Expression Profiling Reveals Novel Pathways in the Transformation of Melanocytes to Melanomas

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ABSTRACT

Affymetrix and spotted oligonucleotide microarrays were used to assess global differential gene expression comparing normal human melanocytes with six independent melanoma cell strains from advanced lesions. The data, validated at the protein level for selected genes, confirmed the overexpression in melanoma cells relative to normal melanocytes of several genes in the growth factor/receptor family that confer growth advantage and metastasis. In addition, novel pathways and patterns of associated expression in melanoma cells not reported before emerged, including the following: (a) activation of the NOTCH pathway; (b) increased Twist expression and altered expression of additional transcriptional regulators implicated in embryonic development and epidermal/mesenchymal transition; (c) coordinated activation of cancer/testis antigens; (d) coordinated down-regulation of several immune modulation genes, in particular in the IFN pathways; (e) down-regulation of several genes implicated in membrane trafficking events; and (f) down-regulation of growth suppressors, such as the Prader-Willi gene NECEDIN, whose function was confirmed by overexpression of ectopic Flag-necdin. Validation of differential expression using melanoma tissue microarrays showed that reduced ubiquitin COOH-terminal esterase L1 in primary melanoma is associated with worse outcome and that increased expression of the basic helix-loop-helix protein Twist is associated with worse outcome. Some differentially expressed genes reside on chromosomal regions displaying common loss or gain in melanomas or are known to be regulated by CpG promoter methylation. These results provide a comprehensive view of changes in advanced melanoma relative to normal melanocytes and reveal new targets that can be used in assessing prognosis, staging, and therapy of melanoma patients.

INTRODUCTION

Wide-spread aberrations in gene expression due to losses and gains in genetic material, suppressive or activating mutations, and dysregulation of transcription factors are rampant in cancer cells, including melanomas. Inactivation of p16INK4a, the cyclin-dependent kinase inhibitor (cyclin-dependent kinase 4/6) that leads to suppression of retinoblastoma protein, is an early event commonly observed in melanomas and other cancer cells as well (reviewed in Refs. 1 and 2). p16INK4a is eliminated in most melanomas not only by point mutations, but also by deletion (Refs. 3 and 4; reviewed in Ref. 5) or silencing through promoter hypermethylation (6). The loss of p16INK4a expression correlates with the invasive stage of melanoma tumor progression (7–9), suggesting that its absence confers growth advantage. Retinoblastoma protein is also likely to be inactivated by signaling pathways that stimulate mitogen-activated protein kinase, such as activating mutation in B-Raf and N-Ras (10, 11), and constitutive stimulation of cell membrane receptors, such as the fibroblast growth factor (FGF) receptor 1 (12) and integrin pathway (13). One of the consequences of retinoblastoma protein inactivation is the release of E2F transcription factor, leading to aberrant expression of E2F-regulated genes that promote cell cycle progression (reviewed in Refs. 14 and 15).

Several high-throughput approaches have been applied to melanomas to assess chromosomal aberrations and gene expression patterns in an unbiased fashion. For example, comparative genome hybridization identified several chromosomal and genetic changes. These include losses of chromosomes 6q, 8p, and 10 and gains in copy number of chromosomes 1q, 6p, 7, and 8 in primary melanomas (16–22); amplification of the cyclin D1 locus in acral melanoma (17, 19); frequent deletion of chromosome 13 and 17p in melanomas arising in chronically sun-damaged skin; and frequent amplification of chromosome 12q in mucosal melanomas that can affect metastatic potential (23, 24). Global gene expression profiling of several melanoma subtypes has implicated dysregulation of the Wnt pathway in contributing to melanoma invasion and motility (25, 26), which was validated by ectopic expression of Wnt5a (26). More recently, serial analysis of gene expression of three melanoma tumors indicated up-regulation of intracellular calcium and G-protein signaling and increased expression of CD74 (27).

Most gene expression analysis studies did not include normal human melanocytes. Therefore, we set out to monitor global gene expression in normal melanocytes compared with melanoma cells. As reported below, gene expression profiling, validated at the protein level for selected gene products, provides a comprehensive view of molecular changes in malignant melanocytes and reveals possible underlying molecular pathogenesis not identified before.

MATERIALS AND METHODS

Cells. Normal human melanocytes from newborn foreskins were grown in Ham’s F-12 medium (GibcoBRL, Invitrogen Corp., Grand Island, NY) supplemented with serum (7% fetal bovine serum from Gemini Bio-Products (Woodland, CA)), termed basal medium, which was further enriched with several ingredients required for optimal proliferation. They included 85 nm 12-O-tetradecanoyl phorbol-13-acetate (TPA), 0.1 nm 3-isobutyl-1-methylxanthine, 2.5 nm cholera toxin, 1 nm Na2VO4, and 0.1 nm N2,2’-O-dibutyryl-adenosine 3:5-cyclic monophosphate (all from Sigma-Aldrich, St. Louis, MO), termed TICVA (28). Early-passage melanocyte cultures (passage 1–2) pooled from three to six Caucasian donors were used to reduce individual donor variations. The normal melanocytes proliferated at a population doubling time of 3–4 days, were highly differentiated, and expressed all known melanocyte-specific genes (12).

The human primary melanoma WW165 (F, 2.25-mm lesion from the back)
and metastatic YUHEIK melanoma cells (F, maxillary gingiva, lymph node metastasis) were grown in basal medium supplemented with 3-isobutyl-1-methylxanthine required for optimal proliferation. The metastatic melanomas MNTI (M, lymph node metastasis; Ref. 29), YUMAC (M, recurrent metastasis at the site of excision), YUCAL (F, soft tissue metastasis), YUSAC2 (M, soft tissue metastasis), YUSAC1 (site unknown), 501 mel (site unknown; Ref. 30), and YUGEN8 (F, brain) were maintained in basal medium as described previously (28). The YUHEIK, YUMAC, and YUCAL melanoma cells were used during early passage in culture (passage 3–8).

