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Id1 expression is transcriptionally regulated in radial growth phase melanomas

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Id genes have been demonstrated to be upregulated in a wide variety of human malignancies and their expression has been correlated with disease prognosis; however, little is known about the mechanisms of Id gene activation in tumors. We have previously shown that the helix-loop-helix transcription factor, Id1, is highly expressed in primary human melanomas during the radial growth phase and that Id1 is a transcriptional repressor of the familial melanoma gene CDKN2A. Here we use a series of melanoma cell lines that recapitulate the phenotypic characteristics of melanomas at varying stages of malignant progression to evaluate the expression levels of Id1 in this model system and determine the mechanism of Id1 dysregulation in these tumor cells. We find elevated protein levels of Id1 to be present consistently in radial growth phase tumor cells in accordance with our primary tumor data. Id1 transcript levels were also found to be elevated in these radial growth phase melanoma cells without any appreciable evidence of gene amplification and Id1 promoter activity was found to correlate with Id expression levels. We therefore conclude that Id1 expression is primarily regulated at the transcriptional level in radial growth phase melanomas and expect that therapies that target Id1 gene expression may be useful in the treatment of Id-associated malignancies.
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Key words: melanoma; Id1; transcription

Id helix-loop-helix transcription factors have been identified as potential protooncogenes since their constitutive expression in primary cells promotes cellular immortalization[1–3]; however, tumor-associated defects in Id genes have not been identified to date and the mechanism of Id gene regulation in tumors has not been well-defined. Over the past several years, there has been growing interest in the role of Id genes, and Id1 in particular, in human malignancies since Id1 gene expression has been shown to be elevated in a variety of primary human tumors *versus* normal control tissue specimens including breast cancers,[4] thyroid cancers,[5] prostate cancers,[6] endometrial cancers,[7] ovarian cancers,[8] cervical cancers,[9] gastric cancers,[10,11] squamous cell carcinomas of the esophagus and nasopharynx[12] and melanomas.[13] Id1, Id2 and Id3 expression are all upregulated in pancreatic cancers, colorectal cancers, astrocytic tumors and squamous cell carcinomas of the head and neck and all 4 Id genes are upregulated in testicular seminomas, further supporting the notion of Id genes as cellular protooncogenes (reviewed in[14–16]).

In general, *in-situ* evaluation of Id expression in human tumors has revealed a correlation between tumor invasiveness/progression and Id expression suggesting that Id genes may be useful therapeutic targets for a variety of malignancies. We have previously shown that Id1 expression is associated with transcriptional repression of the familial melanoma gene, *p16/ink4a*[17] and that Id1 expression is upregulated in primary human radial growth phase melanomas[13]. To investigate the mechanism of Id gene dysregulation in melanoma, we evaluated expression of Id1 in human melanoma cell lines that recapitulate the phenotypic characteristics of melanomas at varying levels of malignant progression.[18] Here we show that Id1 expression is consistently elevated in early, radial-growth phase melanoma cells and that this expression is regulated at the level of gene transcription without any associated gene amplification. We also note that melanoma cells from later stages of malignant progression that do not express elevated levels of Id1 do not possess epigenetic changes associated with Id1 silencing but do display moderate Id1 regulation through proteasomal degradation. We conclude that Id1 expression is transcriptionally upregulated in early-stage human melanomas and suggest that transcription factors that mediate Id gene expression in tumor cells may serve as useful therapeutic targets for Id-associated malignancies.

Material and methods

Cell culture

Melanoma cell lines (WM35, Sbcl2, WM1552C (radial growth phase), WM1341D (early vertical growth phase), WM902B, WM278, WM983A (late vertical growth phase), WM852, WM983B, 1205Lu (metastatic melanomas)) were obtained from Meenhard Herlyn (Wistar Institute, Philadelphia, PA) and cultured in melanocyte growth medium (Cell Applications) without PMA, supplemented with 2% fetal bovine serum (FBS) with the exception of cell lines Sbcl2 and WM902B which were supplemented with 10% FBS. Primary human melanocytes (HMCs) were isolated from neonatal foreskins and propagated in melanocyte growth medium (Cell Applications). Melanoma cells (WM1552C, WM983A, WM852, WM983B, 1205Lu) growing in 10% FBS/DMEM medium were treated with Lactacystin at a concentration of 10 μM for 24 hr. Mock control cells were treated with DMSO.

