Roles of Stem Cell Factor/c-Kit and Effects of Glivec®/STI571 in Human Uveal Melanoma Cell Tumorigenesis*

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The B-RafV599E-mediated constitutive activation of ERK1/2 is involved in establishing the transformed phenotype of some uveal melanoma cells (Calipel, A., Lefèvre, G., Pouponnott, C., Mouriaux, F., Eychene, A., and Mascarelli, F. (2003) J. Biol. Chem. 278, 42409–42418). We have shown that stem cell factor (SCF) is involved in the proliferation of normal uveal melanocytes and that c-Kit is expressed in 75% of primary uveal melanomas. This suggests that the acquisition of autonomous growth during melanoma progression may involve the SCF/c-Kit axis. We used six human uveal melanoma tumor-derived cell lines and normal uveal melanocytes to characterize the SCF/c-Kit system and to assess its specific role in transformation. We investigated the possible roles of activating mutations in c-KIT, the overexpression of this gene, and ligand-dependent c-Kit overactivation in uveal melanoma cell tumorigenesis. Four cell lines (92.1, SP6.5, Mel270, and TP31) expressed both SCF and c-Kit, and none harbored the c-Kit mutations in exons 9, 11, and 13 and 17 that have been shown to induce SCF-independent c-Kit activation. Melanoma cell proliferation was strongly inhibited by small interfering RNA-mediated depletion of c-Kit in these cells, despite the presence of V599E/B-Raf in SP6.5 and TP31 cells. We characterized the signaling pathways involved in SCF/c-Kit-mediated cell growth and survival in normal and tumoral melanocytes and found that constitutive ERK1/2 activation played a key role in both the SCF/c-Kit autocrine loop and the gain of function of V599E/B-Raf for melanoma cell proliferation and transformation. We also provide the first evidence that Glivec®/STI571, a c-Kit tyrosine kinase inhibitor, could be used to treat uveal melanomas.

Many components of the mitogenic signaling pathways in normal and neoplastic cells have been identified. They include the large family of protein kinases, which play central roles in diverse biological processes, such as the control of cell growth, differentiation, and apoptosis (1–4). Protein kinases, including receptor tyrosine kinases, are considered to be prime targets for the development of selective inhibitors for tumor treatment because they are frequently deregulated in human cancers. Constitutive receptor tyrosine kinase activation has been shown to be important for malignant transformation and tumor proliferation and may occur by several mechanisms. In most cases, gene amplification, overexpression, mutations, and ligand-dependent or ligand-independent overactivation are responsible for the acquired transforming potential of oncogenic receptor tyrosine kinases (5, 6).

The proto-oncogene c-KIT is the cellular homolog of the viral oncogene of the feline sarcoma retrovirus, HA-FeSV. c-Kit is a type III receptor tyrosine kinase. This group of kinases includes the platelet-derived growth factor receptor (PDGFRs),1 colony-stimulating factor receptor, and Fit-3. The ligand for c-Kit has been identified as stem cell factor (SCF), which is also referred to as Kit ligand, Steel factor, or mast cell factor. The c-KIT gene product is essential for the maintenance of normal hematopoiesis, melanogenesis, the development of cutaneous melanocytes, gametogenesis, and the growth and differentiation of mast cells and interstitial cells of Cajal (7). The deregulation of c-Kit is thought to play a role in certain tumors, including cutaneous melanomas, germ cell tumors, mast cell tumors, gastrointestinal stromal tumors (GISTs), small cell lung cancers (SCLCs), breast cancers, and neuroblastomas (8–12). The SCF/c-Kit axis may mediate solid tumor development by autocrine or paracrine stimulation of the receptor or specific mutation of c-KIT, resulting in ligand-independent activation of the receptor (13–16). Although SCF stimulation has been shown to activate various intracellular signaling pathways (17, 18), the precise role of c-Kit in solid tumor pathogenesis is unclear. The pharmacological compound Glivec® (formerly known as CGP 57714B, STI571, Imitinib, or Gleevec™), a 2-phenylaminopyrimidine derivative, inhibits the tyrosine kinase activity of c-ABL, BCR-ABL, and PDGFRs (19, 20). It also inhibits c-Kit activation, due to the high level of similarity between the kinase domains of PDGFRs and c-Kit (21). Glivec® inhibits both the autocrine activation of c-Kit in SCLCs and myeloid leukemia cell lines (22, 23) and c-Kit activation by mutation in

1 The abbreviations used are: PDGFR, platelet-derived growth factor receptor; PDGF, platelet-derived growth factor; SCF, stem cell factor; GIST, gastrointestinal stromal tumor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; NUM, normal uveal melanocyte; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; RT, reverse transcriptase; siRNA, small interfering RNA.
GISTs and in the HMC-1 mast cell leukemia cell line (23, 24). However, recent studies have demonstrated limitations to the use of Glivec®, which does not inhibit the kinase activity of the exon 17 c-Kit mutant associated with mastocytosis (25).

Uveal melanocytes are involved in the pathogenesis of various proliferative eye diseases, including malignant melanoma, the most common primary malignant eye tumor in adults. During malignant transformation, cutaneous melanocytes acquire the ability to express and to secrete autocrine growth factors. The oncogenic behavior of cutaneous melanoma results from a combination of autocrine growth factor stimulation and activating mutations in BRAF and RAS. Downstream intracellular signaling pathways, such as the MAPK or ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) module, which controls growth, cell survival, and transformation, are deregulated (26). Few or no mutations have been found in the genes encoding proteins of the major intracellular signaling pathways controlling cell proliferation in human uveal melanomas, including those affected in cutaneous melanoma, with the exception of V599E-Raf, for which mutations have been detected in a few uveal melanoma cell lines (27–33). Thus, the deregulation of growth factor signaling may be more important in uveal melanoma than in cutaneous melanoma tumorigenesis. We have demonstrated that c-Kit is activated by exogenous SCF during normal uveal melanocyte (NUM) proliferation and probably also during pathogenic bilateral and diffuse uveal cell proliferation (34). We have also shown that c-Kit is expressed in 75% of primary choroidal melanomas (35). Mitotic activity and c-Kit immunoreactivity are positively correlated, suggesting that c-Kit is involved in the transformation of normal uveal melanocytes into tumor cells (35). However, the roles of SCF/c-Kit and of the downstream intracellular signaling pathway have been evaluated during the transformation of normal uveal melanocytes. Fiorentini et al. (36) used Glivec® in a few cases of metastatic uveal melanomas and obtained mixed results, suggesting that the molecular mechanisms involved in uveal melanomas were more complex than previously suspected.

