Brn-2 Represses Microphthalmia-Associated Transcription Factor Expression and Marks a Distinct Subpopulation of Microphthalmia-Associated Transcription Factor–Negative Melanoma Cells

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Abstract

The origin of tumor heterogeneity is poorly understood, yet it represents a major barrier to effective therapy. In melanoma and in melanocyte development, the microphthalmia-associated transcription factor (Mitf) controls survival, differentiation, proliferation, and migration/metastasis. The Brn-2 (N-Oct-3, POU3F2) transcription factor also regulates melanoma proliferation and is up-regulated by BRAF and β-catenin, two key melanoma-associated signaling molecules. Here, we show that Brn-2 also regulates invasiveness and directly represses Mitf expression. Remarkably, in melanoma biopsies, Mitf and Brn-2 each mark a distinct subpopulation of melanoma cells, providing a striking illustration of melanoma tumor heterogeneity with implications for melanoma therapy. [Cancer Res 2008;68(19):7788–94]

Introduction

Tumor heterogeneity is likely to represent a major barrier to effective cancer therapy. Yet how different cell identities within a tumor may be established remains poorly understood. In melanoma, around 70% are characterized by activating mutations in BRAF (1), leading to constitutive activation of the mitogen-activated protein kinase (MAPK) pathway. In addition, a substantial minority of melanomas exhibit nuclear accumulation of β-catenin (2, 3). Both melanoma-associated pathways converge on and activate the promoter for the gene encoding the POU domain transcription factor Brn-2 (*N-Oct-3, POU3F2*; refs. 4, 5), leading to its overexpression in melanomas compared with normal melanocytes. A key role for Brn-2 in melanoma proliferation was revealed by the fact that depletion of Brn-2 can lead to hypoproliferation, whereas the converse is observed if Brn-2 is overexpressed in melanocytes (4, 5). However, while Brn-2 clearly regulates proliferation, the mechanism underpinning its effect on the cell cycle is not known.

The microphthalmia-associated transcription factor (Mitf; refs. 6, 7) has been termed a lineage addiction oncogene (8) and is a central regulator of melanoma survival, proliferation, and metastatic potential (8–13). The proproliferative and antiproliferative functions of Mitf can be explained by a model (10) in which cells expressing low levels of Mitf exhibit a stem cell-like phenotype with high $p27^{Kip1}$ expression and low proliferative and high invasive potential, whereas cells expressing Mitf can either proliferate or, if Mitf activity is further increased, undergo differentiation and express $p16^{INK4}$ and $p21^{Cip1}$.

The fact that Brn-2, like Mitf, regulates melanoma proliferation led us to explore the possibility that Brn-2 acts by regulating Mitf expression. Our results show that Brn-2 promotes invasiveness and can bind and directly repress the Mitf promoter. Significantly, the two proteins seem to mark two distinct subpopulations of melanoma cells in melanoma biopsies. The results raise the possibility that melanoma proliferation and metastasis *in vivo* may be controlled by a Brn-2-Mitf axis.

Materials and Methods

Generation and growth of cell lines. All cell lines were grown as described (9). Virus-producing cell lines were produced by transfection of Psi2 cells with pBabe.HA.ER or pBabe.HA.ER-Brn-2 vectors, expressing cells selected with puromycin, and viral supernatants used to infect target cells as described (9). Estrogen receptor (ER) fusion proteins were activated by the addition of 4-hydroxytamoxifen (4OHT) to a final concentration of 300 nmol/L.

Quantitative reverse transcription–PCR. Total cellular RNA was isolated from various melanoma cell lines with the SV Total RNA Isolation System (Promega Corp.). Random-primed cDNA was synthesized from 1 μg of total RNA by reverse transcription at 37°C for 1 h in a 20-μL reaction mixture containing 500 nmol/L of each deoxynucleotide triphosphate, 200 units of reverse transcriptase (MMLV, Life Technologies Invitrogen), 300 μg/mL of random hexamer primers (Amersham Biotech), 40 units of RNase block (Promega), 1× first-strand buffer (Promega), and 10 mmol/L of DTT. Reactions lacking RT served as negative controls.

Real-time quantitative reverse transcription–PCR (RT-PCR) analyses were performed with the following primers: human MITF (5′-ACCGTCTCTCACTGGATTGG-3′ and 5′-TACTTGGTGGGGTTTTCGAG-3′), human N-OCT-3 (5′-CTGGAGAGCCATTTCCTCA-3′ and 5′-GGAGGGGTCATCCTTTTCTC-3′), human TBP (5′-CACGAACCACGGCACTGATT-3′ and 5′-TTTTCTTGCTGCCAGTCTGGAC-3′), mouse Mitf-M (5′-GCCTTGTTTATGGTGCCTTC-3′ and 5′-GTCCTCCTCCCTCTACTTTCTGT-3′), mouse Brn-2 (5′-CGGCGGTTTGCTCTATTC-3′ and 5′-ATGGTGTGGCTCATCGTG-3′), and mouse Hprt (5′-CACAGGACTAGAACACCTGC-3′ and 5′-GCTGGTGAAAAGGACCTCT-3′). Primers were used at 300 nmol/L for all human and murine Brn-2 oligonucleotides and 600 nmol/L for murine Mitf-M and Hprt oligonucleotides. PCR reactions were performed in a Bio-Rad iCycler iQ Multi-Color Real-Time PCR Detection System. Each 25-μL reaction consisted of 2 μL of cDNA, 1× iQ SYBR Green Supermix (Bio-Rad), and appropriate amount of primers. The amount of the target transcript was related to that of a reference gene (*TBP* for human and *Hprt* for mouse) by the Ct method. Each sample was assayed at least in triplicate.

