Melanoma Metastasis Suppression by Chromosome 6: Evidence for a Pathway Regulated by CRSP3 and TXNIP

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ABSTRACT

Loss of genetic material on chromosome 6 has been associated with progression of human melanomas. We showed previously that introducing chromosome 6 into metastatic human melanoma cell lines suppresses metastasis without affecting the ability of the hybrids to form progressively growing tumors. By subtractive hybridization comparing nonmetastatic chromosome 6-containing (neo6/C8161) versus parental (C8161) metastatic cell lines, the KISS1 metastasis suppressor gene was isolated. However, KISS1 expression was more highly in neo6/C8161 and in nonmetastatic melanomas. Increased TXNIP expression inhibited metastasis and up-regulated KISS1. Surprisingly, also mapped to chromosome 1q PCR karyotyping that refined the region on chromosome 6 identified CRSP3/DRIP130, a transcriptional coactivator, as a metastasis suppressor. CRSP3 transfectant cells had up-regulated KISS1 and TXNIP expression and were suppressed for metastasis. Quantitative real-time reverse-transcription PCR of clinical melanoma samples showed that loss of CRSP3 expression correlated with increased KISS1 expression and increased metastasis. Thus, we implicated a specific gene on chromosome 6 in the etiology of melanoma metastasis and identified potential upstream regulators of KISS1 and TXNIP.

INTRODUCTION

Metastasis is the culmination of neoplastic progression toward autonomy from host regulation. Acquiring metastatic potential is apparently “driven” by genomic instability with coincident or superimposed selection pressures (1). To be metastatic, tumor cells must complete a sequential, multistep cascade involving complex interactions between tumor and host. Cells must navigate a series of obstacles, including immune surveillance, loss of original tissue context, invasion through the basement membrane, lymphatic or hematogenous circulation, extravasation, and growth at the secondary site. Any cell shed from the primary neoplasm that fails to complete all requisite steps cannot form metastases (2, 3).

We have shown that the MMCT of human chr6 into metastatic human melanoma cell lines C8161 or MelJuSo suppressed metastasis without blocking tumor formation (4, 5). Metastasis-suppressed hybrids of C8161 (neo6/C8161) were blocked at steps in the metastatic cascade affecting the survival and proliferation at secondary sites (6). We used subtractive hybridization to identify a metastasis suppressor gene, KISS1, which mapped to 1q32 (7–10). Tumor cells transfected with KISS1 cDNA were nonmetastatic, but remained tumorigenic. MMCT of chr6 with a deletion (~40 Mb) spanning 6q16.3-q23 (neo6qdel) did not suppress metastasis (11). Because KISS1 was cloned from C8161 cells, the genetic defect must be at the level of KISS1 regulation, not in KISS1 itself. Also, because neo6qdel hybrids did not express KISS1, the upstream regulator(s) of KISS1 was mapped between 6q16.3-q23 (11). Shirasaki et al. verified this hypothesis in melanoma clinical samples by showing loss of heterozygosity of markers in that region correlated with decreased KISS1 expression (12).

KISS1 is a precursor for secreted neuropeptide ligands [designated Metastin (13) or Kisspeptins (14)] for a G-protein coupled [named hOT7T175 (13), AXOR12 (15), or hGPR54 (14)] receptor-transfected CHO cells (13), as well as phosphorylation of ERK1/2 and weak phosphorylation of p38/MAPK but not of SAPK/JNK (14). Metastin inhibits motility, chemotaxis, and invasion in vitro (13, 16), possibly by repressing the transcription of MMP-9 [via induction of cytosolic IkBα (17)]. Metastin also induces excessive formation of focal adhesions and stress fibers in hOT7T175-transfected B16/BL6 melanoma-injected mice treated with Metastin developed fewer lung metastases (13), the mechanism of KISS1 suppression is still unknown. Recent publications suggest possible mechanisms for KISS1 metastasis suppression. Metastin induces Ca2+ in receptor-transfected CHO cells (13), as well as phosphorylation of ERK1/2 and weak phosphorylation of p38/MAPK but not of SAPK/JNK (14). Metastin inhibits motility, chemotaxis, and invasion in vitro (13, 16), possibly by repressing the transcription of MMP-9 [via induction of cytosolic IkBα (17)]. Metastin also induces excessive formation of focal adhesions and stress fibers in hOT7T175-transfected B16/BL6 and induces the phosphorylation of FAK and paxillin (13), possibly through Rho (14).

The primary objective of the current study was to identify the upstream regulator(s) of KISS1 on chr6. Additionally, microarray hybridizations using metastatic (C8161) and nonmetastatic (neo6/C8161) variants of a human melanoma cell line could be useful in identifying some additional downstream regulators of KISS1.