We therefore investigated the exact role of c-Kit in uveal melanocyte transformation. We investigated c-Kit and SCF expression, the biological effects of exogenous and endogenous SCF, SCF/c-Kit downstream signaling, and the possible involvement of activating mutations of c-Kit in the proliferation, transformation, and survival of normal and tumoral human uveal melanocytes. Finally, we investigated the potential therapeutic role of chemical agents, such as Glivec®, in controlling uveal melanoma cell tumorigenesis.

### EXPERIMENTAL PROCEDURES

#### Cell Cultures

NUM were isolated, as previously described (37), from human enucleated eyes (generously provided by Prof. P. Gain, Faculté de Médecine, Saint-Etienne, France). NUM were cultured in FIC medium (f-12 Ham medium supplemented with 5% FCS, 2.5 μg/ml fungizone/amphotericin B, 2 mM l-glutamine (Invitrogen), 10 ng/ml cholera toxin (Calbiochem), 0.1 mM isobutyl-methylxanthine (Sigma), and 10 ng/ml FGF2 (obtained from Dr. H. Guerin, Centre Hospitalo-Universitaire de Quebec, Canada) cell lines were cultured in RPMI 1640 medium supplemented with 5% FCS, 2.5 μg/ml fungizone/amphotericin B, 50 μg/ml gentamicin, and 2 mM l-glutamine (Invitrogen). NUM and melanoma cells were cultured at 37 °C in a humidified air/CO2 (19:1) atmosphere.

#### Priming Assay

The molecular aspects of cell proliferation were investigated by treating cells with specific pharmacological inhibitors of signaling pathways or immunoblocking antibodies. Stock solutions were made up in Me2SO such that the final concentration of Me2SO in culture medium did not exceed 0.1%, a concentration shown to have no effect on the proliferation of NUM and melanoma cells. Cells were seeded in triplicate in 24-well plates at a density of 3.5 × 104 cells/well for NUM and 1.5 × 105 cells/well for melanoma cells. The plates were incubated for 3 days and were then treated with 1) recombinant human SCF (BIOSOURCE), 2) immunoblocking anti-c-Kit monoclonal antibody (clone K44.2, BIOSOURCE), 3) UO126 and Akt inhibitor (Calbiochem), and 4) Glivec® (kindly provided by W.-M. Weber, Novartis Pharma AG, Basel, Switzerland). With the exception of recombinant human SCF, inhibitors were added 2 h before cell proliferation induction and at induction. The number of viable cells was determined by the MTT colorimetric method after 3 days of culture in low serum conditions (0.5% FCS). The percentage of growth inhibition was calculated with respect to control Me2SO-treated cells.

#### Cell Cycle Progression and Cell Apoptosis Analysis

Cell cycle analysis were performed by determining DNA content with propidium iodide (PI) after 24 h in culture. Cells were washed in PBS and fixed in ice-cold 70% ethanol for at least 2 h at 4 °C. They were rehydrated in cold PBS, treated with 1 mg/ml RNase A (Roche Applied Science), and stained with 50 μg/ml PI for at least 15 min at 4 °C. The stained cells were analyzed by flow cytometry (Epics ALTRA, Beckman Coulter).

### Apoptotic cell death was analyzed with the annexin-V-EGFP and PI double staining method (ApoAlert® Annexin V-EGFP kit, Clontech) according to the manufacturer’s recommendations. Cells were treated for 48 h and then incubated briefly with trypsin, rinsed twice in serum-containing medium, and rinsed once in 1× binding buffer. They were then incubated with annexin-EGFP and PI for 15 min at room temperature. The percentages of apoptotic (annexin V-positive) and necrotic (IP-positive) cells were determined by flow cytometry.

#### Transformation Assay

Cell transformation was analyzed in a clonogenic assay by determining the ability of cells to form colonies in methylcellulose under anchorage-independent conditions. Melanoma cells were suspended in complete medium containing 0.8% methylcellulose (Fluka Methocel MC; Sigma) and either Glivec® or vehicle. They were then plated in 35-mm culture dishes (5500 (OCM-1) or 7000 (Mel270) cells/well) and incubated at 37 °C for 2 weeks. Colonies in three randomly chosen 9-cm2 areas were counted every 2 days from day 10 to day 16 of the culture period.

#### Genomic DNA Purification, PCR, and Mutation Screening

Genomic DNA was prepared from normal melanocytes and melanoma cell lines according to standard procedures, checked by electrophoresis in a 0.8% agarose gel, and quantified by spectrophotometry. We analyzed exons 9, 11, 13, and 17 of the human C-KIT gene, using specific PCR primers designed to amplify each exon plus its flanking intron sequences (Table 1). The primers for amplification of exon 15 of the human BRAF gene have been described elsewhere (31). PCR amplification was carried out in a total volume of 25 μl, containing 250 ng of genomic DNA, 0.5 μM each primer, 0.2 mM each dNTP, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 1.5 mM MgCl2, and 1.25 IU of Platinum High Fidelity TaqDNA polymerase (Invitrogen). DNA was denatured by heating at 95 °C for 5 min and was then amplified by 35 cycles of 95 °C for 30 s, annealing of the primer pair at 56 °C for 30 s, and extension at 72 °C for 60 s. The last cycle was extended by a 10-min elongation step at 72 °C. The amplified products were run on a 1.5% agarose gel and purified with the Qiagen

### Table I

| Exon | Exon size (bp) | Forward (5’–3’) | Reverse (5’–3’) | PCR product size (bp) |
|------|---------------|----------------|----------------|----------------------|
| 9    | 194           | TATGGCACATCCCACAGTG | GGTTGAGTGCATGATTACC | 390 |
| 11   | 127           | GAGTGCTCTAATGACTGAG | CCACCTGGATCTCTAAGAAG | 266 |
| 13   | 111           | GCACTAGCTGCTAAGGTOG | GCAGAGAGCACACAGAG | 293 |
| 17   | 111           | GTGACACATCTACAGGCG | TTACATTATAAATACACAGG | 390 |
MinElute purification kit (Qiagen) before sequencing (MWG Biotech).

