

[23] Haugen BR, Larson LL, Pugazhenthi U, *et al.* Retinoic acid and retinoid X receptors are differentially expressed in thyroid cancer and thyroid carcinoma cell lines and predict response to treatment with retinoids. J Clin Endocrinol Metab 2004; 89: 272-80.

[24] Chang C, Scott G, Kuo W, *et al.* ESX: A structurally unique Ets overexpressed early during human breast tumorigenesis. Oncogene 1997; 14: 1617-22.

[25] Choi S-G, Yi Y, Kim Y-S, *et al.* A novel Ets-related transcription factor, ERT/ESX/ESE-1, regulates expression of the transforming growth factor-ß type II receptor. J Biol Chem 1998; 273: 110-7.

[26] Kim JH, Wilder PJ, Hou J, Nowling T, Rizzino A. Activation of the murine type II transforming growth factor-beta receptor gene: up-regulation and function of the transcription factor Elf-3/Ert/Esx/Ese-1. J Biol Chem 2002; 277: 17520-30.

[27] Park SH, Kim YS, Park BK, Hougaard S, Kim SJ. Sequence-specific enhancer binding protein is responsible for the differential expression of ERT/ESX/ELF-3/ESE-1/jen gene in human gastric cancer cell lines: Implication for the loss of TGF-beta type II receptor expression. Oncogene 2001; 20: 1235-45.

[28] Manavathi B, Rayala SK, Kumar R. Phosphorylation-dependent regulation of stability and transforming potential of ETS transcriptional factor ESE-1 by p21-activated kinase 1. J Biol Chem 2007; 282: 19820-30.

[29] Chang C-H, Scott GK, Baldwin NA, Benz CC. Exon 4-encoded acidic domain in the epithelium-restricted Ets factor, ESX, confers potent transactivating capacity and binds to TATA-binding protein (TBP). Oncogene 1999; 18: 3682-95.

[30] He J, Pan Y, Hu J, Albarracin C, Wu Y, Dai JL. Profile of Ets gene expression in human breast carcinoma. Cancer Biol Ther 2007; 6: 76-82.

[31] Paine TM, Soule HD, Pauley RJ, Dawson PJ. Characterization of epithelial phenotypes in mortal and immortal human breast cells. Int J Cancer 1992; 50(3): 463-73.

[32] Do HJ, Song H, Yang HM, *et al.* Identification of multiple nuclear localization signals in murine Elf3, an ETS transcription factor. FEBS Lett 2006; 580: 1865-71.

[33] Choi Y, Asada S, Uesugi M. Divergent hTAFII31-binding motifs hidden in activation domains. J Biol Chem 2000; 275: 15912-6.

[34] Wang H, Fang R, Cho JY, Libermann TA, Oettgen P. Positive and negative modulation of the transcriptional activity of the ETS factor ESE-1 through interaction with p300, CREB-binding protein, and Ku 70/86. J Biol Chem 2004; 279: 25241-50.

[35] Asada S, Choi Y, Yamada M, *et al.* External control of Her2 expression and cancer cell growth by targeting a Ras-linked coactivator. Proc Natl Acad Sci USA 2002; 99: 12747-52.

[36] Prescott J, Gonzalez M, Gutierrez-Hartmann A. Identification of a novel nuclear localization signal and nuclear export sequence that govern the cytoplasmic-dependent mammary epithelial cell transformation potency of Ese-1. In preparation.

[37] Brown C, Gaspar J, Pettit A, *et al.* ESE-1 is a novel transcriptional mediator of angiopoietin-1 expression in the setting of inflammation. J Biol Chem 2004; 279: 12794-803.

[38] Grall F, Gu X, Tan L, *et al.* Responses to the proinflammatory cytokines interleukin-1 and tumor necrosis factor alpha in cells derived from rheumatoid synovium and other joint tissues involve nuclear factor kappaB-mediated induction of the Ets transcription factor ESE-1. Arthritis Rheum 2003; 48: 1249-60.

[39] Jobling AI, Fang Z, Koleski D, Tymms MJ. Expression of the ETS transcription factor ELF3 in the retinal pigment epithelium. Invest Ophthalmol Vis Sci 2002; 43: 3530-7.

[40] Barrett JM, Puglia MA, Singh G, Tozer RG. Expression of Ets-related transcription factors and matrix metalloproteinase genes in human breast cancer cells. Breast Cancer Res Treat 2002; 72: 227-32.

[41] Lindemann RK, Braig M, Hauser CA, Nordheim A, Dittmer J. Ets2 and protein kinase C epsilon are important regulators of parathyroid hormone-related protein expression in MCF-7 breast cancer cells. Biochem J 2003; 372: 787-97.

[42] Neznanov N, Man A, Yamamoto H, Hauser C, Cardiff R, Oshima R. A Single Targeted Ets2 Allele Restricts Development of Mammary Tumors in Transgenic Mice. Cancer Res 1999; 59: 4242-6.

[43] Man AK, Young LJ, Tynan JA, *et al.* Ets2-dependent stromal regulation of mouse mammary tumors. Mol Cell Biol 2003; 23: 8614-25.

[44] Scott GK, Chang CH, Erny KM, *et al.* Ets regulation of the erbB2 promoter. Oncogene 2000; 19: 6490-502.

[45] Chiang SY, Burli RW, Benz CC, *et al.* Targeting the ets binding site of the HER2/neu promoter with pyrrole-imidazole polyamides. J Biol Chem 2000; 275: 24246-54.

Received: September 12, 2009 Revised: October 30, 2009 Accepted: November 02, 2009

© Walker *et al.*; Licensee *Bentham Open*.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/), which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.