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Understanding the Role of ETS-Mediated Gene Regulation in Complex Biological Processes

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Abstract

Ets factors are members of one of the largest families of evolutionarily conserved transcription factors, regulating critical functions in normal cell homeostasis, which when perturbed contribute to tumor progression. The well-documented alterations in ETS factor expression and function during cancer progression result in pleiotropic effects manifested by the downstream effect on their target genes. Multiple ETS factors bind to the same regulatory sites present on target genes, suggesting redundant or competitive functions. The anti- and prometastatic signatures obtained by examining specific ETS regulatory networks will significantly improve our ability to accurately predict tumor progression and advance our understanding of gene regulation in cancer. Coordination of multiple ETS gene functions also mediates interactions between tumor and stromal cells and thus contributes to the cancer phenotype. As such, these new insights may provide a novel view of the ETS gene family as well as a focal point for studying the complex biological control involved in tumor progression. One of the goals of molecular biology is to elucidate the mechanisms that contribute to the development and progression of cancer. Such an understanding of the molecular basis of cancer will provide new possibilities for: (1) earlier detection, as well as better diagnosis and staging of disease; (2) detection of minimal residual disease recurrences and evaluation of response to therapy; (3) prevention; and (4) novel treatment strategies. Increased understanding of ETS-regulated biological pathways will directly impact these areas.

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1. INTRODUCTION

1.1. The ETS gene family

The oncogene v-ets was discovered in 1983 as part of the transforming fusion protein (p135, gag-myb-ets) of E26, a replication-defective avian retrovirus. Both v-ets and v-myb contribute to the transformation of different lineages and cell types. The name ets is derived from *E*26 *t*ransforming *s*equence or *E*-*t*wenty-*s*ix specific sequence. The v-ets oncogene transforms fibroblasts, myeloblasts, and erythroblasts *in vitro* and causes mixed erythroid–myeloid and lymphoid leukemia *in vivo* (reviewed in Blair & Athanasiou, 2000). Molecular comparisons with the predicted chicken c-Ets1 protein demonstrated that the v-ets contained three internal amino acid substitutions and unique carboxy terminal amino acids. This change resulted from the inversion of the 3′ sequences of the chicken gene during retroviral transduction (Lautenberger & Papas, 1993).

All ETS family members are defined by a conserved sequence that encodes the DNA-binding (ETS) domain (Fig. 1.1 and Table 1.1). Identification of v-ets-related genes from metazoan species has established ETS as one of the largest families of transcriptional regulators, consisting of 28 ETS genes in humans, 27 in mice, 11 in sea urchin, 10 in *Caenorhabditis elegans*, and 9 in *Drosophila* (for reviews, see Gutierrez-Hartmann, Duval, & Bradford, 2007; Hollenhorst, McIntosh, & Graves, 2011; Hsu, Trojanowska, & Watson, 2004; Seth & Watson, 2005; Turner & Watson, 2008; Watson, Turner, Scheiber, Findlay, & Watson, 2010 and references therein). The human ETS factors are classified into 12 subgroups based upon ETS domain sequence homology: ETS, ERG, PEA3, ETV, TCF, GABP, ELF1, SPI1, TEL, ERF, SPDEF, and ESE (Hollenhorst, McIntosh, et al., 2011; Seth & Watson, 2005; Watson et al., 2010; see Table 1.1 for subgroup members). In addition, a subset of four ETS family genes (ELF3, ELF5, EHF, SPDEF) has been placed in a unique subgroup based upon their restricted expression to tissues with high epithelial cell content (Feldman, Sementchenko, & Watson, 2003). In this review, the Unigene Names will be used (alternative nomenclatures are provided in Table 1.1).

1.2. ETS protein domains and DNA-binding specificity

The DNA-binding (ETS) and pointed (PNT) domains are the two most common domains present in ETS proteins and will be discussed briefly below. Other domains present in smaller subsets of ETS proteins have been described in previous reviews, and these include the OST GABPA and B-box (TCF subfamily; ELK1, ELK3, ELK4) domains (Hollenhorst, McIntosh, et al., 2011).

The Ets domain is an ~85-amino acid region that forms the winged helix-turn-helix (wHTH) DNA-binding domain composed of three alpha helices and a four-stranded antiparallel beta sheet that recognizes a core GGAA/T sequence (ETS binding site, EBS). The HTH motif is formed by helices H2 and H3. The third alpha helix makes major groove contacts with the DNA (GGAA/T core). Two invariant arginine residues present in helix H3 make contact with the two guanine residues of the EBS. Interestingly, crystallography data indicate that there are no direct contacts outside of the GGAA/T core. The DNA recognition sequence preference for several family members has been determined by *in vitro* selection

of randomized oligonucleotides and indicates that target site recognition is dependent on sequences flanking the core motif, suggesting that DNA conformation may contribute to specificity for the flanking regions. A recent comprehensive genome-wide analysis of ETS factor binding specificities was conducted for 26 mouse and 27 human ETS genes using transcription factor DNA-binding specificity and protein-binding microarrays (Wei et al., 2010). These data support the model that ETS family DNA-binding specificities fall into four distinct classes, and identify key DNA-contact amino acids that contribute to class specificity based upon the published crystal structures for ETS1, GABPA, ELK1, ELF3, SPI1, and SPDEF. Class I contains 12 family members (ETS, ERG, PEA3, TCF, and ERF subfamilies) and is defined by an ACCGGAAGT consensus. Class II is composed of eight members (ELF, TEL, and ESE subfamilies) and the binding consensus differs in the first nucleotide, with a CCCGGAAGT sequence preference. Class III contains the three members of the SPI1 family, which bind to sites with an adenine-rich sequence 5′ to the core. Class IV contains a single family member, SPDEF, which has a GGAT core sequence rather than the GGAA. It is evident that ETS proteins often interact with EBS sequences that do not conform to the consensus binding site defined by *in vitro* selection experiments. Binding of ETS proteins to such subconsensus sequences is facilitated by the binding of other transacting factors to cis-elements in proximity to the EBS. Indeed, binding is often mediated by synergistic interaction with transcriptional partners on composite DNA elements. The most studied ETS synergistic interactions include those with AP1 (fos/jun), SRF, RUNX (AML), SP1, PAX5, and GATA1 (discussed further below).

DNA binding by multiple ETS factors is inhibited by two regions that flank the DNA-binding domain. Best exemplified by ETS1, this autoinhibition is stabilized by posttranslational modification (serine phosphorylation) on the region encoded by exon VII. Interestingly, exon VII undergoes alternative splicing, resulting in an isoform that binds DNA with higher affinity (Fisher et al., 1994). The alterations at the 3′ end of v-ets are functionally critical to the transforming properties of the virus, since the residues encoded by the 3′ region of c-ets have been shown to be capable of repressing the DNA-binding potential of c-ets; thus, the viral oncoprotein does not undergo autoinhibition and has higher DNA-binding activity. ETS1 autoinhibition is also reduced by interaction with transcriptional cofactors, such as RUNX1 and PAX5.

The second conserved domain found in a subset of ETS genes is the pointed (PNT) domain. This 65–85 amino acid domain belongs to the sterile alpha motif (SAM) family and is found in 11 of 28 human ETS genes and has been shown to function in protein–protein interaction and homo- and heterooligomerization. The PNT domains of several ETS factors also provide the docking site for regulation of extracellular signaling pathways. For example, ERK phosphorylation of ETS1 and ETS2 on threonine phosphor-acceptor sites increases their resultant transcriptional activity through enhanced interaction with the histone acetylase CBP/p300 (Foulds, Nelson, Blaszczak, & Graves, 2004).

In summary, the DNA consensus sequences determined for the different ETS proteins are very similar, and thus specificity is dependent on other factors including interaction with other nuclear factors. Such a dependence of lower affinity ETS binding sequences on

coexpression and binding of cofactors would be anticipated to provide greater biological specificity.

2. MODULATION OF ETS FUNCTION

ETS functional activity is modulated at multiple levels. As noted above, ETS factors are dependent on interaction with other factors for precise transcriptional regulation. Indeed, maximal transcriptional activation of multiple target genes is dependent on simultaneous expression of ETS and other transcription factors. Second, specific intracellular signaling pathways and posttranslational modifications directly affect the activity of several ETS proteins by regulating subcellular compartmentalization, DNA-binding activity, and transactivation potential or stability.

2.1. Regulation by protein–protein interactions

Transcriptional regulation is dependent upon the combinatorial interactions between multiple nuclear proteins. ETS proteins form complexes with many transcription factors and such interactions may strengthen the transcriptional activity and/or define target gene specificity. Tissue-specific combination of ETS with other cofactors also provides a mechanism for proper regulation of relevant target genes in a particular cell type. Many transcription factors have their DNA-binding sites adjacent to EBS (for reviews, see Li, Pei, & Watson, 2000; Verger & Duterque-Coquillaud, 2002). As mentioned above, well-studied ETS interactions with transcriptional cofactors include those with AP1 (fos/jun), SRF, RUNX (AML), PAX5, SP1, and GATA1. Depending on the precise sequence context, binding of an ETS protein near other transcription factors results in higher affinity interaction, synergistic activation, and/or repression of specific target genes.

Among the earliest characterized protein–protein interactions was that between ETS factors and the AP1 transcriptional complex. Interaction was shown to result in synergistic transcriptional activation of promoters containing composite AP1-EBS binding sites, including MMP1, uPA, GM-CSF, maspin, and TIMP-1. In contrast, MafB, an AP1-like protein, inhibits ETS1-mediated transactivation of the AP1-EBS sites (Sieweke, Tekotte, Frampton, & Graf, 1996). ETS/AP1-binding sequences are proto-typical RAS-responsive elements and oncogenic ETS factors (ETV1, ETV4, ETV5, and ERG) have been shown to activate a RAS/MAPK transcriptional program in prostate cells in the absence of MAPK activation (Hollenhorst, Ferris, et al., 2011).

Another well-characterized interaction involves SRF and ELK1 (or ELK3, ELK4, FLI1, EWS-FLI1) that together form a ternary complex with the SRE motif present in several genes, including c-fos, Egr-1, pip92 Mcl-1, and SRF(Buchwalter, Gross, & Wasylyk, 2004).

RUNX1 and ETS1 interaction counteracts autoinhibition of DNA-binding activity (Garvie, Pufall, Graves, & Wolberger, 2002) and homotypic ETS1 interaction enhances binding to palindromic EBS (Baillat, Begue, Stehelin, & Aumercier, 2002). Interaction with PAX5 allows ETS1, as well as other family members, to bind to a nonconsensus EBS present in the early B-cell-specific mb-1 promoter (Fitzsimmons, Lutz, Wheat, Chamberlin, & Hagman, 2001).

SPI1 family proteins can function as activators or repressors of transcription and have been shown to interact with ETS factors with cell- and promoter-specific consequences. For example, functional interaction of FLI1 with SP1 or SP3 is essential for the inhibitory function of Fli1 on the collagen A2 promoter (Czuwara-Ladykowska, Shirasaki, Jackers, Watson, & Trojanowska, 2001).

FLI1 and GATA-1 act synergistically to activate gene transcription of multiple megakaryocytic genes, including *gpIIb*, *gpVI*, *gpIX*, *gpIb*, and *c-mpl* (reviewed in Szalai, LaRue, & Watson, 2006). We and others have demonstrated that FLI1 and GATA1 co-occupy these promoters *in vivo* (Jackers, Szalai, Moussa, & Watson, 2004; Moussa et al., 2010; Pang et al., 2006).

Several proteins that modulate ETS function have been identified, including Daxx (EAP1 (*E*TS1-associated protein 1)), EAPII, and SP100 (Li, Pei, Watson, & Papas, 2000; Pei et al., 2003; Yordy et al., 2004). The notion that loss of corepressor protein expression is relevant to cancer was demonstrated using the NCoR corepressor protein and the coregulators SRC-1 and AIB1, all of which interact with both ETS1 and ETS2 (Myers et al., 2005). The strongest clinical association in breast cancer was for NCoR downregulation in more aggressive hormone-unresponsive tumors (Myers et al., 2005).

2.2. Regulation by posttranslational modification

A common feature of many tumors is deregulation of signal transduction pathways, resulting in constitutive and often ligand-independent activation. As end effectors of these pathways, ETS factor function is significantly altered in cancer. In addition to being downstream of many RTKs (e.g., HER2/neu), ETS factors regulate the expression of multiple receptors, including HER2/neu, M-CSF receptor, MET, c-kit, and VEGF receptor (Sementchenko & Watson, 2000).

ETS factor functions are controlled by phosphorylation, acetylation, sumoylation, ubiquitinylation, and glycosylation (for reviews, see Charlot, Dubois-Pot, Serchov, Tourrette, & Wasylyk, 2010; Tootle & Rebay, 2005; Yordy & Muise-Helmericks, 2000).

One of the best-studied posttranslational modifications is phosphorylation. Phosphorylation of ETS proteins mediates effects on DNA binding, protein–protein interaction, transcriptional activation, and subcellular localization. ERK, JNK, and p38 MAP kinases are downstream components of signaling cascades. ERKs are activated in response to mitogenic signals, while JNKs and p38/SAPKs respond to stress signals. Specific ETS factors, including ETS1, ETS2, ELK1, ELK3, ELK4, GABPA, SPIB, ETV1, ETV4, and ETV5, can be phosphorylated by MAPKs, resulting in increased transcriptional activation (Charlot et al., 2010).

