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ETV1, 4 and 5: An Oncogenic Subfamily of ETS Transcription Factors

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Abstract

The homologous ETV1, ETV4 and ETV5 proteins form the PEA3 subfamily of ETS transcription factors. In Ewing tumors, chromosomal translocations affecting ETV1 or ETV4 are an underlying cause of carcinogenesis. Likewise, chromosomal rearrangements of the ETV1, ETV4 or ETV5 gene occur in prostate tumors and are thought to be one of the major driving forces in the genesis of prostate cancer. In addition, these three ETS proteins are implicated in melanomas, breast and other types of cancer. Complex posttranslational modifications govern the activity of PEA3 factors, which can promote cell proliferation, motility and invasion. Here, we review evidence for a role of ETV1, 4 and 5 as oncoproteins and describe modes of their action. Modulation of their activation or interaction with cofactors as well as inhibiting crucial target gene products may ultimately be exploited to treat various cancers that are dependent on the PEA3 group of ETS transcription factors.

Keywords

Cancer; Chromosomal translocation; ETS; Posttranslational modification; Transcription factor

1. Introduction

The first ETS transcription factor gene was identified three decades ago in the avian E26 erythroblastosis virus, thereby coining the name E-twenty-six or E26 transformation-specific (ETS) gene [1]. Today, we know of 28 ETS genes in humans that have homologs in vertebrates and invertebrates, but are absent in plants, fungi, yeast or bacteria. The defining characteristic of the encoded proteins is the ETS domain, which is composed of ~85 amino acids and binds to DNA sequences with a 5'-GGA(A/T)-3' core [2]. The ETS domain displays three conserved α -helices and a four-stranded antiparallel β -sheet that together form a winged helix-turn-helix motif. Two arginine residues within the third helix make crucial contacts with the two guanine residues of the 5'-GGA(A/T)-3' core binding sequence, while especially the first helix, the β -sheet as well as the turn between the second and third helix are engaged in contacting the DNA backbone within and around the 5'-GGA(A/T)-3' core [3].

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The 28 human ETS proteins are clustered into 12 subgroups. Whereas 25 ETS factors are thereby grouped into nine subfamilies with two or three members, three further ETS proteins are highly divergent, with one of them (ETV2/ER71) even sharing no homology to any other known protein outside its DNA-binding domain [4]. A question still not fully addressed is how these 28 different human ETS proteins specifically regulate gene transcription when they all potentially bind to the same target sequences. One answer is tissue- or cell type-specific expression of ETS proteins. And indeed, in some cell lines and tissues, mRNA from one particular ETS gene is expressed at a much higher level compared to all other ETS mRNAs, suggesting a preponderance of one particular ETS protein. However, in most tissues this is not the case [5]. Another solution to enhance promoter specificity is the ability of ETS proteins to form complexes with transcription factors that bind in close proximity. One example is the ternary complex factor ETS subfamily that only binds to the c-fos promoter in conjunction with the serum response factor [2]. And finally, DNA sequences surrounding the 5'-GGA(A/T)-3' core determine to a certain extent which of the ETS proteins will bind efficiently. Yet, genome-wide studies indicate that there is significant redundancy of ETS protein binding to DNA, suggesting that the relative protein level will often determine which ETS protein binds to a particular site [3]. The nature of the ETS protein(s) bound will influence the transcriptome, as different ETS proteins assemble distinct sets of coactivators or corepressors and thus modulate gene transcription in different ways.

Here, we will focus on the PEA3 subfamily of ETS transcription factors consisting of ETV1, 4 and 5, how these three proteins could contribute to the development of various cancer types and whether they are underlying causes of tumorigenesis.

2. Modular structure of the PEA3 group of transcription factors

The first member of this subfamily cloned was PEA3 (polyomavirus enhancer activator 3), also called E1AF for adenovirus E1A enhancer-binding protein, indicating that cellular PEA3/E1AF can be hijacked by viruses for their replication [6,7]. The rational name for this protein is nowadays ETV4 (ETS variant 4). Two more relatives were identified soon thereafter, ETV1 (also called ER81 for ETS-related 81) and ETV5 (also called ERM for ETS-related molecule) [8,9].

Apart from the DNA-binding ETS motif, the PEA3 family members do not possess any other protein motif present in the Pfam database (<http://pfam.sanger.ac.uk/>). However, they have an acidic domain both at the N- and C-terminus (Fig. 1A), each of which constitutes the core of a transcription activation domain [10,11]. The N-terminal activation domain appears to be more potent than the C-terminal one and sequences flanking both sides of the N-terminal acidic domain of ETV4 inhibit its activity [12]. Likewise, the center of ETV1 exerts a negative effect on both of its activation domains [10].

Systematic analysis of the DNA sequence specificity revealed that the PEA3 family members preferentially bind to 5'-ACCGGAAGT-3', wherein the importance of bases in determining the strength of DNA-binding diminishes with the distance from the 5'-GGAA-3' core [13]. As the activation domains, the ETS domain is regulated in an intramolecular manner: amino acids on both sides of the ETS domain inhibit its DNA-binding ability [11,12,14,15]. Interestingly, antibodies can relieve this intramolecular inhibition of DNA-binding [12]. However, whereas an anti-ETV1 antibody greatly enhanced in vitro binding of ETV1 to the HER2/Neu and Smad7 promoters lacking a 5'-ACCGGAAGT-3' consensus sequence, the same antibody had basically no impact on ETV1 binding to an artificial ETS site containing the 5'-ACCGGAAGT-3' sequence [16,17].

Thus, different modalities of DNA-binding are employed depending on the base composition of the target sequence.