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Small interfering RNA and RT-PCR. Small interfering RNA (siRNA)–mediated down-regulation of Brn-2 was achieved with the following Brn-2–specific target sequences: 5′-GACCCGCACTCGGACGAGGAC-3′ and 5′-CTGGACGGGCGTCTGCAC-3′. The control siRNA sequences used were as described (9). RT-PCR was performed with primers specific for *Brn-2* (5′-GACCCGCACTCGGACGAGGAC-3′ and 5′-CTGGACGGGCGTCTGCAC-3′), Mitf (5′-ATGCTGCAAATGCTAGAATAA-3′ and 5′-CAATCAGGTTGTGATTGTCC-3′), and *G3PDH* (5′-CCAACTGCTTAGCCCCCCTGGCCAAG-3′ and 5′-CTCCTTGGAGGCCATGTAGGCCATG-3′). After siRNA treatment for 3 d, cells were assayed by Western blotting or RT-PCR. Luciferase assays were performed as described (14) and assayed 2 d after transfection.

Matrigel invasion assay. Quantitative invasion assays were performed as described (10). Colo858 melanoma cells were transfected with control or Brn-2–specific siRNA. The invading cells, present on the lower side of the chamber, were stained, air dried, photographed, and counted under the microscope.

Tumor formation assay. Four million or 1 million human melanoma cells from different lines were injected s.c. into 6-wk-old to 8-wk-old female athymic nude mice (nu/nu BALB/c, Charles River strain), and tumor growth was monitored. Tumor size was assessed by caliper measurements every 2 to 3 d, and volume was calculated by the formula length × width × width / 2. Mice were weighed every week. Each injection was performed into the flank of four mice. A melanoma cell line was considered tumorigenic when at least one tumor developed. For ethical reasons, the animals were sacrificed when their tumors reached 400 mm^3. Mice were subjected to autopsy, and metastases were detected by direct observation. A melanoma cell line was considered metastatic when at least one metastasis developed. All animal experiments were approved by the Local Committee on Ethics of Animal Experimentation.

Immunofluorescence microscopy. Immunofluorescence was performed as described (9) with a 1:100 dilution of monoclonal anti-HA (Clone HA-7; Sigma) and with a 1:100 dilution of appropriate secondary antibodies (Vector Laboratories).

Western blot analysis. Western blotting on whole-cell extracts was performed as described (15). The primary antibodies used were anti-Mitf mouse monoclonal (C5, Neomarkers), rabbit polyclonal anti-$p21^{Cip1}$ (Santa Cruz Biotechnology), anti-HA mouse monoclonal (Sigma), anti-Brn-2 (4), anti-Pax3 (Developmental Studies Hybridoma Bank, University of Iowa), and anti-Sox10 (16).

DNA-binding assays. Electrophoretic mobility-shift assays were performed with purified glutathione *S*-transferase (GST)–Brn-2 and ^{32}P-labeled oligonucleotide Mitf promoter probe 1, as described previously (9). The sequences for probe and competitor DNA were as follows: probe 1, 5′-TTTTACATGCATAACTAATTAGCTTAGGTTATTATAAGCAGGGCTTCTGT-3′; 1.m, 5′-TTTTACATGCcgccCTcgagAGCTTAGGTTATTATAAGCAGGGCTTCTGT-3′; 2, 5′-AGCTTAGGTTATTATAAGCAGGGCTTCTG-3′; 2.m, 5′-AGCTTAGGTTcgTATAAGCAGGGCTTCTG-3′; LEF-1, 5′-CTAGAAGGGCACCCTTTGAAGCTCT-3′.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed, as described previously (9), for Brn-2 with 10 μg anti–Brn-2 mouse monoclonal antibody or 10 μg nonspecific IgG (Bio-Rad). The DNA recovered was subjected to amplification by PCR before analysis by agarose gel electrophoresis or quantitative PCR. The primers used for the PCRs were the human *MITF* promoter region, 5′-TCGGAAGTGGCAGTTATTCGC-3′ and 5′-AACAATGTTTTAGGTGGCACCAATCC-3; for the *HSP70* promoter, 5′-CCTCCAGTGAATCCCAGAAGACTCT-3′ and 5′-TGGGACAACGGGAGTCACTCTC-3′. For the RNA PolII ChIP combined with qPCR 501 mel human melanoma, cells were transfected with 5 μg of pCMV5 HA-BRN-2 or pSK (control) using FuGENE Transfection reagent (Roche Diagnostic). Two days after transfection, cells were fixed with 0.4% formaldehyde for 10 min, and the remainder of the ChIP assay was performed by standard procedures as described (9) using HA (12CA5) or RNA polymerase II (H-224 Santa-Cruz) antibodies or nonspecific IgG as control.

Precipitated DNA was quantified by real-time PCR using QuantiTeck SYBR Green PCR kit (QIAGEN) and oligonucleotide primer pairs amplifying around 100 nucleotide fragments at the core promoters for *MITF-M* and *dihydrofolate reductase* (*DHFR*; expressed but not a Brn-2 target). The primers used were MITF 5′-CAAACTCGTAGGGCTTCCAA-3′, 5′-CCACCGGAAACTTTATCACAG-3′ and DHFR 5′-ACCTGGTCGGCTGCACCT-3′, 5′-TTGCCCTGCCATGTCTCG-3′.

