BRN2 is a transcriptional repressor of *CDH13* (T-cadherin) in melanoma cells

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T-cadherin (cadherin 13, H-cadherin, gene name *CDH13*) has been proposed to act as a tumor-suppressor gene as its expression is significantly diminished in several types of carcinomas, including melanomas. Allelic loss and promoter hypermethylation have been proposed as mechanisms for silencing of *CDH13*. However, they do not account for loss of T-cadherin expression in all carcinomas, and other genetic or epigenetic alterations can be presumed. The present study investigated transcriptional regulation of *CDH13* in melanoma. Bioinformatical analysis pointed to the presence of known BRN2 (also known as POU3F2 and N-Oct-3)-binding motifs in the *CDH13* promoter sequence. We found an inverse correlation between BRN2 and T-cadherin protein and transcript expression. Reporter gene analysis and electrophoretic mobility shift assays in melanoma cells demonstrated that *CDH13* is a direct target of BRN2 and that BRN2 is a functional transcriptional repressor of *CDH13* promoter activity. The regulatory binding element of BRN2 was located – 219 bp of the *CDH13* promoter proximal to the start codon and was identified as 5'-CATGCAAAA-3'. Ectopic expression of BRN2 in BRN2-negative/T-cadherin-positive melanoma cells resulted in suppression of *CDH13* promoter activity, whereas BRN2 knockdown in BRN2-positive/T-cadherin-negative melanoma cells resulted in re-expression of T-cadherin transcripts and protein. Transcriptional repression of *CDH13* by BRN2 may participate in malignant transformation of melanoma by increasing invasion and migration potentials of melanoma cells. The study has identified *CDH13* as a novel direct BRN2 transcriptional target gene and has advanced knowledge of mechanisms underlying loss of T-cadherin expression in melanoma.

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Loss of cell surface glycoprotein T-cadherin (H-cadherin, Cadherin-13, gene *CDH13*) has been implicated in the progression of various cancers.^{1–4} A role for T-cadherin as a tumor suppressor was first proposed in a study aimed at identification of genes differentially expressed in human breast cancer cells: T-cadherin transcript was found to be undetectable in all examined breast cancer cell lines as well as in cell lines derived from a variety of other cancers including neuroblastoma, lymphoma, renal carcinoma, squamous cell carcinoma, colon carcinoma, small-cell lung carcinoma, hepatocarcinoma and melanoma.⁴ In addition to a low frequency of *CDH13* loss of heterozygosity due to deletion of chromosome 16q24, *CDH13* promoter hypermethylation is a common phenomenon for inactivation of *CDH13* in several malignancies.^{5–8}

In a study on 48 different human melanoma cell lines, we found reduced or lost T-cadherin transcript and protein expression in $\approx 80\%$ of the cell lines.⁹ However, loss of T-cadherin transcript expression was not ubiquitously attributable to hypermethylation of CpG islands in the promoter region of *CDH13*.⁹ We performed bioinformatical analysis (using Genomatix software) and obtained hints for consensus binding sequences of BRN2 (POU3F2; N-Oct-3) in the proximal promoter region of *CDH13*. The POU (Pit, Oct1, Oct2, Unc 86) domain transcription factors are present in many cell lineages and are important coordinators of cell-fate decisions. BRN2 is present in normal melanocytes where it likely participates in coordination of the complex transcriptional events required for normal melanocytic development, differentiation and homeostasis.^{10,11} BRN2

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expression is upregulated in melanoma cell lines and in melanoma lesions where cells expressing high levels of BRN2 show enhanced invasive and metastatic capacity.^{12–16} Silencing of BRN2 in melanoma cell lines reduces proliferation, invasion and tumorigenesis.^{14–17} In contrast, silencing of T-cadherin in melanocytes increases invasive capacity, whereas ectopic re-expression in several T-cadherin-deficient melanoma cell lines reduces attachment-independent growth, migration and invasion *in vitro* and also their tumorigenicity *in vivo*.⁹ These data invoke some association between BRN2 and *CDH13*.

The POU family of transcription factors have a bipartite DNA-binding domain formed by the conserved POU-specific domain (POUs) and the POU homeodomain (POUh) that are joined by a linker region of variable length.^{18,19} The first POU-binding site to be characterized was the octamer sequence 5'-ATGCAAAT-3'.²⁰ However, the flexible nature of the linker region between the POU-s and POUh subdomains permits considerable diversity in binding sequence motif and target gene recognition by POU family members.²¹ For example, one typical POU3F2 consensus sequence described for the *Mitf-M* promoter in melanoma cells was defined as 5'-CATNNNTAAT-3'.¹⁷ In a genome-wide analysis of BRN2 promoter occupancy in the human melanoma cell line 501mel, 1700 potential target genes with one or more BRN2-binding sites in the proximal regulatory regions were identified.²² Overexpression and silencing studies in 501mel showed that, at least *in vitro*, only a small subset of the identified potential target genes is directly regulated by BRN2 (eg, *Itga4* encoding integrin $\alpha 4$, *Abcb1* encoding the ATP-binding cassette transporter P-glycoprotein, *CD36* encoding the thrombospondin receptor CD36, *Kitl* encoding Kit ligand and *Mitf-M* encoding Microphthalmia-associated transcription factor).²² BRN2 may be an activator for some genes (eg, *Itga4*, *CD36*, *Kitl*) and a repressor for others (eg, *Mitf-M*), depending on promoter context and interactions of BRN2 with coactivators or corepressors.^{10,17,22}

This study has identified *CDH13* as a novel BRN2-regulated target gene in melanoma cells. BRN2 binds to the regulatory element 5'-CATGCAAAA-3' at position -219 proximal of the ATG in the promoter region of *CDH13* and represses *CDH13* promoter activity.

