ARTICLES

KiSS-1, a Novel Human Malignant Melanoma Metastasis-Suppressor Gene

*Jeong-Hyung Lee, Mary E. Miele, Deana J. Hicks, Karen K. Phillips, Jeffery M. Trent, Bernard E. Weissman, Danny R. Welch**

Background: **Microcell-mediated transfer of chromosome 6 into human C8161 and MelJuSo melanoma cells suppresses their ability to metastasize by at least 95% without affecting their tumorigenicity. This observation demonstrates that the ability to metastasize is a phenotype distinct from tumor formation and suggests that tumorigenic cells acquire metastatic capability only after accumulating additional genetic defects. These results also imply that mutations of genes on chromosome 6 are among those late genetic changes responsible for metastatic potential. They further suggest that a melanoma metastasis-suppressor gene(s) is encoded on chromosome 6 or is regulated by genes on chromosome 6.** *Purpose:* **Our objective was to identify the gene(s) responsible for the suppression of metastasis in chromosome 6/melanoma cell hybrids.** *Methods:* **A modified subtractive hybridization technique was used to compare the expression of messenger RNAs (mRNAs), via an analysis of complementary DNAs (cDNAs), in metastatic cells (C8161 or MelJuSo) and nonmetastatic hybrid clones (neo6/C8161 or neo6/MelJuSo).** *Results:* **A novel cDNA, designated KiSS-1, was isolated from malignant melanoma cells that had been suppressed for metastatic potential by the introduction of human chromosome 6. Northern blot analyses comparing mRNAs from a panel of human melanoma cells revealed that KiSS-1 mRNA expression occurred only in nonmetastatic melanoma cells. Expression of this mRNA in normal heart, brain, liver, lung, and skeletal muscle was undetectable by northern blot analysis. Weak expression was found in the kidney and pancreas, but the highest expression was observed in the placenta. The KiSS-1 cDNA encodes a predominantly hydrophilic, 164 amino acid protein with a polyproline-rich domain indicative of an SH3 ligand (binds to the homology 3 domain of the oncoprotein Src) and a putative protein kinase C-α phosphorylation site. Transfection of a full-length KiSS-1 cDNA into C8161 melanoma cells suppressed metastasis in an expression-dependent manner.** *Conclusions:* **These data strongly suggest that KiSS-1 expression may suppress the metastatic potential of malignant melanoma cells.** *Implications:* **KiSS-1 may be a useful marker for distinguishing metastatic melanomas from nonmetastatic melanomas. [J Natl Cancer Inst 1996;88: 1731-7]**

The spread of malignant tumor cells from a primary tumor to form metastases at distant sites is the most life-threatening complication of cancer and is responsible for the majority of deaths in affected individuals. Understanding the regulation of metastasis at the molecular level is required to devise new modalities of cancer therapy, particularly those that improve cure rates when treating metastatic cancer.

Metastasis is a multistep process involving complex interactions between tumor cells and host cells. To metastasize, tumor cells must invade from the primary tumor, dissociate from the tumor mass, and be transported to nearby or distant secondary sites. Single cells, homotypic clusters of cells, or heterotypic emboli then arrest at a distant site with the use of both organ-specific and nonspecific mechanisms, invade into the surrounding tissue, and respond to growth signals at the secondary site (*1-4*). A tumor cell must successfully accomplish each step in the pathway or metastases will not develop. Both positive and negative regulators exist for each step in the metastatic cascade (*5-11*), implicating the involvement of dozens of different genes. This provides the challenging task of identifying critical genes controlling the process for use as targets for therapeutic intervention.

To identify metastasis-controlling genes, we have used a functional assay of metastasis suppression of human tumor cells in athymic nude mice. We previously showed that the introduction of human chromosome 6 into human metastatic melanoma

**Affiliations of authors:* J.-H. Lee, M. E. Miele, D. J. Hicks, D. R. Welch, Department of Experimental Pathology, The Jake Gittlen Cancer Research Institute, College of Medicine, The Pennsylvania State University, Hershey; K. K. Phillips, B.E. Weissman, Department of Pathology, Lineberger Cancer Center, University of North Carolina, Chapel Hill; J. M. Trent, Division of Intramural Research, National Center for Human Genome Research, Bethesda, MD.

Correspondence to: Danny R. Welch, Ph.D., Department of Experimental Pathology (C7810), College of Medicine, The Pennsylvania State University, 500 University Dr., Hershey, PA 17033-0850.

See "Notes" section following "References."

cell lines C8161 or MelJuSo by microcell-mediated chromosome transfer resulted in at least 95% suppression of metastasis without affecting tumorigenicity (*12,13*) or local invasiveness (*14*). Introduction of chromosome 1 also partially suppressed MelJuSo metastasis (*13*). The development of this panel of cell lines provided us with the necessary reagents to isolate genes specifically involved in the control of metastasis.

To identify the gene(s) responsible for metastasis suppression in malignant melanoma, we used a modified subtractive hybridization method (*15-19*). Candidate genes were identified by searching for a minimum 10-fold increase in messenger RNA (mRNA) expression in nonmetastatic chromosome 6-C8161 hybrid cells (neo6/C8161.1) compared with highly metastatic parental C8161 cells grown under similar conditions.

Materials and Methods

Cell Lines and Culture Conditions

C8161 is an amelanotic human melanoma cell line that metastasizes widely following intradermal, subcutaneous, or intravenous injection into athymic nude or severe combined immunodeficient mice (*12,20*). Subclones C8161cl.9 and C8161cl.8 were isolated from C8161 by limiting dilution and were chosen for these studies because they have the highest and lowest metastatic potentials, respectively (*12*). neo6/C8161.1, neo6/C8161.2, and neo6/C8161.3 (*12*) were derived from microcell-mediated chromosome transfer of a single copy of a neomycin-resistance gene-tagged human chromosome 6 with the use of the MCH262A1.D6 donor cell line (*12,21*). Four additional, independently derived neo6/C8161 hybrid cell clones (neo6/C8161.4, neo6/C8161.5b, neo6/C861.6, and neo6/C8161.8) were subsequently developed. None of the neo6/C8161 hybrid cell clones were metastatic in athymic nude mice. neo6(del)(q21-q23) hybrid cell clones were prepared using a related chromosome 6 microcell donor variant A9/6q-cl.1.2 (*22*) and are metastatic in nude mice (Miele ME, Goldberg SF, Hyatt DL, Barbanti-Brodano G, Welch DR: manuscript in preparation).

