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Distinctive Role of the cKit Receptor Tyrosine Kinase Signaling in Mammalian Melanocytes

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The cKit receptor plays a critical role in melanocyte physiology, influencing melanogenesis, proliferation, migration, and survival of the pigment-producing cells. However, pathways of cKit-mediated intracellular signaling and molecular mechanisms, which regulate specific cellular responses to the activation of the receptor in melanocytes, remain incompletely understood. Here, by using the genetically altered mouse melanocytes expressing an endogenous, constitutively active mutant (D814Y) cKit receptor, we investigated physiological cellular responses to the ligand-independent activation of the receptor tyrosine kinase. It was anticipated that such activation would either trigger uncontrolled proliferation of the melanocytes or stimulate melanin biosynthesis. In contrast to the expectation, we found that constitutive signaling from the cKit receptor did not stimulate melanogenesis and proliferation, but significantly promoted migration of the melanocytes both *in vitro* and *in vivo*. We also showed that such signaling is not associated with tumorigenic transformation of the pigment-producing cells. Taken together, our observations suggest that, in mammalian melanocytes, activation of the cKit receptor tyrosine kinase is primarily responsible for transmission of pro-migration signals, which may antagonize proliferation and melanogenesis. Our data also provide an additional explanation as to why malignant melanocytes lose cKit expression during melanoma progression.

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INTRODUCTION

cKit tyrosine kinase receptor for the stem cell factor (SCF), encoded by the proto-oncogene c-*kit*, belongs to the plate-derived growth factor family of the receptor tyrosine kinases (RTK). Structurally, it is characterized by the presence of five immunoglobulin-like motifs in the extracellular domain of the receptor and the cytoplasmic kinase domain, which is split by an insert into the ATP binding region and the phosphotransferase domain (Yarden *et al.*, 1987). Binding of the SCF to the extracellular domain of the receptor launches a cascade of intracellular events that leads to dimerization of receptors, activation of the tyrosine kinase, and subsequent autophosphorylation (Blume-Jensen *et al.*, 1991; Lev *et al.*, 1992). The intracellular signaling from the cKit RTK plays a critical role in the development of a variety of mammalian cell types including melanocytes, hematopoietic progenitor cells, mast cells, primordial germ cells, and intestinal cells of Cajal (Nishikawa *et al.*, 1991; Grabbe *et al.*, 1994; Galli *et al.*, 1995). As demonstrated by genetic studies, naturally occurring inactivating mutations in the proto-oncogene c-*kit* that impair the kinase activity of the receptor lead to various developmental disorders that result in amelanotic congenital patches of white skin, anemia, and sterility often associated with human piebaldism and mouse dominant white spotting (Giebel and Spritz, 1991; Spritz *et al.*, 1992). In contrast, an abnormal activation of cKit due to the autocrine secretion of its ligand, SCF, has been frequently observed in various cancers, including small cell lung cancer, colorectal carcinoma, breast carcinoma, gynecological tumors, and neuroblastomas (Boissan *et al.*, 2000). Additionally, the ligand-independent constitutive cKit RTK activation caused by mutations was found to be responsible for progression of mast cell and gastrointestinal stomal tumors.

Although many studies have contributed to the analyses of the cKit receptor in normal, vitiligo, and malignant melanocytes, the precise role of the cKit-mediated intracellular signaling in melanocyte migration, survival, proliferation, and differentiation remains incompletely understood. Intracellular signaling pathways mediated by cKit receptor have been extensively investigated in various hematopoietic cells, but at present it is not clear where and how cKit activation regulates different aspects of melanocyte physiology. Also, very little is known about how distinct domains of cKit receptor and the different pathways downstream of cKit relate to a receptor's numerous developmental roles.

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Abbreviations: MITF, microphthalmia-associated transcription factor; PI3K, phosphatidyl inositol-3 kinase; RTK, receptor tyrosine kinase; SCF, stem cell factor; SD, standard deviation; TPA, 12-O-tetradecanoylphorbol-13-acetate

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Previously, we introduced an activating point mutation (GAC→TAC, D814Y) into the c-*kit* gene of mouse melanocytes by using the oligonucleotide-mediated strategy of targeted gene alteration. The presence of the mutation in the endogenous c-*kit* gene was confirmed by direct DNA sequencing. The constitutive, ligand-independent activation of cKit receptor was verified by the kinase activity assay and analysis of the receptor autophosphorylation (Alexeev *et al.*, 2002). The introduced activating point mutation (GAC→TAC, D814Y) was first identified and characterized in a murine mastocytoma cell line as responsible for the constitutive activation of the cKit tyrosine kinase in a ligand-independent manner (Tsujimura *et al.*, 1994). It was also shown to be accountable for tumorigenic transformation of mast cells (Piao and Bernstein, 1996). The analogous mutation (GAC→GTC, D816V) was identified and was found to be associated with mast and epithelial cell tumors in humans (Nagata *et al.*, 1995; Longley *et al.*, 1999). Nevertheless, similar activating mutations in the c-*kit* gene have not yet been found in melanomas, nor associated with tumorigenic transformation of melanocytes.

Here, by using genetically altered mouse melanocytes that express endogenous constitutively active mutant cKit, we attempted to characterize physiological responses of melanocytes to the constitutive activation of cKit RTK, and to determine whether such activation induces uncontrolled proliferation of melanocytes promoting tumorigenic transformation, or activates melanogenesis and migration of the pigment-producing cells.

