

# KIT Receptor Activation by Autocrine and Paracrine Stem Cell Factor Stimulates Growth of Merkel Cell Carcinoma In Vitro

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The co-expression of KIT receptor and its ligand stem cell factor (SCF) has been reported in biopsy specimens of Merkel cell carcinoma (MCC). However, the functional role of SCF/KIT in the pathogenesis of this aggressive tumor has not been elucidated. The present study reports expression and effects of SCF and KIT in the Merkel cell carcinoma cell line MCC-1 in vitro. SCF and KIT were endogenously co-expressed in MCC-1 cells. Exogenous soluble SCF modulated KIT receptor mRNA and protein expression, stimulated growth of MCC-1 cells, upregulated endogenous activation of KIT, AKT, and of extracellular signal-regulated kinase (ERK) 1/2 signaling pathway. On the contrary, an inhibitory antibody that neutralized the KIT ligand binding site, reduced growth of MCC-1 cells, as did high doses of the KIT kinase inhibitors imatinib and nilotinib. Also, inhibitors of KIT downstream effectors, U0126 that blocks MEK 1/2 as well as wortmannin and LY294002 that inhibit phosphatidylinositol 3-kinase-dependent AKT phosphorylation, inhibited the proliferation of MCC-1 cells. These data support the hypothesis that KIT is activatable by paracrine or autocrine tumor cell-derived SCF and stimulates growth of Merkel cell carcinoma in vitro. Blockade of KIT and the downstream signaling cascade at various levels results in inhibition of Merkel cell carcinoma growth in vitro, suggesting targets for therapy of this cancer.

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Merkel cell carcinoma or primary neuroendocrine carcinoma of the skin is a tumor with aggressive growth and high propensity for metastases (Hitchcock et al., 1988; Krasagakis et al., 1997). Factors regulating growth of this peculiar tumor remain, however, largely unknown. Several oncogenes and tumor suppressor genes have been implicated in its pathogenesis, including expression of the anti-apoptotic bcl2 oncogene, mutations of the p53 and p73 tumor suppressor genes, hypermethylation of the Ink4a gene coding for p16 and p14ARF, as well as the clonal integration of a new polyoma virus (Merkel cell virus) with presumed oncogenic properties (Plettenberg et al., 1996; Van Gele et al., 2000; Feng et al., 2008; Lassacher et al., 2008).

The proto-oncogene *KIT*, localized to human chromosome 4 (Yarden et al., 1987) encodes a transmembrane receptor, CD117/KIT, which belongs to the class III receptor tyrosine kinase family, including the receptor for colony-stimulating factor 1, and the platelet-derived growth factor receptors type A and B. KIT receptor activation by its ligand stem cell factor (SCF) is accompanied by receptor dimerization, substrate phosphorylation and autophosphorylation, receptor internalization, activation of protein kinases and phospholipases, and transcription of different proto-oncogenes (Roskoski, 2005). Generally, the KIT tyrosine kinase receptor pathway has been shown to be important for tumor growth and progression in several cancers (Miettinen and Lasota, 2005). Mutations in the *KIT* gene leading to ligand-independent phosphorylation (activation) of the KIT tyrosine kinase are believed to play a central pathogenetic role in different types of cancer, that is, gastrointestinal stromal tumors (GIST) and mastocytosis (Hornick and Fletcher, 2007).

KIT has been found expressed in biopsies from primary and metastatic Merkel cell tumors (Su et al., 2002). Importantly, no mutations have been described up to now in KIT exons 9, 11, 13, and 17, which have been reported to result in receptor activation in other tumors (Swick et al., 2007; Kartha and Sundram, 2008). Therefore, the possible mode of activation and the function of KIT in Merkel cell transformation remains subject for further investigation. Recently, co-expression of KIT and its ligand stem cell factor (SCF) has been reported in Merkel cell carcinoma biopsies (Kartha and Sundram, 2008; Krasagakis et al., 2009) suggesting an autocrine, ligand-dependent, activation of KIT in Merkel cell carcinoma. However, no functional studies have been reported up to now that provide evidence for activity of the autocrine loop in Merkel cell carcinoma.

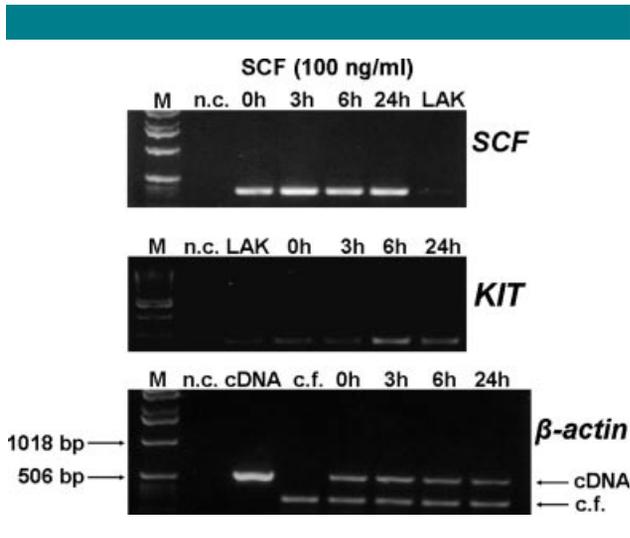
The established Merkel cell carcinoma cell line MCC-1 (Krasagakis et al., 2001) carries a M541L sequence variation of

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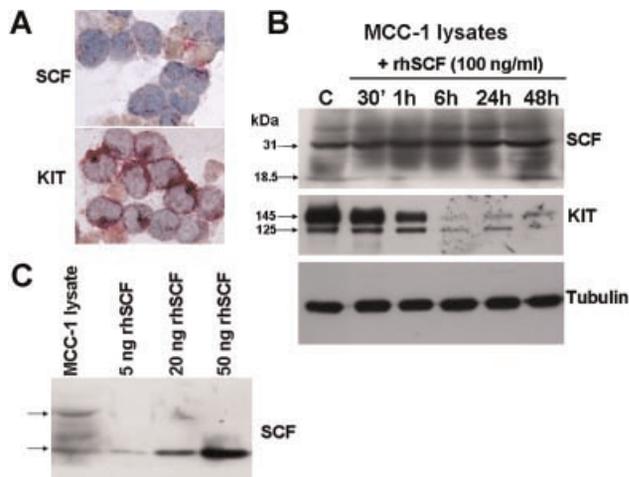
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**Fig. 1.** Semi-quantitative RT-PCR was used to study the expression of *SCF* and *KIT* mRNA and their modulation by exogenous soluble SCF (100 ng/ml) at 3, 6, and 24 h in MCC-1 cells. MCC-1 cells co-expressed *SCF* and *KIT* mRNA and exogenous soluble SCF induced expression of *KIT* mRNA. First lane of ethidium bromide-stained gels shows a DNA marker (M, 1 Kb DNA ladder from Invitrogen). For negative control, cDNA was omitted from PCR reaction (n.c.). As positive control, cDNA from peripheral blood mononuclear cells stimulated with 1,000 IU/ml IL-2 was used (LAK). An appropriately designed  $\beta$ -actin cDNA control fragment was co-amplified in each cDNA specimen of the  $\beta$ -actin samples in order to adjust the cDNAs from different time points to equal amounts (c.f.).