Tissue samples, tissue microarray, immunohistochemistry, and immunofluorescence. Formalin-fixed and paraffin-embedded tissue specimens for tissue microarray (TMA) construction were obtained from Istituto Europeo di Oncologia. TMAs were prepared as described previously (17). Briefly, two representative tumor areas (diameter, 0.6 mm) from each sample, identified previously on H&E-stained sections, were removed from the donor blocks and deposited on the recipient block using a custom-built precision instrument (Tissue Arrayer, Beecher Instruments). Serial sections (3 μm) of the resulting recipient block were treated as described previously (10) and processed for immunohistochemistry with anti-Mitf (1 of 50) monoclonal antibody and anti–Brn-2 rabbit polyclonal (18) or double immunofluorescence with anti-Mitf monoclonal (1 of 100; Dako) and anti–Brn-2 rabbit polyclonal. Results from the melanoma TMA were subjected to semiquantitative visual analysis. In each single sample, the levels of Mitf and Brn-2 expression were calculated as the product of the intensity of staining (scores: 0, no nuclear staining; 1, weak nuclear staining; 2, moderate nuclear staining; 3, strong nuclear staining) and the percentage of reactive cells (0–100%). As a consequence, levels will range from 0 (no staining) to 300 (strong staining in 100% of tumor cells). The mean levels of expression for MITF and Brn-2 in each group (nevi, melanomas, and metastases) was used as cutoff for positivity, as shown in Fig. 4*B* (i.e., below the mean, negative; above the mean, positive).

Results

Brn-2 directly represses Mitf expression. While a role for Brn-2 in melanoma proliferation has been identified previously (4, 5), its potential as a regulator of melanoma invasiveness has not been well characterized. Using a Matrigel assay, siRNA-mediated depletion of Brn-2 in Colo858 cells (Fig. 1*A*) led to a substantial reduction in their invasive capacity (Fig. 1*B*). Because the doubling time of these cells is in the order of 36 hours, the decreased invasiveness observed during the 16-hour incubation in the Matrigel chamber is not a result of altered proliferative capacity. As melanoma proliferation and invasiveness are also regulated by Mitf, we compared the mRNA expression of Brn-2 and Mitf in a panel of melanoma cell lines that we also characterized for their ability to form subcutaneous or metastatic tumors in immunodeficient mice. The results indicated that there was a striking inverse correlation between Brn-2 and Mitf expression (Fig. 1*C*). Intriguingly, using s.c. injection into immunodeficient mice, those cell lines with reduced Mitf and higher levels of Brn-2 were more tumorigenic, whereas the two cell lines, LU1205 and WM825, that expressed the highest levels of Brn-2 and the lowest levels of Mitf were the only ones with metastatic potential in this assay. Determination of the doubling time for each cell line revealed that, in general, lines that expressed higher or lower levels of Mitf had a slower rate of proliferation with the metastatic lines Lu1205 and WM825 exhibiting a doubling time of at least 72 hours. We also examined the murine melanoma cell line B16 C3 which is tumorigenic, but nonmetastatic and two metastatic derivatives, B16 F1 and B16 F10. Consistent with the data obtained for the human melanoma cell lines, the nonmetastatic parental line B16 C3 expressed very low levels of Brn-2, whereas both metastatic derivatives expressed this factor and also exhibited reduced Mitf expression. These observations are consistent with the current models for the role of Mitf in melanoma proliferation and invasiveness. Mitf, that has been termed a lineage addiction oncogene based on its requirement for melanoma proliferation (8), can act both to promote and inhibit proliferation (8–12),

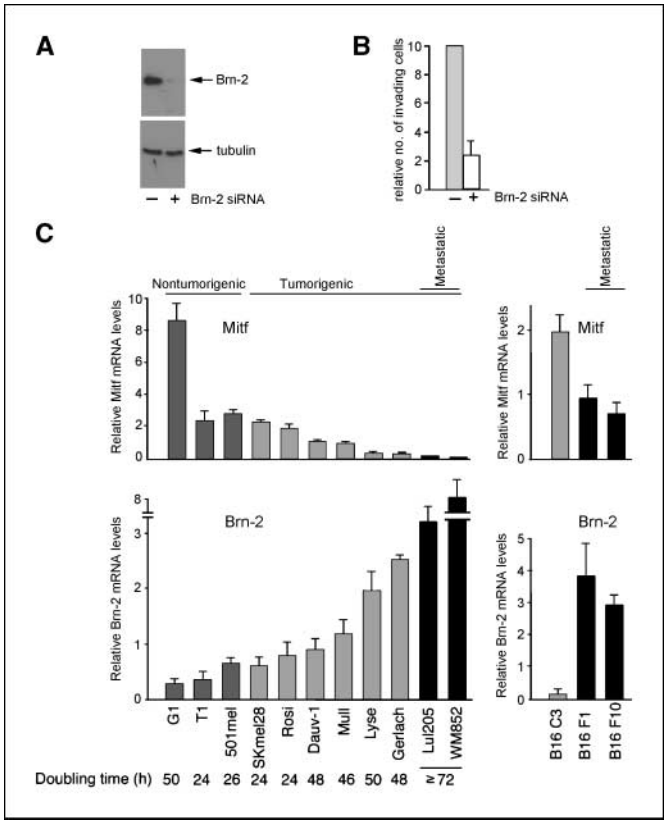

Figure 1. Brn-2 is proinvasive, and its expression is inversely correlated with Mitf. *A,* Western blot of Colo858 melanoma cells transfected with control or Brn-2–specific siRNA. *B,* Colo858 cells transfected with control or Brn-2–specific siRNA, as indicated, were assessed for their invasive potential using a Matrigel assay. The relative number of invading cells means relative to the numbers invading from the control population arbitrarily set to 10. *C,* relative expression of Brn-2 and Mitf mRNA as determined using quantitative real-time RT-PCR from the indicated melanoma cell lines. Values reported are means of three independent experiments performed in triplicates. *Bars,* SD. Doubling times were determined by cell counting with an SD of ±15%.

depending on its activity and expression levels. Thus, elevated Mitf leads to a more differentiated phenotype, whereas low Mitf can lead to a p27-dependent cell cycle arrest and increased invasiveness (10, 19). Proliferation therefore requires that Mitf activity is kept within a specific window, and this is consistent with the cell lines expressing intermediate levels of Mitf having the higher proliferation rates.