MATERIALS AND METHODS

Cell Culture

The human melanoma cell lines 501mel, HMB2 and SKMel28 were derived from metastases of malignant melanomas. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (400 U/ml), streptomycin (50 μ g/ml), L-glutamine (300 μ g/ml) and 10% fetal calf serum (FCS) and split in a 1:5 ratio every 3 days. Long-term melanoma inhibitory activity (MIA)-deficient melanoma cell clones were generated by stable transfection of the melanoma cell line HMB2 with an antisense MIA cDNA expression construct. Two cell clones lacking melanoma

marker MIA expression (HMB2-5 and HMB2-8) were selected for further analysis. A cell clone expressing β -galactosidase (HMB2-lacZ) was used as a mock control to verify the specificity of the MIA-deficient melanoma cell clones HMB2-5 and HMB2-8. To shortly characterize the MIA-deficient cell clones: they form monolayer clusters in cell culture,²³ are pigmented²⁴ and express typical melanocytic genes including fibronectin, E-cadherin, SPARC, TRP1 (tyrosinase-related protein 1) and TYR (tyrosinase).

Azacytidine Treatment

Cells were plated at a low density (7.5×10^5 cells/T-75 flask). After 24 h, cells were further cultured for 72 h in the presence of 5 μ M 5-aza-2'-deoxycytidine (5-azaC; 300 nM or vehicle). Thereafter, medium was refreshed and cells further cultured for 4 h in the presence of 300 nM Trichostatin A (TSA, Sigma Aldrich, Steinheim, Germany) or vehicle. RNA was then harvested for quantitative real-time PCR (qRT-PCR).

Analysis of mRNA Expression by Quantitative PCR

Total cellular RNA was isolated from cultured cells using the RNeasy kit (Qiagen, Hilden, Germany). cDNAs were generated by reverse transcriptase reaction performed in 20 μ l reaction volume containing 500 ng of total cellular RNA. qRT-PCR was performed on a Lightcycler (Roche, Mannheim, Germany). cDNA template (500 ng), 0.5 μ l (20 mM) of forward and reverse primers and 2 μ l of Sybr-Green LightCycler Mix in a total of 20 μ l were applied to the following PCR program: 30 s at 95 °C (initial denaturation); 20 °C/s temperature transition rate up to 95 °C for 15 s, 3 s at 62 °C, 5 s at 72 °C, 81 °C acquisition mode single, repeated for 40 times (amplification). The PCR reaction was evaluated by melting curve analysis and checking the PCR products on 1.8% agarose gels. Oligonucleotide primers used in the PCR were as follows: *CDH13* for: 5'-GGCAATTGACAGTGCGAA CC-3' and *CDH13* rev: 5'-TGCAGGAGCACACTTGTACC-3'; *BRN2* for: 5'-GCGCGGTCCTTTAACCAGAGCGCC-3' and *BRN2* rev: 5'-CTGGTGAGCGTGGCTGAGCGGGTGC-3'.

Transfection Experiments

Cells were plated 2×10^5 cells/well into six-well plates and transfected with 0.5 μ g plasmid DNA using the lipofectamine plus method (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. *CDH13* promoter constructs were cloned as detailed previously.²⁵ The *Renilla* luciferase encoding plasmid pRL-TK (Promega, Mannheim, Germany) was used to control transfection efficiency. The murine BRN2 (mBRN2) expression vector was obtained from Addgene (Cambridge, MA, USA) and the pcDNA3 vector was from Invitrogen. Small interfering RNA (siRNA)-mediated down-regulation of BRN2 was achieved as previously described.¹⁴ BRN2-specific target sequence was BRN2 5'-GCGCAGAGCC UGGUGCAGGUU-3' and its complement, leaving a 3'-UU overhang on both strands (Sigma Aldrich). AllStars negative control siRNA was used for control (Qiagen).

Mutagenesis of the *CDH13* Promoter

Mutagenesis of the *CDH13* promoter was performed using the QuikChange[®] Site-Directed Mutagenesis Kit from Agilent Technologies (Waldbronn, Germany). Primers used for the **A** site (−126 to −157 bp) were as follows: *CDH13* mutated for 5'-CCAATGCTTTCGTGCTGCAGCTGCTGATCTA-3' (including a *Pst*I restriction site) and *CDH13* mutated rev 5'-TAGATCAGCAGCTGCAGCACGAAAGCATTGG-3'. Primers used for the **B** site (−237 to −210 bp) were as follows: *CDH13* mutated for 5'-CCGTATCTGCCATGTACAACGAGGGAGCG-3' (including a *Bsr*GI restriction site) and *CDH13* mutated rev 5'-CGCTCCCTCGTTGTACATGGCAGATACGG-3'.

