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# c-Met Autocrine Activation Induces Development of Malignant Melanoma and Acquisition of the Metastatic Phenotype[1]

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## ABSTRACT

**The molecular and genetic events that contribute to the genesis and progression of cutaneous malignant melanoma, a complex and aggressive disease with a high propensity for metastasis, are poorly understood due in large part to the dearth of relevant experimental animal models. Here we used transgenic mice ectopically expressing hepatocyte growth factor/scatter factor (HGF/SF) to show that the Met signaling pathway is an important *in vivo* regulator of melanocyte function, whose subversion induces malignant melanoma. Tumorigenesis occurred in stages, beginning with the abnormal accumulation of melanocytes in the epidermis and dermis and culminating in the development of metastatic melanoma. Oncogenesis in this model was driven by creation of HGF/SF-Met autocrine loops through forced expression of the transgenic ligand and apparent selection of melanocytes overexpressing endogenous receptor, rather than paracrine stimulation or mutational activation of c-*met*. Preference for liver as a metastatic target correlated with high HGF/SF-Met autocrine activity, consistent with the notion that such activity may influence colonization. Although basic fibroblast growth factor and its receptor were both weakly expressed in the majority of melanomas examined, high levels were found only in those rare neoplasms with low or undetectable HGF/SF and Met expression, suggesting that these two tyrosine kinase receptor autocrine loops serve a critical overlapping function in melanocytic tumorigenesis. Our data support a causal role for HGF/SF-Met signaling in the development of melanoma and acquisition of the metastatic phenotype. Moreover, this transgenic mouse should serve as a highly useful model, facilitating our understanding of mechanisms by which human melanoma progresses to malignancy and expediting the development of efficacious therapeutic modalities designed to constrain metastasis.**

## INTRODUCTION

The incidence of primary cutaneous malignant melanoma is presently increasing in dramatic fashion; it is estimated that by the year 2000, 1 in 90 Caucasians in the United States will develop this potentially fatal disease (1, 2). Despite the fact that the 5-year survival rate of patients with melanoma has almost doubled over the past 50 years, the death rate has also doubled (1, 2), lending some urgency to the efforts of those committed to elucidate the underlying genetic and molecular basis of cutaneous malignant melanoma, a complex disease involving a multitude of genes. Over the last two decades, a number of chromosomal hot spots have been associated with predisposition to melanoma, but only one gene, *INK4a* at the 9p21 locus, has been unambiguously identified as a true melanoma susceptibility gene based on documentation of germ-line and somatic mutations in familial and sporadic melanomas (reviewed in Refs. 3 and 4). Although differential expression studies have implicated a number of other genes in the development and/or progression of melanoma (4), virtually none have been shown to play a critical, rather than a coincidental, *in vivo* role. This is due, in large part, to the difficulty in developing suitable animal models for this disease. Spontaneous melanomas are exceedingly rare in most laboratory animals, including mice, and a relatively small number of experimental models have been described (reviewed in Ref. 5). Of greater consequence, animal models of melanoma presently available are typically not characterized by associated metastasis. For example, *ink4a*-deficient mice, lacking both $p16^{Ink4a}$ and $p19^{ARF}$, in which expression of activated H-*ras* was targeted to melanocytes, developed melanomas with short latency and high penetrance but failed to demonstrate metastatic dissemination (6).

HGF/SF[4] is a multifunctional cytokine able to elicit mitogenic, motogenic, and morphogenic responses in a variety of cultured epithelial cells expressing the transmembrane RTK Met (7–9), including melanocytes (10, 11). HGF/SF is produced in cells of mesenchymal origin, whereas c-*met* is expressed in adult and embryonic epithelium, indicating that HGF/SF functions almost exclusively as a paracrine regulator under normal physiological conditions (12–14). Moreover, gene targeting studies have shown that HGF/SF-Met signaling is required for the development of liver, skeletal muscle, and placenta (15–17).

In addition to participating in a wide variety of normal cellular processes, Met signaling has also been implicated in oncogenesis. c-*met* was first recognized as a proto-oncogene by virtue of its ability to transform NIH 3T3 cells when placed, through chromosomal translocation, under the control of the translocated promoter region, creating the *tpr-met* fusion gene (18, 19). c-*met* has also been shown to be highly expressed in diverse human and mouse tumors (20–25), including melanomas, where in one human study it was correlated with metastatic progression (26). Significantly, a number of these tumor types also coexpress *HGF/SF*, suggesting that establishment of an autocrine signaling loop is associated with tumorigenesis (23, 27, 28). This question has been addressed experimentally *in vitro*, and a variety of cell types have demonstrated a dependency on the establishment of an HGF/SF-Met autocrine loop for efficient transformation and tumorigenicity (22, 27, 29–32). A number of biological activities induced through HGF/SF-Met signaling make it an exceptionally attractive candidate for realizing the metastatic phenotype in primary tumor cells, particularly in those overexpressing both ligand

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[4] The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; RTK, receptor tyrosine kinase; MT, metallothionein; FGF, fibroblast growth factor; bFGF, basic FGF; FGFR, FGF receptor; EGF, epidermal growth factor.

and receptor. HGF/SF can stimulate cellular movement or scatter, extracellular matrix degradation, and angiogenesis, any of which could contribute to tumor cell invasion and metastasis (reviewed in Refs. 33 and 34). In fact, cells expressing both *HGF/SF* and c-*met* show enhanced motility and invasiveness *in vitro* and metastatic activity *in vivo* (35, 36).

The advent of transgenic and gene targeting technologies has permitted the development of uniquely useful model systems to study the underlying mechanisms responsible for oncogenesis. We have shown previously that inappropriate expression of a mouse *HGF/SF* cDNA under the control of the MT-1 promoter and 5′/3′ *MT* gene flanking sequences perturbs the development of multiple tissue types, including neural crest melanocytes (37), and induces the spontaneous formation of a number of epithelial and mesenchymal tumors (38, 39). Here we report the regular occurrence and metastatic spread of cutaneous malignant melanoma in HGF/SF transgenic mice, and provide characterization of these melanomas at the histological and molecular level.

## MATERIALS AND METHODS

**Transgenic Mice.** MT-HGF/SF transgenic mice were generated on an albino FVB/N genetic background as described previously (37). Expression of the mouse *HGF/SF* cDNA was placed under the control of the mouse MT-1 promoter, and the construct included the 5′ and 3′ flanking regions of mouse *MT* genes, which contain locus control regions conferring copy number-dependent and integration site-independent transgene expression (40). In general, zinc water was not required or used to induce tumors in HGF/SF transgenic mice. The melanomas in this study were derived from lines MH19, MH22, and MH37. Mouse work was performed in accordance with The Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Institute for Laboratory Animal Resources, National Research Council, 1996).

**Histopathological and Ultrastructural Analysis.** Tumor tissue for histopathological analysis was fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with H&E. Immunohistochemistry was used to identify and characterize mouse melanomas. HMB45 (Dako Corp) and S-100 (Sigma) rabbit polyclonal antibodies were obtained commercially. Rabbit polyclonal antibodies to TRP1 (αPEP1), tyrosinase (αPEP7), and Pmel 17 (αPEP13) were generously provided by Dr. Vince Hearing, National Cancer Institute (41–43). Dilutions were: 1:150, HMB45; 1:800, S100; 1:500, TRP1; and 1:200, tyrosinase and Pmel 17. The immunoperoxidase/avidin-biotin complex technique was used with either the Vector Rabbit Elite or Mouse Elite kit (Vector Laboratories) as secondary antibody and diaminobenzidine as chromagen. For ultrastructural analysis, tumor tissues and cells were fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer and embedded in eponate 12 resin (Ted Pella, Inc.). Thin sections were prepared, stained with uranyl acetate and lead citrate, and examined under a Philips 201 electron microscope.

