Tbx3 Represses E-Cadherin Expression and Enhances Melanoma Invasiveness

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Abstract

The T-box transcription factors Tbx2 and Tbx3 are over-expressed in many cancers and in melanoma promote proliferation by actively suppressing senescence. Whether they also contribute to tumor progression via other mechanisms is not known. Here, we identify a novel role for these factors, providing evidence that Tbx3, and potentially Tbx2, directly repress the expression of E-cadherin, a keratinocyte-melanoma adhesion molecule whose loss is required for the acquisition of an invasive phenotype. Overexpression of Tbx2 and Tbx3 in melanoma cells down-regulates endogenous E-cadherin expression, whereas depletion of Tbx3, but not Tbx2, increases E-cadherin mRNA and protein levels and decreases melanoma invasiveness in vitro. Consistent with these observations, in melanoma tissue, Tbx3 and E-cadherin expression are inversely correlated. Depletion of Tbx3 also leads to substantial up-regulation of Tbx2. The results suggest that Tbx2 and Tbx3 may play a dual role during the radial to vertical growth phase transition by both inhibiting senescence via repression of p21CIP1 expression, and enhancing melanoma invasiveness by decreasing E-cadherin levels. [Cancer Res 2008;68(19):7872–81]

Introduction

The genetic model of malignant melanoma progression (1) suggests that melanoma initiates with the acquisition by melanocytes or their stem cells of a proliferative phenotype, usually by a gain of function of BRAF or NRAS kinases (2), combined with a suppression of stress or aberrant signaling-induced senescence (3–5). In general, senescence bypass involves a deletion of the CDKN2A locus or any alternative mutation that inactivates the p16INK4a/Retinoblastoma pathway (6, 7), to allow cell proliferation in the presence of activating mutations in BRAF or NRAS. However, a second phase of senescence (replicative senescence) can arise as a consequence of additional rounds of cell division and progressive telomere shortening, leading to up-regulation of p53, which in turn activates the expression of p21CIP1 (1). Intriguingly, as melanomas progress, they maintain high levels of p53, but p21CIP1 levels diminish (5), although the mechanism that uncouples p21CIP1 expression from p53 is unknown.

The transition from radial growth phase (RGP) to vertical growth phase (VGP) with the potential to metastasise is characterized by alterations in levels of adhesion molecules and, in particular, decreased E-cadherin expression that permits the escape of melanomas from keratinocytes (8). Although many of the genetic changes during melanoma progression have been well-documented, the molecular mechanisms involved in the regulation of RGP to VGP transition are poorly characterized.

Tbx2 and Tbx3 proteins are members of the T-box transcription factors family that play an essential role in cell proliferation, fate, and identity during development (9). Tbx2 and Tbx3 are transcription repressors (10–14) and share >90% identity within their DNA-binding domains (15), suggesting that they may regulate similar target genes (11, 13, 14, 16, 17). Significantly, Tbx2 and/or Tbx3 are overexpressed in several cancers including ovarian, breast, pancreatic, cervical, and in melanoma where Tbx2 is amplified in around 40% of cases (11, 14, 18–25). In part, their role in cancer may be related to their capacity to suppress senescence by repressing expression of p14ARF and p21CIP1 (11, 13, 14, 16–18, 26).

The fact that Tbx2 and Tbx3 are overexpressed in melanoma and can repress p21CIP1 expression by targeting histone deacetylase 1 to the p21CIP1 initiator, may indicate they have a role at the RGP/VGP transition, when p21CIP1 expression is down-regulated in the presence of high levels of p53 (5). Here, we show that Tbx3 also enhances melanoma invasiveness and directly represses E-cadherin expression, suggesting that Tbx3 may contribute to the RGP/VGP transition by down-regulating E-cadherin expression.

Materials and Methods

Cell lines and culture conditions. The human melanoma cell lines were cultured in RPMI supplemented with 10% fetal calf serum (SIGMA). The human melanocyte cell line, Hermes, was grown as described (7).

Western blot analysis. For Western blots, the primary antibodies used were as follows: goat polyclonal anti-Tbx3 (clone A:20; Santa Cruz Biotechnology), mouse monoclonal 62-2 anti-Tbx2 (developed in-house), mouse monoclonal anti–E-cadherin (BD Bioscience), mouse monoclonal anti–Fibronectin (BD Bioscience), mouse monoclonal anti-tubulin (clone B-5-1-2; SIGMA), rabbit polyclonal anti–extracellular signal-regulated kinase (ERK)-2 (Santa Cruz Biotechnology), mouse monoclonal anti-Flag M2 (SIGMA), and goat polyclonal anti-Lamin B (clone C:20; Santa Cruz Biotechnology). Quantification of Western blots was obtained by densitometric scanning.

