**KLF6 Gene and Early Melanoma Development in a Collagen I-Rich Extracellular Environment**

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**Background**

A putative tumor suppressor gene at chromosome 10p15, which contains KLF6 and other genes, is predicted to be lost during melanoma development, and its identity is unknown. In this study, we investigated the biological roles and identity of this tumor suppressor gene.

**Methods**

The human UACC 903 melanoma cell line containing introduced DNA fragments from the 10p15 region with (10E6/3, 10E6/11, and 10E6/18) and without (10ER4S.2/1) the tumor suppressor gene was used. Xenograft tumors were generated in a total of 40 mice with melanoma cell lines, and tumor size was measured. Cells were cultured on plastic or a gel of type I collagen. Viability, proliferation, and apoptosis were assessed. Expression of KLF6 protein was assessed by immunohistochemistry and immunoblot analysis. Expression of phosphorylated Erk1/2 and cyclin D1 was assessed by immunoblot analysis. Protein expression of KLF6 was inhibited with small interfering RNA (siRNA). KLF6 protein expression was assessed in 17 human nevi and human melanoma specimens from 29 patients. Statistical analyses were adjusted for multiple comparisons by use of Dunnett method. All statistical tests were two-sided.

**Results**

Melanoma cells containing KLF6 generated smaller subcutaneous xenograft tumors with fewer proliferating cells than control cells. When grown on collagen 1, viability of cells with ectopic KLF6 expression (72%) was lower than that of control cells (100%) (group difference = −28%, 95% confidence interval = −31.3% to −25.2%, *P* < .001). Viability of melanoma cells with or without the KLF6 tumor suppressor gene on plastic dishes was similar. When KLF6 expression was inhibited with KLF6 siRNA, viability of cells with the tumor suppressor gene on collagen I gel increased compared with that of control cells carrying scrambled siRNA. KLF6 protein was detected in all nevi examined but not in human metastatic melanoma tissue examined. Ectopic expression of KLF6 protein in melanoma cells grown on collagen I decreased levels of phosphorylated Erk1/2 and cyclin D1 in the mitogen-activated protein kinase signaling pathway.

**Conclusions**

In melanoma cells, the tumor suppressor gene at 10p15 appears to be KLF6. Signaling from the collagen I-rich extracellular matrix appears to be involved in the tumor suppressive activity of KLF6 protein.


Melanoma is the most aggressive and deadly form of skin cancer (1). Melanoma results from accumulating genetic alterations that lead to gene deregulation, cancer development, and tumor progression (1). Some genetic alterations remove or deregulate proteins that are involved in signaling from the extracellular matrix to enable cancer development (2). Loss of all or part of chromosome 10 is observed in 30%–60% of nonfamilial melanomas (1,3–5). Discrete regions of chromosome 10 at 10q23 and 10p15 that were identified previously as potential sites of cancer suppressor genes in melanoma (3) led to the identification of PTEN tumor suppressor gene at 10q23 (6–8). Cytogenetic analysis, molecular evidence, and functional complementation studies have also demonstrated a second tumor suppressor region on the short arm of chromosome 10 in melanomas (3), prostate cancer (9,10), and gliomas (11–14). In melanomas, location of a putative cancer suppressor gene on the short arm of chromosome 10 has been narrowed to 10p15 between the genetic loci D10S247 and D10S1435 (3). However, the identity of the gene in the 10p15 region and its mechanism of action in melanoma development remain unknown.

The KLF6 gene, which encodes Krüppel-like factor 6 (KLF6), is located in the 10p15 region and has potential to be the putative tumor suppressor. The KLF6 gene, which is a zinc-finger transcription factor, regulates cell development, differentiation, and proliferation (15). It is functionally inactivated in several human carcinomas including those of the prostate (16,17), stomach (18), as well as liver and colon (17,19). Growth suppressive activity mediated by KLF6 protein has been linked to cell cycle regulation via transcriptional activation of the cyclin-dependent kinase (CDK) inhibitor p21<sup>WAF1/Cip1</sup> (16) and have been shown to interact directly with cyclin D1 to reduce its binding to CDK4 (20).
Stimuli from the extracellular matrix (including collagen, fibronectin, and laminin) can provide important negative regulatory signaling in neoplastic tissue that must be overridden for cancer development to occur (2,21,22). Recent studies (23–25) have unraveled the complex biological roles of signals sent from the extracellular matrix and have dissected the processes by which they regulate the transition between the normal and neoplastic cell phenotype. Type I collagen extracellular matrix that has been conditioned by aggressive melanoma cells can stimulate epigenetic transdifferentiation of the normal melanocytic phenotype to that of an aggressive melanoma-like cell (26), with increased expression of genes, such as laminin 5γ2 and urokinase, which are normally associated with extracellular matrix remodeling and invasion.

In the skin, extracellular matrix signals that are mediated through growth factors, ligands, cytokines, or hormones can play key roles regulating cell proliferation and cancer development. Specifically, type I collagen, which constitutes approximately 90% of the dermal extracellular matrix, can regulate integrin cell adhesion, morphology, migration, viability, growth, and differentiation by regulating various proliferative and survival signaling pathways, including the EphA2 and ephrin-A1 pathways (24,27,28). Under normal conditions, cross talk between the extracellular environment of the skin and the cellular milieu is mediated by the focal adhesion kinase pathway. Normally, the interaction with the extracellular matrix is a tightly regulated process that maintains tissue integrity and homeostasis (21). However, during tumorigenesis, cells escape from this regulation by degrading the surrounding extracellular matrix to release growth factors, cytokines, and proteases into the microenvironment (28–32). The integrin family of heterodimeric transmembrane receptors, which are composed of an α and a β subunit, mediates signaling between the cell and its extracellular matrix (31). During different stages of tumor progression, altered expression and surface distribution of various integrin subunits are observed (31). Expression of α5β1, α5β2, and α6β1 integrin receptors (which act as collagen I sensors) is frequently higher in melanomas than in melanocytes, but expression of αβ2 and αβ3 is lower in advanced melanomas than in early-stage melanomas (32). Integrin receptor α5β1 can bind to various components in extracellular matrix, including fibronectin and vitronectin, which leads to the induction of matrix metalloproteinase production and activation. Matrix metalloproteinases then degrade collagen, which promotes detachment of melanoma cells so that they can invade or metastasize (32).

The purpose of this study was to identify the tumor suppressor gene on chromosome 10p15 and identify the underlying mechanism by which it inhibits tumor development. We hypothesized that KLF6 is the putative tumor suppressor gene located in the 10p15 region. We investigated this hypothesis by introducing chromosomal fragments containing the KLF6 gene into melanoma cells and assessing their viability on type I collagen gel and in xenograft tumor models.

Patients, Methods, and Materials

Cell Lines and Culture Conditions

We used four microcell-mediated chromosome transfer hybrid cell lines (10E6/3, 10E6/11, 10E6/18, and 10ER4S.2/1) that had been derived from the human melanoma cell line UACC 903 to identify the tumor suppressor gene on the 10p15 suppressor region and its underlying tumor suppressing mechanism. The creation, characterization, and growth conditions of these cell lines have been reported previously (3). All four cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). Three hybrid cell lines (10E6/3, 10E6/11, and 10E6/18) carry a 10p15 DNA fragment from D10S1435 to D10S2325 that contains the 10p15 tumor suppressor gene, whereas the control hybrid cell line 10ER4S.2/1 carries a smaller fragment of 10p15 DNA from D10S591 to D10S2477 that lacks the tumor suppressor gene.