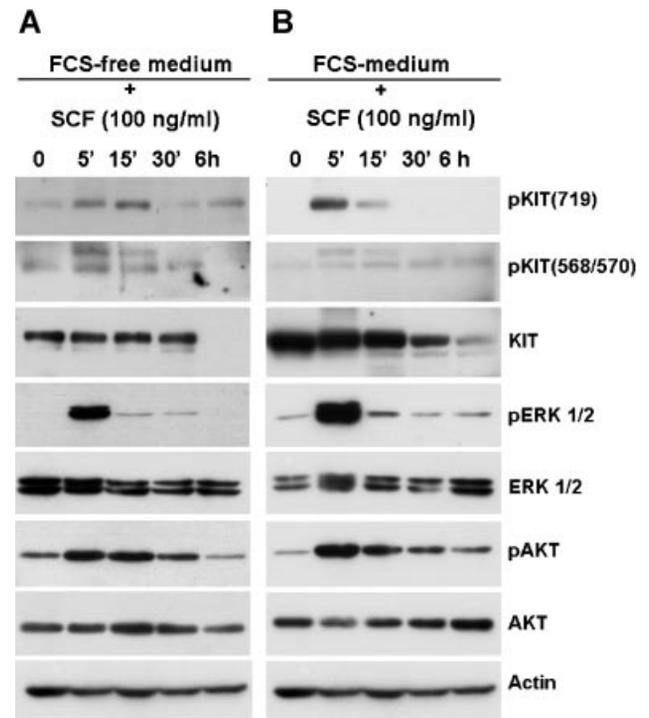
**Fig. 2.** **A:** SCF and KIT protein are expressed in cultured Merkel cell carcinoma cells (MCC-1). Cytospin preparations from MCC-1 cells were stained with anti-SCF and anti-KIT antibodies using immunocytochemistry with Fast red as chromogen. **B:** Endogenous SCF and KIT expression in MCC-1 cells in response to exogenous SCF (100 ng/ml) were determined by Western blot. MCC-1 cells expressed both the membrane-bound 31 kDa and the soluble 18.5 kDa forms of SCF. KIT receptor detected at 145 and 125 kDa was substantially downregulated by SCF after 6 h and partly recovered after 24 and 48 h. Tubulin was included as loading control. **C:** Quantification of endogenous SCF production in MCC-1 cells. SCF in lysates from  $1 \times 10^6$  cells were compared to recombinant human SCF (5, 20, and 50 ng) by Western blot using a mouse monoclonal SCF antibody.

the *KIT* gene (Krasagakis et al., unpublished data), that is, reported to confer an enhanced proliferative response to low levels of SCF but does not support ligand-independent receptor activation (Inokuchi et al., 2002; Foster et al., 2008). Similar to Merkel cell carcinoma biopsies, MCC-1 cells were found to express both SCF and KIT proteins in culture (unpublished data). In the present study, we investigated the autocrine stimulatory mechanism of the SCF/KIT system in the above cell culture model, and examined the effects of exogenous SCF on KIT receptor expression, signaling, and cell proliferation.

## Materials and Methods

### Immunocytochemistry

Immunocytochemistry was performed on air-dried, acetone-fixed, cytospin preparations of MCC-1 cells cultured in the fetal calf serum (FCS)-containing medium described in the DNA-synthesis section. A rabbit polyclonal anti-human antibody against KIT (A4502, dilution 1:50; DAKO Corporation, Carpinteria, CA), a mouse monoclonal antibody against SCF (G-3, dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse monoclonal antibody against Ki67 antigen (M7240, dilution 1:100, MIB-1 clone, DakoCytomation, Glostrup, Denmark) were used. After incubation with the primary antibodies at room temperature, the Ultravision LP Detection



**Fig. 3.** Exogenous SCF upregulates endogenous KIT phosphorylation and activates downstream signaling pathways in Merkel cell carcinoma. Protein lysates of MCC-1 cells starved in FCS-free medium (A) or maintained in FCS-medium (B) were treated for various times with SCF (100 ng/ml) and processed for immunoblotting with phospho-specific antibodies. Western blot analysis showed that in FCS-free medium exogenous SCF increased basal tyrosine 719 phosphorylation of KIT receptor at 5 and 15 min as well as basal tyrosine 568/570 phosphorylation at 5 min. Exogenous SCF induced ERK1/2 phosphorylation at 5 min, and increased basal AKT phosphorylation at 5, 15, and 30 min. In FCS-medium, similar to above, strong, and transient phosphorylation of KIT as well as of ERK1/2 and AKT by SCF was detected. Total KIT, ERK1/2, AKT, and  $\beta$ -actin protein levels are shown as controls.

System with AP Polymer and Fast Red as chromogen (Labvision Corp., Fremont, CA) were used according to the manufacturer's instructions for visualization of antibody binding. Negative controls were prepared by replacing the primary antibody with normal mouse or rabbit IgGs.

#### Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for analysis of SCF and KIT mRNA expression

MCC-1 cell pellets were homogenized after treatment with soluble SCF at various time points in FCS-containing medium as described below, and total cellular RNA was isolated by the thiocyanate/caesium-chloride method. RT-PCR was performed as previously described (Krüger-Krasagakes et al., 1994) using specific primers for SCF: 5' GGG CTG GAT CGC AGC GC and 5' CTC CAC AAG GTC ATC CAC (Longley et al., 1993) and for KIT: 5' CGT TGA CTA TCA GTT CAG CGA G and 5' CTA GGA ATG TGT AAG TGC CTC C (Ratajczak et al., 1992). Peripheral blood mononuclear cells activated with interleukin (IL)-2 were used as positive controls for SCF and KIT expression. The amount of cDNA analyzed in the samples was controlled by amplification of  $\beta$ -actin mRNA using primer sequences published by Yamamura et al. (1991)

and by co-amplification of an appropriately designed  $\beta$ -actin cDNA control fragment as previously described (Krüger-Krasagakes et al., 1994). The use of competitor  $\beta$ -actin control fragments for adjustment of equal amounts of input cDNA in the quantitative PCR has been reported by Uberla et al. (1991). Briefly, for comparison of SCF and KIT mRNA levels in different time points, cDNAs were first adjusted to equal concentrations of  $\beta$ -actin by competitive PCR. Therefore, serial 10-fold and subsequently 2-fold dilutions of cDNA were amplified in the presence of a fixed amount of  $\beta$ -actin control fragment, in order to determine exactly the amount of cDNA required to achieve equal band intensities for both fragments. The so-equalized cDNAs were then analyzed for SCF and KIT mRNA content.

#### Western blot analysis

Cell pellets were lysed after the various treatments in lysis reagent from Promega Corporation (Madison, WI), supplemented with protease inhibitors from Pierce Chemicals. Forty micrograms of protein preparations per lane were resolved by 7.5% SDS-polyacrylamide mini gel electrophoresis. Proteins were transferred by electroblotting to PVDF membranes (Biorad, Hercules, CA). Membranes were blocked for 30 min at RT with 5% skimmed milk diluted in Tris-buffered