One question arising is if and how the intramolecular inhibition of the ETS and transactivation domains is relieved *in vivo*. At least two mechanisms could account for this: (i) binding of other proteins may change the conformation of PEA3 proteins, or (ii) posttranslational modifications may be responsible. In fact, ETV4 can bind to upstream stimulatory factor 1 (USF-1) and USF-1 is capable of stimulating the DNA-binding ability of ETV4. However, this requires that USF-1 binds to DNA by itself in close proximity to ETV4. Conversely, DNA-binding of ETV4 can be further suppressed by the interaction of its ETS domain with the ID2 helix-loop-helix protein [15].

A second way of modulating DNA-binding is via posttranslational modification. In particular, phosphorylation of S334 in ETV1 and S367 in ETV5 inhibits DNA-binding [18,19]. These serine residues are located at the N-terminal boundary of the ETS domain (Fig. 1B) and targeted by protein kinase A (PKA); notably, human ETV4 lacks a homologous serine residue, suggesting that PKA would not inhibit DNA-binding of ETV4. Interestingly, PKA simultaneously stimulates ETV1/ETV5 transcriptional activity, which may not only entail S334/S367 phosphorylation, but likely also phosphorylation at sites utilized by MAPKAPKs (see subsection 4.2) [18,19]. Thus, PKA may selectively stimulate target genes that contain high affinity sites for ETV1/ETV5, since the PKA-induced reduction in DNA-binding of ETV1/ETV5 at such sites should be negligible. In contrast, genes with low affinity binding sites would no longer be targeted by ETV1/ETV5 after PKA phosphorylation. Conversely, acetylation of ETV1 on K116 by the cofactor p300 enhances DNA-binding [20]. Since ETV5, but not ETV4 possesses a lysine residue homologous to K116 in ETV1 (Fig. 1B), DNA-binding of ETV5 is likely also enhanced upon acetylation by p300. In addition, acetylation on both K33 and K116, which flank the Nterminal acidic domain, increases the transactivation potential of ETV1 [20].

3. Physiological roles of ETV1, ETV4 and ETV5

3.1. Functions in morphogenesis

Analysis of ETV1, ETV4 and ETV5 mRNA levels revealed that their genes are expressed in numerous organs both during embryonic development and in adults [5,21,22]. Although there is often overlap of expression, with ETV5 seemingly more ubiquitously expressed, the pattern of ETV1, 4 and 5 mRNA expression can be drastically different in certain tissues, indicating that these PEA3 family members are not regulated in the same fashion and could therefore perform distinct physiological functions.

One emerging role of PEA3 factors is in branching morphogenesis, where primitive epithelial buds bifurcate to generate arborized ducts or acinar structures. This process also involves the adjacent mesenchyme, which interacts with the epithelial cells in complex ways. For instance, both ETV4 and ETV5 are expressed at the tip of the epithelial ducts during mesonephric differentiation in the mouse embryo, while ETV1 is expressed at low levels in the adjoining mesenchyme. After differentiation of the renal glomeruli, ETV4 and ETV5 expression sharply decreases and only remains at sites of poorly differentiated metanephrogenic cap tissue [23]. These data suggest that especially ETV4 and ETV5 are involved in kidney development and perform similar functions. And indeed, this is supported by the analysis of respective knock-out models: mice with inactivation of all four ETV4/ETV5 alleles failed to develop kidneys, while deletion of three or two alleles resulted in less severe phenotypes and deletion of one allele had no effect [24].

Expression of all three PEA3 genes was also reported in the epithelial buds of mammary glands in mouse embryos [21]. Moreover, in situ hybridization revealed that ETV4 and ETV5, but not ETV1, are expressed in mammary epithelial cells particularly in the terminal end buds at puberty, a time when extensive branching of ducts occurs. However, in situ hybridization failed to detect PEA3 gene expression during the mouse pregnancy-lactation-involution cycle, another time when branching morphogenesis occurs within mammary tissue. Since more sensitive RT-PCR analyses still revealed ETV4 and ETV5 mRNA throughout the pregnancy-lactation-involution cycle [23], it remains to be resolved if this low level of PEA3 gene expression is required for the remodeling of breast tissue during sexual maturity.

Similar to kidney and breast, both ETV4 and ETV5 are robustly expressed in developing limb buds, while ETV1 mRNA is expressed at a much lower level, if at all. Whereas individual knock-out of ETV4 or ETV5 had little to no effect on limb development, joint inactivation of ETV4 and ETV5 caused preaxial polydactyly [25,26]. These results further emphasize that PEA3 family members can substitute for each other if being expressed in the same tissue.

3.2. Role of ETV1 in motor coordination

The above described data suggest that ETV4 and ETV5 often perform similar functions during morphogenesis, whereas ETV1 seems to be different. And indeed, ETV1 knock-out mice display overt phenotypes drastically different from ETV4 and ETV5 knock-outs. ETV1 knock-outs are born, yet die approximately one month after birth. They display limb ataxia and abnormal flexor-extensor posturing, which is a consequence of the reduced direct connection of muscle sensory neurons and spinal motor neurons [27]. In addition, ETV1 knock-out mice have defects in muscle spindle formation, with reduced numbers of spindles in proximal limb muscles and supernumerary muscle spindles in distal hindlimbs [27,28]. Also, ETV1 knock-out mice do not form the Pacinian corpuscle limb mechanoreceptors [29]. Collectively, these data suggest that ETV1 plays pleiotropic roles in motor coordination.

Interestingly, ETV4 knock-out mice, which have a normal life-span in contrast to ETV1 knockouts [30], partially phenocopy the deletion of ETV1. They show not a complete, but only a 25% reduction of Pacinian corpuscle numbers [29]. Also, ETV4 knock-out mice display faulty branching of specific motor neurons in their target muscles, yet it remains unclear whether this leads to any obvious disabled motor coordination [31,32]. Thus, ETV1 and ETV4 may have some overlapping physiological functions in motor coordination, yet the more relevant protein in this regard appears to be ETV1.