MATERIALS AND METHODS

Cell Lines and Culture. C8161 is a human melanoma cell line that metastasizes after orthotopic or i.v. injection into athymic mice (18). C8161.9 is a highly metastatic subclone of C8161 (7). Nonmetastatic neo6/C8161.1, neo6/C8161.2, neo6/C8161.3, and neo6/C8161.6 were derived by MMCT using the MCH262A1.D6 donor cell line (4). Metastatic neo6del(q16.3-q23)/C8161 hybrids (neo6qdel/C8161.9.1, neo6qdel/C8161.9.2, neo6qdel/C8161.9.7, neo6qdel/C8161.9.10, and neo6qdel/C8161.9.11) were derived by MMCT using the MCH262A1.D6 donor cell line (4). Metastatic neo6del(q16.3-q23)/C8161 hybrids (neo6qdel/C8161.9.1, neo6qdel/C8161.9.2, neo6qdel/C8161.9.7, neo6qdel/C8161.9.10, and neo6qdel/C8161.9.11) were derived by MMCT using the MCH262A1.D6 donor cell line (4).

Received 8/6/02; accepted 11/14/02.

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1 Supported by NIH Grants CA66128 (to D. R. W.), CA77278 (to D. R. W.), and CA88876 (to M. E. M.); the National Foundation for Cancer Research (to D. R. W.); a Grant-in-Aid for Scientific Research (C2-12670812) from the Japan Society for the Promotion of Science (to N. H. and M. T.); a Japan Foundation Grant-in-Aid for Scientific Research (C2-12670812) from the Japan Society for the Promotion of Science (to N. H. and M. T.); a predoctoral fellowship from the Foreman Foundation (to S. F. G. and D. R. W.); and the Jake Gittlen Memorial Golf Tournament (to D. R. W.).

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and stable transfectants (21) were isolated and cloned by limiting dilution. neo6/C8161.2, and neo6/C8161.6 were used at passages 4, 11, 20, and 10, and 13, respectively. Cells were detached using a solution of 2 mm Tris (pH 8)-1 mM EDTA to a concentration of 50 ng/μl of active G418 (Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO2. None of the cultures were infected with Mycoplasma spp. according to the PCR-based TaKaRa Mycoplasma detection kit (Panvera, Madison, WI). Cells were detached using Oligotex Suspension (Qiagen, Valencia, CA) according to the manufacturer’s “batch” protocol. mRNA was precipitated using the Pellel Pellet coprecipitant (Novagen, Madison, WI), followed by resuspension in 10 mM Tris (pH 8)-1 mM EDTA to a concentration of 50 ng/μl.

RNA Isolation. Total RNA was isolated from cultured cells at 70–90% confluence or from tumor tissue after stabilization in RNA later (Ambion, Austin, TX) and pulverization with a mortar and pestle. Poly(A) RNA was isolated using Oligotex Suspension (Qiagen, Valencia, CA) according to the manufacturer’s “batch” protocol. mRNA was precipitated using the Pellel Pellet coprecipitant (Novagen, Madison, WI), followed by resuspension in 10 mM Tris (pH 8)-1 mM EDTA to a concentration of 50 ng/μl.

Microarray Hyridization. Poly(A) RNA was reverse transcribed with Cy3- and Cy5-end-labeled random 9-mer to generate fluorescent single-stranded cDNA probes. Probes were competitively hybridized to the Human Unigene 1 microarray (Incyte Genomics, Palo Alto, CA), which contained 9182 elements representing 8472 unique annotated genes or expressed sequence tagged clusters arrayed on glass slides. cDNAs on the array are PCR products of 500-5000 bp, with an average size of 1–1.2 kbp. According to the producer, the hybridization is able to detect >1.7-fold changes with 99% confidence, and when 200 ng of starting RNA is used to make a probe, 2 pg of RNA can usually be detected (~1 in 100,000 copies).

For Array 1, 1 μg from RNA isolated from C8161.9, passage 14 (P14), and neo6/C8161.2 (P18) was used for the Cy3 and Cy5 probes, respectively. For Array 2, equal quantities of mRNA from four clones of neo6/C8161 (clones 1, 2, 3 and 6, at passages 16–28) were pooled for the Cy3 probe and four clones of neo6d2el/C8161.9 (clones 1, 2, 7, and 8 at passages 8–10) were pooled for the Cy5 probe. mRNA (600 ng) was used to generate each probe.

The identity of spotted DNA was verified by PCR. A failure in PCR testing resulted in exclusion of the spot measurement. Genes were analyzed if >3 readings had a signal/background ratio >2.5, signal intensity >250 units for one or both of the above criteria and the spot size of ≥40% of the spotted area. A total of 8083 array elements passed these quality control criteria for Array 1, and 7217 genes for Array 2. Cy3 and Cy5 data sets were normalized for median signal values to compensate for differences in labeling and detection efficiency.

Expression Vectors and Cloning. A full-length TXNIP clone in the pNcy vector was obtained from Incyte Genomics (No. 2888464). TXNIP was excised by enzymatic digestion with EcoRI and NotI (Promega, Madison, WI) and subcloned into the pcDNA3 mammalian expression vector (Invitrogen). Full-length CRSP3 was cloned into the pcDNA3 vector.

Transfection and in Vitro Growth Curves. pcDNA3-CRSP3 and pcDNA3-TXNIP were transfected into C8161.9 cells by electroporation using the pICNY vector obtained from Incyte Genomics (No. 2888464). TXNIP was isolated using Oligotex Suspension (Qiagen, Valencia, CA) according to the manufacturer’s “batch” protocol. mRNA was precipitated using the Pellel Pellet coprecipitant (Novagen, Madison, WI), followed by resuspension in 10 mM Tris (pH 8)-1 mM EDTA to a concentration of 50 ng/μl.