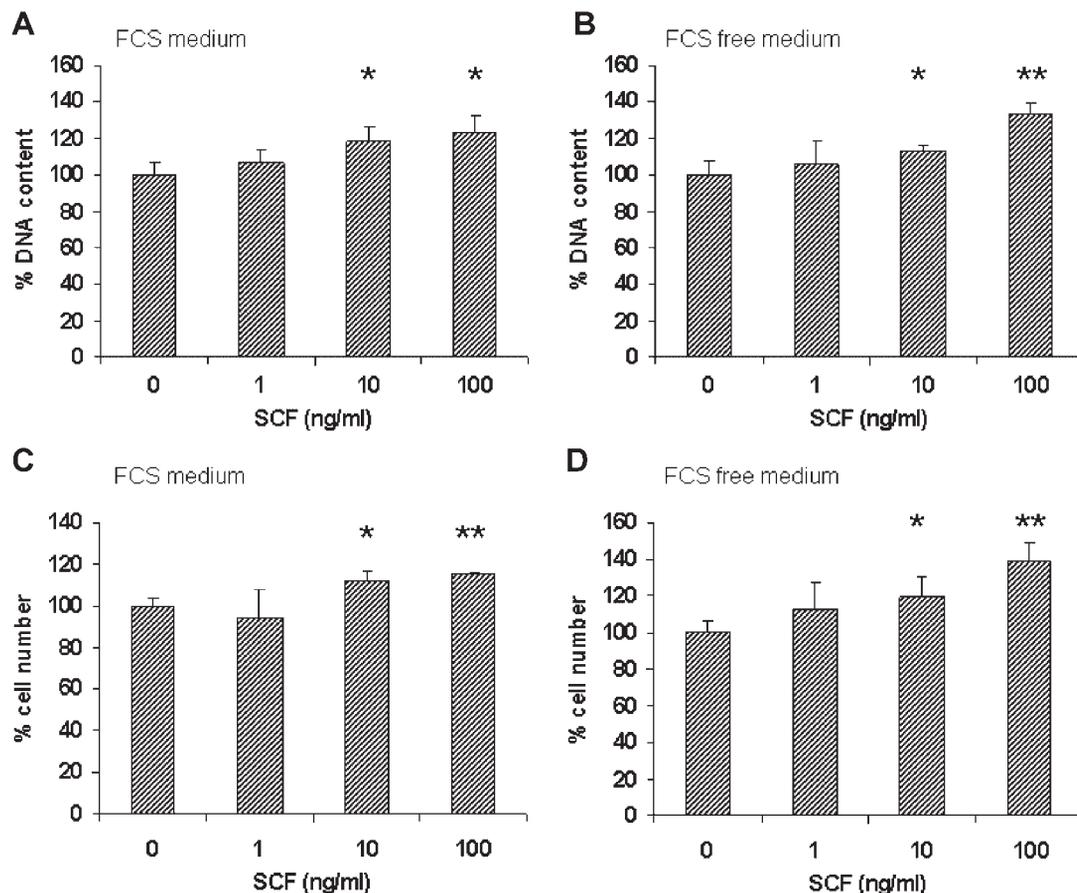


Fig. 4. Dose-dependent stimulation of proliferation (A,B) and growth (C,D) of MCC-1 cells by exogenous soluble SCF. MCC-1 cells were treated for 6 days with various concentrations of SCF (1–100 ng/ml) in FCS-containing (A,C) or FCS-free medium as described in the Materials and Methods Section. Fresh growth medium containing SCF was given every other day. Proliferation was assessed by the DNA-based CyQuant assay (A,B) and cell growth by the MTT assay (C,D). Results are given as mean values and SD of percent DNA-content or percent growth as compared to the respective control cultures (=100%) from four and three independently performed experiments respectively. Asterisks indicate statistically significant differences as compared to the controls (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

saline/Tween-20 (0.1% TBS-T), and subsequently incubated overnight at 4°C with primary antibodies diluted in 1% bovine serum albumin (BSA) in TBS-T. The following primary antibodies were used: mouse monoclonal anti-SCF (clone G-3, Santa Cruz Biotechnology), goat polyclonal anti-KIT (R&D Systems, Minneapolis, MN), two antibodies targeting different phosphorylation sites of KIT, Tyr719 (rabbit polyclonal, Cell Signalling Technologies, Beverly, MA) and Tyr568/570 (goat polyclonal, Santa Cruz Biotechnology), mouse monoclonal antibody against PKC- $\alpha$  or rabbit polyclonal antibody against phosphorylated PKC- $\alpha$  (both from Santa Cruz Biotechnology), rabbit monoclonal antibodies against extracellular regulated kinase (ERK) 1/2, rabbit polyclonal against pMARCKS, phosphorylated ERK1/2, AKT and phosphorylated AKT (all from Cell Signalling Technologies), and mouse monoclonal antibodies against tubulin or  $\beta$ -actin (both from Santa Cruz Biotechnology). After extensive washes, membranes were incubated with 1/5,000 dilution of goat anti-rabbit or goat anti-mouse IgG (both from Chemicon, Temecula, CA) or donkey anti-goat IgG (Santa Cruz Biotechnology) for 1 h at RT in blocking buffer. Membrane bound antibodies were visualized by use of ECL Western blotting detection reagent (Amersham Corp., Arlington Heights, IL) on X-ray films (Hyperfilm ECL, Amersham).

#### DNA-based proliferation assay

To study the effect of exogenous soluble SCF on MCC-1 DNA synthesis, 25,000 cells/ml were seeded in 96-wells in 0.2 ml of FCS-containing medium, composed of RPMI-1640 (Gibco, Paisley, Scotland), supplemented with 10% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany) and antibiotics (Biochrom KG). The same experiments were performed also in FCS-free medium composed of RPMI-1640, supplemented with 5  $\mu$ g/ml insulin, 10  $\mu$ g/ml transferrin, 100  $\mu$ g/ml BSA (Sigma-Aldrich, St. Louis, MO) and antibiotics (Biochrom KG). Recombinant human SCF (Chemicon) in fresh medium (1–100 ng/ml) was given to the cells every other day for 6 days. After treatment, cells were lysed and their number was determined using the fluorometric CyQUANT cell proliferation Assay Kit (Invitrogen, Paisley, UK), which measures DNA content using fluorescent dye binding. For converting sample fluorescence values into cell numbers, whenever necessary, a reference standard curve was created, using serial dilutions of known cell numbers of the MCC-1 cell line. Measurements were performed from five different wells in each experiment, and were repeated in four independent experiments.

#### Cell growth assay

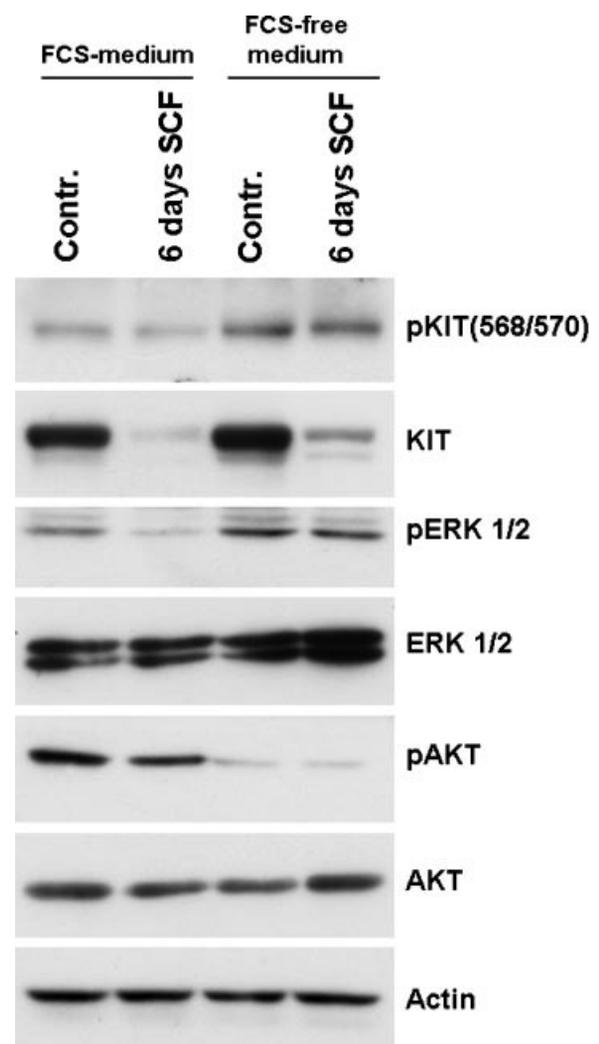
For quantification of cell growth, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Research Organics, Cleveland, OH) was used. Recombinant human SCF (Chemicon) was used as agonist to stimulate KIT in MCC-1 cells. The following KIT and downstream signaling antagonists were studied in MCC-1 cells: a neutralizing goat anti-KIT antibody (R&D Systems), the KIT tyrosine kinase inhibitors imatinib mesylate and nilotinib (also known as AMN107-AA) (both kindly provided by Novartis, Basel, Switzerland), the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)-inhibitor U0126 (Calbiochem, San Diego, CA), as well as the phosphatidylinositol (PI) 3-kinase inhibitors wortmannin (Sigma-Aldrich) and LY294002 (Calbiochem).

To study the effect of exogenous soluble SCF on MCC-1 proliferation, 200,000 cells/ml were seeded in 96-well plates (0.2 ml/well) in FCS-containing or FCS-free medium in the presence of various concentrations of recombinant human SCF (1–100 ng/ml). In case of treatment with anti-KIT antibody, 200,000 cells/ml were seeded in 96-well plates (0.2 ml/well) in

FCS-free medium. Cells were treated with either 1  $\mu$ g/ml of anti-KIT antibody or normal goat IgG (R&D Systems). In case of treatment with the KIT tyrosine kinase inhibitors, the MEK and PI3-K inhibitors, 200,000 cells/ml were seeded in 96-well plates (0.2 ml/well) in FCS-medium containing the various inhibitors, or using water or dimethyl sulfoxide (Sigma-Aldrich) as control solvents. The respective fresh medium with substances was given every other day for a total of 6 days, when 0.5 mg/ml of MTT was added to each well and the plates were incubated for additional 4 h at 37°C. MTT crystals were solubilized by the addition of dimethyl sulfoxide and the absorbance was measured in a microtiter plate reader. MTT assays were repeated in independent experiments three times for SCF and anti-KIT antibody, five to seven times for the KIT kinase inhibitors, and four times for the other inhibitors.

