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Research Article

The Ets-1 transcription factor is involved in the development and invasion of malignant melanoma

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Abstract. The Ets-1 transcription factor plays a role in tumor vascularization and invasion by regulating expression of matrix-degrading proteases in endothelial cells and fibroblasts in the tumor stroma. During early embryogenesis, Ets-1 is expressed in migrating neural crest cells from which melanocytes arise. In the present study, we analyzed Ets-1 expression in various melanocytic lesions and investigated its functional importance in malignant melanomas. We found that Ets-1 was upregulated both in vivo and in vitro in malignant melanoma, compared to benign melanocytic lesions and to primary melanocytes. Assessment of DNA-binding and transactivation assays documented a strong Ets activity in melanoma cells. Using an antisense strategy, the expression and activity of Ets-1 were reduced in the melanoma cell line Mel Im. This correlated with a diminished expression of several Ets-1 target genes known to be involved in invasion, such as MMP1, MMP3, uPA and integrin β3. In line with these findings, the invasive potential of the melanoma cells measured in a Boyden Chamber model was reduced up to 60% after Ets-1 blockade. This can be attributed to the role of Ets-1 in transcriptional regulation of factors involved in invasion of melanoma cells. We conclude that over-expression of Ets-1 during melanoma development contributes to the malignant phenotype.

Key words. Malignant melanoma; Ets-1; proliferation; migration; invasion.

The *ets-1* gene was first characterized as the cellular proto-oncogene of the retroviral *v-ets* oncogene of the avian leukemia retrovirus E26 [1, 2]. *ets-1* is the prototype of the *ets* gene family of transcription factors [3, 4]. This family contains a growing number of transcriptional activators and inhibitors; their activity is regulated by phosphorylation and protein-protein interactions [for reviews see refs. 5–7]. The members of the Ets family are characterized by a conserved ETS domain, which binds to double-stranded DNA containing a GGAA/T core sequence. Some *ets* genes are involved in chromosomal translocations leading to the production of chimeric fusion proteins that are associated with various leukemias and soft-tissue cancers [8–11]. Among the Ets factors, Ets-1, Erg-1 and Fli-1 are expressed in endothelial cells during angiogenesis in normal and pathological conditions [12, 13] [for review see ref. 14]. The human *ets-1* gene has been assigned to chromosome 11q23.3; it encodes a primary transcript of 6.8 kb which can be alternatively spliced into mRNAs with or without exons IV and/or VII [15–17].

Several lines of evidence suggest that Ets-1 plays an important role in differentiation, proliferation, angiogenesis, apoptosis and also tumor vascularization and invasion [7, 12, 13, 18]. Among the target genes of Ets-1, the genes

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encoding urokinase-type plasminogen activator (uPA), various matrix metalloproteinases (MMPs) and integrin β3 are associated with an invasive phenotype. Ets-1 promotes angiogenesis by inducing the expression of integrin β3 and several proteases necessary for early steps in new blood vessel formation [5, 19–22]. Consequently, Ets-1 inhibition by an antisense strategy or a dominant negative molecule was shown to inhibit angiogenesis [14, 18]. Furthermore, during embryogenesis, ets-1 mRNA expression is transiently induced in epithelial structures during the dispersion of somites into the mesenchymal sclerotome and also during emigration of neural crest cells from which mature melanocytes derive [23, 24]. Since cell migration is an important element in tumor invasion and melanocytes are the precursors of melanoma cells, we decided to examine the expression and potential role of Ets-1 in malignant melanoma.

Materials and methods

Human tissues

Ets-1 expression was assessed by in situ hybridization in benign and malignant melanocytic lesions sent to the Institute of Pathology of the University of Bonn for diagnostic purposes (table 1). Patient (25 males and 7 females) age ranged from 3 to 83 years (average 45.0 years).

Cell lines and culture conditions

The melanoma cell lines Mel Im, Mel Ei, Mel Wei, Mel Ho, Mel Juso, Mel Ju, SK Mel 28, SK Mel 3 and HTZ19d have been described in detail previously [25, 26]. Briefly, Mel Ei, Mel Wei, Mel Ho and Mel Juso were derived from a primary cutaneous melanoma, while Mel Im, Mel Ju, SK Mel 28, SK MEL 3 and HTZ19d were from metastases of malignant melanomas. Cells were maintained in DMEM supplemented with penicillin (400 U/ml), streptomycin (50 µg/ml), L-glutamine (300 µg/ml) and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and split at a 1:5 ratio every 3 days.

Human primary melanocytes derived from normal skin were cultured in melanocyte medium MGM-3 (Gibco, Eggenstein, Germany) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells of five different donors were used between passages 6–10. Cells were detached for sub-cultivation or assay using 0.05% trypsin, 0.04% EDTA in PBS.

Table 1. Benign and malignant melanocytic lesions analyzed for Ets-1 expression

	n
Lentigo simplex	4
Junctional melanocytic nevus	3
Compound melanocytic nevus	7
Dermal melanocytic nevus	2
In situ melanoma	
Lentigo maligna	1
Superficial spreading	1
Total	2
Invasive malignant melanoma (Clark level II–V)	
Lentigo maligna	2
Acrolentiginous	2
Not specified	3
Total	7
Metastatic malignant melanoma	
Lymph node metastases	4
Lung metastases	2
Peritoneal cavity	1
Total	7

RNA isolation and reverse transcription

For RT-PCR, total cellular RNA was isolated from cultured cells using the RNeasy kit (QIAGEN, Hilden, Germany). The integrity of the RNA preparations was visualized in 1% agarose/formaldehyde gel electrophoresis and cDNAs were generated by a reverse transcriptase reaction performed in 20 µl reaction volume containing 2 µg of total cellular RNA, 4 µl of 5 × first-strand buffer (Gibco), 2 µl of 0.1 M DTT, 1 µl of dN_6-primer (10 mM), 1 µl of dNTPs (10 mM) and DEPC-water. The reaction mix was incubated for 10 min at 70 °C. Then, 1 µl of Superscript II reverse transcriptase (Gibco) was added and RNAs were transcribed for 1 h at 37 °C. Subsequently, reverse transcriptase was inactivated at 70 °C for 10 min and RNA was degraded by digestion with 1 µl RNase A (10 mg/ml) at 37 °C for 30 min. cDNAs were controlled by PCR amplification of β-actin.