3.3. Roles of ETV4 and ETV5 in fertility

ETV4^{-/-} mice display no obvious phenotype except that males fail to reproduce. This is not due to defects in sexual organs or the production of sperms, which can fertilize eggs in vitro, and also not due to abnormal mating behavior. Rather, the absence of copulatory plugs and sperm in the uterus of mating partners suggests erectile or ejaculatory dysfunction [30]. Likewise, ETV5 knock-out mice are viable but show prominent defects in reproductive organs. Males are sterile and display decreased testicular size. This is due to the fact that ETV5 is required for the self-renewal of spermatogonial stem cells [33,34]. Similarly, female ETV5 knock-out mice are infertile with decreased ovulation and absent interest in mating. Treatment with both estrogen and gonadotrophin partially rescued this phenotype, suggesting that ETV5 deficiency leads to reduced responsiveness to these hormones, whose levels were not significantly changed in ETV5^{-/-} female mice. However, two-cell embryos from these hormone-treated ETV5^{-/-} females often failed to develop into blastocysts in

vitro, suggesting that ETV5 also influences the developmental competence of germ cells [35]. Finally, another group produced a differently inactivated ETV5 gene and reported embryonic lethality; the reason for this different outcome remains to be elucidated [24].

4. A matter of debate: PEA3 factors as oncoproteins in breast cancer

4.1. Overexpression of PEA3 factors in human breast tumors

The first indication that PEA3 factors may be involved in tumorigenesis came from the analysis of transgenic mice overexpressing HER2/Neu in the breast, which leads to tumor formation. All three PEA3 factors became upregulated at the mRNA level in respective mammary tumors, suggesting that they might be downstream effectors of the HER2/Neu oncoprotein [36,37]. HER2/Neu is a member of the epidermal growth factor receptor family and overexpressed in 20%–30% of all human breast tumors. Accordingly, antibodies directed against the extracellular portion of HER2/Neu or small molecules inhibiting its intracellular tyrosine kinase activity have entered the clinic as a mainstay adjuvant therapy [38,39]. Of note, ETV4 overexpression positively correlates with HER2/Neu overexpression, tumor grade and higher recurrence in humans [40,–43]. Likewise, ETV1 and ETV5 overexpression was reportedly associated with HER2/Neu overexpression and adverse outcome, respectively [44,45]. However, two other reports state that there is no correlation between ETV4 expression in human breast tumors and HER2/Neu overexpression or adverse clinicopathologic factors [46,47]. Thus, whereas overexpression of PEA3 factors in breast tumors is firmly established, its relationship with HER2/Neu expression and prognostic value needs further validation.

Breast-specific overexpression of a dominant-negative ETV4 molecule consisting of the DNA-binding C-terminus of ETV4 fused to the *Drosophila* engrailed repression domain counteracted HER2/Neu- or Wnt1-induced breast tumorigenesis by delaying the onset of tumor formation and reducing the number of tumors observed in mice [37,48]. Similarly, downregulation of ETV1 in human MDA-MB-231 breast cancer cells reduced proliferation and tumor formation in nude mice [49], and ETV4 or ETV5 ablation suppressed proliferation, migration and tumor formation by MMT-060562 mouse mammary tumor cells [50]. Together with the observed upregulation of PEA3 factors in breast cancer, these data implicate that PEA3 factor overexpression contributes to the causation of mammary tumors.

4.2. Relationship between HER2/Neu and PEA3 factors

Molecular studies suggest a vicious cycle: HER2/Neu activates the transcriptional activity of PEA3 factors [51,52], which in turn bind to the ETV4 and HER2/Neu gene promoters and stimulate their transcription [16,40,53]. This is controversial, since one report posits that ETV4 binds to and thereby represses the HER2/Neu gene promoter [54]. However, this discrepancy may be due to the fact that different cell lines were employed, in which overexpression of ETV4 may replace more active ETS factors from the HER2/Neu promoter or in which ectopic ETV4 may sequester limiting coactivators, both of which would result in apparent promoter repression.

How could HER2/Neu activate PEA3 transcription factors? It is well established that HER2/Neu stimulates the RAS-RAF-MEK-MAPK (mitogen-activated protein kinase) pathway [38] and all three PEA3 factors are activated upon MAPK stimulation [10,55,56]. In case of ETV1, four MAPK phosphorylation sites (S94, T139, T143, S146) were identified that are part of a MAPK recognition sequence, S/TP (see Fig. 1B), and contribute to ETV1 transcriptional activity [52]. In addition, two further phosphorylation sites were mapped in ETV1, S191 and S216, which are phosphorylated by the p90^{RSK} and MSK1 MAPK-activated protein kinase (MAPKAPK) that are themselves phosphorylated and thereby activated by MAPK [19,57]. Again, phosphorylation at these additional MAPKAPK sites is

required for maximal transcriptional activity of ETV1. Interestingly, several of these six MAPK/MAPKAPK sites are conserved in ETV4 and ETV5 (Fig. 1B), suggesting that MAPK/MAPKAPK phosphorylation is also a mechanism by which these two proteins become activated.

Another pathway leading to the stimulation of PEA3 activity via HER2/Neu may involve SRC (steroid receptor coactivator) proteins. These cofactors themselves are stimulated by MAPK and implicated to be oncoproteins [58]. All three SRCs can stimulate ETV1 activity and SRC-3, also called AIB1 for amplified in breast cancer 1, was even shown to bind to ETV1 and ETV4 [59,60]. Moreover, ETV4 expression was positively correlated with SRC-1 and SRC-3 in human mammary tumors [42,60], further suggesting a physiological significance of PEA3 factors in mammary tumorigenesis.

