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MITF links differentiation with cell cycle arrest in melanocytes by transcriptional activation of INK4A

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Cell cycle exit is required for proper differentiation in most cells and is critical for normal development, tissue homeostasis, and tumor suppression. However, the mechanisms that link cell cycle exit with differentiation remain poorly understood. Here, we show that the master melanocyte differentiation factor, microphthalmia transcription factor (MITF), regulates cell cycle exit by activating the cell cycle inhibitor INK4A, a tumor suppressor that frequently is mutated in melanomas. MITF binds the INK4A promoter, activates p16\(^{ink4a}\) mRNA and protein expression, and induces retinoblastoma protein hypophosphorylation, thereby triggering cell cycle arrest. This activation of INK4A was required for efficient melanocyte differentiation. Interestingly, MITF was also required for maintaining INK4A expression in mature melanocytes, creating a selective pressure to escape growth inhibition by inactivating INK4A. These findings demonstrate that INK4A can be regulated by a differentiation factor, establish a mechanistic link between melanocyte differentiation and cell cycle exit, and potentially explain the tissue-specific tendency for INK4A mutations to occur in melanoma.

Introduction

Terminal differentiation is accompanied by cell cycle exit in most cell types, and this proclivity for mature cells to stop dividing is important for normal development, tissue homeostasis, and tumor suppression (Lipinski and Jacks, 1999). In fact, the normal cellular function of many tumor suppressors is to regulate differentiation and cell cycle exit (Lipinski and Jacks, 1999). Nevertheless, the mechanisms that link differentiation with cell cycle exit remain poorly understood.

Melanocytes are derived from neural crest and differentiate under the control of microphthalmia transcription factor (MITF), a basic helix-loop-helix leucine zipper transcription factor that activates genes involved in pigment production (e.g., DCT, TYR, and TRP1) and melanocyte survival (e.g., BCL2) (McGill et al., 2002; Bear et al., 2003; Widlund and Fisher, 2003). Whether MITF also regulates cell cycle exit and maintenance of the post-mitotic state in melanocytes is unclear. We address that question here by investigating how MITF affects the Rb-p16\(^{ink4a}\) pathway, which plays a key role in cell cycle exit and orderly progression through the differentiation process in many cell types (Lipinski and Jacks, 1999).

Results and discussion

To examine the role of MITF in melanocyte cell cycle regulation, we studied embryonic fibroblasts that differentiate into melanocytes under the influence of ectopic MITF, as previously characterized (Tachibana et al., 1996; Bear et al., 2003). Expression of MITF in 10T1/2 cells, which produce negligible endogenous MITF protein (Fig. 1 A), resulted in marked growth inhibition and morphologic changes consistent with melanocyte differentiation (Fig. 1 B). BrdU incorporation assays indicated that this growth block was due to cell cycle arrest (Fig. 1 C). In contrast, no cell cycle alterations were observed in cells expressing a MITF mutant lacking DNA-binding activity (MITF-DB; Fig. 1 C), indicating that the ability of MITF to negatively regulate the cell cycle is dependent on its DNA-binding activity.

Consequently, we reasoned that the cell cycle inhibitory activity of MITF might be mediated through interaction of the MITF DNA-binding domain with cell cycle gene promoter elements. The canonical MITF recognition motif consists of a distal M box containing a consensus CATGTG near the center and a proximal E box (CANNTG) (Yasumoto et al., 1995). A search of cell cycle regulatory gene promoters revealed multiple M and E boxes in the promoter region of INK4A in both human and mouse (Fig. 2 A). This finding was particularly interesting because INK4A is a tumor suppressor that is frequently inactivated in melanoma (Chin et al., 1998). Using chromatin immunoprecipitation assays, endogenous MITF from normal
melanocytes was detected at the INK4A promoter in vivo (Fig. 2 B). Consistent with this finding, gel mobility shift assays showed binding of endogenous MITF from Mel202 melanoma cells to M and E box elements from the INK4A promoter in vitro (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200410115/DC1). Competition with either the unlabeled M box or the unlabeled E box oligonucleotide inhibited binding of the other labeled oligonucleotides, suggesting that both elements are required for maximal binding of MITF to the INK4A promoter.