Normal human epidermal melanocytes were maintained in MCBD 153 medium (Sigma, St Louis, MO) containing 10% chelated FBS, 2% FBS, 2 mM glutamine, cholera toxin (1.66 µg/mL), stem cell factor (Sigma; 10 ng/mL), 100 nM endothelin-3, and basic fibroblast growth factor (2.5 ng/mL). Chelated FBS was prepared by mixing 15 g of Chelex-100 (Sigma) with 500 mL of FBS and incubating the mixture for 1.5 hours at 4°C with gentle stirring.

Human melanoma cell lines that had been derived previously from melanomas in radial-stage growth (WM35 and WM3211 cells) and vertical-stage growth (WM98.1, WM115, and WM278...
cells) were maintained in a combined medium of 80% MCDB 153 medium (Sigma) supplemented with sodium bicarbonate (1.2 g/L) and 20% Leibovitz L-15 medium (Mediatech, Manassas, VA) supplemented with insulin (Sigma; 5 µg/mL), 2 mM L-glutamine (Mediatech), and 2% heat-inactivated FBS (incubated at 56°C for 30 minutes; Hyclone), as described previously (6,33). Human metastatic melanoma cell lines SK-MEL-24 and UACC 903 were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone). All cells were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Xenograft Tumor Formation in Mice
Female athymic BALB/c (nu/nu) mice (4–6 weeks old) were purchased from Simonsen Laboratories (Gilroy, CA). Experiments with mice were performed according to the protocols approved by the Institutional Animal Care and Use Committee at University of California at San Diego. Briefly, 5.0 × 10³ cells of parental UACC 903 cells or a hybrid cell line (10E6/3, 10E6/11, 10E6/18, or 10ER4S.2/1) in 0.2 mL of DMEM containing 10% FBS were injected subcutaneously into the right side of each mouse. Tumors sizes were measured at day 21 by use of calipers to estimate the volume in cubic millimeters by use of the formula, volume per tumor = width × height × depth.

Tumor Section Processing
Twenty-one days after tumor cell injection, mice were killed by CO₂ asphyxiation and xenograft tumors from UACC 903 and hybrid cell lines (10E6/3, 10E6/11, 10E6/18, or 10ER4S.2/1) were excised and processed for morphological and immunohistological examination. Briefly, tumors were fixed for 24 hours in fresh low-odor 10% buffered formalin phosphate (pH 7.0; Fisher Scientific; Fair Lawn, NJ) and embedded in paraffin. Sections (5 µm thick) were cut and stained with trichrome to visualize the extracellular matrix organization in the tumor. To visualize patterns of type IV collagen, sections were incubated with mouse anti-human type IV collagen monoclonal antibodies (1:100 dilution; Chemicon International, Temecula, CA) followed by incubation with secondary antibody, anti-mouse IgG coupled to avidin–biotin and horseradish peroxidase (1:100 dilution; Vector Laboratories, Burlingame, CA). Brown staining was visualized after incubation with 3,3’-diaminobenzidine (DAKO, Carpinteria, CA). Slides were counterstained with hematoxylin.

Transmission Electron Microscopy
Mice bearing xenograft tumors (from parental UACC 903 cells and the four hybrid cell lines, 10E6/3, 10E6/11, 10E6/18, or 10ER4S.2/1) were killed by CO₂ asphyxiation. Tumors were excised and fixed in 3% formaldehyde (freshly prepared from paraformaldehyde) solution overnight at 4°C. Small pieces of tumor tissue (1 mm³) were then fixed in a solution of 1.5% glutaraldehyde, 0.1 M cacodylate–HCl (pH 7.4), and 5% sucrose for 1 hour at room temperature. After three washes in a solution of 0.1 M cacodylate–HCl (pH 7.4) and 7.5% sucrose, specimens were post-fixed in cacodylate-buffered 1% OsO₄ (pH 7.2) on ice for 1 hour, stained en bloc with uranyl acetate for 1 hour at room temperature, dehydrated, embedded in EPON 812 (Structure Probe, Inc, West Chester, PA), and cured for 18–24 hours at 60°C. Thin sections (50–55 nm) were cut (Reichert-Jung Ultracut E, Vienna, Austria), picked up on copper grids, and stained with uranyl acetate and lead citrate before examination and photography (Philips CM10 electron microscope at 80 kV at the University of California at San Diego Imaging Facility).

Cell Proliferation and Apoptosis in Time- and Size-Matched Tumors
Xenograft tumors from parental UACC 903 cells and the hybrid cells (10E6/3, 10E6/11, 10E6/18, or 10ER4S.2/1) of the same size and developing at same time were used to determine proliferation or apoptotic rates of tumor cells. Tumors of the same size were generated by injecting five times more 10E6/3, 10E6/11, and 10E6/18 cells (which form smaller tumors) than control UACC 903 and 10ER4S.2/1 cell lines. Consequently, we subcutaneously injected 1 × 10³ UACC 903 or 10ER4S.2/1 cells or 5 × 10³ 10E6/3, 10E6/11, or 10E6/18 cells above both the left and right rib cages of female mice (Harlan Sprague-Dawley, Indianapolis, IN; six mice per group). Experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University College of Medicine. Mice were killed by CO₂ asphyxiation at 16, 17, and 18 days; tumors were harvested; and cell proliferation and apoptosis in tumors was assessed. Cell proliferation rates in formalin-fixed tumor sections, as described above, were measured with an RPN 20 cell proliferation kit (GE Health Care, Piscataw, NJ), which uses bromodeoxyuridine incorporation and immunocytochemical detection of bromodeoxyuridine. Briefly, 2 hours before the mice were killed, 0.2 mL of bromodeoxyuridine (3 mg/mL) was injected intraperitoneally into mice, and tumors were processed according to the manufacturer’s instructions. The number of bromodeoxyuridine-stained cells was scored as a percentage of total cells in tumors. Proliferation rates in formalin-fixed paraffin-embedded tumor sections were determined by use of an apoptosis kit (Roche, Mannheim, Germany) that includes terminal deoxy nucleotidyltransferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) and tetramethylrhodamine red staining for deoxyuridine 5-triphosphate. Formalin-fixed paraffin-embedded tumor sections were deparaffinized, rehydrated, digested with proteinase K (Fisher Scientific; 20 µg/mL), and then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyltransferase in a humidified dark chamber for 1 hour at 37°C. Sections were counterstained for 10 minutes with Hoechst-33258 (1 µg/mL) to visualize nuclei and mounted. We counted cells in a minimum of 15–20 fields from three or four different tumor sections, and the number of positive cells was expressed as the percentage of apoptotic cells ([number of apoptotic cells/number of cells in each field] × 100%). Data from the tumors that originated from injection sites to the right and left sides of each mouse were averaged, so that an average number of positive cells per mouse was used in the comparisons.
Gelatin Matrix Metalloproteinase Zymography

To measure matrix metalloproteinase activity in hybrid and parental cells, zymography was performed with Novex Zymogram Gels (Invitrogen, Carlsbad, CA) by following the manufacturer’s protocol. Briefly, UACC 903 cells and the hybrid cells (10E6/3, 10E6/11, 10E6/18, or 10ER4S.2/1) were grown to 80% confluence in complete medium and then incubated for 24 hours in serum-free medium. Serum-free conditioned medium was concentrated by use of Centriprep YM-10 centrifugal devices (Millipore, Billerica, MA). An equal amount of protein from each sample was diluted in 2x sample buffer (125 mM Tris–HCl at pH 6.8, 4% sodium dodecyl sulfate, 0.005% bromophenol blue, and 20% glycerol), applied to 10% zymogram gelatin gels (Invitrogen, Carlsbad, CA), and subjected to electrophoresis. The gels were then incubated in NOVEX Zymogram Renaturing buffer for 30 minutes to remove the sodium dodecyl sulfate and incubated 6 hours in NOVEX Zymogram Developing buffer. Gels were stained for 3 hours in a solution of 30% methanol, 10% glacial acetic acid, and 0.5% Coomassie blue G-250 (Bio-Rad Laboratories, Hercules, CA), destained for 2 hours in 30% methanol–10% glacial acetic acid, and dried. This experiment was independently repeated four times.