As noted above, phosphorylation of a mitogen-activated protein kinase (ERK) site adjacent to the PNT domain has been shown to positively regulate transcriptional activities of ETS1 and ETS2. Although MAP kinase phosphorylation of ETS1 does not affect DNA binding, calcium-induced phosphorylation of ETS1 occurs at serine residues present adjacent to the DNA-binding domain and inhibits ETS1 DNA-binding activity without affecting nuclear

localization. ETS1 and ETS2 activity may also be activated by PKC in invasive breast cancer cells (Lindemann, Braig, Ballschmieter, et al., 2003; Lindemann, Braig, Hauser, Nordheim, & Dittmer, 2003). In contrast, ETV6 activity is negatively regulated by MAPK phosphorylation, which results in its nuclear export and decreased DNA-binding activity. Processes that are reversibly controlled by protein phosphorylation require a balance between protein kinase and protein phosphatase activities. Thus, it is important to assess whether specific protein phosphatases are associated with de-phosphorylation of ETS proteins.

Often associated with phosphorylation, acetylation also regulates ETS gene function. Acetylation of ETV1 enhances its DNA-binding activity and ability to transcriptionally activate target genes (Goel & Janknecht, 2003). In response to TGFβ signaling, ETS1 is acetylated and dissociated from the CBP/p300 complexes (Czuwara-Ladykowska, Sementchenko, Watson, & Trojanowska, 2002). FLI1 activity is repressed through a series of sequential posttranslational modifications (Thr312 phosphorylation and acetylation by p300/CREB binding protein-associated factor), resulting in detachment from target gene (e.g., collagen) promoters in response to TGFβ (Asano et al., 2009; Asano & Trojanowska, 2009).

ETS factors undergo ubiquitination and subsequent proteosomal degradation. ETV1, ETV4, and ETV5 each contain three potential binding motifs for the ubiquitin ligase COP1. ETV1 is degraded after being ubiquitinated by COP1. Data support the notion that COP1 functions as a tumor suppressor mediated by its negative regulation of ETV1, ETV4, and ETV5. Indeed, COP1 deficiency in mouse prostate is correlated with elevated ETV1 and increased cell proliferation, hyperplasia, and early prostate intraepithelial neoplasia (Vitari et al., 2011).

Sumoylation has been shown to affect the stability, activity, and localization of its targets. SUMO modification has been found to alter the function of several transcription factors, including ETS family members. For example, ELK1 is modified by SUMO, and this modification is reversed by ERK–MAP kinase pathway activation. Mechanistically, it has been shown that sumoylation of ELK1 facilitates recruitment of histone deacetylase 2 activity to promoters. This recruitment leads to decreased histone acetylation and altered chromatin structure, resulting in transcriptional repression at ELK1 target genes (Yang & Sharrocks, 2004). In contrast, sumoylation within the pointed domain of ETV6 inhibits ETV6-mediated repression (Chakrabarti & Nucifora, 1999; Chakrabarti et al., 1999), associated with sequestering to subnuclear compartments. Mutation of SUMO acceptor site(s) results in increased transcriptional repression, presumably because of decreased nuclear export (Wood, Irvin, Nucifora, Luce, & Hiebert, 2003). Sumoylation of ETS1, ETV4, and ETV5 leads to reduced transcriptional activity.

Future studies will help elucidate the functional impact of specific post-translational modifications on the activity of ETS transcription factors. As specific antibodies are developed, it will be possible to determine the temporal relationships between specific posttranslational events. Through such analyses, it will also be possible to determine whether specific events work cooperatively or antagonistically.

3. DEFINING AND CHARACTERIZING ETS TARGET GENES

3.1. ETS target genes

The importance of the ETS family of transcription factors in various biological and pathological processes necessitates the identification of downstream cellular target genes of specific ETS proteins. Although some overlap in the biological function of different ETS proteins may exist, the emergence of a family of closely related transcription factors suggests that individual ETS members may have evolved unique roles, manifested through the control of specific target genes. Several key areas are critical for understanding what defines a functionally important ETS target gene: First, the functional importance of the EBS must be demonstrated by mutagenesis. Second, the specific ETS factor or factors responsible for transcriptional control of specific target genes need to be identified. While extensive publications have identified functionally important EBS and thus, ETS target genes (Sementchenko & Watson, 2000), fewer investigations have identified definitive target genes for a specific ETS factor.

ETS factors are known to act as positive or negative regulators of the expression of genes, including those that control response to various signaling cascades, cellular proliferation, differentiation, hematopoiesis, apoptosis, adhesion, migration, invasion and metastasis, tissue remodeling, ECM composition, and angiogenesis (Fig. 1.2). Our earlier literature survey enabled identification of over 200 ETS target genes (Sementchenko & Watson, 2000) and to date, over 700 ETS target genes have been defined, based upon the presence of functional EBS in their regulatory regions (Watson, D.K., unpublished). While most ETS factors were initially characterized as transcriptional activators or repressors, it has become evident that several ETS factors can function as either activators or repressors, depending upon the type of promoter and cellular context.

During cancer progression, the oncogenic and tumor-suppressor activities of ETS factors are likely coordinated by their target genes. In the past few years, we and others have made significant strides in identifying and validating these target genes. Collectively, ETS genes have been shown to regulate the expression of genes that have important roles in malignant and metastatic processes (Fig. 1.2). Among these are those that function in control of cell proliferation (e.g., cyclins and cdks), motility (hepatocyte growth factor, HGF), invasion (uPA & uPAR, PAI, MMPs; TIMPs), extravasation (MMPs, Integrins), micro-metastasis (Osteopontin; BSP and Osteonectin), and establishment and maintenance of distant site metastasis and angiogenesis (Neovascularization and Neoangiogenesis (integrin β3, VEGF, Flt-1/KDR, Tie2; Sementchenko & Watson, 2000)). Aberrant expression of ETS factors results in the altered regulation of their target response genes. For example, upregulated ETS target genes include extracellular matrix (ECM)-degrading proteins (e.g., MMP1, MMP9, uPA), which are associated with clinical features such as lymph node status and prognosis in prostate cancer. Significantly, altered ETS expression also provides a mechanism for the downregulation of response genes that include uPA and survivin. Recent analysis of gene expression signatures allowed correlation between expression of ETS factors, ETS target genes, and prostate cancer progression (Tomlins et al., 2007).

Several studies have demonstrated that a polymorphism that generates a functional EBS within the MMP1 promoter is a negative prognostic indicator (Benbow, Tower, Wyatt, Buttice, & Brinckerhoff, 2002).

Functional studies have demonstrated that SPDEF is a negative regulator of uPA and SLUG mRNA expression. Chromatin immunoprecipitation (ChIP, discussed further below) allows definition of direct target genes for specific ETS factors. However, ChIP alone does not indicate whether the interaction is functional (e.g., causing transcriptional activation or repression). Biological rescue experiments have been used to demonstrate the importance of specific target genes (e.g., SPDEF target genes, uPA, SLUG; Findlay et al., 2011; Turner, Findlay, Kirven, Moussa, & Watson, 2008). Correlation between ChIP and gene expression can further define functional ETS targets. For example, there is an inverse correlation between SPDEF and uPA in primary colon tumors (Moussa et al., 2009).

3.2. ETS gene coexpression

Initial expression analysis supported the notion that while some ETS factors showed rather ubiquitous expression (e.g., ETS2), others had more restricted expression in specific tissues or cells (e.g., ETS1). Subsequent studies have demonstrated the simultaneous expression of 14–25 ETS mRNAs in many human tissues and cell lines. For example, studies examining ETS factor expression profiles in normal and cancerous breast cells have demonstrated that a combination of up to 25 of 28 ETS family members examined is expressed at any one time in these cells (Galang, Muller, Foos, Oshima, & Hauser, 2004; Hollenhorst, Jones, & Graves, 2004). It should be noted that mRNA expression alone does not adequately define the ETS profile, as factors including, but not limited to, alternative splicing, mRNA translation, protein stability, posttranslational modifications, and protein localization ultimately contribute to define the level of functional proteins in a cell. Complete proteomic studies need to be performed to define the relative prevalence of ETS factors in specific tissues. ETS factor function is also highly dependent upon the presence and level of specific coregulatory proteins.

3.3. Whole genome analysis: Redundant and specific binding

Multiple ETS factors bind to the same regulatory sites present on target genes, suggesting redundant or competitive functions. Furthermore, additional events contribute to, or may be necessary for, target gene regulation. As technologies have advanced, it has become possible to identify the true regulatory targets of transcription factors. ChIP has become an established method for the analysis of protein–DNA (gene regulatory elements) interactions *in vivo*. Sequential ChIP is an extension of the ChIP protocol, in which the immunoprecipitated chromatin is subjected to sequential immunoprecipitations with antibodies of different specificity. This provides a method of examining co-occupancy of defined promoters by multiple regulatory proteins. Furthermore, sequential ChIP provides an experimental approach to simultaneously evaluate promoter occupancy and transcriptional status (e.g., histone H3 acetylation, phosphorylated RNAPII-CTD; Jackers et al., 2004). However, ChIP and sequential ChIP methods have been restricted to the analysis of small promoter regions, the boundaries defined by the sequences of the primers designed for the PCR amplification step.

To determine the more global location of *in vivo* promoter binding sites of a specific protein, ChIP protocols have been combined with whole genome analysis methods to produce “ChIP-on-chip” microarrays. ChIP products are amplified and hybridized to arrays consisting of promoter regions, limiting genome coverage. ChIP sequencing (ChIP-seq) is the next generation protocol for defining Protein–DNA transcriptomes (Farnham, 2009; Schmidt et al., 2009; Visel et al., 2009). It combines ChIP with new high throughput sequencing platforms, such as Genome Analyzer (Solexa/Illumina), generating significantly more informative data (Mardis, 2007).

In the context of an ETS transcription network, ChIP-Seq analysis can potentially identify the full transcriptome for each individual ETS family member in any given scenario. Furthermore, by comparing ChIP-Seq data with mRNA expression profiles obtained following modulation of ETS expression, direct and indirect targets for each ETS factor can be ascertained. Recent genome-wide analyses of ETS-factor occupancy have identified genomic regions, both promoters and enhancers, bound by individual ETS proteins in living cells. Nine ETS proteins have been assayed for genome-wide occupancy by either promoter microarrays (ETS1, GABPA, ELF1, ELK1, EWS-FLI1, and SPI1) or high throughput sequencing (GABPA, ETS1, ERG, FLI1, EWS-ERG, EWS-FLI1, SPI1, SPDEF, ETV1, and ELF1; Hollenhorst, McIntosh, et al., 2011).

Genome-wide occupancy data for several ETS proteins have been compared and found to have a high degree of similarity. Genomic targets of ETS transcription factors can be divided into two classes (Hollenhorst et al., 2009; Hollenhorst, McIntosh, et al., 2011; Hollenhorst, Shah, Hopkins, & Graves, 2007). Class 1: redundant binding sites found in the proximal promoters of housekeeping genes. Binding sites in this class are characterized by the consensus ETS sequence (CCGGAAGT) and have the potential to bind any ETS protein with relatively high affinity. DNA regions occupied by multiple ETS proteins are frequently found a short distance (~20–40 bp) upstream of transcription start sites. Class 2: specific binding sites that are found more often in enhancer regions associated with genes that mediate the specific biological functions of an ETS family member. Specific target sites are characterized by a lower-affinity ETS sequence and are sometimes flanked by binding sites for other transcription factors. Many predicted ETS sites are not occupied *in vivo* and conversely, many actual sites of genomic occupancy are not predicted.

4. ETS AND MicroRNA

MicroRNAs (miRNAs) are both upstream modulators and downstream effectors of ETS transcriptional factors. miRNAs are 19–25-nucleotide RNAs that have emerged as a novel class of small, evolutionarily conserved gene regulatory molecules involved in many critical developmental and cellular functions (Wiemer, 2007). miRNAs base-pair with target mRNA sequences primarily in their 3′ untranslated region. Through specific base pairing, miRNAs induce mRNA degradation, translational repression, or both depending upon the complementarity of the miRNA to its mRNA target. Each miRNA can target numerous mRNAs, often in combination with other miRNAs, therefore controlling complex regulatory networks. It is estimated that there are ~1000 miRNAs in mammalian cells, and that approximately one third of all genes are regulated by miRNAs (Rajewsky, 2006; Shilo, Roy,

Khanna, & Sen, 2007). Over 3000 identified mature miRNAs exist in species ranging from plants to humans, suggesting that miRNAs are ancient players in gene regulation (Wang & Li, 2007). Their existence and conservation throughout species support the concept that they perform critical functions in gene regulation (Wang, Stricker, Gou, & Liu, 2007). Indeed, the conserved evolution of both miRNAs and transcription factors highlights their importance in and the complexity of gene regulation (Chen & Rajewsky, 2006). In fact, one of the most widely studied miRNAs is miR-34, which has been shown to be positively and negatively regulated by the transcription factors p53 and myc, respectively (Bui & Mendell, 2010; He, He, Lowe, & Hannon, 2007).