**RT-PCR Analysis—**Total RNA was extracted from uveal melanoma cells by the single-step Trizol method and treated with amplification grade DNase I for 15 min at room temperature. For NUM, mRNA was extracted directly, using the MACS mRNA isolation kit (Miltenyi Biotec). We subjected 1 μg of RNA (for uveal melanoma cells) and 100 ng of mRNA (for normal melanocytes) to reverse transcription for 1 h at 42 °C, using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase. We then determined the level of expression of c-Kit and SCF genes by amplifying the cDNAs obtained by semiquantitative PCR for 35 cycles with annealing at 56 °C. Glyceraldehyde-3-phosphate dehydrogenase and β-actin levels, determined after 22 cycles of amplification, were used to control for loading and constant expression. The following primers were used: c-Kit, forward (5'-TGACTTACGACAGGCTCGTG-3') and reverse (5'-AAGGAGTGAACAGGGTGTAAGG-3') (327 bp); SCF, forward (5'-ATGAAAGAGAAGACAAACTTGGAT-3') and reverse (5'-GGTACCCACTTCTGTAAACTTCACTTCTCT-3') (818 and 734 bp); glyceraldehyde-3-phosphate dehydrogenase, forward (5'-GGCATCACCACGAAAATCTCCA-3') and reverse (5'-GGTGCTTTGGGCCGGGATTAGTC-3') (546 bp); β-actin, forward (5'-AGGAAGAACGCGTGCTAGCTC-3') and reverse (5'-AGGGCCCCGACTGCTATAC-3') (470 bp).

**Immunohistochemistry—**Paraffin-embedded primary tumor specimens of human uveal melanomas were obtained from the Ophthalmology Department (CHRU Lille, France); 4-μm sections were mounted on silane-coated glass slides. Antigen was retrieved by heating in 10 mm citrate buffer, pH 6.0, in an oven. Standard avidin-biotin peroxidase immunohistochemistry was performed with rabbit polyclonal anti-c-Kit and mouse monoclonal anti-SCF antibodies (C19 (1:400) and H-189 (1:20), respectively; Santa Cruz Biotechnology, Inc.). Aminoethylcarbazole was used as the chromogen. The slides were mounted in a water-miscible mounting medium after counterstaining with Mayer's hematoxylin.

**Western Blot Analysis—**NUM and OCM-1 cells were washed twice in PBS, lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, 1% deoxycholate, 50 mM β-glycerophosphate, 0.2 mM sodium orthovanadate, 50 mM sodium fluoride, 1 μg/ml leupeptin, 5 μg/ml pepstatin, 20 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 4 °C for 10,000 × g for 30 min at 4 °C. Protein concentrations were determined with the Bio-Rad protein assay kit. Cell lysates were mixed with 3× Laemmli buffer and heated for 5 min at 95 °C. They were then resolved by SDS-PAGE (10 or 15% polyacrylamide gels) and transferred to polyvinylidene difluoride membrane (Immobilon™; Millipore Corp.) by electrotransfer. To analyze c-Kit and SCF levels, membranes were probed with a rabbit polyclonal antibody against c-Kit and a mouse monoclonal antibody against SCF (1:200; Santa Cruz Biotechnology). Polyclonal antibodies directed against phospho-c-Kit (Y705) (1:100, Zymed Laboratory), phospho-c-Kit (Y719), phospho-Akt (Ser473), and phospho-ERK1/2 (Thr202/Tyr204) (1:1000; Cell Signaling Technology) were used to analyze the activation of these kinases during the proliferation of NUM and melanoma cells. Membranes were probed with a rat monoclonal antibody directed against α-tubulin (1:1000, Serotec) to control for equal loading. These primary antibodies were tagged with specific secondary horseradish peroxidase-conjugated antibodies. Antibody complexes were detected by ECL (Amersham Biosciences), and the membrane was placed against BioMax Light-1 film (Eastman Kodak Co.).

**Inhibition of c-Kit Expression by Small Interfering RNA (siRNA)—**Uveal melanoma cells were plated at 50–60% confluence in complete melanoma cell culture medium in 24-well plates and were incubated for 24 h. They were then transiently transfected in OptiMEM I medium (Invitrogen) with LipofectAMINE 2000 reagent (Invitrogen) and either control or anti-c-Kit siRNAs (800 ng/ml), using the Sure Silencing human KIT siRNA kit, as recommended by the manufacturer (Super-Array BioScience Co.). Five hours later, the Optimem I medium was replaced by complete culture medium. Effects on cell proliferation and death and c-Kit down-regulation were assessed 72 h after transfection, by MTT assay, annexin V-based fluorescence-activated cell sorting analysis, and Western blotting, as previously described.

**Statistics—**Two-tailed Student's t tests (normal distributions with equal variances) and Mann and Whitney tests (nonparametric tests) were used for statistical analysis.

**RESULTS**

**Exogenous SCF Is a Poor Mitogen for Uveal Melanoma Cells, Despite c-Kit Expression—**Mutations in the major intracellular signaling pathway controlling cell proliferation were rare or absent in human uveal melanoma cells, in contrast to what has been observed for cutaneous melanoma cells. This suggests that the deregulation of growth factor signaling may play a central role in uveal melanoma tumorigenesis. Since exogenous SCF stimulates normal human uveal melanocyte proliferation (34), we thought that SCF might be involved in uveal melanoma growth. We tested this hypothesis by analyzing the expression of SCF and its receptor, c-Kit, in six primary uveal melanoma tumors. We demonstrated by RT-PCR that all of the tumors expressed c-Kit and that four also expressed SCF, albeit at very low levels in one patient (patient 11) (Fig. 1A). The expression of these two proteins was confirmed by immunohistochemistry on uveal melanoma cells (Fig. 1B). The large pro-
portion of primary uveal melanomas expressing both SCF and c-Kit suggests the possible involvement of SCF/c-Kit in these tumors.