Western Blotting

Cells (3×10^6) were lysed in 200 μ l RIPA-buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% N-P40, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitor) and incubated for 15 min at 4 °C. Insoluble fragments were removed by centrifugation at 13 000 r.p.m. for 10 min and supernatant lysates were immediately shock-frozen and stored at −80 °C. Lysates (40 μ g protein per lane) were resolved on 10% SDS-PAGE gels and blotted onto polyvinylidene difluoride (PVDF) membranes. Membrane blocking was achieved by incubation with 3% BSA/TBS for 1 h. The following primary antibodies were used: anti-T-cadherin (1 in 1000 dilution; Abcam, Cambridge, UK), anti-BRN2 (1 in 500 dilution, Santa Cruz Biotechnology, Heidelberg, Germany) and anti- β -actin (1 in 5000 dilution; Sigma Aldrich). Incubation with primary antibodies was for 16 h. After three washes with TBS, membranes were incubated for 1 h with an alkaline phosphatase-coupled secondary anti-mouse, anti-goat or anti-rabbit IgG in TBS/0.1% Tween (1 in 3000 dilution; NEB, Frankfurt am Main, Germany). Immunoreactive proteins were visualized by NBT/BCIP (Sigma Aldrich) staining.

Nuclear Extract Preparation and EMSA (Electrophoretic Mobility Shift Assay)

Cells were lysed by incubation (4 °C, 15 min) with Buffer I (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM DTT). After addition of NP-40 (final concentration of 0.1%), the lysates were centrifuged at 4 °C with 10 000 r.p.m. for 1 min. Nuclear pellets were collected and agitated (4 °C, 15 min) with Buffer II (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM DTT) and then centrifuged for 10 min at 13 000 r.p.m. at 4 °C. The supernatant nuclear extracts were N_2 shock-frozen. EMSA was performed using a probe spanning a putative consensus binding element in the *CDH13* promoter (see Figure 2c) with the sequence 5'-CGTATCTGCCATGCAAACGAGGGAGC-3' (−210 to −237 bp) (The bold alphabets signify the more precise consensus binding sequences of BRN-2 in the *CDH-13* promoter sequence.). The oligonucleotide (Sigma Aldrich) was radiolabeled with [γ -³²P]dATP using standard techniques. Binding reactions were performed in a 20- μ l volume reaction mixture containing $1 \times$ binding

buffer (25 mM HEPES, pH 7.9, 250 mM KCl, 25 mM DTT, 1 mM EDTA, 25 mM MgCl₂ and 50% glycerol), 1 μ g poly(dI-dC), 1 ng of radiolabeled probe and 6 μ g of nuclear extract for 20 min at 20 °C. Competition assay was performed using unlabeled octameric DNA consensus sequence oligonucleotide BRN2_1: 5'-TCTCGTGATGCAAATGTGCT-3', classical BRN2 consensus sequence BRN2_2: 5'-TGGTCCGAGATGAATTATTCATGAGCCTGGG-3'.²⁶ Goat anti-BRN2 antibody (Santa Cruz Biotechnology) and goat IgG were used for supershift experiments. Samples were loaded on to non-denaturing 4% (w/v) polyacrylamide gels. Gels were dried onto Whatmann paper and visualized by phosphor-imaging.

Measurement of Migration and Invasion

The xCELLigence System (Roche) is an innovative method based on measurement of electrical impedance and permits real-time analysis of migration and invasion. CIM plates (migration, invasion) were used and basic protocols recommended by the manufacturer were followed. Top chambers were coated with or without Matrigel for measurement of invasion or migration, respectively. The bottom chambers contained culture supernatant from human fibroblasts as chemoattractant. Upper chambers contained serum-free DMEM. After recording background impedance, cells suspended in serum-free DMEM were added to the upper chambers (4×10^3 /well for migration; 2×10^4 /well for invasion). Thereafter, impedance was measured continuously over 24 h. Impedance is represented by the relative and dimensionless parameter named cell index (CI). CI values = $Z_i - Z_0 / 15(\text{Ohm})$, where Z_0 is impedance at the start of the experiment and Z_i is impedance at individual time points during the experiment. The normalized cell index (NCI) was calculated as the cell index CI_{ti} at a given time point (ti) divided by the cell index CI_{nml_time} at the normalization time point (nml_time). The slope is used to describe the steepness of a curve within a given time window (in our case 5 h).