Analysis of *ets-1*-expression by quantitative PCR

Quantitative real-time PCR was performed using a Lightcycler (Roche, Mannheim, Germany). Two microliters cDNA template, 2 µl 25 mM $MgCl_2$, 0.5 µM of forward and reverse primers (5′-CGATCTCAAGCCGACTCT CA-3′, 5′-CGAGGTATAGCGGGATTCTG-3′) and 2 µl of SybrGreen LightCycler Mix in a total of 20 µl were applied to the following PCR program: 30 s 95 °C (initial denaturation); 20 °C/s temperature transition rate up to 95 °C for 15 s, 3 s 65 °C, 5 s 72 °C, 81 °C acquisition mode single, repeated for 40 times (amplification). The PCR reaction was evaluated by melting-curve analysis following the manufacturer's instructions and checking the PCR products on 1.0% agarose gels.

Analysis of Ets-1 splicing

The complete coding region of Ets-1 was amplified by RT-PCR from cDNA using the primers 5′-CGATCT-CAAGCCGACTCTCA-3′ and 5′-GCCACGGCTCAGT TTCTCAT-3′. The PCR reaction was performed in a 50-µl reaction volume containing 5 µl 10 × Taq-buffer, 1 µl of cDNA, 1 µl of each primer, 0.5 µl of dNTPs,

0.5 µl of Taq polymerase and 41 µl of water. The amplification reactions were performed by 33 repetitive cycles of denaturing for 45 s at 94 °C, annealing for 45 s at 65 °C and a final extension step at 72 °C for 45 s. The PCR products were resolved on 1% agarose gels. To analyze possible splice products, two regions of the ets-1 sequence were amplified separately. For amplification from exon 1 to exon 4, the primers 5′- CGATCTCAAGCCGACTCTCA-3′ and 5′- CGAGGTATAGCGGGATTCTG-3′ were used. For amplification from exon 5 to exon 8, the primers 5′- CCCCAGACAACATGTGGATG-3′ and 5′-GCCACGGCTCAGTTTCTCAT-3′ were used. For sequencing, the PCR products amplified from exon 5 to 8 were cut out of a 1% agarose gel and purified with QIAGEN gel extraction kit. The sequencing reaction containing 4 µl of terminator ready reaction mix, 2 µl of PCR product, 1 µl of sequencing primer (5′-CGAGGTATAGCGGGATTCTG-3′) and 20 µl of DEPC-water was performed for 10 s at 95 °C, 5 s at 64 °C, 4 min at 60 °C, 25 cycles. Sequencing products were precipitated with 80 µl of HPLC-water, 10 µl of 3 M sodium citrate solution, 250 µl of 100% ethanol to remove unincorporated terminators and resuspended in 25 µl of template suppression reagent. Products were run on an automatic sequencer from Applied Biosystems (Foster City, Calif.). Both strands were sequenced for each PCR product from at least two independent PCR reactions. Sequences were compared with the gene data bank by means of BLAST search (National Center of Biotechnology Information).

Western blotting

For protein isolation, 2×10^6 cells were washed in 1 × PBS and lysed in 200 µl RIPA buffer (Roche, Mannheim, Germany). The protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, Ill.). Equivalent amounts of cell proteins (20 µg) were denatured at 70 °C for 10 min after addition of Roti-load-buffer (Roth, Karlsruhe, Germany) and subsequently separated on NuPage-SDS gels (Invitrogen, Groningen, The Netherlands). After transferring the proteins onto PVDF membranes (BioRad, Richmond, Calif.), the membranes were blocked in 3% BSA/PBS for 1 h and incubated with a 1:1000 dilution of primary polyclonal anti-Ets-1 antibody (Transduction Laboratories, Lexington, Ken.) overnight at 4 °C. A 1:8000 dilution of rabbit anti-mouse AP (Sigma) was used as secondary antibody. Staining was performed using BCIP/NBT solution (Zymed, San Francisco, Calif.).

Transient transfection of melanoma cells with sense and antisense Ets-1

Mel Im cell clones with reduced Ets-1 expression were established by transient transfection with a pcDNA3 expression vector (Invitrogen NV, Leek, The Netherlands) coding for the Ets-1 antisense or sense cDNA [27]. Controls received pcDNA3 alone. Transfections were performed using the lipofectamine plus method (Gibco) reaching a 40% transfection efficiency. The amount of Ets-1 expression in these clones was determined by reporter gene assays after 3 days of culture.

Transfection experiments for reporter gene analysis

For transient transfections, 2.5×10^5 melanoma cells per well were seeded into six-well plates and transfected with 0.5 µg of pTORU-Luc reporter plasmid [28, 29] using the lipofectamine plus method (Gibco) according to the manufacturer's instructions. To analyze NFκB activity, an NFκB-luciferase construct (Stratagene) was used. Human primary melanocytes were transfected using Amaxa nucleofection [30], gaining a transfection efficiency of over 30%. Twenty-four hours after transfection, the cells were lysed and the luciferase activity in the lysate was measured. To normalize transfection efficiency, 0.2 µg of a pRL-TK plasmid (Promega, Mannheim, Germany) was co-transfected and renilla luciferase activity measured by a luminometric assay (Promega). All transfection experiments were repeated three times.