Short interfering RNA. Depletion of endogenous Tbx3 and Tbx2 mRNA was achieved by using specific small interference RNA (siRNA) oligonucleotides targeting 5’-GACCATGGAGCCCAGAAGA-3’ (Tbx3 siRNA1), 5’-CACGCTCACCCTGAGTCAGCCA-3’ (Tbx3 siRNA2), 5’-GGTCCAAACTCGGCCGCTCT-3’ (Tbx2 siRNA1), and 5’TTCCTGGAATTCTGACGTTACG-3’ (Tbx2 siRNA2; Ambion) of the human coding sequences. The nonspecific siRNA oligonucleotide was directed against a 5’TTCCTGGAACGTGTCACGT-3’ target sequence. siRNAs were transfected into 30% confluent cell cultures using Oligofectamine (Invitrogen) following the manufacturer’s protocol.

Matrigel invasion assay. In vitro invasion assays were performed using the Chemicon cell invasion assay kit (Chemicon) following the recommended protocol. 501mel and Colo679 cells were transfected with siRNAs and incubated 48 h before seeding 1 to 2 × 105 cells into the Matrigel.

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chamber. Cells were allowed to migrate through the matrix for 16 h, and the invading cells were counted under the light microscope.

**Cell-cell adhesion assay.** To preserve the integrity of the cell-cell adhesion molecules, a single-cell suspension was prepared in the absence of trypsin with 3 mmol/L EDTA in PBS. Cells (1–2 × 10^6) were suspended in 5 ml complete medium with 1 mmol/L CaCl₂ (for Ca^2+-dependent cell-cell adhesion) and 3 mmol/L EDTA (for Ca^2+-independent cell-cell adhesion). Cell suspensions were plated on bacterial Petri dishes, incubated at 37°C on a shaking platform for 30 min, and clusters of >4 cells were counted from 10 different fields per dish.

**Reverse transcription-PCR.** Total RNA was made from confluent 501mel cultures. cDNA was synthesized using the AMV reverse transcriptase (Roche) following the manufacturers instructions. PCR reactions were performed using the primers 5'-CCCGAAGAAGAGGTGGAGGACGAC-3' and 5'-GCCTAGCACGGAGGAGGACGAC-3' for Tbx3; 5'-GGCTAGCACGGAGGAGGACGAC-3' and 5'-GCCCTTCGAGGAGGAGGACGAC-3' for Tbx2; 5'-TTTACCTTCCAGCAGCCCCCA3' and 5'-GAGATCTTCGAGGAGGACGAC-3' for Snail 1; and 5'-TTGACCTTGATCAGGAGGACGAC-3' and 5'-GAGATCTTCGAGGAGGACGAC-3' for Snail 2. As control primers, 5'-CCAACTGCTTCGCCCCCTGGCCAAG-3' and 5'-CTCCTTGGAGGCCATGTAGGCCATG-3' for G3PDH were used.

**Transfection experiments and luciferase measurements.** Thirty percent confluent 501mel culture were transfected using FuGENE 6 (Roche) following the manufacturers protocol. Thirty-six hours posttransfection, cells were harvested and luciferase activity was measured using the Luciferase reporter system kit (Promega).

**Electrophoresis mobility shift assays.** DNA-binding mobility assays were performed using purified GST-Tbx2 (1-416), GST-Tbx3 (1-501), and...
GST-Brachyury (1-274) proteins as described (27). The Consensus T-element located in the human E-cadherin promoter (5′-CTCATGGCTCACCT-GAAATCTTACG-3′) were 32P labeled as probes. Quantification of band shift results were obtained by densitometric scanning or using a phosphorimager when appropriate.

Chromatin immunoprecipitation and quantitative PCR. Chromatin immunoprecipitations (ChIP) were performed essentially as described (28). The Consensus T-element located in the human E-cadherin promoter (from −76 to +30 bp); 5′-CCCTGTTGCTCTCTGATTG-3′ and 5′-TGACGCTCACCTGAAATCTTACG-3′ for the transcription initiation region of the human E-cadherin promoter (4); and 5′-CCACCAAAGTCACGCTGAA-3′ and 5′-TGGCTCACACCTGAAATCCT-3′ flanking the half T-element on the E-cadherin promoter (from −647 to −546 bp); and 5′-AACAGTGCTTGGTACGTTG-3′ and 5′-CAAGCCTGG-GAGTTAGTG-3′ for the coding region of human E-cadherin as a negative control.