Immunohistochemistry of Melanoma Tissue Microarray

The immunohistochemical analysis in this study was approved by the medical ethical committee of the University of Regensburg, and as prescribed, patient identity and data privacy was protected. Formalin-fixed, paraffin-embedded human melanoma biopsies were obtained from the Institute of Pathology, University Hospital Regensburg, Germany. A tissue microarray (TMA) was constructed as described before.²⁷ Sections were cut at 4 μ m. Immunohistochemistry protocols have been detailed previously.⁹ Primary antibodies used in this study were polyclonal goat anti-BRN2 antibody (1:50; Santa Cruz Biotechnology) and anti-T-cadherin antibody (1:50; Sigma-Aldrich, St Louis, MO, USA). For negative controls, primary antibodies were replaced by buffer (data not shown). Hematoxylin was used as counterstain.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's instructions

(ChIP-IT™ Express Magnetic Chromatin-Enzymatic Shearing Kit, Active Motif, La Hulpe, Belgium). Chromatin isolation was performed using 501mel cells and for immunoprecipitation 10 µg goat anti-BRN2 polyclonal IgG (Santa Cruz Biotechnology) or rabbit anti-BRN2 polyclonal IgG (Cell Signaling, Danvers, MA, USA) were used. The Kit includes appropriate nonimmune IgG as negative control and RNA polymerase II (PolII) IgG (ChIP-IT control Kit-human; Active Motif). Altogether, five precipitated DNA templates (input DNA (1:5), anti-IgG negative control, anti-BRN2 (Santa Cruz Biotechnology), anti-BRN2 (Cell Signaling) and anti-PolII) were subjected to amplification by PCR. PCR fragments were analyzed on a 2% agarose gel. The following primers were used for the PCRs: the human *Mitf-M* promoter region served as positive control:¹⁷ for 5'-CAAACCTCGTAGGGCTTCCAA-3' and rev 5'-CCACCGGAAACTTTATCACAG-3'; the human *HSP70* promoter region served as negative control:¹⁷ for 5'-CCTCCAGTGAATCCCA GAAGACTCT-3' and rev 5'-TGGGACAACGGGAGTCACTC TC-3'; the *CDH13* promoter region B: for 315 5'-GAGG CTGAGCCCCAACAGTCC-3' and rev 232 5'-GCTCCCTCG TTTTGCTGGCAG-3'.

Statistical Analysis

All experiments were performed on at least three independent occasions. Unless otherwise stated, results are given as mean ± s.d. Comparison between groups was made using Student's paired *t*-test. All calculations were performed using the GraphPad Prism software (GraphPad software, San Diego, CA, USA). A *P*-value of <0.05 was considered significant.

RESULTS

CDH13 and BRN2 Expression

In a previous study that used methylation-specific PCR to investigate association between *CDH13* promoter methylation and loss of T-cadherin transcript expression in melanoma, we found that not all the examined melanoma cell lines showed loss of *CDH13* through hypermethylation.⁹ Here, three exemplary melanoma cell lines lacking T-cadherin transcript expression (SKMel28, HMB2 and 501mel) were sequentially treated with methyltransferase inhibitor 5-azaC and histone deacetylase inhibitor TSA. This treatment led to induction of T-cadherin transcript expression in SKMel28, whereas HMB2 and 501mel were unaffected (Figure 1a). We questioned what might explain *CDH13* inactivation in HMB2 and 501mel, if not hypermethylation.

Bioinformatical analysis of 2300 bp of the *CDH13* promoter using Genomatix software predicted, among others, binding sites for the transcription factor BRN2, leading us to consider that *CDH13* might be a transcriptional target for BRN2. Comparison of endogenous BRN2 expression in SKMel28, HMB2, 501mel and NHEMs (normal human epithelial melanocytes) showed that BRN2 transcript (Figure 1b) and protein levels (Figure 1c) were highest in

HMB2. Levels of BRN2 transcript expression in 501mel and SKMel28 were similarly low (Figure 1b), but surprisingly BRN2 protein expression was not detectable in SKMel28 (Figure 1c). T-cadherin protein was present in NHEM (predominantly the mature 105 kDa protein) and almost undetectable in the melanoma cell lines (Figure 1c).

The combined observations of Figure 1a–c prompted us to use HMB2 as well as HMB2-derived cell clones (HMB2–5 and HMB2–8), previously established and characterized in our laboratory,^{23,24} as model cells for further investigations on transcriptional regulation of *CDH13* by BRN2 in melanoma. HMB2–5 and HMB2–8 cell clones are MIA deficient and have a phenotype comparable to melanocytes (see description in Materials and Methods).

Analysis of T-cadherin and BRN2 expression levels revealed an inverse correlation between the two: compared with the parental cell line HMB2 and the control HMB2-lacZ clone, cell clones HMB2–5 and HMB2–8 exhibited very low BRN2 expression and markedly elevated T-cadherin expression, evident at the level of mRNA (Figure 1d) and protein (Figure 1e). These data point toward some relationship between T-cadherin and BRN2.

CDH13 Regulation Through BRN2

To directly test the association between T-cadherin and BRN2 expression, we selected T-cadherin-negative HMB2 and 501mel melanoma cell lines and transfected them with BRN2-targeted siRNA (siBRN2) or control siRNA (sicontrol). Successful silencing of BRN2 was confirmed by qRT-PCR and western blot analysis in 501mel (Figure 2a and b) and by qRT-PCR in HMB2 (Supplementary Figure 1A). T-cadherin expression was upregulated in siBRN2-transfected 501mel (Figure 2a and b) and HMB2 (Supplementary Figure 1A) as compared with their respective sicontrol transfectants. Using the T-cadherin-positive HMB2–5 cell clone, we performed a reverse experiment and overexpressed mBRN2. Here, we detected a repression of T-cadherin expression in qRT-PCR (Figure 2c) and western blot (Figure 2d) analysis.