Electromobility shift assays

A double-stranded oligonucleotide corresponding to the Ets-1 binding site (5′- GATCTCGAGCAGGAATTCGA-3′ was phospholabeled and used for gel mobility shift assays. Nuclear extracts of the melanoma cell lines (5 µg/lane) were analyzed, and gel shifts were performed as described previously [31].

Invasion assay

Invasion assays were performed in Boyden Chambers containing polycarbonate filters with 8 µm pore size (Costar, Bodenheim, Germany), essentially as described previously [25]. Filters were coated with a commercially available reconstituted basement membrane (Matrigel, diluted 1:3 in H_2O; Becton Dickinson, Heidelberg, Germany). The lower compartment was filled with fibroblast-conditioned medium used as a chemoattractant. Melanoma cells were harvested by trypsinization for 2 min, resuspended in DMEM without FCS at a density of 2×10^5 cells/ml and placed in the upper compartment of the chamber. Transient transfections were performed as described. After incubation at 37 °C for 4 h filters were removed. Cells adhering to the lower surface were fixed, stained and counted.

In situ hybridization

Samples were fixed immediately in 4% paraformaldehyde (for 24 h at 4 °C) for in situ hybridization and then embedded routinely into paraffin. Sections were cut from the paraffin blocks at 4 µm, mounted on positively charged slides (Superfrost) and air-dried in an incubator at 42 °C overnight.

In situ hybridization detection of Ets-1 transcripts was performed as described previously [12, 13]. Briefly, sections were deparaffinized in xylene, rehydrated in graded alcohols, subjected to gentle proteinase K digestion (1 µg /ml proteinase K in 0.1 M Tris HCl pH 8.0, 0.05 M EDTA and 0.1% SDS, 5 min at 37°C), postfixed in 4% PFA (15 min at room temperature (RT), acetylated (10 min at RT) with 0.1 M triethanolamine (pH 8.0) and 25% acetic acid anhydride and finally dehydrated in graded alcohols. ^{35}S-labeled Ets-1 antisense and sense riboprobes (negative control) were synthesized by in vitro transcription from a 825-bp human Ets-1 cDNA template (nucleotides 260–1086) which had been cloned into a pSP64 plasmid vector. SP6 RNA polymerase (Promega) was used for transcription (1 h at 37°C). Transcription was followed by thorough DNAse I digestion of the template [1 U DNAse I/µl, (Promega), 15 min at 37°C], RNA extraction with TRIzol (Gibco) and riboprobe purification by phenol-chloroform extraction and Sephadex G-50 columns (Boehringer). Hybridization was carried out for 16 h at 53°C (riboprobe activity 2×10^4 cpm/µl, hybridization cocktail: 8 mM Tris HCl, pH 8.0, 0.12 M NaCl, 2 mM EDTA, 0.5 M DTT, 50% deionized formamide). Slides were stringently washed in 20 mM Tris HCl pH 8.0, 0.15 M NaCl, 5 mM EDTA, 0.1 M DTT and 50% deionized formamide for 30 min at 64°C. RNAse A digestion (0.02 mg/ml) was then carried out for 45 min at 37°C. After dehydration in graded alcohols, sections were subjected to autoradiography for 10–14 days (photoemulsion NTB2, Kodak). Positive signals were visualized by darkfield illumination of the slides after fluorescent counterstaining of the nuclei with Hoechst 33258.

Results

Ets-1 is up-regulated in melanocytic lesions in vivo

Ets-1 is expressed in migrating neural crest cells from which melanocytes originate during embryogenesis [23]. This early observation suggested that Ets-1 could have a role in melanocyte differentiation in the embryo and, possibly, in transformation of this lineage in the adult. Therefore, we analyzed Ets-1 expression in different melanocytic lesions, including malignant melanomas, using in situ hybridization (fig.1). The results revealed that Ets-1 expression correlates with the proliferative and/or migratory activity of the lesions: normal melanocytes and lentigo simplex, which represents the first stage of melanocytic nevus formation by slight melanocytic hyperplasia, were negative for Ets-1 expression. Junctional, compound and dermal melanocytic nevi showed weak to moderate expression, all junctional melanocytic nevi displaying moderate expression. Lower levels of expression were detected in dermal nevus components when compared to junctional components. In contrast, strong Ets-1 expression was recurrently detected in in situ and invasive melanomas, with no obvious correlation between Clark level of invasion and Ets-1 expression. In hematogenous metastases, intense Ets-1 signals were detected. Interestingly, only focal expression was seen in lymph node metastases. Noteworthy is that positive signals were also found in stromal capillaries during vascularization of the lesions.

Ets-1 is expressed in melanoma cell lines in vitro

We next analyzed Ets-1 expression in nine melanoma cell lines using RT-PCR spanning exons 1–4. Ets-1 transcripts were detected in all melanoma cell lines whereas human primary melanocytes showed no expression (fig. 2). These results were further verified by quantitative RT-PCR. To evaluate the expression of known Ets-1 splice products, RT-PCR spanning the full-length Ets-1 transcript was performed, showing the presence of an additional Ets-1 product in all analyzed melanoma cell lines. These variants were further characterized by RT-PCR spanning exons 5–8 (fig. 3A). Two distinct PCR products of 482 bp and 220 bp were amplified. Both products were isolated and sequenced. The 482 bp product corresponded to full-length Ets-1 while the shorter product of 220 bp corresponded to an exon 6 splice variant (fig. 3B).

In accordance with these observations, protein expression analyzed by Western blotting (fig. 3C) showed strong expression of Ets-1 (50 kDa) in all melanoma cell lines but not in primary melanocytes. Furthermore, a faster-migrating band of 41 kDa was detected which could correspond to the product of the exon 6 spliced transcript.

