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KGF Promotes Paracrine Activation of the SCF/c-KIT Axis from Human Keratinocytes to Melanoma Cells[1]

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Abstract

The paracrine networks of the human melanoma microenvironment are able to influence tumor growth and progression. Among the paracrine growth factors involved in skin homeostasis, the KGF/FGF7 secreted by dermal fibroblasts promotes the epidermal proliferation and differentiation as well as the release from keratinocytes of other paracrine mediators. To evaluate the possible role played by KGF in affecting the behavior of different subtypes of melanoma carrying activating mutations or overexpression of the SCF receptor c-KIT, we used human melanoma cell lines, characterized by different expression levels of c-KIT and opposing responsivity to SCF, and HaCaT keratinocytes. Quantitative real-time reverse transcription–polymerase chain reaction assay and ELISA test on KGF-treated keratinocytes showed enhanced expression and secretion of SCF in response to KGF and dependent on functional KGF receptor. Immunofluorescence microscopy and biochemical analysis showed, in one of the selected melanoma cell models, SCF-dependent c-KIT activation induced by stimulation with the culture supernatants collected from KGF-treated keratinocytes. In keratinocyte-melanoma cocultures stained for the Ki67 proliferation marker, incubation with KGF induced enhanced growth not only of the keratinocytes but also of the melanoma cells, which could be blocked by the c-KIT inhibitor imatinib, demonstrating the establishment of a KGF-induced paracrine signaling network owing to the coexpression of biologically active SCF released from keratinocytes and functional c-KIT on melanoma cells.

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Introduction

Melanoma is a complex neoplasia characterized by high genetic and cell signaling heterogeneity [1,2]. Different activating mutations of critical genes that regulate the signal transduction, such as *BRAF* and *NRAS*, or amplification/overexpression of other oncogenes, such as *CDK4*, occur frequently in distinct subsets of melanomas [1,3–5]. Among these oncogenic alterations, activating mutations and overexpression of c-KIT represent key events in defined subgroups of melanomas, that is, acral and mucosal melanomas of the skin, cutaneous melanomas arising on chronic sun-damaged sites and uveal melanomas [6–11].

The c-KIT protein is a receptor tyrosine kinase that signals in response to its ligand SCF. In normal human melanocytes, binding of paracrine SCF to c-KIT induces receptor dimerization and phosphorylation [12]. Activation of c-KIT leads to the recruitment of signaling proteins and triggering of different pathways, such as the phosphoinositide 3-kinase/protein kinase B (AKT) and the mitogen-activated protein kinase cascade, involved in the control of cell proliferation and survival [12]. It is well known that these activated signaling pathways are critical in melanoma progression [13,14] and the potential targeted therapeutic strategies directed to inhibit them are of great clinical interest [14]. In fact, the small-molecule c-KIT inhibitor imatinib has been used in several clinical trials for c-KIT–positive melanoma therapy, although the results of these studies have raised conflicting results [11,15–18].

Recently, it has been recognized that the tumor microenvironment, with its paracrine network of growth factors and cytokines, may dramatically influence the tumor growth and progression [19]. Therefore, a better knowledge of the melanoma-associated skin microenvironment,

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generated by the dermal-epidermal interactions and by the cell-cell communications, and of the role of fibroblasts and keratinocytes in affecting the tumor behavior is needed. Among the paracrine growth factors that play crucial roles in regulating skin homeostasis and wound healing, the KGF/FGF7 is known to promote epidermal proliferation and differentiation [20,21] as well as cell survival and motility [22–25]. Secreted from mesenchymal cells, KGF acts specifically on epithelial cells through exclusive binding to the KGFR, a spicing transcript variant of the fibroblast growth factor receptor 2 (FGFR2) [26,27]. We have recently reported that KGF is also able to promote the melanosome transfer from melanocytes to keratinocytes by a receptor-mediated stimulation of the phagocytic uptake process in the recipient cells [28,29]. Because of the broad variety of effects exerted by the fibroblast-produced KGF in the skin microenvironment, the aim of the present study was to evaluate the possible contribution of this growth factor in modulating indirectly the growth of melanoma cells, through the activation of a paracrine network. Because c-KIT represents a highly promising therapeutic target and various cultured melanoma cell models that display heterogeneity in c-KIT activating mutations or overexpression are available, we focused our attention on the triggering of the SCF/c-KIT axis in response to paracrine KGF.

Materials and Methods

Cells and Treatments

The human keratinocyte cell line HaCaT and the human melanoma cell line Mel 501 [30] were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. The human melanoma cell lines MST-L and MST-F (kindly provided by Dr. Armando Bartolazzi, Ospedale Sant'Andrea, Rome, Italy) were cultured in RPMI supplemented with 10% FBS and antibiotics. In cocultures, HaCaT and melanoma cells were seeded at a ratio of 20:1 and were maintained in Dulbecco's modified Eagle medium with 10% FBS and antibiotics.

To evaluate SCF messenger RNA (mRNA) expression, HaCaT cells were serum-starved for 12 hours and then incubated for 6 hours at 37°C with 20 ng/ml recombinant human KGF (Upstate Biotechnology, Lake Placid, NY). Alternatively, cells were treated for 12, 24, and 48 hours at 37°C with 20 ng/ml KGF or kept untreated in serum-free medium for the same time points. For the inhibition of KGFR, cells were preincubated with a specific FGFR tyrosine kinase inhibitor SU5402 (25 μM; Calbiochem, Nottingham, UK) for 1 hour before incubating with the KGF diluted in the presence of SU5402. To estimate SCF secretion in the supernatant (SN), HaCaT cells were treated for 24 and 48 hours with 20 ng/ml KGF at 37°C in the presence or absence of SU5402 as above or kept untreated in serum-free medium for the same time points. The SNs were then collected and frozen at −30°C until use.

To induce c-KIT activation, melanoma cells were serum-starved for 12 hours and then treated for 10 minutes at 37°C with 100 ng/ml recombinant human SCF (PeproTech, London, UK). For the inhibition of c-KIT activity, cells were preincubated with the tyrosine kinase inhibitor imatinib (10 μM; Novartis, West Sussex, UK) for 24 hours [31] before incubating with SCF diluted in the presence of imatinib or with imatinib alone. Alternatively, melanoma cells were stimulated for 10 minutes at 37°C with the SNs collected from HaCaT cells untreated, treated with KGF for 48 hours, or treated with KGF for 48 hours in the presence of SU5402 as above. To block c-KIT activity, melanoma cells were pretreated with imatinib for 24 hours and then incubated with the SNs for 10 minutes at 37°C in the presence of imatinib.

For proliferation assays, cocultures of HaCaT and melanoma cells were treated for 48 hours at 37°C with 20 ng/ml KGF or preincubated for 24 hours with imatinib and then incubated with 20 ng/ml KGF for 48 hours in the presence of imatinib. Alternatively, cells were treated with 50 ng/ml SCF for 48 hours or preincubated with SU5402 for 1 hour at 37°C before incubating with SCF for 48 hours in the presence of SU5402.