Next, we performed reporter gene analysis of *CDH13* with seven serially deleted fragments of different lengths within –2304 bp from the translation start site cloned into pGL3basic vector (Figure 3a). The first series of reporter assays were conducted using 501mel because of good transfection efficiency in these cells. 501mel were co-transfected with the deletion fragments and either siBRN2 or sicontrol. Luciferase assays (Figure 3b) demonstrated that transfection with the shortest fragment (–99 bp) very slightly increased reporter activity that was unchanged by BRN2 silencing, ruling out the presence of any BRN2-binding site within the first –99 bp. Transfection of the cells with a –173-bp fragment further increased general reporter activity, but this was also not affected by silencing of BRN2. In cells co-transfected with a –285-bp fragment and siBRN2, reporter activity was maximal and twofold above that of the sicontrol-

Figure 1 Inverse correlation between BRN2 and T-cadherin expression. **(a)** qRT-PCR for the expression level of T-cadherin mRNA following treatment with 5 μ M 5-aza-deoxycytidine for 72 h, followed by 300 nM TSA for 4 h. The results are standardized to housekeeper gene β -actin. Control treated parental cell lines SKMel28, 501mel and HMB2 were set as 1. **(b)** qRT-PCR for BRN2 transcript expression in the melanoma cell lines SKMel28, 501mel and HMB2 compared with normal human epidermal melanocytes (NHEMs) in passage 4 (p4). The results are standardized to housekeeper gene β -actin. NHEM was set as 1. **(c)** Western blot analysis of the protein level of T-cadherin and BRN2 in the melanoma cell lines SKMel28, 501mel, HMB2 and melanocytes (NHEMs). β -Actin was used as loading control. **(d)** qRT-PCR of HMB2 cell clones (HMB2-5 and HMB2-8) for expression of T-cadherin and BRN2 mRNA. HMB2-5 and HMB2-8 were compared with the parental cell line HMB2 and the control HMB2-lacZ. The results are standardized with the housekeeper β -actin. HMB2 was set as 1. **(e)** Western blot analysis of whole-cell lysates from the HMB2 cell clones HMB2-5, HMB2-8, and HMB2-lacZ with antibodies against T-cadherin and BRN2. β -Actin was used as loading control.

transfected cells. Silencing of BRN2 in cells transfected with the -373-bp reporter fragment also resulted in a significant increase of reporter activity compared with that in sicontrol cells. These data suggest that the region of BRN2 binding is located within the first 373 bp of the *CDH13* promoter proximal to the start codon. Transfection with all further

deletion fragments up to -2304 bp resulted in decreased reporter activity either without or with BRN2 silencing, suggesting that alternative transcriptional-repressor-consensus-sequences may be encoded in the following ~1900 bp.

Two possible consensus sequences for BRN2 binding (5'-ATGCTG-3', mentioned as A and 5'-CATGCAAAA-3',

Figure 2 Involvement of BRN2 in *CDH13* regulation. (a) qRT-PCR for BRN2 and T-cadherin transcript expression after transfection of 501mel with BRN2 (POU3F2)-targeted siRNA (siBRN2) or control siRNA (sicontrol). qRT-PCR shows increase expression of T-cadherin mRNA after BRN2 downregulation. The results are standardized to housekeeper β -actin. ** $P < 0.01$ compares siBRN2 and sicontrol. (b) 501mel was transfected with siBRN2 and sicontrol and western blot analysis was performed using antibodies against T-cadherin and BRN2. Knockdown of BRN2 was accompanied by re-expression of T-cadherin protein. NHEMs (melanocytes) served as control for T-cadherin expression. β -Actin was the loading control. (c, d) The HMB2-5 cell clone was transfected with mouse Brn2 (mBRN2) and pcDNA as control and qRT-PCR and western blot analysis were performed. Overexpression of BRN2 was accompanied by loss of T-cadherin expression. The D04 cell line, overexpressing T-cadherin, served as control for T-cadherin protein expression. β -Actin was the loading control in the western blot. The results of the qRT-PCR are standardized to housekeeper β -actin.

mentioned as B) were detected in the first 373 bp of the *CDH13* promoter (Figure 3c).

Transcriptional Regulation of *CDH13* Through the Promoter Region – 373 bp Upstream of the Start Codon

Knowing that a possible BRN2 binding site is located within the *CDH13* promoter region of – 373 bp, we focused on this region. Reporter assays demonstrated that *CDH13* promoter activity of the region – 285 to – 373 bp was higher in BRN2-negative HMB2-5 and HMB2-8 than in the BRN2-positive HMB2 and HMB2-lacZ (Figure 4a). We also validated the reporter activity of fragment – 373 bp after knockdown of BRN2 in the parental cell line HMB2. The – 173 bp promoter fragment is apparently without a BRN2-binding sequence (see Figure 3b) and was used as a ‘negative’ control for *CDH13* promoter activity. This fragment showed no transcriptional activation after BRN2 knockdown (Figure 4b), whereas reporter activities of the – 285 and – 373 bp fragments of the *CDH13* promoter were activated (Figure 4b). Overexpression of mBRN2 in HMB2-5 resulted in decreased reporter activities of the – 285 and – 373 bp fragments (Figure 4c), confirming functionality of BRN2 in repressing *CDH13* promoter activity.