The normal and malignant melanocytes were harvested during the logarithmic growth phase to avoid differences due to cell cycle distribution because normal melanocytes become arrested in the absence of specific growth factors under conditions that allow metastatic melanoma cells to proliferate; likewise, metastatic melanoma cells are arrested in the presence of TPA, MβC, 2′-O-dibutyryladenosine 3′-5′ cyclic monophosphate, and cholera toxin (31, 32).

The immortalized mouse melanocytes derived from a B10BR-black mouse were grown in Ham’s F-12 medium supplemented with antibiotics, 10% horse serum, and TPA (33). RNA isolation and application to DNA microarrays. Approximately 20–30 million cells (normal human melanocytes and melanoma cells/each) were used for mRNA extraction per hybridization. High-quality total RNA was prepared with the TRizol reagent (Invitrogen Life Technologies, Inc., Invitrogen Corp., Carlsbad, CA). The quality of RNA products was determined visually by 1% denaturing agarose gel, and RNA concentration was measured using a spectrophotometer. Poly(A) mRNA was further isolated from ~300 μg total RNA/sample using the PolyATract mRNA isolation system IV (Promega, Madison, WI) following the manufacturer’s instructions. This additional step is required due to the presence of melanin in the total RNA preparation, especially in normal human melanocytes, that suppresses PCR hybridization reactions (our experience as well as that of others; see, for example, Ref. 34). The quality and quantity of the poly(A) RNA products were determined by Bioanalyzer (Agilent Technologies) and spectrophotometric analysis.

The human genome Affymetrix GeneChip system HG-U133A microarray (Affymetrix Inc., Santa Clara, CA) containing 11 pairs of match/mismatch 25-mer oligonucleotide probes for each of ~14,500 transcripts of known genes and the OHU16K human library oligonucleotide array comprising a single 70-mer oligonucleotide probe in duplicate spots for each of 16,659 different gene transcripts (printed by the Keck Microarray Resource at Yale) were used. A description of these slides can be found online.10

For the HG-U133A chip, cDNA templates were generated from purified mRNAs for in vitro transcription of biotin-labeled amplified RNAs. Protocols for hybridizing fragmented biotin-labeled cRNAs, washing unhybridized material from the chips, and scanning them with a GeneArray Scanner (Agilent Technologies) were followed as recommended by Affymetrix Inc.

For the OHU16K microarray we used the two-color hybridization protocols of Drs. Patrick O. Brown and Geoff Childs (35) optimized by the Keck Resource. They are available online.11

The slides were scanned by the Keck Microarray Resource using a GenePix 4000A scanner (Axon Instruments). The Keck array slides was performed with QuantArray 2.0 (Perkin-Elmer, Boston, MA) and Qameger12 (Hongyu Zhao; Laboratory of Statistical Genomics and Proteomics, Yale Medical School, New Haven, CT). The OHU16K data were normalized using the Intensity Dependent (Lowess) method. The values of the raw data were set to be 5-fold higher than the background level of 200. Genes that were differentially expressed between normal human melanocytes and melanoma cells were generated using t tests with a significance level of 0.05.

The two types of microarrays were compared to find the common genes using UniGene as a common identifier and the KARMA program13 (Keck Microarray Resource, Yale Center for Medical Informatics, Yale University). Only the overlapped UniGene clusters were considered for the comparisons. When a UniGene cluster ID has more than one gene, the ratio for that cluster was calculated using the average value of the corresponding genes.

Hierarchical cluster analysis was performed by using the agglomerative clustering algorithm QT_Cluster (37) to determine associations between coexpressed genes allowing for a correlation coefficient of 0.97 as described online.14

Western blot analysis. Normal melanocytes and melanoma cells were lysed in radioimmunoprecipitation assay buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with a mixture of protease (Complete Boehringer Mannheim Corp., Roche Molecular Biochemicals, Indianapolis, IN) and phosphatase (100 μm NaF, 10 μm Na3P2O7, and 1 μm Na2VO4) inhibitors. Total cell extracts (35 μg proteins/lane), measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), were fractionated in precast gels composed of 8% polyacrylamide Tris-glycine or 10% polyacrylamide NuPAGE Bis-Tris (Novex, San Diego, CA) and Western blotted according to standard protocols (38).

Antibodies raised against the following proteins were used for Western blotting: UCHL1 (ubiquitin COOH-terminal esterase L1, also known as protein gene product 9.5; polyclonal antibody AB1761, Chemicon International, Inc.; fibronectin (ab299) and connective tissue growth factor [CTGF (ab6992)], abcam (Cambridge, United Kingdom); tenasin C (H-300), IGBP7 (C-16), ErbB-3 (C-17), anti-Twist (sc-15393), and anti-ZEB/TCF8 (E-020), Santa Cruz Biotechnology (Santa Cruz, CA); ATPas Na+/K+ transporting β1 polypeptide, Upstate Biotechnology (Lake Placid, NY); integrin-linked kinase-associated serine/threonine phosphatase 2C (ILKAP) and Rab27A, BD Transduction Laboratories (San Diego, CA); IFN-inducible MX1/MxA (from Dr. Mark A. McNiven; Center for Basic Research in Digestive Diseases and Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN); and Geoff Childs (35) against capping protein (gelsolin-like, CapG, anti-peptide WKGKANEK-ERQALQVAE) were raised in rabbits by ProteinTech Group, Inc. (Chicago, IL) and against neclin (NC243) by Michio Niinobe (39); anti-RAB32 antibodies were from Dr. R. J. Haslam (Department of Pathology and Molecular Medicine, McMaster University, Ontario, Canada). Anti-tyrosinase (monoclonal antibody T311) was used as a marker for differentiation, and anti-β-actin (A2066; polyclonal antibody; Sigma-Aldrich) was used as a measure for protein loading in each well. In several cases, the same membrane was successively blotted with two or three antibodies, thus sharing the β-actin control.