For in vitro growth curves, C8161.9/TXNIP clones, C8161.9/pCDNA3, neo6/C8161.2, and neo6/C8161.6 were used at passages 4, 11, 20, and 10, respectively. For in vitro growth curves, C8161.9/CRSP3 clones, C8161.9/pcDNA3, neo6/C8161.2, and neo6/C8161.6 were used at passages 8, 19, 23, and 13, respectively. Cells (1 × 106) were seeded on 6-well tissue-culture plates containing 2 ml of cDMEM-F12 + 500 μg/ml G418 per well. Six replicate wells per cell line per time point were detached with 2 mm EDTA and counted using a hemacytometer. Cell numbers were determined daily for 10 days. Morphology was recorded using an inverted microscope (Nikon Diaphot) equipped with a digital camera (DC Viewer Version 3.2.0.0; Leica Microsystems, Ltd., Heerbrugg, Switzerland).

In Vivo Experiments. Female athymic mice 3–4 weeks of age (Harlan Sprague Dawley, Madison, WI) were maintained under the NIH and The Pennsylvania State University College of Medicine guidelines. The Institutional Animal Care and Use Committee approved all protocols. Food and water were provided ad libitum.

For in vivo experiments, C8161.9/pCDNA3 (P6 and 16), neo6/C8161.2 (P20–21), neo6/C8161.6 (P10), C8161.9/TXNIP (P4–5), and C8161.9/CRSP3 (P4–7) were detached at 70–90% confluence, rinsed with CMF-DPBS, and detached with a 2 mm EDTA solution. To minimize aggregation, cells were diluted to the desired concentration in ice-cold HBSS and maintained on ice until they were injected into the mice. Injections were performed using a 27-gauge needle affixed to a 1-cc tuberculin syringe.

Orthotopic injections were made into the dorsolateral flank using 1 × 106 cells in 0.1 ml ice-cold HBSS. Tumor size was measured weekly and geometric MTD was calculated (22). When MTD = 1.0 cm, tumors were surgically removed after Ketamine-Xylazine (80–85 mg/kg;14–16 mg/kg) anesthesia and the wounds closed with sterile stainless-steel 9-mm clips (Becton-Dickinson, Sparks, MD). Tumor size for RT-PCR analysis was obtained when tumors were 0.5–1.0 cm MTD to minimize impact of necrotic tissues. Metastases were quantified 1 month after tumor removal.

For experimental metastasis assays, cells (2 × 106 in 0.2 ml of HBSS) were injected i.v. into the lateral tail veins of nude mice. Animals were killed 1 month after injection and their lungs removed for quantification of metastases.

To quantify lung metastases, lungs were removed and fixed in a diluted Bouin’s solution (20% Bouin’s fixative in neutral buffered formalin). Grossly visible metastases were scored using a dissecting stereomicroscope. Lungs that had metastases too numerous to count, or that had grown into a confluent layer, were scored as having 200 metastases.

Northern Blot Analysis. Total RNA (10 μg/sample) was size separated on a 1% agarose gel containing 2.2 μm formaldehyde at 80 V for 3.5 h. RNA was transferred onto a positively charged Hybond N+ nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) using the Turbobotlysis system (Schleicher and Schuell, Keene, NH) and fixed by UV cross-linking (Stratagene, La Jolla, CA). Full-length KISS1, TXNIP, or a PCR-generated fragment of CRSP3 were radiolabeled ([α32P]-dCTP) using the RediPrime II DNA labeling mix (Amersham Pharmacia Biotech). Blots were prehybridized in ExpressHyb solution (Clontech, Palo Alto, CA) containing 100 μg/ml denatured salmon sperm DNA for 1 h. Labeled probe was added to the hybridization solution and incubated overnight at 68°C. Membranes were rinsed in two changes of low stringency wash buffer (2× SSC + 0.1% SDS) followed by 2× SSC + 0.1% SDS for 15 min at 37°C and a high stringency wash (0.1× SSC + 0.1% SDS) for 15 min at 50–60°C. The membranes were then exposed to Kodak BioMax MR X-ray film (Kodak, Rochester, NY).

RTQ. RTQ was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) located at the Nucleic Acids Facility of the Penn State University Life Sciences Consortium. Relative mRNA levels were determined using the comparative Ct (threshold cycle) method. The expression of TXNIP, KISS1, and CRSP3 (CTRT) was normalized to an endogenous reference gene (G3PDH). Although G3PDH may exhibit differential expression in some cancers (23, 24), it is commonly used as a standard. Our microarray experiments confirmed that expression of G3PDH did not differ significantly among nonmetastatic and metastatic variants of C8161. CTRT was calculated by subtracting the Ct value of the reference (CTRT) from the Ct value of the sample (ΔCTR = CTRT − CT). The relative expression (ΔΔCTR) of a calibrator was determined by subtracting the ΔCTR of the calibrator (ΔCTRcalib) from the ΔCTR of interest (ΔCTRinterest). Samples and internal controls were run in triplicate.