RESULTS

General observations

Using the oligonucleotide-based gene targeting, we have previously corrected an albino mutation (TGT→TCT) in the tyrosinase gene and simultaneously introduced an activating point mutation (GAC→TAC) into the c-*kit* gene of albino mouse melanocytes, Melan-c (Alexeev *et al.*, 2002). To investigate the physiological consequences of constitutive activation of the cKit receptor in pigment-producing cells, we selected two clones expressing the normal, wild-type cKit receptor (c-kit^N) and two clones expressing the mutant, constitutively active cKit (c-kit^A). Initially, we observed some qualitative differences in morphology and degree of pigmentation between these clones (data not shown). Morphologically, c-kit^A melanocytes had spindle-like cell bodies and exhibited fewer dendrites than c-kit^N cells. They were also visibly less pigmented. No significant differences in proliferation rates were seen between c-kit^A and c-kit^N cells.

Melanogenesis

Previously, it has been shown that transient activation of the cKit receptor by its ligand, SCF, induces tyrosinase gene transcription and melanin synthesis in differentiated cultured melanocytes (Luo *et al.*, 1995). Therefore, we investigated whether the ligand-independent activation of the cKit receptor has a similar effect. Surprisingly, based on the melanogenesis assay that correlates well with ^{14}C-labeled 3,4(OH)$_2$-phenylalanine incorporation and with tyrosinase activity Pomerantz assays (Aubock *et al.*, 1983), we determined that melanin content in c-kit^A melanocytes was approximately 30% lower than that in c-kit^N cells (Figure 1a), confirming our initial visual observation. As tyrosinase activity often correlates with the expression of the enzyme, we further compared the levels of transcription and expression of the tyrosinase gene product between c-kit^A and c-kit^N melanocytes. As expected, slightly lower levels of the tyrosinase transcript (Figure 1b) and mature protein (Figure 1c) were detected in c-kit^A cells by Northern and Western blot analyses. Interestingly, constitutive activation of cKit receptor did not appreciably alter the expression of the other melanocyte-specific proteins, TRP2 and Pmel17 (Figure 1d).

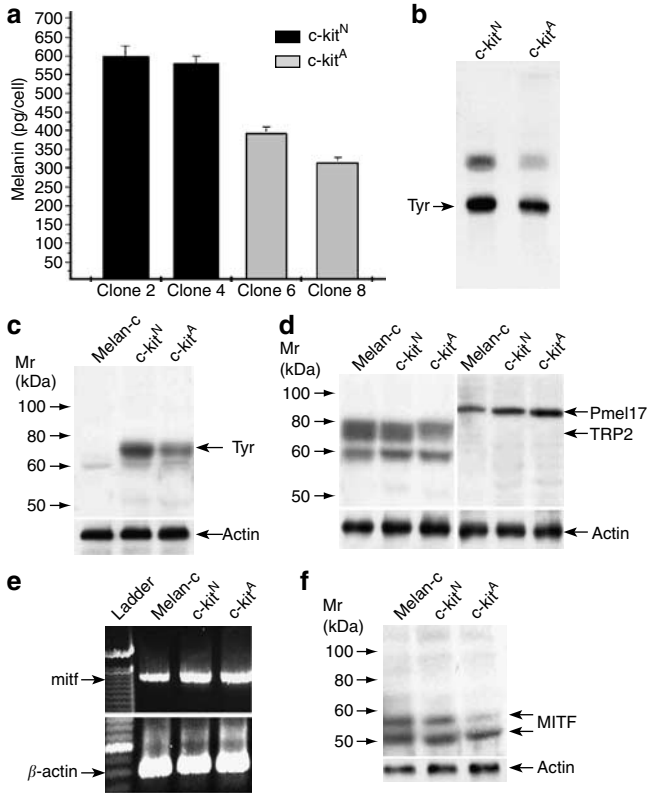

Figure 1. Melanogenesis and expression of the "pigmentation" genes in the c-kit^N and c-kit^A melanocytes. (**a**) Melanin content in c-kit^N and c-kit^A melanocytes. Melanin content (pg/cell) is shown by bars, representing the mean±SD of triplicate measurements. Two distinctive, original clones of the pigmented c-kit^N (clones 2 and 4) and c-kit^A (clones 6 and 8) (described in Alexeev *et al.*, 2002) were used for the analysis. (**b**) Northern blot analysis of the tyrosinase gene expression in c-kit^N and c-kit^A cells. The arrow indicates a full-length tyrosinase transcript. (**c**) Western blot analysis of tyrosinase in c-kit^N and c-kit^A cells. (**d**) Western blot analysis of the TRP2 and Pmel17 (gp100) expression in c-kit^N and c-kit^A cells. (**e**) Reverse transcription-PCR analysis of the mitf gene expression in c-kit^N and c-kit^A melanocytes. Arrows point to the full-length mouse mitf transcript (1,260 bp) (upper panel) and the mouse β-actin (300 bp fragment), used as a loading control. (**f**) Western blot analysis of MITF in c-kit^N and c-kit^A cells detected by MITF-specific monoclonal antibodies. Arrows on the left in panels c, d, and f indicate the apparent molecular weight (kDa). Arrows on the right in panels c, d, and f mark the position of each protein, detected on blots by using protein-specific antibodies.