Quantitative reverse transcription-PCR analysis. Total RNA was isolated with TRIzol reagent (Life Technologies) according to the manufacturer instructions from frozen tissues. Tumor biopsies were classified as nevi and melanomas by routine anatomic-pathologic criteria. Frozen samples were analyzed for tumoral content, and RNA was extracted when the tumoral mass exceed 70% of the entire sample. RNA concentrations were determined by the absorbance at 260 nm. First-strand cDNA was synthesized using the M-MuLV RT (Finnzymes). Quantitative PCR was performed using the Sybr Green PCR Master Mix (Applied Biosystems), signals were detected using a 7,500 Fast Real-Time PCR System (Applied Biosystems), and data were analyzed with the AB Sequence Detection Software, Version 1.4 (Applied Biosystems). The sequences of primer sets used for quantitative reverse transcription-PCR (qRT-PCR) assays are as follows: Tbx2 forward 5′-TTCATGCATTCTTAACTTCAAGTTCG-3′ and reverse 5′-ATGCTGGTGGCTGGCATAT-3′; Tbx3 forward 5′-GCAGCCTTACACTGCTTC-3′ and reverse 5′-TGAGTTGCTGATGCCC-3′; and E-cadherin forward 5′-CCACCAAAGTCACGCTGAA-3′ and reverse 5′-TGTFGGATTCCACAGAAGC-3′. To avoid false-positive results due to amplification of contaminating genomic DNA in the cDNA preparation, we used primers spanning exon-exon junctions. Calibration curves were constructed and linearity was achieved for all primer sets at least in three orders of magnitude under the conditions used in the experiments. All PCR assays were done in triplicate and the data were pooled. Values were normalized to an endogenous reference (RPPO) gene within each sample and expression indicated as relative levels of mRNA, referred to a sample chosen to represent 1× expression of the gene.

Results

Tbx3 and Tbx2 are expressed in melanoma cell lines. Previous work has indicated that Tbx2 or Tbx3 may be overexpressed compared with melanocytes (22). However, no previous study has examined whether both may be coexpressed simultaneously in the same cell line. This is an important issue because although inhibition of Tbx2 function in melanoma cells lacking Tbx3 can lead to senescence (18), in cells expressing both factors, any pro-senescence therapy would need to target both Tbx2 and Tbx3. We therefore examined by Western blot using anti-Tbx2 and Tbx3 antibodies their expression profile in 12 melanoma cell lines (Fig. 1A, left). Tubulin was used as a loading control. The results show that there is a differential pattern of expression of Tbx3 and Tbx2. Whereas some (Colo800, Colo679, SK-Mel1, and 501mel) express both T-box transcription factors, the remaining lines express either factor alone (note that in this blot the 501mel track is underloaded), indicating that Tbx2 and Tbx3 expression are routinely regulated. Moreover, consistent with previous observations (22), 501mel and Colo679 cells overexpress Tbx3 compared with human melanocytes, (Fig. 1A, right) consistent with the fact that Tbx3 is up-regulated by BRAF (29). Although the degree of overexpression may be underestimated because melanocytes are cultured in the presence of 12-O-tetradecanoylphorbol-13-acetate that is known to up-regulate Tbx3 expression.

Endogenous Tbx3 promotes melanoma invasiveness and affects Ca2+-dependent cell-cell adhesion molecules. Previous studies have revealed that melanoma cells at the RGP-VGP transition down-regulate p21CIP1 expression (5). Because Tbx2 and Tbx3 can repress p21CIP1 expression (14, 17) and bypass senescence in melanoma (18), these factors may also play a role at this stage of melanoma progression, which is characterized by acquisition of an invasive phenotype. We asked therefore whether Tbx2 and/or Tbx3 might modulate the invasive capacity of melanoma cells. We initially used siRNA to deplete Tbx3 in 501mel and Colo679 melanoma cells, which express both Tbx2 and Tbx3, and asked whether its down-regulation would affect their invasive capacity using a Matrigel assay. Two different siRNAs that target Tbx3 mRNA were tested (results for 501mel cells shown), with siRNA2 being more effective as determined by Western blotting (Fig. 1B, left). EIRK2 was used as a loading control. Three days after transfection with Tbx3-specific or control siRNA, we seeded the cells into an invasion chamber and allowed them to migrate through the matrix for 16 h. The invasive cells were counted and the result of each experiment was normalized to the number of migrating cells in the control sample. The result, shown in Fig. 1B (right), revealed that in both cell lines, depletion of Tbx3 using either siRNA1 or siRNA2 significantly decreases melanoma invasiveness. Similar experiments were performed using Tbx2-specific siRNA, but no differences in invasive potential were observed (data not shown). The result suggests that although both factors are coexpressed in these cells, it is Tbx3, rather than Tbx2 that regulates invasiveness in this assay.

The capacity of Tbx3 to regulate invasiveness suggests that it is likely to control cell surface adhesion molecules. We therefore tested whether Tbx3 would also affect Ca2+-dependent or Ca2+-independent cell-cell adhesion. After treatment of Colo679 and 501mel cells with either Tbx3 siRNA or control siRNA, we performed an in vitro cell-cell adhesion assay. Figure 1C shows that no difference in the number of cell clusters was observed between the Tbx3-depleted and control populations when the cells were incubated in absence of Ca2+ (in the presence of EDTA). By contrast, when Ca2+ was added to the single-cell suspension, the Tbx3 siRNA-treated cells presented a 3- and 6-fold increase in aggregation compared with the control siRNA population in Colo679 and 501mel cells, respectively (Fig. 1D). The results suggest that Tbx3 represses the expression of Ca2+-dependent cell-cell adhesion molecules.