The ability of MITF to activate the INK4A promoter was tested using an INK4A promoter driving a luciferase reporter in 10T1/2 cells. Expression of MITF in these cells caused a dose-dependent increase in luciferase activity, with up to 20-fold induction compared with cells transfected with MITF-DB expression plasmid or a control empty vector (Fig. 2 C). In contrast, no induction was seen when two nucleotides in the M box sequence were mutated, indicating that activation of the INK4A promoter by MITF requires the MITF-binding motif. Consistent with these results, ectopic expression of MITF (but not MITF-DB) resulted in a substantial increase in endogenous p16\(^{\text{Ink4a}}\) protein and mRNA levels in 10T1/2 cells (Fig. 1 A and Fig. 3 A).

Because p16\(^{\text{Ink4a}}\) induces a G1 cell cycle arrest by inhibiting Cdk4 and allowing the accumulation of hypophosphorylated retinoblastoma protein (Rb) (Serrano et al., 1993), we examined the Rb protein in 10T1/2 cells after ectopic expression...
of MITF or MITF-DB. Neither MITF nor MITF-DB affected total Rb protein levels in 10T1/2 cells, but only MITF induced an accumulation of hypophosphorylated Rb (Fig. 1 A). In particular, MITF expression resulted in loss of phosphorylation at Ser780, a CDK site that is preferentially phosphorylated by the p16
\textsuperscript{Ink4a}
 target Cdk4 and is known to be involved in regulation of cell division and differentiation (Kitagawa et al., 1996; Brugarolas et al., 1999; Ma et al., 2003).

To investigate whether MITF is required to sustain endogenous p16
\textsuperscript{Ink4a}
 expression in melanocytic cells, we depleted MITF in early passage normal melanocytes by RNA interference. Compared with melanocytes transfected with control scrambled siRNA expression vector, those transfected with MITF siRNA expression vector exhibited markedly reduced levels of MITF and p16
\textsuperscript{Ink4a} mRNA and protein (Fig. 3, A and B). These changes were accompanied by an accumulation of hyperphosphorylated Rb (Fig. 3 B). Consistent with these findings, depletion of MITF caused melanocytes to reenter the cell cycle, as evidenced by increased BrdU incorporation (Fig. 3 C), suggesting that the MITF-p16
\textsuperscript{Ink4a}-Rb pathway is also important for maintenance of the post-mitotic state in mature melanocytes. In contrast to normal melanocytes, depletion of MITF in Mel202 melanoma cells, which express low levels of p16
\textsuperscript{Ink4a} (Fig. 3 D) as a result of promoter methylation (van der Velden et al., 2001), had no effect on p16
\textsuperscript{Ink4a} or Rb protein levels, Rb phosphorylation, or BrdU incorporation (Fig. 3, E and F). Thus, MITF induces and maintains cell cycle exit in melanocytes, but this regulatory mechanism can be disrupted in melanoma cells by inactivation of INK4A.

Next, we examined whether melanocyte differentiation and cell cycle arrest are independent processes. MITF and
MITF-DB were expressed in wild-type and INK4A-null primary mouse embryonic fibroblasts (MEFs), and then were monitored for growth rate and morphology. As expected, MITF (but not MITF-DB) inhibited the growth of wild-type cells, but it had no growth inhibitory effect on INK4A-null cells (Fig. 5 A). MITF efficiently blocked cell cycle progression in wild-type cells as measured by BrdU incorporation, whereas no cell cycle inhibition was observed in INK4A-null cells (Fig. 5 B). As with the 10T1/2 cells, MEFs expressing ectopic MITF exhibited morphologic changes consistent with melanocyte differentiation (Fig. 5 C), but MITF did not induce these changes in cells lacking INK4A (Fig. 5 C). Likewise, the melanocyte differentiation marker S100 was detected in MITF-expressing cells that were wild type for INK4A but not in those that were null for INK4A (Fig. 5 D). Importantly, ectopic MITF and MITF-DB levels were similar in wild-type and INK4A-null MEFs (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200410115/DC1). These results suggest that MITF requires INK4A not only for cell cycle inhibition, but also for efficient execution of the melanocyte differentiation program. Indeed, Sviderskaya and coworkers found that melanocytes lacking INK4A were more proliferative and less differentiated than melanocytes having one or both copies of INK4A (Sviderskaya et al., 2002).