#### Cell viability and apoptosis assay

The trypan blue exclusion assay was used to determine viability of MCC-1 cells cultured in the presence or absence of imatinib



**Fig. 5.** Levels and activation status of KIT receptors after 6 days of incubation with exogenous SCF. MCC-1 cells were cultured either in FCS-containing or in FCS-free medium for 6 days in the presence of exogenous SCF (100 ng/ml). At the end of the treatment period, protein lysates of MCC-1 cells were processed for immunoblotting with various antibodies.

or nilotinib according to routine protocol. After trypsinization, cells were mixed with trypan blue solution (Sigma–Aldrich) and counted on hemocytometer. Cell viability was reported as the percentage viable cells in culture. To determine apoptotic changes, DNA fragmentation of apoptotic cells was monitored by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions, as previously described (Krasagakis et al., 2008).

### Statistics

Each growth experiment was performed in five different wells and repeated in independent experiments as indicated. Statistical differences of mean values were validated by the two sided Student's *t*-test.

### Results

#### The Merkel cell carcinoma cell line MCC-I co-expresses SCF and KIT, and exogenous SCF modulates KIT expression

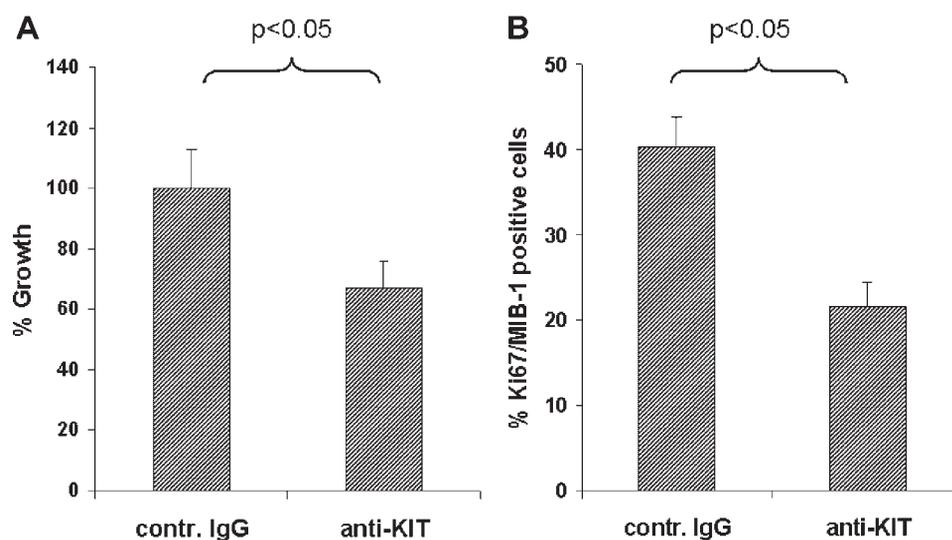
Semiquantitative RT-PCR was first applied to study the expression of *SCF* and *KIT* genes and their modulation in response to treatment with exogenous, soluble SCF. Without treatment, MCC-I cultures co-expressed SCF and KIT mRNA (Fig. 1). The treatment with soluble SCF (100 ng/ml) did not change endogenous levels of SCF mRNA, whereas the levels of KIT mRNA were increased at 6 and 24 h after treatment (Fig. 1). The expression of both SCF and KIT proteins was initially demonstrated by immunocytochemistry using cytospin preparations of MCC-I cells (Fig. 2A). Western blot analysis of protein lysates from MCC-I cells treated with soluble SCF (100 ng/ml) revealed two protein bands at 31 and 18.5 kDa, corresponding to the described membrane-bound and soluble form of SCF, respectively (Fig. 2B). Similar to the mRNA level, expression of endogenous SCF protein was not changed after the addition of exogenous SCF. Endogenous SCF in MCC-I lysates ( $1 \times 10^6$  cells) was quantitated in comparison to

recombinant SCF by Western blot (Fig. 2C). The lower SCF band in MCC-I lysates, corresponding to the soluble form of SCF, was on the order of the 5 ng recombinant SCF. The upper band, corresponding to the membrane-bound SCF, was comparably less than that of Figure 2B, but this might be due to protease sensitive nature of membrane SCF after trypsin-treatment of cultures for cell counting. In contrast to SCF, KIT protein which was expressed in two known isoforms (145 and 125 kDa; Turner et al., 1995; Wypych et al., 1995) in untreated MCC-I cells, was substantially downregulated by SCF after 6 h and partly recovered after 24 and 48 h (Fig. 2B). This effect has been previously reported as ligand-induced degradation of the receptor (Miyazawa et al., 1994). The partial recovery of KIT protein 24 and 48 h after stimulation with SCF is in accordance with the observed upregulation of *KIT* mRNA by SCF at 6 and 24 h.

#### Phosphorylation of KIT and activation of downstream effector molecules after stimulation with soluble SCF

Following binding of its ligand, KIT undergoes dimerization and autophosphorylation of several tyrosine residues. This leads to activation of different signal transduction molecules, including mitogen-activated protein kinases, phosphatidylinositol (PI) 3-kinase and protein kinase C. We used two different antibodies to detect autophosphorylation of KIT at tyrosine residues 568/570 and 719 respectively, after stimulation with soluble SCF (100 ng/ml) in MCC-I cells starved in FCS-free medium. Figure 3A shows that KIT was constitutively phosphorylated, albeit at low levels, at both positions in serum-starved non-stimulated MCC-I cells. Soluble SCF strongly upregulated KIT phosphorylation at both 568/570 and 719 residues immediately after 5 min. In some pKIT(568/570) immunoblots, we detected an additional slower migrating band, closely running to 145 kDa. The phosphorylations were transient and returned to basic levels after 30 min.

We also studied the activation of downstream signaling pathways upon stimulation with SCF. In control cells,



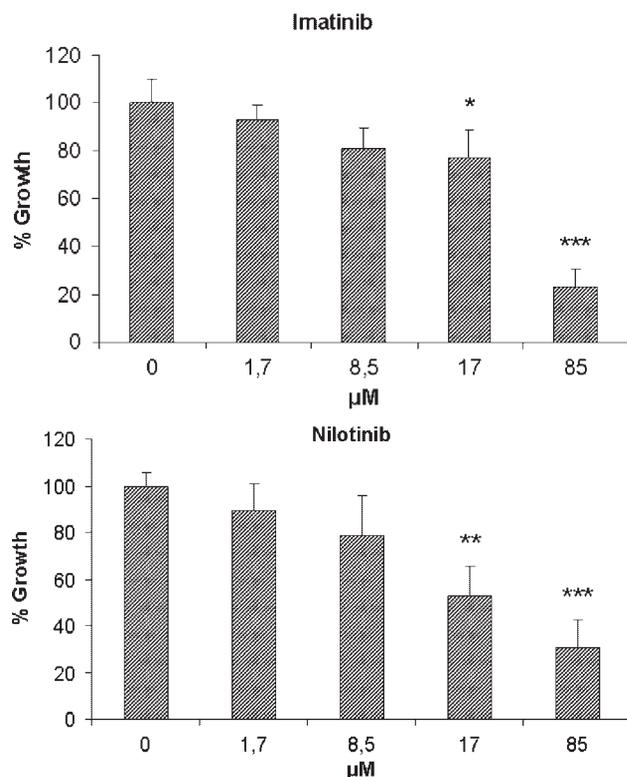
**Fig. 6.** Antagonistic KIT antibody inhibits proliferation of MCC-I cells. MCC-I cells were cultured for 6 days in FCS-free RPMI-1640 supplemented with 5  $\mu$ g/ml insulin, 10  $\mu$ g/ml transferrin, 100  $\mu$ g/ml bovine serum albumin, containing either 1  $\mu$ g/ml of anti-KIT antibody or normal goat IgG. Fresh growth medium containing substances was given every other day. **A:** Cell growth was assessed by the MTT assay and results are given as % mean values and SD compared to the controls (= 100%) from three experiments. **B:** Ki67/MIB-1 labeling index of MCC-I cell line was evaluated by immunocytochemistry performed on cytospin preparations from treated cultures. Results are given as mean values and SD of percent positive stained nuclei from triplicate determinations of randomly selected fields with at least 100 cells each (three independent experiments).

phosphorylated AKT protein was detected, whereas the ERK1/2 protein was not found consistently phosphorylated. Soluble SCF further upregulated AKT phosphorylation and strongly triggered phosphorylation of ERK1/2 after 5 min, without affecting the total amounts of AKT and ERK proteins (Fig. 3A). Upregulation of AKT phosphorylation was more sustained and lasted for 30 min, whereas ERK1/2 phosphorylation revealed a fast turnover and was not detectable after 15 min. Thus, the more sustained AKT phosphorylation timely correlated with the prolonged Tyr719 KIT phosphorylation, which already has been linked to activation of the PI3 kinase/AKT pathway (Serve et al., 1994). In contrast, ERK phosphorylation revealed a fast turnover similar to the Tyr568/570 KIT phosphorylation. The levels of phosphorylated PKC- $\alpha$  remained unaffected after treatment with soluble SCF (data not shown).