4.1. miRNAs targeting ETS factors

A summary of the published studies that identified miR-ETS interactions is provided in Table 1.2. The majority of the studies examining miRNA-regulated ETS factors are for ETS1. miRNA 125b has been shown to be dysregulated in cancer and can act as either a tumor suppressor or oncogene, depending on cellular context. This is true for many miRNAs and adds to the complexity of targeting miRNAs therapeutically. However, in invasive breast cancer, miR-125b is downregulated and predicts poor patient survival (Zhang, Yan, et al., 2011). miR-125b expression inhibits tumor growth *in vivo* and has ETS1 as one of its novel direct targets. Although ETS1 protein levels were decreased by miR-125b, ETS1 mRNA levels were unchanged, suggesting a translational repression mechanism of regulation. Like miR-125b, ETS1 overexpression in invasive breast cancer predicts poor patient prognosis. Another study in hepatocellular carcinoma (HCC) identified ETS1 as a direct target of miR-193b (Xu et al., 2010). miR-193b expression inhibits tumor growth *in vivo* and a negative correlation between miR-193b and ETS1 mRNA levels was defined in HCC tissue samples, suggesting that miR-193b regulation of ETS1 results in mRNA degradation as opposed to translation repression; however, this was not validated *in vitro*. Other studies that identified ETS1 as a direct target of miRs included roles in osteoblast differentiation, inflammation, migration, angiogenesis, and megakaryopoiesis (Table 1.2). Two miRNAs, miR-204 and miR-510, were defined as direct negative regulators of SPDEF by translational repression in breast and prostate cancer (Findlay et al., 2008; Turner et al., 2011). miR-204 and miR-510 expressions are both elevated in tumor samples compared to matched normal in breast cancer and explained the apparent discordance in the literature of reports of SPDEF being elevated or downregulated in breast cancer due to the fact that SPDEF mRNA levels can be elevated in the absence of protein. miR-145 was shown to directly target FLI1 in colon cancer and in pericytes, where it was shown to block migration in response to growth factor gradients (Larsson et al., 2009; Zhang, Guo, et al., 2011). This interaction was also observed in patients with the 5q syndrome, a subtype of myelodysplastic syndrome (MDS), in which the inhibition of FLI1 by miR-145 decreases the production of megakaryocytic cells relative to erythroid cells contributing to the phenotype of the human malignancy (Kumar et al., 2011). Finally, an elegant study by the Ostrowski group showed a link between miR-320 and ETS2 in the stromal fibroblasts (Bronisz et al., 2012). Although many studies have investigated a role for miRNAs in the epithelial tumor cells, very few have focused on their regulation in the stromal compartment. This study showed that miR-320 is a critical target of PTEN in stromal fibroblasts and directly controls ETS2 expression and instructs the tumor microenvironment to suppress many of the aggressive phenotypes

associated with advanced stages of breast cancer, including tumor cell invasiveness and increased angiogenic networks.

4.2. ETS factors targeting miRNAs

Only a handful of studies are published on the role of ETS factor modulated miRNAs (Table 1.3); therefore, we expect many studies in the future in this underdeveloped area of research. In ovarian cancer, a study reported that EGFR signaling leads to transcriptional repression of miR-125a through the ETS family transcription factor ETV4 (Cowden Dahl et al., 2009). It is known that overexpression of EGFR in ovarian cancer correlates with poor disease outcome and induces epithelial–mesenchymal transition (EMT) in ovarian cancer cells (Cowden Dahl et al., 2008; Nicholson, Gee, & Harper, 2001). Overexpression of miR-125a induced a conversion of highly invasive ovarian cancer cells from a mesenchymal to an epithelial morphology, suggesting that miR-125a is a negative regulator of EMT. A study to distinguish serous ovarian cancer from normal ovarian tissue using miRNA profiling identified miR-125a as downregulated (Nam et al., 2008). This correlates well with the previous study; however, miR-21 was also identified as part of the signature in this study and was reported as being upregulated. This is interesting because miR-21 was also shown to be repressed by ETV4 in colon cancer (Kern et al., 2012); therefore, this exemplifies the importance of context when studying miRNAs. Some miRNAs are regulated by the same ETS factor as illustrated by miR-126 in endothelial cells (Harris et al., 2010). miR-126 is abundantly expressed in endothelial cells, and promoter analysis showed that multiple ETS factors led to increased expression, but ETS1 and ETS2 were the most robust.

Some studies have also focused on the role of ETS fusion proteins on miRNAs. In particular, one study performed a genome-wide analysis of miRNAs affected by RNAi-mediated silencing of EWS-FLI1 in Ewing's sarcoma cell lines and identified miR-145 as the top repressed miRNA (Ban et al., 2011), which is interesting as previously mentioned miR-145 is a negative regulator of FLI1 suggesting a possible feedback loop mechanism of regulation of this fusion gene.

5. ETS MOUSE KNOCKOUT AND MUTANT MODELS

5.1. Phenotypes of mice with genetically altered Ets

To date, 23 of the 27 murine Ets genes have been genetically altered (knockouts or mutant mice; Table 1.4). Diverse biological roles of individual ETS family members are supported by the wide range of phenotypes displayed in these models. Most of these models have specific phenotypes, with the exception of Elf1 and Elk1, demonstrating nonredundant functions for the majority of Ets factors. For these, subtle phenotypes have (Elk1, minor defects in neuronal gene activation, and, Elf1, reduced NK-T cell development and function) been identified. Complete or significant embryonic and/or postnatal lethality is observed for 11 family members. Consistent with their tissue expression profiles, the majority have phenotypes that demonstrate their important functions in hematopoiesis, either exclusively or in combination with other lineage defects. There is often a wide range of phenotypes observed even within an Ets subfamily. For example, in the Spi1 subfamily, phenotypes range from Spi1, a principal regulator of myelolymphopoiesis, to SpiB, which regulates

the proper function of terminally differentiated lineages and SpiC that is necessary for the function of a subset of macrophages. Ets1 and Elf4 are important regulators of T cell (T, NK, and NKT) development. Ets family members such as Fli1, Etv2, and Etv6 display functions in hematopoiesis and/or vasculo/angiogenesis. Nonhematopoietic defects were observed for Ets2 which has phenotypes related to extraembryonic development. Etv1, Etv4, and Fev each have defective neurogenesis, Etv4 and Etv5 affect male fertility. Consistent with their restricted epithelial-specific expression, the Ese and Spdef subfamilies (Elf3, Ehf, and Spdef) show tissue-specific (e.g., intestine, mammary gland) phenotypes, albeit at significantly different severities.

These constitutive knockout models reveal only the earliest/most distinct functions of each of these Ets family members. A better understanding of the roles and hierarchies of Ets family members in cellular differentiation and function will come with the generation of new null alleles in untargeted family members, double knockouts, ES cell differentiation and chimera rescue experiments, and tissue-specific inducible knockouts.

Analyses of ES cell differentiation and chimeric and mutant mice were used to evaluate postembryonic phenotypes observed in the constitutive Fli1 knockout mice. These studies demonstrated that Fli1 also plays an important role in multiple non-megakaryocytic hematopoietic lineages, including erythroid, granulocyte, monocyte, and lymphocyte lineages (Zhang et al., 2008). Mutant mice lacking one of two regulatory domains (Fli1ΔCTA) provide novel evidence for the importance of Fli1 in megakaryocytic differentiation and platelet function. These approaches have also established Fli1 as an important regulator of fibroblast functions (Asano et al., 2009; Asano & Trojanowska, 2009; Kubo et al., 2003).

Conditional knockouts have further allowed definition of additional phenotypes. Fli1 conditional knockout mice in combination with Tie2-Cre have shown that mice with reduced endothelial Fli1 expression have compromised vessel integrity, markedly increased vessel permeability, and impaired pericyte/vascular smooth muscle cells coverage (Asano, Stawski, et al., 2010). Conditional Fli1 deletion in the adult results in mild thrombocytopenia associated with a maturation defect of bone marrow megakaryocytes (Starck et al., 2010), as previously observed in fetal liver of constitutive Fli1 knockout mice. In addition to the decrease in megakaryocytic cells, analysis of these mice revealed increases in natural killer (NK) cells and erythrocytic cells and a decrease in granulocytic cells, in agreement with the studies with chimeric Fli1 mice.

Fewer studies have examined the phenotypes of mice with deletions or mutations of two members. Phenotypes support similar roles for ETS subgroup (Ets1 and Ets2) in endothelial cells (Wei et al., 2009), ERG subgroup (Erg and Fli1) in hematopoietic cells (Kruse et al., 2009), TCF subfamily (Elk1 and Elk4) in thymocyte development (Costello et al., 2010), SPI1 subfamily (Spi1 and SpiB) in B-cell function (Garrett-Sinha et al., 1999), and PEA3 subfamily (Etv1 and Etv5) in limb-bud development (Zhang, Verheyden, Hassell, & Sun, 2009).

5.2. Identification of ETS target genes using genetic models

One important experimental approach for identifying Ets targets is the creation of null (knockout) or mutant mice lacking the function of a single or multiple family members. Analysis of these mice provides another means for the identification of genes whose expression or repression is dependent upon an Ets family member. Specific *in vivo* targets for Ets genes have been identified based on the knockout mice (Table 1.4). For example, *c-mpl* (Kawada et al., 2001) and Tie2 (Hart et al., 2000) have reduced expression in knockout Fli1 mice, consistent with the megakaryocytic lineage and vascular defects observed.

Mutant mice lacking one of two regulatory domains (Fli1ΔCTA) are thrombocytopenic and show significantly reduced expression of multiple megakaryocytic genes, including *c-mpl*, *platelet glycoprotein IIb (gpIIb)*, *gpIX*, and *gpV*. These mice also show reduced expression of genes associated with terminal differentiation of megakaryocytes to platelets. As noted above, Fli1 and GATA1 synergistically regulate gene transcription of multiple megakaryocytic genes. Transient-transfection studies indicate that only wild-type (WT) Fli1 can synergize with GATA-1, increasing promoter activity. Consistent with the failure of Fli1ΔCTA and GATA-1 to synergistically activate the *c-mpl* promoter-luciferase reporters *in vitro*, ChIP studies demonstrate that Fli1ΔCTA is not able to efficiently recruit GATA-1 to specific (*c-mpl*, PF4, and *gpIX*) promoters *in vivo* and further define these as Fli1 direct target genes (Moussa et al., 2010).

Fli1 has been shown to repress collagen synthesis in cultured dermal fibroblasts and mouse embryo fibroblasts and Fli1 mutant mice show significant upregulation of fibrillar collagen mRNA and altered expression of matrix-related genes, including decorin, fibromodulin, lumican, procollagen lysine, 2-oxoglutarate 5-dioxgenase 2 (PLOD2), and lysyl oxidase (Asano et al., 2009; Kubo et al., 2003).

Conditional Fli1 knockout mice with Tie2-Cre endothelial cell-specific disruption support the notion that Fli1 may function in maintenance of vascular homeostasis by directly regulating VE-cadherin, PECAM1, Tie2, MMP9, PDGF B, and S1P1 receptor (Asano, Stawski, et al., 2010).

The impact of altered expression of specific Ets response genes can be assessed by performing genetic rescue experiments. This approach is nicely represented by studies of Elf3 knockout mice (Flentjar et al., 2007; Ng et al., 2002). Elf3 knockout mice show significant embryonic and postnatal lethality, due to aberrant morphogenesis and terminal differentiation of the small intestine. Elf3 knockout mice express significantly less TGF-βRII protein. To perform a rescue experiment, transgenic mice that express human TGF-βRII specifically in the intestinal epithelium were crossed to the knockout animals. Significantly, the TGF-βRII transgenic Elf3−/− mice displayed normal small intestinal morphology.

6. ETS FACTORS AND CANCER

The hallmark features of a cancer cell consist of uncontrolled proliferation, loss of differentiation, sustained cell division, increased angiogenesis, loss of apoptosis, and a

capacity to migrate and invade to other tissues and organs. All of these processes are driven by transient and/or permanent changes in gene expression profiles conferred through the activation or repression of cancer-associated genes. It is therefore clear that the role of transcriptional gene regulation in cancer progression cannot be understated and many transcription factors including ETS family members have been assigned as candidate oncogenes or tumor repressors. The importance of ETS genes in human carcinogenesis is supported by the observations that during cancer progression, ETS genes acquire point mutations (e.g., SPI1, ETS1), genomic amplification (ETS1, ETS2, ERG), increased (ETS1, ETS2, ERG) or decreased (e.g., SPDEF, EHF) expression, or rearrangements (ETV6, FLI1, ERG; Seth & Watson, 2005), resulting in altered ETS gene expression which disrupts the regulated control of many complex biological processes, promoting cellular proliferation and inhibiting apoptosis, enhancing cell migration, invasiveness, and metastasis as well as angiogenesis (Fig. 1.2).

6.1. ETS expression in cancer

Altered ETS gene expression levels are correlated with tumor progression in human neoplasias, including thyroid, pancreas, liver, prostate, colon, lung, and breast carcinomas and leukemias (Seth & Watson, 2005; Watson et al., 2010). Furthermore, in breast cancer, upregulation of multiple ETS factors, including ETS1 (Buggy et al., 2004), ETS2 (Buggy et al., 2006), ETV4 (Benz et al., 1997; Chang et al., 1997), ETV5 (Yarden & Sliwkowski, 2001), and ETV1 (Bosc, Goueli, & Janknecht, 2001), is associated with poor prognosis and metastasis. In contrast, other ETS factors including SPDEF (Doane et al., 2006; Feldman, Sementchenko, Gayed, Fraig, & Watson, 2003), ELF5 (Zhou et al., 1998), and EHF (Tugores et al., 2001) are downregulated during breast cancer progression within the same context. The impact of multiple ETS factors (e.g., ETS1, ETS2, ETV4, ELF3, SPDEF, ERG) on phenotypes and molecular regulation in cancer cells has been demonstrated through *in vitro* gain-of-function as well as loss-of-function experiments.