Real-time PCR

High-molecular-weight genomic DNA isolation from short-term cultured primary human melanocytes and 10 melanoma cell lines was performed. Cells growing in monolayer culture were washed twice with PBS and lysed in lysis buffer (10 mM Tris-Cl (pH 8.0), 0.1 M EDTA (pH 8.0), 0.5% (w/v) SDS, and 20 μg/ml DNase-free RNase). After incubation of the lysate at 37°C for 30 min, proteinase K (1.0 mg/ml to a final concentration of 100 μg/ml) was added and the lysate was incubated in a water bath for 3.0 hr at 50°C. Genomic DNA was extracted using Phenol/chloroform methods followed by EtOH precipitation and dissolved in TE buffer.

Real-time quantitative PCR was performed in a 96-well microtiter plate (Applied Biosystems, Foster City, CA) with an ABI PRISM 7900HT Sequence Detector System (Applied Biosystems) using TaqMan™ PCR Core Reagents kit with internal probes labeled by FAM for 5′ and TAMRA for 3′ (Applied Biosystems). The target genes were generated using the following sequences for gene-specific primers and probes: *Id1* sense: 5′-CACCCTCAAC GGCGAGATC-3′; antisense: 5′-CCACAGAGCACGTAATTCC TC-3′; *Id1* probe: 5′-FAM CTTGCACCACTTCCGTCCCATCT

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AMRA-3′. *GAPDH* sense: 5′-GAAGGTGAAGGTCGGAGTCA AC-3′; antisense: 5′-GCACCCGCTGCGCACTAGCAT-3′; GA PDH probe: 5′-FAMCCAGAAACAGGAGGTCCCTACTCCTA MRA-3′. Threshold cycle number was determined using SDS2.0 software. The reactions were performed in triplicate and the results were normalized to glyceraldehydes-3-phosphate (GAPDH) as a reference gene. Genomic DNA levels of Id1 in melanoma cells relative to primary human melanocytes was calculated according to the formula $(R_t - E_t)/R_n - E_n)$ where R_t is the threshold cycle number for the reference gene in the melanoma cell line, E_t is the threshold cycle number for the experimental gene in the melanoma cell line, R_n is the threshold cycle number for the reference gene in the primary human melanocytes and E_n is the threshold cycle number for the experimental gene in the primary human melanocytes.

Total RNA from short-term cultured primary human melanocytes and melanoma cell lines was isolated using the RNeasy mini kit from (Qiagen). Single-stranded cDNA was synthesized using Superscrip™ First-strand Synthesis System (Invitrogen). Primers and probe sequences used are: *Id1* sense: 5′-CACCCTCAAC GGCGAGATC-3′; *Id1* antisense: 5′-CCACAGAGCACGTAA TTCCTC-3′; *Id1* probe: Fam ACGGCCGAGGCGGCATGCGT Tamra. *GAPDH* sense: 5′- GAAGGTGAAGGTCGGAGTCAAC-3′; antisense: 5′-GAAGATGGTGATGGGATTT CCAG-3′; GAP DH probe: 5′-Fam TGACAAGCTTCCCGTTCT CAGCC Tamra-3′. Quantitative PCR and calculation of relative fold difference between primary melanocytes and melanoma cell lines was conducted as described earlier.