Tbx3 down-regulates both E-cadherin and Tbx2 expression in melanoma cells. During the RGP to VGP transition the pattern of cell-cell adhesion molecules on the potentially invading cells change. In particular, the repression of the Ca2+-dependent cell-cell adhesion molecule E-cadherin is a critical step in this transition (8). We therefore analyzed whether the depletion of endogenous Tbx3

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4 S. Prince, personal communication.
Figure 2. Tbx3 and Tbx2 repress the E-cadherin promoter. A, depletion of endogenous Tbx3 or Tbx2 using specific or control siRNAs from 501mel and Colo679 melanoma cells as indicated was followed by Western blotting using indicated antibodies or RT-PCR using primers specific for the indicated genes. ERK2 or tubulin are used as loading controls for the Western blots and G3PDH for the RT-PCR. Note that for the RT-PCR both isoforms of Tbx3 (+2a and −2a) are detected. B, 501mel cells were transfected with Flag-epitope–tagged expression vectors for Tbx2, Tbx2 (R122E, R123E), Tbx3, and Tbx3 (R130G, R131G). Twenty-four hours after transfection, cells were trypsinized to remove the E-cadherin protein from the cell membrane. A sample of nontransfected trypsinized 501mel cells was taken to monitor the efficiency of the E-cadherin degradation process, whereas the rest of the cell cultures were incubated for 36 h more before being harvested to obtain the respective whole cell extracts. A Western blot was performed and E-cadherin expression after trypsinisation was determined. Expression of WT and DNA-binding mutant (mut) T-box proteins was determined using anti-Flag mouse monoclonal antibody. 501mel cells transfected with an empty Flag expression vector was added as a negative control. Lamin B was used as a loading control. C, 501mel cells were cotransfected with 1.5 kb mouse E-cadherin promoter (from −1500 to +93 bp) luciferase reporter (0.9 μg) and increasing amounts of Flag-tagged Tbx2, Tbx3, and Snail expression vectors (0.3, 0.5, and 0.8 μg in a total amount of 1.7 μg of DNA). Each transfection was done in triplicate. A Western blot using anti-Flag antibody shows the relative expression of each Flag-tagged transcription factor (insert). D, the ability of WT and non–DNA-binding mutant Tbx3 and Tbx2 to repress the 1.5 kb E-cadherin promoter (0.3 and 0.5 μg of T-box expression vectors) was compared. Columns, mean of independent experiments; bars, SD.
from 501mel or Colo679 cells would affect the expression of E-cadherin.

501mel cells were therefore treated with control or Tbx3 siRNAs and assessed by Western blot for levels of E-cadherin and Tbx3 protein expression. The expression levels of tubulin, fibronectin, and Tbx2 were used as controls. Depletion of Tbx3 from 501mel cells resulted in a 4-fold increase in E-cadherin protein levels, whereas fibronectin and tubulin were unchanged (Fig. 2A, left). The increase in E-cadherin is consistent with the previous results showing that depletion of Tbx3 decreased invasiveness and Ca²⁺-dependent cell-cell adhesion. Surprisingly, depletion of Tbx3 also led to an up to 10-fold increase in Tbx2 expression, raising the possibility that Tbx3 is a repressor of the Tbx2 promoter. The potential for Tbx3 to repress Tbx2 may partly explain that in many of the melanoma cell lines tested (Fig. 1A), Tbx2 and Tbx3 are expressed in a mutually exclusive fashion. By contrast, depletion of endogenous Tbx2 failed to affect significantly the level of E-cadherin expressed, Tbx2 expression is substantially increased on depletion of Tbx3, and it was possible that high amounts of Tbx2 would be able to target the E-cadherin gene. To assess whether increasing Tbx2 and Tbx3 would lead to repression of E-cadherin by both proteins, we transfected 501mel cells with expression vectors for either Flag-tagged Tbx2 or Tbx3 and assessed the levels of endogenous E-cadherin by Western blotting. As a control, we used a non–DNA-binding mutant of Tbx2 in which two consecutive arginine residues (R122 and R123) of the T-domain that were substituted for glutamic acids (R122E and R123E mutations; refs. 12, 13) and an equivalent mutant in Tbx3. Note that the long half-life of E-cadherin means that a substantial decrease in the level of the endogenous RNA would not be reflected in rapid changes in protein level. To circumvent this problem, after transfection with the Tbx2 and Tbx3 expression vectors, cells were trypsinized to remove cell surface E-cadherin, and then assessed for its re-expression 24 hours later. In this case, repression of the endogenous E-cadherin promoter would lead to reduced de novo expression of E-cadherin. The results obtained (Fig. 3B) reveal clearly that ectopic expression of wild-type (WT) Tbx2 and Tbx3 inhibit the expression of E-cadherin in melanoma cells, whereas the non–DNA-binding mutants that were expressed to similar levels as their WT transcription factors (Fig. 2A, third panel). On the other hand, depletion of TBX3 mRNA led to 3-fold increase in both E-cadherin and TBX2 mRNA. No change was observed in the level of GAPDH expression. Consistent with these observations, depletion of Tbx3 in the Colo679 melanoma cells also resulted in up-regulation of E-cadherin expression (Fig. 2A, right) in agreement with the decrease in invasiveness observed in these cells on depletion of Tbx3.
counterparts, repressed less efficiently. Lamin B was used as a loading control.