As transcription of tyrosinase is controlled by the microphthalmia-associated transcription factor (MITF), we also analyzed the MITF status of c-kit^N and c-kit^A melanocytes. By using mouse mitf-specific primers, we reverse transcribed and amplified full-length mitf transcripts from the total RNA isolated from c-kit^N and c-kit^A melanocytes. Qualitative comparison (Figure 1e) showed no significant differences in the mitf transcription levels in the analyzed cells. On the contrary, we found rather a lower level of the MITF protein in the c-kit^A cells, detected as an almost complete loss of the 56-kDa MITF isoform (Figure 1f).

Proliferation

Previously, it has been shown that ligand-independent constitutive activation of the cKit receptor is responsible for progression of the mast cell and epithelial cell tumors (Boissan *et al.*, 2000). Therefore, we anticipated that expression of the mutant cKit receptor in c-kit^A cells would have a similar impact in melanocytes. In contrast to our expectation, continuous signaling from the cKit RTK in these cells did not stimulate proliferation, but resulted in slower cell cycle progression (Figure 2). During 8 days, the c-kit^N cells had undergone approximately seven divisions and formed compact, rounded colonies with an average of 128 cells per clone. During the same period, the c-kit^A cells divided approximately six times and formed scattered, amorphous colonies with an average of 64 cells per individual clone (Figure 2a). At the end of the 2-week period, there were approximately 2.5 times less c-kit^A cells per clone than c-kit^N melanocytes (Figure 2a and b). This difference could be explained either by slower cycling or by elevated level of cell death of the c-kit^A cells in given tissue culture conditions. To exclude one of these possibilities, we counted dead cells in culture media collected from both c-kit^N and c-kit^A cells. The number of dead cells was approximately equal and did not change significantly over a 2-week period (data not shown).

We also performed the proliferation assay in a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-free culture medium without any supplementation. Under these conditions, c-kit^A melanocytes did not proliferate and remained mostly as a single cell or divided once during 2 weeks, while c-kit^N cells proliferated slowly and divided three times forming small colonies of approximately eight cells per clone by the end of the second week (Figure 2c and d). These observations clearly demonstrated that the constitutive intracellular signaling from the cKit receptor does not trigger uncontrolled proliferation and tumorigenic transformation of melanocytes. On the contrary, it slows down cell cycle progression from 27 hours for c-kit^N cells to 32 hours for c-kit^A melanocytes. The smaller sizes and amorphous shapes of the c-kit^A colonies raised the question of whether expression of the constitutively active cKit may induce migration of melanocytic cells.

***In vitro* migration**

Ligand-dependent activation of the cKit receptor is required for migration of melanoblasts and melanocytes during embryonic development and in adulthood (Nishikawa *et al.*, 1991; Botchkareva *et al.*, 2001). We investigated whether the constitutive activation of the cKit receptor differentially affected migration of c-kit^A and c-kit^N cells by using *in vitro* migration assay. As presented in Figure 3, the

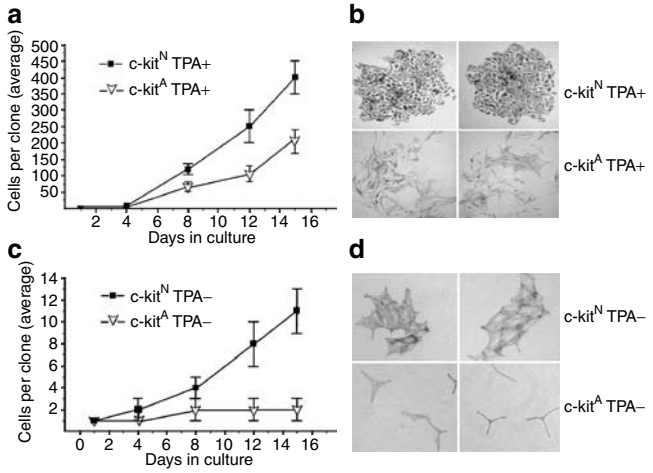

Figure 2. Proliferation of c-kit^N and c-kit^A melanocytes. (**a**) Proliferation of c-kit^N and c-kit^A melanocytes cultured in TPA-containing culture media. (**b**) Photographs of representative individual c-kit^N and c-kit^A clones cultured in TPA-containing culture media, 2 weeks after plating. Bars represent mean ± SD of triplicate experiments. (**c**) Proliferation of c-kit^N and c-kit^A melanocytes cultured in the absence of TPA from culture media. (**d**) Photographs of representative individual c-kit^N and c-kit^A clones cultured in the absence of TPA from culture media, 2 weeks after plating.

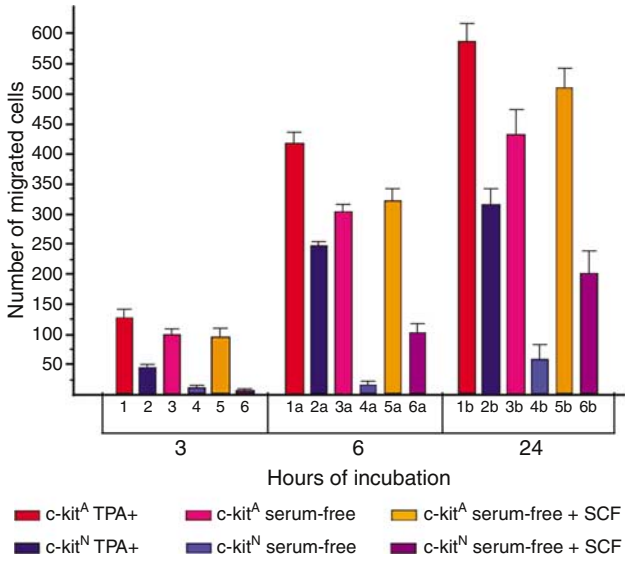

Figure 3. *In vitro* migration of c-kit^N and c-kit^A melanocytes. Both c-kit^N or c-kit^A melanocytes were plated onto the porous membranes of the migration chamber in different tissue culture conditions, as indicated below the graph. The number of migrated cells was counted 3, 6, and 24 hours after plating. Columns represent the number of migrated c-kit^N or c-kit^A cells. Each culture condition is marked in a different color and numbered 1–6. In addition, various time points for each culture condition are demarcated: 1–6 (3 hours after plating), 1a–6a (6 hours after plating), and 1b–6b (24 hours after plating). Bars represent the mean ± SD of triplicate experiments.