The biological consequence of mBRN2 overexpression in HMB2-5 was investigated using the xCELLigence system. Both invasion and migration were increased after overexpression of BRN2 (Figure 4d). This is consistent with our previous observations that siRNA-mediated knockdown of T-cadherin increases invasive potential of melanocytes. BRN2-dependent *CDH13* promoter regulation might therefore be assumed to be a functional participant in the mechanisms determining migration and invasion of melanoma cells.

Direct Binding of BRN2 to the *CDH13* Promoter

Having detected two potential consensus sequences (5'-ATG CTG-3', mentioned as A and 5'-CATGCAAAA-3', mentioned as B) for BRN2 binding within the first – 373 bp of the *CDH13* promoter proximal to the start codon (see Figure 3c), we investigated their functionality in BRN2 binding. First, we mutated both sequences in the luciferase vector and performed reporter assays in HMB2 and HMB2-lacZ (Figure 5a). Mutation of potential binding site A had no effect on reporter activity, whereas mutation of potential binding site B resulted in activation of reporter activity, indicating inhibition of BRN2 binding and thereby prevention of

Figure 3 Promoter of *CDH13*. (a) Illustration of the deletion fragments of the *CDH13* promoter cloned into pGL3basic. (b) Luciferase assays were performed after co-transfection of 501mel with sicontrol or siBRN2 and with various deletion fragments of the *CDH13* promoter sequence cloned into pGL3basic. A total of 100 ng of pRLTK transfection was used as internal control. pGL3basic activities in sicontrol or siBRN2 were set as 1. ** $P < 0.01$, *** $P < 0.001$ compares siBRN2 and sicontrol. (c) Illustration of the *CDH13* promoter sequence with regulatory elements for BRN2 binding named as **A** (5'-ATGCTG-3') and **B** (5'-CATGCAAAA-3').

transcriptional repression (Figure 5a). This is in accordance with the result of Figure 2d where it was shown that the region > 173 bp is under the control of BRN2.

To determine physical interaction between regulatory element **B** and BRN2, we performed EMSA using nuclear extracts from HMB2-5 and HMB2-8 and a radiolabeled oligonucleotide designed from the *CDH13* promoter and spanning the consensus sequence **B** (-210 to -237 bp; see Materials and Methods). Nuclear extracts from HMB2-5 and HMB2-8 transfected to overexpress (mouse) mBRN2 were also tested. EMSA revealed three striking nucleoprotein complexes on the *CDH13* oligonucleotide, each of which was more prominent in cells overexpressing mBRN2 (Figure 5b) indicating binding of mBRN2 to regulatory element **B**. Binding *in vivo* was confirmed using a ChIP assay in which a specific signal was reproducibly detected using two different anti-BRN2 antibodies (Figure 5c). As additional experimental controls to those provided by the kit (nonimmune IgG and anti-PolII IgG), we tested for BRN2 binding to the promoter sequences of *Mitf-M* (positive) and of *HSP70* (negative), as previously published by Goodall *et al*¹⁷

(Figure 5c). To further confirm binding *in vivo* we performed EMSA in which nuclear extracts of NHEMs from two different human donors, parental HMB2, HMB2-5, HMB2-8 cell clones and 501mel were tested for binding to the -210 to -237 bp oligonucleotide. Figure 5d demonstrates markedly more prominent nucleoprotein complex formation in extracts from HMB2 and 501mel (BRN2-positive/T-cadherin-negative) compared with extracts from NHEM, HMB2-5 and HMB2-8 (BRN2-negative/T-cadherin-positive).

To validate BRN2-specific nucleoprotein complex formation competition, EMSAs were performed using nuclear extracts from parental HMB2 (Figure 5e), the radiolabeled *CDH13* oligonucleotide (-210 to -237 bp) **B**, and either of two unlabeled consensus sequence oligonucleotides BRN2_1 and BRN2_2 (see Materials and Methods) or unlabeled *CDH13* oligonucleotide (-210 to -237 bp) **B** as competitors. Competition was visible for the classical octamer binding sequence (BRN2_1), and the unlabeled *CDH13* oligonucleotide. The oligonucleotide BRN2_2 (specific for BRN2 binding) displaces the bands 1, 2 and 3. For a more precise identification of the different bands arising with

Figure 4 Involvement of BRN2 in *CDH13* promoter suppression and cell function. (a) Luciferase assays were performed with HMB2, HMB2-5, HMB2-8 and HMB2-lacZ following transfection with various deletion fragments of the *CDH13* promoter sequence cloned in pGL3basic. For each deletion fragment tested HMB2 was set as 1. $^{**}P < 0.01$, $^{***}P < 0.001$ compares HMB2-5 and HMB2-8 with the parental cell line HMB2. (b) Luciferase assays were performed after co-transfection of HMB2 with sicontrol or siBRN2 and various deletion fragments of the *CDH13* promoter sequence cloned into pGL3basic. pGL3basic activities in sicontrol or siBRN2 were set as 1. $^{**}P < 0.01$ compares siBRN2 and sicontrol. (c) Luciferase assays were performed after co-transfection of HMB2-5 with pcDNA3 or mBRN2 and various deletion fragments of the *CDH13* promoter sequence. pGL3basic activity was set as 1. $^{**}P < 0.01$ compares pcDNA3 and mBRN2. (d) Invasion and migration potentials of HMB2-5 transfected with mBRN2 or control pcDNA3 (set at 100%) were analyzed using the xCELLigence System (see Materials and Methods). $^{*}P < 0.05$ compares pcDNA3- and mBRN2-transfected cells.