**Analysis of RNA Transcripts.** Total RNA was isolated from melanoma tissue using guanidine thiocyanate (44). For Northern blot analysis, total RNA (10 μg) was resolved on a denaturing 1% agarose/formaldehyde gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was prehybridized and hybridized at 42°C in a solution the contents of which included 50% formamide and 6× SSC, as described previously (44), washed, and subjected to autoradiography. To control for RNA loading and transfer variation, filters were routinely rehybridized with a glyceraldehyde-3-phosphate dehydrogenase probe. Both the 2.2-kb mouse *HGF/SF* cDNA probe and the 1.5-kb mouse c-*met* cDNA probe were synthesized by PCR (39). The 622-bp mouse *FGFR-1* probe was synthesized by PCR using as template a cDNA derived from NIH 3T3 cells and the following primer set: 5′-TTGACGTCGTGGAACGAT-3′ and 5′-CTGGAGGAGAGCCGTGAG-3′. The 0.9-kb *Xba*I fragment for mouse Fas ligand (FasL) cDNA was isolated from the plasmid pBL-MFLW4 (45).

For analysis by PCR, total tissue RNA was reverse transcribed to cDNA using the SuperScript Preamplification System for First Strand cDNA Synthesis kit (Life Technologies, Inc.) according to the manufacturer's protocol. cDNA was amplified by PCR generating a 424-bp fragment specific for mouse bFGF (forward primer, 5′-GCATCACCTCGCTTCC-3′; reverse primer, 5′-AGTATGGCCTTCTGTC-3′), a 622-bp fragment specific for mouse FGFR-1 as described above, or a 625-bp fragment specific for mouse FasL (forward primer, 5′-TCATCTTGGGCTCCTC-3′; reverse primer, 5′-TCAACCTCT-TCTCCTC-3′). A β-actin primer set (Stratagene) was used as control. The following conditions were routinely used: 1 cycle at 94°C for 5 min, then 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Finally, the reaction mixture was kept at 72°C for 10 min to achieve complete extension. The products were resolved on a 2% agarose gel.

**Analysis of Met and Met Activation.** Quantification of Met and Met tyrosine phosphorylation was performed as described (39). Briefly, frozen tumor tissues or cultured cells were solubilized in RIPA buffer consisting of 50 mM Tris (pH 7.4), 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM sodium $PP_i$ (Sigma), 50 mM sodium fluoride (Sigma), 1 mM sodium orthovanadate (Sigma), 1 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim), 10 μg/ml leupeptin (Boehringer Mannheim), 10 μg/ml pepstatin (Boehringer Mannheim), and 10 μg/ml aprotinin (Boehringer Mannheim). Protein concentration in the resulting lysates was determined using Albumin Standard (Pierce), and equivalent amounts of lysate were incubated with anti-Met antibody (Santa Cruz Biotechnology) for 2 h. After the addition of Gamma-Bind G Sepharose (Pharmacia Biotech) and washing in RIPA buffer, samples were fractionated on reducing SDS/7.5% polyacrylamide gels. After electrophoretic transfer to Immobilon P membranes (Millipore), filters were blocked and then incubated with anti-Met antibody overnight. Met was visualized by incubation with anti-rabbit antibody conjugated to horseradish peroxidase and by using enhanced chemiluminescence (Amersham) according to manufacturer's instruction. Subsequently, filters were stripped with buffer consisting of 100 mM 2-mercaptoethanol (Sigma), 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) at 50°C for 30 min. Filters were reblocked and incubated overnight with phosphotyrosine monoclonal antibody (Upstate Biotechnology). Met was visualized using an anti-mouse antibody conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham), according to the manufacturer's instructions.

***In Vivo* Growth and Metastasis Assays.** To determine *in vivo* growth rates, pieces of ~1 $mm^3$ of freshly removed melanoma tissue from 10- to 17-month-old HGF/SF transgenic mice were aseptically transplanted under the skin of Swiss athymic nude (strain designation, NCr-*nu/nu*) or FVB/N mice. Recipient mice were observed for 3 weeks. Tumor diameters were measured every 3 days with calipers, and tumor volume was calculated according to the formula $V = a \times b^2/2$.

To prepare primary cell culture, pieces of tumor tissue were suspended in complete growth media consisting of high glucose DMEM (Life Technologies, Inc.) supplemented with 15% FBS (Life Technologies, Inc.), 100 IU/ml penicillin (Life Technologies, Inc.), 100 μg/ml streptomycin (Life Technologies, Inc.), 2 mM L-glutamine (Life Technologies, Inc.), 5 μg/ml insulin (United Biomedical, Inc.), and 5 ng/ml EGF (United Biomedical, Inc.) and minced with scalpels to small pieces under sterile condition. Minced pieces were placed in complete media containing 1 mg/ml collagenase and rocked at 37°C in a humidified atmosphere of 5% $CO_2$ for 18 h. The resulting suspension of single cells was washed with PBS, and 1.5–3 million were seeded in 10-cm Petri dishes with 10 ml complete growth media or 35-mm Petri dishes with 5 ml complete growth media and incubated at 37°C with 5% $CO_2$. When cells became confluent, a conservative transfer of 1:5 was performed. The NK2 growth inhibition assay was performed on melanoma cells as described by Rubin *et al.* (46).

To determine their metastatic potential, trypsinized melanoma cell cultures were neutralized with complete growth media and washed with PBS, and one million cells in 0.2 ml of PBS was injected into the tail vein of Swiss athymic nude mice. Typically, recipient animals were killed with $CO_2$ between 3 and 4 weeks or earlier if they appeared distressed. Metastatic spread was assessed at gross necropsy and histologically, and micrometastatic lesions were identified by histopathological examination.

## RESULTS

**Altered Distribution and Number of Melanocytes in HGF/SF Transgenic Skin.** Albino FVB/N mice ectopically expressing an MT-HGF/SF transgene during development and in a wide variety of tissues, including skin, had aberrant localization of melanoblasts