Cell line nomenclature has been chosen to identify the origin and nature of each cell line as unambiguously as possible. Single-cell clones are preceded by a "." (e.g., C8161cl.9 is a single-cell clone), and pooled, uncloned populations are identified by a "-" (e.g., C8161-KiSS-1-P1 is an uncloned population No. 1 of C8161 cells transfected with KiSS-1). Microcell hybrids are identified by the tagged chromosome followed by a "/" [e.g., neo6/C8161.3 is single-cell clone 3 derived from a fusion with a neomycin-resistance gene-tagged human chromosome 6; neo6(del)(q21-q23)/C8161cl.8 is single-cell clone 8 derived from a microcell fusion with a neotagged chromosome 6 containing deletion of the q21-q23 bands].

All cells were grown in Dulbecco's modified minimal Eagle's medium and Ham's F-12 medium (DME-F12) (Irvine Scientific Co., Santa Ana, CA) supplemented with 10% fetal bovine serum (cDME-F12). Neo-resistant cells, neo6/C8161, and neo6(del)(q21-q23)/C8161 hybrids were maintained in cDME-F12 containing 500 μg/mL geneticin (G-418; Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). Nearly 70%-90% confluent cultures were passaged in Corning (Oneonta, NY) tissue culture dishes following detachment with a solution of calcium- and magnesium-free Dulbecco's phosphate-buffered saline containing 2 m*M* EDTA at split ratios of 1:10 to 1:20. All cultures were routinely tested and found to be negative for *Mycoplasma* spp. infection using a polymerase chain reaction (PCR)-based test kit (PanVera, Madison, WI).

Subtractive Hybridization

Subtractive hybridization was done as described (*18,19,23*) with minor modifications. Briefly, mRNAs were isolated from 80% to 90% confluent cells using the FastTrack mRNA isolation kit (Invitrogen Corp., San Diego, CA). A complementary DNA (cDNA) library was constructed from neo6/C8161.1 cells using a λZAP-cDNA Gigapack II Gold cloning kit (Stratagene, La Jolla, CA). Subtractive hybridization was carried out between the first-strand cDNA from nonmetastatic neo6/C8161.1 cells and mRNA from metastatic parental C8161 cells. cDNA was synthesized from 1 μg polyadenylic acid [poly(A)$^+$]-enriched RNA from neo6/C8161.1 using oligo(dT) primers. Biotinylation of mRNA from parental C8161 cells was performed using the Photobiotin-labeling system (Life Technologies, Inc.) according to manufacturer's instructions. To subtract, biotinylated mRNA (20 μg) was mixed with cDNA (1 μg) from neo6/C8161.1 and ethanol precipitated. The pellet was resuspended in diethylpyrocarbonate-treated water (20.5 μL), then added to 2× hybridization buffer (80% formamide, 100 m*M* hydroxyethylpiperazine ethane sulfonic acid [pH 7.5], and 2% sodium dodecyl sulfate [SDS], 22.5 μL). This mixture was boiled for 2 minutes, chilled on ice for 5 minutes, and followed by the addition of NaCl (5 *M*, 2 μL). Hybridization was performed at 42 °C for 48 hours. Streptavidin (25 μg; Sigma Chemical Co., St. Louis, MO) was added to the hybridization mixture, and the mixture was incubated at room temperature for 5 minutes before extraction with phenol–chloroform–isoamyl alcohol (25:24:1). The organic phase was back-extracted twice with 50 μL streptavidin-binding buffer (100 m*M* Tris–HCl [pH 8.0]; 1 m*M* EDTA, and 500 m*M* NaCl). The aqueous phases were then pooled. Unhybridized cDNA target was ethanol precipitated. This subtracted cDNA was used as a probe for the neo6/C8161.1 cDNA library screening following random primer labeling (Amersham Life Sciences, Inc., Arlington Heights, IL). Positive clones were isolated and used for evaluation of expression in northern blot analysis.

Screening Candidate cDNAs and Isolation of Full-Length KiSS-1

Candidate cDNAs were initially evaluated for differential expression by northern blot analysis using poly(A)$^+$-enriched mRNAs from C8161 and neo6/C8161.1 cells. If the expression was greater in neo6/C8161.1 cells by at least 10-fold, the same cDNA was used to probe a more extensive blot containing RNAs from a panel of cell lines with differing metastatic potentials. If the pattern of expression correlated with metastatic potential, full-length cDNAs were isolated and further characterized.

Full-length KiSS-1 was obtained from a λZAP-cDNA library (7.5×10^6 plaques) prepared from neo6/C8161.1 cells. After third-round screening, seven positive clones were isolated and identified as a same gene by restriction mapping and sequence analysis. Library screening was done using a 0.5-kilobase (kb) partial KiSS-1 probe obtained from the subtractive hybridization. The longest cDNA insert was sequenced on both strands by the dideoxy chain termination method with Sequenase version 2.0 (Amersham Life Sciences, Inc.).

Northern Blot Analysis

For northern blot analysis, poly(A)$^+$-enriched mRNA was isolated from 80% to 90% confluent cells with the use of a FastTrack mRNA isolation kit (Invitrogen). Poly(A)$^+$ mRNA (2.5 μg) was loaded and electrophoresed on 1% agarose gel containing 2.2 *M* formaldehyde at 78 V for 3.5 hours. RNA was transferred onto a nylon membrane using the Turboblotter system (Schleicher & Schuell, Keene, NH) and fixed by UV cross-linking (Stratagene). Full-length KiSS-1 cDNA probe was radiolabeled using random priming (Amersham Life Sciences, Inc.). Prehybridization was done in 48% formamide, 4.8× sodium chloride/sodium citrate (SSC) (1× SSC = 0.15 *M* NaCl and 0.015 *M* sodium citrate), 20 m*M* Tris (pH 7.6), 1× Denhardt's solution (0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone), 10% dextran sulfate, and 0.1% SDS. Hybridization was carried out in the same buffer plus herring sperm DNA (100 μg/mL) for 24 hours at 42 °C. The membrane was rinsed twice with 2× SSC containing 0.1% SDS for 15 minutes at 42 °C, followed by washing twice with 0.1× SSC containing 0.1% SDS for 15 minutes at 42 °C. The rinsed membrane was exposed to x-ray film (Du Pont-NEN, Boston, MA) for desired intensity.