Ets-1 binds to DNA and activates transcription in melanoma cells

To investigate the potential binding of endogenous Ets-1 to DNA in melanoma cells, gel shift experiments were performed using a double-stranded oligonucleotide probe containing an Ets-1 DNA-binding site. Two major products were resolved by electromobility shift assay performed with nuclear extracts of melanoma cell lines. In contrast, no shift was detectable when using nuclear extracts from primary human melanocytes (fig. 4A).

Furthermore, the ETS transcriptional activity in Mel lm, Mel Ju, Mel Ho, Mel Ei, Mel Juso, SK Mel 28 and normal melanocytes was assessed by transient transfection with an ETS reporter vector. Strong transcriptional activity, comparable to the strong NFκB activity in melanoma cells, was measured in melanoma cells (fig. 4B), whereas no activity was detected in human primary melanocytes.

In addition, transient transfection of increasing amounts of an antisense *ets-1* construct in melanoma cells strongly reduced the ETS transcriptional activity (fig. 5), suggesting that a significant part of the ETS activity detected in these cells corresponded to endogenous Ets-1. Transient transfection of a sense expression vector only led to mod-

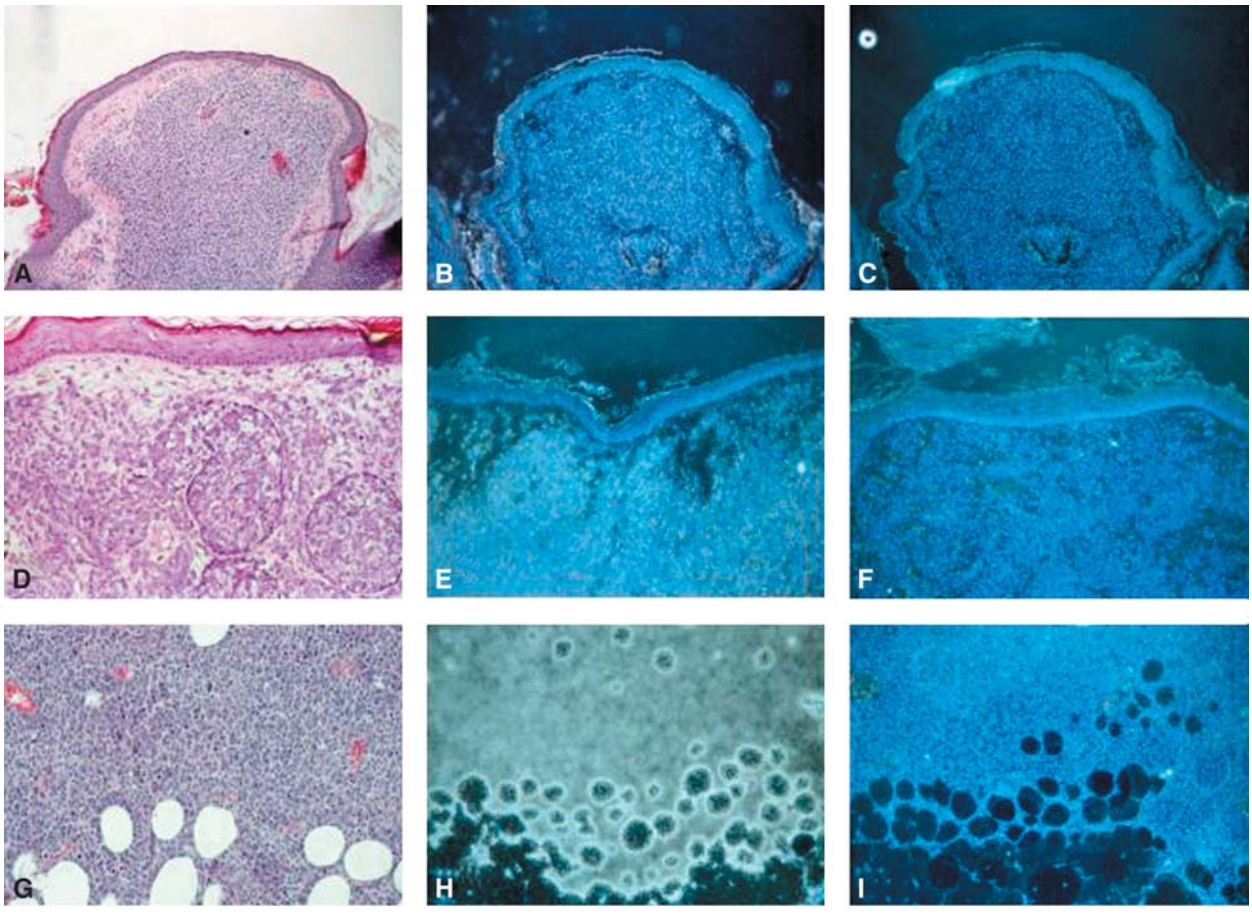

Figure 1: *Ets-1* in situ hybridization of human melanocytic lesions. Shown are dermal melanocytic nevus (*A*–*C*), invasive malignant melanoma of the skin (*D*–*F*) and metastatic melanoma within peritoneal fat tissue (*G*–*I*). Ets-1 transcripts (light grains) are seen in melanocytic nevus (*B*). Expression is strong in invasive malignant melanoma (*E*) and maximal in neoplastic cells of the metastasis (*H*). No positive signals are seen in negative control sections (*C*, *F*, *I*); (*ets-1* sense riboprobe). Histomorphology of the lesions is shown in H&E-stained slides (*A*, *D*, *G*). Specific hybridization signals and negative controls are demonstrated in darkfield illuminations of the slides after fluorescent counterstaining of the nuclei with Hoechst 33258 (bar, 100 µm).