In addition to ETS-mediated transcriptional activation of multiple genes associated with cancer progression, analysis of androgen receptor (AR) genomic targets demonstrated an enrichment of ETS transcription factor family members and, more specifically, an interaction between the AR and ETS1 at a subset of the AR promoter targets was found (Massie et al., 2007). These studies support the model that ETS proteins, including ETS1, regulate genes, including androgen response genes, which contribute to prostate cancer progression.

6.2. ETS translocations

Cancer involves many chromosomal aberrations, the most studied being nonrandom chromosomal translocations resulting in recombinant chromosomes. Tumor cell formation results from the translocation associated production of FLI1 chimeric proteins as has been shown for Ewing’s sarcomas (EWS) and related primitive neuroectodermal tumors (PNET; reviewed in Arvand & Denny, 2001). In this instance, chimeric transcripts result from the fusion of the amino terminal region of the EWS gene with the carboxyl terminal DNA-binding domain of the FLI1 gene (Delattre et al., 1992; Zucman et al., 1992). The chimeric fusion protein lacks the putative RNA-binding domain of EWS and one of the

transactivation domains of FLI1. It has also been shown that the EWS-FLI1 fusion is a more potent transcriptional activator than the FLI1 protein. In other Ewing's sarcoma and PNET tumors, translocations fuse the EWS gene to other members of the ETS family, including ERG, ETV1, ETV4, and FEV.

ETV6 was originally identified by its rearrangement in specific cases of chronic myelomonocytic leukemia (CMML) presenting a t(5,12)(q33; p13) chromosomal translocation (Golub, Barker, Lovett, & Gilliland, 1994). ETV6 is rearranged in CMML, acute myelogenous leukemia (AML), acute myeloblastic leukemia (AML-M2), MDS, and acute lymphoblastic leukemia. Either the PNT domain or the ETS domain or both domains of ETV6 have been identified in over 20 different translocations observed in human leukemia and more rarely solid tumors (reviewed in Mavrothalassitis & Ghysdael, 2000). Fusions involving the PNT domain of ETV6 often lead to oligomerization that is necessary for constitutive activation of kinase activity of receptor or protein tyrosine kinases. Fusions that retain the DNA-binding domain of ETV6 are expected to result in aberrant regulation of ETS target genes.

ERG is highly expressed in over 60% of prostate tumor cells relative to benign tissues. A molecular mechanism to account for ERG overexpression in prostate cancer was subsequently provided by the identification of chromosomal rearrangements that result in the fusion between the 5′ end of the androgen-regulated, prostate-specific transmembrane serine protease TMPRSS2 gene to ERG (Soller et al., 2006; Tomlins et al., 2005). Collective studies show that the TMPRSS2-ERG fusion is present in 40–80% of prostate cancers (recently reviewed in Kumar-Sinha, Tomlins, & Chinnaiyan, 2008; Shah & Chinnaiyan, 2009; Shah & Small, 2010). TMPRSS2 gene fusions involving other ETS transcription factors ETV1, ETV4, or ETV5 have been identified in prostate cancer; however, TMPRSS2-ERG fusion and mRNA overexpression accounts for the majority of cases. Possible mechanistic insights are provided by observation that TMPRSS2-ERG fusion activates MYC and abrogates prostate epithelial cell differentiation. An 87-gene signature has been associated with TMPRSS2:ERG fusion tumors. Collective data suggest that the TMPRSS2-ERG fusions define a subset of prostate cancer and specific fusions predict poor prognosis and survival.

6.3. ETS target gene expression and function

Functional studies demonstrate the impact of such altered expression on the regulation of genes associated with proliferation, transformation, migration, invasion, anti-apoptosis, and angiogenesis (Seth & Watson, 2005) and include but are not exclusive to Her2/neu, uPA, MMPs, TIMPs, MET, Bcl2, maspin, and VEGFR (Sementchenko & Watson, 2000; Fig. 1.2).

Alterations in cell cycle control are a critical step in carcinogenesis. Cell cycle arrest at the G_1–S transition by upregulation of the cyclin-dependent kinase inhibitor p21 occurs in response to DNA damage or oncogenic insult. The elevated level of p21 is known to be mediated through p53 and we have demonstrated SPDEF-mediated regulation of p21 expression is associated with inhibition of growth *in vitro* (Feldman, Sementchenko, Gayed, et al., 2003) and *in vivo* (Schaefer et al., 2010). Indeed, SPDEF-mediated inhibition of breast cancer xenograft growth can be reversed by shRNA targeting of p21 (Schaefer et al.,

2010). These observations combined with ChIP demonstrate that p21 is a key direct target of SPDEF used to control cellular growth. The increased expression of the p21-activated kinase (PAK1) has been shown to be correlated with more aggressive breast cancer (Salh, Marotta, Wagey, Sayed, & Pelech, 2002). Furthermore, studies have shown that PAK1 regulates the activity of ELF3 by phosphorylation (Manavathi, Rayala, & Kumar, 2007). This novel finding raises the possibility that using a specific inhibitor to the upstream effector of ELF3 (e.g., PAK1-specific inhibitor CEP-1347) may represent a novel approach for targeting a transcription factor in breast cancer.

Migration and invasion, critical steps in the metastatic process, requires changes in cell-to-cell adhesion as well as cell adhesion to the ECM. Migration and invasion are often associated with EMT and resultant down-regulation of E-cadherin. Invasion is mediated in part by proteolytic degradation of the ECM by MMPs and uPA. Indeed, activation of the uPA system is associated with a poor prognosis in breast cancer. Significantly, we and others have shown that ETS factors are critical regulators of EMT, protease expression, and ECM (discussed further below, microenvironment). For example, studies using breast, prostate, colon, or ovarian cancer cells have demonstrated the antimigratory and antiinvasive properties of SPDEF, by negative regulation of the EMT regulator SLUG and mesenchymal genes, proteases (uPA, MMPs).

6.4. ETS conversion

To date, ETS research has mainly focused on the molecular mechanisms and functions of individual transcription factors and has produced insights into ETS factor function in both normal and cancer cells. In many cells, multiple ETS factors with similar or opposite functions are present simultaneously and the cell's fate may depend ultimately on the balance between the activities of distinct ETS factors.

ETS factor dysregulation disturbs normal cellular homeostasis, increasing cancer growth, invasion, and metastasis. While some ETS factors are lost during cancer progression, others show increased expression: tumor suppressive and oncogenic ETS factors. We hypothesize that the balance of "tumor suppressor" and "oncogenic" ETS factors could be a marker for aggressive cancer. Taken together, accumulating evidence suggests that multiple ETS factors act in concert to positively and negatively regulate the pathways that control progression to metastatic cancer. This indicates a possible ETS conversion mechanism of gene regulation which provides the cell with an integrated mechanism by which to respond to a variety of intra- and extracellular signals efficiently (Hsu et al., 2004; Turner, Findlay, Moussa, & Watson, 2007; Watson et al., 2010). Several Ets factors are deregulated in the development of breast cancers. During cancer progression, the expression of some ETS factors (e.g., ETS1, ETS2, ETV4, ETV5, ELF3) is often increased, while the expression of other ETS factors (SPDEF, EHF) is reduced or lost (Turner, Findlay, et al., 2007; Watson et al., 2010). The ETS conversion model further hypothesizes that the change in expression pattern from what is observed in normal or benign tissues (e.g., SPDEF expression) to that observed in invasive cancer (e.g., elevated ETS1) is necessary for cancer progression to proceed.

Reciprocal ETS regulation of a metastasis-associated gene can be illustrated by the uPA promoter. ETS regulation of uPA has both positive and negative effects on cancer

progression depending on the specific Ets factor expressed. ETS1 is overexpressed in invasive breast and aggressive prostate cancer and associated with increased uPA expression. In noninvasive (ETS1 negative) breast cancer cells, reexpression of ETS1 increases uPA levels leading to more aggressive tumorigenic phenotypes, including increased cell growth, migration, and invasion. In contrast, the expression of another ETS family member, SPDEF is present in noninvasive, but lost in invasive, breast cancer cells. SPDEF reexpression in invasive cells represses endogenous uPA transcription leading to an inhibition of cell migration and invasion and an antimetastatic phenotype (Feldman, Sementchenko, Gayed, et al., 2003; Turner, Moussa, Sauane, Fisher, & Watson, 2007). Significantly, a statistically significant inverse correlation between SPDEF and uPA expression is observed in colon cancer clinical specimens (Moussa et al., 2009). Intriguingly, although several potential EBS are found in the uPA promoter, both ETS1 and SPDEF have been demonstrated to bind at the same consensus EBS *in vivo*.

Many ETS factors (including ETS1, ETS2, ETV4) transcriptionally activate multiple MMPs, most commonly in cooperation with AP1 complexes. In contrast, SPDEF is a repressor of MMP7 (Moussa et al., 2009) and MMP9 (Johnson et al., 2010)

Another example of reciprocal regulation is provided by the maspin promoter. Maspin is a type II tumor-suppressor gene that has been shown to have antimetastatic properties when expressed in invasive breast and prostate cancer cells (Zou et al., 1994). The maspin promoter has been shown to be regulated by SPDEF (Feldman, Sementchenko, Gayed, et al., 2003; Yamada, Tamai, Miyamoto, & Nozaki, 2000). Significantly, this activation appeared to be specific for SPDEF, since neither FLI1 nor ETS1 was able to activate this promoter. Indeed, ETS1 expression inhibited SPDEF-mediated transactivation of the maspin promoter.

6.5. ETS regulatory network

Taken as a whole, this evidence strongly suggests the existence of distinct ETS expression regulatory networks that act in concert to positively or negatively regulate cancer-associated genes. Significantly, each ETS network would result in distinct patterns of target gene expression, the elucidation of which may identify prometastatic and antimetastatic signatures of gene expression that may predict the aggressive behavior of cancer cells. The ETS Regulatory Network is comprised of the ETS factors themselves, their upstream modulators, their coregulatory proteins, and their target genes (Fig. 1.3). Inflammatory cells are recruited by tumors through their secretion of chemokines, cytokines, and growth factors (1). In response, the recruited inflammatory cells and other cells of the microenvironment (e.g., fibroblasts promote tumor proliferation and progression through additional secretion of biologically active molecules). This in turn results in the activation of intracellular signaling cascades via ligand binding at the cell surface of epithelial cells (2). The activated cascades directly or indirectly (through crosstalk) result in the expression and repression of varying combinations of the 28 ETS family members (3). ETS factors can regulate their own expression and/or that of other family members (4). The composition of Ets factors defines the transcriptional regulation of their target genes, many known to be involved in cancer progression (5). The altered expression of these genes has profound consequences on many cancer-related pathways (6).

6.5.1 ETS-mediated anti- and prometastatic signatures—

Gene expression signatures consist of sets of gene profiles that are known to be predictive of a disease state and/or patient response to treatment. The combined statistical analysis of multiple gene sets obtained from independent gene micro-array studies has resulted in an increased number of putative and validated “metastatic signatures” that predict the outcome of disease in cancer. In addition, comparison of gene expression profiles from primary and metastatic tumors in multiple cancer types reveals highly specific signatures that allow discrimination between primary and metastatic tumors. Similarly, by elucidating the expression networks conferred by ETS family members that elicit a prometastatic response (ETS1, etc.) and an antimetastatic response (SPDEF, etc.), improved pro- and antimetastatic signatures may be isolated which predict the aggressive behavior of cancer cells. As such, these new insights may provide a novel view of the ETS gene family as well as a focal point for studying the complex biological control involved in tumor progression.

7. THE ROLE OF ETS FACTORS IN THE MICROENVIRONMENT

The majority of cancer-related deaths are due to tumor progression, whereby cells from the primary tumor migrate, invade, and reestablish at distant metastatic sites (Guarino, Rubino, & Ballabio, 2007; Turner, Moussa, et al., 2007). The progression of solid tumors corresponds with progressive alterations in the tumor microenvironment, suggesting crosstalk between epithelium and stroma. Increasing evidence suggests that these stromal–epithelial interactions play a critical role in regulating tumor growth and progression. However, this aspect of tumorigenesis remains little understood. Previous studies have indicated that members of the ETS transcription factor family are abnormally expressed in both tumor and stromal compartments. This aberrant expression of ETS factors has been associated with cancer progression and frequently correlates with poor prognosis. For example, ETS1 is frequently overexpressed in epithelial, endothelial, and stromal cells in various tumors (Behrens, Rothe, Florin, Wellmann, & Wernert, 2001; Behrens, Rothe, Wellmann, Krischler, & Wernert, 2001; Takai, Miyazaki, Nishida, Nasu, & Miyakawa, 2002; Trojanowska, 2000). Studies have demonstrated that ETS1 is a strong independent predictor of poor prognosis in breast cancer (Myers et al., 2005; Span et al., 2002). Further, drug-resistant breast cancer cells have been shown to overexpress ETS1 (Kars, Iseri, & Gunduz, 2010), suggesting that ETS factors may play a significant role in tumor aggressiveness and contribute to failed therapies. These studies highlight the importance of understanding the mechanisms by which ETS factors function in both the epithelium and microenvironment.