Expression of KiSS-1 in normal human tissues was evaluated by use of a multiple-tissue RNA blot [2 μg of poly(A) RNA per lane] purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). The blot was hybridized by use of a full-length KiSS-1 probe as above. Expression of KiSS-1 in normal human melanocytes (CLONTECH Laboratories, Inc.) was determined by reverse transcription–polymerase chain reaction (RT-PCR). Detection of KiSS-1 message (1.0 kb) in placenta was possible following overnight exposure. KiSS-1 mRNA was detectable in the pancreas (0.8 kb) and kidney (0.9 kb) only after a 3-7-day exposure at –70 °C with two intensifying screens.

Transfections and Metastasis Assays

A full length of KiSS-1 cDNA was cloned into the pcDNA3 expression vector (Invitrogen Corp.) in which transcription is driven by human cytomegalovirus promoter. The resultant plasmid pcDNA3-KiSS-1 was transfected

into C8161 cells using Lipofectin (Life Technologies, Inc.). Individual transfectants were isolated and cloned following growth in cDME-F12 containing G-418 (500 μg/mL). Poly(A)$^+$ mRNA was isolated from each transfectant by use of the Micro-FastTrack mRNA isolation kit (Invitrogen Corp.), and northern blot analysis was done as described above. C8161-neomix was isolated as a control, uncloned population of C8161 cells transfected with the pcDNA3 vector only.

Transfectants were evaluated for growth in vitro. Cells (2×10^4) were inoculated into 24-well tissue culture plates (Corning) in cDME-F12 medium. After 24, 48, 72, and 96 hours, cells were trypsinized and counted using a hemacytometer.

For spontaneous metastasis assays, cells (1×10^6) were injected intradermally into the dorsolateral flank of athymic nude mice (four to 12 mice per group). Female mice (3-4 weeks old) (Harlan Sprague-Dawley, Inc., Madison, WI) were used for these studies. Animals were maintained under the guidelines of the National Institutes of Health and The Pennsylvania State University College of Medicine. All protocols were approved by the Institutional Animal Care and Use Committee. Food and water were provided ad libitum. Tumor size was measured weekly by taking orthogonal measurements and were expressed as mean tumor diameter. Mean tumor diameter was calculated as described (*12,13*) by use of the following equation:

$$\sqrt{(\text{diameter}_x) \times (\text{diameter}_y)}\ .$$

After the mean tumor diameter reached 1.5-2.0 cm, mice were killed, necropsied, and visible metastases were counted (*20,24*).

In the C8161 human melanoma model, the rank orders of spontaneous and experimental metastasis assays for multiple cell variants are equivalent [i.e., poorly metastatic clones in the spontaneous metastasis assay are poorly metastatic in experimental metastasis assays. Likewise, individual clones are highly metastatic in both assays (*20*)]. For experimental metastasis assays, cells (2×10^5 to 3×10^5) suspended in ice-cold Hanks' balanced salt solution (0.2 mL) were injected into the lateral tail vein of 3- to 4-week-old female athymic nude mice. After 4 weeks, the mice were killed by cervical dislocation, and metastatic lesions were scored as described (*20,24*). Quantification of metastasis formation was identical, regardless of the metastasis assay used. Briefly, the lungs from each mouse were removed, fixed in a mixture of formalin and Bouin's fixative (5:1 vol/vol), and examined under a dissecting stereomicroscope (*24*). Unless otherwise noted, all other tissues were examined and found to be free of metastases.

Statistical Analysis

The number of lung metastases was compared in KiSS-1 transfectants and parental C8161 cells. For experimental metastasis assays, one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference post-test was used. For spontaneous metastasis assays, a Kruskal–Wallis ANOVA of ranks procedure was used. Calculations were performed using SigmaStat statistical analysis software (Jandel Scientific, San Rafael, CA). Statistical significance was defined as $P \leq .05$ using two-sided tests.

Results and Discussion

Several candidate metastasis-suppressor cDNAs were identified by subtractive hybridization and differential display comparing C8161 and neo6/C8161.1 cells (*25*). One clone, designated KiSS-1, was expressed only in nonmetastatic neo6/C8161.1 cells. The cDNA designation combines interim laboratory nomenclature for putative Suppressor Sequences with acknowledgment of the gene's discovery in Hershey. KiSS-1 cDNA sequence was submitted to GenBank as a novel gene with an accession number of U43527.

Lack of Expression of KiSS-1 in Metastatic Melanoma Cells

The expression pattern of KiSS-1 was confirmed by northern blot analysis using an extensive panel of cell lines derived from the C8161 melanoma with widely ranging metastatic potentials. KiSS-1 mRNA expression could not be detected in any metastatic melanoma cell line (Fig. 1, A). Expression was not detectable, even in overexposed blots or by RT-PCR, in parental C8161 or two subclones, C8161cl.9 and C8161cl.8, representing the highest and lowest metastatic potential among clones, respectively (*12*). KiSS-1 mRNA expression was also undetectable in C8161cl.9 microcell hybrids containing human chromosome 6 bearing a complex deletion within the region 6q21→q23 that remained metastatic in nude mice (Miele ME, Goldberg SF, Hyatt DL, Barbanti-Brodano G, Welch DR: manuscript in preparation).

Seven independently prepared hybrids that contained an intact copy of human chromosome 6 in C8161 expressed high levels of a 1.0-kb transcript of KiSS-1 mRNA. Equal loading of lanes was confirmed by measuring glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. All neo6/C8161 hybrid clones failed to metastasize following intravenous (experimental metastasis assay), subcutaneous, or intradermal (spontaneous metastasis assay) injection into 3- to 4-week-old female athymic nude mice. Therefore, KiSS-1 mRNA expression appeared to be a qualitative marker—i.e., exclusive to nonmetastatic C8161 cell populations.

Expression of KiSS-1 mRNA was evaluated by northern blotting of other, unrelated human metastatic melanoma cell lines (Fig. 1, B). To test for the possibility that truncated forms of KiSS-1 may have existed, a full-length cDNA probe was used. We could not detect the 1.0-kb KiSS-1 transcript or bands of other sizes in any cells capable of metastasizing in athymic nude mice.