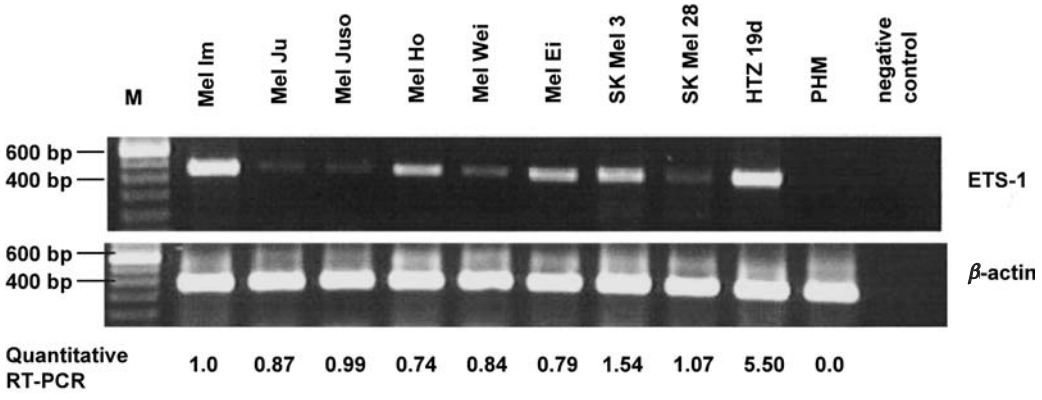

Figure 2. Amplification of the coding region of Ets-1 mRNA by PCR in melanoma cell lines compared to primary melanocytes. The coding region from exon 1 to 4 was specifically amplified by RT-PCR. All melanoma cell lines analyzed (Mel Im, Mel Ju, Mel Juso, Mel Ho, Mel Wei, Mel Ei, SK Mel 3, SK Mel 28 and HTZ19d) express Ets-1 mRNA. Human primary melanocytes show no Ets-1 expression. Relative levels of expression cannot be estimated here as the PCR was carried out to saturation. To more precisely compare levels of Ets-1 expression, quantitative RT-PCR was performed. The expression levels are given underneath the figure (Mel Im is set as 1).