Analysis of Anchorage-Independent Cell Proliferation

Anchorage-independent cell proliferation was assessed by use of poly-2-hydroxyethyl methacrylate (PolyHEMA)–coated 96-well plates (Corning Life Sciences, Corning, NY). Briefly, plates were coated with a 1:10 dilution of PolyHEMA at 120 mg/mL (Sigma) in 95% ethanol and dried on a heat plate overnight. Before use, plates were washed with phosphate-buffered saline. UACC 903 and hybrid cells (10E6/3, 10E6/11, 10E6/18, or 10ER4S.2/1) were seeded at 1 × 10^4 cells per well in DMEM supplemented with 10% FBS for 5 days. Cell proliferation was assessed by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt (MTS) assay (CellTiter 96 AQueous Cell Proliferation Assay; Promega, Madison, WI). This experiment was repeated three times, with eight samples per group.

Preparation of Collagen Gel Culture Plates

We prepared a neutralized collagen solution (10 mL; 3.0 mg/mL) on ice under sterile conditions by mixing 7.5 mL of type I collagen from rat tail tendon (BD Biosciences, Bedford, MA; 4.0 mg/mL), 1 mL of sterile 10× phosphate-buffered saline, 172.5 µL of 1 N NaOH (for a final concentration of 0.02 N NaOH), and 1.3 mL of sterile distilled H₂O. The collagen solution was added to 96-well plates (50 µL per well) or to 60-mm plates (1 mL per well) and was polymerized at 37°C in an atmosphere of 5% CO₂ and 95% air for 1 hour. Cells were then plated immediately on the collagen gel for all experiments. We used UACC 903, 10E6/3, 10E6/11, 10E6/18, and 10ER4S.2/1 cells; UACC 903 cells that ectopically express constructs and SK-MEL-24 that ectopically express constructs; and/or UACC 903, 10E6/3, 10E6/11, 10E6/18, and 10ER4S.2/1 cells transfected with siRNA in various experiments.

cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

We used UACC 903, 10E6/3, 10E6/11, 10E6/18, and 10ER4S.2/1 cells for this experiment. Total RNA was extracted with Qiagen RNeasy mini kit (Qiagen, Valencia, CA) from these cells in logarithmic growth phase at 70% confluency, and cDNA was synthesized from total RNA extracted from each cell line. Briefly, first-strand cDNA synthesis used 5 µg of total RNA, 1 µL of oligo(dT) primers (0.5 µg/µL), 1 µL of a solution of all four deoxyribonucleoside triphosphates (each at 10 mM), and 10× Superscript III reverse transcriptase (Invitrogen, Gaithersburg, MD; 200 U/µL). For TaqMan-based qRT-PCR, 50 ng of cDNA was added to 10 µL of 2× Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 1 µL of 20× KLF6 primers and the probe set (Applied Biosystems). For real-time fluorescence monitoring of probe, the ABI-7900HT Fast Real-Time PCR System (Applied Biosystems) was used. The thermal cycling conditions were incubated in a 384-well plate at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Human GAPDH mRNA was amplified for use as an internal control. The amount of KLF6 mRNA was normalized to that of GAPDH mRNA from three independent experiments. KLF6 expression levels in 10p15 hybrid cell lines were analyzed by the comparative Cₘ method (ΔΔCₘ) (34). This experiment was repeated three times, with triplicate samples.

Ectopic Expression Studies and KLF6 Constructs

Full-length wild-type KLF6 cDNA and two mutant KLF6 cDNAs, K209R and W162X, were used as reported previously (19). A hemagglutinin A tag was attached to all KLF6 cDNAs, and the tagged cDNAs were subcloned into the EcoRI and XhoI sites of pcDNA3.1-puro vectors containing cytomegalovirus promoter. We also subcloned KLF6 cDNA without a hemagglutinin A tag into the EcoRI and XhoI sites of pcDNA3.1-puro vectors. We then introduced 5 µg of each construct into a 1 × 10⁶ UACC 903 cells or 2 × 10⁶ SK-MEL-24 cells by nucleofection (an electroporation technique that uses an Amaxa Nucleofector; Amaxa, Köln, Germany) as controlled by a K-17 or U-20 electroporation program, respectively. Nucleofected UACC 903 or SK-MEL-24 cells were allowed to recover in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS on 60-mm Petri dishes for 2 days, and then, cells expressing plasmids were selected for 3 days in puromycin at 0.5 µg/µL. UACC 903-puro, UACC 903-KLF6, UACC 903-HAKLFL6, UACC 903-HAK209R, UACC 903-HAW162X, SK-MEL-24-puro, SK-MEL-24-KLF6, SK-MEL-24-HAKLFL6, SK-MEL-24-HAK209R, and SK-MEL-24-HAW162X were selected. For immunoblot analysis of KLF6, cyclin D1, and α-enolase protein expression, 1 × 10⁴ selected cells were cultured on 60-mm plates. For cell proliferation analysis, selected cells were cultured on 60-mm plates at 1.0 × 10⁵ cells per well or on collagen-coated 96-well plates at 2.0 × 10⁵ cells per well, and proliferation was assessed by use of MTS assays (Promega). Experiments were repeated three times, with eight samples per group.

Immunoblot Analysis of Mitogen-Associated Protein (MAP) Kinase Pathway Signaling Proteins

Protein lysates were collected from UACC 903, 10E6/3, 10E6/11, 10E6/18, or 10ER4S.2/1 cells; UACC 903 cells that ectopically express constructs; SK-MEL-24 that ectopically express constructs; or UACC 903, 10E6/3, 10E6/11, 10E6/18, or 10ER4S.2/1 cells.
cells transfected with siRNAs. Protein was quantitated with a BCA assay from Pierce (Rockford, IL), and immunoblots were analyzed, as described previously (33). Briefly, we used the following primary antibodies: mouse monoclonal anti-hemagglutinin A (1:2000 dilution), rabbit polyclonal anti-human KLF6 (1:500 dilution), rabbit polyclonal anti-human cyclin D1 (1:1000 dilution), mouse monoclonal anti-human extracellular-related kinase 2 (ERK2) (1:2000 dilution), and goat polyclonal anti-human α-enolase (1:1000 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA); and rabbit polyclonal anti-human phosphorylated extracellular-related kinase (1:1000 dilution) and mouse monoclonal anti-human CDK4 (1:1000 dilution) from Cell Signaling Technology (Danvers, MA). Secondary antibodies, including horseradish peroxidase–conjugated anti-mouse IgG (1:4000 dilution), horseradish peroxidase–conjugated anti-rabbit IgG (1:1000 dilution), and horseradish peroxidase–conjugated anti-goat IgG (1:1000 dilution) were from Santa Cruz Biotechnology. Antibody binding was detected by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

Small Interfering RNA (siRNA) Analysis
For these analyses, we used UACC 903 cells and 10E6/3, 10E6/11, 10E6/18, and 10ER4S.2/1 hybrid cells and duplexed Stealth siRNAs (Invitrogen, Carlsbad, CA). Sequences of the siRNAs used were control scrambled siRNA (5′-AAUUCUCCGAACGUGUCACGUGAGA-3′) and KLF6 siRNA (5′-CACACAGGAGAAAGCCUUACGAGA-3′). siRNAs (100 pmol) were introduced into 1.0 × 10^6 cells by nucleofection with an Amza M nucleofector that used the Solution R program K-17. The transfection efficiency was determined to be greater than 70% by use of pMaxGFP as a green fluorescent protein expression plasmid control. After nucleofection, cells were allowed to recover for 2 days in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS on 60-mm Petri dishes for 2 days, and then, 1 × 10^6 cells were transfected to collagen-coated 96-well plates in DMEM supplemented with 10% FBS. Six days later, cell proliferation was measured with an MTS assay. This experiment was repeated three times, with eight samples per group.