The stromal compartment consists of fibroblasts, endothelial cells, perivascular cells, blood-borne cells, nerves, and intervening ECM. The fibroblasts of the tumor microenvironment, termed carcinoma-associated fibroblasts (CAFs), are thought to promote tumor progression by establishing a reactive tumor stroma, stimulating growth, sustaining angiogenesis, inhibiting the immune response, promoting the malignant phenotype, and promoting invasion and metastasis (Hanahan & Coussens, 2012; Hanahan & Weinberg, 2000, 2011; Karnoub et al., 2007; Orimo & Weinberg, 2006). While these previous studies have identified important functions for CAFs, the factors regulating the functions of these cells are undefined.

Growing evidence suggests that ETS family members are critical regulators of stromal activation. The reactive tumor stroma is characterized by excessive remodeling of the ECM via CAF production of matrix molecules (i.e., collagen-1), matrix-degrading factors (i.e., matrix metalloproteinases, MMPs), and growth factors (i.e., TGFβ). Fli1 has been established as a regulator of fibroblast function (Asano et al., 2009; Kubo et al., 2003; Truong & Ben-David, 2000; Watson et al., 1992). A hallmark of the CAF is the expression of alpha-smooth muscle actin (αSMA) and studies have shown that Fli1 reduction leads to αSMA upregulation (Nakerakanti, Kapanadze, Yamasaki, Markiewicz, & Trojanowska, 2006). Fli1 has also shown to function as a physiological transcription repressor of collagen type I gene *in vivo* (Asano, Bujor, & Trojanowska, 2010; Czuwara-Ladykowska et al., 2001). The absence of Fli1 correlates with elevated collagen synthesis (Kubo et al., 2003), a second hallmark of the activated tumor stroma. Fli1 has also been shown to regulate expression of tenascin-C, an additional ECM protein associated with wound healing and tumor stroma activation (Shirasaki, Makhluf, LeRoy, Watson, & Trojanowska, 1999). At least part of the Ets functions in tumor stromal cells is the regulation of ECM-degrading enzymes including MMPs, uPA, and collagenases, molecules that are crucial for the establishment of a reactive stroma and onset of metastasis (Westermarck, Seth, & Kahari, 1997). ETS1 and FLI1 have been shown to modulate MMP1 expression (Gavrilov, Kenzior, Evans, Calaluce, & Folk, 2001; Nakerakanti et al., 2006). ETS1 and ETS2 have also been implicated in uPA and MMP9 activation (Watabe et al., 1998). A recent study identified novel ETS1 target genes by subtractive hybridization in stromal fibroblasts under bFGF stimulation (Hahne, Fuchs, et al., 2011; Hahne, Okuducu, Fuchs, Florin, & Wernert, 2011). MMP1, MMP3, PAI-1, and collagen Iα2 were confirmed as ETS1 target genes. Several additional targets were identified which may play a role in generation of the activated tumor stroma: cathepsin, a lysosomal proteinase whose elevated expression is associated with several cancers; lumican, a proteoglycan that binds collagen I and II to sequester growth factors in matrix; decorin, a proteoglycan that binds collagen I during matrix assembly and interacts with fibronectin, thrombospondin, epidermal growth factor receptor, and TGFβ to affect their functions; gremlin, a secreted antagonist of BMPs that promotes cancer cell survival and proliferation and is overexpressed in stroma of many cancers; HSP-90, a heat shock protein that acts to stabilize various growth factor receptors, is required for the induction of VEGF and nitric oxide synthase, and assists MMP2 to promote invasion/metastasis. While these studies did not experimentally demonstrate effects of ETS1 on the promoters of potential ETS1 target genes identified, promoter analysis showed the presence of potential EBS in the promoter regions of each gene identified.

ETS factors have also been shown to directly regulate the expression of cytokines as well as the response to specific growth factors and chemokines (Turner et al., 2008; Turner, Moussa, et al., 2007; Turner & Watson, 2008). For example, ETS1 is a downstream effector of the stroma-derived EMT-promoting HGF and an activator of its receptor, c-Met, thereby regulating a positive feedback loop whereby HGF/c-Met affects both tumor stroma and tumor cells (Hsu et al., 2004). HGF has also been shown to induce MMP1 protein expression in cultured human dermal fibroblasts. Studies showed that the balance of ETS1 and FLI1 binding to the EBS in the MMP1 promoter regulated the effects of HGF, with ETS1 binding leading to upregulation of MMP1 and FLI1 antagonizing this expression

(Jinnin, Ihn, Mimura, et al., 2005). The activities of FLI1 and ETS1 toward the expression of Tenascin-C and connective tissue growth factor (CTGF/CCN2), novel Ets target genes (Jinnin et al., 2006; Nakerakanti et al., 2006; Shirasaki et al., 1999), are modulated by acetylation in a TGFβ-dependent manner (Asano, Czuwara, & Trojanowska, 2007; Asano et al., 2009; Asano & Trojanowska, 2009). These data suggest that ETS1 and FLI1 are the effectors of the TGFβ signaling pathway through novel, previously undescribed regulatory mechanisms. Elevated ETS1 has also been shown to be an antagonist of TGF-β functions in stromal cells (Czuwara-Ladykowska et al., 2002). Significantly, ETS1 and FLI1 are targets of the TGF-β signaling pathway, the primary regulator of fibroblast maturation, activation, and function. HGF-activated ETS1 has also been shown to regulate CXCL12/CXCR4-dependent promotion of tumor cell chemoinvasion (Maroni, Bendinelli, Matteucci, & Desiderio, 2007).

Several *in vivo* studies have demonstrated a correlation between stromal expression of ETS factors, dysregulation of matrix factors, and tumor progression. For example, stromal upregulation of ETS1, MMP1, and MMP9 has been observed in invasive ductal and lobular breast cancers (Behrens, Rothe, Wellmann, et al., 2001) and in invasive HNPCC and sporadic colon cancer (Behrens et al., 2003). Stromal cell expression of a specific ETS target gene (MMP9) has been shown to play a critical role in angiogenesis and growth of ovarian tumors in mice (Huang et al., 2002). Together, these studies suggest that targeting Ets factors in cells of the microenvironment may be an effective antitumor therapy. To demonstrate this potential, specific inactivation of Ets2 in the CAF population in a Pten murine mammary tumor model led to a reduction in tumor size (Li, Wallace, & Ostrowski, 2010). The absence of Ets2 in fibroblasts led to decreased epithelial cell proliferation and delayed tumor progress, illustrating the ability of ETS factors to regulate crosstalk between epithelial and stromal compartments and the importance of targeting this interaction.

In addition to their role in the fibroblastic and ECM components of tumor stroma, the altered expression of several ETS factors has been suggested to regulate angiogenesis, another key step in tumor progression and metastasis. FLI1 is normally expressed in vascular cells including hematopoietic cells, perivascular cells, and endothelial cells (Jinnin, Ihn, Yamane, et al., 2005; Kubo et al., 2003; Lelievre, Lionneton, & Soncin, 2001; Lelievre, Lionneton, Soncin, & Vandenbunder, 2001; Liu, Walmsley, Rodaway, & Patient, 2008; Pimanda et al., 2007; Spyropoulos et al., 2000; Truong & Ben-David, 2000; Watson et al., 1992). Loss of Fli1 results in embryonic lethality due in part to the absence of megakaryocytes, aberrant vasculogenesis, and disruption of tissue integrity (Kawada et al., 2001; Spyropoulos et al., 2000). FLI1 expression is reduced or lost in stromal cells in epithelial tumors, suggesting that this loss of FLI1 could have a direct effect on tumor vasculogenesis. Stromal-derived VEGF can induce ETS1 expression in endothelial cells (Lavenburg, Ivey, Hsu, & Muise-Helmericks, 2003) and activated transcription of VEGFR2/Flt-1 in concert with HIF-2α (Elvert et al., 2003). In addition, expression of ERG and FLI1 has been correlated with Tie2 gene expression, which is involved in the formation and remodeling of normal vascular networks (Mattot, Vercamer, Soncin, Fafeur, & Vandenbunder, 1999).

Evolving data indicate that Fli1 plays an important role in multiple hematopoietic lineages, including erythroid, granulocyte, monocyte, and lymphocyte lineages (Hart et al., 2000;

Kawada et al., 2001; Masuya et al., 2005; Nowling, Fulton, Chike-Harris, & Gilkeson, 2008; Spyropoulos et al., 2000; Zhang et al., 2008). Tumor-associated macrophages (TAMs), a cell of monocyte origin, have been implicated in tumor progression by mediating angiogenesis, invasion, and immunosuppression (Sica et al., 2008). Studies have demonstrated that ETS2 is an important downstream mediator of CSF1-R (colony stimulating factor-1, a growth factor that regulates macrophage survival, proliferation, and differentiation) signaling in TAMs. Macrophage-specific ablation of Ets2 in the *PyMT* tumor model resulted in significant decrease in mammary tumor metastasis to lung (Lin, Nguyen, Russell, & Pollard, 2001; Zabuawala et al., 2010). Gene expression profiling studies have demonstrated that Ets2 target genes are not only tumor specific, but compartment specific between CAFs and TAMs (reviewed in Li et al., 2010). These studies reinforce the idea that cellular context defines the direction and magnitude of response to ETS factors.

8. ETS FACTORS AND OTHER DISEASES

While less attention has been directed toward the elucidation of the roles for ETS transcription factors in diseases other than cancer, clear roles for ETS factors in autoimmune diseases have been defined; these and some other diseases will be briefly discussed below.

Transgenic mice overexpressing Fli1 develop a lupus-like disease (Zhang et al., 1995). It was also previously demonstrated that FLI1 is overexpressed in peripheral blood lymphocytes of systemic lupus erythematosis (SLE) patients compared to normal healthy controls and that NZB/NZW mice, a murine lupus model, have higher Fli1 mRNA expression in splenic lymphocytes than normal control mice (Georgiou et al., 1996). When Fli1 heterozygous mice were crossed with MRL/*lpr* mice, another model of SLE, Fli-1+/− MRL/*lpr* mice had significantly decreased serum levels of total IgG and anti-dsDNA antibodies as disease progressed. In addition, these mice had significantly increased splenic CD8+ and naive T cells and markedly decreased proteinuria and significantly lower pathologic renal scores compared to Fli1+/+ MRL/*lpr* mice. At 48 weeks of age, survival was significantly increased in the Fli1+/− MRL/*lpr* mice as 100% were alive, in contrast to only 27% of Fli1+/+ mice. Both *in vivo* and *in vitro* production of MCP-1 were significantly decreased in Fli1+/− MRL/*lpr* mice (Zhang et al., 2004). Similar findings were obtained in NZM2410 mice (derived from NZB X NZW F1 hybrids), where 93% of Fli1+/− NZM2410 mice survived to the age of 52 weeks compared to only 35% of WT NZM2410 mice (Mathenia et al., 2010).The primary endothelial cells isolated from the kidneys of Fli1+/− NZM2410 mice produced significantly less MCP-1. ChIP analysis demonstrated that Fli1 directly binds to the promoter of the MCP-1 gene. These data indicate that Fli1 impacts glomerulonephritis development by regulating expression of inflammatory chemokine MCP-1 and inflammatory cell infiltration in the kidneys. Together, these findings indicate that FLI1 expression is important in lupus-like disease development. The length of a GA microsatellite in the FLI1 promoter has been shown to be inversely correlated to promoter activity and is associated with SLE patients without nephritis (Morris et al., 2010). Recent genome-wide association studies have identified genetic variants of ETS1 associated with SLE (Yang et al., 2010) and Ets1 knockout mice develop lupus-like disease (high IgM and IgG autoantibodies, glomerulonephritis, and local complement activation; Wang, John, et al., 2005). It has been recently suggested that some of these phenotypes could be related

to ETS1 functions, including negative regulation of Th17 and B-cell differentiation (Pan, Leng, Tao, Li, & Ye, 2011).

Systemic sclerosis (SSc) or scleroderma is an autoimmune inflammatory disease characterized by fibrosis of the skin and internal organs as well as microvessel injury. The importance of reduced FLI1 expression in the pathogenesis of SSc has been reviewed recently (Asano, Bujor, et al., 2010). Although FLI1 expression in dermal fibroblasts is relatively low, studies have shown that FLI1 plays a critical role in the regulation of ECM genes, including type I collagen (Jinnin, Ihn, Yamane, et al., 2005) and the multifunctional matricellular factor CTGF/CCN2 (Nakerakanti et al., 2006). Importantly, FLI1 has been shown to be a potent inhibitor of collagen biosynthesis in dermal fibroblasts and its aberrant expression has been implicated in the pathogenesis of cutaneous fibrosis in SSc (Kubo et al., 2003; Wang, Fan, & Kahaleh, 2006). Interestingly, MCP-1 (regulated by FLI1 in SLE) also has been shown to play a role in SSc fibrosis (Artlett, 2010). In humans, FLI1 is expressed in the healthy skin microvasculature; however, its presence is greatly reduced in endothelial and periendothelial cells in SSc skin (Kubo et al., 2003). Conditional deletion of Fli1 in the endothelium of mice results in vascular defects observed in SSc vasculature (Asano, Stawski, et al., 2010).