4.3. Action of PEA3 factors in breast tumor cells

Apart from the HER2/Neu gene, PEA3 factors regulate a variety of other genes in breast cells. Most prominently, MMP (matrix metalloproteinase) genes have been shown to be activated by PEA3 factors, including MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), MMP-7 (matrilysin) and MMP-9 (gelatinase B) [52,60–62]. Upregulation of MMPs is involved in tumor cell migration and invasion [63] and accordingly ETV4 overexpression conferred an invasive phenotype on MCF-7 breast cancer cells [64]. Similarly, overexpression of ETV4 or ETV5 in TAC-2.1 mammary epithelial cells not only led to more branching duct-like structures when grown in collagen gels, but also to more invasion into the collagen matrix [23]. Another important target gene that is coordinately upregulated by HER2/Neu and ETV1 is telomerase reverse transcriptase [65,66], the catalytic subunit of telomerase whose overexpression is required for the immortalization of the vast majority of tumor cells [67,68]. Also, PEA3 factors are capable of stimulating the transcription of cyclooxygenase-2 (COX-2) [69–71], which is involved in the synthesis of prostaglandins. Notably, COX-2 expression is higher in HER2/Neu-positive breast tumors and correlates with poor prognosis. Accordingly, pharmacological inhibition of COX-2 may be useful in preventing and treating breast cancer [72]. Collectively, these examples show how dysregulation of transcription upon PEA3 factor overexpression could contribute to breast tumor formation.

However, despite the large body of literature studying PEA3 factors in breast tumorigenesis, it is still unresolved whether PEA3 factor upregulation is a cause or a consequence of breast tumorigenesis. A comprehensive screen of ETS genes in HER2/Neu-induced mouse mammary tumors indicated that not only the PEA3 group becomes upregulated, but also other ETS genes, albeit the PEA3 group shows the highest difference in mRNA molecules per cell between normal and tumor tissue; in addition, most of the ETS genes are expressed in mammary tissue [73]. Accordingly, a dominant-negative ETV4 molecule may not only block PEA3 factors, but also many other ETS proteins in mammary tumors. Thus, it is unclear if the reported [37,48] ability of dominant-negative ETV4 to suppress HER2/Neu- and Wnt1-mediated breast tumorigenesis is due to blocking PEA3 factor activity or other ETS proteins, which can bind to similar sites as PEA3 proteins. Transgenic mouse models are urgently needed to resolve whether PEA3 factor overexpression leads to mammary tumorigenesis. This is not the case for ETV1, since even two-year-old respective transgenic mice did not display any histopathological abnormalities, although MMP-3 and urokinase plasminogen activator transcription were enhanced in the breast [74]. However, this does not exclude that ETV1 is a promoter, rather than an initiator of breast tumorigenesis. For instance, would ETV1 overexpression aggravate HER2/Neu-mediated breast tumorigenesis? Unless such in vivo proof is obtained, a causative role of PEA3 proteins in breast tumor development should not be taken for granted.

5. Chromosomal translocations in Ewing tumors

Ewing's sarcoma is a small round blue-cell tumor that is very aggressive, infrequent with an estimated 200–300 cases annually in the US and mostly affecting children and adolescents. The defining feature of these tumors are chromosomal translocations involving the Ewing's sarcoma (EWS) gene (or in rare instances a homolog of EWS, FUS) and one out of five ETS genes: FLI1, ERG, FEV, ETV1 or ETV4 [75]. In addition to Ewing's sarcomas, these translocations and the resulting EWS-ETS fusion proteins are also found in other small round blue-cell malignancies such as peripheral primitive neuroectodermal and Askin's tumors, which are therefore subsumed together with Ewing's sarcomas into the Ewing family of tumors. The most frequent chromosomal translocation observed (~85%) is the EWS-FLI1 t(11;22)(q24;q12) one, followed by EWS-ERG t(21;22)(q22;q12) translocations in ~10% of all Ewing tumors [76]. In contrast, EWS-FEV t(2;22)(q33;q12), EWS-ETV1 t(7;22)(p22;q12) and EWS-ETV4 t(17;22)(q12;q12) translocations are quite rare [77–79]. Currently, it is unresolved why the majority of Ewing tumors displays an EWS-FLI1 translocation: this could be due to hot-spots of recombination being more frequent within the FLI1 gene than in other ETS genes, or due to EWS-FLI1 being a more potent oncoprotein than other EWS-ETS fusion proteins and thus having a higher chance to elicit tumor formation after the chromosomal translocation has occurred.

EWS-ETS fusion proteins consist of the N-terminal part of EWS, which represents a potent transactivation domain [80,81], and a C-terminal part of an ETS protein, including its DNA-binding domain. The fusion proteins are highly effective transforming agents and can strongly activate gene transcription. However, microarray studies revealed that many genes are also downregulated by EWS-ETS fusion proteins, suggesting that they also have a repressive function [82]. But it remains unclear whether this repression of gene transcription is a direct or indirect effect, for instance through the upregulation of a transcriptional repressor. Similar to PEA3 factors, FLI1, ERG and FEV form one subfamily of ETS proteins [3]. And moreover, FLI1, ERG, FEV and the PEA3 factors have a nearly identical in vitro DNA-binding specificity [13], which might explain why all of the resultant EWS-ETS fusion proteins could occupy the same sites within the genome. However, genome-wide studies are needed to confirm that indeed all five EWS-ETS fusion proteins bind to and regulate the same target genes. In addition, FLI1, ERG, FEV and the PEA3 factors share the same in vitro DNA-binding consensus with nine other ETS proteins [13], begging the question why fusion proteins of these nine ETS factors are not found in Ewing tumors. Could it be that the ETS domain exerts another crucial function aside from DNA-binding, for instance the recruitment of Ewing tumor-critical transcriptional cofactors that interact solely with the DNA-binding domains of FLI1, ERG, FEV and PEA3 factors? Another possibility is that fusion of EWS to ETS proteins may alter the DNA-binding specificity of exclusively FLI1, ERG, FEV and PEA3 factors. Indeed, it was found that EWS-FLI1, but not FLI1, can also bind to GGAA microsatellite repeats in cells, and transcription of genes regulated by such microsatellites might be important for Ewing tumor genesis [13,83,84].