The expression of KiSS-1 mRNA in the human heart, brain, placenta, liver, lung, skeletal muscle, kidney, or pancreas was also examined by northern blot analyses (Fig. 1, C). Abundant KiSS-1 transcript (1.0 kb) was found in the placenta, with very weak expression in the kidney (detectable only after exposure for ≥3 days using two intensifying screens). Upon longer exposure, different transcript sizes of KiSS-1 were detected in the pancreas (0.8 kb) and the kidney (0.9 kb), suggesting that alternative splicing may take place in different tissues. Expression of KiSS-1 could also be detected in normal human melanocytes by RT-PCR (data not shown). Expression in melanocytes suggests that KiSS-1 functions in normal precursor cells and may therefore be useful in staging melanoma progression. The precise role of KiSS-1 in melanocyte function has not yet been determined.

KiSS-1 cDNA has a single open reading frame that encodes a protein of 164 amino acids with a predicted molecular mass of 18 kd (Fig. 2). The initiation codon and surrounding nucleotides fit the Kozak consensus, and there is a consensus polyadenylation site downstream of the termination codon. In vitro transcription and translation of a full-length KiSS-1 cDNA resulted in a single band at 18 kd (data not shown). A search of the GenBank and European Molecular Biology Laboratory databases revealed no significant homology with known genes. A similar lack of homology was seen for protein sequences in the SwissBank database.

Metastasis Suppressed in C8161 KiSS-1 Transfectants

To demonstrate a functional basis for the correlation of KiSS-1 expression and the lack of metastatic potential, full-length