Melanoma tissue microarrays (TMAs). The TMA comprised 570 tissue cores (553 melanomas, 17 normal skin samples) measuring 0.6 mm in diameter, spaced 0.8 mm apart on a single glass slide. The cohort was constructed from paraffin-embedded formalin-fixed tissue blocks obtained from the archives of Yale University Department of Pathology as described previously (40, 41). The specimens were resected between 1959 and 1994, with a follow-up period ranging between 2 months and 38 years. The TMA slides were treated, probed, and scored as described previously (42). The primary

12 http://bioinformatics.med.yale.edu/.
13 http://ymd.med.yale.edu/karma/cgi-bin/karma.pl.
14 http://www.genome.org/cgi/content/full/9/11/1106.
antibodies anti-UCHL1 and anti-Twist, validated by Western blotting, were applied at 1:500 and 1:100 dilution, respectively. The prognostic significance of the parameters was assessed for predictive value using the Cox proportional hazards model with overall survival as an end point. Survival curves were calculated using the Kaplan-Meier method, with significance evaluated using the Mantel-Cox log-rank test.

**Plasmid and Transfection.** The mouse HEY1/HESR1-pRES2-EGFP plasmid (43) was from Drs. C. C. Hughes and M. Henderson (Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, CA), and pcDNA3.Flag-necdin (44) was from Drs. Timothy M. Wright and Bo Hu (Department of Medicine, University of Pittsburgh, Pittsburgh, PA). The HEY1/HESR1-pRES2-EGFP plasmid was transfected into immortalized TPA-dependent normal mouse melanocytes (33), and the pcDNA3.Flag-necdin was transfected into human melanoma cells (501 mel) using Lipo-fectAMINE 2000 reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) following the manufacturer’s instructions. Parallel cultures were transfected with pcDNA.GFP as a control. Transfection efficiencies were in the order of 30% as evaluated by sorting for green fluorescing cells. The level of the ectopic protein was also assessed 1 day or 2 days after transfection by Western blotting of cell lysates (35 μg protein/assay) with anti HEY1/HESR1 antibodies (AB5714; Chemicon International, Inc.) or anti-Flag M2 monoclonal antibody (Sigma-Aldrich). The HEY1/HESR1-transfected mouse melanocytes were grown in medium deprived of growth factor (TPA) 4 days after transfection for over 3 months to evaluate changes in growth properties and differentiation. The growth properties of pcDNA3.Flag-necdin as compared with green fluorescing protein transfecants were evaluated by DNA synthesis assay (28) after 2 days of incubation in G418-supplemented medium (400 μg/ml; 30,000 cells/well; 24-well plate in triplicates) applied 2 days after transfection and by cell proliferation after 8 days of selection in G418 starting with a seed cell number (200,000 cells each seeded in 5-cm2 Petri dishes) each transfection was repeated three times with similar results.

**Immunofluorescence Microscopy.** Melanoma cells and normal human melanocytes were grown on coverslips and processed for immunofluorescence analysis with anti-necdin and anti-Flag antibodies as described previously (45).

**Reverse Transcription-PCR.** Poly(A) tracked mRNA was isolated from total RNA using the PolyATtract mRNA isolation system IV (Promega) following the manufacturer’s instructions. Complementary DNA template was generated from the poly(A)-selected RNA using Invitrogen SuperScript II Reverse Transcriptase according to the manufacturer’s instructions, and the levels of Rab33A and FGFR3 gene transcripts compared with β-actin control were assessed by PCR using the following primer pairs synthesized by the oligonucleotide synthesis facility at the Department of Pathology, Yale University School of Medicine (Table 1).

**DNA Sequencing.** Sequencing of selected cDNA amplified from normal melanocytes and melanoma cells with gene-specific primers by PCR was performed by the Keck DNA Sequencing Resource at Yale.

**RESULTS**

**Global Changes in Gene Expression and Hierarchical Clustering**

Global probing for differences in gene expression between normal human melanocytes and melanoma cells showed statistically significant altered expression of 589 genes in the Affymetrix U133A data set using a 2.5-fold change cutoff and of 186 genes in the OHU16K arrays using a 2-fold change cutoff. These cutoffs were determined as the least stringent when compared with expression of known genes and published information. Of these, 315 and 274 genes were up- and down-regulated, respectively, in the Affymetrix U133A data set, and 99 and 87 genes were up- and down-regulated, respectively, in the OHU16K data set.

A comparison between the gene expression data from the Affymetrix U133A and OHU16K microarrays using a P of <0.05 without fold change cutoff restriction showed that the expression levels of only five genes were altered in the opposite direction in the two types of microarrays. Among these genes, ISG15 (IFN-α-inducible protein clone IFI-15K, also known as GIP2) showed significantly reduced expression in five of six melanoma cell strains compared with normal melanocytes in the Affymetrix data set (~21-fold) but was slightly up-regulated in the OHU16K microarray (1.44-fold). As shown in Fig. 5B, Western blot analysis revealed up-regulation of ISG15 in the melanoma cells, consistent with previously published data (46). The change in expression of the other four genes was in the range of 1.3–2, below that used in our analysis.