Human SCF ELISA

HaCaT cells grown to confluence were incubated with 20 ng/ml KGF for 24 and 48 hours in the presence or absence of SU5402 as above. SCF in SNs from HaCaT cells was quantified using the SCF ELISA kit (Quantikine High Sensitivity Human; R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The human SCF Quantikine Kit was a Sandwich ELISA–based quantification assay, and the absorbance was measured at a wavelength of 450 nm, with the correction at 540 nm, within 30 minutes using a microplate reader (μQuant; BioTek Instruments, Inc, Winooski, VT). A standard curve for each ELISA experiment was prepared from triplicate wells with increasing concentrations of SCF (0-7, 8-15, 6-31, 2-62, 5-125-250-500 pg/ml), using the protocol described above. As negative controls, the primary antibodies were omitted and replaced with phosphate-buffered saline (PBS). The results were normalized for the number of cells contained in each sample and were expressed as picograms per 1×10^6 cells. Each sample was analyzed in triplicate. Student's *t* test was performed to evaluate significant differences. $P < .05$ was considered statistically significant.

Real-time Polymerase Chain Reaction Primer Design

Oligonucleotide primers for *SCF* target gene and *GAPDH* housekeeping gene were chosen with the assistance of the Oligo 5.0 computer program (National Biosciences, Plymouth, MN). For *SCF* gene, the primers 5′-AAGAGGATAATGAGATAAGTATGTTGC-3′ (sense; position 863-245) and 5′-TTACCAGCCAATGTACGAAAGT-3′ (antisense; position 966-987) were used, yielding a complementary DNA (cDNA) product of 104 bp in length, as given by GenBank accession number NM_003994. *GAPDH* amplification was performed with 5′-TCACCATCTTCCAGGAGCGA-3′ (sense; position 323-342) and 5′-CAAATGAGCCCCAGCCTTCT-3′ (antisense; position 438-457) primer pairs, giving an amplicon of 116 bp in length (NM_002046.3). To exclude amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons. Gel electrophoresis was used to verify the specificity of polymerase chain reaction (PCR) amplicons. For each primer pair, we performed no-template control and no-reverse-transcriptase control (RT-negative) assays, which produced negligible signals (usually >45 in threshold cycle [C_t] value), suggesting that primer-dimer formation and genomic DNA contamination effects were negligible.

RNA Extraction

RNA was extracted using the TRIzol method (Invitrogen, Milan, Italy) according to the manufacturer's procedure. Briefly, cells were homogenized in 1 ml of TRIzol reagent and extracted with 0.2 ml of $CHCl_3$; 0.5 ml of isopropanol was added to the aqueous phase. RNA pellet was washed in 75% ethanol and eluted with 0.1% diethylpyrocarbonate-treated water. Total RNA quantity, purity, and absence of ribonuclease digestion were assessed by measuring the optical density ratio at

260/280 nm and by agarose gel electrophoresis. Total RNA samples were stored at −80°C.

cDNA Synthesis

After denaturation in diethylpyrocarbonate-treated water at 70°C for 10 minutes, 1 μg of total RNA was reverse-transcribed using a reaction mix of ImProm-II 5× reaction buffer containing 1.5 mM $MgCl_2$, 0.5 mM dNTP, 20 U of RNase inhibitor (Promega, Madison, WI), 1 μl of ImProm-II Reverse Transcriptase (Promega), and 0.5 μg of oligo(dT) primers. First-strand cDNA synthesis was carried out in a final volume of 20 μl, incubating at 25°C for 5 minutes and at 42°C for 60 minutes, and inactivating reverse transcriptase by heating at 70°C for 15 minutes and cooling at 5°C for 5 minutes.

PCR Amplification

Real-time PCR was performed using the iCycler Real-time Detection System (iQ5; Bio-Rad, Hercules, CA) with optimized PCR conditions. The reaction was carried out in a 96-well plate using iQ SYBER Green Supermix 2× (Bio-Rad) adding each forward and reverse primers and 1 μl of diluted template cDNA to a final reaction volume of 15 μl. Oligonucleotide primers were purchased from Invitrogen. All assays included a negative control, and all were replicated three times. The relative expression of *GAPDH* was used for standardizing the reaction. The thermal cycling conditions comprised an initial denaturation step at 95°C for 3 minutes, followed by 45 cycles at 95°C for 10 seconds and at 60°C for 30 seconds.

Data Analyses

Real-time quantitation was performed by using SYBR Green dye as fluorescent signal, with the help of the iCycler IQ optical system software version 3.0a (Bio-Rad) according to the manufacturer's manual. Quantitative values are obtained from the C_t number at which the increase in signal associated with exponential growth of PCR products starts to be detected. Target gene (*SCF*) amplification was compared with simultaneous amplification of an endogenous reference gene (*GAPDH*), and each sample was normalized on the basis of its *GAPDH* content. For data analyses, the C_t values were exported into a Microsoft Excel Worksheet (Microsoft Corporation, Redmond, WA). Results, expressed as the amount of target gene expression relative to the housekeeping gene, were calculated with the $\Delta\Delta C_t$ method and indicated as $2^{-\Delta\Delta Ct}$. The ΔC_t value of the sample was determined by subtracting the average C_t value of the *SCF* target gene from the average C_t value of the *GAPDH* gene and normalized on the ΔC_t value of the untreated control sample. For the untreated control sample, the amount of target gene expression equals 1.0, by definition. Results are reported as mean ± SD from three different experiments in triplicate. Statistical analysis was performed using Student's *t* test. $P < .05$ was considered statistically significant.

Western Blot Analysis

Subconfluent cultures of HaCaT, Mel 501, MST-L, and MST-F cells were lysed in a buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM EGTA, supplemented with protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethyl sulfonyl fluoride, 10 μg/ml leupeptin), and phosphatase inhibitors (1 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 0.5 M NaF); 50 μg of total protein was resolved under reducing conditions by 7% SDS-PAGE and transferred to a reinforced nitrocellulose (BA-S 83; Schleider & Schuell, Keene, NH). The membranes were blocked with 5% nonfat dry milk in PBS 0.1% Tween 20 and incubated with anti–c-KIT, CD117 (Dako, Carpinteria, CA) or with anti–phospho-c-KIT Tyr719 (Cell Signaling, Cambridge, MA), polyclonal antibodies followed by enhanced chemiluminescence detection (ECL; Amersham, Arlington Heights, IL). To estimate the protein equal loading, the membranes were rehydrated by being washed in PBS–Tween 20, stripped with 100 mM mercaptoethanol and 2% SDS for 30 minutes at 55°C, and probed again with antiactin (Sigma Chemicals, St Louis, MO) monoclonal antibody.