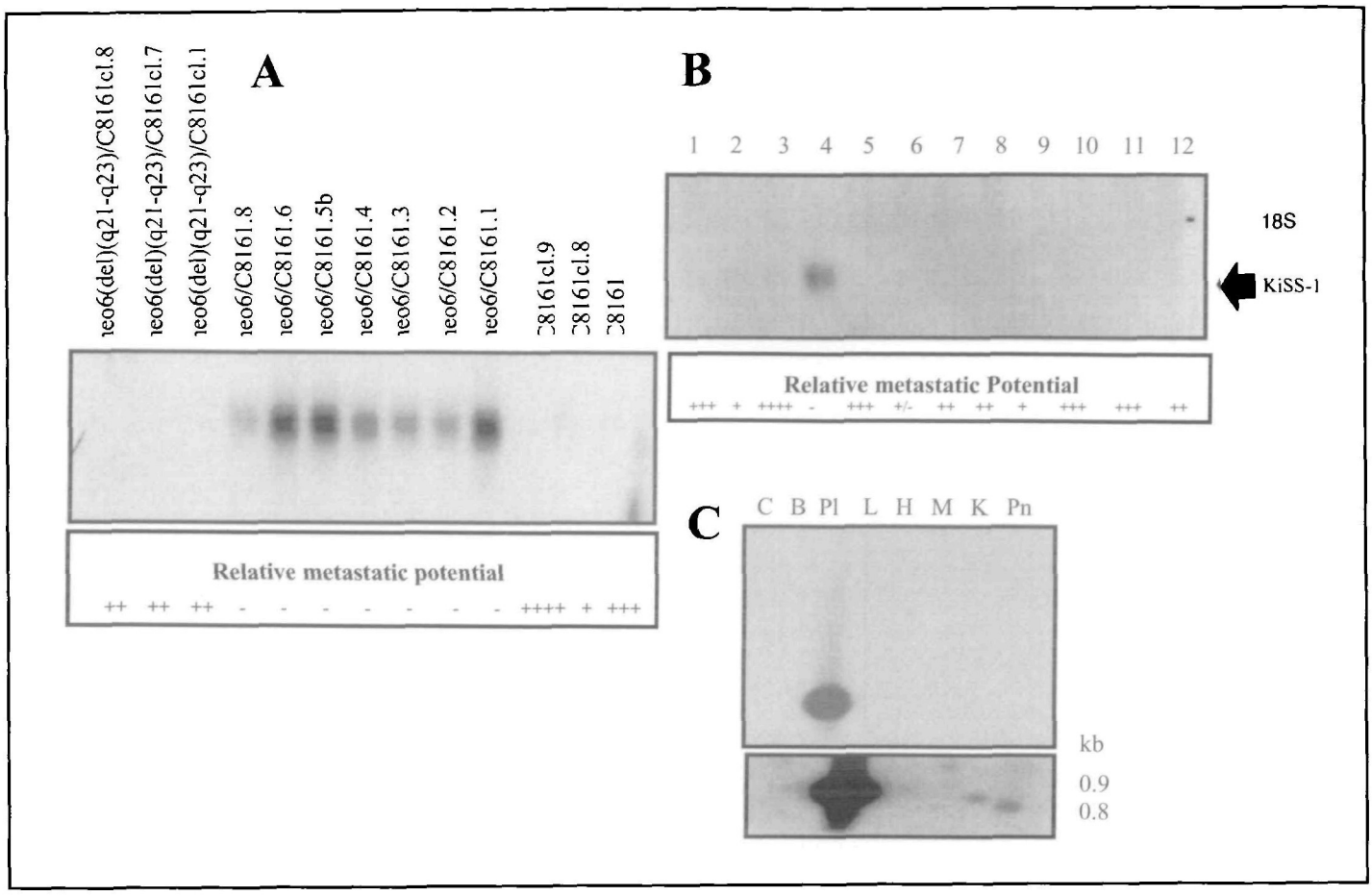

Fig. 1. Northern blot analyses of KiSS-1. **A)** Expression of KiSS-1 messenger RNA (mRNA) in cell clones derived from C8161, neo6/C8161, and neo6(del)(q21-q23)/C8161 cell clones. neo6/C8161 hybrids were prepared using MCH262A1.D6 as a microcell chromosome donor (*21*), and neo6(del)(q21-q23) hybrids were prepared using a related variant, A9/6q$^-$Cl.1.2 (*22*), as a microcell chromosome donor (Miele ME, Goldberg SF, Hyatt DL, Barbanti-Brodano G, Welch DR: manuscript in preparation). Relative metastatic potentials are based on both experimental (intravenous inoculation into lateral tail vein) and spontaneous (intradermal inoculation into dorsolateral flank) metastasis assays. Poly(A)$^+$ mRNA (2.5 μg) was electrophoresed on denatured agarose gel, transferred onto a nylon membrane and fixed and probed with full-length KiSS-1 cDNA. KiSS-1 was undetectable in any cell line possessing metastatic potential, whether low or high. **B)** Expression of KiSS-1 mRNA in human metastatic melanoma cell lines. Lane 1 = C8161; 2 = C8161cl.8; 3 = C8161cl.9; 4 = non-metastatic hybrid cell clone neo6/C8161.1; 5 = MelJuSo, 6 = A375P; 7 = A375M; 8 = MeWo; 9 = MeWo-3S5; 10 = MeWo-70W; 11 = M24met; and 12 = OM431. Poly(A)$^+$ RNA was isolated from each cell culture, and northern blot analysis was performed as above. All cell lines having metastatic potential did not express KiSS-1. **C)** Expression of KiSS-1 mRNA in normal human tissues. The multiple-tissue RNA blot [2 μg of poly(A)$^+$ RNA per lane] was purchased from CLONTECH Laboratories, Inc., Palo Alto, CA. The blot was hybridized by use of a full-length KiSS-1 probe as above. Detection of KiSS-1 message (1.0 kilobase [kb]) in the placenta was possible following overnight exposure. KiSS-1 mRNA was detectable in the pancreas (0.8 kb) and in the kidney (0.9 kb) only after a 3-7-day exposure at −70 °C with two intensifying screens. Besides differential organ expression, these results suggest that alternative splice variants of KiSS-1 may exist in different cells.

cDNA of KiSS-1 was subcloned into the pcDNA3 constitutive expression vector and transfected into C8161. The vector alone was transfected as a negative control. Several clones were randomly selected and analyzed for expression of KiSS-1 by RNA blotting (Fig. 3). The KiSS-1 transcript in transfectants is larger (1.3 kb) because of additional sequences in the expression vector. The in vitro growth rates of the selected clones were not significantly ($P>.05$) different compared with parental metastatic C8161 cell clones (Fig. 4). Clones expressing differing levels of KiSS-1 transcript were tested for metastatic ability in athymic nude mice by use of the experimental and spontaneous metastasis assays.

In the spontaneous metastasis assay, which measures the ability of cells injected intradermally or subcutaneously into the dorsolateral flank to metastasize to distant sites (*20,24*), KiSS-1 transfectant clones 2, 3, and 9 were less able to colonize lung or regional lymph nodes than concomitantly injected C8161 cells (Table 1). Differences in metastatic potential could not be explained by slower in vivo primary tumor growth rate, since several more metastatic subclones of C8161 grow more slowly than KiSS-1 transfectants (*12*). Parental C8161 cells yielded an average of 50 lung metastases per mouse, and every mouse had regional lymph node metastases. In contrast, transfectant clone KiSS-1cl.2, which highly expressed KiSS-1 RNA, produced an average of only one metastasis per mouse. None of the mice had more than three metastases, and only two had lymph node metastases. KiSS-1 cl.3 produced a mean of three metastases per mouse (all mice yielded less than six metastases) and slightly higher incidences of lymph node metastases. Similar results were obtained following direct inoculation into the lateral tail veins of athymic nude mice (experimental metastasis assay, Table 2).

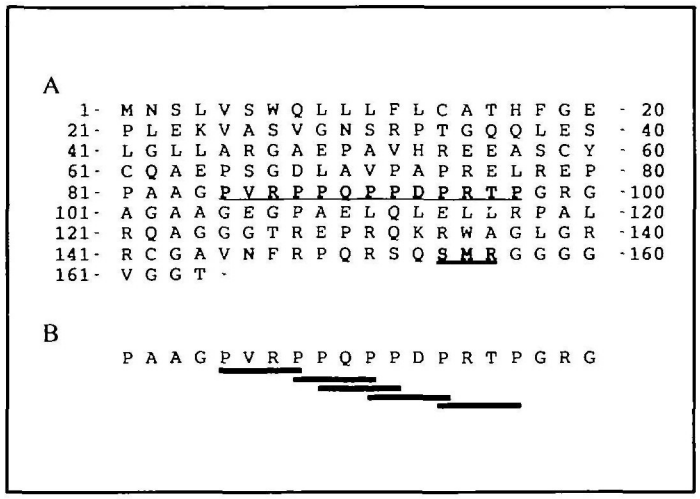

Fig. 2. A) Predicted protein sequence of KiSS-1. The nucleotide sequence has been submitted to GenBank (accession number U43527). Abbreviations for amino acid residues are: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; and Y = Tyr. Full-length KiSS-1 was obtained from by the complementary DNA (cDNA) library screening using a neo6/C8161.1 λZAP-cDNA library (7.5×10^6 plaques). After third-round screening, seven positive clones were isolated and identified as the same gene by restriction mapping and sequence analysis The longest cDNA insert was sequenced on both strands by the dideoxy chain termination method with Sequenase version 2.0 (Amersham Life Sciences, Inc., Arlington Heights, IL). KiSS-1 has a polyproline-rich domain (underlined) and a putative protein kinase C-α phosphorylation site (double underlined). **B)** Amino acids 81-100 showing PXXP consensus minimal SH3 binding motif (underlined).