Transient transfection and luciferase assays

A dual reporter luciferase assay was carried out to assess Id1 promoter activity in melanoma cells. Approximately 80% confluent cells were transiently cotransfected using 0.8 μg of plasmid DNA (pID1SB-Luc)[19] or control plasmid DNA (pGL2-Basic) with 10 ng of pRL-SV40 Renilla plasmid using the Lipofectmine™ 2000 reagent (Invitrogen). Thirty hours after transfection, cells were lysed with 100 μl of lysis buffer, and luciferase activity was determined using the dual luciferase reporter assay system (Promega) according to manufacturer's protocol. The firefly luciferase activities were normalized by renilla luciferase activity. The normalized luciferase activities were then divided again by the protein content of the assayed samples in order to compare the ID1 promoter activities of each melanoma cell line. All transfections were repeated in triplicate and relative luciferase activities were expressed as the mean ± S.D. of each experiment. The results shown are representative of 3 independent experiments.

Western blot analysis

Cells were grown in the above noted growth media and supplemented with 10% FBS prior to lysis. Cells were lysed using 0.1% NP-40 lysis buffer (0.1% NP-40, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM DTT, 0.5 mM AEBSF, 1 μg/ml aprotini/leupeptin, 2 mM NaF, 0.5 mM Na-Vanadate) and 100 μg of protein was run on an SDS-PAGE gel and blotted onto PVDF membranes (Immobilon-P, Millipore) as previously described.[17] Western blotting was performed according to standard procedures. Primary antibodies used included anti-Id1, p16 and PCNA antibodies (SC-488, SC-1661, SC-56, Santa Cruz Biotechnology), and anti-actin antibody (N350, Amersham).

Methylation-specific PCR

Genomic DNA from melanoma cell lines and primary human melanocytes was bisulfite modified as previously described.[20] MSP primers were designed according to genomic sequences in the proximal promoter region immediately 5′ to the transcription start site (+1), which is at position 2,114 (Genbank sequence U57645). Primer sequences were oligo-synthesized (IDT, Coralville, IA) to allow MSP to detect bisulfite-induced changes affecting unmethylated (U) and methylated (M) alleles. MSP primers are as follows: ID1-M-forward, 5′-ATTTTTGTTGTTTTGAAA TTCGGGTC-3′; ID1-M-reverse, 5′-CGACATAAAAACGTAA CCCGCG-3′, amplifying a 101 bp fragment corresponding to bases 1,923–2,024 in Genbank sequence U57645, and ID1-UN-forward, 5′-TTTATTTTTGTTGTTTTGAAATTTGGGTT-3′; ID1-UN-reverse: 5′-CCAATAAACAACATAAAAACATAACCCAC A-3′; amplifying a 112 bp fragment corresponding to bases 1,920–2,032 in Genbank sequence U57645. Each MSP reaction incorporated ~100 ng of bisulfite-treated DNA, 25 pmoles of each primer, 100 pmoles dNTPs, 2.5 μl 10× PCR buffer, and 1 unit of JumpStart Red Taq Polymerase (Sigma) in a final reaction volume of 25 μl. Cycle conditions were: 95°C × 5 min; 35 cycles × (95°C × 30 sec, 60°C × 30 sec, 72°C × 30 sec); 72°C × 5 min. MSP products were analyzed using 6% polyacrylamide gel electrophoresis.