We have shown previously that anti-sense–mediated inhibition of Brn-2 expression in a melanoma cell line led to reduced invasiveness and tumor formation *in vivo* (20), emphasizing the likely importance of Brn-2 in tumor progression. The inverse correlation between Mitf and Brn-2 expression and their reciprocal roles in melanoma invasiveness raised the possibility of a regulatory link between the two factors. Because examination of the Brn-2 promoter failed to identify any potential Mitf binding sites, we explored the possibility that Brn-2 would regulate Mitf expression. We therefore expressed Brn-2 in B16 C3 melanoma cells that express Mitf but which express relatively low levels of Brn-2 compared with other melanoma cell lines (4, 5). To circumvent the possibility that constitutive expression of Brn-2 might lead to the selection of cells in which Mitf was reduced incidentally, we expressed Brn-2 as a fusion with the HA epitope and the 4OHT-responsive ligand-binding domain of the ER (21). Immunofluores-

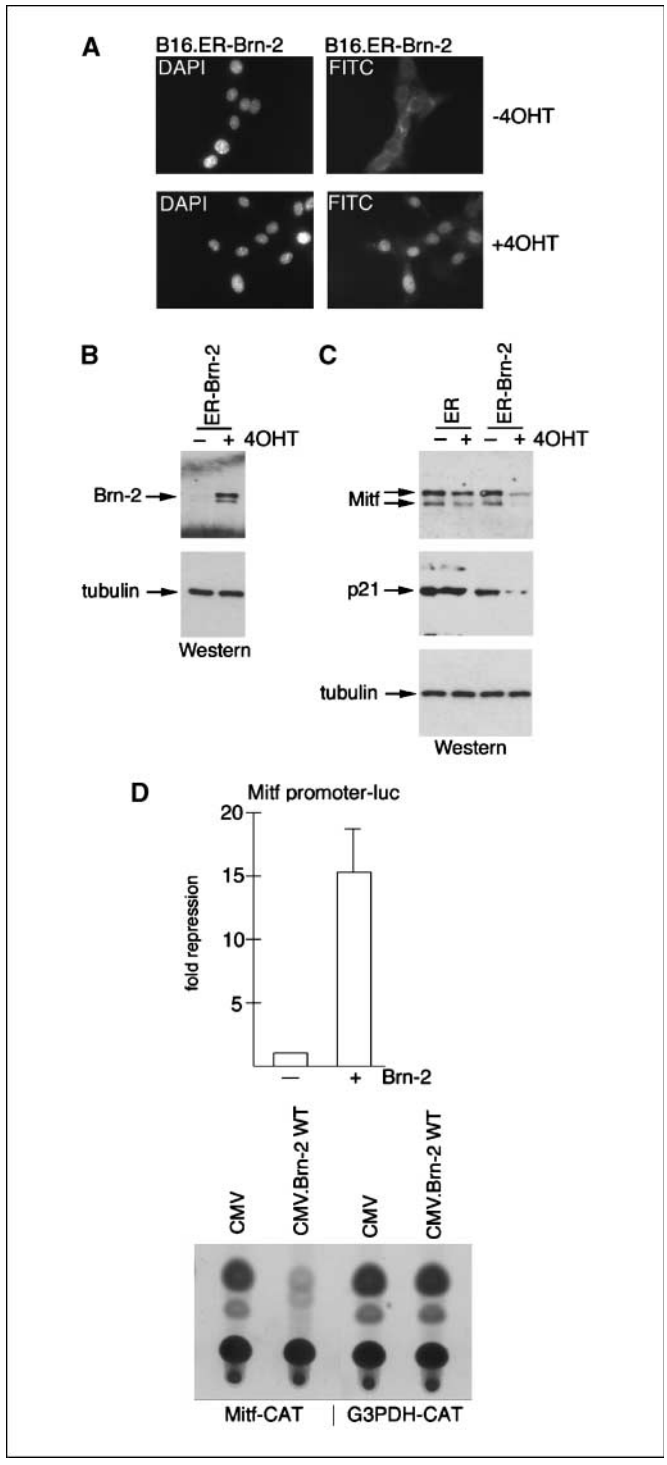

Figure 2. Brn-2 can repress Mitf expression. *A,* B16 C3 melanoma cells were infected with a retrovirus expressing HA epitope–tagged Brn-2 fused to the 4OHT-responsive ER ligand–binding domain or ER-HA alone. Clones were isolated and subjected to treatment with or without 4OHT, as indicated before being subjected to immunofluorescence using anti-HA antibody. 4′,6-diamidino-2-phenylindole (*DAPI*) was used to stain DNA. *B,* Western blot using anti-HA antibody of ER-HA–Brn-2 cells treated or not with 4OHT. Tubulin was used as a loading control. *C,* Western blot using anti-Mitf or anti-p21 antibodies of B16 cells expressing ER-HA or ER-HA–Brn-2 grown in the presence or absence of 4OHT as indicated. Tubulin was used as a loading control. *D,* luciferase assay of 501 mel cells transfected with an Mitf-luciferase reporter alone or together with a Brn-2 expression vector as indicated (*top*) or with *Mitf* or *G3PDH* promoter-CAT reporters (*bottom*). 501 mel cells were used, as they express high levels of Mitf protein and mRNA. Results represent the mean of three experiments and SD.