CDH13 element B, an antibody specifically directed against BRN2 (Santa Cruz Biotechnology) was included in the mixture. This resulted in a drastic decrease of the BRN2/DNA complex formation of band 3. The remaining bands possibly reflect the binding of further transcription factors of the POU family.

Taken together, these data support that BRN2 binds to the regulatory element 5'-CATGCAAA-3' in the promoter region of *CDH13* and is likely responsible for silencing of *CDH13* promoter activity.

Possible *In Vivo* Relevance of Transcriptional T-Cadherin Repression by BRN2

We have previously demonstrated the occurrence of T-cadherin protein and transcript expression downregulation in human tumor melanoma tissues and cell lines.⁹ Here we performed an immunohistochemical analysis of BRN2 and of T-cadherin expression in consecutive sections of a small TMA comprising human primary and metastatic melanomas. Representative images are shown in Figure 6a. Scoring of BRN2 staining in the tissue samples was adjusted on

Figure 5 Direct transcriptional regulation of *CDH13* through BRN2. (a) *CDH13* promoter fragment – 373 bp proximal to the start codon was mutated in the putative BRN2-binding elements **A** (5'-ATGCTG-3') and **B** (5'-CATGCAAA-3'). Promoter activity was measured by luciferase assay after transfection of HMB2 and HMB2-lacZ with native (promoter activity set at 1) and mutated fragments of the T-cadherin promoter. (b) EMSA was performed with nuclear extracts from HMB2-5 and HMB2-8 and after transient transfection with pcDNA3 and mBRN2. The radiolabeled oligonucleotide has the sequence 5'-CGTATCTGCC**CATGCAAA**ACGAGGGAGC-3'. (c) Agarose gel after ChIP using the indicated antibodies from Santa Cruz Biotechnology (SC) and Cell Signaling (CS), respectively. As negative control, an IgG antibody was used. As positive control, a RNA Polymerase II (PolII) antibody was used. The primers were specific for *Mitf-M*, *HSP70* and *CDH13* element **B**. (d) EMSA was performed with nuclear extracts from HMB2-5, HMB2-8, 501mel, HMB2, SKMel28 and NHEM from two different donors. The radiolabeled oligonucleotide has the above-mentioned sequence of element **B** (5'-CGTATCTGCC**CATGCAAA**ACGAGGGAGC-3'). (e) Competition EMSA performed with nuclear extracts from HMB2. The transcriptional binding was quenched with nonlabeled classical sequences BRN2_1 and BRN2_2, (see Materials and Methods) and with nonlabeled oligonucleotide *CDH13* **B**. The specific binding of BRN2 (band 3) was shown by using BRN2 antibody (1 μg/6 μg nuclear extract; Santa Cruz Biotechnology) in a competition assay.

T-cadherin-negative tissues (Figure 6b). Of the 13 primary melanomas, 11 were T-cadherin negative, and 7 of these 11 T-cadherin-negative tissues stained positive for BRN2. All 14

melanoma metastases were T-cadherin negative and 9 of these 14 stained positive for BRN2. Scoring of the immuno-histochemical staining outcome in both primary and meta-

Figure 6 (a) Representative immunohistochemistry images of a TMA with 13 primary melanoma tissues and 14 melanoma metastases. The analysis was performed with anti-T-cadherin and anti-BRN2 antibodies. (b) Graphical scoring of the immunohistochemistry shown in **a** according to T-cadherin-negative tissue samples (in summary (Σ), 11 primary melanomas and 14 melanoma metastases were stained).

Figure 6 Continued.

static melanoma tissues (Figure 6b) implies that approximately half of the tumors might be T-cadherin negative because of suppressive regulation via expression of BRN2.

DISCUSSION

The promoter of *CDH13* lacks a TATA-box similar to other cadherins such as E-cadherin and P-cadherin. The proximal 5'-flanking region contains a CAAT-box at -154 bp and two inverted CAAT-boxes at -179 and -450 bp from the translation start site. Although many studies⁵⁻⁸ including our own⁹ have demonstrated anomalous hypermethylation of *CDH13* promoter in various cancers, this does not ubiquitously account for loss of T-cadherin expression. In this and our previous⁹ investigation on melanoma, DNA demethylation and histone deacetylase inhibition failed to induce T-cadherin re-expression in all melanoma cell lines examined. Therefore, and as is well known for E-cadherin, where methylation of the *E-cadherin* promoter is not always correlated with silencing,^{28,29} additional genetic or epigenetic modifications likely also contribute to T-cadherin down-regulation. The zinc-finger transcription factors Snail,³⁰ Slug and ZEB1³¹ are potent repressors of E-cadherin expression in melanoma. There are only few investigations on transcriptional regulation of *CDH13*. In gallbladder cancer cells, the transcriptional regulator ZEB1 was shown to repress *CDH13* expression in an E-box-like sequence-dependent fashion.³² In rat vascular smooth muscle cells, location of an AHR (aryl hydrocarbon)-responsive element (GCGTG) at -45 bp was putatively correlated with AHR-ligand-dependent repression of *CDH13*.³³ Another study on vascular smooth muscle cells reported downmodulation of T-cadherin protein in response to growth factors such as platelet-derived growth factor, epidermal growth factor or insulin-like growth factor, but transcriptional mechanisms were not investigated.³⁴ In endothelial cells, a requirement of thioredoxin-1 in redox-sensitive modulation of T-cadherin expression was described and a thioredoxin-1-regulated region between -156 and -203 bp upstream of the translational start site of *CDH13* was identified.²⁵ In osteosarcoma cells, analysis of the 1500 bp proximal 5'-flanking region of *CDH13* identified