Primer and fluorogenic probe sets for TXNIP, KISS1, CRSP3, and G3PDH were designed using Primer Express V1.0 (Applied Biosystems). The TNIP forward and reverse primers and fluorescent-labeled probe were 5′-GGCTTAAAGATGGCAGACC-3′, 5′-TCCAACAACACCCCCGTGA-TCA-3′, and 5′-ATCTCTACGACGCGCCATG-3′, respectively. The sequences of the CRSP3 forward and reverse primers and fluorescent-labeled probe were 5′-GCCCAAGCTTGGACTTG-3′, 5′-TGCAAGCGAAGTGAAATCAAAGAG-3′, and 5′-AACAGATTGCGTGCTATCCATCGC-3′.
respectively. The sequence of the KISS1 forward and reverse primers and fluorescent-labeled probe were 5'-TGCTGTTGACGCTGGA-3', 5'-CGAACGTACGGCCG-3', and 5'-AGGACCCGACACTCAACTTGGAAC-3', respectively. The 5' fluorogenic reporter probe was FAM, and the 3' fluorogenic quencher was BlackHole (Biosearch Technologies, Inc., Novato, CA). The G3PDH forward and reverse primers and fluorescent-labeled probe were 5'-GAAGGTGAGTGGATTGAG-3', 5'-GAAGGTGAGTGGATTGAG-3', and 5'-CAAGGTTCCCAGTCAAGC-3', respectively. The 5' fluorogenic reporter probe was VIC (Biosearch Technologies, Inc.) and the probes obtained from Biosearch Technologies, Inc.

RTQ was performed with the Taqman Universal PCR Assay Mix in a 96-well reaction plate. G3PDH was amplified at the same time and used as a reference gene. Reverse transcription using the murine leukemia virus reverse transcriptase was performed at 42°C for 1 h, followed by a 5-min extension at 72°C. Each RT-PCR contained the following: 10 μM each TXNIP- and G3PDH-specific primers, 1 μM each TXNIP/G3PDH/KISS1 and G3PDH fluorogenic probe, and 8 μl of the reverse transcriptase product. After incubation for 2 min at 50°C and 10 min at 95°C, PCR amplification was performed for 40 cycles (95°C for 15 s and 60°C for 1 min) using the AmpliTag Gold DNA polymerase (Applied Biosystems).

Clinical Samples and Analysis of KISS1 and CRSP3 Expression. Twenty-four cutaneous melanoma specimens (11 primary and 13 metastasis) were obtained from 20 Japanese patients undergoing surgery at Kanazawa University Hospital. The specimens were frozen immediately in liquid nitrogen and used for RNA extraction. Total RNA was extracted from frozen tumors using a silica-gel-based membrane system (Rneasy; Qiagen, Hilden, Germany). To avoid false positives from contaminating genomic DNA, RNA samples were treated with DNase I (Qiagen) for 15 min. The first strand cDNA synthesis was performed from 1 μg of total RNA using avian myeloblastosis virus reverse transcriptase and oligodeoxynucleotide primers (Promega, Madison, WI). All protocols were approved by both the Kanazawa University and Penn State University Institutional Review Boards. Patient identifiers were removed for reporting of this experimental data.

RT-PCR. Total RNA (2.5 μg) was reverse transcribed and amplified using the Advantage Titanium One-step RT-PCR kit (Clontech) according to the manufacturer's instructions. Reverse transcription proceeded at 50°C for 1 h, followed by 35 cycles of PCR (94°C, 45 s; 60°C, 45 s; 72°C, 60 s) and a final extension step of 10 min at 45°C. TXNIP was amplified with the human-specific primers 5'-CAAGGAGTTTCTCCTGATTG-3' and 5'-TTGAGAGATGTCCACAGGAG-3' yielding an expected product size of 422 bp. CRSP3 was amplified with the human-specific primers 5'-GGCTTTTCCTGT-3' and 5'-AACACTGTTGACGAGCAG-3', giving an expected product size of 540 bp. Human G3PDH was amplified a control amplimer set (Clontech), giving an expected product size of 983 bp. The products were resolved on a 1% agarose gel and visualized by ethidium bromide staining.

RT-PCR for clinical samples was performed as reported previously (12). Analyses were made using a Prism 7700 sequence Detector (PE Biosystems). The sequences of primers and fluorescence-labeled probes were as follows: KISS1: forward 5'-ACCATCTGTTTTTGGGAGG-3', reverse 5'-ACTGTTTTCTTCTCCTGTCGAG-3', and probe 5'-FAM-ACTGTTTTCTTCTCCTGTCGAG-TAMRA-3'; CRSP3: forward 5'-GCCCGACGT-3', reverse 5'-TTGACAGACGTAATCAAGAG-3', and probe 5'-FAM-AACACAGTGTTTGGGCTATCATTCC-3'; β-actin: forward 5'-TC ACCCAACATGGGCACACTG-3', reverse 5'-AGGGAAGGAGGGGACTG-3', and probe 5'-FAM-CTTGCAACGACCTGCATG-3'. RT-PCR for clinical samples was performed as reported previously (12). Analyses were made using a Prism 7700 sequence Detector (PE Biosystems). The sequences of primers and fluorescence-labeled probes were as follows: KISS1: forward 5'-ACCATCTGTTTTTGGGAGG-3', reverse 5'-ACTGTTTTCTTCTCCTGTCGAG-3', and probe 5'-FAM-ACTGTTTTCTTCTCCTGTCGAG-TAMRA-3'; CRSP3: forward 5'-GCCCGACGT-3', reverse 5'-TTGACAGACGTAATCAAGAG-3', and probe 5'-FAM-AACACAGTGTTTGGGCTATCATTCC-3'; β-actin: forward 5'-TC ACCCAACATGGGCACACTG-3', reverse 5'-AGGGAAGGAGGGGACTG-3', and probe 5'-FAM-CTTGCAACGACCTGCATG-3'.
Statistical Analysis. The number of lung metastases was compared in CRSP3 transfectants and control cells. A one-way ANOVA followed by the Student-Newman-Keuls post-test was used to determine significance. Statistical significance was defined as $P \leq 0.05$ using two-sided tests. The SE was calculated by dividing the SD by the square root of the sample size. For analysis of clinical samples, correlations between KISS1- and CRSP3-transcript expression by RT-PCR were compared by Spearman’s correlation test by ranks.