We further studied KIT phosphorylation and activation of downstream signaling pathways ERK1/2 and AKT by SCF in the presence of FCS. As can be seen from Figure 3B, the pattern of KIT, ERK1/2, and AKT activation in the presence of FCS was similar to that in the absence of FCS, with only small differences in basal levels and kinetics of phosphorylation of the proteins.

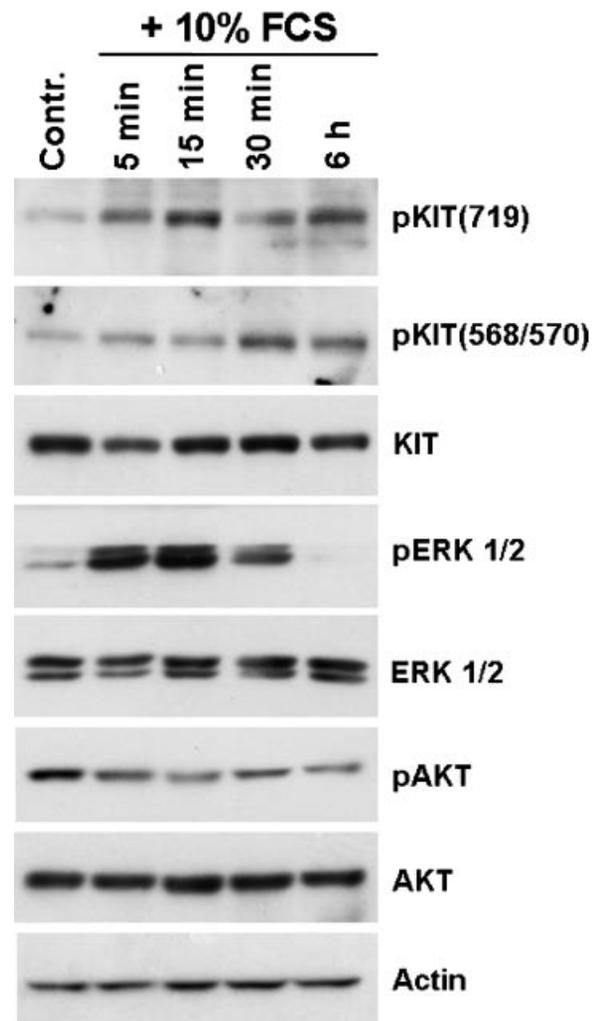
### Soluble SCF stimulates MCC-I cell proliferation

Treatment of MCC-I cells for 6 days with various concentrations of exogenous soluble SCF stimulated dose-dependently proliferation and growth both in FCS-



**Fig. 7.** Inhibition of MCC-I cell proliferation by the KIT kinase inhibitors. MCC-I cells were treated for 6 days with increasing concentrations of imatinib or nilotinib. Fresh growth medium containing inhibitors or solvent was given every other day. Cell growth was assessed by the MTT assay and was given as % of controls (=100%). Data represent mean values and SE of five (nilotinib) to seven (imatinib) independent experiments each performed in five wells. Asterisks indicate statistically significant differences as compared to the controls (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

containing and FCS-free medium. Figure 4A,B demonstrate results from four independent experiments with the CyQuant assay showing a moderate increase of DNA content at 100 ng/ml SCF, which was more prominent in the FCS-free medium than in FCS-containing medium (135% of the controls,  $P < 0.001$  vs. 121% of the controls,  $P < 0.05$ ). The increase of DNA content at lower SCF concentrations was less prominent and statistically significant only in the FCS-free medium (119% at 10 ng/ml;  $P < 0.05$ ). Figure 4C,D demonstrates the results obtained by the MTT assay from three independently performed experiments. Similarly to the findings of the CyQuant assay, a notable increase was observed only at 100 ng/ml SCF in FCS-free medium (139%;  $P < 0.001$ ) and to a lesser extent in FCS-containing medium (115%;  $P < 0.01$ ). SCF at 10 ng/ml also stimulated cell growth (Fig. 4C,D). The observed rather moderate pro-mitotic effects of exogenous SCF may be attributed to the presence of endogenous, autocrine-produced SCF in MCC-I cultures, but also might be due to the exogenous SCF-induced KIT degradation that would restrict KIT-dependent signaling. We therefore examined levels and



**Fig. 8.** FCS activates KIT signaling. Protein lysates of MCC-I cells starved in FCS-free medium and treated for various times with 10% FCS were processed for immunoblotting with phospho-specific antibodies. Western blots showed phosphorylation of KIT as well as of ERK1/2 by FCS. Total KIT, ERK1/2, AKT, and  $\beta$ -actin protein levels served as controls.

activation status of KIT receptors after 6 days of incubation with exogenous SCF. Figure 5 demonstrates that KIT levels are partly restored by this time, and that the restitution is more prominent in the FCS-free medium, where we also observed that the effect of exogenous SCF on cell proliferation was stronger. Interestingly, the level of pKIT(568/570) was equal between 0 and 6 days of treatment, suggesting that the restored KIT receptors are present mainly in their phosphorylated forms. Also, the levels of ERK1/2 and AKT phosphorylation were similar between 0 and 6 days of treatment, except those of pERK1/2 after 6 days in FCS-medium. The levels of pKIT(719) were hardly detectable either in control or SCF-treated cultures.

#### Antibody-mediated KIT neutralization inhibits MCC-I growth

For studying the effect of endogenous autocrine SCF activity on Merkel cell carcinoma growth we used a goat antibody directed against the extracellular domain of KIT, able to neutralize SCF receptor-mediated bioactivity. Incubation of MCC-I cells for 6 days with the neutralizing KIT antibody reduced cell proliferation. Figure 6A shows results from three independent experiments demonstrating reduction of cell proliferation by 33% compared to the controls (Fig. 6A,  $P < 0.05$ ). No cytotoxicity was observed by the trypan blue exclusion test, suggesting solely a cytostatic effect of the KIT antagonistic antibody. Accordingly, the proliferating fraction of MCC-I cells decreased from 41% to 22%, as determined by nuclear staining with the Ki67/MIB-I antibody (Fig. 6B, results from three independent experiments,  $P < 0.05$ ). The Ki67/MIB-I proliferation index correlates with the growth fraction of the cells (Scott et al., 1991; Schlüter et al., 1993).

#### Imatinib and nilotinib inhibit growth of MCC-I cells and block phosphorylation of KIT

To further investigate the role of KIT on autocrine stimulation of MCC-I cells, two pharmacologic inhibitors of KIT kinase were used and the effects on cell proliferation were studied. Imatinib is known to bind to the adenine-binding portion of the enzyme (Mol et al., 2004), and nilotinib, a second generation compound structurally related to imatinib, also inhibits KIT kinase activity (Roberts et al., 2007). As demonstrated in Figure 7, imatinib at 85  $\mu\text{M}$  was able to reduce growth of MCC-I cells by 77% ( $P < 0.001$ ,  $n = 7$ ), and nilotinib by 69% at 85  $\mu\text{M}$  ( $P < 0.001$ ,  $n = 5$ ). MCC-I cells were relatively resistant to both KIT kinase inhibitors, since concentrations lower than 17  $\mu\text{M}$  were not effective.