Immunofluorescence

Melanoma cells grown on coverslips and incubated for 10 minutes at 37°C with 100 ng/ml SCF in the presence or absence of imatinib as above or with SNs from HaCaT monolayers (collected from untreated, treated with KGF or with KGF + SU5402 cells) in the presence or absence of imatinib as above were fixed in methanol for 4 minutes. Cocultures of HaCaT and melanoma cells grown on coverslips incubated for 48 hours at 37°C with 20 ng/ml KGF in the presence or absence of imatinib or incubated with 50 ng/ml SCF in the presence or absence of SU5402 were fixed with 4% paraformaldehyde followed by treatment with 0.1 M glycine for 20 minutes at 25°C and with 0.1% Triton X-100 for an additional 5 minutes at 25°C to allow permeabilization. Cells were then incubated for 1 hour at 25°C with the following primary antibodies: anti–c-KIT (1:50 in PBS; Dako) antityrosinase (1:50 in PBS; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and anti-Ki67 (1:50 in PBS; Zymed Laboratories, Inc, San Francisco, CA) polyclonal antibodies and antiphosphotyrosine (1:100 in PBS; Upstate) and anticytokeratins (1:100 in PBS; clone MNF116; Dako) monoclonal antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole (1:10,000 in PBS; Sigma). The primary antibodies were visualized, after appropriate washing with PBS, using goat antimouse immunoglobulin G (IgG)–fluorescein isothiocyanate (FITC, 1:50 in PBS; Cappel Research Products, Durham, NC), goat antirabbit IgG-FITC (1:500 in PBS; Cappel), donkey antirabbit IgG–Alexa Fluor 594 (1:1000 in PBS; Molecular Probes, Eugene, OR), and chicken antigoat IgG–Alexa Fluor 488 (1:1000 in PBS; Molecular Probes). Coverslips were finally mounted with Mowiol (Calbiochem, Merck, Darmstadt, Germany) for observation. Fluorescence signals were analyzed by recording and merging stained images using a CCD device SPOT-2 camera (Diagnostic Instruments, Inc, Sterling Heights, MI) and IAS2000/H1 software (Delta Sistemi, Rome, Italy). Quantitative analysis of the phosphotyrosine fluorescence intensity was performed by the analysis of 50 cells for each sample in five different fields randomly taken from three different experiments. Percentage of Ki67-positive cells in cocultures of keratinocytes and melanoma cells was analyzed counting for each treatment a total of 500 cells, randomly observed in 10 microscopic fields from three different experiments. Results have been expressed as mean values ± SE. Significance was calculated using Student's *t* test: *P* values < .05 were considered statistically significant. Alternatively, cells were scanned in a series of 0.5-μm sequential sections with an ApoTome System (Zeiss, Ober Kochen, Germany) connected with an Axiovert 200 inverted microscope (Zeiss); image analysis was then performed by the Axiovision software (Zeiss), and a three-dimensional reconstruction was obtained.

Results

KGF Treatment of Human Keratinocytes Induces SCF Expression and Release

The keratinocytes of human skin produce both membrane type and soluble SCF [32,33]. In particular, HaCaT keratinocytes represent an

in vitro source of SCF and its release is increased by cell differentiation [33]. To evaluate if treatment of HaCaT cells with KGF would induce SCF production, we assessed the presence of *SCF* transcripts in HaCaT cells by quantitative real-time reverse transcription–PCR (RT-PCR) and *GAPDH* was used as housekeeping gene: *SCF* mRNA extracted from cells stimulated with KGF for different time points (6, 12, 24, and 48 hours) was quantitated and compared with that from untreated control HaCaT cells. In KGF-stimulated cells, a significant increase in mRNA *SCF* expression compared with control cells was observed at 12 hours of treatment ($P < .05$). After 24 hours, the transcript levels appeared decreased but still significantly higher in comparison to untreated cells ($P < .05$). No differences were observed after 48 hours (Figure 1*A*). To demonstrate the direct effect of KGF in inducing *SCF* expression, the quantitative RT-PCR assay was also performed in cells treated with the growth factor in the presence or absence of the specific FGFR tyrosine kinase inhibitor SU5402: the results showed that the presence of the inhibitor was able to inhibit the *SCF* up-regulation ($P < .05$; Figure 1*A*).

The enhanced expression of *SCF* induced by KGF was also validated at the protein level by ELISA assay evaluating the amount of