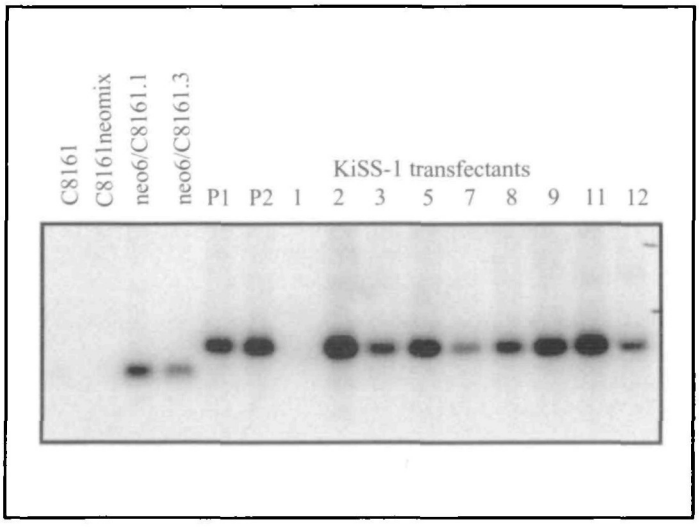

Fig. 3. Expression of KiSS-1 messenger RNA (mRNA) in KiSS-1 transfectants. A full length of KiSS-1 complementary DNA was cloned into pcDNA3 (Invitrogen Corp., San Diego, CA) in which transcription is driven by human cytomegalovirus promoter. The resultant plasmid pcDNA3-KiSS-1 was transfected into C8161 cells using Lipofectin (Life Technologies, Inc., [GIBCO-BRL] Gaithersburg, MD). Individual transfectants were isolated and cloned following growth in the neomycin analog G-418 (600 μg/mL). Polyadenylic acid [Poly(A)] mRNA was isolated from each transfectant using Micro-Fast-Track mRNA isolation kit (Invitrogen Corp.), and northern blot analysis was done as described above. C8161neomix is an uncloned population of C8161 cells transfected with pcDNA3 vector only. Two different-sized transcripts are observed. KiSS-1 (1.0 kilobase [kb]) is smaller than exogenous KiSS-1 (1.3 kb) because of vector sequences in the latter. Lanes labeled P1 and P2 are uncloned populations of C8161 cells transfected with pcDNA3-KiSS-1 construct.

Predicted KiSS-1 Protein Possibly an SH3 Ligand or Phosphorylated by PKC-α

The predicted protein sequence encoded by KiSS-1 cDNA was analyzed, and the following homologies were found: a putative PKC-α phosphorylation site (single-letter amino acid code, SMR, corresponding to amino acid positions 154-156), and a proline-rich region with five overlapping minimal SH3 binding domains (binding to the homology 3 domain of the oncoprotein Src) (*26,27*) (PXXP motif, Fig. 2, B, corresponding to amino acids 85-97, where P is proline and X can be any amino acid). This suggests that KiSS-1 may be a ligand for a protein possessing an SH3 domain and/or may be a substrate for protein kinase C-α phosphorylation. Additionally, there are four cysteines, leaving the possibility for intramolecular or intermolecular disulfide linkages. The N-terminal 70 amino acids contain seven serines and a tyrosine in position 60 as possible phosphorylation sites. There are also five threonines in the KiSS-1 molecule. There are no apparent transmembrane, glycosylation, nuclear translocation, or domains indicative of KiSS-1 being a kinase. Although predominantly hydrophilic, it is unlikely that KiSS-1 is a secreted protein on the basis of the rules of von Heijne (*28*).

The PXXP motif is a constant feature of all SH3 ligands. The presence of five PXXP sequences in a 13 amino acid stretch is further evidence that KiSS-1 is an SH3 ligand (*26,27,29,30*). Rules governing specificity of SH3 binding are currently being elucidated (*26,27,31-35*), but for the most part, they remain unknown. However, the putative KiSS-1 SH3 binding domain shares many features of the RLP-type (class I) ligand orientation.

SH3 domains are 50-70 amino acid modules present in a variety of intracellular proteins that mediate protein–protein in-

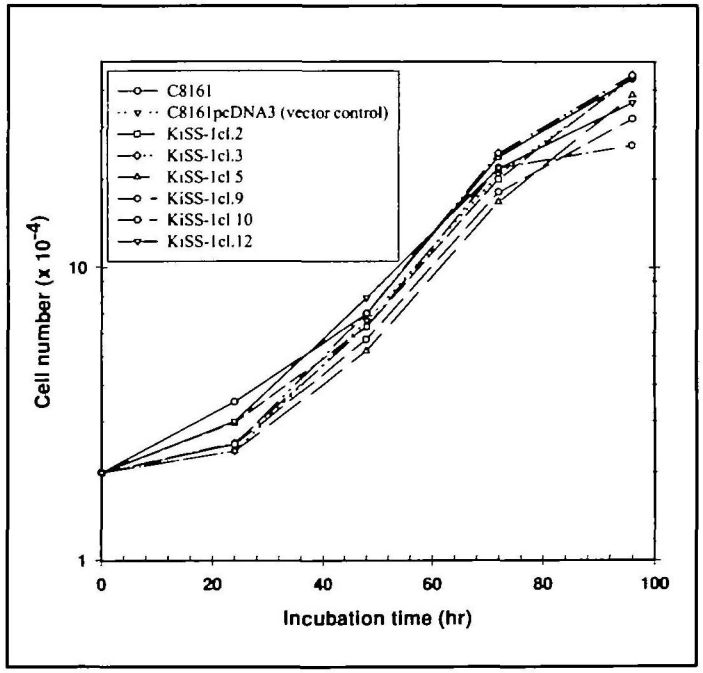

Fig. 4. Growth of KiSS-1 transfectants in vitro. Cells (2×10^4) were inoculated into a 24-well tissue culture plate in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 10% fetal bovine serum. After 24, 48, 72, and 96 hours, cells were trypsinized and counted with a hemacytometer. Data are shown as mean ± standard error.

Table 1. Suppression of spontaneous metastasis of C8161 human melanoma cells by KiSS-1*

Cell line	Relative KiSS-1 expression	Metastases: Lung: No. of mice with metastases/total No. of mice	Metastases: Lung: Mean ± SEM	Metastases: Lung: Median (range)	Metastases: Extrapulmonary: No. of mice with metastases/total No. of mice
C8161	–	16/16	50 ± 25	9.5 (2 to >200)	8/8
KiSS-1cl.2	+++	5/7	1.1 ± 0.4	1 (0 to 3)	2/8
KiSS-1cl.3	+	6/7	2.7 ± 0.7	3 (0 to 6)	4/8
KiSS-1cl.9	++	0/7	0	0	2/7

*Cells (1×10^6) were injected intradermally into the dorsolateral flank of 3- to 4-week-old female athymic nude mice (seven to eight mice per group, Harlan Sprague-Dawley). When the mean tumor diameter (square root of the product of orthogonal measurements) reached 1.5-2.0 cm, mice were killed. All organs were examined for the presence of metastases, and many were confirmed by microscopic examination of hematoxylin–eosin-stained paraffin-embedded sections (4-6 μm). Macroscopic lung metastases were quantified after being stained with a mixture of neutral-buffered formalin and Bouin's fixative (5:1) and were counted with the aid of a dissecting microscope as previously described (*20,24*). SEM = standard error of the mean.

teractions that are important for intracellular signaling and cytoskeletal organization (*36-43*). Many of these interactions involving SH3 domains have been directly or indirectly associated with various steps in the metastatic cascade. Therefore, KiSS-1 could suppress metastasis by regulating key signaling pathways important to one or more of these steps. KiSS-1 transfectants did not display significantly ($P>.05$) influenced adhesion to the extracellular matrix components type-I collagen or fibronectin nor was adhesion to a complex basement membrane-like mixture, Matrigel, affected (data not shown).