RESULTS

Microarray Hybridization. To identify metastasis suppressor genes on chr6, a dual microarray strategy was used. In Array 1, highly metastatic subclone C8161.9 (4) was used in a competitive hybridization with metastasis-suppressed neo6/C8161.2. The vast majority (>96%) of genes showed similar expression – the mean fold-difference was 1.25 ± 0.525 (mean ± SD; Fig. 1A). To minimize the impacts of clonal heterogeneity and gene expression attributable to gene dosage, Array 2 probes comprised equal mixtures of four clones each from neo6/C8161 and neo6qdel/C8161.9. Again, >96% of genes showed <2-fold difference in expression. In metastatic cells, 218 genes were expressed >2-fold higher. Fifty-two were expressed ≥2-fold higher in the nonmetastatic cells. The average fold-difference was 1.25 ± 0.316 (Fig. 1B). Applying a strict ±3 SD threshold, the pool of candidate effectors was only 12 genes.

Genes Expression and Validation. Eleven genes were more highly expressed in the metastatic cells (i.e., down-regulated by chr6; Table 1). Three genes encoded proteins involved in cell replication: CENP-E (centromere-associated protein E), CDC2, and MAD2 (mitotic arrest deficient 2 homologue-like 1). Six genes encoded proteins that are involved in signal transduction: DKK1 (dickkopf-1), SFRP1 (secreted frizzled-related protein-1), β-catenin-like 1-α-catenin, SOC52 (STAT-induced STAT inhibitor-2), and the gene encoding the catalytic subunit C-β of PKA. Interestingly, the first three are involved in the Wnt pathway. On the basis of their roles in extracellular signaling, embryonic development and neoplastic progression (26–28), it is conceivable that these genes may promote steps in the metastatic cascade. Finally, two genes (PLOD2 and Proteoglycan H9252) are involved in adhesion or extracellular matrix interactions.

Surprisingly, none of the genes ≥3-fold more highly expressed in nonmetastatic neo6/C8161 cells mapped to chr6. Thus, metastasis suppressors on chr6 were not directly identified by microarray, likely because of the constraints inherent in microarrays (array coverage ~14–25% of the genome). None of the known metastasis-suppressor genes KISS1, BRMS1, KAI1, and MKK4, were among the array elements. Only one gene showed ≥3-fold higher expression in neo6/C8161 cells in both arrays (Table 1). TXNIP (also known as VDUP1) was expressed 27-fold higher in the Array 1, and 5-fold higher in Array 2. TXNIP is a TRX-binding protein induced by 1α,25-dihydroxyvitamin D$_3$ (calcitriol), intracellular Ca$^{2+}$, oxidative stress and heat shock (29, 30). By binding thioredoxin (31) at the redox-critical site (30, 32), TXNIP antagonizes the interactions between TRX and proliferation-associated peroxiredoxin I or apoptosis-stimulating kinase (ASK-1/MAPKKK5). Association with metastasis is inferred because ASK1 phosphorylates and activates MKK4, a pro-taste carcinoma metastasis suppressor (33). TRX controls cancer cell growth through regulation of DNA synthesis and transcription factor activity, and is over-expressed by, and stimulates the proliferation of, many human solid tumor cells (reviewed in (34, 35).

TXNIP Expression. Differential expression of TXNIP was validated by RNA blotting using a panel of metastatic and nonmetastatic variants of C8161 (Fig. 1C). TXNIP expression was virtually undetectable in the vector-only control, but was readily detectable in all four neo6/C8161 clones examined. Expression in neo6qdel/C8161 clones was detectable, but significantly reduced compared with neo6/C8161 clones. The magnitudes of differential expression were consistent between Northern blots and microarrays. Verification of protein expression was not possible because antibodies were not available.

TXNIP expression was evaluated relative to melanoma progression using a panel of cell lines representing different stages of tumor progression using RTQ. Two independent experiments, each containing samples in triplicate, were averaged. A strong inverse correlation between melanoma progression and TXNIP expression was observed (Fig. 1D).