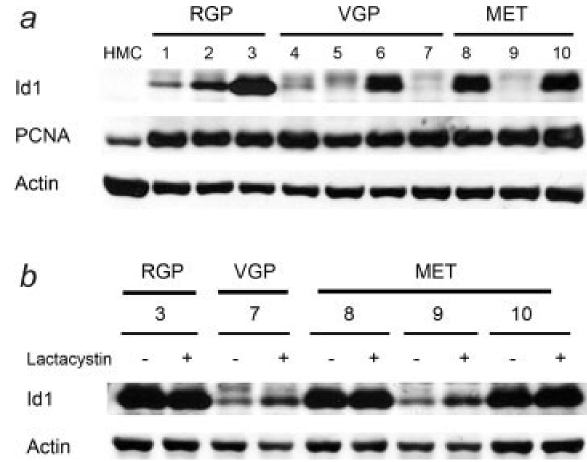

Figure 1 – Id1 protein levels are elevated in radial growth phase melanomas (*a*) Melanoma cell lines (HMC = primary human melanocytes, 1 = WM35, 2 = Sbc12, 3 = WM1552C (RGP-radial growth phase), 4 = WM1341D (VGP-early vertical growth phase), 5 = WM902B, 6 = WM278, 7 = WM983A (VGP-late vertical growth phase), 8 = WM852, 9 = WM983B, 10 = 1205Lu (MET-metastatic melanomas)) were evaluated for expression of Id1, proliferating cell nuclear antigen (PCNA), and actin (loading control) by Western blotting. M=molecular weight marker at 20 kD. (*b*) Melanoma cell lines noted above were treated with the proteasome inhibitor lactacystin and evaluated for Id1 expression by Western blotting. Actin appears as a loading control.

Results

Id1 expression is elevated in radial-growth-phase melanomas

Since we had previously evaluated Id1 expression in a small set of melanoma tissue specimens and found Id1 expression to be elevated in radial growth phase tumors *in vivo*,[13] we sought to evaluate whether cultured melanoma cells also maintained Id1 gene expression in a stage-specific manner. We therefore evaluated a series of melanoma cell lines from different stages of progression[18] for protein levels of Id1 using Western analysis. Melanoma cell lines [WM35, SBcl2 and WM1552C (radial growth phase), WM1341D (early vertical growth phase), WM902B, WM278, WM983A (late vertical growth phase), WM852, WM983B, 1205Lu (metastatic melanomas)] were obtained from Meenhard Herlyn (Wistar Institute, Philadelphia, PA) and cultured as described. Remarkably, we found that 2/3 of radial-growth-phase melanoma cells consistently expressed elevated levels of Id1 protein compared to primary human melanocytes (HMCs) while later stages of malignant progression displayed heterogeneous Id1 expression with 1/4 of all vertical growth phase melanomas expressing elevated levels of Id1 (Fig. 1*a*). As expected, all tumor cell lines expressed high levels of the proliferating cell nuclear antigen (PCNA) relative to primary human melanocytes. We also sought to determine whether Id1 expression inversely correlated with p16/CDKN2A expression in melanoma cells as we had noted

previously in primary melanocytic lesions.[13,17] We found no detectable levels of p16/INK4a in our melanoma cell lines and only noted expression in our primary human melanocytes confirming the loss of p16 expression in the melanoma cell lines as previously described[18] (data not shown). Since Id gene expression has been demonstrated to be tightly regulated by ubiquitination and proteasomal degradation,[21] we evaluated Id1 expression in melanoma cell lines exposed to lactacystin, a specific inhibitor of the 26S proteasome. We noted elevation of Id1 protein levels in two late-stage melanoma cell lines expressing lower amounts of Id1 suggesting some degree of posttranslational regulation of Id1 expression by proteasomal degradation in aggressive melanomas (Fig. 1*b*).