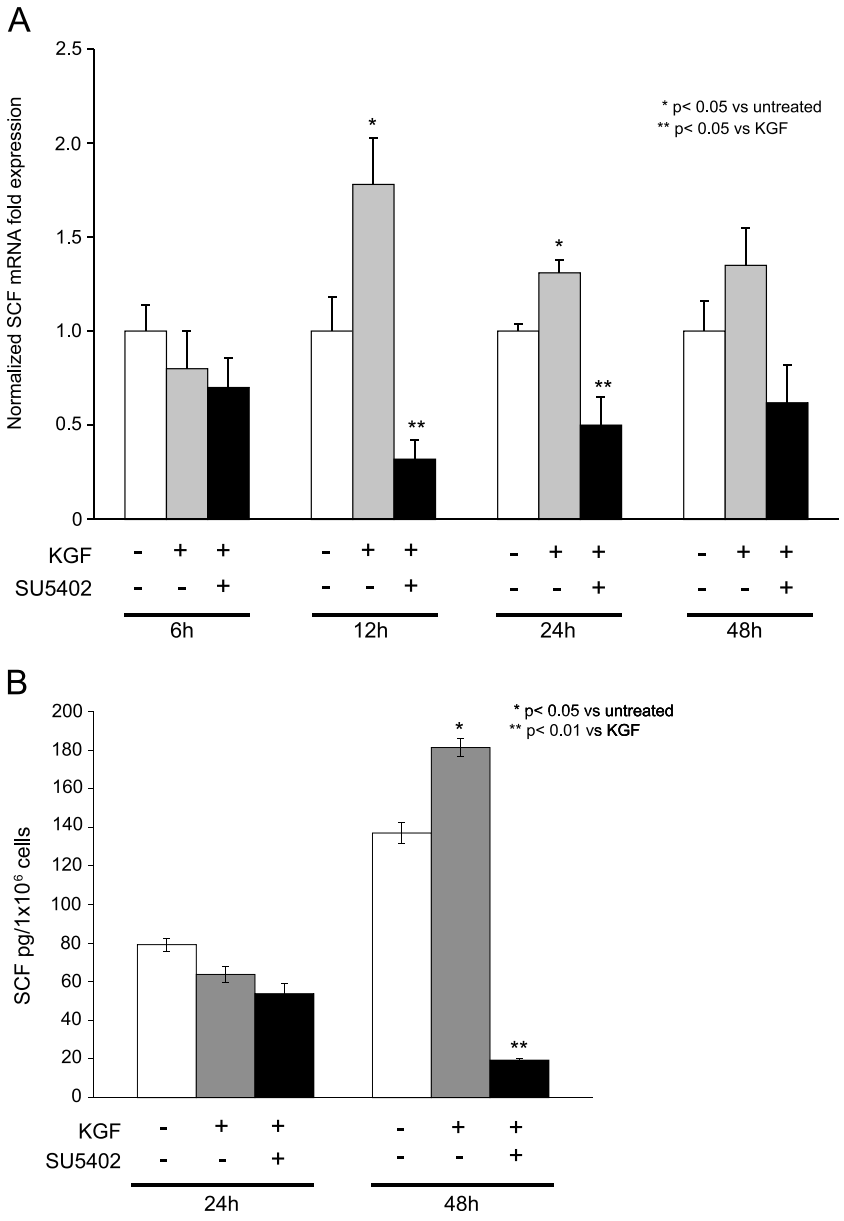


Figure 1. Expression of SCF in HaCaT cells treated with 20 ng/ml KGF for different time points (6, 12, 24, and 48 hours) in the presence or absence of the KGFR inhibitor SU5402 (25 μM). (A) Relative expression level of SCF evaluated by real-time quantitative RT-PCR. *SCF*/*GAPDH* mRNA ratio was calculated with the $\Delta\Delta C_t$ method as described in Materials and Methods. The values of samples treated with KGF for 6, 12, 24, and 48 hours were expressed relative to untreated control samples as normalized fold expression. A significant increase in mRNA SCF expression was observed at 12 and 24 hours. Results represent the mean values ± SD from three different experiments. Student's *t* test was performed to evaluate significant differences. (B) SCF quantitation by ELISA assay on supernatants from HaCaT cells treated as described. The amount of SCF released in the medium resulted to be significantly increased after the treatment with KGF for 48 hours. The presence of the inhibitor SU5402 was able to revert such stimulation. Student's *t* test was performed to evaluate significant differences.

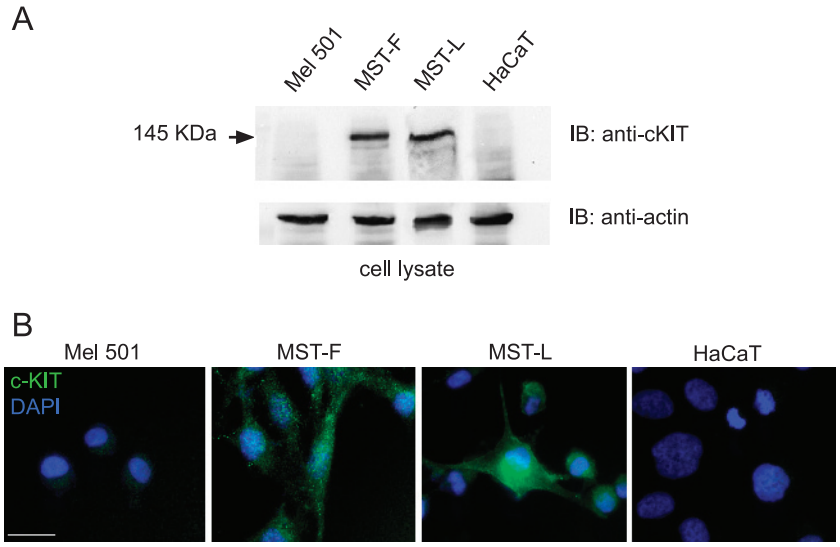
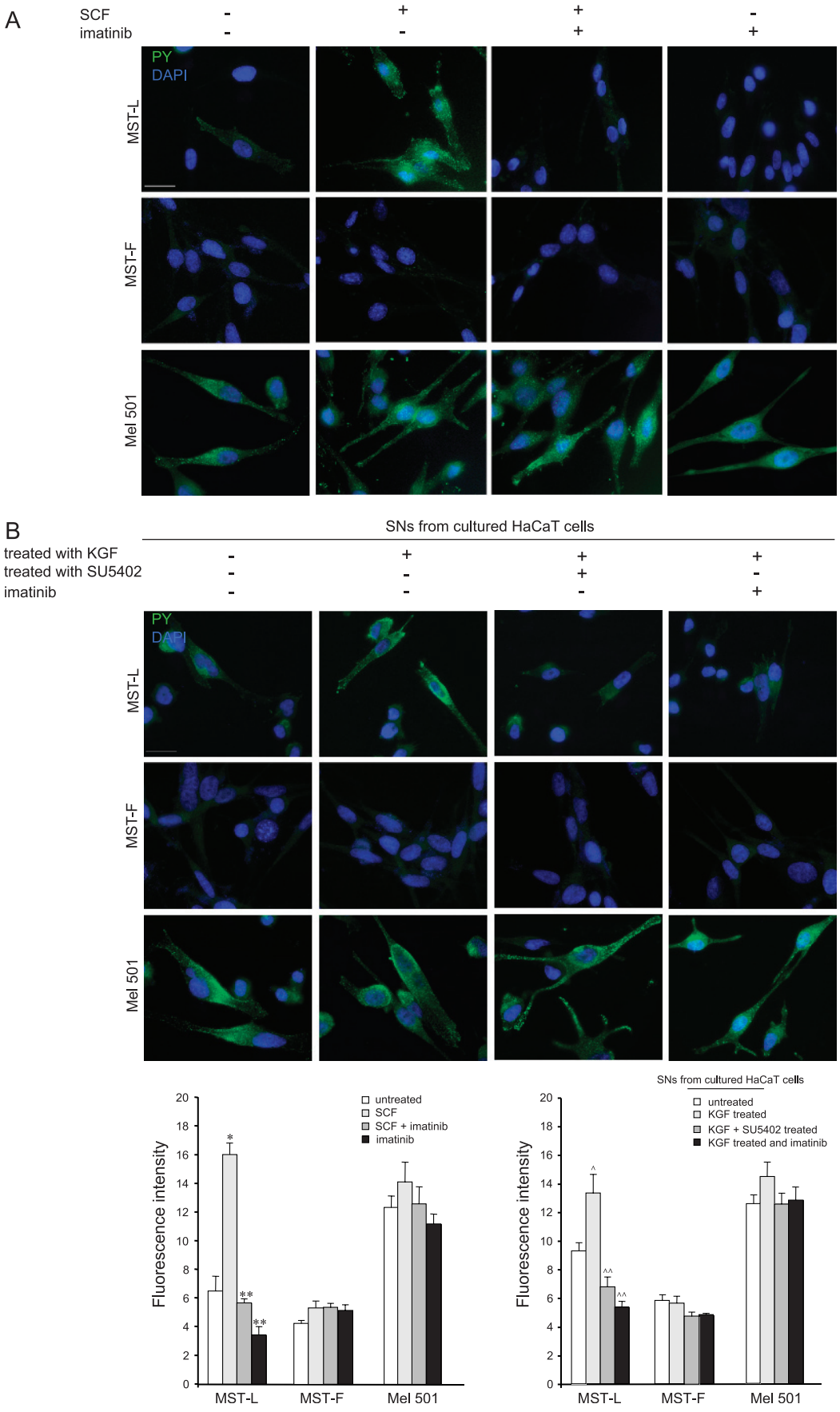