cence (Fig. 2*A*) and immunoblotting using anti-HA antibody (Fig. 2*B*) showed that in the absence of 4OHT, ER–Brn-2 was expressed at low levels and was cytoplasmic, whereas addition of 4OHT led to increased levels of ER-Brn-2, presumably as a result of increased stability of the fusion protein, and its accumulation in the nucleus. Immunoblotting using an anti-Mitf antibody on extracts control B16 cells expressing the ER ligand–binding domain alone revealed that the addition of 4OHT had little effect on Mitf expression (Fig. 2*C*). In contrast, in cells expressing ER–Brn-2, addition of 4OHT substantially reduced Mitf levels. Consistent with our previous observation that Mitf can activate $p21^{Cip1}$ expression (9), the reduced levels of Mitf expression in these cells upon 4OHT treatment correlated with down-regulation of $p21^{Cip1}$. Tubulin was used as a loading control. To confirm that Brn-2 could target the melanocyte-specific Mitf-M promoter, cotransfection assays revealed that ectopic expression of Brn-2 led to substantial repression of Mitf-M promoter luciferase or Mitf-M promoter-CAT reporters, but not a *G3PDH*-CAT reporter (Fig. 2*D*).

Initial examination of the Mitf-M promoter revealed a consensus Brn-2 binding site CAT NNN TAAT (22) located immediately upstream from the TATA box and downstream from other known activators of the promoter (Fig. 3*A*). To confirm that this sequence could bind Brn-2, we performed *in vitro* DNA binding assays using a probe spanning this element together with bacterially expressed and purified Brn-2. The results (Fig. 3*B*) showed that Brn-2 bound the probe and that binding was competed with wild-type (WT) oligonucleotide (1) corresponding to the probe sequence, but not by a mutated element 1.m that competed as poorly as an unrelated competitor containing a consensus Lef1 binding site. Weak binding was also detected using a competitor (2) spanning the TATA box that was abolished by a 2-bp substitution within the TATA motif (2m). Mutation of the Brn-2 binding site in the Mitf promoter luciferase reporter led to an over 8-fold increase in activity (Fig. 3*C*), strongly suggesting that endogenous Brn-2 was able to bind and repress *Mitf* expression. Binding *in vivo* was confirmed using a ChIP assay (Fig. 4*A*) in which a strong signal was detected using anti–Brn-2 antibody, whereas only a background signal was observed using either no antibody or nonspecific IgG as controls. As an additional negative control, no Brn-2 was detected at the *HSP70* promoter even after further cycles of PCR amplification. The results from the ChIP using endogenous Brn-2 were further substantiated by ectopically expressing an HA epitope-tagged Brn-2. In this case, HA-tagged Brn-2 was readily detected at the *Mitf* promoter using anti-HA antibody, whereas no significant signal was observed using either nonspecific IgG or chromatin from untransfected cells or if primers for the *DHFR* promoter were used (Fig. 4*B*). To verify that endogenous Brn-2 can repress *Mitf* expression, we depleted Brn-2 using a specific siRNA that we have characterized previously (4, 5). RT-PCR and Western blotting of control or Brn-2–depleted cells (Fig. 4*C*) revealed that the decrease in Brn-2 mRNA or protein levels were mirrored by an increase in Mitf mRNA and protein. Expression of *G3PDH* or lamin B were used as negative controls for RNA and protein, respectively. Anti-sense–mediated silencing of Brn-2 has been reported to lead to reduced expression of Sox10 (23), a positive regulator of the *Mitf* promoter. We therefore checked whether siRNA-mediated depletion of Brn-2 might affect Sox10 levels and thereby indirectly affect Mitf. However, Western blotting revealed that depletion of Brn-2 using two different siRNAs failed to affect expression of either Sox10 or Pax3, another positive regulator of the *Mitf* promoter (Fig. 4*D*). Tubulin was used as a loading control. Taken together, the data suggest that Brn-2 directly regulates the *Mitf* promoter.

Brn-2 and Mitf mark distinct melanoma cell populations. The data thus far revealed that Brn-2 can bind the *Mitf* promoter and repress *Mitf* expression. Our previous results (10) revealed that individual tumors are heterogeneous and contain both Mitf-negative and Mitf-positive melanoma cells. We therefore sought to examine the expression pattern of Brn-2 in human melanomas using a melanoma TMA and correlate it with already available Mitf