Id1 gene expression is transcriptionally regulated in melanoma cells

Since we were intrigued by the correlation of Id1 protein expression with the radial-growth-phase in our melanoma cells lines and the potential replication of expression patterns analogous to those seen *in vivo*, we sought to determine the level of gene regulation of Id1 in the cell lines evaluated. Quantitative RT-PCR of Id1 transcripts and quantitative PCR of Id1 genomic DNA were standardized to expression levels and genomic DNA levels in primary human melanocytes. Of note, tumor cell lines were grown in media containing 2% FBS as detailed in the materials and methods since Id genes are notoriously serum–responsive. These studies revealed nearly 75-fold elevated expression of Id1 mRNA in the radial-growth-phase melanoma cell line Mel-3 (WM1552c) and 15-fold expression of Id1 mRNA in the Mel-2 (Sbcl2) radial-growth-phase cell line (Fig. 2*a*). Elevated Id1 transcript levels were also noted in the metastatic melanoma cell lines Mel-8 (WM852) and Mel-10 (1202Lu) which also demonstrated appreciable Id1 protein expression on Western analysis (Fig. 1*a*); however, transcript levels for the invasive melanoma cell line Mel-6 (WM278) were not particularly elevated despite increased protein levels suggesting serum-responsive expression of Id1 protein in these cells. Relative genomic DNA copy numbers for Id1 were normalized to GAPDH copy number and evaluated in all cell lines (Fig. 2*b*). Cell lines demonstrated a maximal 4-fold copy number increase in Id1 genomic DNA which did appear to correlate with protein and transcript levels of Id1 in the associated cell lines suggesting correlation with DNA copy number but lack of genomic DNA amplification. Further studies of Id1 promoter activity using an Id1 luciferase reporter construct confirmed the association of Id1 protein levels with transcriptional regulation of Id1 with highest promoter activity and transcript levels noted in the radial growth phase melanoma cell line Mel-3 (Fig. 2*c*).

Id1 gene silencing in melanomas is not associated with promoter methylation

Since both *in vivo* and *in vitro* evaluation of Id1 expression in melanomas suggests early transcriptional upregulation with subsequent gene silencing at later stages of disease progression, we sought to identify the mechanism of Id1 gene silencing in the melanoma cell lines evaluated. A putative CpG island was identified at the 5′ region of the Id1 gene and evaluated for epigenetic silencing using methylation-specific PCR (Fig. 3*a*). Methylation-specific PCR at the above-noted CpG island in all cell lines failed to demonstrate methylated CpG sites within the Id1 genomic DNA from any of the melanoma cell lines evaluated suggesting alternative mechanisms of Id1 inactivation in late-stage melanomas (Fig. 3*b*).

Discussion

Id genes have been implicated in the development of a variety of malignancies because of their deregulated expression in tumors *versus* normal tissues[14]; however, the mechanisms underlying regulation of Id gene expression in cancers have not been well delineated. Here we examine the expression of Id1 in human melanoma cell lines that recapitulate tumors of varying stages of malignant progression. We find elevated Id1 protein expression in radial growth phase melanoma cell lines (2/3) in correlation with our previous findings in *in situ* analyses of primary melanocytic lesions and melanomas.[13] We go on to evaluate the level of dysregulation of Id1 in melanomas and find that Id1 is transcriptionally activated in radial-growth-phase melanomas in association with elevated Id1 mRNA and increased Id1 promoter activity but without evidence of gene amplification. Since Id1 is expressed in early primary melanomas and often silenced at later stages of the disease,[13] we sought to determine the role of epigenetic gene silencing through promoter methylation as a means of Id1 deactivation. Our studies show that in all 10 melanoma cell lines tested, putative methylation sites within the Id1 promoter remain unmethylated suggesting other means of Id1 silencing in later stage melanomas. Further evaluation of Id1 ubiquitination and proteasomal degradation using the specific inhibitor of the 26S proteasome, lactacystin, demonstrated evidence for Id1 degradation by the proteasome in metastatic melanomas (Fig. 1*b*). On the basis of the above data, we suggest that Id1 gene activation plays a role in the development of radial growth phase melanomas but that later stages of the disease may be attributable to other genetic and epigenetic alterations that occur as a result of early activation of Id1. Interestingly, we did see elevated Id1 protein and transcript levels in 2 of 3 metastatic melanoma cell lines; however, data from our previous *in vivo* studies only showed perivascular expression of Id1 in met-