Figure 2. Different expression of c-KIT in human melanoma cell lines. (A) Mel 501, MST-F, and MST-L melanoma cells were lysed and processed for Western blot analysis using anti–c-KIT polyclonal antibodies to detect the endogenous protein: a band of 145 kDa corresponding to the molecular weight of c-KIT protein is visible in MST-F and MST-L melanoma cells but not in Mel 501 cells, and in HaCaT human keratinocytes used as negative control. Equal loading was assessed by stripping the blots and reprobing with antiactin monoclonal antibody. (B) Mel 501, MST-F, and MST-L cells and HaCaT keratinocytes were fixed, permeabilized as described in Materials and Methods, and immunolabeled with anti–c-KIT polyclonal antibodies followed by FITC-conjugated secondary antibodies. Cell nuclei were visualized by DAPI. Immunofluorescence microscopical analysis shows that in both MST-F and MST-L cells, the staining for c-KIT protein is intense and localized on the cell plasma membrane as well as in the juxtanuclear Golgi area or in small dots scattered throughout the cell periphery. In contrast, no c-KIT immunostaining is detectable in Mel 501 and HaCaT cells. Bar, 10 μm.

secreted SCF in the culture SNs at 24 and 48 hours of treatment with KGF. We observed a significant increase in *SCF* released at 48 hours (181.38 pg/1 × 10^6 cells) with respect to untreated control cells (137.09 pg/1 × 10^6 cells; $P < .05$). Pretreatment with SU5402 showed to repress the augmented secretion of SCF (19.31 pg/1 × 10^6 cells; $P < .01$ *vs* KGF; Figure 1*B*), indicating that KGFR and its signaling are specifically involved in the modulation of *SCF*. Although at 24 hours, the *SCF* mRNA expression level in KGF-stimulated cells is higher than that in control cells, the SCF secretion is lower in KGF-treated cells with respect to the untreated cells (Figure 1*B*): because HaCaT cells are known to produce both membrane type and soluble SCF [33], the transcript expression increased by KGF at 24 hours could correlate better with the

Figure 3. Tyrosine phosphorylation in melanoma cells induced by incubation with SCF or with supernatants collected from KGF-treated HaCaT cells. (A) MST-L, MST-F, and Mel 501 cells were serum-starved for 12 hours, treated with 100 ng/ml SCF for 10 minutes or preincubated for 24 hours with the c-KIT inhibitor imatinib (10 μM), and then treated with SCF as above in presence of imatinib. Cells were stained with anti-PY monoclonal antibody followed by FITC-conjugated secondary antibodies and with DAPI to visualize the cell nuclei. Quantitative immunofluorescence analysis of the fluorescence intensity, performed as described in Materials and Methods, shows in MST-L cells an increase of the phosphotyrosine staining at the cell plasma membrane induced by SCF stimulation, which is drastically reduced by pretreatment with imatinib, demonstrating that the signal is mostly due to c-KIT activation induced by the SCF ligand. Either the low phosphotyrosine fluorescence staining observed in MST-F cells or the basal intense staining evident in Mel 501 cells does not seem modified by SCF stimulation or imatinib treatment. Bar, 10 μm. (B) MST-L, MST-F, and Mel 501 cells were serum-starved for 12 hours and treated for 10 minutes with different SNs collected from confluent cultures of HaCaT cells. Alternatively, cells were serum-starved for 12 hours, preincubated for 24 hours with imatinib, and then treated with the SNs collected from KGF-treated cells. Cells were then stained with antiphosphotyrosine monoclonal antibody. Immunofluorescence microscopical analysis and quantitative analysis of the fluorescence intensity in MST-L cells reveals an increase in the phosphotyrosine signal upon stimulation with SNs from untreated HaCaT cells and, much more, with SNs from KGF-treated HaCaT cells compared with the basal signal of serum-starved MST-L cells. The staining obtained by incubating with SNs from KGF-treated HaCaT cultures is strongly decreased by imatinib pretreatment, demonstrating that the phosphotyrosine signal is dependent on SCF present in the SN and corresponds to c-KIT activation. No significant increase in the signal is evident upon stimulation with SNs from HaCaT cells treated with KGF in the presence of the KGFR inhibitor SU5402, testifying the specificity of the KGF's effect on SCF secretion from HaCaT keratinocytes. In contrast, either the basal weak phosphotyrosine fluorescence signal observed in MST-F cells or the basal intense staining evident in Mel 501 cells does not seem to be significantly affected by stimulation with the different SNs. Bar, 10 μm. Student's *t* test was performed, and significance level has been defined as $^*P < .05$ *versus* the corresponding untreated cells; $^{**}P < .05$ *versus* the corresponding SCF treated cells; $^\wedge P < .05$ *versus* the corresponding cells incubated with the SNs from untreated HaCaT cells; $^\wedge\wedge P < .05$ *versus* the corresponding cells incubated with the SNs from KGF-treated HaCaT cells. (C) MST-L melanoma cells were lysed and processed for Western blot analysis using anti–phospho-c-KIT and anti–c-KIT antibodies to detect the active form and the total protein: c-KIT phosphorylation is visible after incubating with SCF or with the SN from KGF-treated HaCaT cells but not in control unstimulated cells or in cells treated with SCF in the presence of imatinib. Equal loading was assessed with antiactin monoclonal antibody.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SNs from cultured HaCaT cells
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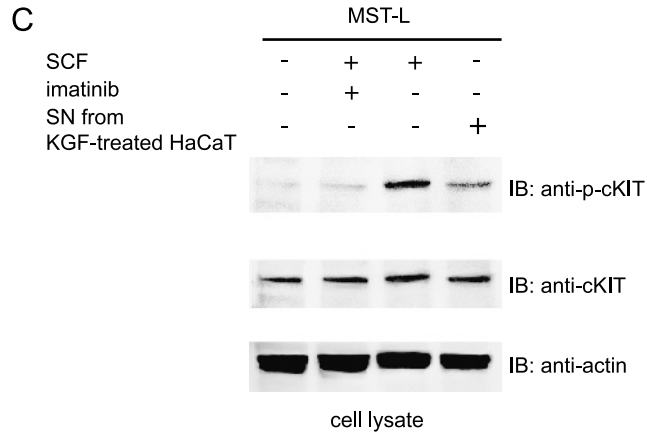


Figure 3. (*continued*).

membrane form than with the secreted form of SCF. In addition, the increased SCF release observed in HaCaT cells at 48 hours compared with that observed at 24 hours is consistent with previous report that describes a higher secretion of SCF in differentiating *versus* proliferating cells [33].

Thus, KGF is able to induce up-regulation of *SCF* gene expression in HaCaT cells and increased release of soluble SCF, which, in turn, might exert a paracrine action on melanocytes.