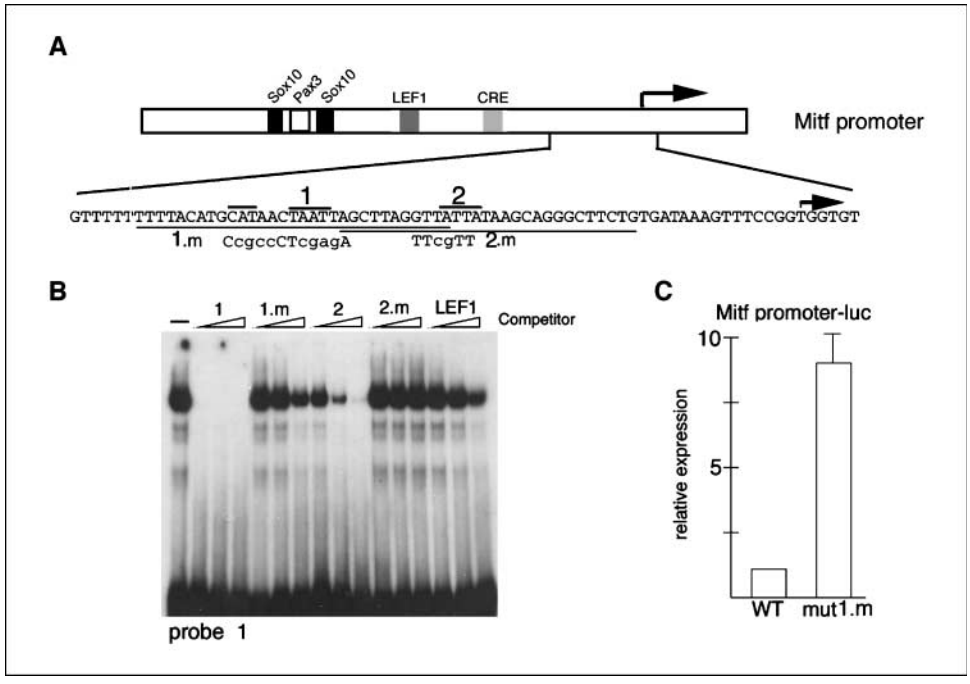

Figure 3. Brn-2 directly binds and represses the *Mitf* promoter. *A,* a schematic of the *Mitf* promoter showing binding sites for known regulators. The insert shows the sequence containing the Brn-2 binding site CATNNNTAAT (*1*) and the TATA box (*2*) overlined. The sequences of mutations introduced into each element are shown below, and the underlined sequences correspond to the probes and competitors used in the *in vitro* DNA-binding assays. *B,* band shift assay using probe 1 containing the Brn-2 binding site together with bacterially expressed and purified GST–Brn-2. The indicated competitors were used at 10, 50, and 250 ng. *C,* luciferase assay using WT Mitf-luciferase reporter or a derivative bearing a mutation (1.m in *A*) transfected into 501 mel cells. *Columns,* mean of three experiments; *bar,* SD.

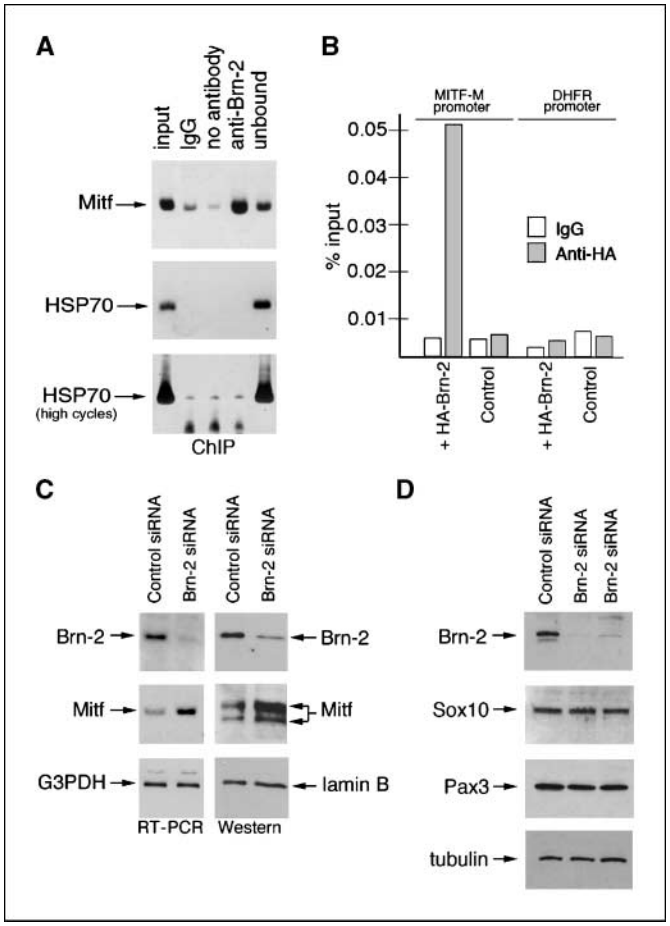

Figure 4. Brn-2 directly represses the Mitf promoter. *A,* ChIP using the indicated antibodies and primers specific for the *MITF* or *HSP70* promoters. HSP70 (high cycles) indicates that the PCR reaction (*middle*) was further amplified to show unequivocally no immunoprecipitation of Brn-2 at the *HSP70* promoter. *B,* ChIP using IgG or anti-HA or anti-RNA polymerase II antibodies as indicated on 501 mel cells transfected or not with an HA–Brn-2 expression vector. Precipitated material was subject to qPCR with primers specific for the Mitf promoter. *C,* Western blot using anti–Brn-2 and anti-Mitf antibodies and RT-PCR (as indicated) of melanoma cells transfected with control or Brn-2–specific siRNA. Lamin B or G3PDH were used as controls for the Western blot and RT-PCR, respectively. *D,* Western blot using indicated antibodies of melanoma cells transfected with two different Brn-2 siRNAs or control siRNA as indicated.