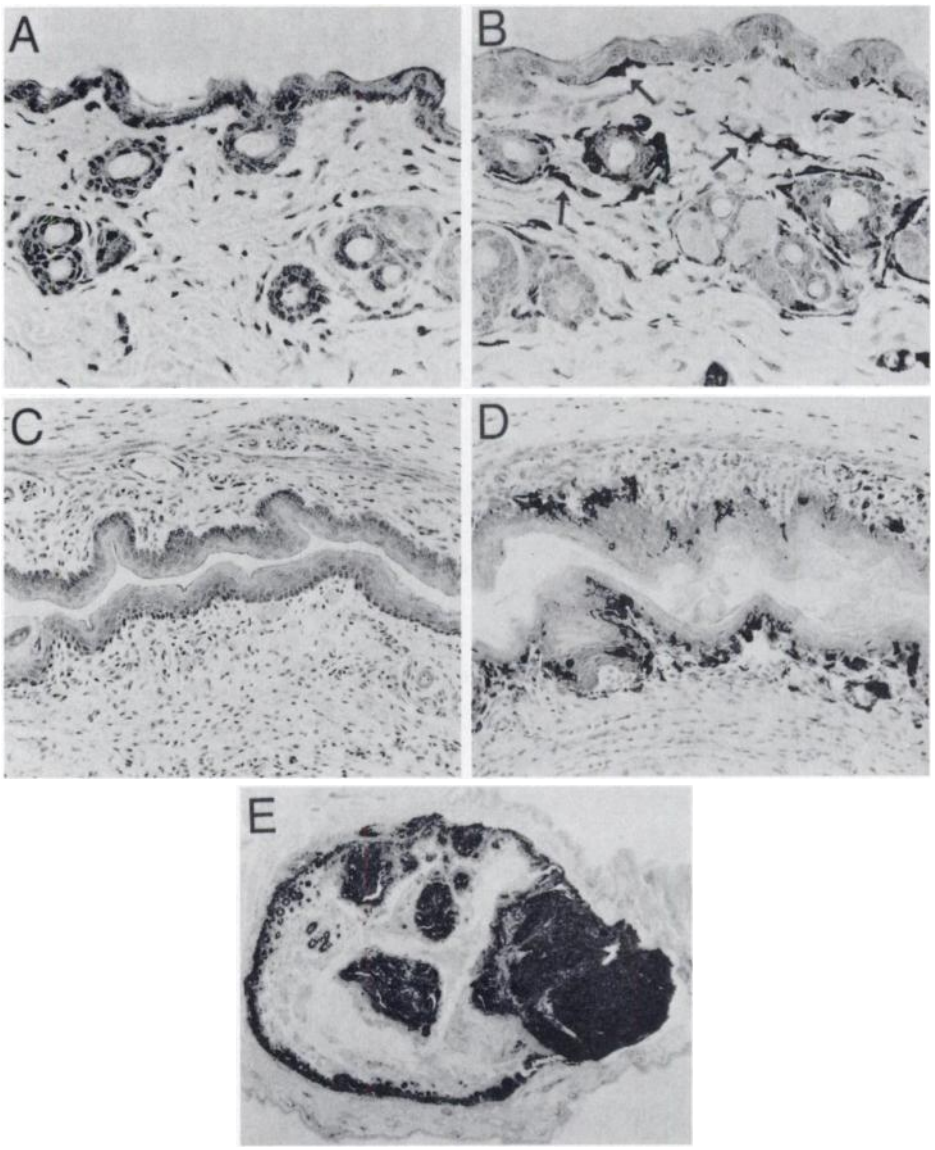


Fig. 1. Immunohistochemical localization of melanocytes in the dorsal skin (*A* and *B*) and prepuce (*C* and *D*) of wild-type (*A* and *C*) and HGF/SF transgenic (*B* and *D*) adult mice using an anti-TRP1 antibody. In the transgenic skin, melanocytes were aberrantly clustered in the epidermis, dermis, and at the junction (*arrows*). Note the great abundance of melanocytes in the transgenic prepuce relative to the controls. *E*, anti-TRP1 immunohistochemical staining of a melanoma that formed in the prepuce. *A* and *B*, ×400; *C* and *D*, ×200; *E*, ×25.

during embryogenesis, and upon cross-breeding to C57BL/6, mice showed patterned hyperpigmentation of the skin (37). Immunohistological examination of the transgenic skin using an antibody to the melanocytic marker TRP1 revealed that melanocytes were inappropriately abundant in the dermis, at the dermal-epidermal junction, and less consistently in the basal layer of the epidermis (Fig. 1B). In contrast, melanocytes in the skin of wild-type mice of the same age were confined to the hair shafts. Melanocytes in transgenic mice were most heavily concentrated in the skin of the paws, tail, ears, muzzle, and penis but were also abundant in the dorsal skin. In addition, many melanocytes were aberrantly localized to the prepuce (Fig. 1*D*) and central nervous system and lymph nodes (37).

Table 1 *Incidence of malignant amelanotic melanoma in HGF/SF transgenic mice*[a]

| Mouse genotype | Mean age ± SD (mo) | Total mice | No. with tumors | M/F ratio | No. metastatic |
|---|---|---|---|---|---|
| Transgenic[b] | 15.6 ± 4.3 | 87 | 19 (22%) | 17/2 | 4 (21%) |
| Wild type | 14.9 ± 5.6 | 53 | 0 (0%) | 0/0 | 0 |

[a] Based on mice ≥6 months of age.
[b] The 19 mice reported here with melanomas were from four different albino lines; 10 were from line MH19, 7 from MH37, 1 from MH31, and 1 from MH22.

**Induction of Malignant Melanomas in HGF/SF Transgenic Mice.** HGF/SF transgenic mice were predisposed to a wide variety of carcinomas and sarcomas, demonstrating the broad influence of c-*met* overstimulation on cells of different lineages (39). Of these tumors, cutaneous malignant melanoma, which is exceedingly rare in wild-type mice, was among the most prevalent. Table 1 shows that 22% (19 of 87) of HGF/SF transgenic mice 6 months of age or older that were examined developed malignant melanoma; wild-type controls of comparable mean age had no melanomas. The average age of onset was 15.6 months, but these tumors occurred as early as 6 months of age. All melanomas were amelanotic because the HGF/SF transgenic mice were created on an albino FVB/N genetic background in which tyrosinase is mutationally inactivated (47). Melanocytic tumors occurred predominantly in the skin and subcutaneum of areas characterized by a heavy population of melanocytes, including the back, neck, tail, and ears. More infrequently, melanomas appeared in the prepuce (Fig. 1*E*) and the exorbital and mammary glands. Surprisingly, melanocytic tumors arose preferentially in male mice; 17 of 19 mice with melanomas were male (Table 1). The reason for this male preference is unknown; molecular analysis of eight melanomas revealed

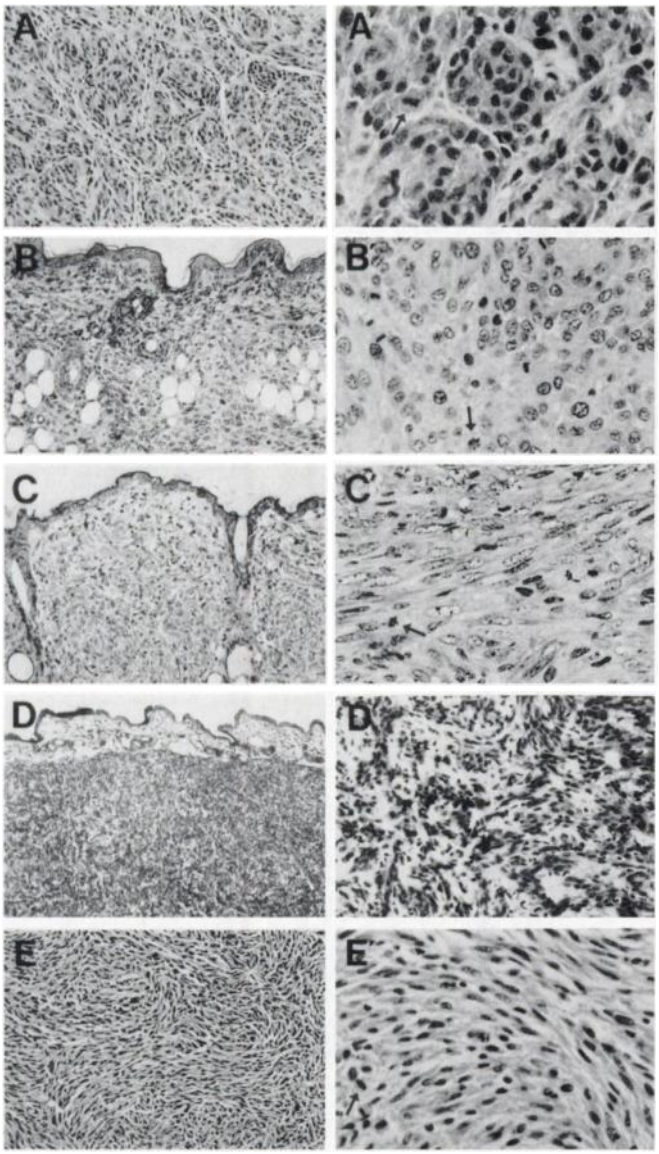


Fig. 2. Diverse morphology of HGF/SF-induced amelanotic melanomas shown at low (*A–E*) and high (*A'–E'*) magnification. *A* and *A'*, highly metastatic melanoma 37-32 showing mixed population of cells with epitheloid and schwannomatous features, forming a whorling pattern. *B* and *B'*, same tumor as in *A*, arising in adjacent skin after resection of original primary tumor, showing predominantly epitheloid characteristics. *C* and *C'*, highly invasive but more poorly differentiated tumor 37-7 with a fibrosarcomatous appearance. *D* and *D'*, noninvasive melanoma with schwannomatous characteristics, including arrangement of spindle-shaped cells in interlacing fasicles (*D*), nuclear palli-sading, and a suggestion of Verocay body formation (*D'*). *E* and *E'*, lymph node metastasis consisting of melanoma cells with fibrosarcomatous appearance. *A–C, E, D'*, ×200; *A'–C', E'*, ×630; *D*, ×50. *Arrows*, examples of numerous mitotic figures.

that only one overexpressed the androgen receptor (data not shown).