KiSS-1 maps to chromosome 1q32-q41 by fluorescence in situ hybridization (data not shown). Its mapping to another location within the genome compels the hypothesis that KiSS-1 is regulated by the gene(s) on chromosome 6. Our data also suggest that KiSS-1 is an important downstream effector of a gene(s) encoded on chromosome 6 and are consistent with metastasis suppression following introduction of chromosome 1 into MelJuSo melanoma cells (*13*). This hypothesis can be further refined because of data failing to detect KiSS-1 mRNA in metastatic neo6(del)(q21-q23)/C8161 hybrids. The latter can be explained by: 1) KiSS-1 mutations, 2) KiSS-1 deletions in subclones of C8161, or 3) mutation or deletion of regulatory gene(s) mapping to 6q21-q23. These hypotheses will require additional studies. The latter explanation is consistent with high-frequency loss of heterozygosity observed in late stage melanomas (*44-46*).

In summary, we cloned a new human melanoma metastasis-suppressor gene by subtractive hybridization comparing metastatic C8161 cells and nonmetastatic neo6/C8161.1 cells. Loss of expression correlates with metastatic potential in human melanoma cells, and its expression results in significant suppression of melanoma metastasis in athymic nude mice. It will be necessary to further analyze the KiSS-1 gene in melanoma progression. The predicted KiSS-1 protein has a proline-rich region with homology to SH3-binding domain that could predict a mechanism for melanoma metastasis suppression. KiSS-1 may function in cell signaling and/or cytoskeletal organization on the basis of the usual role of SH3 domains in these processes; however, there is no evidence directly supporting these hypotheses. Taken together, these data strongly support a critical role for KiSS-1 in the regulation of cancer metastasis in human malignant melanoma. Additional experiments will be required to determine a role for KiSS-1 in other cancers or whether KiSS-1 may be a useful marker for staging melanomas in a clinical setting.

Table 2. Suppression of experimental metastasis of C8161 human melanoma cells by KiSS-1*

Cell line	Relative KiSS-1 expression	Lung metastases: No. of mice with lung metastases/ total No. of mice	Lung metastases: Mean ± SEM	Lung metastases: Median (range)	*P*
C8161	–	12/12	152 ± 25	>200 (2 to >200)	
C8161c1.9	–	4/4	200 ± 41	>200 (98 to >200)	NSD
C8161-neomix	–	4/4	160 ± 26	174 (93 to >200)	NSD
KiSS-1cl.12	+	8/8	81 ± 31	40 (1 to >200)	$P<.05$
KiSS-1cl.3	+	16/16	82 ± 49	101 (2 to >200)	$P<.05$
KiSS-1cl.9	++	15/16	21 ± 13	24.5 (0 to 80)	$P<.001$
KiSS-1cl.10	+++	4/12	1 ± 1	0 (0 to 4)	$P<.0001$
KiSS-1cl.2	+++	9/16	0.9 ± 0.4	0.5 (0 to 3)	$P<.001$

*Cells (3×10^5) were injected intravenously into the lateral tail vein of 3- to 4-week-old female athymic nude mice (seven to eight mice per group, Harlan Sprague-Dawley). Mice were killed 26 days after inoculation. All organs were examined for the presence of metastases, and many were confirmed by microscopic examination of hematoxylin–eosin-stained paraffin-embedded sections (4-6 μm). Macroscopic lung metastases were quantified with the aid of a dissecting microscope after staining with a mixture of neutral-buffered formalin and Bouin's fixative (5:1) as described (*20,24*). *P* values were calculated by one-way analysis of variance using Tukey's Honestly Significant Difference post-test. Values shown compare each group with parental C8161. SEM = standard error of the mean; NSD = no significant difference.

References

(1) Liotta LA, Stetler-Stevenson WG. Tumor invasion and metastasis: an imbalance of positive and negative regulation. Cancer Res 1991;51(18 Suppl):5054s-9s.

(2) Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 1991; 64:327-36.

(3) Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis [*see* comment citation in Medline]. Cell 1994; 79:185-8.

(4) Nicolson GL. Cancer progression and growth: relationship of paracrine and autocrine growth mechanisms to organ preference of metastasis. Exp Cell Res 1993;204:171-80.

(5) Dear TN, Kefford RF. Molecular oncogenetics of metastasis. Mol Aspects Med 1990;11:243-324.

(6) Wright JA, Egan SE, Greenberg AH. Genetic regulation of metastatic progression. Anticancer Res 1990;10:1247-55.

(7) MacDougall JR, Matrisian LM. Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. Cancer Metastasis Rev 1995;14:351-62.

(8) Fodstad O, Kjonniksen I. Microenvironment revisited: time for reappraisal of some prevailing concepts of cancer metastasis. J Cell Biochem 1994; 56:23-8.

(9) Ponta H, Hofmann M, Herrlich P. Recent advances in the genetics of metastasis. Eur J Cancer 1994;30A:1995-2001.

(10) Degen WG, Weterman MA, van Groningen JJ, Cornelissen IM, Lemmers JP, Agterbos MA, et al. Expression of nma, a novel gene, inversely correlates with the metastatic potential of human melanoma cell lines and xenografts. Int J Cancer 1996;65:460-5.

(11) Safarians S, Sternlicht MD, Freiman CJ, Huaman JA, Barsky SH. The primary tumor is the primary source of metastasis in a human melanoma/scid model. Implications for the direct autocrine and paracrine epigenetic regulation of the metastatic process. Int J Cancer 1996;66:151-8.

(12) Welch DR, Chen P, Miele ME, McGary CT, Bower JM, Stanbridge EJ, et al. Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. Oncogene 1994;9:255-62.

(13) Miele ME, Robertson G, Lee JH, Coleman A, McGary CT, Fisher PB, et al. Metastasis suppressed, but tumorigenicity and local invasiveness unaffected, in the human melanoma cell line Me/JuSo after introduction of human chromosomes 1 or 6. Mol Carcinog 1996;15:284-99.