KLF6 DNA Sequence Analysis
Genomic DNAs that were isolated from UACC 903, 10E6/3, 10E6/11, 10E6/18, and 10ER4S.2/1 cells, as described previously (35), were amplified by PCR with a high-fidelity Pfu polymerase (Roche). The following intronic primers were used to amplify the coding region and the intron–exon boundaries of the KLF6 gene: exon 1 = forward 5′-TCTGCGAGCCCTGGAGATT-3′ and reverse 5′-TCTGAACCCAAACAGGCAG-3′, exon 2 = forward 5′-CGGGCAGCAATGTTATCTGTGGCCTC-3′ and reverse 5′-CCCTCCAGGGCTGTGGA-3′, exon 3 = forward 5′-TGTGTGTTACCGATGCCAGAAG-3′ and reverse 5′-CAATGTGCAGGTTGATGTGGAAAGG-3′, and exon 4 = forward 5′-CCTATGCTAGGTGGTCTC-3′ and reverse 5′-GGTACAGCCTGGCTCTACAGGAC-3′. PCR cycling conditions were 10 minutes at 94°C for one cycle; 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C for 45 cycles; and a final extension of 5 minutes at 72°C. PCR products were analyzed by electrophoresis and then purified (Qiagen, QIAquick Gel Extraction kit) for sequencing. By use of the same primers as listed above, purified products were sequenced with an ABI 3130XL capillary sequencer (Applied Biosystems) at the Penn State Functional Genomic Core. The sequencing reactions were performed by cycle sequencing that used fluorescent dye-labeled dye terminator and a modified Taq polymerase.

Melanoma Patient Tumors and Immunohistochemistry
Melanoma tumor specimens from human patients were randomly selected according to the protocols approved by the Institutional Review Board at The Pennsylvania State University and the Cooperative Human Tissue Network. Informed consent was provided according to the Declaration of Helsinki. Tissue samples from 29 patients with metastatic melanoma had been surgically removed, snap-frozen in liquid nitrogen, and stored at −80°C until RNA extraction or preparation of protein lysate. To prepare RNA and protein, tumors were pulverized with a mortar and pestle that had been chilled in liquid nitrogen. To determine RNA expression in these tumors, RNA was isolated from 30 mg of pulverized tumor tissue by using Qiagen RNEasy Mini kit (Qiagen). Protein lysates were extracted from pulverized tumors, as described previously (33). Briefly, 1 mL of protein lysis buffer was added for every 200 mg of tissue powder and sonicated for 2 minutes (with 15-second intervals) in an ice-filled sonicator bath. The samples were centrifuged (12 000g) at 4°C for 10 minutes. The supernatant was transferred to a clean tube, and protein was measured by use of a Bio-Rad protein assay. Levels of KLF6 and ITIH5 proteins were measured by immunoblot analysis.

Sections (5 µm thick) from 17 samples of atypical nevi, sections (<0.75 mm thick) from five samples of melanomas, and sections (5 µm thick) from four samples of malignant melanomas were stained with rabbit polyclonal anti-human KLF6 antibody (Santa Cruz Biotechnology) that had been diluted 1:50 in 1.5% goat serum and incubated at 4°C overnight. Slides without KLF6 antibody incubation were included as a negative control. Antibody binding was localized by use of a rabbit ABC staining system (Santa Cruz Biotechnology). For epitope retrieval, slides were incubated in a water bath at 95°C in 10 mM sodium citrate buffer (pH 7.0) for 10 minutes and blocked in 1.5% goat serum for 1 hour at room temperature.

Statistical Analysis
All experiments were independently repeated at least three times to confirm results. Data were tested for normality. Different experimental groups were compared by use of one-way analysis of variance. Statistical analyses of xenograft tumor experiments were done on a per mouse basis. For all analyses, P values and 95% confidence intervals (CIs) were adjusted for multiple comparisons by use of the Dunnett method. SAS version 9.2 (SAS Institute, Inc, Cary, NC) was used for the statistical analysis. All statistical tests were two-sided.

Results
Characteristics of Xenograft Tumors With or Without Chromosome 10p15
We used three microcell hybrid cell lines (10E6/3, 10E6/11, and 10E6/18) that carried fragments of the 10p15 region containing
the tumor suppressor gene and a control cell line (10ER4S.2/1) that carried a fragment of the 10p15 region lacking the putative tumor suppressor gene (3) to identify the candidate 10p15 tumor suppressor gene and to characterize its functional role in melanoma. All four cell lines had been derived previously from UACC 903 melanoma cells (3).

We examined the effect of the 10p15 fragment on tumorigenesis by subcutaneously injecting nude mice with control parental UACC 903 cells, control 10ER4S.2/1 cells, or one of the three microcell hybrid cell lines (10E6/3, 10E6/11, and 10E6/18) that contain the 10p15 tumor suppressor gene. Control UACC 903 cells and 10ER4S.2/1 cells formed tumors that were statistically significantly larger than those formed by 10E6/3, 10E6/11, and 10E6/18 cells that carry the tumor suppressor gene (Figure 1, A).

For example, the mean volume on day 21 of UACC 903 tumors was 2900 mm$^3$ and that of 10E6/11 tumors was 370 mm$^3$ (difference = 2530 mm$^3$, 95% CI = 1800 to 3200 mm$^3$, P < .001). Histological analysis of tumors showed substantial differences in the extracellular matrix between tumors generated from cells carrying the tumor suppressor gene and tumors generated from the control cells (Figure 1, B). Control cells generated xenograft tumors with less extracellular matrix material that was poorly organized and contained randomly distributed collagen IV molecules. In these control tumors, erythrocytes could be found between tumor cells, demonstrating the presence of blood vessels in these areas. In contrast, xenograft tumors generated from cells that carried the tumor suppressor gene fragment had a well-organized basement membrane that was rich in collagen IV and other extracellular matrix materials, and the cells were in direct contact with collagen IV and extracellular matrix (Figure 1, B).

The cellular organization of these tumors resembled that of early melanocytic lesions (36). Furthermore, electron microscopy showed that tumors generated by cells that carried the tumor suppressor gene fragment were surrounded by more bundles of type I collagen fibers than tumors generated by control cells (Figure 1, C). Thus, the presence of the 10p15 chromosomal fragment containing the tumor suppressor gene appears to have decreased the tumorigenic potential of melanoma cells by altering...
the growth and extracellular matrix of tumors through an undefined mechanism.

**Cell Proliferation and Apoptosis in Tumors With or Without the 10p15 TumorSuppressor DNA Fragment**

We next investigated the mechanism by which the tumor suppressor gene inhibits tumor growth by examining cell proliferation and apoptosis in time- and size-matched xenograft tumors generated by control UACC 903 or 10ER4S.2/1 cells or cells with the tumor suppressor fragment (10E6/3, 10E6/11, and 10E6/18) at days 16, 17, and 18 after subcutaneous injections of cells. The use of time- and size-matched tumors allowed us to estimate proliferation and apoptosis rates in tumors formed from control cells and in tumors from experimental cells, which carry tumor suppressor fragments. We found that tumors generated by control cells had more proliferating cells than tumors generated by cells with the tumor suppressor fragment (Figure 2, A and B). For example, at day 17, the cell proliferation rate, as measured by bromodeoxyuridine incorporation, was 4.15% in UACC 903 tumors and 0.61% in 10E6/11 tumors (difference = 3.54%, 95% CI = 2.43% to 4.65%, \( P < .001 \)) (Figure 2, B). In contrast, the percentage of apoptotic cells in all five tumors were similar at days 16–18 (Figure 2, C). Thus, the chromosome 10p15 tumor suppressor gene appeared to inhibit tumor growth by reducing cell proliferation rather than by promoting apoptosis.