Amelanotic melanomas were diagnosed based on a number of criteria: histomorphology, immunohistochemical positivity to specific melanocytic and S100 markers, and presence of melanosomes as determined by electron microscopy. Morphologically, melanomas could in most instances be placed into one of three categories: epitheloid cell type (Figs. 2*B* and 3*A*), resembling pigmented melanomas described in some other animal models; spindle cell type (Fig. 2*D*), resembling schwannomas with the Antonini type A pattern reported previously in albino Fischer 344 rats (48); or a mixed population with epitheloid and schwannomatous components (Fig. 2*A*). Rarely, melanoma cells had a more poorly differentiated, fibrosarcomatous appearance (Fig. 2*C*). Standard immunohistochemical analyses used to confirm sarcomatous tumors as amelanotic melanomas included positivity to S100, which was frequently nuclear as well as cytoplasmic (Fig. 3*B*), and one or more of the following markers, TRP1 (Fig. 3*C*), tyrosinase (Fig. 3*D*), and Pmel 17 (Fig. 3, *E* and *F*). Positivity to individual markers varied greatly among the melanomas examined, although almost all tumors were positive for S100. Some tumors were positive for all three melanocytic markers (Fig. 3, *A–F*), whereas others were completely devoid of specific staining for one or two (Fig. 3, *G* and *H*). Electron microscopy was used to further characterize selected melanomas. Typical melanomas, such as 37-32 and 19-106, possessed an unusually high concentration of organelles, including Golgi, mitochondria, and melanosomes, shown in Fig. 4, *A* and *B*, respectively. Occasionally, more schwannomatous features were observed, including intertwining cell processes (Fig. 4*D*), and the formation of a basal lamina (Fig. 4, *B* and *D*). An atypical, more poorly differentiated tumor (37-7) is shown in Fig. 4*C*.

Histopathological examination of primary cutaneous melanomas revealed that some spread diffusely through the dermis (Figs. 2, *B* and *C*, and 3*A*) and/or invaded muscle, whereas others were clearly localized although not encapsulated (Figs. 2*D* and 3*G*). Significantly, 21% of the mice (4 of 19) had melanomas that metastasized to various sites, including liver (Fig. 5*A*), lung (Fig. 5*C*), pancreas, epididymis, femur (Fig. 5*D*), lymph nodes (Figs. 2*E* and 5*B*), and spleen. Metastatic lesions tested were positive for the presence of melanocytic markers (data not shown).

**Molecular and Biochemical Characterization of Melanomas.** We showed previously that transgenic HGF/SF RNA transcripts were abundant, and endogenous c-*met* transcripts were elevated relative to normal tissue in a wide variety of tumor types arising in HGF/SF transgenic mice, including a small sample of melanomas (39). To determine whether melanoma genesis is universally associated with autocrine HGF/SF-Met activation, we examined the levels of expression of HGF/SF and c-*met* RNA in melanomas arising in these transgenic animals (Table 2). We found overexpression of transcripts encoding both the ligand and receptor in seven of nine (78%) primary tumors (six positive samples are shown in Fig. 6), suggesting that the establishment of HGF/SF-Met autocrine loops was a common mechanism. Two of these seven (37-32 and 19-106) had a metastatic phenotype. Subsequent analysis of overtly pure populations of cells derived from a subset of these tumors (see below) confirmed that the melanoma cells were indeed expressing both ligand and receptor mRNA (data not shown). Very low or undetectable expression of both HGF/SF and its receptor were observed in two other neoplasms (37-7 and 19-43), neither exhibiting metastases (Fig. 6). One of these (37-7) was a poorly differentiated s.c. tumor with both fibrosarcomatous and melanomatous characteristics (Figs. 2*C* and 4*C*).

To quantify levels of Met protein, extracts from this same group of primary melanomas were subjected to immunoprecipitation using an anti-Met antibody, followed by electrophoretic fractionation and immunoblotting using the anti-Met antibody. Fig. 7*A* shows that levels of Met in most primary tumors were in general agreement with the c-*met* RNA data. As exceptions, Met in 37-84 and 37-32 was lower and higher than expected, respectively. To confirm that high Met expression in these HGF/SF transgenic cells actually translated into functional autocrine loops, Met tyrosine phosphorylation was determined in extracts obtained from representative primary cells cultured from a highly expressing (37-32) and a poorly expressing (37-7) melanoma. Met was immunoprecipitated from cellular extracts and visualized with anti-Met antibody, after which tyrosine phosphorylation was assessed by reblotting with an anti-phosphotyrosine antibody. Fig. 7*B* shows that high Met tyrosine phosphorylation was associated with melanoma cells highly expressing c-*met* (37–32) but