(14) You J, Miele ME, Dong C, Welch DR. Suppression of human melanoma metastasis by introduction of chromosome 6 may be partially due to inhibition of motility, but not to inhibition of invasion. Biochem Biophys Res Commun 1995;208:476-84.

(15) Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction [*see* comment citation in Medline]. Science 1992;257:967-71.

(16) Liang P, Averboukh L, Pardee AB. Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acids Res 1993;21:3269-75.

(17) van Groningen JJ, Bloemers HP, Swart GW. Identification of melanoma inhibitory activity and other differentially expressed messenger RNAs in human melanoma cell lines with different metastatic capacity by messenger RNA differential display. Cancer Res 1995;55:6237-43.

(18) Lee SW, Tomasetto C, Sager R. Positive selection of candidate tumor-suppressor genes by subtractive hybridization. Proc Natl Acad Sci U S A 1991;88:2825-9.

(19) Duguid JR, Rohwer RG, Seed B. Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library. Proc Natl Acad Sci U S A 1988;85:5738-42.

(20) Welch DR, Bisi JE, Miller BE, Conaway D, Seftor EA, Yohem KH, et al. Characterization of a highly invasive and spontaneously metastatic human malignant melanoma cell line. Int J Cancer 1991;47:227-37.

(21) Trent JM, Stanbridge EJ, McBride HL, Meese EU, Casey G, Araujo DE, et al. Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. Science 1990;247:568-71.

(22) Gualandi F, Morelli C, Pavan JV, Rimessi P, Sensi A, Bonfatti A, et al. Induction of senescence and control of tumorigenicity in BK virus transformed mouse cells by human chromosome 6. Genes Chromosomes Cancer 1994;10:77-84.

(23) Schweinfest CW, Henderson KW, Gu JR, Kottaridis SD, Besbeas S, Panotopoulou E, et al. Subtraction hybridization cDNA libraries from colon carcinoma and hepatic cancer. Genet Anal Tech Appl 1990;7:64-70.

(24) Welch DR, Neri A, Nicolson GL. Comparison of spontaneous and 'experimental' metastasis using rat 13762 mammary adenocarcinoma cell clones. Invasion Metastasis 1983;3:65-80.

(25) Lee JH, Welch DR. Use of differential display and subtractive hybridization to identify highly expressed genes in metastasis-suppressed chromosome 6/human melanoma hybrids. Proc Am Assoc Cancer Res 1996; 37:530.

(26) Cohen GB, Ren R, Baltimore D. Modular binding domains in signal transduction proteins. Cell 1995;80:237-48.

(27) Alexandropoulos K, Cheng G, Baltimore D. Proline-rich sequences that bind to Src homology 3 domains with individual specificities. Proc Natl Acad Sci U S A 1995;92:3110-4.

(28) von Heijne G. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 1986;14:4683-90.

(29) Hennessey C, Henry JA, May FE, Westley BR, Angus B, Lennard TW. Expression of the antimetastatic gene nm23 in human breast cancer: an association with good prognosis. J Natl Cancer Inst 1991;83:281-5.

(30) Musacchio A, Wilmanns M, Saraste M. Structure and function of the SH3 domain. Prog Biophys Mol Biol 1994;61:283-97.

(31) Joseph G, Pick E. Peptide walking is a novel method for mapping functional domains in proteins. Its application to the Rac1-dependent activation of NADPH oxidase. J Biol Chem 1995;270:29079-82.

(32) Feng S, Chen JK, Yu H, Simon JA, Schreiber SL. Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions Science 1994;266:1241-7.

(33) Mayer BJ, Eck MJ. SH3 domains. Minding your p's and q's. Curr Biol 1995;5:364-7.

(34) Rickles RJ, Botfield MC, Zhou XM, Henry PA, Brugge JS, Zoller MJ. Phage display selection of ligand residues important for Src homology 3 domain binding specificity. Proc Natl Acad Sci U S A 1995;92:10909-13.

(35) Ishino M, Ohba T, Sasaki H, Sasaki T. Molecular cloning of a cDNA encoding a phosphoprotein, Efs, which contains a Src homology 3 domain and associates with Fyn. Oncogene 1995;11:2331-8.

(36) Koch CA, Anderson D, Moran MF, Ellis C, Pawson T. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 1991;252:668-74.

(37) Musacchio A, Noble M, Pauptit R, Wierenga R, Saraste M. Crystal structure of a Src-homology 3 (SH3) domain [*see* comment citation in Medline]. Nature 1992;359:851-5.

(38) Pawson T, Gish GD. SH2 and SH3 domains: from structure to function. Cell 1992;71:359-62.

(39) Pawson T, Schlessinger J. SH2 and SH3 domains. Curr Biol 1993;3:434-42.

(40) Feller SM, Ren R, Hanafusa H, Baltimore D. SH2 and SH3 domains as molecular adhesives: the interactions of Crk and Abl. TIBS Trends Biochem Sci 1994;19:453-8.

(41) Nobes CD, Hall A. Rho, Rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 1995;81:53-62.

(42) Ridley AJ. Membrane ruffling and signal transduction. Bioessays 1994; 16:321-7.

(43) Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, et al. Oncogenes and signal transduction [published erratum appears in Cell 1991;65:following 914]. Cell 1991;64:281-302.

(44) Trent JM, Thompson FH, Meyskens FL Jr. Identification of a recurring translocation site involving chromosome 6 in human malignant melanoma. Cancer Res 1989;49:420-3.

(45) Guan XY, Meltzer PS, Cao J, Trent JM. Rapid generation of region-specific genomic clones by chromosome microdissection: isolation of DNA from a region frequently deleted in malignant melanoma. Genomics 1992;14:680-4.

(46) Millikin D, Meese E, Vogelstein B, Witkowski C, Trent JM. Loss of heterozygosity for loci on the long arm of chromosome 6 in human malignant melanoma. Cancer Res 1991;51:5449-53.

Notes

Present address: M. E. Miele, Department of Medical Technology, University of Delaware, Newark.

Supported by Public Health Service grants CA62168 and CA44470 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, by the National Foundation for Cancer Research, and by gifts from the Jake Gittlen Memorial Golf Tournament.

This paper is dedicated to the memory of Ray C. Welch, who was always supportive and encouraging.

Manuscript received May 10, 1996; revised August 14, 1996; accepted September 10, 1996.