The results obtained thus far indicate that depletion of Tbx3 leads to up-regulation of E-cadherin mRNA and protein expression, and that increased expression of both Tbx2 and Tbx3 will also down-regulate E-cadherin protein levels. To determine whether the regulation observed arose via targeting the E-cadherin promoter, we transfected 501mel cells with an E-cadherin promoter–luciferase (from −1,500 to +93 bp) reporter vector and either the Tbx2 or Tbx3 Flag-tagged expression vectors. An

Figure 4. Tbx3 binds to the transcription initiation region of the E-cadherin promoter in vivo. A, luciferase assay in 501mel cells using either the proximal human E-cadherin promoter (from −176 to +93 bp) or 1.5 kb full-length promoter driving the expression of the reporter gene (0.9 μg) and increasing amounts of Tbx2- and Tbx3-Flag expression vectors (0.5 and 0.8 μg in a total amount of 1.7 μg of DNA). Three independent transfections were performed. The protein levels of ectopically expressed Tbx3 and Tbx2 were compared by Western blotting using anti-Flag antibody and tubulin as a loading control (on the right of the graph). B, bandshift assay using purified GST-Tbx3 (1-501) and the human E-cadherin half T-element as a probe. Competition reactions were performed with the consensus T-element, the half T-element from the mouse E-cadherin promoter and its mutant version in which the element was substituted by a HinDIII site, in addition with the transcription initiation site of the same promoter and its corresponding HinD III mutated version (10, 50, and 250 ng of each competitor). C, the HinDIII mutations from B were introduced into the 1.5 kb mouse E-cadherin promoter-luciferase reporter vector and different combinations of mutant promoters were obtained (half T-element mutant promoter, transcription initiation region, and double mutant promoters). A luciferase assay was performed in 501mel cells with the different mouse E-cadherin promoter vectors (0.9 μg) together with the Tbx2- and Tbx3-Flag expression vectors (0.5 μg). Right, protein levels of each T-box transcription factor expressed during the reporter gene assay are presented on the right. D, quantitative real-time PCR analysis after Tbx3 immunoprecipitation from the chromatin of 501mel cells. ChIP was performed using a 10 μg of rabbit polyclonal anti-Tbx3 (Invitrogen) and IgG rabbit as a negative control. The recovered input served as a substrate for real-time PCR using a set of primers flanking the human E-cadherin transcription initiation region, the half T-element and as a negative control a pair of primers for the coding region of the gene.
expression vector for Flag-tagged Snail1, a known repressor of E-cadherin promoter activity (30, 31) was used as a positive control. Cotransfection of the E-cadherin–luciferase reporter with WT Tbx2 or Tbx3 expression vectors led to up to 12-fold repression of the E-cadherin promoter, whereas Snail1 repressed to a maximal 3.8-fold (Fig. 2C, left). Western blotting using anti-Flag antibody revealed that the Tbx2, Tbx3, and Snail1 proteins were expressed to similar levels (Fig. 2C, right). To verify that the repression observed required T-box factor DNA-binding, we also compared the WT and non-DNA-binding versions of Tbx2 and Tbx3 for their capacity to repress the E-cadherin promoter reporter. Compared with the 12-fold repression obtained using WT Tbx2, the mutant Tbx2 expression vector repressed a maximum 4-fold. For Tbx3, the WT and mutant proteins repressed maximal 9- and 5-fold, respectively (Fig. 2D). Note that although the R122/R123E mutants abolish binding of Tbx2 and Tbx3 in vitro, T-box factors can interact with a number of proteins/cofactors (12, 32–37) and the histone H3 NH₂-terminal tail that facilitate their recruitment to DNA (38). Interaction of the mutant T-box factors with any potential dimerization partners or histones could lead to some residual capacity for target gene repression.