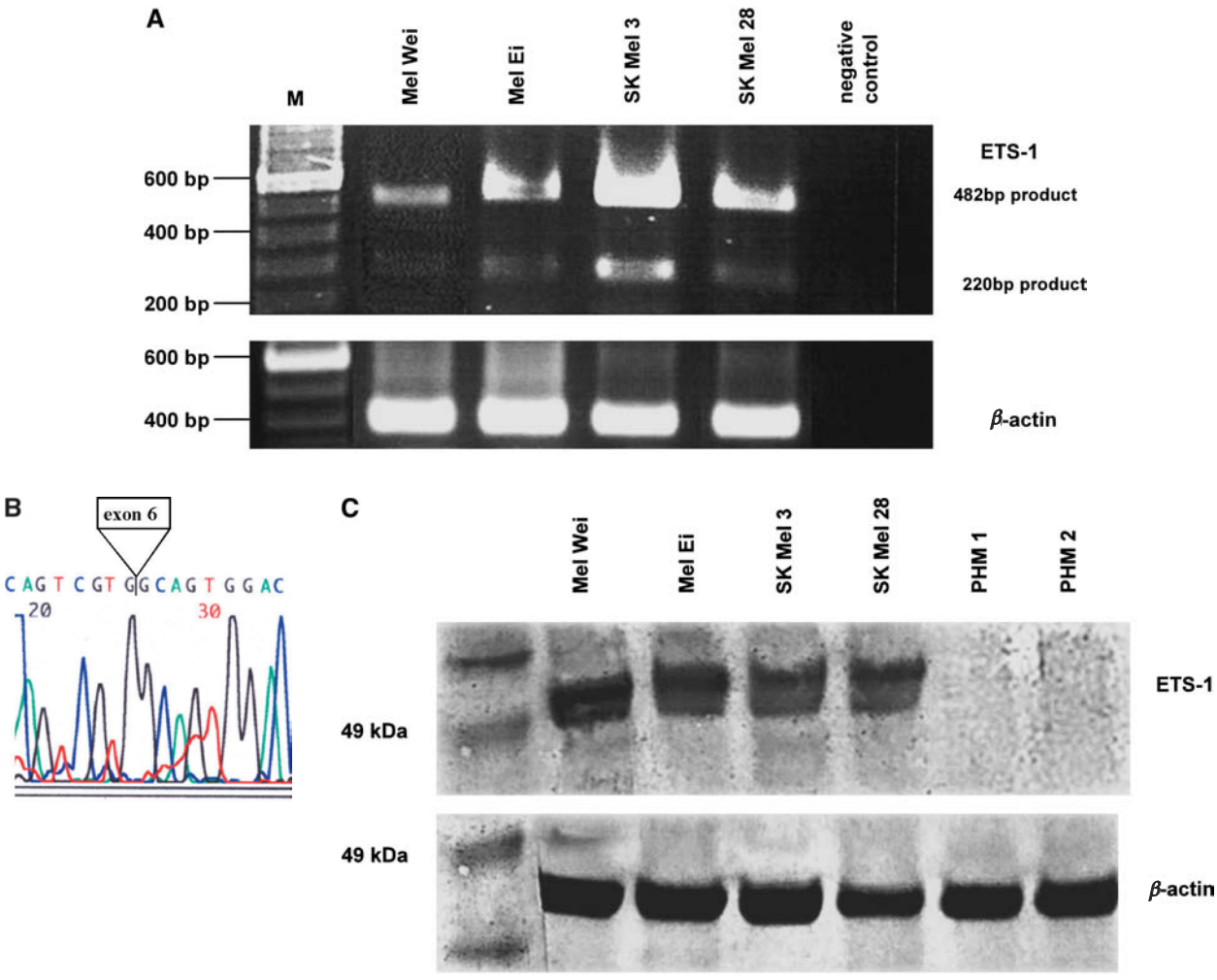

Figure 3. Analysis of Ets-1 alternative splicing. (*A*) RT-PCR from exons 5–8 revealed two distinct PCR products of 482 and 220 bp. The 482-bp product contains exon 6, whereas exon 6, a 262-bp fragment, is missing in the smaller PCR product. The expression level of the shorter 220-bp splice product is about 30% compared to the 482-bp product. (*B*) Sequencing of the shorter PCR product revealed a splice product missing exon 6. (*C*) Western blot analysis of Ets-1 protein expression in melanoma cell lines. Expression of Ets-1 protein was detected in melanoma cell lines but not in primary human melanocytes (for the human primary melanocytes, two separate preparations were analyzed). Both products, the full-length and the alternative splicing products, were detectable. Counterstaining of β-actin serves as loading control.

est induction of ETS activity due to constant high endogenous ETS activity in melanoma cells.

uPA, MMP1, MMP3, and integrin β3 are Ets-1 target genes in melanoma cell lines

Next, melanoma cells were transiently transfected with the *ets-1*-antisense construct to clarify whether inhibition of Ets-1 expression correlates with reduced expression of the known Ets-1 target genes uPA, MMPs 1 and 3, as well as integrin β3. Compared to the mock-transfected control cells, expression of these genes was strongly reduced in the antisense *ets-1*-transfected cells (fig. 6). Transient antisense transfection using the most effective dose (0.5 µg; see fig. 5) resulted in a strong reduction of Ets-1 expression and activity, consequently leading to a reduction in target gene expression.

Ets-1 blockade inhibits melanoma cell migration and invasion

We next assessed whether inhibition of Ets-1 expression affected melanoma cell migration and invasion. Functional in vitro cell migration and invasion assays were performed using the Boyden chamber invasion assay. A marked repression of the invasive potential of the cells transfected with the *ets-1* antisense construct was obtained in comparison to the mock-transfected Mel Im cells (fig. 7).

Discussion

Our previous studies on human tumors using in situ hybridization revealed that Ets-1 was expressed within stro-

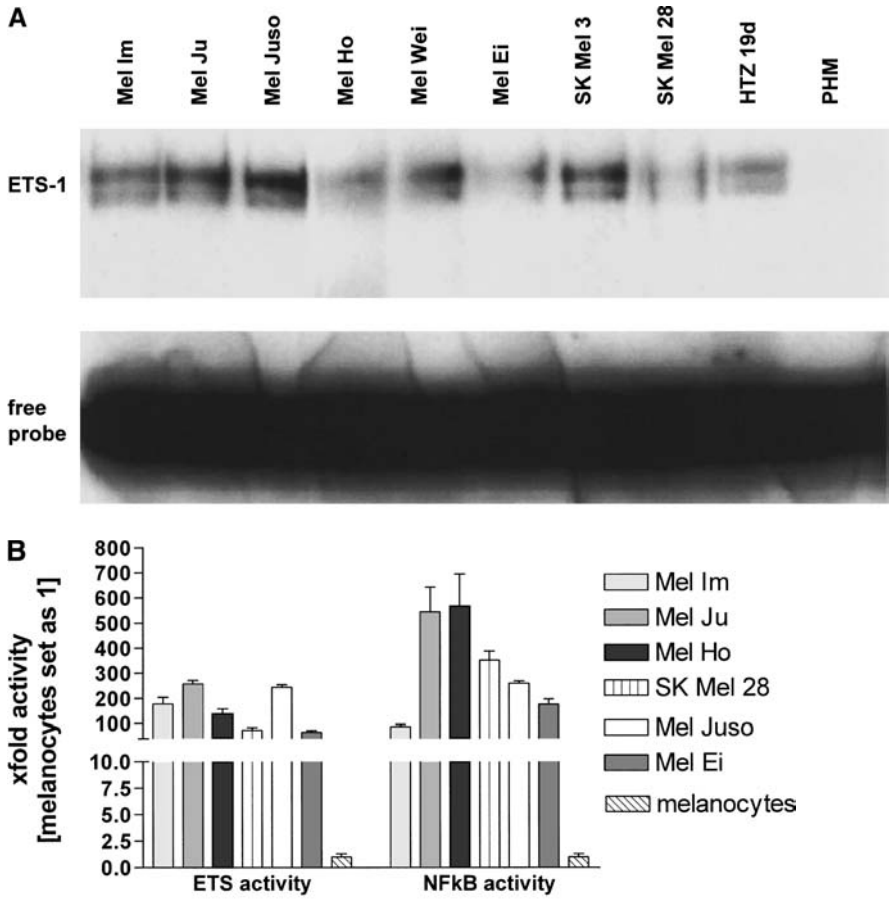

Figure 4. Analysis of Ets-1 activity in malignant melanoma. (*A*) In gel shift assays using an Ets-1 binding site containing oligonucleotide probe, shifts appeared with nuclear extracts from melanoma cell lines (Mel Im, Mel Ju, Mel Juso, Mel Ho, Mel Wei, Mel Ei, SK Mel 3, SK Mel 28 and HTZ19d) but not from human primary melanocytes (PHM). (*B*) Reporter gene assays revealed strong ETS transcriptional activity in the melanoma cell lines Mel Im, Mel Ju, Mel Ho, Mel Juso, Mel Ei and SK Mel 28 but not in human primary melanocytes. The ETS transcriptional activity is similar to the activity of NFκB in the melanoma cells analyzed.