Ewing tumors need aggressive treatment, starting with surgery and complemented by radio- and chemotherapy. Despite improvements in clinical care, 30–50% of patients will eventually succumb to this disease, in part due to secondary malignancies arising from aggressive therapy [85]. This indicates the need for improved therapy, and multiple new avenues of treatment are considered. One new treatment option focuses on anti-angiogenic therapy, based on the observation that EWS-ETS fusion proteins upregulate the transcription of vascular endothelial growth factor (VEGF), which is overexpressed in Ewing tumors and whose inhibition can suppress growth of Ewing tumor cells in vitro [86–88]. Respective clinical trials are still ongoing and their outcomes are eagerly awaited [89].

Ewing-like sarcomas were observed that do not possess EWS-ETS fusion proteins, but are characterized by a t(4;19)(q35;q13) chromosomal translocation. Here, a fusion protein is generated consisting of most of the high mobility group box CIC protein and a small portion of DUX4, excluding its DNA-binding double homeodomain. In contrast to CIC that is a transcriptional repressor, the CIC-DUX4 fusion protein is a potent transcriptional activator and capable of transforming NIH3T3 cells. By virtue of the CIC high mobility group box, the CIC-DUX4 protein binds to and upregulates the ETV1 and ETV5 gene promoters, and the same is predicted for ETV4 that also has CIC binding-sites in its promoter region [90]. Consistently, downregulation of CIC in SKMEL13 melanoma cells simultaneously increased ETV1, 4 and 5 mRNA levels [91]. These studies suggest that overexpression of ETV1, 4 and/or 5 may be sufficient to induce Ewing tumors. However, it needs to be determined whether the oncogenic activity of CIC-DUX4 is solely due to the upregulation of PEA3 group members and if the Ewing-like sarcomas characterized by the t(4;19)(q35;q13) chromosomal translocation are true Ewing tumors.

6. Role in prostate cancer

6.1. ETS translocations in prostate cancer

Prostate cancer is the second most frequent cause of cancer mortality in males in the US [92]. Apart from surgery and radiation therapy, androgen ablation is a cornerstone of advanced prostate cancer treatment [93]. Initially, this leads to the regression of the disease, but nearly invariably patients with metastases relapse and die within three years. Despite of the pivotal role of the androgen receptor (AR) in prostate cancer, mutations in the AR gene are only observed in a minority of prostate tumors and rarely at the onset of the disease, suggesting that other genetic lesions contribute to prostate tumorigenesis [94,95]. It is well established that chromosomal translocations can be underlying causes of cancer, but such translocations were not known in any major solid carcinoma. This was fundamentally changed in 2005 with the discovery that the majority of prostate tumors display chromosomal translocations involving TMPRSS2 and either ERG or ETV1 [96]. Thereafter, it was found that also ETV4 and ETV5 can be fused to TMPRSS2 in prostate tumors [97,98]. The consequence of such a translocation is the generation of a TMPRSS2-ETS fusion gene, in which the expression of the ETS protein is controlled by the TMPRSS2 gene promoter/enhancer. Since the TMPRSS2 serine protease gene is androgen-inducible and highly expressed in the prostate [99], its translocations induce the androgen-dependent expression of ETS proteins in this organ, thus guaranteeing that ETS proteins become overexpressed in prostate tumors.

While TMPRSS2 is the most frequent translocation partner for ETS proteins in prostate tumors, other gene fusions were observed that put ETS expression under the control of various androgen-dependent or constitutively active gene promoters [100]. The ETS gene rearrangements observed result in the expression of full-length or slightly truncated ETS proteins, which may or may not have small stretches of TMPRSS2 or other fusion partner amino acids at their N-termini. All fusion proteins retain the DNA-binding domain of the ETS protein, thus causing dysregulation of gene transcription. ERG has a DNA-binding specificity very similar to the PEA3 group [13], explaining why ERG as well as ETV1, 4 and 5 translocations may all cause prostate cancer. One may even speculate that FLI1 and FEV, which together with ERG form one subfamily of ETS proteins and are all involved in Ewing tumors (see section 5), await discovery as translocated genes in prostate tumors.

Currently, there is no consensus which fusion gene is correlated with more aggressive prostate cancer or whether translocation-positive prostate tumors are more or less lethal than the ~30% of prostate tumors without ETS translocations [101]. With ~50% of all cases, ERG is the most frequently rearranged ETS gene in prostate tumors, followed by ETV1

(~5–10%) and ETV4 or ETV5 (~1–2%). The reason for this preponderance of ERG translocations is most likely that the ERG gene is located in close proximity to the TMPRSS2 gene on chromosome 21, whereas the PEA3 family genes are localized on different chromosomes. Furthermore, TMPRSS2 fusions to ERG or ETV1 are non-random. Ligand-bound AR was shown to bind to intronic regions of these genes at translocation loci, thereby promoting the spatial proximity of TMPRSS2 to either the ERG or ETV1 gene. Moreover, ligand-bound AR also promotes the recruitment of enzymes involved in DNA double-strand breaks [102–104]. Thus, the prostate as an androgen-dependent organ is predestined for TMPRSS2-ETS fusions to occur. Further, the number of AR binding-sites within the PEA3 group genes may determine the frequency with which TMPRSS2 becomes translocated onto ETV1, ETV4 or ETV5.