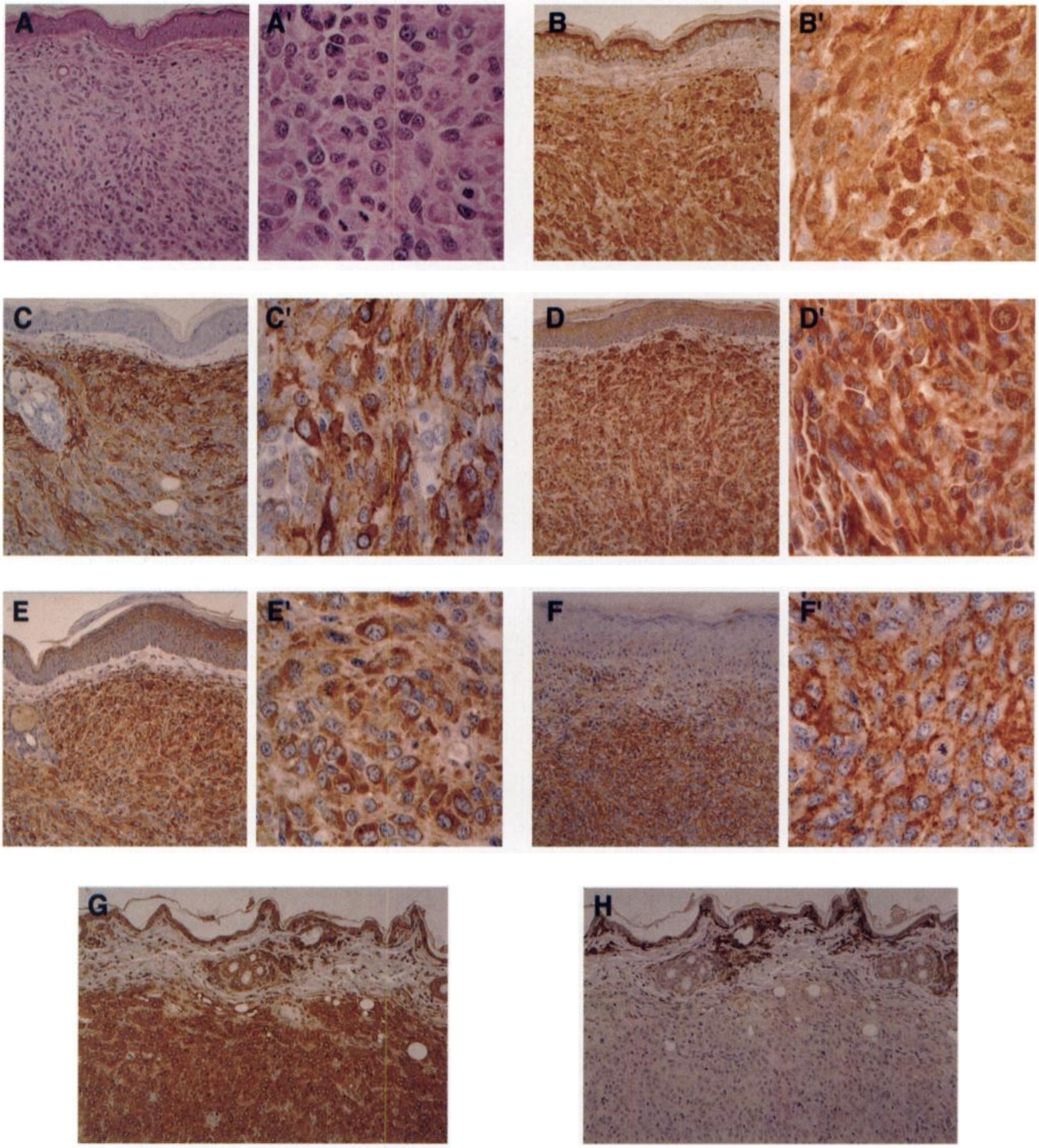


Fig. 3. Immunohistochemical characterization of HGF/SF-induced cutaneous melanomas. *A–F*, analysis of the more typical, metastatic neoplasm 19-106, which is positive for all melanocytic markers tested. Shown are panels of H&E staining with diffuse infiltration of the dermis (*A*), mitotic figures and epitheloid appearance of 19-106 (*A'*), and positive immunohistochemical detection using S100 (*B*), anti-TRP1 (*C*), anti-tyrosinase (*D*), anti-Pmel 17 (*E*), and HMB45, which also recognizes Pmel 17 (*F*). *G* and *H*, nonmetastatic melanoma 19-154 showing strong positivity for tyrosinase (*G*) but no staining for TRP1 (*H*). Note obvious TRP1 staining of abundant melanocytes in surrounding normal skin (*H*). *A–H*, ×200; *A'–F'*, ×630.

not in cells characterized by poor expression (37-7). DNA sequence analysis of the Met kinase domain in primary tumors suggested that increased Met activity was not the consequence of activating mutations in c-*met* (data not shown). Strong Met activation appeared to be required for the optimal *in vitro* growth of at least some melanoma cells; 37-32 cells were 59 and 62% growth inhibited by 0.3 and 1.0 μg/ml, respectively, of the growth antagonist NK2, a natural splice variant of HGF/SF (49–51).

FGF signaling, also initiated through potent RTK activity, has been implicated in both normal melanocyte function and melanoma development (reviewed in Refs. 52 and 53). For example, Halaban *et al.* (54) first showed that normal melanocytes do not produce bFGF; however, many human melanomas are characterized by bFGF overexpression. We therefore determined whether bFGF and FGFR1 transcripts were altered in melanomas induced by the HGF/SF transgene using Northern blot and RT-PCR analyses. Both bFGF and FGFR1

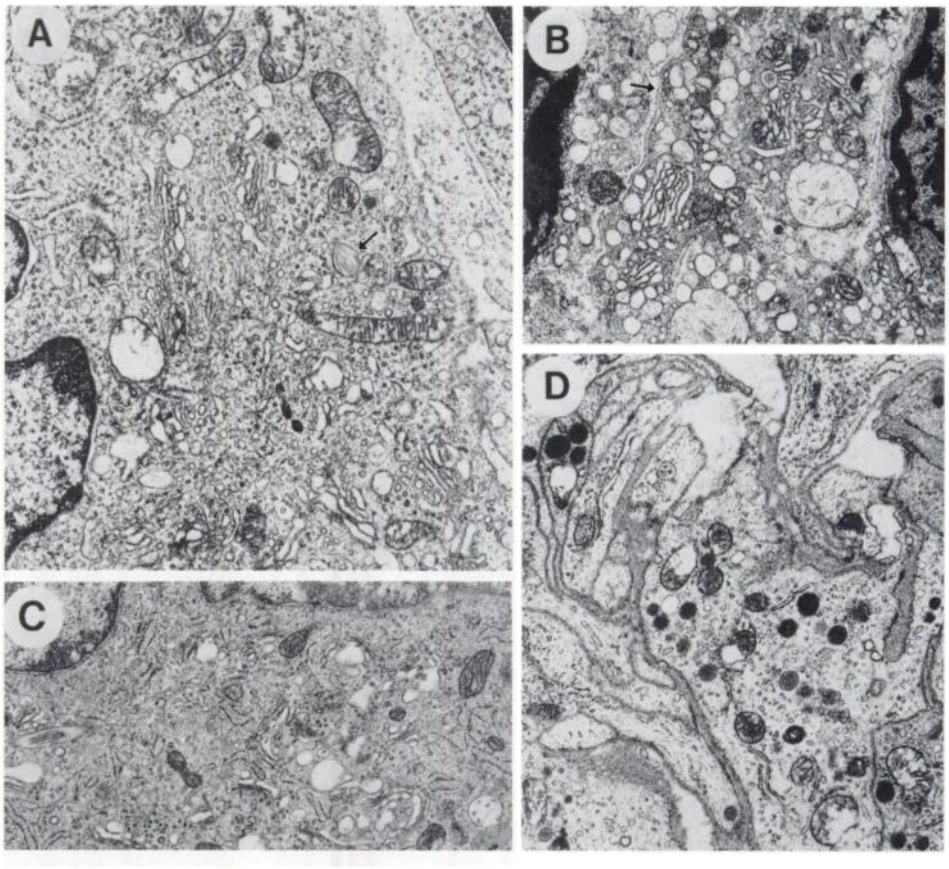


Fig. 4. Ultrastructure of amelanotic melanomas. Cells contain an abundance of polyribosomes, prominent Golgi apparatus and rough endoplasmic reticulum, and mitochondria, characteristic of melanoma. *A*, metastatic amelanotic melanoma (37-32) with typical features, showing presence of numerous type II melanosomes (*arrow*). *B*, metastatic amelanotic melanoma (19-106) with an abundance of type II melanosomes and the presence of a basal lamina (*arrow*). *C*, cultured primary cells from a more poorly differentiated tumor (37-7) with both melanomatous and fibrosarcomatous characteristics, *i.e.*, the cytoplasm had prominent Golgi apparatus, mitochondria, and polyribosomes but was also rich in dilated rough endoplasmic reticulum. *D*, melanoma with Schwann cell morphology; note the formation of basal lamina and cell processes. *A–D*, ×15,400.

mRNAs were weakly detected in the majority of melanomas, but were notably elevated in the two tumors characterized by low expression of HGF/SF and Met (Table 2). Perhaps not coincidentally, one of these (37–7) was more poorly differentiated, consistent with the findings of Dotto *et al.* (55), who showed that bFGF expression can suppress melanocytic markers. As reported in human melanomas, this coexpression of *bFGF* and *FGFR1* would create a potent autocrine loop.

In contrast, FasL, which has been implicated in the ability of melanoma cells to evade the immune system (56), was also examined by RT-PCR and found to be expressed in only one of eight transgenic melanomas (Table 2). These data suggest that FasL overexpression was not a general mechanism whereby melanomas were permitted to develop in this model system.

**Growth and Metastasis of Malignant Melanomas.** Three representative primary malignant amelanotic melanomas were selected for transplantation analysis to determine their true oncogenic potential. Tumor 37-32, which arose from dorsal skin and demonstrated multiorgan metastasis in the transgenic parent mouse (Fig. 5*A*), consisted of a mixed epitheloid/schwannomatous population of cells (Fig. 2*A*) that was strongly positive for all melanocytic markers. Cells from tumor 19-106, which arose from neck skin and metastasized only to the mandibular lymph node (Fig. 5*B*), were epitheloid in appearance and strongly positive for all markers as well (Fig. 3, *A–F*). Both primary tumors 37-32 and 19-106 coexpressed strongly both *HGF/SF* and c-*met* transcripts (Fig. 6). In contrast, the primary tumor 37-7 weakly expressed HGF/SF and Met (Figs. 6 and 7), appeared to be more poorly differentiated (Fig. 2C) with weak or no staining for melanocytic markers, and although highly invasive was not metastatic.

Upon s.c. transplantation, fragments from two of these melanomas, 37-32 and 37-7, rapidly grew in nude mice (Fig. 8). In general, phenotypic characteristics of each of these two primary tumors were maintained at their sites of s.c. transplantation, including histomorphological and invasive features. In contrast, only a fraction of the transplanted fragments of melanoma 19-106 formed s.c. tumors, and tumor growth was significantly reduced (Fig. 8). Expression levels of both *HGF/SF* and c-*met* were also determined for these three s.c. transplants. In all three cases, the expressions of ligand and receptor were unchanged relative to the original primary tumors (Fig. 6).