expression data (10). We initially stained adjacent sections with either anti–Brn-2 or anti-Mitf antibody and scored for expression of either protein. Some examples are shown in Fig. 5*A*. The results obtained revealed that only one nevus expressed Brn-2, wherein, surprisingly, it was localized in the cytoplasm (not shown). Although, in a previous study, we were unable to detect significant staining for Mitf in nevi (10), here, using a different batch of anti-Mitf antibody that seemed to provide increased sensitivity, around 40% of nevi scored positive. The results for primary tumors and metastases showed that the expression of these two proteins was extremely heterogeneous among melanoma cells, exhibiting clear staining frequently in a small fraction of cells in the tumor core. The results for primaries and metastases were broadly similar, with some apparently expressing neither protein, some expressing one or the other, and a further substantial proportion exhibiting staining for both factors (Fig. 5*B*). The conclusion from this analysis, based on the statistics arising from the expression patterns, suggested that there was a positive correlation between Brn-2 expression and Mitf. Because this was clearly at odds with our cell-based assays and because of the heterogeneous pattern of expression of the two proteins, we performed further analysis to clarify the TMA results. We first considered that, those samples in which neither protein could be detected might be negative for any of several reasons, in particular sensitivity of the Brn-2 or Mitf epitopes to loss or expression of these proteins below the level of immunohistochemical sensitivity, for example. The samples that stained for one or the other factor were unequivocal and in agreement with our cell-based assays. However, those positive for both Mitf and Brn-2 posed a key question: because the antibodies were used to stain adjacent sections, could we be sure that the double positives (see Fig. 5*C* for example) in fact represented staining of the same cells, or did each antibody highlight a different population within the same tumor? To test the possibility that Brn-2 and Mitf, in fact, were expressed in distinct subpopulations, we performed a double immunofluorescence assay on the same TMA used for immunohistochemical analyses with the rabbit anti–Brn-2 and the mouse anti-Mitf antibodies. The results (Fig. 5*D*) were striking. In double-positive samples (examples from six different patients are presented), Mitf and Brn-2 were clearly expressed in different populations of cells. The data obtained highlight the importance of using double immunofluorescence to obtain an accurate picture of coexpression of different markers in the TMAs and the power of combining tissue analysis with cell-based assays.

Discussion

Brn-2 and the rheostat model for Mitf function. Mitf controls melanoma/melanocyte survival, differentiation, proliferation, and invasiveness and is highly regulated (for review, see ref. 24) by extracellular cues or by constitutively activated melanoma-associated signaling pathways at both the transcriptional and posttranslational levels. Because Brn-2 expression is up-regulated by both BRAF and β-catenin (4, 5) that may both be constitutively activated in melanoma, its ability to regulate Mitf expression may be especially important. The effect of Brn-2 on Mitf and regulation of melanoma proliferation and invasiveness should be viewed in the context of the "rheostat" model proposed previously (10), in which low levels of Mitf lead to a slow proliferating cell with high invasive potential, whereas increased Mitf activity marks either proliferating or differentiating cells. In cells in which Mitf may be promoting a more differentiated phenotype, Brn-2–mediated repression of Mitf could lead to increased proliferation, as seen by us previously (4, 5). By contrast, in cells in which Mitf is promoting proliferation, Brn-2 repression of Mitf would increase invasive potential, consistent with our observations in this paper. It is important to note, however, that, although in the experiments that are described here Brn-2 is clearly a potent repressor of Mitf expression, Brn-2 can interact with the p300 transcription coactivator (25), and consistent with this, Brn-2 can activate transcription in some melanoma cell lines (26). Thus, like many transcription factors, it would seem that Brn-2 may act as either an activator or repressor, depending on context. However, the mutually exclusive pattern of Brn-2 and Mitf expression in melanoma tissue clearly suggests that *in vivo* Brn-2 is more likely to be an Mitf repressor, at least in those melanomas examined here.

Although Brn-2 expression is up-regulated by MAPK signaling downstream from activated BRAF (5) that is found in around 70% of melanomas (1), the fact that Brn-2 is not expressed in all melanoma cells within a tumor *in vivo* suggests that other factors

will contribute to whether Brn-2 is expressed and, consequently, its effect on *Mitf* expression. For example, in the absence of Wnt signaling or activated β-catenin, LEF1, which can target the Brn-2 promoter (4) may function as a repressor and thereby override any positive effect of activation of BRAF. It also seems likely that additional, as yet uncharacterized, signaling pathways activated by microenvironmental cues will also play a role in the regulation of both *Brn-2* and *Mitf* expression. Thus, whether a specific cell will express Brn-2 or not is likely to depend both on cell intrinsic factors, such as activation of BRAF or NRAS, and cell extrinsic factors, such as growth factors, cytokines, interactions with stroma, and oxygen and nutrient availability. Determining how the microenvironment will regulate Brn-2 expression in cells harboring BRAF mutations will be an important future goal.