constitutive activation of the cKit receptor significantly enhanced melanocytic migration. In TPA-containing culture media, twice more c-kit^{A} melanocytes migrated to the underside of the porous membrane and to the lower compartment of the migration chamber within the first 3 hours (columns 1 and 2). This difference (2:1 on the average) remained the same for longer time periods up to 24 hours (columns 1a and 2a, and 1b and 2b). In TPA-free media, a more dramatic difference in migration patterns was observed between c-kit^{A} and c-kit^{N} melanocytes (columns 3 and 4, 3a and 4a, and 3b and 4b). On average, 12 times more c-kit^{A} melanocytes migrated to the lower compartment of the chamber within 3, 6, or 24 hours compared to c-kit^{N} cells. As expected, addition of SCF to the culture media of the lower compartment stimulated migration of the c-kit^{N} cells (columns 6, 6a, and 6b). However, even in the presence of SCF, the migration rate of the c-kit^{N} cells remained 2–5 times lower than that of the c-kit^{A} cells under similar conditions (columns 5, 5a, and 5b).

In vivo migration

As c-kit^{A} melanocytes exhibited elevated migratory abilities *in vitro*, we transplanted these cells into mouse skin to test whether the constitutive activation of the cKit receptor will also support elevated melanocytic migration *in vivo*. To facilitate identification of the transplanted cells in live animals and skin sections, c-kit^{N} and c-kit^{A} cells were transplanted into the dorsal skin of albino mice (Balb/c). After transplantation, animals were monitored visually for the presence and distribution of pigment every week during 12 weeks. Transplantation of c-kit^{A} or c-kit^{N} melanocytes did not result in donation of melanin from transplanted melanocytes to follicular keratinocytes, as determined by the absence of any pigmented hairs. Nevertheless, the pigmented spots at the injection sites were clearly seen in both groups of animals ($n=3$ for each group) for a period of approximately 2 weeks (Figure 4a and b). During the next 2 weeks, these spots faded in all mice injected with c-kit^{N} cells (Figure 4c and d). By 12 weeks, the pigmented spots were hardly distinguishable in all mice injected with c-kit^{N} melanocytes. In contrast, the pigmented spots remained visible throughout the entire period of observations in all mice injected with c-kit^{A} cells, thus indicating better survival and possible migration of c-kit^{A} melanocytes toward the epidermis (Figure 4g and h).

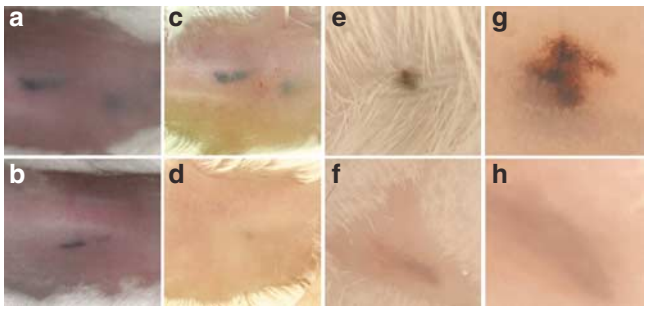

Figure 4. Photographs of mouse skin at various time points after transplantation of c-kit^{N} and c-kit^{A} melanocytes. (**a**, **c**, **e**, **g**) Visual detection of pigmentation in mouse skin after transplantation of c-kit^{A} melanocytes. (**b**, **d**, **f**, **h**) Visual detection of pigmentation in mouse skin after transplantation of c-kit^{N} melanocytes. Photographs were taken at different time points: (**a**, **b**) 2 weeks after transplantation; (**c**, **d**) 4 weeks after transplantation; (**e**, **f**) 12 weeks after transplantation; (**g**, **h**) magnified fields from (**e**, **f**).

Histological analysis of biopsy tissues during this time period also confirmed the progressional upward migration of c-kit^{A}, but not c-kit^{N}, cells. In 2 weeks after transplantation, c-kit^{N} and c-kit^{A} melanocytes were mostly associated with the connective tissue and lower dermis and did not show notable differences (Figure 5c and d). In 4 weeks, differences in the distribution of c-kit^{N} and c-kit^{A} cells were detected (Figure 5e and f). In each group ($n=3$) of mice examined, c-kit^{A} cells started to migrate from the connective tissue/lower dermis toward the dermal–epidermal border, whereas c-kit^{N} cells remained associated with the connective tissue and lower dermis (Figure 5i and j). At a higher magnification, upward migration of c-kit^{A} cells was seen along the side of the hair follicle, as illustrated in Figure 5j and k. During the additional 8 weeks, c-kit^{N} cells remained in the connective tissue/lower dermis, whereas c-kit^{A} melanocytes continued to migrate toward the epidermis (Figure 5g and h). By the end of the 12th week, c-kit^{A} cells were seen in a close vicinity of the epidermis (Figure 5l and m) in all treated animals ($n=6$). In four out of six mice injected with c-kit^{A} cells, pigmented melanocytes were detected near the dermal–epidermal border and in the epidermal layer of skin (Figure 5n and p). No melanocytes were detected in this area in c-kit^{N}-treated mice (Figure 5o).