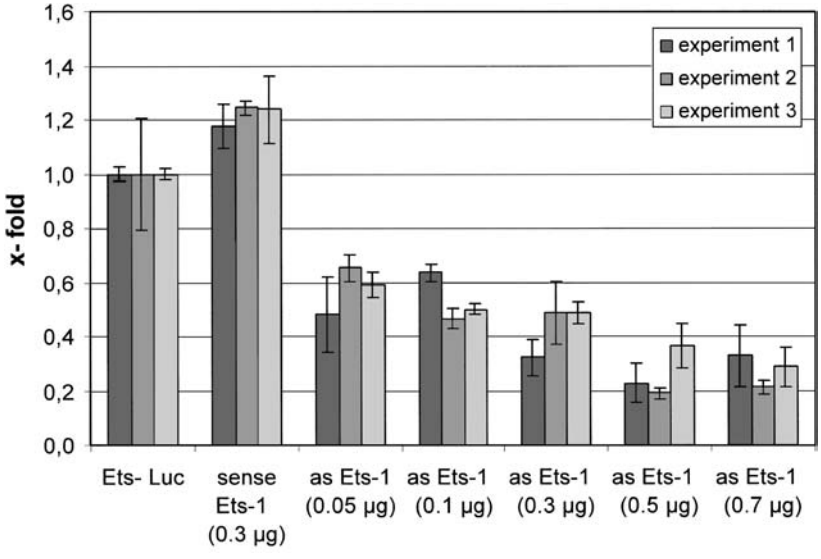

Figure 5. Transient transfection of an antisense and sense *ets-1* construct. Defined amounts of antisense *ets-1* expression plasmid (0.05–0.7 µg) were transfected into the melanoma cell line Mel Im. With reporter gene assays, Ets-1 activity was measured in the transiently transfected cells. A dose-dependent downregulation of Ets-1 activity was achieved. Transfecting sense ets-1 expression plasmid (0.3 µg) only led to modest induction of *ets-1* activity in the melanoma cells due to constant high activity. The results of three independent experiments are shown.

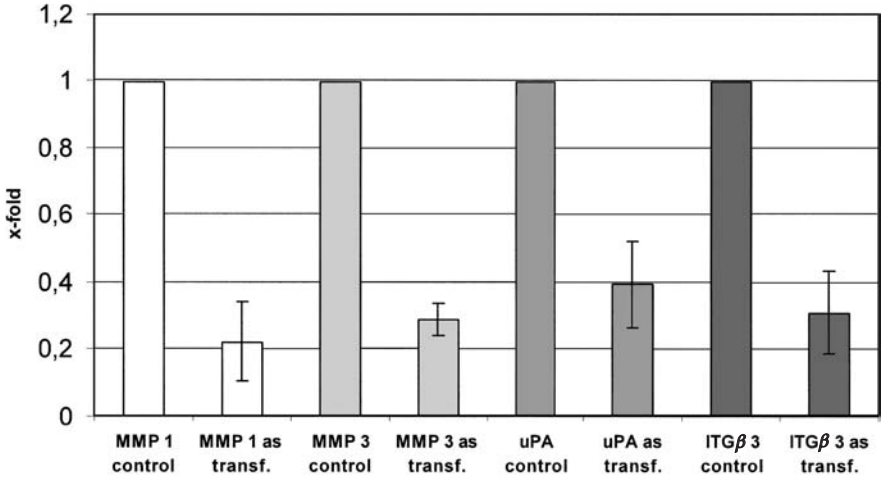

Figure 6. Expression of Ets-1 target genes in malignant melanoma. Melanoma cells Mel Im were transiently transfected with 0.5 µg antisense *ets-1* expression plasmid which was determined to be the most effective dose to repress Ets-1 activity. As a control, Mel Im cells were transfected with pcDNA3. Expression of MMP1, MMP3, uPA and integrin β3 (INTGβ3) was measured by quantitative PCR. The data are presented with expression of the analyzed gene in the control cells set as 1. MMP1 showed a 4.5-, MMP3 a 3.4-, uPA a 2.5- and integrin β3 a 3.2-fold downregulation compared to the negative control.

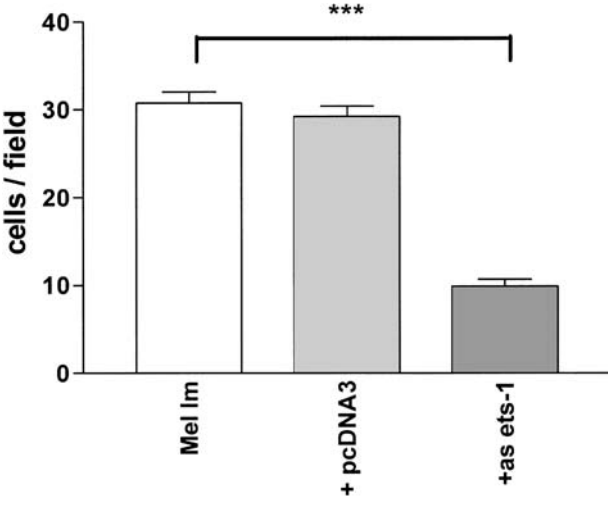

Figure 7. Influence of Ets-1 expression on melanoma cell invasion. Melanoma cells Mel Im were transiently transfected with 0.5 µg antisense Ets-1 expression plasmid (+as ets-1) or control vector (+pcDNA3). Invasive capacity was evaluated in the invasion assay after 2 days of culture. Invasion of the cells was inhibited in the Ets-1 reduced transfectants.