Jacobsen syndrome (11q-) is a rare chromosomal disorder caused by deletions in distal 11q. Individuals have thrombocytopenia with a subpopulation of cells having enlarged α-granules. In addition to platelet effects, Jacobsen syndrome patients also present with a wide spectrum of the most common congenital heart defects, including an unprecedented high frequency of hypoplastic left heart syndrome (HLHS). Both of these conditions are associated with deletions on the long arm of chromosome 11, including 11q23, where ETS1 and FLI1 are located. Thus, these patients have only one copy of these ETS genes due to a heterozygous loss of regions in Chromosome 11. FLI1 monoallelic expression combined with its hemizygous loss underlies Jacobsen thrombocytopenia (Raslova et al., 2004). Significantly, overexpression of FLI1 in patient CD34(+) cells restores the megakaryopoiesis *in vitro*, indicating that FLI1 hemizygous deletion contributes to the hematopoietic defects (Raslova et al., 2004). Ets1 is expressed in the endocardium and neural crest during early mouse heart development. Ets1 knockout mice show large membranous ventricular septal defects and a bifid cardiac apex, and less frequently a nonapex-forming left ventricle (one of the hallmarks of HLHS). These results implicate an important role for ETS1 in mammalian heart development and some of the most common forms of congenital heart disease (Ye et al., 2010).

The functional polymorphism in the MMP1 promoter affecting ETS binding may contribute to the pathogenesis of osteomyelitis by increased MMP1 expression (Montes et al., 2010) as well as higher disease severity in recessive dystrophic epidermolysis bullosa (Titeux et al., 2008). It has also been hypothesized that the ETS2 activation of Bcl-xL may protect glia from constitutive oxidative stress that is believed to be a key mechanism for amyotropic lateral sclerosis, an adult-onset neurodegenerative disease (Lee, Kannagi, Ferrante, Kowall, & Ryu, 2009).

Phenotypes of several of the knockout, mutant, and transgenic mice support the notion that ETS factors have roles in several diseases. For example, the importance of Fli1 and Erg in megakaryopoiesis would support a possible role for these ETS factors in other diseases affecting megakaryopoiesis (thrombocytopenia, megakaryocytopenia) or other conditions associated with thrombocytopenia (e.g., chronic liver disease, acquired immunodeficiency syndrome). Many ETS factors would be expected to have a critical role in other hematopoietic, vascular, and respiratory (e.g., asthma, cystic fibrosis, chronic obstructive pulmonary disease) diseases. ETS1 has been shown to be a mediator of inflammation and neointima formation in a model of carotid artery balloon injury (endoluminal vascular injury; Feng et al., 2010). Spdef is required for differentiation of pulmonary goblet cell and regulates genes associated with mucus production, supporting the model that Spdef plays a critical role in regulating a transcriptional network mediating the goblet cell differentiation and mucus hyperproduction associated with chronic pulmonary disorders (Park et al., 2007).

9. TARGETING THE ETS NETWORK

9.1. Therapeutic targeting of ETS transcription factors

Targeting transcription factors for therapeutic gain is the focus of intense research as being able to manipulate transcriptional expression patterns would provide a novel approach for the treatment of many human diseases. The primary limitations to targeting transcription factors are the potential for off-target effects and insufficient delivery within the cell. Overwhelming evidence suggests that the number of transcription factors whose aberrant function supports tumorigenesis is limited (Darnell, 2002). Additionally, this limited number of transcription factors function at critical focal points controlling many of the genes involved in cancer-associated processes. Therefore, targeting transcription factors has great potential for therapeutic gain.

9.2. Targeting ETS factor biology

ETS factor family members are associated with the positive and negative regulation of gene expression profiles affecting all the classic hallmarks of cancer, including sustaining proliferative signaling, evading growth suppressors, resisting apoptosis, replicative immortality, activated angiogenesis, and induced invasion and metastasis (Turner & Watson, 2008). Critically, an ever growing body of evidence demonstrates many genetic and epigenetic alterations of ETS transcription factor function and activity in cancer. ETS factors, therefore, provide potential targets for cancer therapy. Pharmacological intervention may be used to inhibit the altered expression of oncogenic ETS factors such as ETS1, ETS2, ELF3, and/or activate the expression of tumor-suppressive members such as SPDEF and ETV6 (Turner & Watson, 2008). Several of the multifaceted aspects of ETS factor biology have been explored in order to assess the potential of therapeutically targeting these proteins. Strategies have included: directly inhibiting the promoter of oncogenic ETS factors (Carbone, McGuffie, Collier, & Catapano, 2003; Carbone et al., 2004; Miwa et al., 2005; Sahin et al., 2005) or directly targeting specific ETS factor mRNA (Dohjima, Lee, Li, Ohno, & Rossi, 2003; Hu-Lieskovan, Heidel, Bartlett, Davis, & Triche, 2005; Kitange et al., 1999; Tomlins et al., 2007; Wernert et al., 1999) to prevent the expression of their target genes; directly targeting the ETS protein itself (Turner & Watson, 2008)

or indirectly targeting ETS responsive promoters of transcriptional target genes (Hewett et al., 2006; Pourtier-Manzanedo et al., 2003; Sementchenko, Schweinfest, Papas, & Watson, 1998; summarized in Table 1.5; Fig. 1.4; and in Turner & Watson, 2008).

9.3. Therapeutic drugs to target ETS factor regulation

A growing body of evidence shows that many of the drugs being tested for cancer treatment alter the activity of ETS factors and provide further rationale for developing ETS targeting therapeutics. The polyphenolic flavone Luteolin demonstrates antitumor activity in several types of cancer. In prostate cancer, Luteolin treatment has been show to increase SPDEF expression while decreasing AR expression to lower the levels of prostate-specific antigen (Tsui, Chung, Feng, Chang, & Juang, 2012). Increased SPDEF expression in this model induced BTG2, NDRG1, and Maspin gene expression to inhibit proliferation and induce apoptosis. A recent study demonstrated that the therapeutic blockade of angiotensin II type1 receptor (AGTR1) inhibits CRPC through the inhibition of ETS1 (Kosaka et al., 2010). Knockdown of AGTR1 inhibits cell proliferation and influences AR expression levels in prostate cancer cells. Wang et al. has successfully used a nanoparticle drug delivery system to deliver the natural extract gambogic acid (GA) into pancreatic cancer cells (Wang, Zhang, Chen, Shi, & Chen, 2012). GA is a potent anticancer agent that inhibits cell growth and the ability for motile function in a wide variety of tumor cells. GA-treated cells show significantly decreased expression of ETS1 as well as its downstream target genes cyclin D1, uPA, and VEGF (Wang et al., 2012).

9.4. Translational ETS targeting

While these studies demonstrate the potential of targeting ETS factors for therapeutic gain, translating this success to the bedside has been limited by the challenges of directly targeting transcriptional factors in complex physiological systems. Such challenges include successfully targeting their nuclear localization and the successful design of small-molecule inhibitors given the large surface area of their DNA–protein and protein–protein interactions (Konstantinopoulos & Papavassiliou, 2011). However, the potential benefits of being able to target transcription factors have been demonstrated for the nuclear hormone-receptor family (e.g. estrogen receptor targeting by tamoxifen in breast cancer patients) as they can be targeted on the cell surface before translocation to the nucleus (Jordan, 2003). The notion that transcription factors represent undruggable targets is slowly being eroded. Technological advances in drug delivery systems and drug design provide insight into how we may overcome these challenges (Konstantinopoulos & Papavassiliou, 2011). Recently, a cell-penetrating synthetic peptide has been developed to disrupt the ERG–DNA interaction in prostate cancer. The peptide is a potent inhibitor of ERG *in vitro* and *in vivo* to inhibit DNA damage, cell growth, invasion, and metastasis. Further structure/function studies are being performed to allow further optimization of the peptide (http://www.faqs.org/patents/app/20110207675). The transcriptional activity of ETS factors themselves is regulated by multiple ligands, coregulatory proteins, transcriptional cofactors, and chromatin remodeling components, which determine not only the expression status of a target gene but also the magnitude and duration of activation or repression. The binding sites of several other key transcription factors are found adjacent to ETS binding sites (e.g. ETS/AP-1, ETS/NFκB, ETS/AR) and such composite binding sites mediate the

synergistic activation or repression of target genes (Turner & Watson, 2008). The ETS protein:cofactor binding interface is therefore crucial in the regulation of DNA binding, subcellular localization, target gene specificity, and transcriptional activity. The in-depth knowledge of both the overall structure and functional domains of specific ETS family members, including SPDEF (Y. Wang, Feng, et al., 2005) and ETS1 (G. M. Lee et al., 2005), makes targeting their interactions particularly attractive for drug discovery using small-molecule inhibitors. A series of small-molecule inhibitors have been shown to inhibit the interaction between the ETS factor ELF3 and its coactivator Sur2 (a RAS-linked subunit of the mediator complex). ELF3 with its coactivator Sur2 are required for the high HER2 promoter activation observed in cancer. The ELF3-Sur2 protein interaction is mediated by one face of an eight-amino acid alpha-helical region in the ELF3 activation domain (Shimogawa et al., 2004). Screening of an indole-mimicking π-electron-rich chemical library led to the development of wrenchnolol, a small inhibitor that mimics the alpha-helical region of the ELF3 activation domain (Shimogawa et al., 2004). *In vivo* studies using wrenchnolol in mice have been reported to be promising (Jung, Choi, & Uesugi, 2006). Secreted alkaline phosphatase screening has also identified a fluoroquinophenoxazine derivative that also disrupts the ELF3-Sur2 binding interface. A-62176 treatment arrests the cell cycle in the G1 phase via the downregulation of cyclin D1 and the upregulation of p27Kip1 in NCI-N87 gastric cancer cells (Kim et al., 2012). Disruption of the ELF3-Sur2 binding interface in cancer cells impairs the expression of HER2, inhibits HER2-mediated phosphorylation of MAPK/AKT, and restrains the activity of topoisomerase IIa (Kim et al., 2012).

There is a growing interest in developing miRNAs as therapeutic targets due to their ability to regulate multiple genes and networks of proteins. Multiple companies have been formed to exclusively pursue this research goal. One such company is Mirna Therapeutics, Inc., a biopharmaceutical company focused on miRNA-directed oncology therapies (http://mirnatherapeutics.com). Mirna Therapeutics currently has eight lead compounds in various phases of development with their lead compound, MRX34, an miR-34-mimic, due to be the first miRNA replacement therapy in Phase I clinic trials in 2013. This is an exciting area of research and one hopes to see great advances in the very near future.

9.5. Targeting ETS fusion proteins

ETS fusion proteins offer unique therapeutic targets as they are found only in certain cancer types and not in normal cells. Ewing's sarcoma ETS family fusion, EWS–FLI1, has been targeted using siRNA leading to more than 80% reduction in the EWS-FLI11 transcript and cell growth inhibition (Dohjima et al., 2003). Limitations to the use of siRNA approaches *in vivo* have been addressed by coating siRNA with a cyclodextrin-containing polycation to increase stability and by conjugating transferrin to siRNA to target to transferrin receptor-expressing tumor cells. Such modifications have successfully allowed the systemic delivery of EWS–FLI1 fusion targeting siRNA into a murine model of metastatic Ewing's sarcoma, inhibiting tumor growth with no observed side effects (Hu-Lieskovan et al., 2005). Recent research has used a gene expression approach to identify a 14-gene expression signature for the attenuation of EWS–FLI1 expression in Ewing's sarcoma (Stegmaier et al., 2007). The signature was then used to screen a small-molecule library highly enriched for FDA-

approved drugs and identified cytosine arabinoside as a modulator of EWS–FLI1 protein expression and this compound reduced cell viability, transformation, and tumor growth in a xenograft model.

Oncogenic ETS fusion proteins are also therapeutic targets which have been targeted using small-molecule inhibitors. EWS-FLI1 fusions in Ewing's Sarcoma have been targeted using the compound YK-4-279 which inhibits EWS-FLI1 activity, induces apoptosis in cell lines and slows down tumor growth in mouse xenograft models (Barber-Rotenberg et al., 2012; Rahim et al., 2011). The potential of YK-4-279 has also been assessed against ETS fusions found in prostate cancer. It was found to inhibit the biological activity of both ERG and ETV1 in fusion positive cell lines to decrease migration and invasion (Rahim et al., 2011).

Given the successes in targeting ETS transcription factors for therapeutic gain, an intense examination of possible additional therapeutic approaches to target these factors is warranted.

10. CONCLUDING REMARKS

The ETS family is one of a limited number of fundamentally important gene families (Hsu et al., 2004; Turner, Findlay, et al., 2007; Watson, Ascione, & Papas, 1990; Watson et al., 2010). Although considerable work has been done on individual members of this family, little effort have been made in understanding the interrelationships between the family members and thus, why they exist as a family. Progress is being made toward addressing this fundamental question. We are just beginning to define unique versus redundant ETS functions. This will require understanding which proteins interact with each family member, which signal transduction pathways are ETS family members involved in, target genes of each family member, and the roles of ETS regulatory network in oncogenesis, tumor suppression, cell proliferation/death, and differentiation/development. During normal development, ETS factor expression is tightly controlled to regulate many biological processes including cell proliferation, differentiation, hematopoiesis, apoptosis, metastasis, tissue remodeling, angiogenesis, and transformation. In cancer, aberrant ETS factor expression results in the upregulation of genes known to drive cancer and the downregulation of genes known to suppress cancer. It is becoming increasingly evident that cellular context defines the direction and magnitude of response to ETS factors. In order to advance our understanding of the ETS-dependent regulation of cancer progression and metastasis, future studies should be directed toward elucidation of the effects of simultaneous expression of multiple transcription factors on the transcriptome of nonmetastatic and metastatic cancer. Collectively, we are beginning to define the molecular mechanisms that determine which ETS family member will regulate a particular target gene and are developing appropriate approaches to determine which target genes are necessary for ETS-dependent phenotypes.