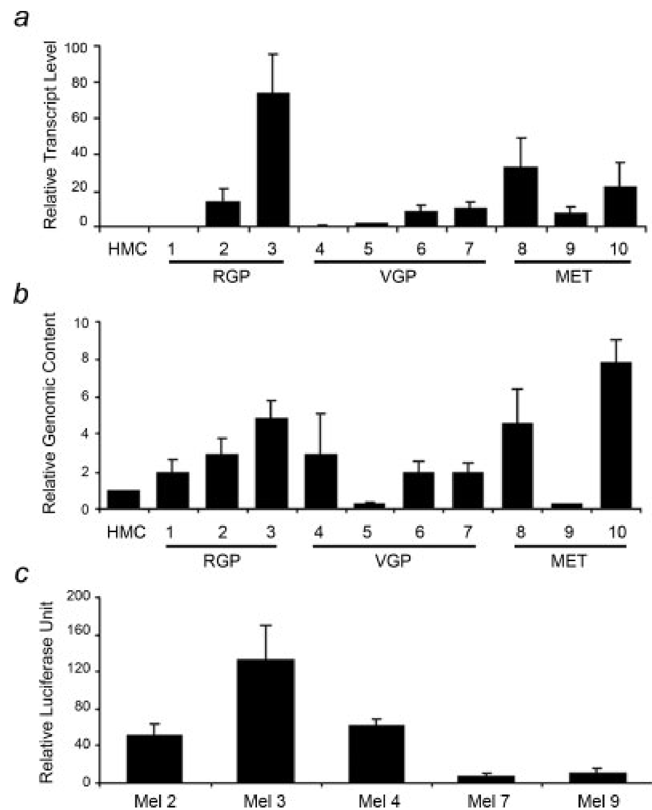

Figure 2 – Id1 expression is transcriptionally regulated in human melanoma (*a*) Quantitative RT-PCR for Id1 normalized to GAPDH in melanoma cell lines (HMC = primary human melanocytes, 1 = WM35, 2 = Sbc12, 3 = WM1552C (RGP-radial growth phase), 4 = WM1341D (VGP-early vertical growth phase), 5 = WM902B, 6 = WM278, 7 = WM983A (VGP-late vertical growth phase), 8 = WM852, 9 = WM983B, 10 = 1205Lu (MET-metastatic melanomas)) (*b*) Quantitative PCR normalized to GAPDH for Id1 genomic DNA. Note no cell lines evaluated had >3-fold amplification of the Id1 locus. (*c*) Id1 promoter activity for melanoma cell lines of varying stages of progression. Id1 reporter activity is assessed using relative luciferase units for the melanoma cell lines depicted.