mal capillaries and fibroblasts of different tumors such as breast, lung, colon, ovarian and renal cancers [12, 13, 16, 32]. Well in line with these findings, we also observed Ets-1 expression in the stromal capillaries of melanomas during tumor vascularization. Stromal capillaries and fibroblasts carry out two major functions in the tumor stroma: the capillaries are necessary for tumor vascularization and for continuous tumor growth while the fibroblasts participate in tumor invasion by secreting various matrix-degrading proteases [for review see ref. 33]. Functional studies confirmed the roles of Ets-1 in these processes, as Ets-1 regulates the expression of several angiogenesis-related genes and various proteases involved in matrix degradation and tumor invasion [5, 18, 19, 20]. In this study, we report that Ets-1 is strongly expressed in human melanocytic lesions, including malignant melanoma, but not in melanocytes. Since the levels of Ets-1 expression correlate with the proliferative and/or migratory activity of melanocytic lesions, our results also suggest that Ets-1 plays a role in the development and aggressiveness of melanoma. While lentigo simplex lesions, the first stage of melanocytic nevus formation, were negative for Ets-1 expression, Ets-1 transcripts are upregulated in melanocytic nevi and strongly expressed in malignant melanoma, reaching maximum levels in hematogenous metastases. These results are also observed in vitro, where melanoma cells show strong Ets-1 expression while normal primary melanocytes do not express Ets-1. Over-expression of Ets-1 is consequently correlated with higher transcriptional activity, as assessed in vitro by electromobility shift assay, reporter gene assays and the Ets-1 antisense strategy. All these results confirm that Ets-1 is one of the major ETS factors in melanoma cells.

Interestingly, we found that melanoma cells express a previously unknown splice variant of Ets-1. Mammalian Ets-1 is expressed in two main variants, resulting from alternative splicing of exon 7 found in human [15, 17], rat [34] and mouse [unpublished results]. Unlike the full-length ETS-1, the splice variant missing exon 7 was shown to specifically induce colon cancer cell apoptosis by over-expression of caspase-1/ICE [29]. In contrast, we found that melanoma cells express a splice variant missing exon 6 in addition to the full-length Ets-1 protein. Whether the exon 6 alternative-spliced Ets-1 reported in

this study displays distinct properties not shared by the full-length Ets-1 and is relevant in melanocyte transformation remains to be investigated.

Since Ets-1 is not expressed in normal melanocytes, our results raise a number of questions regarding Ets-1 gene regulation during transformation. Although several inducers of Ets-1 expression are known, the regulation of the gene has not been addressed in detail. Ets-1 is expressed in endothelial cells of forming vessels throughout embryonic development but is not expressed in quiescent endothelial cells in adult tissues [13]. Ets-1 expression is induced in endothelial cells during the formation of new blood vessels in tumors, as well as in granulation tissue during wound healing, and after denudation of the aortic endothelium [13, 35]. In vitro, Ets-1 is expressed by proliferating and migrating endothelial cells, but not by confluent endothelial cells [13, 35]. Expression of Ets-1 is also increased by angiogenic factors in vitro [13, 20] and retinoic acid [36]. Several responsive elements have been localized in the promoter of the *ets-1* gene [15]. In correlation with these observations, the *ets-1* promoter is activated when cells are treated with serum or transfected with the AP-1 complex [36]. Ets-1 is also able to stimulate its own gene expression [37], as observed for the ETS family member, Fli-1 [38], possibly feeding a positive auto-regulatory loop once expression is started. In addition, the *ets-1* gene contains negative regulatory elements which repress expression [15]. The regulatory signals that mediate re-expression of Ets-1 in melanoma remain to be elucidated. Of particular interest will be to investigate whether induction of Ets-1 expression in melanoma cells is caused by over-expression of various activators of ets-1 or by loss of essential negative regulators during transformation.

As expected when detecting upregulation of a transcription factor, a number of Ets-1 target genes were found to be upregulated in melanoma cells. Initially, we were mainly interested in the genes encoding integrin β3, MMP1, MMP3 and uPA because these were known to be strongly upregulated in malignant melanoma [39]. Furthermore, we previously found several (uPA, MMP1 and MMP9) to be co-expressed with Ets-1 in the fibroblastic stroma of human lung, colon and breast cancers or upregulated in preinvasive cancer stages [12, 16]. Integrin β3, MMP1, MMP3 and uPA genes were found to be downregulated in melanoma cells using an antisense strategy inhibiting Ets-1 expression, further suggesting that Ets-1 is a main actor in transformation. A large number of other Ets-1 target genes which have not been analyzed in this study are expected to be upregulated as well, including transcription factors such as, for example, myc [40] and jun-B [41]. Thus, the re-expression of Ets-1 in melanoma cells could trigger a cascade of other gene activators which could then further participate in cellular transformation.

Several lines of evidence suggest that Ets-1 regulates cell migration and invasion. In vitro, Ets-1 is involved in scatter factor/hepatocyte growth factor-induced dispersion of MDCK epithelial cells [24]. Glioma cells show a reduced invasive phenotype after inhibition of Ets-1 expression using an antisense strategy or over-expression of a dominant negative Ets-1 mutant [42, 43]. Here, we found that inhibition of Ets-1 expression in melanoma cells induced a 51% reduction of the invasive potential of these cells compared to wild-type melanoma. In addition, we also found that the level of expression of Ets-1 reached its maximum in hematogeneous melanoma metastasis in vivo. All this suggests that Ets-1 could be used as a prognosis marker for this type of cancer as has been proposed for breast [44], oral [45], ovarian [46] and cervical cancers.

The findings presented in this study show that upregulation of Ets-1 contributes to melanoma progression by promoting the invasive properties of the neoplastic cells. Antisense inhibition of Ets-1 may therefore provide a potential and exciting tool for the treatment of this tumor entity.

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