6.2. The case for ETS factors as oncoproteins

Proof that overexpression of ETS proteins as a consequence of chromosomal translocations causes neoplastic transformation comes from transgenic mouse models. Androgen-inducible, prostate-specific overexpression of ERG or ETV1 induced prostatic intraepithelial neoplasia (PIN) in 3–12 months old transgenic mice, whereas no carcinoma formation was observable [105–108]. PIN is a non-malignant precursor of prostate cancer, implicating that ETS overexpression on its own leads to the initiation, but not establishment of prostate carcinoma. However, two other reports failed to observe PIN formation in ERG transgenic mice [109,110]. Reasons for this discrepancy could be a different design of the transgenic ERG vector, different sites of chromosomal integration causing different expression levels, or the different genetic background of the transgenic mice. But crossing these ERG transgenic mice onto a PTEN^{+/-} background resulted in one of these studies in the induction of invasive carcinomas at 6 months of age, whereas PTEN^{+/-} mice developed just PIN by roughly 8 months of age [109]. In the other study, all ERG/PTEN^{+/-} compound mice developed PIN at 6 months of age, whereas only 12.5% of PTEN^{+/-} littermates displayed PIN [110]. These data suggest that ETS translocations cooperate with other genetic lesions to induce prostate cancer in humans. In particular, loss of the tumor suppressor PTEN is very often observed in prostate cancer [111], TMPRSS2-ERG translocations are positively correlated with loss of PTEN, and the combination of TMPRSS2-ERG and loss of PTEN is associated with a poor prognosis of prostate cancer patients [109,110,112,113]. Future studies may show that ETS translocations cooperate with various other genetic lesions in prostate tumor formation, an attractive one being the loss of the Nkx3.1 homeobox tumor suppressor gene as frequently found in human prostate tumors [114].

The continuous overexpression of ETS proteins in human prostate tumors indicates that ETS proteins play a crucial role not only in the initial formation, but also in the maintenance and later stage progression of prostate cancer [100,101]. Consistently, downregulation of ERG or ETV1 in prostate cancer cells harboring respective translocations impairs cell proliferation, invasion and tumor formation in nude mice [105,107,115–117]. Conversely, overexpression of ERG, ETV1, ETV4 or ETV5 in immortalized, non-transformed prostate epithelial cells can lead to enhanced proliferation and invasion [98,105–107,117–119]. Interestingly, whereas effects of ETS proteins on prostate cell proliferation were often modest or non-existing, they significantly promoted cell invasion in nearly all reported studies. Accordingly, microarray analyses indicated that an invasion program is directed by ETS protein overexpression [105, which includes the upregulation of MMP-1, -3, -7, -9, urokinase plasminogen activator and its receptor, all of which are key proteins in the remodeling of the extracellular matrix during cell migration and invasion. This invasion program parallels one observed in Ewing's sarcoma cells [120,], implicating that increased ETS factor activity can cause similar consequences in various tumors.

Furthermore, chromatin immunoprecipitation studies revealed that ETV1, ETV4 and ERG occupy a common set of genomic regions, which are in close proximity to the transcription start sites of various genes [121]. These genes regulate blood vessel formation, which is reminiscent of the role of PEA3 proteins in branching morphogenesis (see subsection 3.1) and also suggests that PEA3 factors facilitate tumorigenesis by promoting angiogenesis. Furthermore, ETV1, ETV4 and ERG appear to bind to genes that are involved in cell differentiation, growth and multicellular organismal development [121]. Although the relevance of these findings needs to be further studied, one may speculate that PEA3 factors thereby induce a de-differentiation program conferring stem cell-like properties on cancer cells, reminiscent of ETV5's role as a required factor for spermatogonial stem cell renewal [33].

6.3. The relationship between androgen receptor and ETS proteins

Genome-wide analyses revealed that AR binding sites often colocalize with ETS sites [122,123], suggesting that AR and ETS proteins may coregulate gene transcription. In fact, ETV1, ETV5 and ERG all bind to AR [108, 123,124]. However, whereas ETV1 appears to cooperate with AR in the activation of gene transcription, ERG antagonizes AR signaling by inhibiting AR activity and suppressing AR expression. The repressive action of ERG on the AR axis is somewhat counterintuitive, since AR is a pivotal driver of prostate tumorigenesis [95]. However, it is speculated that ERG may specifically inhibit one facet of AR activity, namely pro-differentiation, and thereby promote tumorigenesis by facilitating a de-differentiated cellular phenotype [123]. In concordance with this, ERG and AR synergize in a transplant model to induce mouse prostate epithelial cells into developing adenocarcinomas [125].

Notably, ETV1 and AR may recruit the c-JUN oncoprotein into a common complex [126]. Since PEA3 factors were shown to cooperate with c-JUN [62,127,128], this may further modulate AR-dependent gene transcription. In addition, binding of AP-1 (which consists of FOS/JUN dimers) was frequently observed in close proximity to PEA3 factors or ERG in the genome of PC3 prostate cancer cells, further underscoring a potential cooperativity between c-JUN and ETS proteins in prostate tumorigenesis [121].

6.4. Posttranslational modifications of PEA3 factors and prostate cancer

As mentioned before (see subsection 4.2), all three PEA3 factors are activated by MAPKs [10,55,56], whereas ERG is not known to be a target for MAPKs. Recent studies indicate that upstream activators of MAPKs, RAS and RAF, are hyperactivated in ~40% of primary and ~90% of metastatic prostate tumors. This can be due to gene mutations, rearrangements, copy-number alterations or expression changes [129,130]. Moreover, HER2/Neu is overexpressed in prostate tumors and appears to correlate with the progression of the disease [131,132]. Accordingly, it is predicted that MAPK-dependent phosphorylation and activation of PEA3 factors will occur in prostate cancer and promote the deleterious function of PEA3 factors, but not necessarily of ERG. And indeed, PEA3 factors synergized with MAPK activation in stimulating gene transcription, whereas ERG did not [121].