To characterize their metastatic potential, cells from the two most rapidly growing melanoma transplants, 37-32 and 37-7, were introduced into athymic nude mice via tail vein injection. These two tumors were also chosen because they exhibited a number of very different morphological and molecular characteristics, including high (37-32) and poor (37-7) HGF/SF-Met autocrine signaling (see above). Cells from the 37-32 melanoma disseminated preferentially to the liver (Fig. 5*F*), spleen (Fig. 5*E*), and skin, positive metastatic sites in the MH37 mouse from which this particular tumor originally arose, as well as the lung, lymph node, and ovary (Table 3). High coexpression of *HGF/SF* and c-*met* was found to be maintained in 37-32-derived tumors that colonized both the liver and spleen (data not shown). In contrast, cells from the 37-7 tumor demonstrated more inefficient colonization that was restricted to the lungs (Table 3). These results clearly show that at least some melanomas arising in transgenic mice overexpressing *HGF/SF* were functionally malignant, possessing the ability to extravasate, colonize, and grow with great efficiency at specific metastatic sites.

## DISCUSSION

**Association of HGF/SF-Met Autocrine Activation with Melanoma Genesis.** Melanocytes express a variety of RTKs, including Met, Kit, and the FGFR, but require a combination of growth factors

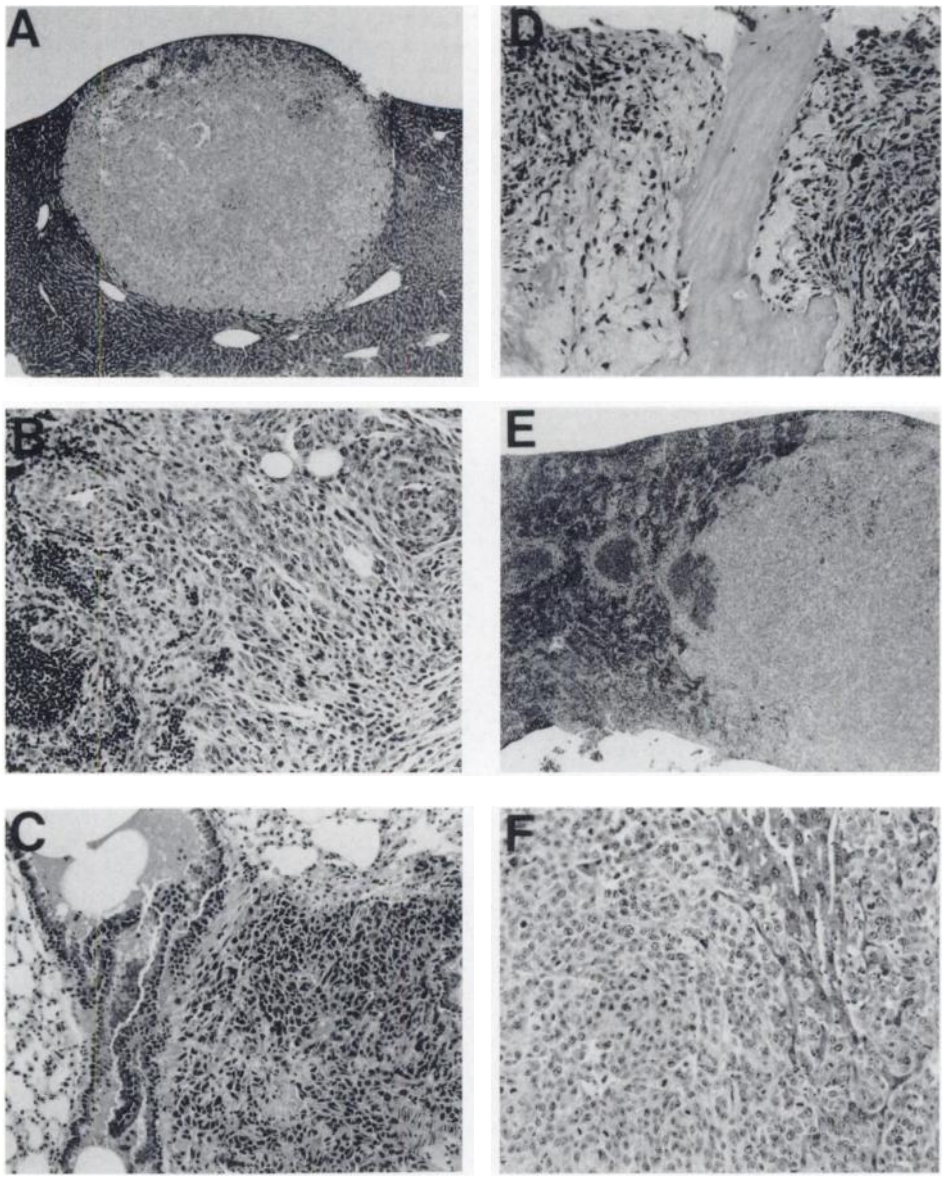


Fig. 5. Melanomas induced by ectopic *HGF/SF* expression can acquire a metastatic phenotype. *A*, metastatic liver neoplasm in transgenic mouse carrying primary melanoma 37-32. *B*, lymph node metastasis in transgenic mouse carrying primary melanoma 19-106. *C* and *D*, metastatic dissemination to lung and femur, respectively, of primary melanoma 22-74 in transgenic mouse. *E* and *F*, metastasis to spleen and liver, respectively, resulting from injection of cultured 37-32 melanoma cells into tail vein of athymic nude mice. *A* and *E*, ×50; *B–D*, *F*, ×200.

to propagate *in vitro* (57). The acquisition of growth factor autonomy has been strongly implicated in the development and progression of melanoma (reviewed in Refs. 52 and 53). However, the role of the HGF/SF-Met signaling pathway in melanocytic oncogenesis has not been clearly established because of somewhat conflicting reports in the literature (26, 57–61). In the present study, we demonstrated that ectopic expression of mouse HGF/SF induces at high incidence the development of malignant melanoma in transgenic mice, and that tumorigenesis is typically associated with the overexpression of endogenous c-*met* and the resulting establishment of chronic HGF/SF-Met autocrine signaling.

Table 2 *Expression of ligand and receptor RNA transcripts in melanomas*

| Tumor | Transgenic HGF/SF[a] | Met[a] | bFGF[b] | FGFR1[a] | FGFR1[b] | Fas ligand[b] |
|---|---|---|---|---|---|---|
| 37-32[c] | +++ | ++ | + | − | + | − |
| 37-84 | +++ | ++ | + | − | + | − |
| 37-154 | +++ | ++ | − | − | + | − |
| 37-117 | ++ | + | + | − | + | − |
| 19-106[c] | ++ | ++ | + | − | + | − |
| 19-601 | ++ | ++ | + | − | + | − |
| 37-7 | + | +/− | ++ | + | ++ | − |
| 19-43 | +/− | − | + | ++ | ++ | + |

[a] Northern blot analysis.
[b] Reverse transcription-PCR analysis.
[c] Metastatic melanoma.