SCF Induces c-KIT Activation in Melanoma Cells

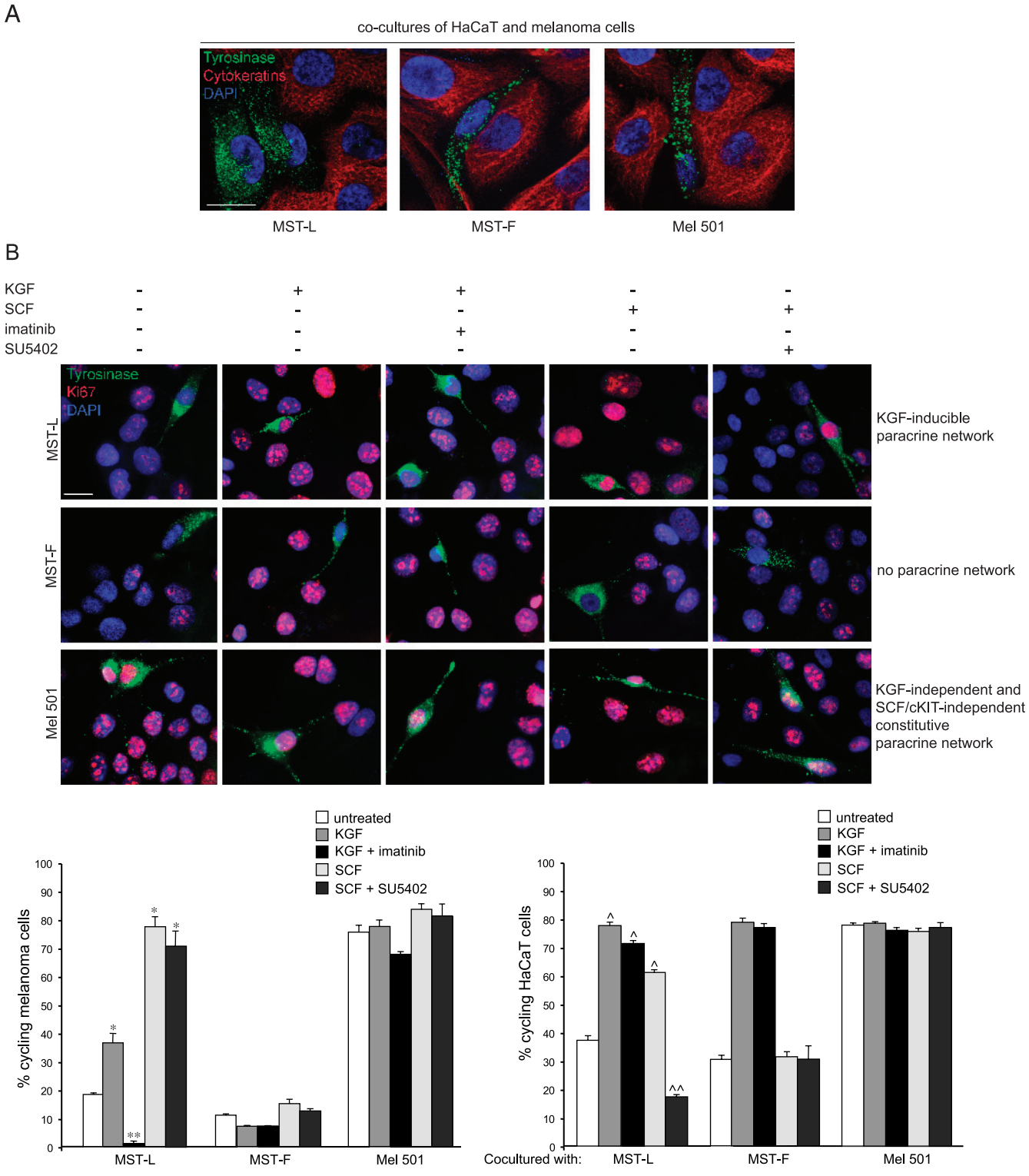
To analyze the possible role of soluble SCF secreted by KGF-treated keratinocytes on melanoma cells, we selected three different melanoma cell lines, Mel 501, MST-L, and MST-F cells, expressing high or low levels of c-KIT. The expression of c-KIT protein was first evaluated by Western blot analysis using anti–c-KIT polyclonal antibodies: a specific band of 145 kDa corresponding to the molecular weight of c-KIT protein was visible in MST-F and MST-L melanoma cells, but not in Mel 501 cells and in HaCaT human keratinocytes used as negative control (Figure 2*A*). To assess equal loading, blots were stripped and reprobed with antiactin monoclonal antibody (Figure 2*A*). To confirm the different expression of c-KIT in the selected cell models and to verify the correct localization of the receptor on the plasma membranes of the positive cells, we performed an immunofluorescence microscopical analysis with the anti–c-KIT antibodies: in MST-F and MST-L cells, the immunostaining appeared intense and localized on the cell plasma membrane and in the juxtanuclear Golgi area. Immunolabeling was also visible in small dots scattered throughout the cell periphery, possibly corresponding to intracellular transport structures of the exocytic or endocytic compartments (Figure 2*B*). In contrast, no c-KIT immunostaining was detectable in Mel 501 and HaCaT cells (Figure 2*B*), confirming the biochemical results described above.

Binding of SCF to the c-KIT tyrosine kinase is known to induce tyrosine autophosphorylation and activation of the receptor. To determine whether the c-KIT proteins expressed on MST-L and MST-F melanoma cells would be constitutively activated, as it occurs in a subset of melanomas expressing oncogenic mutations of the *KIT* gene [6], or would undergo tyrosine phosphorylation only in response to SCF, we performed a quantitative immunofluorescence microscopical analysis of MST-L, MST-F, and Mel 501 cells with antiphosphotyrosine monoclonal antibody: cells were serum-starved and treated with SCF for 10 minutes at 37°C before immunolabeling or with SCF in the presence of the small-molecule c-KIT inhibitor imatinib [9]. In both untreated MST-L and MST-F cells, a very weak intensity of the signal was observed, suggesting a low amount of constitutive activation of receptor tyrosine kinases, whereas the PY signal of Mel 501 was intense also in untreated serum-starved conditions, revealing constitutive phosphotyrosine activation of receptors other than c-KIT (Figure 3*A*). SCF stimulation of MST-L cells induced a high increase of the PY staining at the cell plasma membranes, which was drastically reduced by pretreatment with imatinib (Figure 3*A*), suggesting that the immunofluorescence signal decorating the cell surface is mostly due to functional c-KIT activation induced by the SCF ligand. In contrast, treatment with SCF of MST-F cells did not seem to trigger c-KIT activation (Figure 3*A*) and the Mel 501 cell line was not significantly affected by SCF stimulation or imatinib treatment. Thus, the three selected melanoma cell lines seem to represent three different models of melanoma behavior: 1) MST-L as melanoma cells with