Differential expression of multiple genes raised the question of whether coordinate gene regulation and/or a common pathway might involve TXNIP and KISS1. To determine whether TXNIP transfectants exhibited increased KISS1 expression, RTQ was performed (Fig. 1E). KISS1 expression was undetectable in C8161, vector-only transfectants and in neo6qdel/C8161 cells. However, KISS1 was detected in all neo6/C8161 clones and TXNIP transfectants. Thus, we hypothesized that TXNIP might be an upstream regulator of KISS1 expression.

Identification of CRSP3. Because TXNIP was mapped to chr1, the TXNIP and KISS1 regulatory gene(s) on chr6 remained unidentified. However, data from several other experiments assisted in identification of candidate genes on chr6q. neo6qdel hybrids refined the metastasis suppressor locus to a region between markers D6S300 and D6S457. CRSP3 was a compelling candidate because antibodies were not available.

The gene mapping nearest D6S457 was CRSP3 (DRIP130, Vitamin D Receptor Interacting Protein, 130 kDa). CRSP3 was a compelling candidate because it encodes a required element in a coactivator complex necessary for vitamin D receptor-regulated transcription (36). CRSP3 is a component of other coactivator complexes, including

<table>
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<th>Gene</th>
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<th>Locus vs. neo6$^a$</th>
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$^a$ Two genes were expressed more than 3 standard deviations above or below the mean in both arrays. Mean fold-difference in both arrays is shown.

$^b$ mRNA was isolated from a mixture of four cell clones from neo6/C8161 (neo6) and neo6qdel/C8161 (6qdel). See Materials and Methods for details.
NAT and ARC, indicating a potential role in mediating transcription by several factors (30). Given the up-regulation of a vitamin D-responsive gene (TXNIP) in metastasis-suppressed neo6/C8161 cells, we hypothesized that loss of CRSP3 might affect metastasis by down-regulation of TXNIP. Furthermore, because CRSP3 is required for SP1-mediated transcription (37) and because the KISS1 promoter contains SP1 binding elements, additional evidence pointed toward CRSP3 being a strong candidate for the metastasis suppressor locus on human chr6q.

**CRSP3 Expression Is Inversely Correlated with Melanoma Progression.** RTQ was performed to evaluate CRSP3 expression in the same panel of cell lines representing melanoma progression described above. CRSP3 expression also shows an inverse correlation with melanoma progression (Fig. 2A). Whether the higher level of expression observed for CRSP3 in early VGP is universal will require more detailed and extensive studies. Loss of expression occurs during VGP, the time where melanomas acquire metastatic potential. As with TXNIP, validation of protein expression was not possible because antibodies were unavailable.

**CRSP3 Up-regulates TXNIP and KISS1.** If CRSP3 were indeed responsible for suppressing metastasis, we reasoned that it would do so by inducing expression of TXNIP and/or KISS1 (i.e., CRSP3 transfection should increase KISS1 and VDUP1 expression). C8161.9 cells were transfected with pcDNA3-CRSP3. By RNA blot TXNIP was undetectable in C8161.9 cells, but was highly expressed in CRSP3 transfectants (CRSPmix). Mixed pools (uncloned) of CRSP3 transfectants, CRSPmix, were used to determine relative mRNA levels using total RNA extracted from cell lines derived from different stages of melanoma progression (See Fig. 1D for details). CRSP3 expression was normalized to G3PDH. Relative expression is plotted as a percent of melanocyte expression. C8161.9/KISS1, a KISS1 transfectant; neo6 = neo6/C8161 transfectant clones; neo6/del = neo6/del/C8161.9 transfectant clones; TXNIP = TXNIP transfectant clones; CRSPmix = mixed pools (uncloned) of CRSP3 transfectants; DRIP1-# = isolated colonies (presumed clones) of CRSP3 transfectants. A, RTQ of CRSP3 Transfectants of C8161.9. RTQ was used to evaluate expression levels of CRSP3 relative to an endogenous control (G3PDH). Data are combined from two independent experiments of samples and controls run in triplicate. Expression has been normalized to vector-only (pcDNA3) transfectants, shown here as an expression level of 1. Samples represent the pcDNA3 vector-only control, two independently derived mixed pools of CRSP3 transfectants (CRSPmix), and clonal CRSP3 transfectants (clones 1-5, 1-9, 1-13, 1-20, and 2-8). B, RNA Blot (total RNA, 10μg) of TXNIP expression in TXNIP transfectant clones. C8161.9 cells were transfected with TXNIP in the pcDNA3 vector. TXNIP was undetectable in the parental cells, highly expressed in the neo6/C8161 (neo6) clones, and exhibited variable expression in transfectant clones.

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8 R. S. Samant and D. R. Welch, unpublished observations.
neoa/C8161 clones, a mixed pool of CRSP3 transfectants, and TXNIP transfectants (Fig. 2B). Similarly, KISS1 was undetectable in C8161.9, vector-only transfectants, and neo6qdel/C8161 hybrids, but was expressed in a KISS1 transfectant clone of C8161.9, neo6/C8161 cells, and CRSP3 transfectants (Fig. 2, C and D).