We used primary uveal melanoma tumor-derived cell lines to characterize the role of the SCF/c-Kit signaling in transformation. We investigated the effects of exogenous SCF on uveal melanoma cells by analyzing the mitogenic activity of this molecule in six human uveal melanoma cell lines (OCM-1, MKT-BR, 92.1, SP6.5, Mel270, and TP31) and in control normal uveal melanocytes. NUM proliferation was stimulated by 50 ng/ml SCF (Fig. 2A), confirming our previous results (34). Three of the six uveal melanoma cell lines (OCM-1, MKT-BR, and 92.1) were insensitive to SCF stimulation, whereas the other three (SP6.5, Mel270, and TP31) responded to SCF stimulation but much more weakly than normal melanocytes (Fig. 2A). The surprising inefficiency of high concentrations of exogenous SCF in the stimulation of uveal melanoma cell proliferation may be due to 1) a lack of c-Kit expression on the cell surface in the melanoma cell lines, 2) the presence of activating mutations in c-Kit, conferring ligand independence, 3) the uncoupling of SCF stimulation of melanoma cells by secreted endogenous SCF, rendering cells insensitive to further stimulation with exogenous SCF, or 4) the presence of the V600E-Raf activating mutation, rendering stimulation with exogenous SCF unnecessary for maximal rates of cell proliferation.

**Fig. 2. Effects of SCF on cell proliferation and expression of c-Kit and SCF mRNA and protein in normal uveal melanocytes and uveal melanoma cells.**

A. Effects of exogenous SCF on the proliferation of NUM and six uveal melanoma cell lines were investigated, using the MTT colorimetric method. Cells were cultured with FIC medium supplemented with 20% FCS (NUM) or RPMI 1640 medium supplemented with 5% FCS (melanoma cells) for 3 days. Cell cultures were then stimulated with FIC medium supplemented with 0.5% FCS (NUM) or RPMI 1640 medium supplemented with 0.5% FCS (melanoma cells) in the presence or absence of SCF (50 ng/ml) for 3 days. The percentage growth induction was calculated with respect to control PBS-treated cells. The levels of c-Kit and SCF mRNAs were determined by RT-PCR (B) (see the legend to Fig. 1 for an explanation of the two bands for SCF), and those of c-Kit and SCF proteins were determined by Western blotting (C), as described under Experimental Procedures. The 145- and 125-kDa isoforms of c-Kit correspond to the mature and immature forms of the receptor, respectively. The 43- and 31-kDa isoforms of SCF are the translation products of the alternatively spliced mRNAs. The data presented are representative of three independent experiments performed in triplicate.

We therefore cannot rule out the possibility that there are SCF-independent activating mutations of c-Kit, an autocrine activation loop involving secreted SCF, or BRAF gain-of-function mutations in the four c-Kit-positive melanoma cell lines. Activating point mutations in exons 9, 11, 13, and 17 of c-Kit, inducing the SCF-independent activation of c-Kit, were associated with a number of malignancies such as GISTs, mastocytosis, and some leukemias. Such mutations have therefore been implicated in tumorigenesis (23, 24). We screened c-Kit exons 9, 11, 13, and 17 and their respective flanking intron sequences for mutations in the 92.1, SP6.5, Mel270, and TP31 cell lines to exogenous SCF. However, these data did not account for the weak responses of the 92.1, SP6.5, Mel270, and TP31 cell lines to exogenous SCF.

**V600E-Raf and SCF/c-Kit Expression Is Not Mutually Exclusive in Uveal Melanoma Cells**—We investigated the expression of c-Kit and SCF in NUM and in the six melanoma cell lines. RT-PCR confirmed that SCF-responsive normal melanocytes expressed c-Kit mRNA (Fig. 2B). C-Kit mRNA was also detected in the 92.1, SP6.5, Mel270, and TP31 cell lines but not in the OCM-1 and MKT-BR cell lines (Fig. 2B). These results were confirmed by Western blotting, which showed that c-Kit protein and transcript profiles were similar, both in NUM and in melanoma cell line cultures (Fig. 2C). In subsequent experiments, the OCM-1 and MKT-BR cell lines were therefore considered to be c-Kit-negative and the 92.1, SP6.5, Mel270, and TP31 cell lines were considered to be c-Kit-positive. The high rate of melanoma cell proliferation in basal culture medium (without SCF) cannot be due to c-Kit overproduction, because NUM produced similar amounts of c-Kit to TP31 melanoma cells and more c-Kit than the other five melanoma cell lines. Furthermore, c-Kit levels generally mirrored the proliferative responses of melanoma cells to exogenous SCF. This is consistent with the lack of a mitogenic effect of exogenous SCF in the OCM-1 and MKT-BR cell lines, due to the absence of c-Kit.

However, these data did not account for the weak responses of the 92.1, SP6.5, Mel270, and TP31 cell lines to exogenous SCF. We therefore cannot rule out the possibility that there are SCF-independent activating mutations of c-Kit, an autocrine activation loop involving secreted SCF, or BRAF gain-of-function mutations in the four c-Kit-positive melanoma cell lines.

We then investigated whether there was an SCF/c-Kit autocrine loop in uveal melanoma cells. RT-PCR and Western blot analyses showed that SCF mRNAs and proteins were present in NUM and in the six melanoma cell lines (Fig. 2 B and C). However, enzyme-linked immunosorbent assay showed that only melanoma cells secreted significant amounts of SCF (50.8–120 pg/ml of conditioned medium) (Table II). No SCF was detected in the culture medium of NUM, confirming the absence of an SCF/c-Kit autocrine loop in normal melanocyte proliferation (Table II). Thus, an autocrine activation loop, involving the production and secretion of endogenous SCF, may be involved in the high rate of proliferation of 92.1, SP6.5, Mel270, and TP31 uveal melanoma cells.
The sequences of exon 15 of \(BRAF\) and exons 9, 11, 13, and 17 of \(C-KIT\) were analyzed in NUM and uveal melanoma cell lines after PCR amplification and sequencing of genomic DNA, as described under “Experimental Procedures.” SCF secretion into the culture media of NUM and melanoma cells was analyzed by enzyme-linked immunosorbent assay (R&D Systems). SCF secretion into the cell-conditioned culture media was quantified after 48 h of culture and is expressed in pg/ml culture media. +, presence; −, absence.