In summary, while expression and promoter arrays will allow identification of new cancer-associated target genes that are regulated by ETS transcription factors, concomitant molecular studies will increase our understanding of the mechanisms by which ETS transcription factors act as oncogenes and tumor-suppressor genes. The holy grail of

any therapeutic cancer regime is the reactivation of tumor-suppressor function and/or the inhibition of oncogene activation. Direct or indirect therapeutic intervention of ETS factor function or regulation offers intriguing possibilities in order to achieve this.

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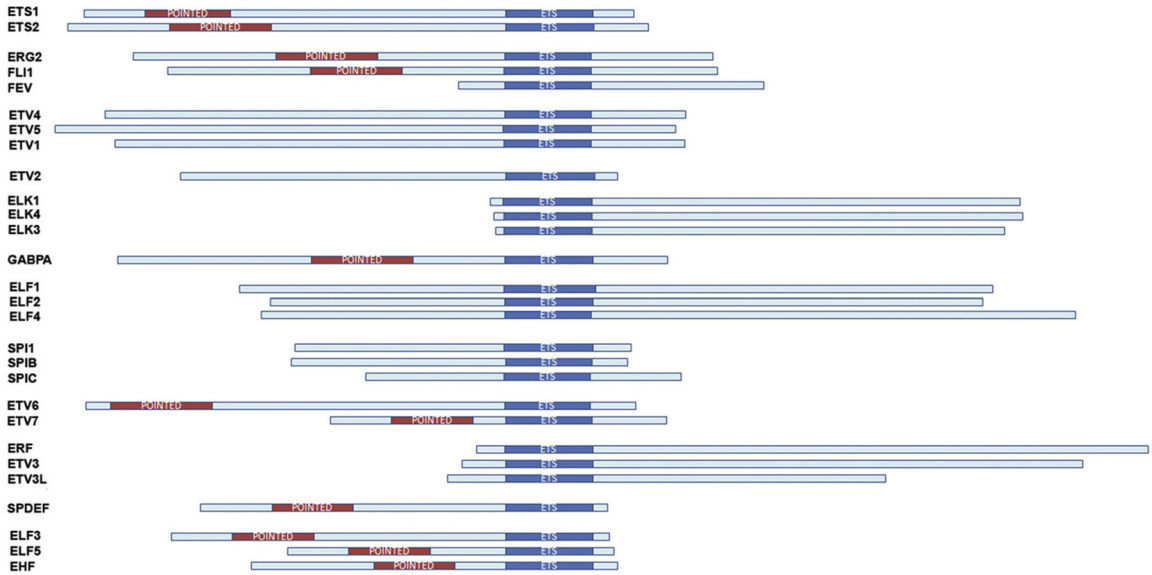

Figure 1.1.

The human ETS family of transcription factors. The main structural organization of each human ETS protein by subfamily (see Table 1.1) is depicted. The ETS and Pointed domains are indicated.

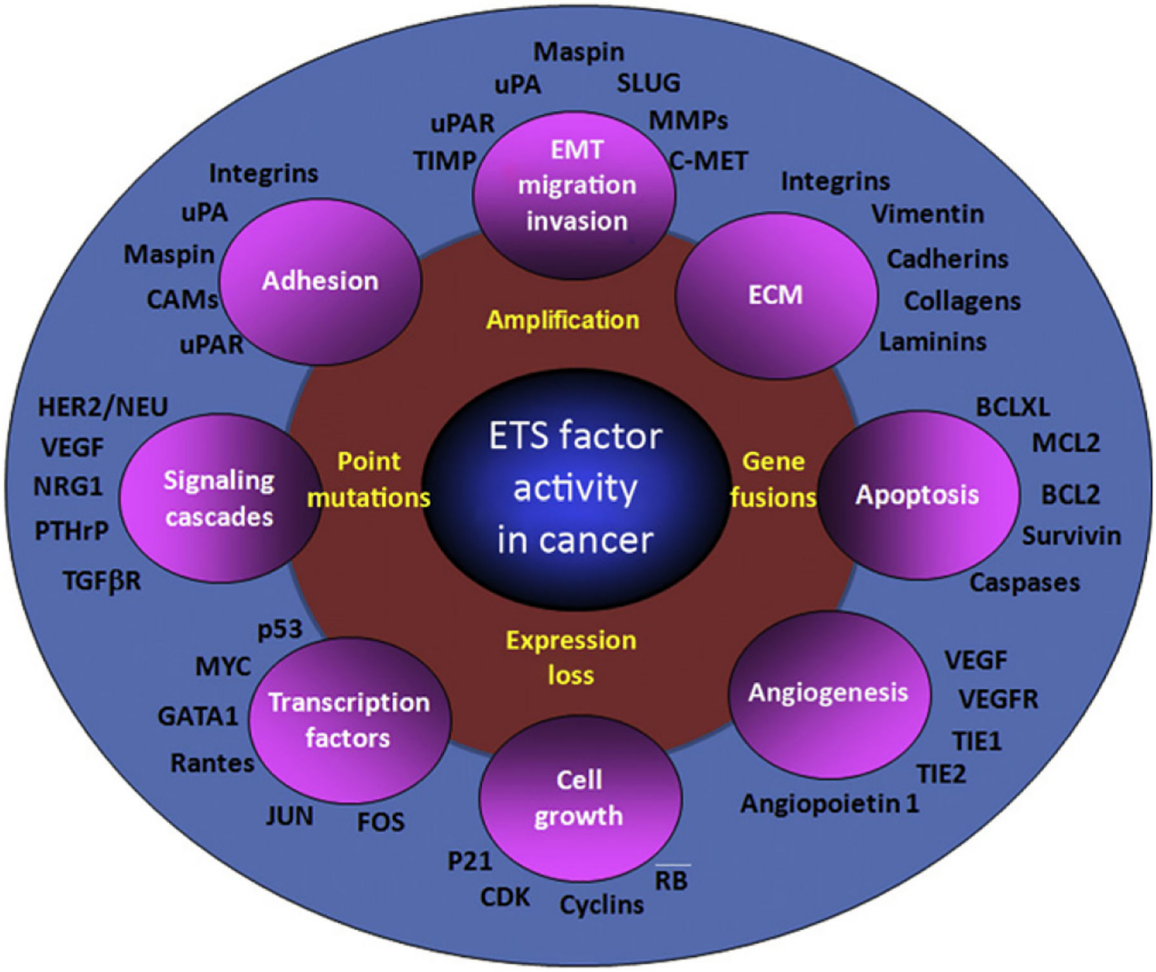

Figure 1.2.

ETS factors regulate the expression of genes associated with cancer progression. Dysregulated ETS factor function leads to the altered expression of multiple target genes that are known to play critical roles in many of the processes required for cancer progression. While each of the target genes highlighted has functional EBS(s) in their regulatory regions, the role and relative affinities of specific ETS factors have only been examined in a limited subset.

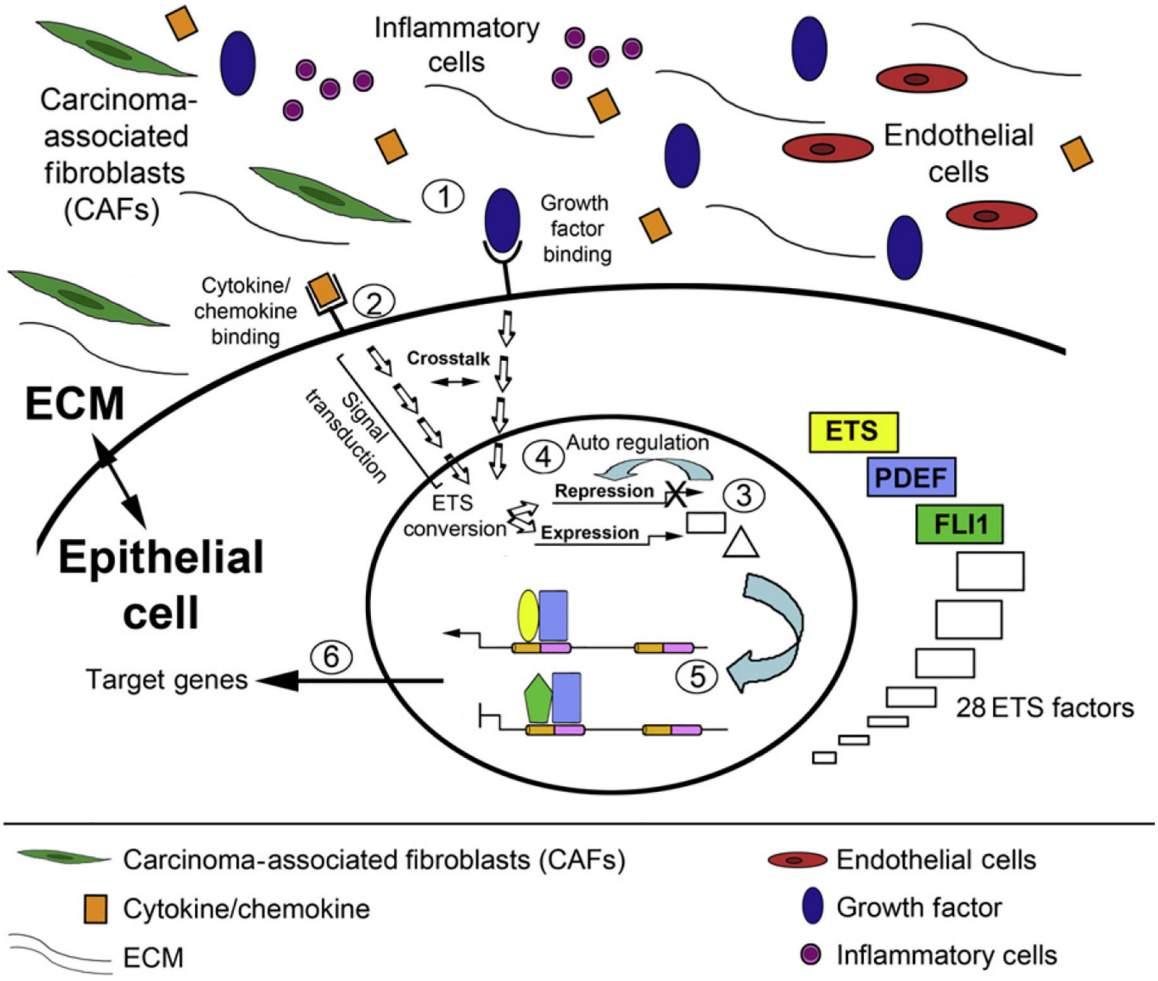

Figure 1.3.
Hypothetical model of the ETS regulatory network in cancer. See text for details.

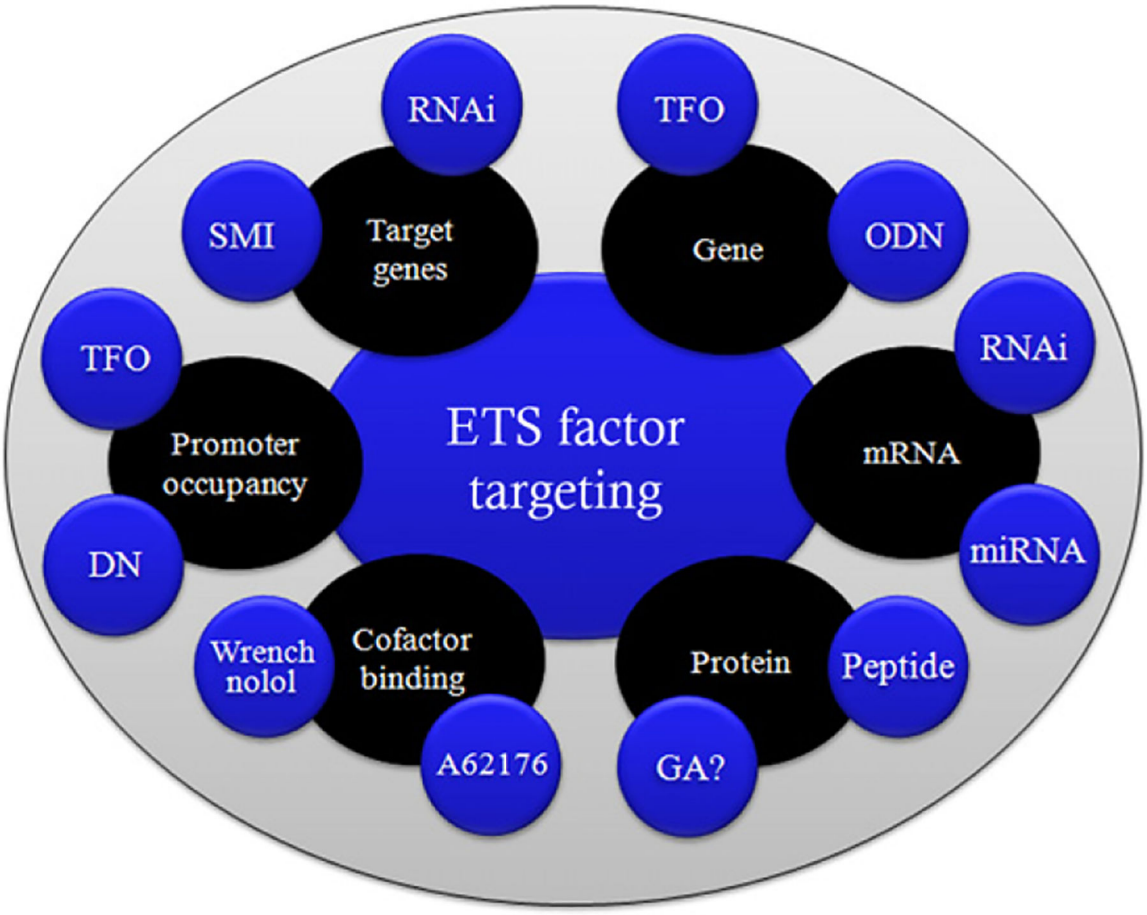

Figure 1.4.