**Tumor Cell Viability and MAP Kinase Signaling as a Function of Culture System**

Because differences in type I collagen organization were found between tumors with and without the tumor suppressor gene, we investigated whether type I collagen played a role in the tumor suppressor inhibition of tumor cell viability. We used control UACC 903 and 10ER4S.2/1 cells and cells with the tumor suppressor fragment (10E6/3, 10E6/11, and 10E6/18) for these experiments. Viability of cells that were grown on collagen I gel or plastic or in anchorage-independent cultures was determined. When cultured on plastic in DMEM supplemented with either 10% FBS or 1% FBS or grown in anchorage-independent

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**Figure 2.** Cell proliferation and apoptosis in xenograft tumors containing or lacking the tumor suppressor fragment. **A** Proliferating cells in xenograft tumors at day 16 after injection. Proliferative cells were stained by use of the bromodeoxyuridine (BrdUrd) method. **Arrows** = proliferating tumor cells. Scale bars = 50 \( \mu \)m. **B** BrdUrd incorporation in tumors at days 16–18 after injection. Data are the mean percentage of BrdUrd-positive staining tumor cells of total tumor cells. For each tumor type, at least four different tumors and six fields per tumor were assessed (from minimum of 15 fields per group). * = \( P < .001 \) (F \( F_{1,30} \) = 19.90 for day 16, 30.94 for day 17, and 18.30 for day 18) two-sided one-way analysis of variance test followed by Dunnett multiple comparison test for the comparisons of UACC 903 control tumor cells with 10E6/3, 10E6/11, and 10E6/18 hybrid tumor cells. **C** Apoptosis rate of tumors containing or lacking the 10p15 suppressor gene at days 16–18. Data are the mean value from minimum of 15 fields per group. **Bars** are as described in panel (B). Data in panels (B and C) are presented as the mean per mouse. Error bars = upper 95% confidence intervals.
cultures, similar levels of viability were observed for all cell types under these culture conditions (Figure 3, A and B). In contrast, when cultured on collagen I gel, the viability of the cells containing the 10p15 tumor suppressor fragment was approximately 60% lower than that of control cells lacking the tumor suppressor gene (Figure 3, C). For example, in collagen culture, viability of

Figure 3. Viability of melanoma cells containing the 10p15 tumor suppressor gene in different culture systems. Cell viability was measured by use of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay and is reported as the percentage of control UACC 903 cell cultures. UACC 903 and hybrid cells were cultured in 96-well dishes. A) Viability of cells cultured in plastic dishes. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing either 10% or 1% fetal bovine serum (FBS). B) Viability of cells in anchorage-independent culture. Cells were cultured in polyHEMA-coated 96-well plates. C) Viability of cells in type I collagen culture. (A–C) Data are the mean value from eight wells per group. Experiments were repeated three times. * = P < .001 (F = 129.31, two-sided one-way analysis of variance test followed by Dunnett multiple comparison test to compare UACC 903 control cells with 10E6/3, 10E6/11, and 10E6/18 hybrid cells that carry the tumor suppressor gene fragment); error bars = 95% confidence intervals. D) Mitogen-activated protein (MAP) kinase pathway signaling. Protein lysates were collected from cells growing in DMEM containing 10% FBS on plastic or type I collagen. Levels of phosphorylated extracellular-related kinase (pErk1/2), cyclin D1, and cyclin-dependent kinase 4 (CDK4) were assessed by immunoblot analysis. α-Enolase was the control for equal protein loading. Experiments were repeated three times.
10E6/11 cells that carry the tumor suppressor gene was 30.8% of the viability of control UACC cells (100%) (difference = −69.2%, 95% CI = −80.9% to −57.5%, P < .001). To eliminate the possibility that lack of the chromosome 10p15 region leads to increased production of matrix metalloproteinases, which degrade type I collagen, we analyzed the activity of matrix metalloproteinases 2 and 9 with gelatin zymograph assays and found that these activities were similar in all cells analyzed (data not shown). Thus, collagen organization and cell viability do not appear to be regulated by altered matrix metalloproteinase activation. Furthermore, the pattern of decreased growth of tumors derived from cells carrying the tumor suppressor gene that were subcutaneously injected into collagen I-rich mouse skin (Figure 1) and the pattern of decreased viability of the same cells in a collagen I-rich culture are similar, supporting the hypothesis that the 10p15 suppressor protein may directly or indirectly use signals from the collagen I-rich extracellular environment to regulate cellular proliferation and tumor growth.

The MAP kinase pathway is a major signaling pathway that regulates melanoma cell proliferation (37,38). To determine whether signals from the MAP kinase pathway participate in the growth inhibitory effect of collagen I, total protein lysates were made from control UACC 903 and 10ER4S.2/1 cells and three hybrid cells with the tumor suppressor gene (10E6/3, 10E6/11, and 10E6/18) that were cultured on plastic or collagen I. All cell types cultured on plastic had similar levels of phosphorylated extracellular-related kinase, cyclin D1, or CDK4. However, when cultured on type I collagen, cells with the tumor suppressor gene had lower levels of all three proteins than control cells (Figure 3, D). Thus, cells with the tumor suppressor gene that were grown in a type I collagen-rich environment appeared to have reduced activity of the MAP kinase pathway.

**KLF6 Gene and the Regulation of Collagen I-Mediated Inhibition of Tumor Cell Growth**

The 10p15 fragments carried by 10E6/3, 10E6/11, and 10E6/18 cells contain the KLF6 gene, which is mutated or functionally inactivated in various cancers, including prostate, stomach, liver, and colon (20), indicating that KLF6 is a good candidate to be the tumor suppressor gene in this region of chromosome 10. To explore this possibility, we first examined KLF6 protein expression in seven human melanoma cell lines that were isolated from human melanomas at different stages of development and in cultured normal human epidermal melanocytes. The seven melanoma cell lines used had been derived from tumors in the radial (WM35 and WM3211), vertical (WM115, WM98.1, and WM278), and metastatic (UACC 903 and SK-MEL-24) stage of growth. We found that KLF6 protein expression was lower in six of the seven melanoma cell lines examined than in normal human melanocytes (Figure 4, A); the exception was the WM35 cell line, which is a very early-stage melanoma derived from tumors in the radial growth phase and which has KLF6 protein expression levels similar to that of normal human epidermal melanocytes. The level of KLF6 protein expression in metastatic UACC 903 cells was approximately 35% lower than that in normal human epidermal melanocytes (difference = −0.35%, 95% CI = −0.75% to 0.05%, P = .022; note that the inconsistency in statistical significance between the confidence interval and the P value is from SAS Proc Mixed Dunnett multiple comparisons adjustment). This result raised the possibility that UACC 903 cells had a KLF6 gene with mutations that produced an inactive KLF6 protein or a lower than normal level of KLF6 protein. We ruled out the presence of a KLF6 mutation in the parental UACC 903 cells and all three hybrid cells containing the tumor suppressor fragment by sequencing the KLF6 genes from these cells (data not shown). Thus, having a lower than normal level of KLF6 protein may be sufficient to increase cell proliferation.