| Cells          | c-Kit Mutations in Exons 9, 11, 13, or 17 | SCF Mutations in Exon 15 | SCF Expression in pg/ml | B-Raf mutation in exon 15 | SCF Secretion in pg/ml |
|----------------|------------------------------------------|--------------------------|--------------------------|--------------------------|------------------------|
| NUM            | +                                       | +                        | +                        | +                        | +                      |
| OCM-1          | −                                       | −                        | +                        | 99.2 V599E               | +                      |
| MKT-BR         | −                                       | −                        | +                        | 80.8 V599E               | +                      |
| 92.1           | +                                       | +                        | +                        | 95.8 V599E               | +                      |
| SP6.5          | +                                       | +                        | +                        | 61.7 V599E               | +                      |
| Me1270         | +                                       | +                        | +                        | 120.0 V599E              | +                      |
| TP31           | +                                       | +                        | +                        | 50.8 V599E               | +                      |

The lack of such an SCF/c-Kit autocrine activation loop for cell proliferation in the OCM-1 and MKT-BR cell lines may be accounted for by the presence of the \(V599E\) B-Raf activating mutation, which is involved in the acquisition of growth autonomy (31). The c-Kit-positive cell line SP6.5 also harbors the \(V599E\) B-Raf mutation. We cannot rule out a cooperative effect between the gain-of-function B-Raf mutation and SCF/c-Kit autocrine loop activation in the acquisition of self-sufficiency in growth signals in transformed cells. We therefore screened for mutations in exons 11 and 15 of the \(BRAF\) gene in the other three c-Kit-positive cell lines: Mel270, 92.1, and TP31. BRAF exons 11 and 15 were amplified by PCR from genomic DNA and sequenced. No mutations were detected in exon 11 in any of these melanoma cell lines (Table II). In contrast, a single-base substitution was detected in exon 15 in the TP31 melanoma cell line (Table II). This T1796A substitution leads to the replacement of a valine by a glutamic acid at position 599 (V599E). No mutation in exon 15 of the \(BRAF\) gene was detected in the Mel270 and 92.1 cell lines (Table II).

These data suggest that there is an autocrine activation loop, involving the production of SCF and c-Kit and the secretion of endogenous SCF, in some melanoma cells. This SCF/c-Kit autocrine loop may be specifically involved in the acquisition of autonomous growth in 92.1 and Me1270 uveal melanoma cells. In the SP6.5 and TP31 cell lines, the presence of the \(V599E\) B-Raf mutation does not exclude SCF/c-Kit autocrine loop activation, since these two phenomena may cooperate in the acquisition of autonomous growth. In contrast, for the two c-Kit-negative cell lines, OCM-1 and MKT-BR, the \(V599E\) B-Raf mutation seems to be the major mechanism underlying oncogenic behavior. These data demonstrate considerable heterogeneity between the various uveal melanoma cells, which expressed different combinations of wild-type B-Raf, \(V599E\) B-Raf, wild-type c-Kit, and SCF (Table II).

The Autocrine Loop of SCF/c-Kit Activation Controls Uveal Melanoma Cell Proliferation and Survival—To confirm the existence of an SCF/c-Kit autocrine activation loop in uveal melanoma cells, we first treated NUM and the melanoma cell lines with a monoclonal anti-c-Kit immunoblocking antibody and then stimulated them with serum. The inhibition of c-Kit tyrosine kinase activity by incubation with 250 ng/ml anti-c-Kit immunoblocking antibody reduced cell proliferation by 22, 42, 58, and 54% in the 92.1, SP6.5, Me1270, and TP31 cell lines (Fig. 3A). In contrast, treatment of the c-Kit-negative cells MKT-BR and OCM-1 reduced cell proliferation by only 3 and 8%, respectively, confirming that SCF has no significant effect in these two cell lines and demonstrating the specificity of the c-Kit immunoblocking antibody (Fig. 3A). The lack of such an SCF/c-Kit autocrine activation loop in normal uveal melanocytes and in uveal melanoma cell lines did not lead to an SCF/c-Kit autocrine activation loop (Fig. 3A). Control experiments with nonimmune antibody at the same concentration did not affect cell proliferation (data not shown). No significant difference in the inhibitory efficiency of c-Kit blockade was observed between the \(V599E\) B-Raf-mutated (SP6.5 and TP31) and wild-type B-Raf (Mel270 and 92.1) c-Kit-positive cell lines.

We used an siRNA-based approach to down-regulate c-Kit protein expression rather than blocking c-Kit activity. The transfection of c-Kit-positive cells with control siRNA had no effect on c-Kit levels. In contrast, c-Kit-specific siRNA strongly down-regulated c-Kit protein levels and reduced the rate of cell proliferation by 66 and 62% in SP6.5 and Me1270, respectively (Fig. 3, B and C). The efficiency of cell proliferation inhibition following c-Kit down-regulation was similar in \(V599E\) B-Raf mutation-harboring (SP6.5) and \(V599E\) B-Raf mutation-free (Me1270) cell lines. Thus, c-Kit plays a key role in the direct control of uveal melanoma cell proliferation, and c-Kit activation is necessary and sufficient for the acquisition of autonomous growth. Flow cytometry analysis showed that inhibition of the SCF/c-Kit autocrine activation loop by siRNA also induced melanoma cell death (44.2 versus 16.9% dead cells in treated versus untreated cells) (Fig. 3D).

Thus, the SCF/c-Kit autocrine activation loop plays a key role in controlling uveal melanoma cell proliferation and survival and could therefore be used as a target in the treatment of uveal melanomas.

Determination of the Intracellular Signaling Pathways Involved in the SCF/c-Kit Autocrine Activation Loop Controlling Uveal Melanoma Tumorigenesis—We investigated the specific intracellular signaling pathways involved in the maintenance of SCF/c-Kit stimulation of uveal melanoma cell growth, with a view to developing a c-Kit-based strategy for treating uveal melanomas. The stimulation of normal and tumor cells with SCF activates two major intracellular signaling pathways (the ERK and protein kinase B/Akt pathways), and the pattern of activation of these two pathways depends on cell type (17, 18).