cKit-dependent migration of various cell types was previously shown to be associated with activation of the phosphatidyl inositol-3 kinase (PI3K) (Dudek *et al.*, 1997). We examined the status of this enzyme in c-kit^{N} and c-kit^{A} melanocytes. As shown in Figure 6, the ligand-independent constitutive activation of the cKit RTK constitutively activates PI3K via its phosphorylation. The level of PI3K phosphorylation in the c-kit^{A} cells is even higher than that in the c-kit^{N} cells cultured in the presence of 5 ng/ml of SCF in the media for 2 weeks. Interestingly, one of the PI3K downstream molecules, protein kinase Akt (AKT), was found to be highly phosphorylated only when cKit receptor was transiently activated by the exogenous SCF. Prolonged exposure of the c-kit^{N} cells to the cKit ligand or constitutive activation of the receptor in the c-kit^{A} cells results only in a slight activation of the AKT (Figure 6).

As migration of melanocytes was shown to be associated with the changes in expression of adhesion proteins such as E-cadherin and N-cadherin (Hsu *et al.*, 2000), we examined expression of these proteins in c-kit^{N} and c-kit^{A} melanocytes. As determined by Western blotting, the constitutive activation of the cKit receptor indeed resulted in an elevated level of N-cadherin expression, which was even higher than that in c-kit^{N} cells cultured in the presence of 5 ng/ml of SCF. Interestingly, the cKit-stimulated upregulation of N-cadherin expression was not accompanied by downregulation of E-cadherin, in contrast to the previous observations made in malignant melanocytes (Figure 6b).