The level of KLF6 mRNA expression was examined in cells with (10E6/3, 10E6/11, and 10E6/18) or without (UACC 903 and 10ER4S.2/1) the 10p15 suppressor gene and also in WM35 cells and SK-MEL-24 cells, which had high and low levels of KLF6 protein, respectively. qRT-PCR was used to measure KLF6 mRNA levels in cells. KLF6 mRNA levels were approximately 28% higher in cells carrying the 10p15 fragment with the tumor suppressor gene than in cells lacking this gene (Figure 4, B). For example, the mean KLF6 mRNA level in 10E6/11 cells was 28% (95% CI = 17% to 39%, P < .001) higher than that in UACC 903 cells. Thus, UACC 903 melanoma cells have decreased KLF6 mRNA expression compared with cells carrying the 10p15 fragment with the tumor suppressor gene.

**Ectopic Expression of KLF6 Protein in Melanoma Cells**

To determine whether the relatively modest changes in KLF6 expression were functionally important, KLF6 protein was ectopically expressed in two independently derived melanoma cell lines, UACC 903 and SK-MEL-24, which had lower levels of endogenous KLF6 protein than normal melanocytes. Both hemagglutinin A-tagged and nontagged KLF6 proteins were expressed in cells to determine whether the growth effect was due to ectopic expression of KLF6 because UACC 903 cells expressed endogenous KLF6 protein. The pcDNA3.1-puro empty vector was used as a control. In addition, two hemagglutinin A-tagged nonfunctional KLF6 mutant proteins (HA3K209R and HA3W162X, where HA is hemagglutinin) were also expressed in UACC 903 and SK-MEL-24 cells to determine whether wild-type or mutant KLF6 protein could decrease cell viability. We showed that these cells did express wild-type KLF6 protein, hemagglutinin A-tagged KLF6 protein, or nonfunctional hemagglutinin A-tagged KLF6 mutant proteins (HA3K209R and HA3W162X) by use of immunoblot analysis with HA and KLF6 antibodies (Figure 5, A). It should be noted that functional KLF6 proteins (wild-type or hemagglutinin A-tagged KLF6 protein) could only be expressed ectopically at levels in the normal physiological range (approximately 1.5 relative units) compared with that of WM35 control cells (1.0 relative units), even though we expected to express higher levels of KLF6 protein by using the cytomegalovirus promoter in the pcDNA 3.1 vector (Figure 5, A). Thus, levels of ectopically expressed KLF6 protein were 23%–40% higher in UACC 903 cells carrying KLF6 or HA-KLF6 vectors than in control UACC 903 cells carrying empty vectors (Figure 5, A, left) and 39%–100% higher in SK-MEL-24 cells carrying the KLF6 or HA-KLF6 vectors than in SK-MEL-24 cells carrying empty vectors (Figure 5, A, right). Because ectopic expression of KLF6 protein in UACC 903 and SK-MEL-24 cells could restore the level of KLF6 protein to
approximately that in normal melanocytes, the level of KLF6 protein expression in melanoma cells appears to be tightly regulated and small differences in expression may be biologically important.

**Melanoma Cell Viability and Culture on Plastic or Type I Collagen**

To investigate whether KLF6 expression is associated with the viability of melanoma cell growth on collagen I, UACC 903 and SK-MEL-24 melanoma cells ectopically expressing KLF6 (KLF6 and HA3KLF6) were cultured on plastic or type I collagen for 6 days and then cell viability measured with an MTS assay. In type I collagen gel cultures, numbers of viable cells that ectopically expressed KLF6 protein were statistically significant lower than control cells carrying empty vectors. However, in cultures on plastic dishes, numbers of viable cells were similar, independent of the level of KLF6 protein expression (Figure 5, B).

![Figure 4](image-url)

Figure 4. KLF6 protein and mRNA expression in melanoma cell lines. A) Level of KLF6 protein in normal melanocytes (NHEM) and melanoma cell lines established from primary tumors at the radial (WM35 and WM3211), vertical (WM115, WM98.1, and WM278), and metastatic (UACC 903 and SK-MEL-24) growth phase of melanoma progression. Data are levels of KLF6 protein normalized to that of extracellular-related kinase 2 (the control for protein loading), which were averaged from three independent experiments. $\# = P = .014$; $\& = P = .22$, $^* = P < .001$ ($F_{7,16} = 14.15$, two-sided one-way analysis of variance [ANOVA] test followed by Dunnett multiple comparison test to compare melanocyte control cells with WM35, WM3211, WM98, WM115, WM278, UACC 903, and SK-MEL-24 cells); arrows = decreased expression of KLF6 compared with normal melanocytes. B) KLF6 mRNA levels in control UACC 903 and 10ER4S.2/1 cells and in 10E6/3, 10E6/11, and 10E6/18 hybrid cells with the tumor suppressor gene fragment, as well as WM35 and SK-MEL-24 melanoma cells. Human GAPDH mRNA was the internal control, and level of KLF6 mRNA was normalized to that of GAPDH mRNA. Data are the mean of triplicate samples from three independent experiments. Square bracket = percentage of increased expression in hybrid cells; $^* = P < .001$ ($F_{4,40} = 27.33$, two-sided one-way ANOVA test followed by Dunnett multiple comparison test to compare UACC 903 control with 10E6/3, 10E6/11, and 10E6/18 hybrid cells); error bars = 95% confidence intervals. Experiments were repeated three times.
dishes was similar. Thus, increasing expression of KLF6 in melanoma cells to approximately the level observed in normal melanocytes or early-stage WM35 melanoma cells resulted in decreased cell proliferation on type I collagen.

Next, we silenced the expression of KLF6 protein by introducing KLF6 siRNA into UACC 903 melanoma cells and hybrid cells (10E6/3, 10E6/11, 10E6/18, and 10ER4S.2/1) and investigated the expression of KLF6 protein by immunoblot analysis and cell viability on type I collagen gel culture cells by using an MTS assay. In all cells, transfection with KLF6 siRNA reduced KLF6 protein expression by approximately 85% (range = 82%-94%) compared with that in control cells (Figure 6, A). In addition, for 10E6/3, 10E6/11, and 10E6/18 hybrid cells with the 10p15 fragment carrying the tumor suppressor gene, transfection with KLF6 siRNA resulted in a statistically significant increase in the number of viable cells in type I collagen cultures compared with transfection with a control scrambled siRNA (Figure 6, B). For example, in type I collagen gel culture, viability of 10E6/3 cells was initially 38.8% and viability of KLF6 siRNA-transfected 10E6/3 cells was 76.9%, both compared with viability for untransfected UACC 903 cells (difference = −38.1%, 95% CI = −42.6% to −33.6%, P < .001). Thus, siRNA-mediated inhibition of KLF6 protein expression in hybrid cells that contain a 10p15 fragment with a copy of the KLF6 gene increased viability of cells in type I collagen gel culture.

**MAP Kinase Pathway Signaling and KLF6 Protein Expression in Type I Collagen-Rich Culture**

We investigated whether the MAP kinase signaling pathway was involved in the growth inhibitory effects of type I collagen that are mediated by KLF6 protein. Specifically, we measured the levels of two components of the MAP kinase pathway, phosphorylated Erk1/2 and cyclin D1, by use of immunoblot analysis. We used total cell lysates from UACC 903 and SK-MEL-24 melanoma cells that carried an empty puro vector (control) or expression vectors carrying wild-type KLF6, hemagglutinin A-tagged wild-type KLF6, or hemagglutinin A-tagged inactive KLF6 (HA3K209R and HA3W162X). Cells were cultured on plastic or on type I collagen. When cultured on plastic, all cell lines had similar levels of phosphorylated Erk1/2 or cyclin D1. However, when cultured on type I collagen, expression of phosphorylated Erk1/2 or cyclin D1 was lower in cells that expressed wild-type KLF6 or hemagglutinin A-tagged wild-type KLF6 than in cells that carried an empty vector or expressed inactive KLF6 proteins (HA3K209R and HA3W162X) (Figure 7). Thus, in melanoma cells, MAP kinase pathway signaling appears to be involved in the growth inhibitory effects of type I collagen mediated by KLF6.