We first tried to determine which pathway was activated during exogenous SCF-stimulated NUM proliferation by studying the activation of c-Kit and of the two potential downstream intracellular signaling pathways: ERK1/2 and Akt. These two independent signaling pathways are activated following the SCF-induced activation of c-Kit via the phosphorylation of specific tyrosine residues: mainly Tyr703 and Tyr719 for ERK1/2 and Akt, respectively (18, 38). We investigated the phosphorylation status of c-Kit by Western blot analysis, using two antibodies that specifically recognize active c-Kit, phosphorylated on Tyr703 and Tyr719, respectively. No phosphorylated c-Kit Tyr703 or c-Kit Tyr719 was detected in the absence of NUM stimulation with exogenous SCF, confirming the absence of the SCF/c-Kit autocrine activation loop in normal cells (Fig. 4A). Conversely, phosphorylation of both the c-Kit Tyr703 and c-Kit Tyr719 forms was rapidly detected (within 5 min) after SCF stimulation (Fig. 4A), suggesting that both downstream signaling pathways, ERK1/2 and Akt, were simultaneously activated in NUM following stimulation with exogenous SCF. We investigated ERK1/2 and Akt phosphorylation status by Western blot analysis, using two antibodies specifically recognizing active ERK1/2 and Akt, respectively. No ERK1/2 phosphorylation/activation and only slight basal phosphorylation/activation of Akt could be detected in the absence of SCF stimulation.
FIG. 3. Effects of c-Kit inhibition and depletion on the proliferation and death of normal uveal melanocytes and uveal melanoma cells. A, to investigate the effects of c-Kit immunoblocking, NUM and uveal melanoma cell lines were cultured in their respective complete culture media for 3 days. Cells were then cultured in the presence of 0.5% FCS and treated with an anti-c-Kit immunoblocking antibody at the indicated concentrations. The proliferation of NUM and of the six uveal melanoma cell lines was investigated after 3 days of treatment, using the MTT colorimetric method. Control experiments were performed with a nonimmune antibody at the same concentrations. The percentage of growth inhibition was calculated with respect to control cells. (B–D). We investigated the effects of c-Kit depletion by siRNA on c-Kit-positive uveal
in NUM (Fig. 4A). In contrast, the rapid activation of both ERK1/2 and Akt was observed within 5 min of NUM stimulation with SCF (Fig. 4A), consistent with the activation of c-Kit by phosphorylation of both c-Kit Tyr703 and Tyr719 after SCF stimulation.

We then investigated whether similar signaling pathways were activated during the SCF/c-Kit-stimulated proliferation of the c-Kit-positive uveal melanoma cell line, Mel270. Surprisingly, no activated forms of c-Kit Tyr719 were found in proliferating melanoma cells, suggesting that Akt signaling is not constitutively activated by the SCF/c-Kit autocrine activation loop in melanoma cells (Fig. 4B). In contrast, the c-Kit Tyr703 form was strongly activated in Mel270, consistent with constitutive ERK1/2 activation by the SCF/c-Kit autocrine loop in uveal melanoma cells (Fig. 4B). This hypothesis was confirmed by an analysis of ERK1/2 activation (Fig. 4B). Thus, ERK1/2 activation seems to be necessary and sufficient for the acquisition of autonomous growth induced by the SCF/c-Kit autocrine loop in uveal melanoma cells, whereas both the ERK1/2 and Akt signaling pathways are activated in SCF-stimulated NUM proliferation.

To demonstrate that the activation of ERK1/2, but not of Akt, was involved in SCF/c-Kit-controlled melanoma cell proliferation, we inhibited each of these two signaling pathways separately and then analyzed melanoma cell proliferation. The four c-Kit-positive melanoma cells were treated with either U0126 (a specific inhibitor of MEK1/2 (39), the kinases directly upstream of ERK1/2) or a specific Akt inhibitor (40). Cell proliferation analysis with the MTT assay showed that the inhibition of MEK1/2 by 2 μM U0126 completely abolished melanoma cell proliferation (92–98% inhibition) (Fig. 5A). Conversely, melanoma cell treatment with 5 μM Akt inhibitor had no significant effect on melanoma cell proliferation (2–13% inhibition) (Fig. 5).

Thus, the SCF/c-Kit autocrine loop controlled cell proliferation via exclusive, constitutive activation of the MEK/ERK module in melanoma cells, suggesting that this module is sufficient for melanoma cell proliferation. Conversely, the stimulation of c-Kit induced the activation of ERK1/2 and Akt in NUM, suggesting that both signaling pathways are required for exogenous SCF-stimulated NUM proliferation.

**Effects of Glivec® on Cell Growth and Transformation and Characterization of the Glivec®-targeted Intracellular Signaling Pathway Downstream from the SCF/c-Kit Activation Loop—Signal transduction pathway components have become targets for the development of novel treatments designed to block the proliferation of cancer cells. Glivec®/STI571 was optimized for selective inhibition of the Bcr-Abl tyrosine kinase fusion protein and is currently used to treat chronic myelogenous leukemia (11, 19). In addition to its considerable potential for the treatment of chronic myelogenous leukemia, Glivec® is of potential therapeutic value for other cancers, since it also inhibits c-Kit in vitro (23, 24). We therefore investigated the effects of Glivec® on uveal melanoma cells.

We first assessed the effects of Glivec® on uveal melanoma cell proliferation and survival. Cells were treated with various concentrations of Glivec® and analyzed by the MTT method. Glivec® reduced cell proliferation in a concentration-dependent manner but with various efficiencies (Fig. 6A). The efficiency of Glivec®, determined by the concentration of Glivec® necessary to inhibit cell proliferation by 50% (IC50), depended on c-Kit levels in the six uveal melanoma cell lines. SP6.5, Mel270, TP31, and 92.1 were the most sensitive to Glivec® treatment, with IC50 of 1.7, 1.9, 2.4, and 4.4 μM, respectively (Fig. 6A). Consistent with the hypothesized role of the SCF/c-Kit autocrine activation loop in the control of uveal melanoma cell proliferation, the OCM-1 and MKT-BR cell lines were much less sensitive than the c-Kit-positive cells to Glivec® treatment. Cell proliferation was only partly inhibited, even with 10 μM Glivec®, in c-Kit-negative melanoma cells (Fig. 6A). The complete insensitivity of normal melanocytes to melanoma cells by plating wild-type B-Raf (Mel270) and mutated v599E-B-Raf (SP6.5) melanoma cells in complete melanoma cell culture medium at 50–60% confluence and incubating for 24 h. Cells were transiently transfected, using the Sure Silencing human KIT siRNA kit, as described under “Experimental Procedures.” B, C-Kit down-regulation was assessed 72 h post-transfection by Western blot analysis, as previously described. C, melanoma cell proliferation was investigated after 3 days of treatment, using the MTT colorimetric method. Percentage growth inhibition was calculated with respect to control LipofectAMINE 2000-treated cells. D, cell death was analyzed by fluorescence-activated cell sorting analysis after a 72-h period of treatment, as described under “Experimental Procedures.” Similar results were obtained in three independent experiments.
Glivec® treatment confirmed our previous data on the lack of an SCF/c-Kit activation loop in the control of cell proliferation in normal cells. c-Kit-positive (Mel270 and SP6.5) and c-Kit-negative (OCM-1 and MKT-BR) melanoma cell lines were also treated with various concentrations of the DNA topoisomerase inhibitor, VP16 (etoposide), to determine whether the differ-