**Biological Properties of TXNIP and CRSP3 Transfectants.** To test whether CRSP3 and TXNIP would suppress metastasis, we transfected C8161.9 cells and isolated transfectants with various levels of expression (Fig. 2, E and F). Whereas CRSP3 mRNA was detectable in the vector-only control, it was expressed at significantly higher levels in the transfectants. CRSP3-transfectant clones 1-5, 1-9, 1-13, 1-20, and 2-8 were chosen for more extensive analysis. By RNA blot, TXNIP (1.2 Kb) was virtually undetectable in C8161.9, but was highly expressed in neo6/C8161 clones 2 and 6. TXNIP-transfectant clones 3, 5, 11, and 13 were chosen for further analysis.

**CRSP3/TXNIP/KISS1: METASTASIS SUPPRESSION PATHWAY**

**Morphology of TXNIP or CRSP3 transfectants was not grossly affected in vitro (data not shown), consistent with previous observations involving neo6/C8161 and KISS1. No differences for growth in culture or saturation density were observed (data not shown).**

TXNIP and CRSP3 transfectants were injected at an orthotopic site in athymic mice and tumor growth was measured weekly. After tumors reached ~1 cm, they were removed, and the mice were allowed to recover 4 weeks before quantifying metastasis. The time to tumor removal was as follows: 32–36 days for C8161.9/pcDNA (vector control); 42–46 days for neo6/C8161.2, CRSP3-transfectant clones 1-13 and 1-20 and TXNIP transfectant clone 5; and 54–61 days for neo6/C8161.6, CRSP3 transfectant clones 1-5, 1-9, and 2-8 and TXNIP transfectant clones 3, 11, and 13 (Fig. 3, A and B).

Although CRSP3 and TXNIP transfectants initially grew slower than vector-only controls, the growth rate was comparable with neo6/C8161 cells. Once established, transfectants grew at rates comparable with parental or vector-only controls. TXNIP and CRSP3 expression was maintained during orthotopic tumor growth as assessed by RT-PCR (Fig. 3, C and D). Furthermore, CRSP3 was still functional because TXNIP expression was found in the tumors (Fig. 3D).

CRSP3 and TXNIP transfectants formed significantly fewer metastases than controls. C8161.9/pcDNA3 mice developed an average of 111 metastases/lung. (Fig. 4, top). One neo6/C8161-injected mouse developed a single metastasis. Only two mice each in the C8161.9/TXNIP and C8161.9/CRSP3 groups had detectable metastases (one metastasis per mouse). The low level of lung metastasis was impressive considering that most mice injected with CRSP3 or TXNIP cells recurred at the site of local tumor, consistent with observations that the tumors were still locally invasive. One mouse in the C8161.9/CRSP3–1 group developed 23 lung metastases and also had axillary lymph node involvement.

Morphology of TXNIP or CRSP3 transfectants was also injected i.v. to assess metastasis. Two independent experiments were performed with comparable results. Fig. 4, B and C, shows data combined from both studies (n = 16–17 mice/group) for TXNIP and CRSP3 transfectants, respectively. neo6/C8161 cells were used as positive controls. For the TXNIP experiment, vector-only transfectants formed lung metastases in all mice (mean = 172). neo6/C8161.2 cells produced an uncharacteristically and unexplained high number of lung metastases (mean = 5) with previously (or subsequently) unobserved incidence (11 of 16 mice). Nonetheless, suppression was significant. neo6/C8161.6 developed no metastases. Only 8 of 64 mice injected with
C8161.9/TXNIP cells had any macroscopic metastases (maximum = 2 per lung). CRSP3 likewise suppressed metastasis (Fig. 4C). Only 30% of mice had any metastases. Of those, only 10% (8 of 80) developed more than a single lesion.

To compensate for in vivo latency of growth, mice injected with CRSP3 clones were euthanized at 8 weeks, rather than 4 weeks, after i.v. injection. Lung metastasis by two CRSP3 and two TXNIP transfectants was significantly suppressed (data not shown). The lower incidence of metastasis cannot, therefore, be explained by slower growth alone.

**Clinical Correlations.** To address whether CRSP3 expression predicts melanoma progression toward metastasis and whether CRSP3 expression correlates with KISS1 expression in vivo, clinical samples from 20 patients undergoing melanoma surgery at Kanazawa University were examined by RTQ. KISS1 and CRSP3 were normalized to β-actin expression. Although the sample size was limited, there was a 62.9% correlation between CRSP3 expression and KISS1 expression (Fig. 5). This result was highly significant (P < 0.0023) by Spearman’s correlation test by ranks. Although these results are consistent with the hypothesis that CRSP3 is a metastasis suppressor, a correlation coefficient of 63% must not be over-interpreted. Discordant data do not account fully for presence of mutations within CRSP3, “contamination” by normal tissue, other interactors, or protein half-life.

**DISCUSSION**

Metastasis suppressors are defined as genes that block metastasis without eradicating a cell’s ability to form a tumor (38). Several laboratories have identified metastasis suppressors using MMCT (reviewed in 39–41). When we introduced chr6 into the C8161 and MelJuSo, we observed significant suppression of metastasis without tumor suppression. However, the metastasis suppressor gene identified by subtractive hybridization (i.e., KISS1) mapped to chr1, rather than chr6 (7, 8), implying that a gene(s) on chr6 regulates an effector on chr1. This hypothesis was supported by two independent studies mapping the putative regulatory gene to 6q16.3-q23 (11, 12).