The involvement of HGF/SF-Met autocrine loops in tumorigenesis in other cell types has been clearly established. A multitude of human cell lines and tumors, particularly sarcomas, overexpress both Met and its ligand (22–24, 28). Moreover, cotransfection of human *HGF/SF* and c-*met* expression constructs conferred a malignant phenotype to NIH 3T3 and C127 cells (27, 36). Melanomas have been shown to express c-*met*, and results from some studies have suggested a role in invasiveness (26, 57–61). Although tumors of melanocytic origin can show a loss of responsiveness to exogenous HGF/SF, the reason for this is unclear; endogenous HGF/SF expression in either melanocytes or melanomas has not been demonstrated (57, 58). Although activating mutations in c-*met* have been found to be associated with papillary renal carcinoma (62), they have not been reported for melanoma, and we have not detected such mutations in our tumors. In HGF/SF transgenic mice, melanoma genesis and progression is associated with the apparent selection of cells overexpressing both the transgene and endogenous c-*met*. This is reminiscent of the behavior of NIH 3T3 cells cotransfected with constructs expressing *HGF/SF* and c-*met*, which demonstrated both a greatly strengthened transformed pheno-

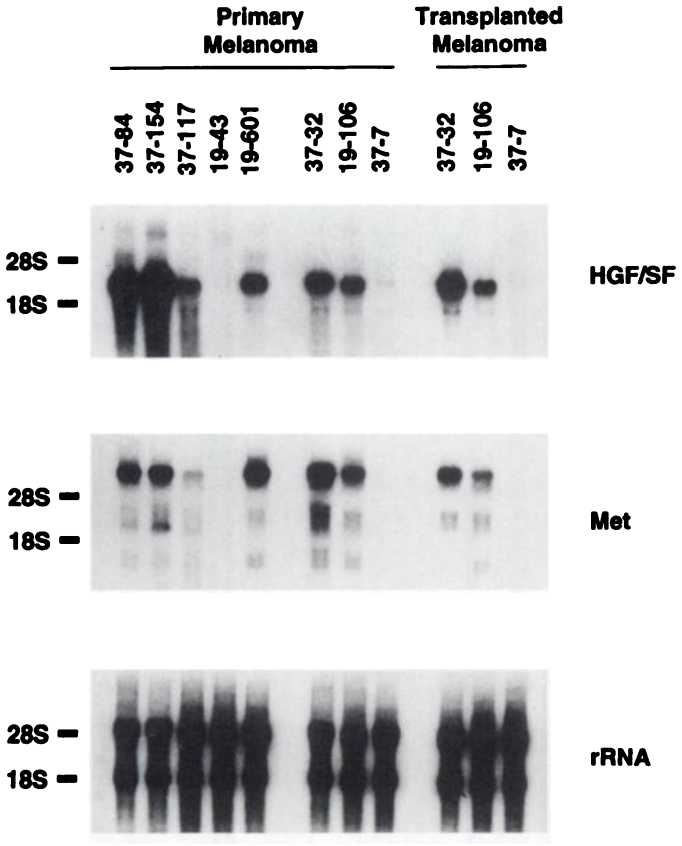


Fig. 6. Expression in primary and transplanted melanoma of transgenic HGF/SF and native c-*met* transcripts using Northern blot hybridization. The *upper panel* was probed with a *HGF/SF*-specific cDNA probe, and the *middle panel* with a c-*met*-specific cDNA probe. Each lane contains equivalent 10-$\mu$g samples of total RNA, as confirmed by visualization of rRNA with ethidium bromide (*lower panel*). *Left*, expression in eight primary melanomas; *right*, expression in three representative transplants. The positions of the 18S (2.0 kb) and 28S (4.7 kb) rRNA species are indicated.

type and enhanced expression of both transgenes upon serial passage through nude mice (27).

A number of studies have shown that human melanocytes express FGFR1 but require exogenous bFGF for normal growth, whereas melanomas express their own bFGF (54, 63–65). Indeed, interference with FGFR signaling using either antisense oligonucleotides, antisense cDNAs, or dominant-negative receptor mutants inhibits melanoma cell proliferation and/or angiogenesis (65–67). We noted that the establishment of Met autocrine signaling in our HGF/SF transgenic mice did not completely obviate the apparent need for the creation of at least a minimally active bFGF/FGFR1 autocrine loop, suggesting that these two RTKs may behave cooperatively at some level. This result mirrors studies of Halaban *et al.* (57), who showed that only together can HGF/SF and bFGF efficiently support melanocyte growth *in vitro*. However, it may be significant that the only two tumors characterized by poor HGF/SF and Met expression (37-7 and 19-43) demonstrated elevated expression of both bFGF and its receptor, raising the possibility that some shared component(s) of the Met and FGFR signaling pathways are required for melanoma genesis. Although present knowledge of biochemical pathways of FGFR signaling is rather limited, phospholipase C$\gamma$ and the Ras/mitogen-activated protein kinase pathway have been implicated in FGFR activity (68–71) as well as Met activity (57, 72).

Constitutive HGF/SF-Met signaling clearly induced the development of malignant melanoma in our transgenic model; wild-type mice with normal Met signaling did not develop melanoma. However, greatly elevated levels of ligand and receptor were neither necessary nor sufficient for melanoma growth *in vivo*, as evidenced by the relatively rapid and poor s.c. growth of transplants 37-7 and 19-106, respectively. Moreover, based on the relatively long latency demonstrated by most melanomas, autocrine Met activation alone was not enough to induce malignant conversion; additional genetic events were required. This conclusion is in agreement with Halaban *et al.* (57), who showed that constitutive activation of HGF/SF-Met alone did not confer the malignant phenotype to cultured mouse melanocytes. However, it is notable that all metastatic melanomas examined demonstrated high coexpression of HGF/SF and Met, suggesting that the metastatic phenotype was facilitated by strong autocrine signaling.

**HGF/SF-Met Signaling in Manifestation of the Metastatic Phenotype.** Significantly, >20% of HGF/SF-induced primary melanomas were metastatic to lymph nodes and/or visceral organs. Because metastatic melanoma is exceedingly rare in mice, these results indicate that one or more of the biological activities intrinsic to HGF/SF may be critical for the realization of the metastatic phenotype. For example, HGF/SF can stimulate the dissociation, movement, and invasion of isolated epithelial cells (73, 74). In addition, HGF/SF can induce the expression of both urokinase and its receptor and thereby activate focal degradation of extracellular matrix required for cellular invasion (75–78). Moreover, because endothelial cells express c-*met* and can respond to HGF/SF by proliferating, migrating, or stimulating the formation of capillary-like tubes, this cytokine can profoundly influence angiogenesis (reviewed in Ref. 34). These potent properties of HGF/SF could, either singly or in combination, contribute to tumor cell invasion and metastasis. In fact, fibroblastic NIH 3T3 and mammary C127 cells expressing both *HGF/SF* and c-*met* show enhanced motility and invasiveness *in vitro* and metastatic activity when reintroduced into athymic nude mice (35, 36). Moreover, HGF/SF stimulates a very strong invasive response in melanocytic cells as judged by movement through Matrigel (57). In contrast, we saw no apparent correlation between the growth rate of primary melanomas *in vivo* and their metastatic potential.

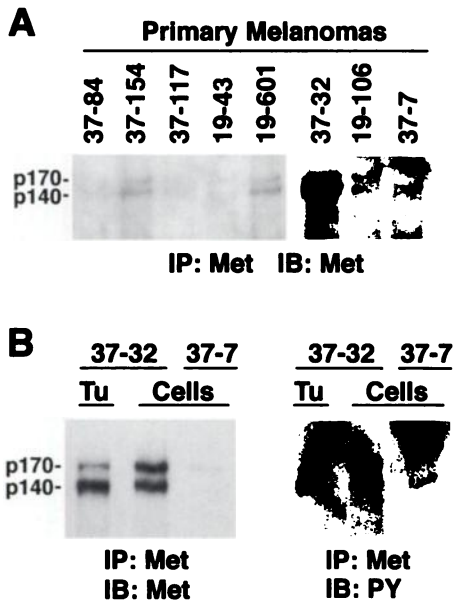


Fig. 7. Evaluation of Met protein levels and activity in HGF/SF-induced melanoma using Western blotting. In *A*, extracts were prepared from the same eight primary tumors shown in Fig. 6, and 200 $\mu$g of each were subjected to immunoprecipitation (*IP*) and then immunoblotting (*IB*) with an anti-Met antibody. In *B*, extracts from transplanted 37-32 tumor tissue (*Tu*) or primary cultured cells from both 37-32 and 37-7 were prepared, and 1 mg of each was subjected to immunoprecipitation with anti-Met antibody, and then immunoblotting was performed with either an anti-Met (*Met*) or an anti-phosphotyrosine (*PY*) antibody. The expected bands of $M_r$ 170,000 and $M_r$ 140,000 were observed.