![Diagram](https://via.placeholder.com/150)
phosphorylation of the c-Kit Tyr 719 form in SCF-stimulated the c-Kit Tyr703 form and, accordingly, that of ERK1/2 in uveal melanoma cells. Glivec® downstream signaling pathways, ERK1/2 and Akt, in uveal melanoma cell proliferation via the ERK1/2 or the Akt signal-

We then investigated the precise mode of action of Glivec® in uveal melanoma cells. We compared the effects of 5 μM Glivec® on the survival of c-Kit-negative (OCM-1) and c-Kit-positive (Mel270) melanoma cells. Flow cytometry analysis showed that Mel270 underwent massive apoptosis after 48 h of treatment, with cell death rates increasing by a factor of 9 (Fig. 6B). Conversely, treatment with Glivec® did not induce cell death in the c-Kit-negative melanoma cell line, OCM-1 (Fig. 6B). The efficiency of Glivec® to affect the ability of cells to proliferate under anchorage-independent conditions and to form colonies in soft agar was also tested in c-Kit-positive and c-Kit-negative melanoma cells. Glivec® (10 μM) greatly reduced the number of colonies of the c-Kit-positive melanoma cell line, Mel270, in methyl-cellulose, whereas it had only a minor effect on the c-Kit-negative melanoma cell line, OCM-1 (Fig. 7). Thus, Glivec® is of potential value for use in the treatment of c-Kit positive uveal melanoma.

Finally, we investigated whether Glivec® inhibited uveal melanoma cell proliferation via the ERK1/2 or the Akt signaling pathway. Western blotting showed that Glivec® inhibited phosphorylation of the c-Kit Tyr719 form in SCF-stimulated normal cells, suggesting that the Akt signaling pathway is the target of Glivec® in NUM (Fig. 8A). Akt phosphorylation was also reduced by Glivec® in SCF-stimulated NUM (Fig. 8A). However, Glivec® also inhibited phosphorylation of the c-Kit Tyr703 form and, accordingly, that of ERK1/2, in SCF-stimulated normal cells, suggesting that both the ERK and Akt signaling pathways are targeted by Glivec® in NUM (Fig. 8A). These findings also confirm that the activation of both ERK1/2 and Akt is required for the SCF-stimulated proliferation of NUM and provide evidence that the inhibitory effects of Glivec® are mediated by the inhibition of exogenous SCF-stimulated c-Kit activation in NUM. We then analyzed the effects of Glivec® on the activation of c-Kit and its two major downstream signaling pathways, ERK1/2 and Akt, in uveal melanoma cells. Glivec® greatly decreased phosphorylation of the c-Kit Tyr703 form and, accordingly, that of ERK1/2 in uveal melanoma cells (Fig. 8B). This strongly suggests that Glivec® inhibits melanoma cell proliferation and induces cell death by inhibiting ERK1/2 activation. These results are also consistent with the inhibitory effect of Glivec® being mediated by inhibition of the activated SCF/c-Kit autocrine loop in melanoma cells.

Thus, normal melanocytes were completely insensitive to Glivec® treatment, whereas c-Kit-negative uveal melanoma cells were resistant. Conversely, in c-Kit-positive uveal melano-

**DISCUSSION**

**ERK1/2 Is a Key Signaling Pathway for the Acquisition of Oncogenic Behavior in Melanoma Cells**—The Ras/Raf/MEK/ERK cascade is a major intracellular mediator of mitogenic signaling that regulates numerous biological processes (41, 42). Activating point mutations in RAS and BRAF are found in 9–15% and 60–66% of cutaneous melanomas, respectively (43, 44). It has been suggested that a gain-of-function mutation in BRAFV600EB-Raf is largely responsible for the constitutive activation of ERK1/2 and therefore for the acquisition by cutane-

However, during malignant transformation, cutaneous melanoma cells also acquire the ability to express and to secrete growth factors activating the Ras/Raf/MEK/ERK cascade. It is therefore likely that autocrine growth factor activation loops are involved in the acquisition of autonomous growth, via the constitutive activation of ERK1/2 in cutaneous melanomas (45). The V600EB-Raf mutation is also present in the OCM-1, MKT-BR, and SP6.5 uveal melanoma cell lines (31). The direct link between the constitutive activation of ERK1/2 and cell proliferation in uveal melanoma cells demonstrates that constitutive ERK1/2 activation following a gain-of-function mutation is common to the oncogenic behavior of cutaneous and uveal melanoma cells.
The activation of c-Kit, by specific mutation or autocrine stimulation, results in activation of the ERK1/2 and Akt signaling pathways (17, 18, 46). We demonstrate here that only the ERK1/2 signaling pathway is activated by the SCF/c-Kit autocrine activation loop in uveal melanoma cells. Our data strongly suggest that, due to the overactivation of ERK1/2, uveal melanoma cells became exclusively dependent on this pathway for their oncogenic behavior. The constitutive activation of ERK1/2 was not exclusively dependent on the presence of the V599E-B-Raf mutation, since c-Kit-positive melanoma cells with wild-type B-Raf also displayed constitutive ERK1/2 activation. While this work was under way, constitutive ERK1/2 activation was detected in primary uveal melanomas in the absence of BRAF mutations (47), confirming our hypothesis that ERK1/2 activation plays a key role in melanoma cell tumorigenesis.