Due to the inhibitory effect of imatinib and nilotinib on MCC-I proliferation in the presence of FCS, we investigated whether FCS activates KIT receptor and ERK1/2 or AKT signaling. It should be noted that FCS is a poor source for SCF, since SCF levels are below 100 pg/ml (Hikono et al., 2002). We stimulated serum-starved cells with 10% FCS for various time points and performed Western blot (Fig. 8). As can be seen, FCS induced phosphorylation of KIT at both Tyr719 and 568/570 positions, which was more protracted than the stimulation with soluble SCF. Also, FCS stimulated transient phosphorylation of ERK1/2, but not of AKT, which appeared under phosphorylated after FCS.

We also aimed to investigate the specificity of 85  $\mu\text{M}$  imatinib for KIT tyrosine kinase inhibition in the presence of FCS. We therefore stimulated MCC-I cells for 15 min with FCS in the presence or absence of 85  $\mu\text{M}$  imatinib. As it can be observed in Figure 9, imatinib inhibited phosphorylation of KIT at Tyr719 and 568/570 positions, and of AKT. Interestingly, an increased phosphorylation of ERK1/2 was observed in FCS-treated cultures in the presence of imatinib compared to FCS-only treated cultures. The phosphorylation of PKC- $\alpha$  and of

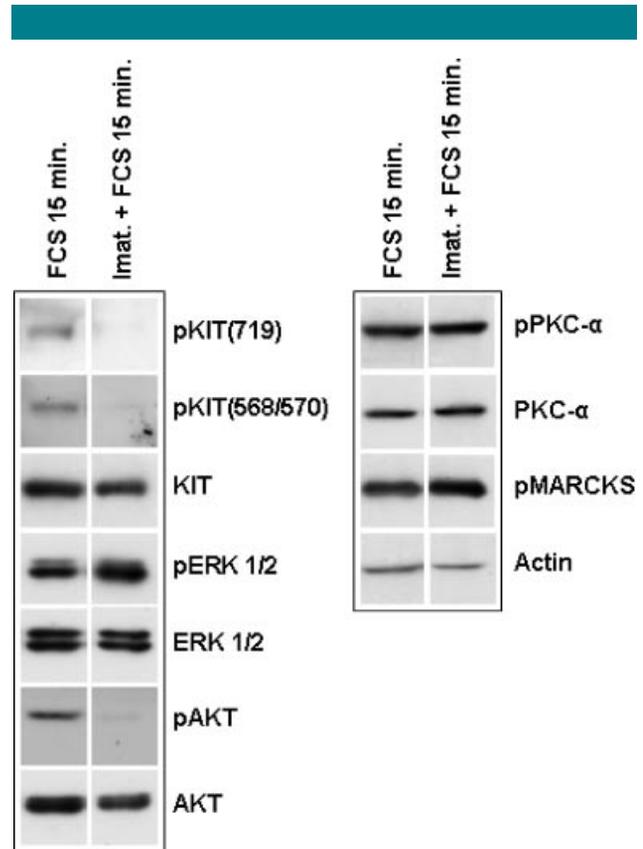
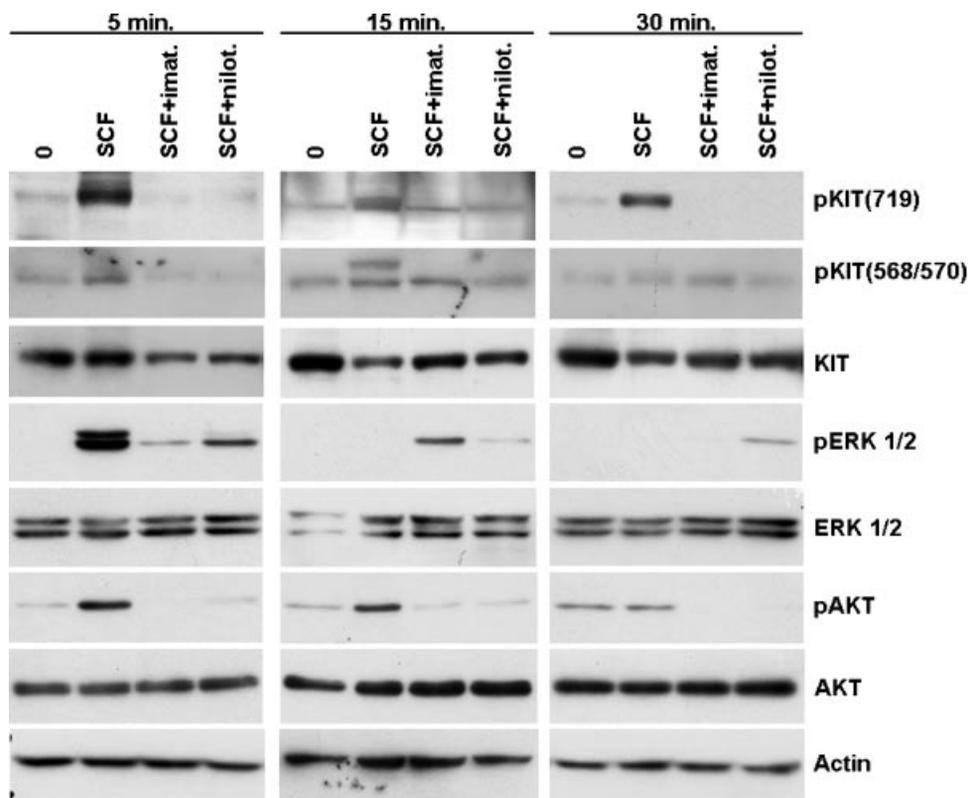


Fig. 9. Specificity of KIT kinase inhibition by imatinib (85  $\mu\text{M}$ ) in the presence of FCS. MCC-I cells starved in FCS-free medium were preincubated for 2 h with 85  $\mu\text{M}$  imatinib and were then treated for 15 min with 10% FCS and processed for immunoblotting with phospho-specific antibodies. Western blots showed inhibition of phosphorylation of KIT as well as of AKT by imatinib but not of PKC- $\alpha$  or MARCKS. ERK1/2 phosphorylation was increased by imatinib. Total KIT, ERK1/2, AKT, PKC- $\alpha$ , and  $\beta$ -actin protein levels were used as controls.

MARCKS, a substrate of PKC- $\alpha$ , were not influenced by imatinib.

Since the cell line carries the KIT M541L sequence variation which may influence imatinib or nilotinib sensitivity we also examined KIT phosphorylation in the presence of exogenous soluble SCF. Both imatinib and nilotinib effectively blocked SCF-induced KIT and AKT phosphorylation in MCC-I cells (Fig. 10). According to these findings, the inhibition of KIT and AKT phosphorylation is linked to the regulation of Merkel cell carcinoma growth. Also, imatinib and nilotinib partially inhibited SCF-induced early (5 min) upregulation of ERK1/2. Similar to the findings in FCS-stimulated cells, a persistence of ERK1/2 phosphorylation at later time points (15 or 30 min) was seen in imatinib- and nilotinib-treated cells, when ERK1/2 phosphorylation was already downregulated in cells treated with SCF alone.

We investigated whether late ERK1/2 phosphorylation was a survival signal due to the toxicity of high dose KIT kinase inhibitors. Therefore, we performed an experiment to control whether induction of cell death was part of the antiproliferative effect of KIT inhibitors, as already reported in other cell types. We determined cell viability of MCC-I cells treated by imatinib (17 and 85  $\mu\text{M}$ ) by the trypan blue exclusion assay at 6, 24, and 96 h. The results shown in Figure 11 demonstrated significant cytotoxicity observed after 24 h of treatment with 85  $\mu\text{M}$  KIT inhibitors ( $P < 0.01$ ), whereas at 96 h both concentrations used



**Fig. 10.** Imatinib and nilotinib inhibit SCF-induced activation of KIT, AKT, and ERK1/2. MCC-1 cells were preincubated for 2 h with 85  $\mu$ M imatinib or nilotinib and were then treated for 5, 15, or 30 min with 100 ng/ml soluble SCF. Protein lysates were processed for Western blotting using antibodies against phospho-KIT (pKIT), KIT, phospho-ERK1/2 (pERK1/2), phospho-AKT (pAKT), and  $\beta$ -actin.

had a cytotoxic effect. Viability decreased from initially 90%, down to 30% and 16% by 85  $\mu$ M imatinib and nilotinib respectively (96 h,  $P < 0.001$ ). At 6 h the cytotoxic effect of 85  $\mu$ M KIT inhibitors was marginal, since viability decreased by 7% compared to the controls. Cytotoxicity was not due to apoptosis but rather due to necrosis, since TUNEL assays at 6, 24, and 96 h did not reveal more than 3% apoptotic cells at the high dose of KIT inhibitors (data not shown).