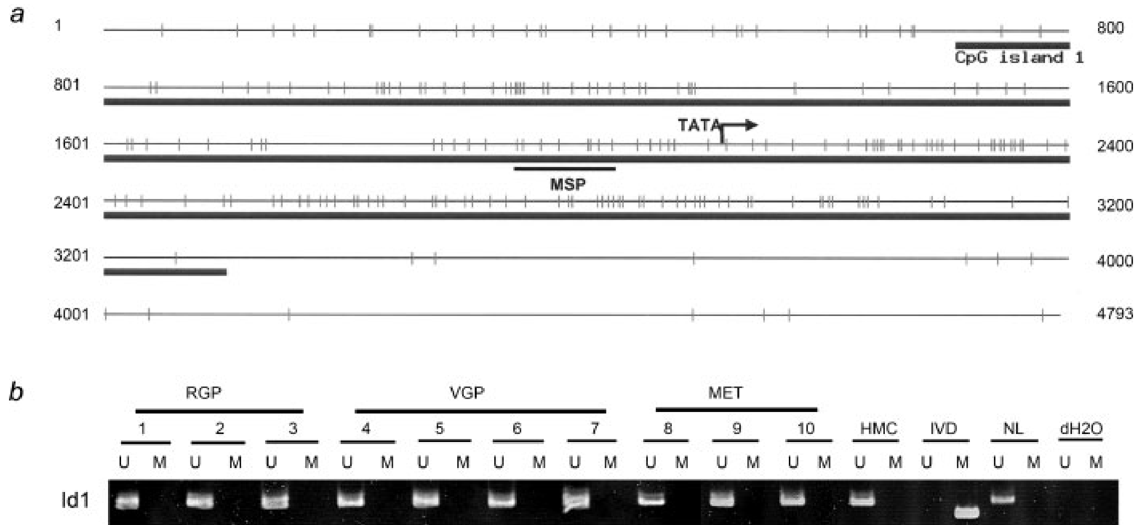

Figure 3 – Id1 is not methylated during melanoma progression (*a*) Genomic map depicting the 4,793 base pair region of the ID-1 gene, including the proximal promoter and exons 1 and 2, from Genbank sequence U57645. A broken arrow indicates the transcription start site (+1), which is at position 2,114, following a TATA box (2080–2086). A CpG island, identified by CpG Island Searcher program is shown by the thick horizontal bar and extends from nucleotide 700–3,300. CpG dinucleotides are indicated by vertical bars. The region examined by MSP to determine ID-1 promoter methylation status is noted (nucleotides 1,923 to 2,024 for methylated reaction and 1,920–2,032 for unmethylated reaction). (*b*) Methylation status of the ID-1 promoter in melanoma cell lines. Melanoma (MEL) cell lines (1 = WM35, 2 = Sbc12, 3 = WM1552C (RGP-radial growth phase), 4 = WM1341D (VGP-early vertical growth phase), 5 = WM902B, 6 = WM278, 7 = WM983A (VGP-late vertical growth phase), 8 = WM852, 9 = WM983B, 10 = 1205Lu (MET-metastatic melanomas) were evaluated for Id1 promoter methylation. The presence of a band exclusively in the U lane or M lane indicates that promoter CpG dinucleotides are fully unmethylated or fully methylated, respectively. *In vitro* methylated DNA (IVD) serves as a positive methylation control. Normal lymphocytes (NL) and primary human melanocytes (HMC) serve as positive unmethylated controls. Water (dH_2O) serves as a negative control.