Hierarchical clustering of the Affymetrix U133A data set indicated that the two independent cultures of normal human melanocytes grown from different donors closely resembled each other (Fig. 1A, NM). Therefore, the average expression values for specific genes of the two data sets were used for further comparisons. In addition, the hierarchical clustering showed that among the six melanoma cell strains, the MNT1 gene expression pattern was the least divergent from that of normal human melanocytes, followed by the primary melanoma WW165 and the metastatic melanomas YUMAC, YUSIT1, YUHEIK, and YUCAL (Fig. 1A). The similarity between the metastatic MNT1 melanoma cells and normal melanocytes could not be attributed solely to differentiated state, being highly pigmented, and expressing normal levels of melanocyte-specific proteins such as tyrosinase (Fig. 1B) because YUHEIK and YUMAC melanoma cells have similar characteristics (Fig. 1B).

The bioinformatic and QT_Clust analysis of the combined data set revealed differential expression, in some cases in a coordinated manner, of genes representing several functional groups. We report here the analysis derived mostly from the Affymetrix data set, as corroborated or not conflicted by the OHU16K data set when applicable. The Affymetrix chip incorporates mismatch controls for cross-hybridization and was thus deemed more reliable.

Below is a description of changes in selected functional groups following, in most cases, the guidance set by Gene Ontology. The full scope of differential gene expression can be accessed in the supplementary data.

**Receptor Activity**

The oligonucleotide microarray results showed changes in the expression of receptors, ligands, cell surface and adhesion molecules that affect proliferation, angiogenesis, tissue remodeling, cell-cell communication, ion channels, attachment, motility, and invasion (Fig. 2A; Supplementary Table 1, A and B). Some of these changes, as observed also at the protein level (Fig. 2B), confirmed published observations. These include integrin signaling (47), insulin-like growth factor (IGF1) receptor (48), FGFR1 receptor (reviewed in Ref. 31), transforming growth factor β (49), ErbB-3 (50), EphA3 (reviewed in Ref. 51), melanoma-inhibitory activity (52), N-cadherin and protocadherin, down-regulation of E and P-cadherins (53, 54), and the transmembrane protease dipetidyl-peptidase IV (55). However, there were also changes in the expression of additional genes, in some cases in a coordinated manner, that had not been observed previously.

We found increased expression of NOTCH2 and HEY1/HESR1, the transcription factor activated by NOTCH signaling (Ref. 56; Fig. 2; Table 1: Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Product size (bp)</th>
</tr>
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<tbody>
<tr>
<td>Rab33A</td>
<td>F: 5'-GACAGATGGGCCGCACTCCAT-3'</td>
<td>835</td>
</tr>
<tr>
<td></td>
<td>B: 5'-TTTTTTTTATACAATTTTGATGGAAC-3'</td>
<td>835</td>
</tr>
<tr>
<td>FGF13</td>
<td>F: 5'-CACAATGAAGAAGGGGAGACAT-3'</td>
<td>429</td>
</tr>
<tr>
<td></td>
<td>B: 5'-TTCAGGACCCGACAGACTCC-3'</td>
<td>429</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: 5'-GTGGGGGCGCCCGCCGACCA-3'</td>
<td>540</td>
</tr>
<tr>
<td></td>
<td>B: 5'-CCTTAAATGTCAAGGACAGATT-3'</td>
<td>540</td>
</tr>
</tbody>
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* F: forward; B: backward.
A metalloproteinase 3 (TIMP3, YUCAL, YUSIT1, 501 mel, YUSAC, and YUGEN8) were amelanotic or very slightly tyrosinase levels were also highly pigmented. The other melanoma cells (WW165, melanocyte (and melanoma cells (YUHEIK, YUMAC, and MNT1) with high Supplementary Table 1).

**Cancer-Testis Antigens (CTAs)**

The results not only confirmed the up-regulation of several genes encoding CTAs (reviewed in Ref. 61) but also unraveled different patterns of coexpression for MAGE and GAGE family members (Fig. 4, A and B). The reexpression of at least some of these antigens in tumors is driven by demethylation of selected CpG in the promoter regions (reviewed in Refs. 61–63), and the unique pattern of expression for each group suggests a coordinated chromatin modification. Although the function of these antigens is not known, they are recognized by autologous CTLs and are targets for CTL-directed immunotherapy (reviewed in Ref. 61). The coordinated expression of specific CTA members revealed by our data may help in assessing antigen expression and selection of patients for immunotherapy.

**Immune Modulation**

In this category, we included the IFN-regulated genes, immune complements, and histocompatibility antigens, as well as genes encoding proteins in the vesicular and ubiquitin pathways (Supplementary Tables 2–4). The latter two categories may provide melanoma cells with an immune escape mechanism by reducing the level of antigenic peptides (see, for example, Ref. 64).

**IFN-Regulated Genes.** Genes that belong to the IFN pathway were predominantly down-regulated (21 of 22 genes), composing ~8% of all down-regulated genes (Supplementary Table 2A). Furthermore, 14 of these genes were coordinately down-regulated, as revealed by QT_Clust analysis (Fig. 5A). The down-regulation of MX1/IFIT7 was confirmed by Western blotting, showing nice correlation with the Affymetrix data set. MX1/IFIT7 was expressed in normal melanocytes (NM) and YUHEIK (Fig. 5B), the only melanoma cell strain with intensity levels similar to that in normal melanocytes (Fig. 5A, gray diamonds). On the other hand, the expression screed by the overexpression of several integrins, in particular ITGA7, ITGA4, ITGA6, and ITGB3 (integrins α7, α4, α6, and β3; Fig. 2C); the four integrin ligands TNC1, FN1, CTGF, and Cyr61 (cysteine-rich); and two tetraspanins, TM4SF13 and TM4SF1, known to complex with integrins (Fig. 2D) and the underexpression of ILKAP (Fig. 2E; Supplementary Table 1, A and B). Here again we confirmed the overexpression of CTGF and the underexpression of ILKAP at the protein level, the latter correlated nicely with the Affymetrix gene transcript hybridization intensity (compare Fig. 2F, ILKAP with Fig. 2E).