Figure 4. Proliferation of melanoma cells cocultured with HaCaT keratinocytes. Cocultures of MST-L, MST-F, or Mel 501 cells with HaCaT keratinocytes were plated at a seeding ratio of 1:20. (A) Double immunofluorescence staining was performed with anti–tyrosinase polyclonal antibodies (green) to unequivocally identify melanoma cells and with anticytokeratin monoclonal antibody (red) to recognize HaCaT keratinocytes. Cell nuclei were visualized by DAPI. The immunofluorescence analysis was performed by serial optical sectioning and three-dimensional reconstruction as described in Materials and Methods. Images obtained by three-dimensional reconstruction of a selection of three of the total number of the serial optical sections are shown: the selected sequential sections are central and crossing the nucleus. The tyrosinase-positive dots corresponding to melanosomes are localized in the cytoplasm of melanoma cells, which are surrounded by HaCaT keratinocytes containing bundles of cytoplasmic keratin filaments. Bar, 10 μm. (B) Cocultured cells were serum-starved for 12 hours, treated with 20 ng/ml KGF for 48 hours or with 50 ng/ml SCF for 48 hours also in the presence of imatinib or SU5402 to inhibit c-KIT expressed on melanoma cells or KGFR expressed on HaCaT keratinocytes, respectively. Cocultures were stained with anti-Ki67 monoclonal antibody (red) to detect the proliferating cells and with anti-tyrosinase polyclonal antibody (green) to identify the melanoma cells. Quantitative analysis of the percentage of cycling cells displaying the nuclear Ki67 positivity was performed as described in Materials and Methods. In cocultures of MST-L and HaCaT cells, addition of KGF causes a drastic increase in the percentage of cycling Ki67-positive nuclei in both types of cells, demonstrating that KGF is able to induce a paracrine network. Cotreatment of KGF with imatinib specifically blocks melanoma proliferation confirming the involvement of SCF release by keratinocytes in the activation of MST-L cells. Addition of SCF induces not only melanoma cell growth, as expected, but also proliferation of HaCaT keratinocytes that is selectively inhibited by cotreatment with SU5402, suggesting the possible release of KGFR ligands from melanoma cells. In cocultures of MST-F and HaCaT cells, addition of KGF causes exclusively proliferation of HaCaT cells, and incubation with SCF does not stimulate melanoma cells, revealing no induction of a paracrine network and confirming the inability of c-KIT expressed in these cells to respond to SCF. In contrast, the coculture of Mel 501 with HaCaT cells leads to the induction of a paracrine network also in untreated conditions, demonstrating that such cell-cell interaction is independent of KGF and SCF/c-KIT. Bar, 10 μm. Student's *t* test was performed and significance level has been defined as $*P < .05$ *versus* the corresponding untreated cells; $**P < .05$ *versus* the corresponding KGF-treated cells; $\hat{}P < .05$ *versus* the untreated cells; $\hat{}\hat{}P < .05$ *versus* the SCF-treated cells.

SCF-inducible c-KIT activation, 2) MST-F as cells with c-KIT not responding to the ligand SCF, and 3) Mel 501 as cells with constitutive tyrosine phosphorylation and signaling activity independent of SCF and c-KIT.

To ascertain if the soluble SCF released by HaCaT cells on KGF stimulation would be biologically active in triggering c-KIT on melanoma cells, we used the three cell lines characterized above for their distinct SCF/c-KIT axis and we incubated them with SNs collected from KGF-treated HaCaT cultures. Melanoma cells were then stained with antiphosphotyrosine monoclonal antibody as above. Quantitative analysis of the fluorescence intensity revealed that incubation of MST-L cells with the SNs from untreated HaCaT cultures induced an increase of the phosphotyrosine signal compared to the basal PY staining of serum-starved cells (Figure 3*B*) and the positive signal was further raised by incubating with SNs from KGF-treated HaCaT cells (Figure 3*B*). This intense staining obtained on stimulation of MST-L cells with SNs from KGF-treated HaCaT cultures was strongly reduced by imatinib, suggesting that the PY signal is



dependent on the SCF released in the SNs and that it corresponds to c-KIT activation (Figure 3*B*). Again, to demonstrate the direct role of KGF in the up-modulation of SCF secreted in the SNs from cultures of HaCaT keratinocytes, a specific block of KGFR signaling was achieved through treatment of the HaCaT cells with KGF in the presence of the KGFR inhibitor SU5402 before the collection of the culture SNs: the immunofluorescence analysis showed no significant increase of the signal, confirming the specificity of the KGF effect on SCF secretion (Figure 3*B*). The weak PY staining observed in MST-F cells and the basal intense signal evident in Mel 501 cells were not significantly affected by stimulation with the different SNs (Figure 3*B*).

To confirm in MST-L cells the c-KIT activation induced by SCF released by the KGF-treated HaCaT cells and its specific inhibition by imatinib, we performed a Western blot analysis using anti–phospho-c-KIT antibodies directed against the Tyr719 and recognizing the active protein: c-KIT phosphorylation was evident in MST-L cells treated with SCF or with the SNs from KGF-treated HaCaT cells and drastically inhibited by imatinib (Figure 3*C*). Equal loading was assessing with antiactin monoclonal antibody (Figure 3*C*).

Thus, c-KIT present on the MST-L melanoma cells is activated in a paracrine manner by keratinocyte-derived SCF, which, in turn, is secreted in response to the paracrine action of KGF.

KGF and SCF Paracrine Loop in Cocultures

Cell-cell interactions and paracrine networks have been widely studied *in vitro* using coculture models of different cell types. Therefore, to verify our hypothesis of the role of KGF in inducing the SCF/c-KIT axis from the HaCaT keratinocytes to the selected melanoma cells characterized above for their behavior on SCF stimulation and c-KIT activation, we cocultured MST-L, MST-F, or Mel 501 cells with HaCaT cells at a seeding ratio of 1:20. To evaluate the cell growth, we performed an immunofluorescence analysis for the quantitative detection of cells positive for the Ki67 proliferation marker. To unequivocally identify melanoma cells, we used antityrosinase polyclonal antibodies that decorate the membranes of melanosomes, whereas to recognize the HaCaT keratinocytes, we labeled the cultures with anticytokeratin monoclonal antibody. Cells were then analyzed by serial optical sectioning and three-dimensional reconstruction as described in Materials and Methods. The double immunofluorescence staining revealed tyrosinase-positive dots corresponding to melanosomes in the cytoplasm of melanoma cells and bundles of cytoplasmic keratin filaments in the HaCaT keratinocytes (Figure 4*A*).

Cocultured cells were treated with KGF (20 ng/ml for 48 hours) or with SCF (50 ng/ml for 48 hours). Alternatively, cocultures were incubated with KGF in the presence of imatinib to inhibit c-KIT expressed on melanoma cells or with SCF in the presence of SU5402 to block the KGFR expressed on HaCaT keratinocytes. Cocultures were then immunolabeled with anti-Ki67 monoclonal antibody to detect all cycling cells and with antityrosinase antibody to visualize the melanoma cells. Quantitative analysis of the percentage of cycling cells displaying the nuclear Ki67 positivity was performed as described in Materials and Methods. In cocultures of HaCaT with MST-L cells, the addition of KGF caused a drastic increase in the percentage of Ki67-positive nuclei not only in the keratinocytes expressing the receptor but also in the melanoma cells not expressing the KGFR, demonstrating that KGF is able to induce a paracrine network (Figure 4*B*). Cotreatment of KGF with imatinib specifically blocked melanoma proliferation, confirming the involvement of SCF released by keratinocytes in the paracrine stimulation of MST-L cells. Interestingly, the addition of SCF induced not only melanoma cell growth, as expected from the expression of functional c-KIT in these cells, but also proliferation of HaCaT keratinocytes, which was selectively inhibited by cotreatment with SU5402, suggesting the possible release of KGFR ligands from melanoma cells. In contrast, the addition of KGF to cocultures of HaCaT with MST-F cells was able to cause proliferation of HaCaT cells only (Figure 4*B*), revealing no induction of a KGF-dependent paracrine network. Moreover, the incubation with SCF did not stimulate either HaCaT or MST-F cells, confirming the inability of c-KIT expressed in these cells to respond to SCF. Finally, the coculture of HaCaT with Mel 501 cells led to growth of both cell types also in untreated conditions, demonstrating the existence of a constitutive paracrine network independent of KGF and SCF/c-KIT.