**Tbx3 and Tbx2 bind to the E-cadherin promoter.** Although Tbx3 and Tbx2 clearly were able to repress E-cadherin expression, it was not evident whether this was a direct effect on its promoter. T-box factors are known to bind a consensus sequence known as the T-element comprising a palindromic AGGTGTGA motif (39), although in vivo known target sites tend to contain a single half T-element (10, 12–14). We therefore scanned the human and mouse E-cadherin promoters for consensus T-box factor binding sites. In the human promoter at −644 upstream from the transcription start site, we observed a full consensus half T-element, and the mouse promoter at also contained a similar sequence at −679. To determine whether these motifs were target sites for Tbx3 and Tbx2, we used a radiolabeled consensus T-element as a probe in a band shift assay together with bacterially expressed and purified GST fusion proteins (Fig. 3A) containing the NH₂-terminal regions of Tbx2 (aa 1–416), Tbx3 (aa 1–501), and Brachyury (aa 1–274), which contain the T-box DNA binding domain. DNA-protein complexes were competed using cold consensus T-element and the E-cadherin promoter half T-element. The results (Fig. 3B) revealed that all three proteins bound efficiently to the consensus T-element, but that for Tbx2 and Tbx3, the E-cadherin half T-element competed 5-fold less efficiently than the full consensus, consistent with the fact that the consensus T-element contains two T-box factor binding sites. No DNA binding was observed using GST alone (data not shown). In agreement with previous work that has shown that Brachyury has a strong preference for the full consensus compared with a half T-element (39, 40), Brachyury was competed substantially less well by the E-cadherin T-element motif than by the consensus. To confirm the binding of these factors to the E-cadherin promoter element, we performed similar assays using a radiolabeled E-cadherin binding element. In this case, in agreement with the competition studies, Tbx3 and Tbx2, but not Brachyury, bound well (Fig. 3C).

The presence of a half T-element within the mouse and human E-cadherin promoters, reinforced the notion that the promoter was a direct target for Tbx3 and potentially Tbx2. We next performed a luciferase assay using a truncated E-cadherin promoter (from −176 to +93 pb) in which the consensus T-element half site was absent. Surprisingly, the lack of an upstream T-box factor binding motif did not substantially affect the capacity of Tbx3 and Tbx2 to repress expression (Fig. 4A). This result indicated that additional, nonconsensus T-box factor target sites were likely to reside in the proximal promoter. Note that although the consensus sequence was identified for Brachyury, other T-box factors exhibit a range of binding specificities, and indeed, a substantial component of their ability to target DNA may reside in their interacting proteins and/or cofactors (12, 32–37). Preliminary DNA binding assays scanning

**Figure 5.** Expression of Tbx2, Tbx3, and E-cadherin in melanoma tissue. The expression levels of Tbx2, Tbx3, and E-cadherin mRNAs are differentially regulated during melanoma progression. Expression levels of the Tbx2, Tbx3, and E-CADHERIN genes were determined by qRT-PCR in nevi and melanoma samples. RPPO rRNA was used as an internal control. Expression levels are expressed relative to the Dysplastic Nevus sample chosen to represent 1/C2 expression of the gene. The data shown are the means of two independent experiments done in triplicate.
700 bp of the human and mouse E-cadherin promoters (data not shown) revealed a second putative target T-box factor—binding site close to the transcription initiation site. Experiments designed to characterize this binding motif in more detail are shown in Fig. 4B. Using the E-cadherin consensus half T-element as a probe, we performed a bandshift assay using purified GST-Tbx3 (1-501) protein. The result indicated that the consensus T-element and the E-cadherin half T-element compete very efficiently for the binding of purified Tbx3, whereas the mutant version of the half T-element fail to compete. Competition was also observed using an oligonucleotide spanning the E-cadherin initiator, although substantially less than the upstream element. Mutation of the initiator sequence abolished competition, indicating the specificity of the interaction.

To verify which elements were responsible for repression of the E-cadherin promoter by Tbx2 and Tbx3, we mutated either the consensus half T-element, the putative initiator binding site, or both and assessed their ability to be repressed by Tbx2 and Tbx3 in cotransfection assays. Note that the mutations introduced into the E-cadherin promoter corresponded to those used in the band shift assay shown in Fig. 4B. The results, Fig. 4C, reveal that mutation of the upstream consensus T-element failed to affect the repression by ectopically expressed Tbx2 or Tbx3, consistent with the results of the deletion analysis (Fig. 4A). By contrast, mutation of the initiator element, or the double mutation, reduced the ability of either Tbx3 or Tbx2 to repress the promoter. Both Tbx2 and Tbx3-Flag were expressed to similar levels (Fig. 5C, right).