There was up-regulation of IGF1 (~4-fold, in four of six melanoma cells) and several insulin-like growth factor-binding proteins (IGFBPs), with IGFBP3 being the most prominent (Fig. 3). The expression of IGFBPs appeared to be nonrandom, with higher levels in metastatic cells, such as MNT1, YUHEIK, YUSIT1 and YUCAL, and to a lesser degree, YUMAC, compared with normal melanocytes and the primary melanoma WW165 (Fig. 3). Interestingly, the cytokines Cyr61 and CTGF discussed above also belong to the family of IGFBPs (IGFBP10 and IGFBP8, respectively), and their expression is similarly coordinated with the other IGFBPs (Fig. 3).

We show for the first time the up-regulation of FGF13/FHF2 (Fig. 4A), confirmed by semi quantitative reverse transcription-PCR (Fig. 4C). FGF13 maps to chromosomal region Xq26.3, and QT_Clust analysis revealed that it is coordinately expressed in the different melanoma cell strains in a pattern similar to that of MAGE gene family members located on Xq26.3, suggesting a similar mechanism of activation. FGF2, another member of this family of growth factors up-regulated in melanoma cells (57, 58) and known to confer growth advantage (59, 60), was not identified here in the microarray analysis probably due to low mRNA levels (57).

**Supplementary Table 1. NOTCH2 levels were coordinately upregulated with genes belonging to the family of receptor activity, such as Tenascin C (TNC1), Fibronectin 1 (FN1), Tissue inhibitor of metalloproteinase 3 (TIMP3), and other adhesion/receptor molecules (Fig. 2A). However, overexpression of HEY1/HERSI-pIRES2-EGFP in immortalized mouse melanocytes did not facilitate release from TPA-dependent mode of growth, suggesting that, by itself, HEY1/HERSI does not confer growth advantage.

The immunoblotting confirmed the general trend of up-regulation of this class of genes (Fig. 2B). However, there was no consistent correlation between transcript levels, as reflected by hybridization intensity and cellular proteins. TNC1, for example, was up-regulated in all six melanoma cell strains examined with the Affymetrix microarray (Fig. 2A), but the protein was not detected in YUHEIK and MNT1 melanoma cells (Fig. 2B).

The role of integrin signaling in melanoma progression was under-
of ISG15, which was conflicted between the Affymetrix HG-U133A and the Operon 70-mer 16K data sets (see above), was shown to be up-regulated (Fig. 5B), in agreement with previously published reports (46).

Leukemia Inhibitory Factor (LIF). The immune modulator LIF (also known as melanoma-derived LPL inhibitor) was up-regulated in four of six melanoma cell strains (Fig. 5C; Supplementary Table 2B). LIF is a cytokine that activates monocytes and macrophages and induces chemotaxis in immune cells (reviewed in Ref. 65). Its production in melanomas was suggested to induce cancer cachexia (66, 67). LIF also stimulated the human HLA-G promoter in JEG3 choriocarcinoma cells (68). In our QT_Cluster analysis, LIF expression strongly correlated with the expression of genes encoding several class II major histocompatibility complexes, HLA-DQβ1, HLA-DRα, HLA-DMα, and HLA-DRB4 (Fig. 5C), suggesting a common mechanism by which this group of genes is regulated.

Vesicular Pathway. Several genes in the membrane transport pathway were down-regulated. These include VAMP8, TRS20, HPS1, and several Ras-related GTP-binding proteins (Fig. 5D; Supplementary Table 3). The products of some of these genes participate in the transport of proteins into melanosomes and/or the transport of melanosomes to the plasma membrane (reviewed in Refs. 69–71). Based on the hybridization intensity data, Rab38, Rab27A, and Rab32 were the most abundant, and Rab31 and Rab33A were the least abundant family members in normal human melanocytes (Fig. 5D). As confirmed by Western blotting, Rab38 and Rab27A were down-regulated in several melanomas but remained at high levels in the melanotic MNT1 melanoma cells, the line least divergent from normal human melanocytes (Fig. 5B). In contrast, reverse transcription-PCR analysis revealed that Rab33A was easily detected in normal melanocytes but not in the five melanoma cell strains tested, including MNT1 (Fig. 5E).

RAGA (Ras-related GTP-binding protein, also known as FIP-1) was also about 3-fold down-regulated in the six melanoma cell strains tested (Supplementary Table 3). RAGA is involved in transport of viruses and cytoplasmic organelles and in chromosome segregation through its binding to the light chain of cytoplasmic dynein (72), and therefore its down-regulation may abrogate these processes. Because RAGA localizes to 3p21.3, a region commonly lost in melanomas, we sequenced the cDNA from normal melanocytes and several melanoma cell strains (501 mel, YUMAC, and YUSIT1), but we ruled out the presence of any mutation.

Ubiquitin Pathway/Proteolysis. The ubiquitin pathway encompasses an additional group of genes that was predominantly down-
regulated in melanoma cells compared with normal melanocytes (12 of 14 genes; Supplementary Table 4).

GRAIL (also known as RNF128), an E3 ubiquitin ligase, was up-regulated in five of the seven melanoma cell strains tested (Fig. 6A). Western blot analysis also showed up-regulation at the protein level in five of nine melanoma cell strains (Fig. 6B). The two data sets were in general agreement, with the exception of YUSIT1 (Fig. 6, compare hybridization intensity levels in A and protein level in B).