**KLF6 Protein Expression in Human Melanoma Tumors and Nevi**

We next investigated KLF6 protein expression in human nevi and melanoma tumors by using immunohistochemistry to assess KLF6 protein expression. We tested tissue sections from 17 nevi, five early melanomas, and four metastatic melanomas. All 17 atypical nevi had high levels of KLF6 protein staining in the type I collagen-rich dermis (Figure 8, A). Also, normal keratinocytes, which are the primary constituent of the epidermis, had high levels of KLF6 protein expression and served as an internal staining control. KLF6 protein expression was found in the periphery of the melanoma tumor nodules in tumors that were greater than 0.75 mm in depth but not in the center of the tumors (Figure 8, A). In contrast, metastatic melanomas had negligible KLF6 protein expression throughout the lesions (Figure 8, A). The distribution of KLF6 protein in melanoma tissues was consistent with that observed for UACC 903-xenografted tumors, which had negligible expression in the tumor interior but slightly higher expression at the tumor edge that was in contact with the type I collagen-rich extracellular matrix (Figure 8, B). Thus, increasingly more aggressive tumors (from nevi, to early melanomas >75 mm in depth), to metastatic melanomas) appear to have increasingly lower KLF6 protein expression.

Expression of KLF6 protein was next measured by immunoblot analysis, and expression of KLF6 mRNA was measured by qRT-PCR in malignant melanomas that were flash-frozen after removal from patients (Table 1) (29 tumor specimen were used for the protein expression experiment and five tumor specimens in which KLF6 protein expression could not be detected were used for mRNA expression experiments). Protein lysates from WM35 cells served as the positive control for high KLF6 protein expression and lysates from SK-MEL-24 cells served as the negative control. We detected no KLF6 protein expression in the 29 malignant melanomas, indicating that loss of this gene may be associated with melanoma development (Figure 8, C). We also detected various levels of protein expression for ITIH5, a control gene that is located within the same 10p15 region as KLF6, and for PTEN, which is located at 10q23, in these 29 tumors, indicating that loss of the KLF6 gene may be associated with metastatic melanoma (Figure 8, C; representative example of 13 tumors). To determine whether low levels of KLF6 protein expression in metastatic melanomas resulted from decreased mRNA levels, we examined the mRNA levels in these tumors by qRT-PCR. In the five metastatic melanoma tumor specimens lacking KLF6 protein expression that were selected for this analysis, KLF6 mRNA levels were found to be similar to or higher than that in control WM35 cells (Figure 8, D). The level of KLF6 protein expression in WM35 cells is similar to that in normal melanocytes. Thus, metastatic melanomas appear to have lost KLF6 protein expression through a posttranscriptional or posttranslational mechanism.

**Discussion**

In this study, we have identified KLF6 as a tumor suppressor gene on chromosome 10 that is lost during melanoma development. When UACC 903 cells that carry a fragment of the 10p15 region with the KLF6 gene (ie, 10E6/3, 10E6/11, and 10E6/18), two melanoma cell lines that ectopically express KLF6 protein (ie, UACC 903 and SK-MEL-24), and control cells were cultured on plastic dishes, proliferation was essentially the same for all cells. However, when cells were cultured on type I collagen, proliferation of cells carrying the KLF6 gene was decreased compared with that of control cells. This collagen-mediated inhibition appears to be mediated through decreased signaling via the MAP kinase pathway. When cells carrying the KLF6 gene or their control cells were injected into mice, growth of xenograft tumors that were generated...
Figure 5. Ectopic expression of KLF6 and viability of UACC 903 and SK-MEL-24 melanoma cells on type I collagen and on plastic. Expression of wild-type and mutant KLF6 protein in melanoma cells on type I collagen. **A, Upper** Immunoblots of total lysates collected from UACC 903 or SK-MEL-24 cells ectopically expressing wild-type KLF6, hemagglutinin A (HA)-tagged KLF6 (HA3KLF6), and mutant forms of KLF6. Cells carrying empty puro vectors were the control. Blots were probed with antibodies against the HA tag or wild-type KLF6 protein. **Intact** = ectopic expression of wild-type HA-KLF6; **truncated** = mutant KLF6 form HA-W162X. Endogenous levels of KLF6 were probed with an antibody against KLF6 protein. α-Enolase was the control for equal protein loading. **A, Lower** Relative levels of KLF6 protein. Levels of KLF6 protein in blots in panel (A) were normalized to that of α-enolase, and the relative expression was plotted. Endogenous levels of KLF6 in UACC 903 vector control were set as 1 (dash line). The normal physiological range of KLF6 protein is 1.5 as shown in WM35 cells, which has similar expression.

### Table

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<th>Condition</th>
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(continued)
from cells carrying the KLF6 gene was decreased compared with that of control xenograft tumors. Finally, KLF6 protein was present in nevi but was not detectable in more aggressive melanomas from patients, which is consistent with KLF6 protein being a tumor suppressor.

For more than a decade, loss of an intact copy of chromosome 10 in early melanoma development has driven the search for tumor suppressor genes in chromosome 10 (3–5). Two tumor suppressor genes reside on this chromosome (3). PTEN was the first tumor suppressor gene identified (39). Its identification on the long arm of chromosome 10 provided a partial explanation for loss of the entire chromosome because signaling by PTEN and Akt3 proteins has been shown to play a key role in melanomas by regulating cell apoptosis, but other processes are also required for melanoma tumorigenesis (6–8,33,37). The second tumor suppressor gene was mapped to 10p15 (3), but its identity remained elusive, until now.

We found that the type I collagen-mediated inhibition of cell proliferation could be uncoupled during melanoma tumorigenesis, possibly through loss of the tumor suppressor gene on chromosome 10. In addition, we found that the KLF6 gene, which is located in the 10p15 region of chromosome 10, appeared to have characteristics that are consistent with those of the tumor suppressor gene. Specifically, when DNA fragments containing the KLF6 gene

Figure 6. KLF6 small interfering RNA (siRNA), melanoma cells containing chromosome 10p15 fragments, and viability on type I collagen of UACC 903, 10E6/3, 10E6/11, 10E6/18, and 10ER4S.2/1 cells. A) KLF6 siRNA and KLF6 protein expression. siRNA (100 pmol) of was introduced by nucleofection with an Amaxa Nucleofector, and 2 days later, protein lysates were prepared and KLF6 protein expression was assessed by immunoblot analysis with KLF6 antibody. Untransfected or cells nucleofected with scrambled siRNA were used as controls, and α-enolase was the control for equal protein loading. Arrow = reduction of KLF6 expression compared with control. Experiments were repeated three times. B) Cell viability at day 5 of cells cultured on type I collagen. Data are the mean value of eight samples; the experiment was repeated three times. * = P < .001 (F4,35 = 361.7 for 10E6/3 cells, 161.2 for 10E6/11 cells, and 260.6 for 10E6/18 cells; two-sided one-way analysis of variance test followed by Dunnett multiple comparison test to compare the effect of siRNA-mediated targeting KLF6 with that of untransfected and scrambled siRNA controls); error bars = 95% confidence intervals; arrow = increased cell viability compared with controls.

expression levels as normal melanocytes. Blots were repeated twice, and both showed similar results. B) Expression of KLF6 and viability of melanoma cells on collagen I. Viability of UACC 903 or SK-MEL-24 cells ectopically expressing wild-type or HA-tagged KLF6 (HA3KLFL6) was assessed in type I collagen cell culture and compared with that of control cells expressing inactive (HA3K209R and HA3W162X) protein or carrying the empty puro vector, by use of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay. Data are the mean value from eight samples; the experiment was repeated three times. * = P < .001 (F4,35 = 641.17 for UACC 903 cells and F4,35 = 102.76 for SK-MEL-24 cells; two-sided one-way analysis of variance test followed by Dunnett multiple comparison test to compare UACC 903 or SK-MEL-24 puro vector control cells with KLF6 ectopically expressing cells); error bars = 95% confidence intervals.