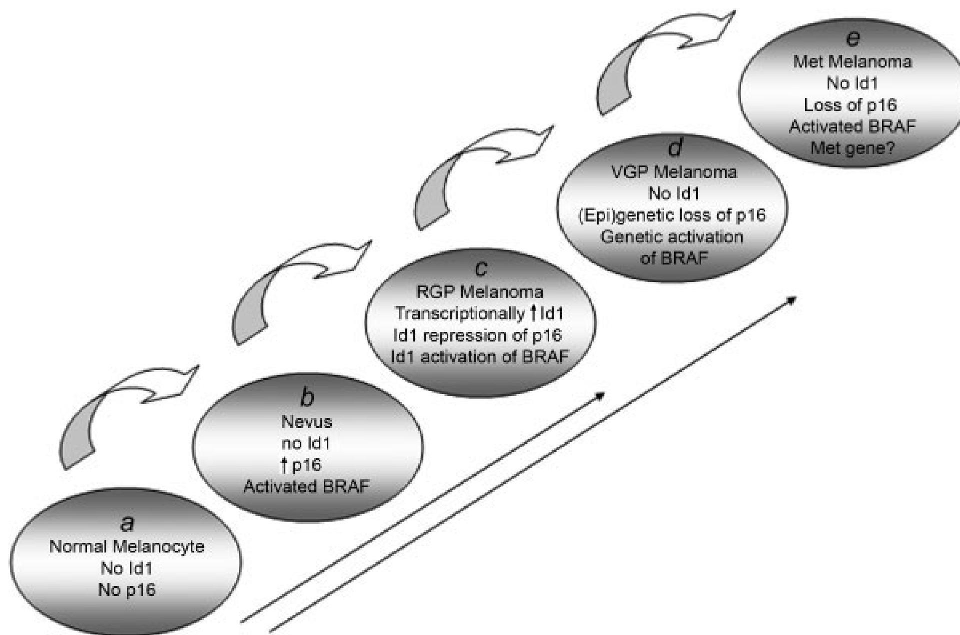

Figure 4 – Model for the role of Id1 in melanoma development and progression. Transcriptional activation of Id1 appears to be an early event in melanoma development. Unlike BRAF kinase activity which is activated in benign nevi (*b*), Id1 activation appears to be specifically associated with the earliest stages of melanoma development (*c*) and can activate the RAF kinase pathway and inactivate the p16/INK4a tumor suppressor. Id1 activation promotes cell cycle progression and cellular proliferation through activation of the RAF kinase pathway and inactivation of the retinoblastoma tumor suppressor pathway *via* p16/INK4a repression. Subsequent genetic or epigenetic events targeting BRAF (or other genes in the MAP kinase pathway) and p16/INK4a allow for sustained melanoma development and progression in the absence of Id1 (*d*). Melanoma metastasis proceeds through additional, as yet undefined, pathways (*e*). While the progression of melanoma development may proceed along the above noted pathway (*a–e*), additional pathways that lead to melanoma development are indicated by the black arrows below the diagram.

astatic melanomas without significant intratumoral Id1 expression.[13] We hypothesize that the elevated Id1 protein levels seen in the metastatic melanoma cell lines reflects molecular changes that have been acquired *in vitro* through serial passage of genomically unstable tumor cells since Id1 protein levels are elevated in radial growth phase cell lines but not in invasive melanoma cell lines and again appear to be elevated in metastatic melanoma cell lines.

Much data has accrued over the past few years implicating the BRAF kinase in melanoma development since 60–70% of benign nevi and melanomas possess activating BRAF mutations (reviewed in[22]). Surprisingly, BRAF mutations are relatively rare in radial growth phase melanomas suggesting an alternative pathway for melanoma development in some early, preinvasive lesions.[23] Although this alternative pathway is likely to involve activation of the functionally redundant N-RAS pathway, recent studies suggest that Id1 can also activate the MAP kinase signaling pathway through activation of RAF/ERK kinase.[24] We therefore propose a model for melanoma development that, in some cases, involves the early transcriptional activation of Id1 with associated activation of RAF kinase and repression of p16/INK4a. Id1-associated activation of Raf kinase and repression of p16/CDKN2A could allow for enhanced cellular proliferation which predisposes affected melanocytes to acquire additional genetic mutations. Thus, more advanced, invasive stages of melanoma do not express elevated Id1 because of subsequent genetic or epigenetic changes involving the p16/INK4a tumor suppressor pathway and the MAP kinase pathway (Fig. 4). What are the clinical implications of these findings? Our model presupposes that Id1 expression in tumor cells indicates early, radial-growth-phase melanoma without invasive potential which would therefore represent a tumor with good prognosis. Additionally, previous studies showed no significant Id1 expression in benign nevi or atypical nevi supporting the hypothesis that Id1 expression is important for early malignant transformation of melanocytes.[13] An alternative hypothesis would suggest that Id1 expression in a melanocytic tumor represents a proliferative "dead end" and that such clonal Id-expressing melanocytes will never go on to become invasive tumors. In either case, Id1 expression would be expected to portend a good outcome for patients diagnosed with melanoma. Large-scale studies are currently underway to evaluate the diagnostic and prognostic utility of Id1 expression in melanomas.

We conclude that Id1 expression is transcriptionally upregulated in early, radial growth phase melanomas but is often inactivated in later stages of the disease in association with genetic/epigenetic alterations in the BRAF kinase and retinoblastoma tumor suppressor pathways. The critical question that remains to be answered in all Id-associated malignancies continues to be: what is the source of transcriptional activation of Id1 in such a broad spectrum of tumors and are Id genes the relevant therapeutic targets in these malignancies? This question has been raised in the past[15] and its answer remains elusive. While transgenic model systems suggest a causative role for Id genes in select tumors (reviewed in[14]) such proof-of-principle experiments remain to be developed more broadly. It is likely that the true significance of Id gene expression in human cancers will be determined through genetic analysis of Id gene function in a wide range of malignancies.

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