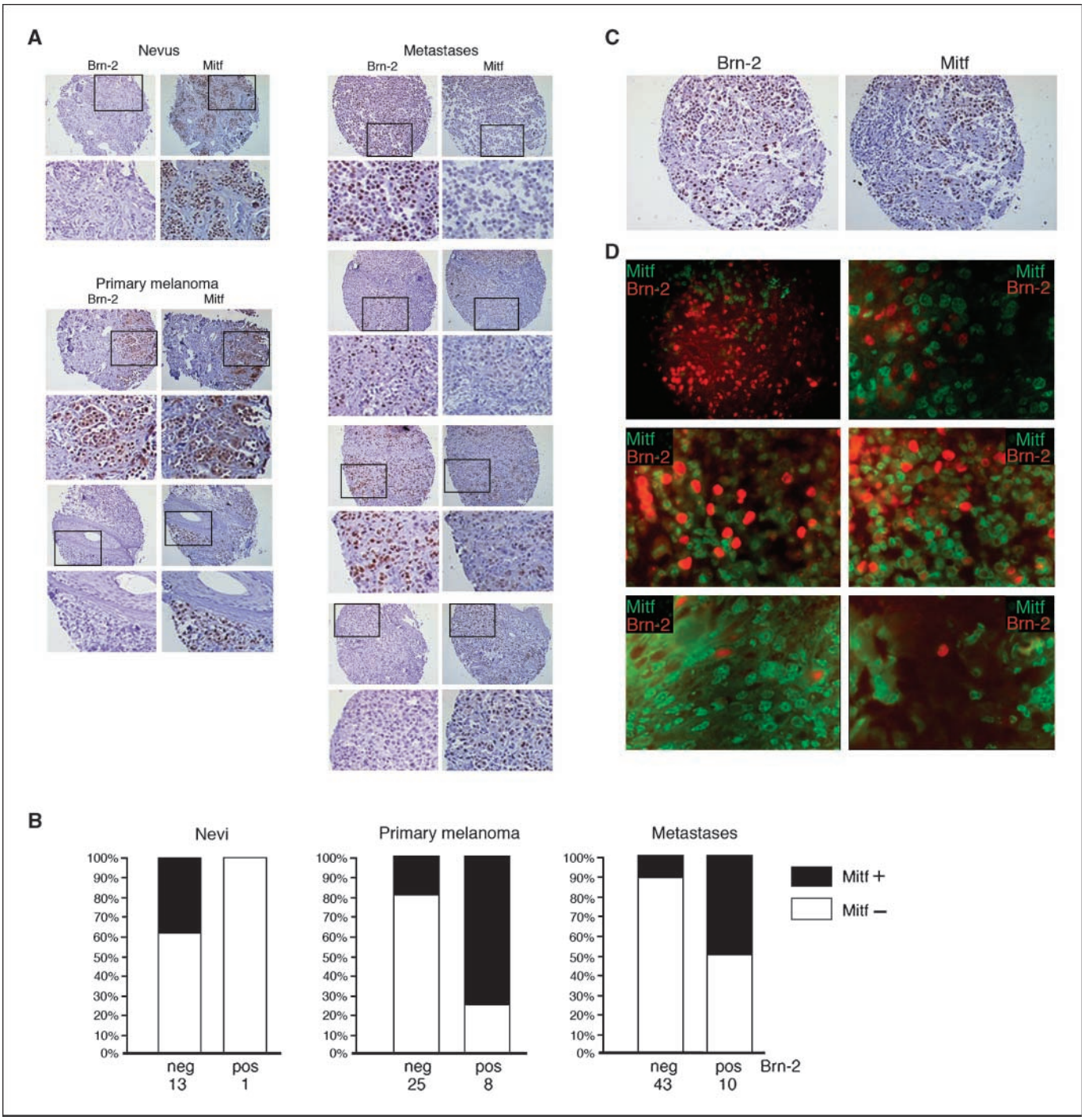

Figure 5. Mitf and Brn-2 mark distinct populations of melanoma cells in human tumors. *A,* representative samples showing results of immunohistochemistry of melanoma TMAs performed with the indicated antibodies on adjacent sections of the same array. The insets below each sample correspond to the boxed area. *B,* statistical analysis of the data from a melanoma microarray that included the samples shown in *A. C,* an example of a double-positive sample of a metastatic melanoma. *D,* double immunofluorescence images showing Mitf (*green*) and Brn-2 (*red*) of metastases from different patients. The sample on the top left is shown at lower magnification.

The microenvironment will vary substantially within a specific tumor, as well as when a cell metastasizes. As such and in contrast to the irreversible nature of genetic lesions, changing microenvironmental conditions are expected to lead to dynamic and reversible alternations in gene expression, as we proposed previously in our rheostat model for Mitf function (10), with $Mitf^{+}/Brn\text{-}2^{-}$ and $Mitf^{-}/Brn\text{-}2^{+}$ cells being able to switch profiles, depending on extracellular cues. Thus, consistent with the recent results from Hoek and colleagues (27), who showed that *in vivo* the gene expression profiles of melanomas can switch, we do not expect that one population or the other will reach clonal dominance within a tumor.

Brn-2 and Mitf as melanoma markers. One important lesson from these studies is that correlating the expression of different tumor markers based on staining adjacent sections is unreliable for heterogeneously expressed proteins, such us Brn-2 and Mitf; the double immunofluorescence assay used here showed unequivocally that Mitf and Brn-2 are expressed in different cells on the same section. Remarkably, we do not see significant levels of coexpression, as observed in melanoma cell lines, suggesting that *in vivo* there may be a feedback mechanism that enables cells to adopt either an Mitf-negative or Mitf-positive profile, although we cannot exclude that, in the samples of immunohistochemistry negative for both genes, some coexpression might be detected using more sensitive methods. Importantly, the presence of two distinct melanoma cell populations has major implications for melanoma therapy. Because low Mitf cells are highly invasive (10), we would predict that the Brn-2–positive, Mitf-negative population is more likely to be slow proliferating with high-invasive potential. Because most melanoma therapies are designed to target proliferating (chemotherapy) or differentiated (immunotherapy) cells, the presence of an Mitf-negative population poses particular problems. Whether the Mitf–positive/Brn-2–positive cells represent melanoma stem cells is yet to be investigated, and their characterization will depend on the availability of stem cell sufficient markers. Nevertheless, the identification of Brn-2 as a marker for the Mitf-negative cells will allow us to dissect the characteristics of the different melanoma cell populations and consequently design therapeutic strategies that take into account the tumor heterogeneity detected here.

Disclosure of Potential Conflicts of Interest

The authors declare they have no competing financial interests.

Acknowledgments

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