These findings provide new mechanistic insights into several biological problems. First, they provide an example of how differentiation can be linked with cell cycle arrest. By activating genes involved in cell cycle exit (i.e., INK4A) as...
well as melanocyte cell fate, MITF efficiently coregulates these interrelated processes. Analogous to MITF, MyoD is a helix-loop-helix transcription factor that induces both cell cycle exit and differentiation in myoblasts (Sorrentino et al., 1990). However, unlike our findings for MITF, MyoD-induced cell cycle arrest does not appear to be a prerequisite for myoblast differentiation. Second, our results show that INK4A can be regulated directly by a differentiation factor. This is a particularly relevant point because surprisingly little is known about the transcriptional control of this important cell cycle regulator. Third, our results provide new insights into one of the most perplexing questions in cancer biology (Sherr, 2004): why are specific tumor suppressors (many of which are differentiation factors) associated with cancer in certain cell types but not others? Our findings would suggest that at least part of the answer lies in the mechanisms linking differentiation and cell cycle exit in a given cell type. The direct link between MITF and INK4A provides an attractive explanation for the strong association between INK4A mutation and melanoma (Ranade et al., 1995; Monzon et al., 1998). Why do melanocytes mutate INK4A rather than MITF or Rb? One potential explanation is that both MITF and Rb are required for melanocyte survival (Yavuzer et al., 1995; McGill et al., 2002; Yu et al., 2003), whereas INK4A appears only to regulate cell cycle.

A critical question that remains unanswered is how differentiation is prohibited in the absence of cell cycle exit. One speculation, based on the observation that E2F1 becomes bound to hypophosphorylated Rb during differentiation (Ikeda et al., 1996; Kastner et al., 1998), is that free E2F inhibits the differentiation program. Rb-E2F may then serve as a checkpoint to allow differentiation only when Rb is hypophosphorylated and able to induce G1 arrest. Further work is underway to address this question.

Materials and methods

Cells and transfection

MEL202 human melanoma cells were a gift of B. Ksander (Harvard, Boston, MA). C3H 1071/2 and primary MEFs were obtained from American Type Culture Collection. Normal human melanocytes were described previously (Harbour et al., 2002). INK4A-null MEFs were generated at E13.5 from FVB.CDKN2am12.C1dp mice (from Mouse Models of Human Cancers Consortium, Frederick, MD). Cells were maintained in DMEM with 10% FCS. MITF expression vector in pPT73D-Pool was obtained from American Type Culture Collection. MITF-DB was generated by introducing a T-to-A nucleotide substitution in the MITF DNA-bind-

ing domain using site-directed mutagenesis with QuickChange II (Stratagene), resulting in an E212N substitution, which inhibits DNA binding (Takebayashi et al., 1996). RNA interference was achieved using s12lancer plasmid [Ambion] expressing MITF siRNA or scrambled control (Takebayashi et al., 1996). RNA interference was achieved using pSi-

BrdU and immunofluorescence assays

BrdU and immunofluorescence assays were performed as described previously (Ma et al., 2003). In brief, 10^5 cells were transfected with the indicated expression vectors, along with an GFP vector (10:1 ratio), on cover-

slips. For BrdU assays, cells were labeled with BrdU (1:1,000 dilution; Amersham Biosciences) 24-72 h after transfection. After a further 12-36 h, cells were rinsed with 1 x PBS, fixed in 3.7% formaldehyde for 15 min, permeabilized in 0.3% Triton X-100 for 15 min, and probed with an anti-BrdU antibody (BD Biosciences) at 1:200 dilution in immunofluorescence buffer for 1 h at 37°C in a humidified chamber. After washing the coverslips twice with 1 ml/well PBS, the cells were incubated with Alexa Fluor-conjugated secondary antibody (Molecular Probes, Inc.), at 1:1,000 dilution for 1 h. Cells were stained with DAPI, mounted onto slides using Vectashield mounting medium (Vector Laboratories) and analyzed by fluorescence microscopy. The percentage of cells incorporating BrdU was calculated from the fraction of GFP-positive transfected cells that were positive for BrdU staining.

For other immunofluorescence assays, cells were incubated with anti-

S100, anti-Dct, or anti-TRP1 antibodies and Texas-Red-conjugated secondary antibodies (all from Santa Cruz Biotechnology, Inc.) and analyzed by fluorescence microscopy in a similar fashion.

Image acquisition

Microscopy was performed with an Olympus BX60 microscope (brightfield) and an Olympus BH2 microscope (fluorescence) using an Olympus UPLan FL 40 x magnification objective lens with 0.75 aperture. Photomicro-

graphs were obtained at RT under air using the Olympus DP70 camera (brightfield) and the Insight Spot RT camera (fluorescence). The fluorochrome was Oregon green 488 (Molecular Probes, Inc.). Image acquisition was achieved with Olympus DP controller software. Minimal image processing was performed with Adobe Photoshop.