Apart from transcriptional upregulation caused by chromosomal rearrangements, PEA3 factor overexpression could also be (partially) due to increased protein stability in prostate tumors. In fact, PEA3 factors are degraded through the proteasome pathway [131,134]. One E3 ubiquitin ligase, COP1 [135], was identified to bind to all three PEA3 factors, induce their polyubiquitylation and thereby decrease their protein levels [136,137]. Interestingly, COP1 does not affect the stability of ERG nor of a truncated ETV1 molecule, Δ ETV1, which lacks the first 131 amino acids and has been found overexpressed in prostate tumors like full-length ETV1 [96,100]. This is not surprising, since Δ ETV1 lacks the two degrons that are

recognized by COP1 and encompass the critical VPD/E signatures at ETV1 amino acids 63–65 and 71–73 (see Fig. 1B). Similar degrons are present in ETV4 and ETV5 [136]. Knocking out COP1 in the mouse prostate led to early PIN formation at one year of age and cooperated with loss of PTEN in enhancing the invasion of carcinomas. However, only 5 out of 166 human prostate tumors analyzed displayed a loss of COP1, suggesting that COP1 loss is an infrequent mechanism to enhance PEA3 factor expression in prostate tumors [137].

Ubiquitylation of ETV4 appears to be promoted by its modification with SUMO [138], a protein that is structurally related to ubiquitin and similarly attached to lysine residues [139]. In vitro and in vivo studies revealed that sumoylation can occur on four lysine residues in ETV4 and ETV5 (see Fig. 1B) [140–142]. These four sites are also conserved in ETV1 and conform to the sumoylation consensus sequence Ψ KxE, where Ψ is a large hydrophobic residue [143]. Attachment of SUMO reduces the transactivation potential of ETV4 and ETV5 [140–142,144], although one dissenting report suggests that sumoylation activates ETV4 [138]. How does sumoylation relate to prostate cancer? Similar to phosphorylation or acetylation, posttranslational modification with SUMO is a dynamic process that is governed by a balance of the UBC9 conjugating enzyme and SUMO ligases on the one hand and SUMO proteases on the other hand [145]. One SUMO protease is SENP1, and its expression is enhanced by AR [146]. Thus, SENP1 may become upregulated in prostate tumors that are characterized by high AR activity. In accordance, SENP1 protein levels in human prostate tumors appear to increase with the severity of the disease. Further, ectopic expression of SENP1 in the prostate of mice led to the formation of PIN [147]. Thus, it is tempting to speculate that PEA3 factor sumoylation becomes reduced in prostate tumors, which could increase their stability by preventing ubiquitylation and additionally stimulate their transactivation potential.

Another way to affect PEA3 protein stability may involve the acetyltransferase p300. This protein is capable of acetylating histones and thereby stimulate gene transcription [148]. Moreover, ETV1, ETV4 and AR were shown to bind to and cooperate with p300 in activating gene transcription [20,149–151]. Interestingly, acetylation of ETV1 on K33 and K116 enhances its stability and transactivation potential [20]. Acetylation can thereby contribute to ETV1 overactivity, especially since p300 itself is overexpressed in prostate tumors [152] and its catalytic activity is additionally enhanced by MAPKs [20]. Whereas lysine residues homologous to K33 and K116 in ETV1 are conserved in ETV5, only a lysine homologous to K33 is present in ETV4 (Fig. 1B). This suggests that ETV5, but possibly not ETV4, is regulated in the same manner by p300-mediated acetylation as ETV1.

Yet alternatively, ETV4 seems to be activated through acetylation especially on those lysine residues that also become sumoylated [151]. Since acetylation and sumoylation are mutually exclusive at a particular lysine residue, it remains to be determined how these two posttranslational modifications affect each other and the ETV4 protein. Also, the N-terminal sumoylation of ETV4 on K96 is likely affected by phosphorylation on S101, suggesting an even more complex interplay of posttranslational modifications on PEA3 factors [138]. Eventually, the importance of phosphorylation, acetylation and/or sumoylation sites needs to be tested in vivo. And since transgenic ETV1 mice develop PIN [105,108], it should be possible to assess the importance of posttranslational modifications for the oncogenic activity of ETV1 by comparing wild-type ETV1 transgenic mice to those overexpressing ETV1 that is mutated at its phosphorylation, acetylation and/or sumoylation sites.

7. Role in other cancers

One indication for an oncogenic protein is its overexpression in tumors and multiple studies report so for PEA3 factors. For instance, ETV4 is overexpressed at the mRNA and protein

level in colorectal tumors and ETV4 overexpression correlates with a shorter survival period of affected patients [153–159]. ETV1 and ETV5 mRNA upregulation is also observable in colorectal tumors, but does not correlate with survival [153]. Similarly, ETV4 mRNA overexpression was reported in 64% of human gastric tumors and was indicative of a shorter disease-free survival. In contrast, ETV1 and ETV5 mRNA were only overexpressed in 31% and 26%, respectively, of gastric tumors and their overexpression did not correlate with survival [160]. Immunohistochemical analysis of tissue microarrays confirmed that ETV4 is overexpressed at the protein level in gastric adenocarcinomas and that ETV4 overexpression, in combination with activation of MAPKs, is a marker of poor prognosis [161]. Likewise, ETV4 and ETV1 are overexpressed in esophageal adenocarcinomas and may cooperate with activated MAPKs in stimulating proliferation and invasion of OE33 esophageal squamous cancer cells [162]. Another report posits that ETV4 and ETV5, but not ETV1, are overexpressed in esophageal squamous cell carcinomas and that ETV4 expression correlates with a shorter overall survival [163]. Also, ETV4 is upregulated in ovarian tumors and its expression correlated with reduced survival [41,164]. Analysis of endometrioid endometrial carcinomas showed ETV5 overexpression to be restricted to myometrial invading tumors [165]. Consistently, ETV5 enhances migration of the human endometrial Hec-1A cancer cell line and induces a more aggressive and infiltrative pattern of myometrial invasion in an orthotopic tumor model [166]. Moreover, ETV4 is overexpressed at the mRNA level in oral squamous cell carcinomas and non-small-cell lung cancer and appears to promote cell motility and invasion [167–169]. Finally, comparative genomic hybridization of lung adenocarcinomas in never smokers revealed gains on both the short and long arm of chromosome 7 that contain the ETV1 and BRAF genes, respectively [170], reminiscent of melanomas that also display joint copy number gain of 7p and 7q (see below) [171]. Altogether, these reports are indicative that PEA3 factors may exert oncogenic functions in many different tumors, yet this circumstantial evidence requires substantiation through animal models and further genetic analyses.