Down-regulation of UCHL1 at the gene transcription level, as reflected by the Affymetrix hybridization intensity values, correlated nicely with the protein level (compare NM, YUHEIK, and YUCAL expression in Fig. 6, A and B, panel marked UCHL1). Assessment of UCHL1 expression in the 553 case melanoma TMA showed a trend toward decreased survival with decreased levels that was not statistically significant (Fig. 6C; P = 0.0671). However, in primary melanomas, there was a significant association between poor survival and decreased UCHL1 expression (Fig. 6D; P = 0.0361). High UCHL1 expression was more abundant in primary lesions compared with metastases (P = 0.01) and in lesions thinner than 2 mm (P = 0.04), suggesting that it is lost as malignancy progresses (see Fig. 6E for histospots representing high and low UCHL1 expression). There was no significant association between UCHL1 expression and Clark level, presence of ulceration, patient age, and gender. On multivariate analysis, UCHL1 was not an independent predictor of survival among primary melanomas (P = 0.1), and the only two independent predictors were Breslow depth and Clark level.

Transcription Factors and Growth Suppressors

There were changes in the expression of genes encoding highly conserved families of transcriptional regulators critical for developmental programs, mesoderm/ectoderm transition, and differentiation (Supplementary Table 5). In addition to HEY1/HERS1 mentioned above, ASXL1, SHOX2, and FOXD1 were persistently up-regulated in melanoma cells (Fig. 7A; Supplementary Table 5). FOXD1 transcription factor regulates the expression of growth factors secreted by stromal cells that modulate the differentiation of neighboring epithelial cells, such as vascular endothelial growth factor and placental growth factor (73). In our QT_Clust analysis, FOXD1 expression clearly correlated with vascular endothelial growth factor and heparin-binding epidermal growth factor-like growth factor (Fig. 7A, VEGF and HBEGF), implying a cause and effect. On the other hand, four HOX genes were down-regulated (Supplementary Table 4). The HOX genes are sequentially activated during embryogenesis, setting in motion correct embryonic patterning (see, for example, Ref. 74), suggesting that the down-regulation in melanoma may be the reversal of this process.

The Affymetrix data showed that Twist was up-regulated in five of six melanoma cell strains and displayed a pattern of expression similar to that of TBX3 (Fig. 7B). Western blot analysis revealed that TWIST
was up-regulated in five of nine melanoma cell strains (Fig. 7C). Here again, there was no direct correlation between the Affymetrix intensity values and the levels of the protein in two melanoma cell strains (YUHEIK and YUSIT1). Probing of the melanoma TMA with anti-Twist antibodies indicated that Twist overexpression was associated with worse patient survival in the entire cohort \( (P = 0.0037) \) and within the subset of primary melanomas \( (P = 0.0028; \text{Fig. 7, D and E}) \), highlighting the significance of its involvement in tumorigenesis in vivo. Intense Twist staining was more prominent in metastatic lesions (including nodal and distant metastases) than in primary lesions \( (P = 0.0053; \text{Fig. 7E}) \). Among the primary lesions, there was no significant association between high Twist expression and Clark level, Breslow depth, presence of ulceration in the lesion, patient age, or gender. On multivariate analysis using the Cox model, high Twist expression was an independent predictor of poor outcome \( \text{hazard ratio, 3.2; 95\% confidence interval, 1.16–8.99; } P = 0.024) \), as were Breslow depth of \( >1 \) mm and a Clark level of IV or V. Other variables included in this analysis that were not of significant predictive value were presence of ulceration on primary tumors, patient age, and gender.

Three genes encoding growth suppressors, NECDIN (neurally differentiated embryonal carcinoma cell-derived factor), NBL1 (neuroblastoma suppressor of tumorigenicity 1 precursor), and CIRBP (cold inducible RNA-binding protein) were also down-regulated in melanoma cells (Supplementary Table 5), but in an uncoordinated manner (Fig. 8A). Reduced Necdin expression was confirmed by Western blotting in seven of nine melanoma cell strains (Fig. 8, B and D), but attempts to stain melanoma TMA failed due to the nonspecific binding of the anti-Necdin antibodies to the tissues on the microarray (data not shown). Overexpression of Flag-tagged Necdin in melanoma cells (501 mel cells, Fig. 8C, inset) suppressed growth, as observed by a 30\% reduction in \([\text{H}]\text{thymidine incorporation 4 days after transfection (data not shown) and by a cell count determined 10 days after transfection (Fig. 8C). Immunofluorescence analysis revealed that endogenous ncedin was cytoplasmic in normal and malignant cells but displayed a patchy and aggregated pattern in melanoma compared with even distribution in normal pigment cells (Fig. 8E). However, sequencing NECDIN in 501 mel cells did not indicate any mutation, suggesting that the different pattern was due to other factors.}

DISCUSSION

We report here a comprehensive analysis of changes in gene expression in human melanoma cells compared with normal melanocytes at a scope not demonstrated previously. These changes were observed by analyzing proliferating melanoma cells from six advanced lesions and normal human melanocytes grown in culture. The melanoma cells were selected to represent strains with high and low levels of pigmentation expressing a wide range of tyrosinase, the melanocyte-specific gene product, as a measure of differentiation. Several of the differences between normal and malignant melanocytes detected by our analysis have already been reported in cells and tumors, supporting the notion that most of them were not generated during growth in culture and do not reflect a response to ingredients in the culture medium. The use of proliferating normal and malignant melanocytes proved that several critical changes in gene expression...
characteristic to the malignant state are independent of proliferation status.