We demonstrated, using the c-Kit inhibitor Glivec®/STI571, that the endogenous SCF-mediated activation of c-Kit and of ERK1/2 was necessary for uveal melanoma cell proliferation, survival, and transformation. This confirms the importance of c-Kit-mediated ERK1/2 activation for tumor cell proliferation (48). However, the lack of phosphorylation of c-Kit on the Tyr719 residue and the weak inhibition of cell proliferation following Akt inhibition show that Akt is not involved in uveal melanoma cell survival. These data contrast with the commonly accepted role of Akt in cell survival in c-Kit-positive cells (7). They also conflict with the demonstrated activation of Akt in other tumor cells expressing wild-type c-Kit (23). However, they confirm other recent data demonstrating that the Tyr719 residue is not the ligand-induced activation site of c-Kit (49), suggesting that the involvement of Akt in cell survival is a cell-type-dependent mechanism in c-Kit-positive tumor cells. Thus, the constitutive activation of ERK1/2, due to a gain-of-function mutation in BRAF or autocrine activation of SCF/c-Kit, plays a key role in the acquisition of autonomous growth in both cutaneous and uveal melanoma cells.

It would be useful to determine whether the downstream targets of ERK1/2 signaling responsible for the oncogenic behavior of melanomas are similar in uveal and cutaneous melanomas. We recently demonstrated that the inhibition of constitutive ERK1/2 activation leads to up-regulation of p27Kip1 expression and down-regulation of cyclin D1, together with cyclin A and Cdk1/Cdc2 expression in uveal melanoma cells, producing B-RafV599E (31, 50). The blockade of constitutive ERK1/2 activation, leading to the inhibition of cell proliferation in cutaneous melanoma cells, is also accompanied by the up-regulation of p27Kip1 expression and the down-regulation of Cdk2 activity (51), suggesting that at least some of the key components of cell cycle regulation may be common to cutaneous and uveal melanoma cells.

Is c-Kit the Only Type III Tyrosine Kinase Receptor Involved in Uveal Melanoma Cell Tumorigenesis?—The efficiency of Glivec® depends on the type of growth factor autocrine activation loop. Efficiency was highest in the four uveal melanoma cells harboring the SCF/c-Kit autocrine loop, and lowest in the two c-Kit-negative uveal melanoma cell lines. Glivec® has no effect on NUM. Four main conclusions can be drawn from our data. 1) SP6.5, Mel270, and TP31 cells were the melanoma cells harboring the SCF/c-Kit autocrine loop that were most sensitive to Glivec® treatment. The response of 92.1 cells was only half as strong, suggesting that uveal melanoma may be heterogeneous in terms of response to Glivec®. One possible reason for this is that 92.1 cells express less c-Kit than do SP6.5, Mel270, and TP31 cells, possibly rendering these cells less sensitive to the drug. The activating V599E-B-Raf mutation is not responsible for the difference in sensitivity to Glivec®, since 92.1 cells do not harbor this mutation, whereas SP6.5 and TP31 cells do. In contrast, differences in cell sensitivity may result from a phenotypic feature of 92.1 cells: their epitheloid shape. However, epitheloid melanoma cells are the most aggressive in vivo, whereas activating c-Kit mutations in GISTs have been shown to occur preferentially in spindle rather than in epitheloid cell variants (52). 2) Glivec® inhibited cell proliferation and induced cell apoptosis in wild-type c-Kit-positive cells. This contrasts with previously formulated hypotheses concerning the apoptotic effect of Glivec® on cells preferentially expressing constitutively activated mutant forms of c-Kit (24, 46). 3) Although the MTK-BR and OCM-1 cell lines were c-Kit-negative, they responded weakly to Glivec®, suggesting that other Glivec® targets are involved in regulating the proliferation of c-Kit-negative uveal melanoma cells. Indeed, a recurrent gain-of-function mutation in PDGFRα was recently reported in cells with wild-type c-Kit in GISTs (53). Interestingly, c-Kit and PDGFRα mutations appear to be mutually exclusive (54), suggesting that PDGFRα mutations may be involved in uveal melanomas that do not harbor c-Kit mutations (55). Although Glivec® efficiently inhibits constitutive activation of the PDGFRα mutant, its effect on cell proliferation remains to be determined (54). We also cannot exclude the possibility of a PDGF/PDGFR autocrine loop, with no gain-of-function mutation of PDGFR, in uveal melanomas. This is the case in osteosarcomas, which express wild-type PDGFRα and β (56). The efficiency of Glivec® for inhibiting PDGF-BB-stimulated osteosarcoma cell proliferation is much lower than that observed for gain-of-function mutations in PDGFR or c-Kit in mast cell leukemia lines. Conversely, it is very similar to that observed in uveal melanoma cells with a SCF/c-Kit autocrine activation loop (22, 23, 25, 56). These data strongly suggest that uveal melanomas may also express PDGFRαβ and harbor a PDGF/PDGFR autocrine activation loop. 4) Despite the expression of c-Kit and the activation of both ERK1/2 and Akt after SCF stimulation, Glivec® had no effect on the proliferation and survival of NUM. This does not exclude a role for c-Kit in other SCF-mediated biological processes, such as melanogenesis, differentiation, or chemotaxis, in normal melanocytes. Nevertheless, the use of Glivec® to treat uveal melanoma would affect the survival but melanoma cells but not that of NUM.

In conclusion, we demonstrate here that the ligand-dependent stimulation of c-Kit is specifically involved in the proliferation and transformation of uveal melanoma cells via SCFc-Kit autocrine activation loop. We also showed that a loss of dependence on the antiapoptotic Akt signaling pathway may be a key event in the oncogenic behavior of uveal melanoma cells. These data, which explain the key role of ERK1/2 activation in the acquisition of autonomous growth and malignant transformation, may have important implications for the design of specific therapeutic strategies for the control of melanoma progression. The demonstration that c-Kit can be targeted by an siRNA approach and Glivec®/STI571 in melanoma cells therefore provides promising possibilities for future treatment.

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Roles of Stem Cell Factor/c-Kit and Effects of Glivec®/STI571 in Human Uveal Melanoma Cell Tumorigenesis
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