Thus, KGF promotes the establishment of a paracrine SCF/c-KIT axis from the HaCaT keratinocytes to melanoma cells expressing functional c-KIT and a reverse cell-cell paracrine interaction leading to keratinocyte growth.

Discussion

Genetic alterations of c-KIT are known to be associated with several neoplastic disorders including gastrointestinal stromal tumors, seminomas, and mast cell leukemias [11]. Interestingly, the *c-KIT* oncogene seems to play opposing effects on different subtypes of melanoma: in fact, in some tumors, c-KIT negatively regulates the growth and seems to be down-modulated during progression [11], whereas in other subgroups of melanomas, c-KIT activating mutations or overexpression results in aberrant signaling that drives tumorigenesis [6–11]. In addition, melanoma cells might either lose dependence on paracrine growth factors, acquiring autocrine pathways of stimulation [19], or might be able to respond to paracrine cross talk with an enhanced proliferation [19,34]. Among the paracrine growth factors that may influence melanoma behavior, the c-KIT ligand SCF is one of the major mitogens for melanocytes [32]. Here we have selected three distinct cell lines that represent models of melanomas characterized by different expression levels of c-KIT and different responsivity to the c-KIT ligand SCF: the three cell models reflect the possible ways of involvement of the SCF/c-KIT axis in the paracrine signaling network between normal keratinocytes and melanoma cells, ranging from total independency to dependency and inducibility by other networks. Because of this heterogeneity in SCF/c-KIT and the availability of therapeutic strategies based on the treatment with the specific c-KIT inhibitor imatinib, which are currently under clinical investigations, a deeper investigation on the epidermal-derived or dermal-derived growth factors that may trigger the SCF/c-KIT cross talk is needed.

Therefore, having characterized our cell models of melanoma and their cocultures with keratinocytes, we wondered if one of the most critical mediator of epidermal proliferation and differentiation, the KGF/FGF7 derived from dermal fibroblasts, could be able to induce an SCF/c-KIT–dependent signaling network. The results showed that KGF, through the promotion of SCF expression and secretion from confluent differentiating keratinocytes, indirectly stimulates the growth of melanoma cells in which c-KIT is expressed and is functionally active by its ligand (Figure 5). This stimulation in two steps is justified by the presence of KGF receptor exclusively on epithelial cells and its absence on melanocytes. Moreover, the specificity of this network is demonstrated by the selective inhibition of the KGF receptor on the keratinocytes or of c-KIT on the melanoma cells (Figure 5).

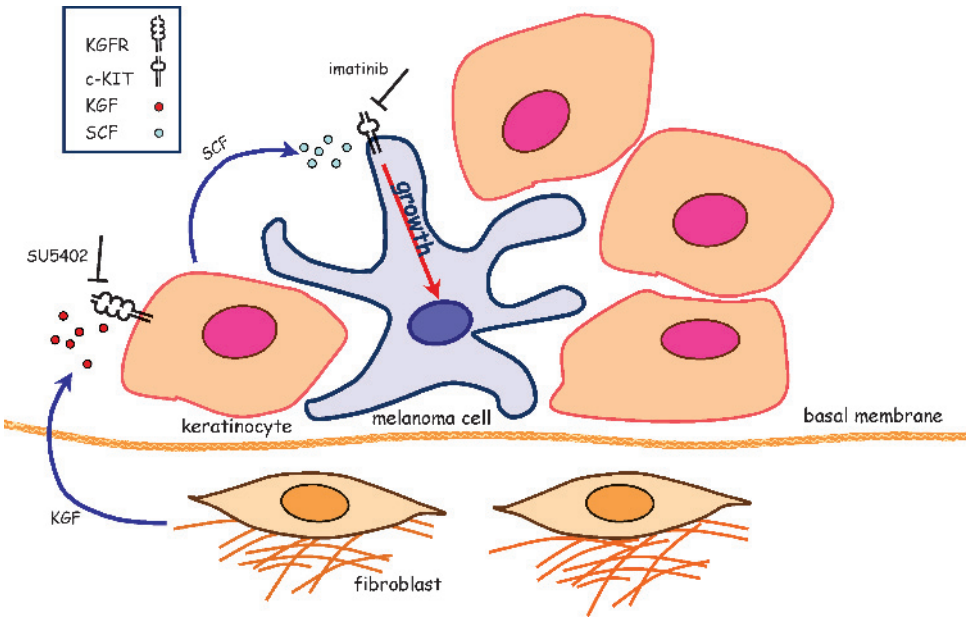


Figure 5. Schematic drawing of the proposed KGF-dependent paracrine SCF/c-KIT axis from keratinocytes to melanoma cells: KGF secreted by dermal fibroblasts binds to KGFR exposed on epidermal keratinocytes, which, in turn, produce SCF. Through binding to functional c-KIT expressed on melanoma cells, SCF stimulates tumor cell growth.

Multiple cytokines and growth factors are suspected to play roles in the induction or progression of melanoma. Similar to KGF, the hepatocyte growth factor (HGF) is also secreted by dermal fibroblasts and acts on epidermal cells; however, different from KGF, HGF is able to directly exert its scattering activity on melanocytes and melanoma cells promoting tumorigenesis and invasion [34,35]. In addition, whereas several growth factors, in general, and HGF, in particular, frequently become responsible for autocrine loops of stimulation in tumor cells or in cells of the tumor microenvironment [19], KGF has not been reported as involved in known autocrine pathways. However, the possible contribution of KGF secreted in a paracrine manner by stromal cells surrounding a tumor with specific molecular gene signatures, such as the SCF/c-KIT–dependent melanoma subgroups, remains to be established.

Finally, our results obtained by the coculture experiments clearly indicate the existence of a reverse inducible paracrine interaction from melanocytes to keratinocytes involving KGFR ligands. Although we cannot exclude the release of other known ligands for KGFR, such as FGF10, evidence for the expression of KGF in human malignant melanoma has been reported [36], suggesting the possible release of KGF from the tumor that can stimulate keratinocyte proliferation. Because it is well known that both KGF and FGF10 also induce keratinocyte differentiation [21,37] and that SCF is released by more differentiated keratinocytes [33], the suggested reverse signaling cross talk involving KGFR ligands from melanoma to keratinocytes might further activate and deregulate the SCF/c-KIT axis.

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