The results of the point mutational analysis of the E-cadherin promoter suggested that like the previously identified targets for Tbx2, the Tyrp-1, p14ARF, and p21CIP1 promoters (10, 13, 14), repression might be mediated by binding to the initiator region. To verify that Tbx3 was bound to the E-cadherin initiator in vivo, we performed a ChIP assay using anti-Tbx3 antibody and sonicated chromatin from 501mel cells. The results (Fig. 4D) reveal Tbx3 bound to the initiator region of the E-cadherin promoter. No significant signal was detected either at the region containing the half T-element of the E-cadherin promoter or in the coding region. Similarly, no signal above background was observed using either no antibody or nonspecific IgG as controls. In agreement with luciferase assay results (Fig. 4D), the ChIP analysis showed that Tbx3 binds in vivo to the transcription initiation region of the human E-cadherin promoter rather than the upstream consensus half T-element. Using an anti-Tbx2 antibody, we were unable to obtain any signal above background (data not shown), consistent with the failure of siRNA-mediated depletion of Tbx2 to affect endogenous E-cadherin expression, although we cannot rule out the possibility that the Tbx2 antibody was simply inefficient in the ChIP assay.

Tbx3, Tbx2, and E-cadherin expression in melanoma tumors. The results suggest strongly that in cultured melanoma cells E-cadherin is repressed by Tbx3, leading to increased invasiveness. If the same held true in melanomas in vivo, we might expect to see an inverse correlation between Tbx3 and E-cadherin expression. In the absence of anti-Tbx2 and Tbx3 antibodies that work for immunohistochemistry on paraffin-embedded melanoma material, we sought to analyze mRNA expression levels for Tbx2, Tbx3, and E-cadherin using qRT-PCR. To this end, melanoma specimens were examined microscopically, and tumor mass containing a minimum 70% melanoma cells were used to extract RNA. The results of qRT PCR using primers specific for Tbx2, Tbx3, and E-cadherin are shown in Fig. 5 where the levels of gene expression are presented relative to that of the ribosomal protein gene RPP0 with the level obtained in a dysplastic nevus given the arbitrary value of 1. Although the sample number is small, the data obtained suggest that in melanoma in vivo, there is indeed an inverse correlation between E-cadherin expression and Tbx3 or Tbx2. Thus, the dysplastic nevus exhibits low Tbx2 and Tbx3 expression and expresses a high level of E-cadherin. Both primary tumors fail to express significant levels of Tbx2, although Tbx3 is expressed in both but at higher levels in the thicker tumor. By contrast, E-cadherin expression in the two primary melanomas is lower in the thicker tumor and inversely correlates with Tbx3 expression. A similar inverse correlation between Tbx3 and E-cadherin expression is observed in the metastases, with the subcutaneous metastasis expressing the lowest level of E-cadherin also expressing the highest level of Tbx3, whereas the lung metastasis exhibits the opposite pattern of Tbx3 and E-cadherin expression. Interestingly, in contrast to the primary tumors, Tbx2 is expressed in the metastases, with the lowest levels of Tbx2 also apparent in the lung metastasis that exhibits the most E-cadherin expression. The expression of E-cadherin in the lung metastasis is consistent with current models that suggest that microenvironmental factors will determine whether E-cadherin remains repressed or is re-expressed once cells have metastasized.

Although the sample size is relatively small, the results tend to suggest that in melanoma in vivo Tbx3 and E-cadherin expression is inversely correlated and are consistent with the cell based assays that indicate that Tbx3 can directly repress the E-cadherin promoter. Confirmation of these in vivo data will await the development of specific anti-Tbx2 and Tbx3 antibodies that will enable the expression of these factors to be correlated with E-cadherin expression at the single cell level.

Discussion

During the past decade, increasing evidence has related Tbx2 and Tbx3 expression with cancer progression. Overexpression and/or amplification of these T-box genes has been identified in ovarian carcinoma(23), pancreatic cancer (20), breast cancer (11, 21, 23, 24), uterine cervical cancer (25), and melanoma (14, 18, 19, 22). Ectopic overexpression of Tbx2 or Tbx3 results in the bypass of senescence in mouse embryo fibroblasts (11, 13) or striatal cells (16) in which direct repression of the p19ARF/p14ARF gene was the suggested mechanism. In melanoma cells, the CDKN2A locus (which encodes p19ARF/p14ARF) is frequently mutated or deleted (41), and Tbx2 (and potentially Tbx3) inhibits senescence by a mechanism that involves the repression of the p21CIP1 promoter (14, 17, 18).