We further aimed to determine whether cell cycle inhibition contributed to the imatinib and nilotinib-mediated growth inhibitory effect. MCC-1 cells treated with active doses of the KIT inhibitors (17 and 85  $\mu$ M) were stained with the Ki67/MIB-1 antibody to determine the growth fraction of the cultures. Figure 11 demonstrates a significant cytostatic effect of KIT inhibitors in all concentrations at 24 and 96 h ( $P < 0.001$ ). We observed that the proliferating fraction of MCC-1 cells treated with inhibitors was higher in 96 h than 24 h. Since in 96 h the cytotoxicity rates in treated cultures were high, it can be concluded that a significant proportion of the few cells surviving from cytotoxicity were actively proliferating. This probably represents a mechanism of resistance to cytotoxic therapy.

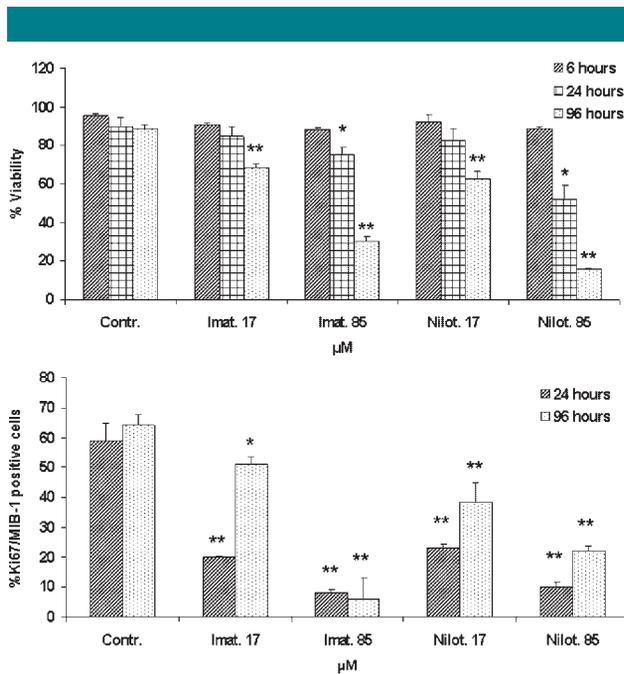
#### Effect of inhibition of MAPK/ERK and PI3K/AKT pathway on growth of MCC-1 cells

Since ERK1/2 and AKT were both activated by SCF in MCC-1 cells, additional small molecule inhibitors for MEK and PI3K were used in order to discriminate the contribution of the MAPK/ERK and PI3K/AKT pathways to growth control. As depicted in Figure 12A, the MEK inhibitor U0126 significantly inhibited proliferation of MCC-1 cells by 66% (average of four

independent experiments,  $P < 0.001$ ). Similarly, wortmannin and LY294002, two different inhibitors of PI3-kinase, both inhibited growth of MCC-1 cells by 44% and 56% respectively (average of four independent experiments,  $P < 0.001$  for both). In addition, we examined the specificity of these kinase inhibitors by determining ERK1/2 and AKT phosphorylation. Therefore, FCS-starved MCC-1 cells were preincubated for 2 h with 50  $\mu$ M U0126, 10  $\mu$ M wortmannin, or 50  $\mu$ M LY294002 and then treated for 5 min with SCF (100 ng/ml). Cell lysates were processed for immunoblotting. Figure 12B shows that the inhibitors U0126, wortmannin and LY294002 were highly active for inhibiting ERK1/2 and AKT phosphorylation respectively, with little unspecific activity in case of wortmannin. These results suggest the contribution of both MAPK/ERK and PI3K/AKT pathways in growth of MCC-1 Merkel cell carcinoma cells.

#### Discussion

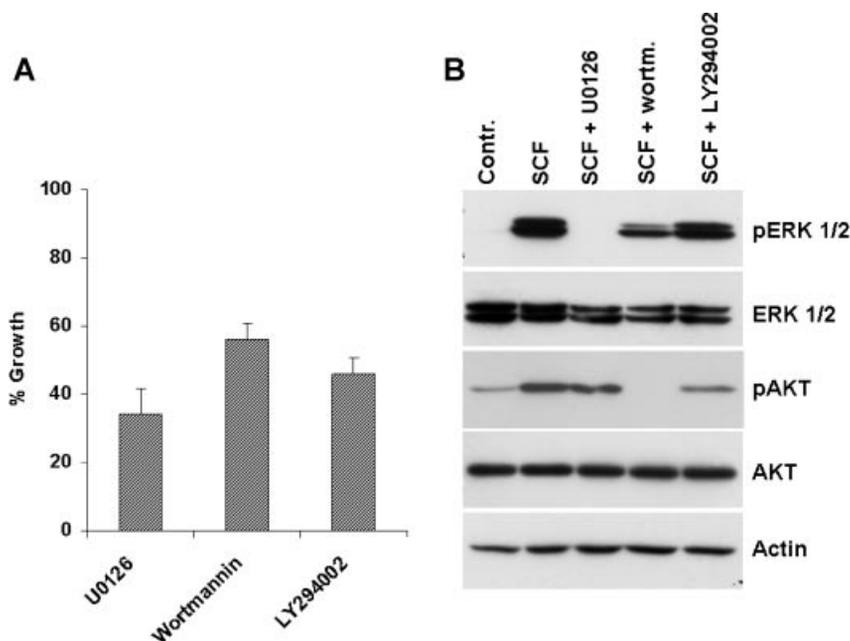
The present paper is the first study reporting the activation of KIT by exogenous SCF and the presence of an endogenous autocrine stimulatory loop of SCF and KIT in Merkel cell carcinoma cells. KIT activation in tumors is accomplished by two major mechanisms: (a) autocrine or paracrine stimulation of KIT by its ligand SCF, and (b) acquisition of KIT activating mutations. SCF-mediated stimulation has been reported in several types of cancer such as small cell lung and colon carcinoma (Krystal et al., 1996; Bellone et al., 2001), whereas KIT activating mutations were found in mastocytosis and GIST (Nagata et al., 1995; Rubin et al., 2001). Autonomous proliferation of tumor cells may also depend on a combination of both (Théou-Anton et al., 2006; Hirano et al., 2008).



**Fig. 11.** Cytotoxic and cytostatic effect of KIT kinase inhibitors. MCC-I cells were treated for 96 h with 17 or 85  $\mu\text{M}$  imatinib or nilotinib. Fresh growth medium containing inhibitors or solvent was given every other day. Cytotoxicity was assessed by determining viability rates using the trypan blue exclusion test at 6, 24, and 96 h. Cytostasis was assessed by determining the growth fraction of the cultures with the Ki67/MIB-1 labeling index at 24 and 96 h as described in Figure 7. Data represent mean values and SD from triplicate determinations. Asterisks indicate statistically significant differences as compared to the controls (\* $P < 0.01$ ; \*\* $P < 0.001$ ).

The MCC-I cell line expressed both KIT and SCF, as previously described for Merkel cell carcinoma tumors (Kartharou and Sundram, 2008; Krasagakis et al., 2009). Similar to the findings in gastrointestinal stromal tumors (Hirano et al., 2008), the membrane-bound form of SCF was more abundantly expressed than the soluble in MCC-I cells. Both SCF forms may contribute to autocrine activity in MCC-I cell line, since cells grow in multicellular floating aggregates with direct cell-to-cell contacts, thus permitting the interaction of membrane-bound SCF with cell surface KIT receptors. Interestingly, the membrane-bound SCF form has been related to constitutive KIT activation by causing persistent KIT phosphorylation (Miyazawa et al., 1995), as observed in serum-starved non-stimulated MCC-I cells with KIT phosphorylated at 568/570 and 719 tyrosines. Phosphorylation at 568/570 is known to activate Src family kinases, which contribute through the adaptor proteins Grb2 and Shc to Ras and mitogen-activated protein kinase (MAPK) activation (Ueda et al., 2002; Roskoski, 2005). Phosphorylation at Tyr719 is important for recruitment of PI3-kinase and subsequent activation of the central survival and growth regulator AKT (Blume-Jensen et al., 1998; Roskoski, 2005). Interestingly, only AKT was constitutively activated in MCC-I cells, whereas ERK was not found consistently phosphorylated in unstimulated cells.