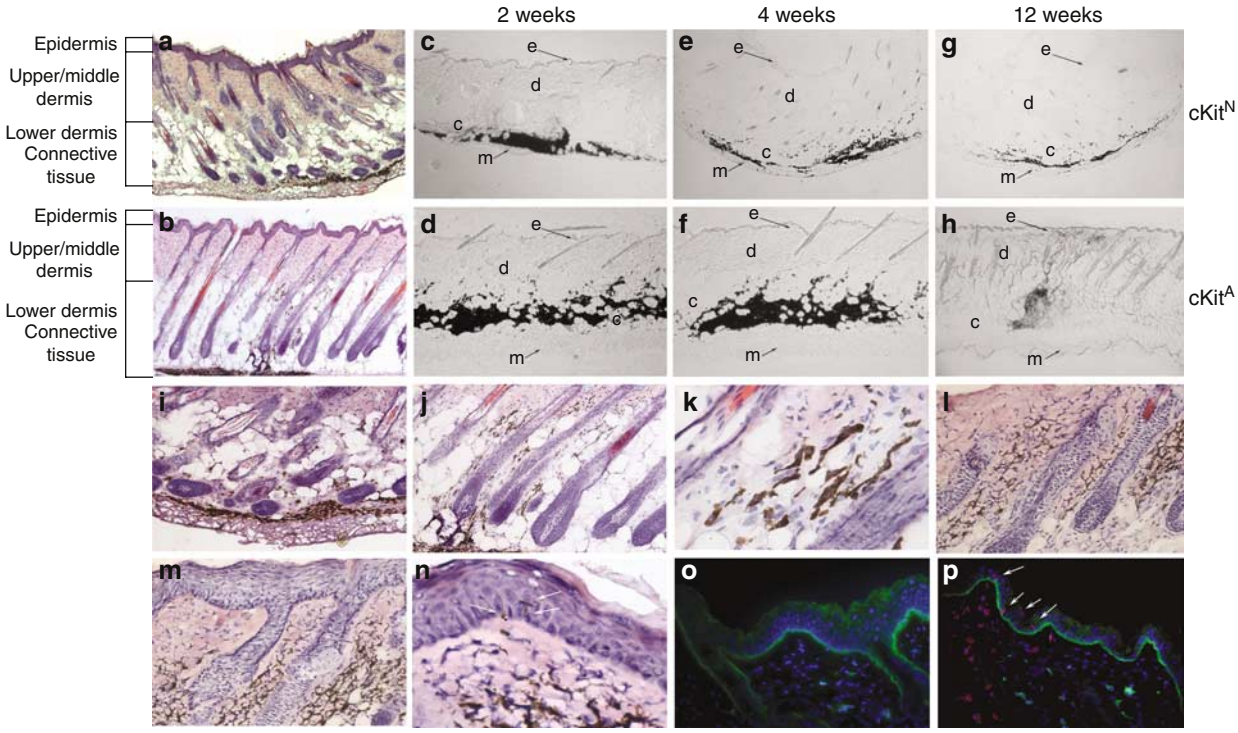

Figure 5. Histological examination of the cross-sections of skin biopsies taken from mice injected with either c-kit^N or c-kit^A melanocytes. (**a**, **b**) Cross-sections of skin biopsies taken from mice injected with c-kit^N and c-ki^A melanocytes and virtual division of the cross-section to score migration. (**c**, **d**, **e**–**h**) Cross-sections of skin biopsies taken from mice injected with c-kit^N and c-kit^A melanocytes at indicated time points (2, 4, and 12 weeks); original magnification $\times 10$. e: epidermis; d: dermis; c: connective tissue; m: muscle layer. (**i**, **j**) Photographs of representative cross-sections of skin biopsies taken from mice injected with c-kit^N (**i**) and c-kit^A (**j**), 4 weeks after injection; original magnification $\times 20$. (**k**) Migration of c-kit^A melanocytes along the sides of the hair follicles; original magnification $\times 40$. (**l**, **m**) Considerable migration of c-kit^A melanocytes toward the dermal–epidermal border during 12 weeks after transplantation. Pigmented melanocytes are seen in the interfollicular dermis and in a close vicinity to the dermal–epidermal border; original magnification $\times 20$. (**n**) The ectopic presence of transplanted c-kit^A melanocytes 12 weeks after transplantation. Arrows point to the pigmented cells present on the dermal–epidermal border and in the epidermis; original magnification $\times 40$. (**o**, **p**) Immunodetection of transplanted melanocytes on cross-sections taken from c-kit^N- and c-kit^A-treated mice. Basement membrane zone (BMZ) is outlined by using anti-collagen VII antibodies (Chemicon International, Temecula, CA), and melanocytes are detected by using tyrosinase-specific antibodies (αPEP7). Green: BMZ; red: tyrosinase-positive melanocytic cells: blue: DAPI nuclear staining; original magnification $\times 20$. Arrows point to melanocytic cells (c-kit^A) in the epidermis.

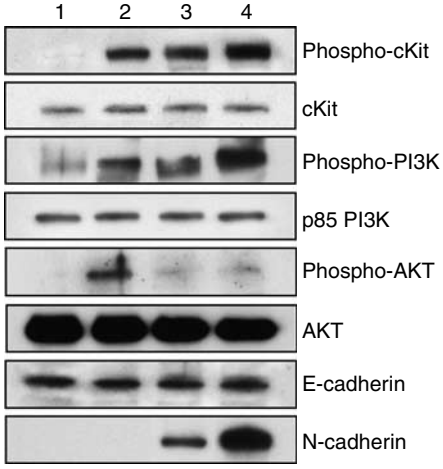

Figure 6. cKit-mediated activation of PI3K and AKT and alteration of cadherins expression. Lane 1: untreated c-kit^N melanocytes; lane 2: c-kit^N cells pulsed with 100 ng/ml soluble SCF at 37°C for 10 minutes; lane 3: c-kit^N melanocytes cultured in the presence of 5 ng/ml soluble SCF for 2 weeks; lane 4: untreated c-kit^A melanocytes.

Importantly, during the overall 12-week period, neither visual observations nor histological analyses revealed any inflammatory immune responses or formation of transient nodules and tumors in any of the 15 animals injected with c-kit^A cells. The absence of tumor initiation/formation in these mice additionally supports the hypothesis that activation of cKit is not associated with stimulation of uncontrolled proliferation and tumorigenic transformation of melanocytes.

DISCUSSION

In various cell types including melanocytes, cKit receptor transmits a variety of signals associated with stimulation of proliferation, differentiation, migration, and survival. At present, it is not clear whether the activated cKit RTK transmits all these signals simultaneously and independently, or whether there is a crosstalk between them to enhance or oppose each other and regulate/coordinate the receptor's numerous roles. Here, we systemically investigated the consequences of constitutive activation of the cKit receptor

in the genetically altered mouse melanocytes containing the activating point mutation (GAC→TAC, D814Y) in the coding sequence of the c-*kit* gene. We demonstrate that the constitutively active cKit RTK primarily transmits pro-migration signals, which may antagonize pro-proliferation and pro-differentiation signals. These findings are in agreement with previous data indicating that activation of cKit by soluble SCF promoted the survival and proliferation of cKit-positive and/or 3,4$(OH)_2$-phenylalanine-positive neural crest-derived cells (NCCs), but not proliferation of fully pigmented melanocytes, and that such activation of cKit alone does not support melanogenesis in neural crest-derived cells (Kawa *et al.*, 2000).

At first, we found that expression of the mutant cKit was associated with lesser pigmentation of melanocytes, presumably caused by downregulation of the tyrosinase gene expression. Such downregulation could be caused by cKit-dependent inactivation of MITF, a basic helix–loop–helix leucine zipper transcription factor that regulates transcription of tyrosinase. Normally, a ligand-dependent activation of the cKit RTK results in activation of mitogen-activated protein and Rsk-1 kinases that triggers phosphorylation of Ser 73 and Ser 409 on MITF, respectively. Both phosphorylation events stimulate the MITF transcriptional activity and, simultaneously, the rapid covalent addition of the polyubiquitin chains to MITF, resulting in subsequent degradation of the transcription factor (Hemesath *et al.*, 1998; Price *et al.*, 1998; Wu *et al.*, 2000; Xu *et al.*, 2000; Kim *et al.*, 2003). Based on this model of coupled activation and degradation of MITF, the data obtained in this study suggest that constitutive activation of cKit in c-kit^A cells may trigger incessant degradation of MITF, which prevails over its activation via cKit-dependent recruitment and/or activation of the ubiquitin ligases (Wollberg *et al.*, 2003).