Hierarchical clustering showed that the primary melanoma cell strain WW165 isolated from an advanced lesion was more similar to the metastatic melanoma cells than to normal melanocytes, confirming an observation made almost two decades ago (75). Indeed, the WW165 cells, unlike primary melanoma cells from superficial spreading or thin lesions, proliferate in culture independently of most of the growth factors required for normal human melanocytes (76). The MNT1 melanoma cells, on the other hand, isolated from lymph node metastasis, were the least divergent from normal human melanocytes. The similarity included not only high pigmentation, which was observed in at least two other melanoma cell strains (YUHEIK and YUMAC), but also genes in the receptor activity group, such as TNC1, TNFRSF12A, TIMP3, ITGA6, SDC2, and in the Rab family (Rab38 and Rab27A). MNT1 is a melanoma cell strain that was established in culture more than a decade ago (29), and information regarding the patient’s clinical outcome is not available.

Although extensive activation of cell surface receptors in melanoma cells has been reported previously, our analysis revealed several novel details. We show, for example, the increased expression of FGF13 as a possible mediator of FGF receptor 1 stimulation. FGF13 was originally cloned from an ovarian cancer cell library, and its expression is particularly high in the brain. The growth factor is likely to be mitogenic for the melanoma cells because it stimulates the mitogen-activated protein kinase pathway (Ref. 77 and the references therein).

We also demonstrated that several components of the integrin pathway are overexpressed in a synergistic manner, which include integrins (ITGA7, ITGA4, ITGA6, ITGB3), integrin ligands (Cyr61, TNC1, FN1, CTGF), and two tetraspanins (TM4SF13 and TM4SF1), and demonstrated down-regulation of the serine/threonine phosphatase ILKAP. ILKAP selectively suppresses the activity of integrin-linked kinase 1 (78), a mediator of integrin signal transduction that promotes angiogenesis (see, for example, Ref. 79). More recently, it was demonstrated that integrin-linked kinase 1 protein expression increases as melanoma progresses, with strong positive correlation between expression and lymph node invasion and with decreased 5-year survival (80). Suppression of ILKAP by small interfering RNA increased entry of cells into S phase, and overexpression of ILKAP inhibited anchorage-independent growth of LNCaP prostate carcinoma cells (81). Reduced ILKAP expression, therefore, in combination with high integrin-linked kinase expression, is likely to enhance the aggressive state of melanomas.

Other changes in the integrin pathway shown here have been observed previously in melanoma tumors, such as Tenascin C (82–84). Tenascin C is also present in the serum of patients with stage IV melanoma, but not in healthy donors (85), suggesting that it can be used as a marker for early transformation and metastatic load. Cyr61, on the other hand, is expressed in uveal melanomas (86). It is an angiogenic factor that induces migration and stimulates mitogenesis by interacting with integrins (87). Cyr61 confers resistance to apoptosis by increasing nuclear factor-κB activity (88), and the expression of Cyr61 and CTGF, in addition to vascular endothelial growth factor, is likely to contribute to the angiogenic activity of melanoma cells. These three factors are produced by other cancer cells as well and are targets for tumor therapy [see, for example, Ref. 89; Lester F. Lau (University of Illinois, Chicago, IL and FibroGen, South San Francisco, CA)].
Francisco, CA). TM4SF1 is also being evaluated as a diagnostic marker for colorectal and gastric cancers and as a target for therapy with monoclonal antibodies (90, 91).

We demonstrated overexpression at the gene transcript level of NOTCH2 and HEY1, the latter of which is a downstream effector of the NOTCH receptor. The increased HEY1/HERS1 expression in the least divergent melanocytic lesions, i.e., MNT1 and the primary melanoma WW165, suggests a role in early transformation. Based on a limited microarray analysis, Seykora et al. (92) also found increased NOTCH2 expression in two advanced primary melanoma tumors compared with benign nevi. However, ectopic expression of HEY1/HERS1 in immortalized benign mouse melanocytes did not release the cells from their dependence on the external growth factor TPA, suggesting that HEY1/HERS1, by itself, is not sufficient to confer malignancy. This observation is in agreement with previously published results showing that collaborating genetic events are required for tumorigenic transformation by NOTCH, in particular Ras-mediated activation of Erk/mitogen-activated protein kinase and phosphatidylinositol 3'-kinase (Ref. 93; reviewed in Ref. 94).

A surprising finding was the extent of changes in genes whose products affect immune response. In particular, we observed extensive suppression of IFN-regulated genes (21 of 22 genes; 14 were suppressed in a coordinated manner). Among the down-regulated genes was OAS-1. OAS-1 activity is required for RNase endoribonuclease activation and the antiviral and apoptotic activity of IFNs (Refs. 95 and 96; reviewed in Refs. 97 and 98). Several mutations in RNase endoribonuclease are associated with prostate cancer (98), and IFN-responsive genes are down-regulated in colon polyps and tumors as compared with normal colon tissue (99, 100). The repression of IFN signaling genes might reflect immortalization because the predominant genes silenced by DNA methylation in spontaneously immortal Li-Fraumeni fibroblasts belonged to this pathway (46% of 85 genes; Ref. 101). The coordinated down-regulation of IFN-responsive genes is of particular importance because IFN α,β is frequently administered as an adjuvant in immunotherapy for melanoma (102, 103). The effectiveness of this treatment is likely to be conditional on the ability to induce the expression of at least some of these genes.

We also demonstrated that genes in the ubiquitin pathway were mostly down-regulated (12 of 14 genes). One of them, UCHL1,
Suppressed in all of the melanoma strains tested, and its expression was also reduced in metastatic melanoma lesions, as seen by probing the melanoma TMA. Reduction in UCHL1 in primary melanomas was significantly associated with poor survival. UCHL1 has a dual function, a hydrolase activity that removes small COOH-terminal ubiquitin to generate the ubiquitin monomer and a dimerization-dependent ubiquitin ligase activity. Several UCHL1 variants associated with Parkinson disease may play roles in proteasomal protein degradation (reviewed in Ref. 104). The UCHL1 enzymatic activity most consequential for melanocyte tumorigenesis is not yet clear.