To complement the subtractive hybridization study, microarray analyses were performed. The experimental design took advantage of genetically related cell lines (to minimize the likelihood of differences attributable to unrelated cell lines) and the availability of multiple clones (to minimize spurious results attributable to clonal heterogeneity). Array 1 compared two clones, C8161.9 and neo6/C8161.2, which were metastatic and nonmetastatic, respectively. Array 2 compared metastatic and nonmetastatic variants as well; however, equal proportions of mRNA from four clones were mixed to minimize the impact of clonal heterogeneity on interpretation. In both arrays, TXNIP had the greatest differential expression (27- and 5-fold, respectively) between the populations.

TXNIP expression inversely correlates with melanoma progression and exerted an antimetastatic, but not tumor suppressing, effect upon transfection into melanoma cells. However, as with KISS1, TXNIP maps to chromosome 1q, implying that regulatory elements on chr6 remained unidentified. Candidate genes on chr6 were evaluated taking into consideration updated genome maps, refinement of the metastasis suppressor locus in revertants, information about transcription regulatory elements in the KISS1 promoter and an association of TXNIP with vitamin D. CRSP3 met all criteria and was evaluated for its metastasis-suppressor capabilities. CRSP3 transfectants were suppressed for metastasis but remain tumorigenic. Furthermore, CRSP3 transfection resulted in up-regulation of both TXNIP and KISS1. Taken together, these data implied that CRSP3 was the underlying defect resulting in acquisition of metastatic potential. Loss or decreased expression of CRSP3 would result in diminished expression of TXNIP and KISS1, which would, in turn, allow cells to metastasize. A model putting all three components is depicted in Fig. 6. Because CRSP3 and TXNIP are known components of transcriptional regulation, involvement of other genes remains a possibility. Indeed, given the complex nature of metastasis regulation of other genes is quite likely.

**TXNIP: Possible Mechanisms of Suppression.** A role for TXNIP in controlling metastasis is consistent with previously published properties. TXNIP was first identified as a gene up-regulated in cells after exposure to vitamin D3/calcitriol (29). A relationship between vitamin D3 and metastasis is possible, but is not addressed in the current study. Whether exogenous vitamin D3 modulates C8161 metastasis by inducing transcription of TXNIP remains to be determined. However, in a single experiment using a single dose of calcitriol, we did not observe an effect on in vitro morphology or proliferation.9

9 Note: there may be alterations in primary tumor growth kinetics, but masses still develop and continue to express the metastasis suppressor gene.
TXNIP could also exert effects independent of vitamin D$_3$ (reviewed in Ref. 42). Accumulating evidence suggests that redox regulation is important in gene regulation. TRX can control cell growth via regulation of DNA synthesis and transcription factor activity. Therefore, TXNIP binding to TRX at its active site would antagonize TRX interactions with other proteins which, in turn, could affect multiple downstream effectors. In the present report, we show that enforced expression of TXNIP increases KISS1 expression. It is reasonable to speculate that TXNIP, via TRX binding, is an upstream regulator of KISS1. However, because TXNIP was expressed at low levels in metastatic cells, whereas KISS1 expression was undetectable, such an explanation is not straightforward. Perhaps, a threshold of TXNIP must be exceeded to inactivate all of the TRX in the cell.

**CRSP3: Possible Mechanisms of Metastasis Suppression.**

In C8161, loss or mutation of either KISS1 or TXNIP is unlikely. Rather, dysregulation of their expression by genes on chr6 is implied by the data. Whether a single gene on chr6 is responsible for coordinate regulation of TXNIP and KISS1, or whether KISS1 regulation is (ind)irect, remains to be determined. Our working hypothesis is that CRSP3 is the beginning of a common cascade regulating both TXNIP and KISS1. CRSP3 is part of vitamin D receptor-related coactivator complexes, indicating a potential role in mediating transcription by several factors (reviewed in Ref. 43). Conceivably, loss or mutation of CRSP3 could explain the breadth of differential expression observed between metastatic and nonmetastatic cells.

In summary, we demonstrated inverse correlations between metastatic potential of human melanomas and expression of CRSP3, TXNIP, and KISS1. Although further validation of CRSP3 and TXNIP in melanoma samples will be required, the data imply that these molecules are involved in the regulation of metastasis. The data also implicate a complex and intricate network whereby gene expression can be regulated by these genes. Further genomic and proteomic analysis of these cells may permit the elucidation of specific transcriptional and regulatory pathways that are altered in metastasis. Importantly, we have provided preliminary evidence that the three functional metastasis suppressors (CRSP3, TXNIP, and KISS1) are serially regulated. This data suggests a compelling, albeit incomplete, model for a metastasis suppression pathway (Fig. 6), among the first such pathways elucidated. According to this model, deletions on chr6, which are frequent in melanoma, result in loss of CRSP3/TXNIP/KISS1: METASTASIS SUPPRESSION PATHWAY

**ACKNOWLEDGMENTS**

We wish to acknowledge Dr. Meenhard Herlyn (Wistar Institute, Philadelphia) for providing the panel of melanoma cell lines. We are grateful to all members of the Welch lab for helpful advice and discussions.

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Melanoma Metastasis Suppression by Chromosome 6: Evidence for a Pathway Regulated by CRSP3 and TXNIP

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