In this study, we also present evidences that activation of the cKit receptor is primarily responsible for stimulation of migration rather than proliferation of melanocytes. The morphological changes observed in c-kit^A cells, such as spindle-shape cell bodies and reduced number of dendrites, reflect higher migratory properties of melanocytes that could have resulted from the cKit-dependent reorganization of actin cytoskeleton. This is in agreement with the previous report that cKit activation triggered a rapid increase in actin stress fiber formation and an elevated melanocyte migration on fibronectin (Scott *et al.*, 1996). In addition, the elevated level of N-cadherin expression found in c-kit^A cells also supports the notion that the cKit receptor is primarily responsible for stimulation of migration. The primary role of cKit in melanocyte migration is further supported by the observations that, in the mouse embryo, only the cKit-positive subpopulation of cells on the dorsomedial aspect of the neural tube immigrated exclusively into developing dermis, and then expressed definitive markers of the melanocyte lineage (Wilson *et al.*, 2004). In addition, they are in agreement with the recent report demonstrating that cKit receptor signaling in zebrafish larvae promotes migration of melanocytes through distinct cKit-dependent mechanisms (Rawls and Johnson, 2003). In contrast to the findings in zebrafish, in which melanocytic migration was shown to be dependent on the extracellular, ligand-binding domain of the receptor by mutation analysis, we found that the cKit RTK itself transmits primarily pro-migration signals independent of ligand–receptor interaction. Although the requirement of cKit activation for melanocyte migration is not unexpected, it is surprising that constitutive activation of the RTK resulted exclusively in elevated migration rather than proliferation of the cells. Nevertheless, it is quite possible that such selectiveness is determined by the specific signals transmitted by the cKit RTK.

It is well known that activation of cKit results in autophosphorylation of tyrosine residues located in the intracellular domain of the receptor and in subsequent activation of src-homology 2 domain-containing proteins that further transduce signals that lead to various cellular responses. For instance, Src family kinases that bind to the pY568 and pY570 residues of the cKit receptor (Lennartsson *et al.*, 1999) activate p38 mitogen-activated protein kinase, which in turn inhibits proliferation (Merighi *et al.*, 2005) and induces apoptosis of malignant melanocytes (An *et al.*, 2005; Xia *et al.*, 2005). Activation of the Tec family kinases, which interact with pY703 of cKit, results in phosphorylation of the Dok-1 protein (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997), which negatively regulates SCF-triggered hyperproliferation (Yamanashi *et al.*, 2000; Di Cristofano *et al.*, 2001) via a not yet characterized mechanism. Another tyrosine residue, pY719, located in the kinase insert domain of the cKit receptor serves as the only binding site for PI3K (Serve *et al.*, 1995). cKit-dependent phosphorylation and activation of PI3K alone is sufficient for transmission of survival and pro-migration signals (Dudek *et al.*, 1997), but not enough for induction of proliferation (Timokhina *et al.*, 1998). Most recently, by using a functionally and biochemically inert cKit receptor that lacks the binding sites for seven early signaling pathways, Hong *et al.* (2004) demonstrated that restoration of the binding sites for Src family kinases stimulated migration and survival of erythroid cells. Similarly, restoration of PI3K binding sites did not affect cellular proliferation, but resulted in increased survival and migration of cells. Restoration of binding sites for phospholipase Cγ had no effect on cell growth, survival, and migration. As demonstrated in this study, ligand-independent activation of the cKit receptor in c-kit^A melanocytes leads to constitutive activation of PI3K, but, surprisingly, a barely detectable activation of one of its downstream molecules, AKT. As AKT plays a significant role in the survival of various cell types (Downward, 2004), our findings suggest that cKit-mediated constitutive or continuous activation of PI3K primarily supports migration rather than survival of melanocytic cells. Collectively, our current data and the analysis of cKit-mediated intracellular signaling studies suggest that ligand-independent, constitutive activation of the cKit RTK in mammalian melanocytes primarily results in the transmission of pro-migration and, possibly, antiproliferation signals. Further analysis of specific cKit-mediated signaling pathways is required for dissecting numerous roles of the cKit receptor in melanocytic physiology.

Predominant pro-migration activity of cKit is also supported by our *in vivo* studies, which clearly demonstrated that genetically altered c-*kit* mutant melanocytes acquire elevated migration abilities. When transplanted into the dorsal skin of albino mice, c-kit^{A} cells demonstrated distinctive migration from the injected sites to the upper dermis and dermal–epidermal border, mimicking an ectopic migration of melanocytes in the footpad epidermis in the K14-SCF transgenic animals that express SCF in basal keratinocytes (Kunisada *et al*., 1998a, b). As the intra-skin gradient of SCF is not likely to affect the direction of migration in our experimental system, the observed distinct upward migration pattern of the c-kit^{A} melanocytes throughout the dermis, as detected on all examined skin sections, suggests that the follicular and/or interfollicular keratinocytes, not dermal fibroblasts, provide other ''chemotactic'' signals that influence the homing of melanocytes to the epidermis. To some extent, the observed migration of the c-*kit*-altered melanocytes resembles the higher migratory properties of the c-*kit* mutant mast cells in the skin, previously associated with mastocytomas (Tharp and Longley, 2001).

This study also addressed the role of cKit in tumorigenic transformation of malignant melanocytes. Previously, it was hypothesized that activation of cKit RTK may play a role in the induction of uncontrolled proliferation of pigment-producing cells and in metastasis of melanomas. Here, we present evidences that contradict both hypotheses. First, we demonstrated that constitutive activation of the cKit RTK did not stimulate proliferation of melanocytic cells. Second, we showed that c-kit^{A} melanocytes, unlike c-kit^{N} cells, directionally migrated toward the epidermis. Our observations suggest that in order to acquire proliferative advantage and escape from the epidermal boundaries, malignant cells should lose cKit expression. This idea correlates extremely well with the findings that cKit expression in tumorigenic melanocytes and primary melanoma lesions is often down-regulated (Lassam and Bickford, 1992; Natali *et al*., 1992).