Phosphorylation of ERK1/2 and AKT paralleled KIT autophosphorylation by SCF. It has been reported previously that MAPK cascade was not activated in the majority of Merkel cell carcinomas, with only a subset of them expressing constitutively active, phosphorylated ERK1/2 (Houben et al., 2006). Similarly, we did not find any significant ERK1/2 phosphorylation in starved MCC-I cells, but that ERK1/2 was strongly activated by exogenous SCF. This implies that the ERK pathway may become of significance for Merkel cell tumor growth, once it is activated by exogenous factors such as SCF or serum growth factors. This is further supported by the MEK



**Fig. 12.** Inhibition of KIT downstream signaling pathways results in growth inhibition of Merkel cell carcinoma cells in vitro. **A:** MCC-I cells were treated for 6 days with 50  $\mu\text{M}$  U0126, 10  $\mu\text{M}$  wortmannin, or 50  $\mu\text{M}$  LY294002. Fresh medium containing inhibitors or solvent was given every other day. Cell growth was assessed by the MTT assay. Mean values and SE of percent growth compared to untreated controls (=100%) from four independently performed experiments are shown.  $P$  values were in all cases at the level of 0.001. **B:** Specificity of MEK and PI3-K inhibitors was controlled by determining ERK1/2 and AKT phosphorylation. FCS-starved MCC-I cells were preincubated for 2 h with 50  $\mu\text{M}$  U0126, 10  $\mu\text{M}$  wortmannin, or 50  $\mu\text{M}$  LY294002 and then treated for 5 min with SCF (100 ng/ml). Cell lysates were processed for immunoblotting.

inhibitor U0126. MEK is upstream of ERK and its inhibition significantly reduced proliferation of serum-stimulated MCC-I cells. Thus, the MAPK pathway inhibition may be considered as therapeutic target for Merkel cell carcinomas. In this respect, our findings differ from those of Houben et al. (2007), who reported that overexpression of c-Raf-1 triggered MAPK activation and induced apoptosis in Merkel cell carcinoma cells, which was blocked by U0126. However, MAPK activation remained constantly at high levels in c-Raf-1-overexpressing cells, which may initiate also different programs of gene activation. In contrast, stimulation with soluble SCF or serum produces only a short and transient activation of ERK 1/2, as also seen in our experiments.

AKT protein was also activated by SCF, which may modify the signal to promote proliferation and inhibit apoptosis in Merkel carcinoma cells. In line with this, two different inhibitors of PI3-kinase, the enzyme that lies upstream and activates AKT protein, reduced proliferation of MCC-I cells. Activated AKT may enable cells to overcome cell cycle arrest in G1 and G2 phases and thus to permit cell proliferation (Ramaswamy et al., 1999; Kandel et al., 2002). Anti-apoptotic signals can be also induced by AKT, that is, by the phosphorylation of Bad by KIT that leads to its dissociation from the Bcl-2/Bcl-X complex (Blume-Jensen et al., 1998). In MCC-I cells, activation of AKT remained three times longer than the activation of ERK 1/2.

Proliferation and growth of MCC-I cells promoted by exogenous SCF were rather moderate, even in the absence of serum that activated KIT signaling in MCC-I cells. This might be either due to endogenous KIT activation from autocrine-produced SCF or to exogenous SCF-induced KIT degradation that would restrict KIT-dependent signaling. When we examined KIT levels in later time points, we found that KIT activation and levels were partly restored, suggesting that KIT receptors are functional during long-term incubation by SCF.

The importance of autocrine SCF in the stimulation of MCC-I proliferation was demonstrated by the growth inhibitory effect of a neutralizing KIT antibody and of the KIT kinase inhibitors imatinib and nilotinib (Roskoski, 2005). Imatinib has been reported to inhibit the autocrine activation of KIT in small cell lung carcinomas and myeloid leukemia cell lines (Heinrich et al., 2000; Krystal et al., 2000). Nilotinib is a phenylpyrimidine compound with superior to imatinib activity against mutant KIT V560G in neoplastic mast cells (Gleixner et al., 2006). The present study revealed equipotent activities of the two compounds as reported previously by Verstovsek et al. (2006), although higher doses were needed here. High doses of imatinib were also required in another study for moderate inhibition of Merkel cell carcinoma cells with unknown KIT mutational status (Fenig et al., 2004). The M541L KIT sequence variation, also found in MCC-I cells, has been reported to enhance the sensitivity of myeloid M541L-transfected cells to imatinib by a factor of two (Foster et al., 2008). The decreased sensitivity of the MCC-I cell line used here may depend on crosstalk with other signaling pathways. Nevertheless, these compounds effectively inhibited KIT activation and downstream signaling induced either by SCF or by serum in MCC-I cells. However, we can not exclude that other receptor kinases, such as of the PDGFA/PDGFR system that is expressed in Merkel cell carcinoma (Brunner et al., 2008; Kartha and Sundram 2008), may also be inhibited by imatinib or nilotinib and contribute to their antiproliferative effect. This is a possible limitation of our study compounds. Notwithstanding, there are no data known on the activity of PDGFA/PDGFR in Merkel cell carcinoma. The possibility that the growth inhibitory effect of these compounds is solely due to inhibition of PDGFR is not supported by the growth inhibitory effect of the antagonistic KIT-antibody.

A recent study to test the clinical effect of imatinib therapy in advanced MCC showed lack of efficacy (Samlowski et al., 2009).

The doses that were effective in our model are beyond the range of 1–2  $\mu$ M that can be attained in the plasma of imatinib or nilotinib-treated patients (Kantarjian et al., 2006; Larson et al., 2008). It is therefore of clinical significance to identify possible reasons for imatinib or nilotinib resistance. Although these compounds clearly inhibited SCF-induced AKT signaling in MCC-I cells, they only partially inhibited the ERK 1/2 phosphorylation in the presence of SCF. By contrast, increased phosphorylation of ERK 1/2 was observed 15 min after SCF- or FCS-treatment in the presence of imatinib. Since no clear correlation between Tyr568/570 KIT and ERK 1/2 phosphorylation in imatinib or nilotinib-treated cultures was observed, KIT inhibitors probably induce ERK phosphorylation independently of KIT status. A reason might be that late pERK 1/2 was a survival signal due to toxicity of high dose KIT inhibitors. Different assays showed that the antiproliferative effect of imatinib and nilotinib is due to both cytotoxicity (necrosis) and cytostasis with no apoptosis, whereas cytotoxicity became evident after 24 h. Although these findings do not exclude, they do not give strong support that pERK 1/2 by KIT inhibitors is a survival signal. Paradoxical activation of the ERK 1/2 route has been reported in myeloma cells, which also require high doses of imatinib for inhibition (Pandiella et al., 2003). Interestingly, we observed that MCC-I cells escaping KIT kinase inhibitor cytotoxicity were still proliferating, suggesting possible survival of resistant cells.

Summarizing, the present study reports that Merkel cell carcinoma cells may gain autonomous proliferation by an autocrine mechanism of the SCF/KIT system. This pathway activates the pro-mitogenic and anti-apoptotic signaling cascades of ERK 1/2 and AKT. The blockade of the SCF/KIT activation loop by a KIT neutralizing antibody and several inhibitors of intracellular signaling (KIT, MEK, PI3-K) resulted in a substantial inhibition of cell proliferation. Thus, SCF/KIT activation and its downstream effector molecules ERK 1/2 and AKT seem to be important for the acquisition of either autonomous or exogenously stimulated growth of Merkel carcinoma cells. Disruption of autocrine SCF/KIT axis in Merkel cell carcinoma by pharmacologic inhibitors may be of potential value for treatment of this tumor. However, specific considerations have to be taken regarding the possible presence of KIT genetic changes or pathway cross-talk that may affect sensitivity to well established KIT inhibitors. Nevertheless, the blockade of downstream kinases (ERK 1/2 or AKT) that mediate KIT action may overcome the existence of any KIT sequence alterations or pathway interactions that affect drug sensitivity.

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