ETV1 has also been implicated in the development of gastrointestinal stromal tumors (GISTs), which are primarily characterized by mutations in the KIT or PDGFRA receptor tyrosine kinase [172]. ETV1 is highly expressed in interstitial cells of Cajal, which are sensitive to KIT-mediated transformation, as well as in GISTs. Furthermore, ETV1 was required for growth of GIST cell lines and cooperated with KIT in the transformation of NIH3T3 cells. Interestingly, ETV1 protein stability may be enhanced by KIT through the MAPK pathway [173]. Although this suggests that ETV1 is important for GIST formation, it does not prove that ETV1 is an underlying cause of disease development. Rather, ETV1 seems to be just one of the required proteins for KIT to exert its oncogenic functions.

More compelling evidence for an oncogenic function of ETV1 comes from the analyses of melanomas. 40% of melanomas display ETV1 gene copy gains, with 13% of primary and 18% of metastatic tumors even having more than six copies per cell. In addition, ETV1 mRNA is also upregulated in melanomas without copy-number change [171]. A key oncoprotein in melanomas is BRAF, and inhibitors targeting the BRAF^{V600E} mutant present in more than half of all melanomas are now utilized in the clinic [174]. And ETV1 synergized with BRAF^{V600E} to transform primary melanocytes and promote tumor growth in nude mice. Conversely, downregulation of ETV1 suppressed proliferation and anchorage-independent growth of several melanoma cell lines [171]. The mechanism by which ETV1 may contribute to melanoma formation could be the reported [171] upregulation of the microphthalmia-associated transcription factor. This is a master regulator of melanocyte lineage development and proven melanoma oncoprotein and, like ETV1, cooperates with BRAF^{V600E} in neoplastic transformation [175].

8. Perspective

It is now well established that PEA3 factors are causally involved in prostate and Ewing tumors as a consequence of chromosomal translocations, and further evidence points to critical roles of PEA3 factors in melanomas, breast and other cancers. So how can this knowledge be utilized in cancer therapy? Presently, it is hard to imagine targeting ETV1, 4 or 5 directly with, for instance, siRNA. Rather, it is more promising to focus on PEA3 factor activation, target genes and interaction partners (Fig. 2). Inhibition of the RAS-RAF-MEK-MAPK axis or upstream receptor tyrosine kinases should reduce PEA3 factor activity and could thus be an important adjuvant therapy. Likewise, inhibition of target genes such as MMPs, COX-2 and VEGF may be included in the therapy of PEA3 factor-dependent tumors. But such therapies are attempted in many cancers and not specifically tailored to PEA3 factors.

More specificity may come from focusing on interaction partners. For instance, enzymes like p300 or poly(ADP-ribose) polymerase 1 (PARP1) bind to ETV1 and appear to be required for ETV1 activity [20,176]; as such, respective enzymatic inhibitors could be beneficial in the treatment of ETV1-dependent tumors. Even binding partners of PEA3 factors that are not enzymes could be a focus for therapy, if one could develop small molecule drugs that obstruct their interaction. Precedence has been reported for MLL-fusion leukemia, where small molecules binding to BET proteins prevent their recruitment to chromatin and thereby affect epigenetic changes that are therapeutically effective [177,178]. However, such an approach requires the identification of the key interaction partner(s) of PEA3 proteins and the additional identification of inhibitors disrupting respective interactions. One step in this direction has been the discovery of YK-4-279, a small molecule that abrogates the interaction of EWS-FLI1 with RNA helicase A and inhibits tumor growth of Ewing's sarcoma cells in nude mice [179]. YK-4-279 also binds to ETV1 and ERG and inhibits invasion of ETV1-overexpressing LNCaP and ERG-overexpressing VCaP prostate cancer cells. How YK-4-279 does so remains unclear, as this small compound does not prevent RNA helicase A from binding to ERG nor does it affect DNA-binding of ERG [180]. And more preclinical studies are needed to determine whether YK-4-279 solely acts through ETV1/ERG and if it would be beneficial in prostate cancer therapy.

Despite two decades of research, there are still large gaps in knowledge about the mechanisms of PEA3 factor action. Yet, given the rapidly developing tools in genomics and proteomics, the next two decades are poised to greatly enhance our understanding of the roles of PEA3 factors in normal and diseased cells. This may show that ETV1, 4 and 5 are the most "rocking" ETS proteins in cancer research, as 1-4-5 is probably the most common chord progression in all of pop music.

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in ETV4 is a minor in vitro sumoylation site and likely not sumoylated in vivo); and acetylation (Ac) of lysine residues by p300 (only the four most important ETV4 acetylation sites are shown, which happen to overlap with its sumoylation sites).

Fig. 2.
Potential avenues of inhibiting PEA3 factor action.