Twist was up-regulated in ~55% of the melanoma cell strains tested, and its overexpression in melanoma tumors correlated with poor survival. Twist is a basic helix-loop-helix transcription factor that is required for mesodermal cell fates, and its pattern of expression in melanomas was similar to that of TBX3, a transcriptional repressor required for normal breast development, with mutations in the TBX3 gene linked to ulnar-mammary syndrome (Ref. 105 and the references therein). TBX3 and Twist suppress senescence by down-regulating ARF, a protein that inhibits MDM2-mediated proteolysis of p53 (106–108). Twist also blocks myogenic differentiation and is inappropriately expressed in 50% of rhabdomyosarcomas (109). In our samples, there was no correlation between Twist expression and differentiation, as determined by the levels of tyrosinase and pigment formation. In another report, increased Twist protein levels were associated with resistance to Taxol and vincristine in nasopharyngeal, bladder, ovarian, and prostate cancers (110). We showed that Twist expression correlated with poor survival, and we can only speculate that it contributes to melanoma resistance to this type of drug.

Necdin was one of the growth suppressors down-regulated in six of eight melanoma cell strains tested. Low expression in melanomas can still confer advantage for tumor growth, as shown for other tumor suppressors. For example, tumors developed in animals lacking one copy of p27Kip1 expressing half the levels of functional wild type p27Kip1 (111). Similar observations were gleaned from p53, and the phenomenon of gene dosage sensitivity is termed haploinsufficiency (Ref. 112 and the references therein). Furthermore, Necdin in melanoma cells is localized to a different cytoplasmic compartment compared with normal melanocytes, suggesting that it is inactivated by sequestration. Its growth-suppressive function was validated by ectopic overexpression that led to rapid and efficient arrest of melanoma cell proliferation. Necdin is expressed predominantly in postmitotic cells, such as neurons and muscle cells (Ref. 113 and the references therein), and in melanocytes, as demonstrated here for the first time. NEDDIN is on chromosomal region 15q11.2-q12, and is one of four genes encoding the Prader-Willi syndrome. This is an autosomal dominant neurodevelopmental disorder caused by mutations or deletions because the respective genes on the maternal chromosome in this region are inactive through imprinting. In the case of melanoma, the expressed necdin was not mutated in the coding region. The exact function of necdin has not yet been determined. It binds to the transcription factors E2F1 and E2F4 and to p75 neurotrophin receptor (113). Ectopic coexpression of necdin with E2F1 in U2OS osteosarcoma cells suppressed E2F1-stimulated transcription. On the other hand, coexpression of necdin with E2F1 or p75 neurotrophin receptor in N1E-115 neuroblastoma cells sequestered the protein in the nucleus of the cell membrane, respectively (113). Therefore, identifying the necdin-binding proteins in melanoma cells and normal melanocytes is likely to shed light on the molecular basis for the growth-arresting function of necdin in this cellular system.

Some of the down-regulated genes are localized at chromosomal regions known to be altered in melanomas. For example, NBL1 is on 1p36.3–p36.2, and GBP1 (guanylate-binding protein 1, IFN-inducible, 67 kDa) is on 1p22.2, regions harboring melanoma suppressor genes (114). Another example is RRAGA (Ras-related GTP-binding protein) on 9p21.3, a region frequently deleted in melanomas that may possess more than one tumor suppressor gene (115). However, sequencing of RRAGA in normal melanocytes and several melanoma cell strains failed to identify any mutation, suggesting that if down-regulation is due to loss of chromosomal material, then the remaining gene is normal.

Expression or reexpression of some genes might be driven by demethylation/methylation of selected CpG in the promoter regions, processes common in cancer cells (reviewed in Ref. 116). Although the majority of the activated genes on the X chromosome encoded CTAs known to be reexpressed due to demethylation (reviewed in Refs. 61–63), other X-linked genes were also activated, such as TRAG1 (Taxol resistance-associated gene 3; Xq28) and FGF13 (Xq26.3), and might be affected by the same process. DNA methylation/demethylation processes of promoter Cpg-rich regions might cause the change in expression of HOXB genes (HOXB2, HOXB7, and HOXB13; 17q21.2; Ref. 117), MXI1/FIT18 (12q24.2; Ref. 101), and dipetidylpeptidase IV (2q24.3; Ref. 99; reviewed in Refs. 100 and 118–120). Indeed, preliminary sequence analysis of the promoter region and exon 1 using the MethPrime program of Li and Dahiya (121) available online revealed that 64% (of 35 genes) and 70% (of 48 genes) of the top down- and up-regulated genes, respectively,

15 http://itsa.ucsf.edu/~urolab/methprimer/index1.html.
contain at least one CpG-rich island (data not shown). Monitoring CpG methylation patterns and the genes they affect can be used for assessing tumor progression, drug resistance, and immunological responses. This information is particularly needed to assess drug efficacy of novel DNA demethylating agents, such as 5-aza-2-deoxoxygen-tidine (commercially known as decitabine), antisense to DNA methyltransferases such as DNMT1 (MG98), and histone deacetylase inhibitor depsipeptide FR901228, which are in Phase II trials (see, for example, Refs. 122 and 123; reviewed in Ref. 124).

In summary, we have identified a number of novel genes and pathways that are up- or down-regulated in metastatic melanoma cell strains compared with normal melanocytes. The data provide new insights into immune modulation and possible causes for failed IFN or chemotherapy treatments common in melanomas. We identified FGF13 as a new autocrine factor, Twist as clinically valuable prognostic markers, and genes involved in embryonic tissue remodeling. These data can serve as a basis for further evaluation of pathways leading to malignant transformation, prognostic markers, and/or drug targets.

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