Table 3 *Incidence and colonization site selection of experimental metastases*[a]

| | Whole body | | Individual organs | | | | | |
|---|---|---|---|---|---|---|---|---|
| Tumor cells | Gross | Microscopic | Liver | Lung | Spleen | Skin | Lymph node | Ovary |
| 37-32 | 6/7 | 7/7 | 7/7 | 7/7 | 3/7 | 1/7 | 1/7 | 1/7 |
| 37-7 | 2/7 | 7/7 | 0/7 | 7/7 | 0/7 | 0/7 | 0/7 | 0/7 |

[a] Number of athymic nude mice with metastases per number of mice receiving i.v. injections of $10^6$ cells after an average of 26 days. The preferred metastatic site for the 37-32 cells was the liver, in terms of incidence, number of colonies per tissue, and average size of tumors. Relative to the liver, lung tumors were usually much smaller and fewer in number in mice receiving injections of either 37-32 or 37-7 cells.

A number of lines of evidence indicate that non-Ras pathways in Met signaling are likely responsible for the relatively rare ability of HGF/SF to induce metastasis in melanoma cells. For example, phosphatidylinositol 3-kinase has been shown to be critically involved in Met-associated scattering, branching morphogenesis, proliferation, urokinase proteolysis, and tumor invasiveness (79–83). Moreover, a point mutation in c-*met* that superactivates Ras while inhibiting optimal interactions with other transducers, such as phosphatidylinositol 3-kinase, elevates transforming capability while eliminating metastatic potential (84). Perhaps most interestingly, the mice expressing activated c-H-*ras* in melanocytes on an *ink4a*-deficient background developed melanomas with short latency but without metastatic capacity (6).

It is noteworthy that upon introduction into nude mice by i.v. tail vein injection, 37-32 melanoma cells, which demonstrated very high c-*met* expression and activity, predominantly and aggressively colonized the liver, whereas 37-7 cells, which contained no detectable c-*met* activity, metastasized only to the lung. Although a small sample, this result is consistent with data generated by Rusciano *et al.* (60), who showed that B16 murine melanoma cells with enhanced expression of activated c-*met* (B16-LS9) were more motile and invasive and demonstrated a preference for metastatic spread to liver over lung. The two model systems differ in that HGF/SF transgenic melanoma cells activate endogenous Met through an autocrine mechanism, whereas the B16-LS9 cells are characterized by paracrine receptor activation. In another transgenic model not based on c-*met*, targeted expression of the viral oncogene T-antigen to melanocytes induced melanomas of ocular and cutaneous origin that metastasized predominantly to lymph nodes, lung, bone, muscle, and brain but apparently not to liver or spleen (85). Whether c-*met* activity specifically influences organ targeting or simply enhances overall colonization efficiency remains to be determined.

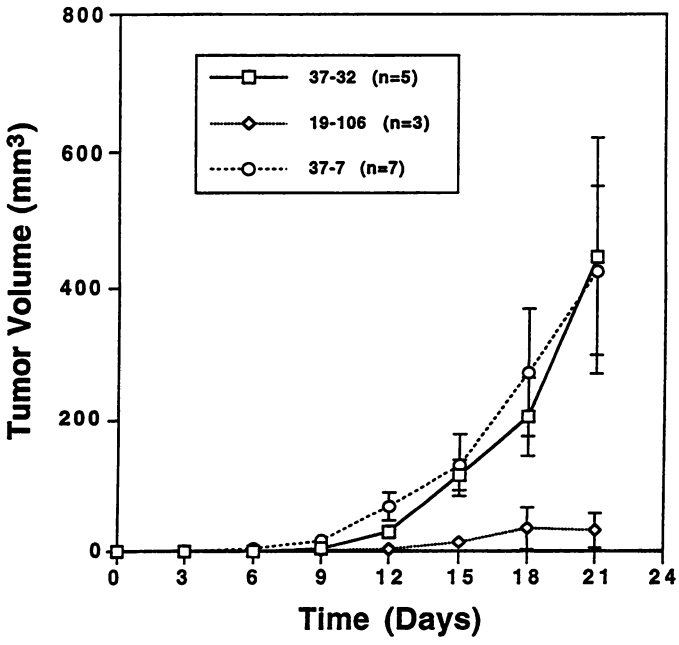


Fig. 8. Melanoma growth *in vivo*. Five to seven pieces of each of three different melanomas were transplanted s.c. into the flank of two to three athymic nude mice. The sizes of three to seven tumors for each type of melanoma were measured every 3 days; *bars*, SEM.

**HGF/SF Transgenic Mice as a Model for Human Malignant Melanoma.** The HGF/SF transgenic mouse represents a relatively rare and useful mammalian animal model for cutaneous melanocytic tumorigenesis. We believe this model is relevant because it is driven through the subversion of a mechanism whose molecular components already exist and function within the normal melanocyte. As described above, c-*met* is a proto-oncogene that has clearly been associated with malignant transformation, is sensitive to autocrine stimulation, and is expressed in wild-type melanocytes and melanomas. Moreover, a number of biological properties ascribed to HGF/SF-Met signaling could readily account for the metastatic capabilities of melanomas in these transgenic mice and in humans, including invasion and angiogenesis. Although the dermal origin of tumors in our transgenic mice are perhaps more comparable with malignant cellular nevus tumors and perhaps less like the more common malignant melanomas in humans, which are of epidermal melanocytic origin, the availability of this transgenic model may well permit the study of critical aspects common to all malignant melanocytic tumors.

As in humans, malignant melanoma in HGF/SF transgenic mice develops through a series of sequential stages that begin with an accumulation of melanocytes in the epidermis, dermis, and junction, which manifests as patterned hyperpigmentation in nonalbino strains. This initial phenotype alone may well predispose HGF/SF transgenic mice to melanoma. The enhanced melanocyte population provides a much larger target base for mutational events associated with tumorigenesis. Moreover, the relocation of melanocytes, usually confined to the follicular epithelia of the hair shaft in adult wild-type mouse skin, to an anomalous milieu may also relieve them of normal constraints on growth and movement, facilitating melanoma formation (86). Importantly, malignant melanoma in this model can then progress, becoming highly invasive and metastatic, permitting detailed study of these processes.

Because spontaneous melanomas are relatively rare, it may be significant that two of the other described animal models of melanoma are driven through mechanisms that perturb RTK function. One model is a fish of the genus *Xiphophorus*, which develops melanomas when aberrantly expressing the activated *Xmrk* gene, encoding a novel transmembrane RTK related to the EGF receptor (87, 88). In the other, a more closely related mouse model, ectopic expression of the activated *ret* oncogene, derived from the RTK encoded by c-*ret*, induced widespread hyperpigmentation of skin and development of melanocytic tumors at multiple sites (89). Spontaneous metastasis of primary tumors was not observed, although s.c. transplants of established cultured cells from *ret* transgenic tumors were found to possess metastatic capability (90). These results were somewhat surprising because c-*ret* expression has not been detected in melanocytes (91–93); however, these data support the notion that different RTKs can serve redundant roles in melanoma genesis by activating similar

critical signaling pathways. Finally, the involvement of RTKs in human melanoma is strongly supported by the observation that amplification and overexpression of the EGF receptor has been associated with melanoma progression (94, 95). Together, these studies indicate that subversion of RTK function may play an important role in the development and/or progression of malignant melanoma.

The HGF/SF transgenic mouse model, in concert with recently improved technical approaches to gene discovery, should prove to be a valuable asset in the continuing search for genes whose products contribute to the genesis of malignant melanoma, and especially to the acquisition of those phenotypic characteristics that permit select tumor cells to successfully disseminate and colonize metastatic sites. This search is paramount, because although it is widely appreciated that metastatic capability is both the most important determinant of lethality in human melanoma and the best target for efficacious therapeutics, the underlying mechanistic basis for metastasis is still poorly understood.

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