Figure 5 (continued).
were transferred into melanoma cells, cell proliferation decreased apparently through inhibitory signaling by the MAP kinase pathway, as indicated by decreased levels of Erk and cyclin D proteins. KLF6 protein appeared to be able to transmit the inhibitory signal from type I collagen in the extracellular matrix of the skin or in culture as shown by plating cells on a type I collagen gel. Type I collagen has been shown to regulate proteins involved in cell proliferation (including p27, tyrosine kinase discoidin domain receptor 2, and focal adhesion complex proteins) in cancer development (23,29,40), but the molecular mechanisms involved have not been fully elucidated. In addition, type I collagen have been shown to inhibit cell migration and proliferation (29,41). In ad

Figure 7. Mitogen-activated protein (MAP) kinase pathway signaling, KLF6 expression, and culture on type I collagen or plastic in UACC 903 or SK-MEL-24. A) Cell lines derived from UACC 903 melanoma cells. B) Cell lines derived from SK-MEL-24 melanoma cells. Levels of phosphorylated extracellular-related kinase (pErk1/2) and cyclin D1 were assessed in lysates collected from UACC 903 and SK-MEL-24 cells ectopically expressing wild-type KLF6, HA-KLF6, or mutant forms of KLF6 by immunoblot analysis with anti-phosphorylated Erk1/2 and anti-cyclin D1 antibodies. Numerical values below each blot are the normalized averages of pErk1/2 and cyclin D1 expression from two independent experiments. Averages were normalized to the value for α-enolase (control for protein loading). Experiments were repeated two times.

We provide evidence that KLF6 protein is involved in inhibitory signaling from type I collagen through both gain- and loss-of-function analyses. Silencing of KLF6 expression by introducing KLF6 siRNA into hybrid cells carrying a 10p15 chromosomal fragment with the KLF6 gene increased cell proliferation in collagen I-rich cultures compared with such cells containing a scrambled siRNA; however, when KLF6 protein was ectopically expressed in UACC 903 or SK-MEL-24 cells, KLF6 protein was expressed to only the level found in normal melanocytes, indicating that the KLF6 expression level may be tightly controlled and may be an important component in this signaling pathway. Cells that expressed KLF6 protein at approximately normal levels also expressed decreased levels of components of the MAP kinase signaling pathway. In contrast, when cells were grown on plastic or in anchorage-independent culture, no differences in proliferation or MAP kinase pathway signaling were observed between cells that expressed KLF6 protein and control cells. Thus, a KLF6 level similar to that in normal melanocytes appears to be necessary for coupling collagen I signaling with decreased melanoma cell proliferation.

Members of the KLF family have been associated with cancers of the prostate, intestine, and colon presumably by regulating cell growth and tumor progression (15,46). However, this is, to our knowledge, the first report that a KLF family member is involved with signaling from the extracellular matrix that inhibits cell proliferation. Type I collagen-mediated signaling through KLF6 protein may also be active during the transition from radial to vertical phase growth in melanomas, which is associated with tumor growth within the type I collagen-rich dermis, but may not be active in metastatic cells, which are no longer in contact with type I collagen.

Interaction of KLF6 protein with other deregulated pathways, including MAP kinase signaling pathway, in melanoma also appears to be involved in melanoma development. For example, in early melanocytic lesions, BRAF is mutated to V600E, which constitutively activates the MAP kinase signaling pathway (47), V600E BRAF alone may be necessary for melanocyte proliferation but causes oncogene-induced senescence in cells via high MAP kinase pathway activity (37,48), indicating that other events, such as activation of Akt3 (37) or loss of KLF6 function, might be required to promote early melanoma development by fine-tuning MAP kinase pathway activity. In addition, ectopic expression of KLF6 in melanoma cells containing V600E BRAF decreased expression of key indicator proteins, including phosphorylated Erk and cyclin D1 downstream in the MAP kinase signaling cascade in a collagen I-dependent manner (49). Thus, KLF6 might be necessary for inhibiting early melanoma development by regulating or fine-tuning MAP kinase activity.

Loss of chromosome 10 in early melanocytic lesions reduces the expression of PTEN and KLF6, which promotes melanoma development (3). Human UACC 903 melanoma cells have lost a copy of chromosome 10 followed by duplication of the remaining copy of chromosome 10, resulting in a condition called uniparental disomy (3). These cells expressed approximately 35% less KLF6 protein than normal melanocytes. The level of KLF6 protein in the tumor from which UACC 903 cells were isolated is, of course, unknown but could have been considerably lower because we found that all 29 tumors from patients with malignant melanoma had negligible KLF6 protein expression.
Loss of a copy of KLF6 with the loss of chromosome 10 in early melanoma development appears to be the primary mechanism that disrupts the activity of KLF6; however, other mechanisms might also play a role. KLF6 splice variants, such as KLF6-SV1, may lead to inactivation. For example, in prostate and lung cancer, increased expression of KLF6-SV1 antagonizes the suppressive effects of KLF6 protein (50–53). It is also possible that collagen I can influence posttranscriptional and posttranslational modification of KLF6 protein to regulate its level, which are reduced in patient tumors. However, levels of KLF6 mRNA in metastatic melanomas were found to be similar to or even higher than those in early-stage melanocytic lesion cells (Figure 8, D).

This study had several limitations. The direct mechanism through which type I collagen regulates KLF6 function has not been determined. Posttranscriptional or posttranslational modifications of KLF6 were not studied and might be important because the levels of KLF6 mRNA and protein in cultured cells and in tumors did not correlate. In future studies, it will be important to determine whether type I collagen regulates KLF6 expression by posttranscriptional or posttranslational modification. Tumor growth was studied in immunodeficient mice, and there were only four mice per experimental group. No information was available about individuals who contributed the 17 nevi for this study. Only 29 human melanoma specimens were studied.

Table 1. Characteristics of the 29 melanoma patients analyzed in this study

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* Characteristics for the 17 subjects who contributed atypical nevi was not determined. ND = not determined.
† Data on age at diagnosis were missing for two patients.
In conclusion, we have identified KLF6 as a tumor suppressor gene at 10p15 that with PTEN, the tumor suppressor gene at 10q23, appears to be involved in the development of melanoma. When expressed in melanoma cells, KLF6 protein mediated signaling between collagen I in the extracellular matrix and the MAP kinase pathway, thereby inhibiting cell proliferation. Loss or decreased expression of KLF6 protein (approximately 35% lower than KLF6 expression in normal melanocytes) appears to uncouple this signaling so that cell proliferation in the collagen I-rich environment of the skin is increased. Reconnecting signaling between aggressive melanoma tumor cells and collagen-rich microenvironment of the tumor, by inserting a KLF6 expression construct, can decrease cell proliferation. Thus, restoring KLF6 expression may be a potential target for the prevention or treatment of melanoma.

References


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