Western blot

Western blots were performed as described previously (Ma et al., 2003) using antibodies that recognize MITF (Oncogene Research Products), Rb (sc102; Santa Cruz Biotechnology, Inc.), Rb-phospho-Ser780 (Cell Signaling), p16\(^{ink4a}\) (Cell Signaling), and gamma-tubulin (Sigma-Aldrich).

Quantitative PCR

Total RNA was collected using Trizol (Invitrogen), cDNA was generated using Superscript First Strand Synthesis (Invitrogen), and amplification was performed using the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen) according to manufacturer’s instructions in an iCycler (Bio-Rad Laboratories). LUX Fluorogenic primers for MITF-M and INK4A transcripts were: MITF-forward 5'-CCCTGCGCGCTTCTGATG-3', MITF-reverse 5'-CAACAATCACGTGCTTACTGGAGG-3', INK4A forward 5'-CGGTCCCTCCAGAGGATTG3'-3', INK4A-reverse 5'-ACCGCTCTTTTCTTCTTCCGG-3'.

Methylation-specific PCR

Genomic DNA was isolated using the DNeasy Tissue kit (Qiagen), was bisulfite modified, and was amplified with methylation-specific primers for INK4A promoter CpG islands. Primer sequences are as follows: methylated-forward 5'-TTTAAATAGTTTTTGTTTGAAGG-3', methylated-reverse 5'-ACGAATTATAACCACCCCTTAAACC-3', unmethylated-forward 5'-TTTTAAATAGTTTTTGTTTGAAGG-3', and unmethylated-reverse 5'-AAAAATATACCCCCTTAAATACC-3'. PCR was performed using a 0.5 μg template, 20 pmol each primer, 1.25 mM dNTP, 6.75 mM MgCl\(_2\), 10x PCR buffer (Invitrogen), and Taq Polymerase (Invitrogen). Reactions were run at 94°C for 5 min, followed by 35 cycles of 94°C (30 s), 53°C (30 s), and 72°C (30 s). PCR products were visualized on a 2.0% agarose gel containing ethidium bromide. MEL202 cells were used as a positive control.

Chromatin immunoprecipitation assays

ChIP assays were performed using a commercial kit (Upstate Biotechnology) as described by the manufacturer. In brief, 10^6 normal melanocytes were cross-linked in PBS containing proteinase inhibitors and 1% formaldehyde for 15 min at 37°C, scraped and centrifuged (4 min, 2,000 rpm, 4°C), resuspended in SDS lysis buffer with proteinase inhibitors, sonicated, and centrifuged (4 min, 2,000 rpm, 4°C). Dilution buffer was added, and supernatants were precleared with 75 μl salmon sperm DNA/protein A 1 μl MITF antibody [Oncogene Research Products] or mouse IgG (negative control) was added overnight at 4°C, then 60 μl salmon sperm DNA/protein A was added for 1 h at 4°C. The slurry was pelleted (1 min, 1,000 rpm, 4°C), and was washed 5 min each with low salt wash, high salt wash, LiCl buffer, and TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Samples were treated with 250 μl elution buffer (1% SDS/0.1 M NaHCO\(_3\)) at RT for 15 min, then with 20 μl of 5M NaCl at 65°C for 4 h, then with 0.5M EDTA/Tris-HCl and 2 μg proteinase K at 45°C for 1 h. DNA was purified using the DNEasy kit (Qiagen). PCR to detect the INK4A and SIL promoters was performed with the following primers: INK4A-S'-AGGAAGCAGGAGCCTGTAATCCCAGCTA-3'; INK4A-S'-TGGGAGAGACACGAGCCAGCGAGCAGCGG-3'.

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CAAGAGGCGAACCTTG3′, SILV-5′-CATGGAGAACCTCCAAAGGT-GG3′, and SILV-5′-TACTCTCCCAAGGGTATAAGT3′.

**Online supplemental material**

Gel mobility shift assay demonstrates that MITF binds to the INK4A promoter elements, and that efficient binding requires both the E box and the M box (Fig. S1). Western blot analysis (Fig. S2 A) and immunofluorescence assays (Fig. S2 B) demonstrate that ectopic MITF is expressed at similar levels in wild-type and INK4A-null MEFs. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200410115/DC1.

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