Here, we provide evidence to suggest that Tbx3, and most likely Tbx2, contribute to the acquisition of an invasive melanoma phenotype. Thus, treatment of melanoma cells with Tbx3-specific siRNAs decreases the ability of the cells to migrate through a matrix in vitro and affects Ca2+-dependent cell-cell adhesion molecules. Moreover, overexpression of Tbx3 and Tbx2 down-regulate the expression of the Ca2+-dependent cell-cell adhesion E-cadherin protein and bind directly to the E-cadherin promoter in vitro and in vivo. The results raise the possibility that Tbx3 and Tbx2 may contribute to the acquisition of the invasive phenotype by repressing E-cadherin expression during the RGSP-VGP transition at the same time as they repress p21 expression. This notion is consistent with our qRT PCR data that suggest that in vivo, there is an inverse correlation between Tbx3 and E-cadherin expression.
The repression of E-cadherin by endogenous Tbx3 seems to be direct, although the nature of DNA recognition by members of the T-box family makes it difficult to unequivocally identify target sites in vitro. Thus, the crystal structure of Tbx3 and Brachyury indicates only two base-specific contacts per consensus half site (15, 40), with DNA target specificity most likely being dictated by a combination of interaction with sequence-specific DNA-binding proteins (12, 32, 34, 35), as well as specific chromatin determinants (38). Any in vitro DNA-binding assays performed in the absence of either chromatin, or the yet-to-be-identified interacting partners for Tbx2 and Tbx3, may give misleading results. For the E-cadherin promoter, two potential Tbx2 and Tbx3 binding motifs were identified. The upstream consensus half T-box element bound Tbx2 and Tbx3 efficiently in vitro but did not seem to play a role in regulating E-cadherin expression in transfection assays. However, we cannot rule out a role for this site in the chromosomal context where the chromatin assembled across the E-cadherin promoter would be more authentic, or at a specific stage of the cell cycle when Tbx2 (42) and Tbx3 are up-regulated. By contrast we found that weak in vitro binding to an element near the transcription initiation site, confirmed by ChIP assays, correlated to repression of E-cadherin by Tbx3. The position of this element close to the transcription initiation site is reminiscent of the location of the well-defined Tbx3. The position of this element close to the transcription initiation site is reminiscent of the location of the well-defined Tbx2 and Tbx3 binding sites at the initiators of the Tyrp1, p14ARF, p21CIP1 promoters (10, 13, 14, 17), although confirmation that this is indeed a binding site for T-box factors E-cadherin promoter and these factors play a major role in identification of Tbx2 and Tbx3-interacting DNA-binding cofactors.

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Unexpectedly we found that the treatment of 501mel cells with siRNA specific for Tbx3 leads to up-regulation of Tbx2 mRNA and protein levels, suggesting that Tbx3 represses the expression of Tbx2 in melanoma cells. Several previous studies provide evidence for hierarchical T-box gene regulation (43–46). In particular, in Tbx4 null embryos, the expression of Tbx2 is absent in allantois and limb development (43), whereas by contrast, Tbx20 null mouse embryos exhibit ectopic expression of Tbx2 in the developing heart (45). The ability of Tbx3 to regulate Tbx2 and their coexpression in several cell lines is particularly important for melanoma. We have shown previously that both Tbx2 (14, 18) and Tbx3 (17) can directly repress the p21CIP1 promoter and may act to prevent p53-mediated activation of p21CIP1 during melanoma progression. Strikingly, inhibition of Tbx2 (and Tbx3) leads to melanoma senescence (18). The fact that Tbx2 and Tbx3 can be coexpressed means that for any pro-senescence therapy to be effective, the activity of both factors would need to be targeted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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We thank Lionel Larue for the E-cadherin promoter-luciferase reporter vectors and Dorothy Bennett for the HERMES human melanocyte cell line.

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12. Halbert PE, Moorman AF, Clout DE, et al. Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression with DNA target specificity most likely being dictated by a combination of interaction with sequence-specific DNA-binding proteins (12, 32, 34, 35), as well as specific chromatin determinants (38). Any in vitro DNA-binding assays performed in the absence of either chromatin, or the yet-to-be-identified interacting partners for Tbx2 and Tbx3, may give misleading results. For the E-cadherin promoter, two potential Tbx2 and Tbx3 binding motifs were identified. The upstream consensus half T-box element bound Tbx2 and Tbx3 efficiently in vitro but did not seem to play a role in regulating E-cadherin expression in transfection assays. However, we cannot rule out a role for this site in the chromosomal context where the chromatin assembled across the E-cadherin promoter would be more authentic, or at a specific stage of the cell cycle when Tbx2 (42) and Tbx3 are up-regulated. By contrast we found that weak in vitro binding to an element near the transcription initiation site, confirmed by ChIP assays, correlated to repression of E-cadherin by Tbx3. The position of this element close to the transcription initiation site is reminiscent of the location of the well-defined Tbx2 and Tbx3 binding sites at the initiators of the Tyrp1, p14ARF, p21CIP1 promoters (10, 13, 14, 17), although confirmation that this is indeed a binding site for T-box factors E-cadherin promoter and these factors play a major role in identification of Tbx2 and Tbx3-interacting DNA-binding cofactors. In addition to Tbx3, both Slug and Snail also down-regulate the E-cadherin promoter and these factors play a major role in regulating E-cadherin expression in vivo (30, 31). However, the effect of Tbx3 on E-cadherin expression is unlikely to be mediated by Slug or Snail because the up-regulation of E-cadherin on depletion of Tbx3 was not accompanied by an increase in Slug or Snail expression. Nevertheless the fact that depletion of Slug/Snail or Tbx3 leads to increased E-cadherin expression suggests that both are likely to contribute to maintaining low levels of E-cadherin.

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