Therapeutic strategies for targeting ETS factor biology. Strategies have included directly inhibiting the promoter of oncogenic ETS factors; directly targeting specific ETS factor mRNA to prevent the expression of their target genes; directly targeting the ETS protein itself or indirectly targeting ETS responsive promoters of transcriptional target genes. See text for details. RNAi, RNA interference; miRNA, microRNA; GA, gambogic acid nanoparticles; ?, direct versus indirect effect; DN, dominant negative; ETS, E26 transforming sequence; ODN, decoy oligonucleotide; SMI, small-molecule inhibitor; TFO, triplex-forming oligonucleotide.

Table 1.1

The human ETS gene family

	Group	Name	Unigene name	Original name	Alternative names	Locus	Size	ETS domain	Pointed domain
1	ETS	ETS1	ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1	EWSR1	11q23.3	441	331–416	54–135
2		ETS2	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)		21q22.3	469	369–443	88–168
3	ERG	ERG2	ERG	v-ets erythroblastosis virus E26 oncogene-like (avian)	erg-3, p55	21q22.3	462	290–375	120–201
4		FLI1	FLI1	Friend leukemia integration 1 transcription factor	ERGB, EWSR2, SIC-1	11q24.1–q24.3	452	277–361	115–196
5		FEV	FEV	Fifth Ewing variant	PET-1, HSRNAFEV	2q23	238	43–126	None
6	PEA3	PEA3	ETV4	Polyoma enhancer binding	E1AF, PEAS3	17q21	462	315–399	None
7		ERM	ETV5	Ets-related molecule		3q28	510	368–449	None
8		ER81	ETV1	Ets variant gene 1		7p21.3	458	314–397	None
9	ETV	ER71	ETV2	Ets variant gene 2	ETSRP71	19q13.12	370	265–350	None
10	TCF	ELK1	ELK1			Xp11.2	428	7–92	None
11		SAP1	ELK4	SRF accessory protein 1A		1q32	431	4–89	None
12		NET	ELK3	Net transcription factor	SAP2, ERP	12q23	407	5–85	None
13	GABP	GABPα	GABPA	GA binding protein transcription factor A	E4TF1, NFT2, NRF2	21q21.3	454	318–400	171–249
14	ELF1	ELF1	ELF1	E74-like factor 1		13q13	619	207–289	None
15		NERF	ELF2	E74-like factor 2	NERF1, NERF2, EU32	4q21	581	198–277	None
16		MEF	ELF4	E74-like factor 4	ELFR	Xq26	663	204–290	None
17	SPI1	SPI1	SPI1	Spleen focus forming virus (SFFV) proviral integration oncogene spi1	PU.1, SFPI1, SPIA	11p11.2	264	168–240	None
18		SPIB	SPIB	SpiB transcription factor		19q13.3–q13.4	262	169–251	None
19		SPIC	SPIC	SpiC transcription factor		12q23.2	248	111–193	None
20	TEL	TEL	ETV6	Ets variant gene 6		12p13	452	340–419	38–119
21		TEL2	ETV7	Ets variant gene 7	TEL-B, TREF	6p21	264	149–228	49–114
22	ERF	ERF	ERF	Ets2 repressor factor		19q13	548	26–106	None
23		ETV3	ETV3	Ets variant gene 3	METS, PE1, bA110J1.4	1q21–q23	512	34–118	None
24		ETV3L	ETV3L	Ets variant gene 3 like		1q23.1	361	38–121	None
25	PDEF	PDEF	SPDEF	SAM pointed domain containing Ets transcription factor	PSE	6p21.3	335	248–332	138–211
26	ESE	ESE1	ELF3	Epithelium-specific Ets transcription factor 1	ESX, JEN, ERT, EPR1	1q32.2	371	275–354	47–132

	Group	Name	Unigene name	Original name	Alternative names	Locus	Size	ETS domain	Pointed domain
27		ESE2	ELF5	Epithelium-specific Ets transcription factor 2		11p13–p12	255	165–243	46–115
28		ESE3	EHF	Epithelium-specific Ets transcription factor 3	ESEJ	11p12	300	209–288	42–112

List of the known human ETS genes (grouped by subfamily), including gene names and alternative nomenclature, chromosomal location, size of protein (amino acids), approximate boundaries of the Ets domain (~85 amino acids) and approximate boundaries of the Pointed domain (65–80 amino acids, if present).

Table 1.2

microRNAs targeting ETS factors

microRNA	ETS factor	Function/disease	References
569	SPI1	Systemic lupus erythematosis	Hikami et al. (2011)
510	SPDEF	Breast and prostate cancer	Findlay et al. (2008) and Turner et al. (2011)
204		Breast cancer	Findlay et al. (2008)
145, 214	ELK1	Smooth muscle cell proliferation	Park et al. (2011)
7	ERF	Lung cancer	Chou et al. (2010)
196a, 196b	ERG	Acute leukemia	Coskun et al. (2011)
145	FLI1	Megakaryocyte and erythroid differentiation	Kumar et al. (2011)
		Colon cancer	Zhang, Guo, et al. (2011)
		Migration of microvascular cells (pericytes)	Larsson et al. (2009)
125b	ETS1	Breast cancer	Zhang, Yan, et al. (2011)
193b		Hepatocellular carcinoma	Xu et al. (2010)
370		Osteoblast differentiation	Itoh, Ando, Tsukamasa, and Akao (2012)
155, 221/222		Inflammation, migration of endothelial cells	Wu et al. (2011) and Zhu et al. (2011)
200b		Angiogenesis	Chun et al. (2011)
208		Preosteoblast differentiation	Itoh, Takeda, and Akao (2010)
155		Megakaryopoiesis	Romania et al. (2008)
320	ETS2	Stroma breast cancer	Bronisz et al. (2012)
221		Endothelial cell motility	Wu et al. (2011)
378	GABPA	Metabolic shift breast cancer	Eichner et al. (2010)

Table 1.3

ETS factors targeting microRNAs

ETS factor	microRNA	Action	Function/disease	References
SPI1	29b	Activation	Neutrophil differentiation (PML)	Batliner et al. (2012)
ETV5	21	Activation	Spermatogonial stem cell self-renewal	Niu et al. (2011)
ETV4	125a	Repression	EMT in ovarian cancer	Cowden Dahl, Dahl, Kruichak, and Hudson (2009)
	21	Repression	Colorectal cancer	Kern et al. (2012)
ELK1	34a	Activation	Oncogene-induced senescence	Christoffersen et al. (2010)
ETS1	126	Activation	Endothelial cells	Harris et al. (2010)
ETS2	126	Activation	Endothelial cells	Harris et al. (2010)
	196b	Repression	Gastric cancer	Liao et al. (2012)
Fusions				
TEL/AML	494, 320a	Repression	Leukemia	Diakos et al. (2010)
EWS/FLI1	30a-5p	Activation	Ewings sarcoma	Franzetti et al. (2012)
	let-7a			De Vito et al. (2011)
	145, 100, 125b	Repression	Ewings sarcoma	Ban et al. (2011)
	22, 221/222			McKinsey et al. (2011)
	27a, 29a, 145			Riggi et al. (2010)

Table 1.4

Mouse Ets gene knockout and mutant mice

Gene name	Severity of mutation	Phenotype	Gene targets identified
Ets1	Viable and fertile with 50% neonatal lethality at 4 weeks	Hematopoietic cell defects. Reduced number of B, T, and NK cells	MDM2; Cyclin G (Xu et al., 2002), Foxp3 (Mouly et al., 2010), T-bet (Nguyen et al., 2012), MMP2, 3, and 13, TIMP1, 2, and 3 (Hahne et al., 2006)
Ets2	Embryonic lethal (<E8.5)	Extraembryonic tissue defect. Rescued neomorph has hair follicle defect	MMP3, MMP9, MMP13 (Yamamoto et al., 1998)
Erg	Embryonic lethal at E11–12.5	Lack of definitive hematopoiesis; defective thrombopoiesis in heterozygotes	Igfr2, MMP3 (Loughran et al., 2008)
Fli1	Knockout is embryonic lethal at E12.5.	Thrombocytopenia, reduced erythroid progenitors, defective vascular genesis, and B-cell maturation	Spyropoulos et al. (2000) and Starck et al. (2010)
Fev	Viable with reduced numbers of homozygous knockouts	Defective development of 5-HT neurons; increased anxiety and aggression	TPH, SERT, AADC (Hendricks et al., 2003)
Etv4	Viable; males infertile	Male sexual defect; lack of dendrite patterning in motor neurons	
Etv5	Viable; males infertile	Defective sperm production	CXCL12, CXCL5, CCL7 (Chen et al., 2005)
Etv1	Viable	Lack of motor coordination, synaptic defect, limb ataxia	
Etv2	Embryonic lethal E8–9.5	Defects in hematopoiesis and vasculogenesis. Lack of endothelial and endocardial lineages.	Tie-2 (Ferdous et al., 2009), SOX9 (DiTacchio et al., 2012)
Elk1	Viable; no phenotype	Phenotypically normal, minor effects on neuronal gene activation	Cesari et al. (2004)
Elk4	Viable	Defects in thymocytes and peripheral T cells	Costello, Nicolas, Watanabe, Rosewell, and Treisman (2004)
Elk3	Hypomorphic mutant dies at birth	Vascular defect leading to respiratory failure	EGR-1 (Ayadi et al., 2001)
Gabpa	Lethal prior to implantation	Mitochondrial defects; T cells	AChRδ and AChRε; AChE; Utrophin (O'Leary et al., 2007); IL7Rα (Yu, Zhao, Jothi, & Xue, 2010)
Elf1	Minor defect	Reduced NK-T cell development and function	(Choi et al., 2011)
Elf4	Viable and fertile	Reduced NK and NK-T cell development and function	Perforin (Lacorazza et al., 2002)
Spi1	Embryonic lethal at E18.5	Lack of hematopoiesis; no B, T cells or monocytes	Colucci et al. (2001)
SpiB	Viable	Defective B-cell responses; reduced intestinal immunity	Schotte, Nagasawa, Weijer, Spits, and Blom (2004), de Lau et al. (2012), and Kanaya et al. (2012)
SpiC	Viable	Defect in red pulp macrophages and red blood cell recycling and iron homeostasis	Vcam1 (Kohyama et al., 2009)
Etv6	Embryonic lethal E11	Lack of embryonic angiogenesis; defective hematopoiesis	Wang et al. (1998) and Ciau-Uitz, Pinheiro, Gupta, Enver, and Patient (2010)
Erf	Embryonic lethal E10.5	Defective placental development	Papadaki et al. (2007)

Gene name	Severity of mutation	Phenotype	Gene targets identified
Spdef	Viable; fertile	Defective mucosal development in the gastrointestinal tract and respiratory tract. Increased gastrointestinal malignancies	Muc6,Tff (Horst et al., 2010)
Elf3	30% Embryonic lethality E11.5	Abnormal morphogenesis and differentiation of intestinal epithelium	TGFβ (Ng et al., 2002)
Elf5	Embryonic lethal E7.5	Embryonic patterning defect; heterozygotes deficient in mammary gland development	WAP, casein (Zhou et al., 2005); Notch pathway (Chakrabarti, Wei, et al., 2012); Snai2 (Chakrabarti, Hwang, et al., 2012)
Ets1 −/−; Ets2A72/A72	Embryonic lethal E11.5–15.5	Endothelial cell defects leading to failure of vascular branching	MMP9, BCL-xL, cIAP2 (Wei et al., 2009)

Table 1.5

Approaches used to investigate the multifaceted aspects of ETS factor biology in order to assess the potential of therapeutically targeting these proteins

Approach	Mode of action	Method	Molecular target	References
ETS promoter occupancy	Competition for binding to promoters	Decoy oligonucleotides	ETS1, ETS2	Miwa et al. (2005)
		Triplex-forming oligonucleotides		Carbone et al. (2003, 2004) and Sahin et al. (2005)
ETS mRNA	Prevention of expression	Antisense oligonucleotides, RNA interference	Multiple ETS factors, EWS-FLI1	Dohjima et al. (2003), Hu-Lieskovan et al. (2005), Kitange et al. (1999), Tomlins et al. (2007), and Wernert et al. (1999)
ETS protein	Functional inhibition	Small-molecule inhibitor	ESE1-SER2, ETS fusions, ERG	Barber-Rotenberg et al. (2012), Rahim et al. (2011), Stegmaier et al. (2007), Turner and Watson (2008), and Patent app # 20110207675
ETS responsive promoters	Prevention of ETS transcriptional regulation	Triplex-forming oligonucleotides, dominant negative	TIE1, FGF2	Hewett et al. (2006), Pourtier-Manzanedo et al. (2003), and Sementchenko et al. (1998)