potential binding sites/consensus sequences for several transcription factors, including AHR, the glucocorticoid receptor, progesterone receptor and estradiol receptor.³⁵

Theoretical analysis of *CDH13* promoter using Genomatix software showed that at least 44 different transcription factors may bind to the first -380 bp of the *CDH13* promoter proximal to the start codon. They include ETS-1, SOX9, STAT6, CREB, the TWIST/SNAIL family, the LEF/TCF family and BRN2, which are transcription factors already identified as being relevant to melanoma progression. We have excluded SNAIL and ETS-1 as responsible factors for regulating *CDH13* expression (data not shown). This study focused on whether *CDH13* promoter activity might be under the control of transcription factor BRN2, based on the *in silico* detection of consensus binding sequences of BRN2 in the proximal promoter region of *CDH13*, on observations of increased levels of BRN2¹²⁻¹⁶ and decreased levels of T-cadherin⁹ expression in melanomas, and on observations of opposing effects of BRN2¹²⁻¹⁶ and T-cadherin⁹ on melanoma cell behavior.

Our results support that *CDH13* is a target gene for BRN2 in melanoma cells. The regulatory binding element of BRN2, located -219 bp of the *CDH13* promoter proximal to the start codon, was identified as 5'-CATGCAAAA-3'. Ectopic expression of BRN2 in BRN2-negative/T-cadherin-positive melanoma cells (HMB2-5 and HMB2-8) resulted in suppression of *CDH13* promoter activity, whereas BRN2 knock-down in BRN2-positive/T-cadherin-negative melanoma cells (HMB2 and 501mel) resulted in re-expression of T-cadherin transcripts and protein. Therefore, BRN2 is a *bona fide* functional transcriptional repressor of *CDH13* promoter activity in melanoma cell lines. Such BRN2-dependent repression of *CDH13* promoter activity could represent a mechanism of T-cadherin expression regulation in melanoma progression alternative to that of epigenetic silencing by *CDH13* promoter hypermethylation.⁹

Modulation of the properties of melanoma cells by BRN2 is complex. One major mechanism proposed to account for the proliferative and proinvasive functions of BRN2 in melanoma cells is through regulation of the melanocyte lineage determining transcription factor MITF (microphthalmia-associated transcription factor) that acts as a master regulator of melanocyte development, function and survival by modulating various differentiation and cell-cycle progression genes.³⁶ BRN2 represses *Mitf-M* in melanoma cells through binding directly to the proximal internal promoter driving expression of the MITF-M isoform.¹⁷ Another mechanism by which BRN2 may enhance the motile and malignant properties of melanoma cells is through regulation of the cGMP-specific phosphodiesterase PDE5A.³⁷ BRN2 binds to two sites in the *PDE5A* proximal promoter upstream of the TSS to repress its expression; PDE5A downregulation leading to increased cGMP and cytosolic calcium has only a minor effect on proliferation, but potentially increases melanoma cell invasion.³⁷ Another pathway involves activation of

Kitl (Kit ligand) expression by BRN2 through a cluster of binding sites in the proximal promoter.²² Kit ligand, also known as steel factor (stem cell factor and mast cell growth factor), exerts survival, proliferation and migration functions in Kit receptor-expressing melanocytes.³⁸ As amplification and/or activating mutations of *Kit*, encoding the receptor for Kit ligand, have been associated with melanoma progression,³⁹ BRN2 might modulate the properties of melanoma cells via autocrine Kit ligand signaling. In a genome-wide analysis of *BRN2* promoter occupancy in human melanoma cell line 501mel, many of the potential *BRN2* target genes were found to be associated with the membrane, membrane signaling and interactions with the extracellular matrix.²² *CDH13*, whose gene product T-cadherin is mainly located on the cell surface, was not identified as a potential target gene in that study. Our study provides both indirect and direct evidence that *BRN2* is a transcriptional repressor of *CDH13*.

In conclusion, *CDH13* is not only regulated by promoter hypermethylation⁹ but also by *BRN2* in melanoma. Transcriptional repression of *CDH13* promoter activity by *BRN2* in melanoma cells increases their migratory and invasive potential. We conclude that *BRN2* overexpression and T-cadherin downregulation are relevant mechanisms contributing to the complex regulation of melanoma migration and invasion.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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