Overall, our studies demonstrated that constitutive activation of the cKit RTK in mouse melanocytes, in contrast to other cell types, does not implicate the uncontrolled proliferation and tumorigenic transformation of the pigment-producing cells, but rather transmits pro-migration signals, which allow melanocytic migration *in vitro* and *in vivo*. Our findings are consistent with the model of formation of the pigmentation system, and also provide additional explanation for the fact that malignant melanocytes lose cKit expression during progression of melanoma.

MATERIALS AND METHODS

Cell culture

Spontaneously immortalized non-tumorigenic pigmented mouse melanocytes expressing normal and mutant constitutively active cKit receptors were obtained from mouse albino melanocytes (Melan-c) via oligonucleotide-based targeted alteration of the tyrosinase and c-*kit* genes as described previously (Alexeev *et al*., 2002). Both c-*kit* normal (c-kit^{N}) and c-*kit*-altered (c-kit^{A}) cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 10 mg/ml penicillin/streptomycin, 200 nM phorbol myristate acetate, and 100 μM β-mercaptoethanol. All reagents were purchased from Invitrogen (Valencia, CA).

Melanogenesis assay

The melanin content of c-kit^{N} and c-kit^{A} cells was determined by the method that correlates with ^{14}C-labeled 3,4(OH)$_2$-phenylalanine incorporation and with tyrosinase activity Pomerantz assay as described previously (Naeyaert *et al*., 1991). Briefly, pigmented melanocytes were harvested by trypsinization, and washed in isotonic saline. Cells (1×10^6) were dissolved in 1 ml of 1 N NaOH. Melanin concentration was calculated by determining the optical density at 475 nm wavelength and comparing with a standard curve of synthetic melanin (Sigma, St Louis, MO). The melanin content is expressed as picogram per cell.

Proliferation and colony forming assay

To investigate the ability of c-kit^{N} and c-kit^{A} melanocytes to proliferate and form colonies, cells were plated onto the wells of six-well plates in a clonal density (1×10^3 cells/well) in melanocyte culture media. Individual cells were allowed to proliferate and form colonies in either full or TPA-free growth media. At 1, 4, 8, 12, and 15 days after plating, the number of colonies was counted and individual clones were photographed. The number of cells per individual clones was counted. All assays were performed in triplicate. Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was calculated using two-sided Student's *t*-test.

Migration assay

For melanocyte migration assay, we used 8 μm pore size Transwell migration chambers (Costar/Corning, Corning, NY). Cells were plated onto the porous membrane of the upper chamber in RPMI culture media containing 10 μg/ml BSA. Cell migration was allowed to proceed at 37°C in a standard tissue culture incubator for 3, 6, or 12 hours. At the indicated time points, cells were removed from the upper side of the membrane with a cotton swab, and cells that migrated to the underside and to the lower compartment were counted. At least 10 random high-power fields were counted from each membrane and the lower compartment of the migration chamber. All assays were performed in triplicate. Data are presented as mean $\pm$ SD. Statistical significance was calculated using two-sided Student's *t*-test.

Western blot analysis

Primary polyclonal αPEP7, αPEP8′, and αPEP13 antibodies, specific to tyrosinase, TRP2, and Pmel17 (gp100), respectively, were kindly provided by Dr V. Hering (NIH, Bethesda, MA) and were used for Western blotting at dilution of 1:20,000. MITF-specific monoclonal antibodies were purchased from NeoMarkers (Fremont, CA) and used for Western blotting at a dilution of 1:2,000. Antibodies, specific to N- and E-cadherin, were purchased from BD Transduction Laboratories (Lexington, KY) and used at a dilution of 1:5,000. Anti-cKit, anti-(phospho)cKit, anti-PI3K, anti-(phospho)PI3K, anti-AKT, and anti-(phospho)AKT antibodies were purchased from Cell Signaling Technologies (Beverly, MA) and used for Western blotting at a dilution of 1:2,000.

Mouse melanocytes grown to 80% confluency were lysed in a buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA,

1% Triton X-100, 0.5% NP-40, 1 mM phenylmethyl sulfonyl fluoride, protease, and phosphatase inhibitors. The amount of protein in lysates was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA), and 10 μg of total protein per lane was separated on a 10% SDS-PAGE, followed by a transfer onto polyvinylidene difluoride membrane. Membranes were blocked with 5% BSA in Tris/Tween 20 buffer saline and probed with protein-specific antibodies. Immunocomplexes were detected by using horseradish peroxidase-labeled secondary antibodies with subsequent visualization with the Supersignal WestFemto detection kit (Pierce, Rockford, IL). Secondary, anti-rabbit and anti-mouse horseradish peroxidase-conjugated antibodies were purchased from Promega (Madison, WI) and used for detection of immunocomplexes at a dilution of 1:100,000.

RNA isolation and Northern and reverse transcription-PCR analyses

Total cellular RNA was isolated from cultured c-kit^N or c-kit^A cells using the RNeasy kit (Qiagen, Valencia, CA). For Northern blot analysis, the isolated RNA was subjected to electrophoresis (15 μg per lane) and transferred onto a Zeta-Probe blotting membrane (Bio-Rad Laboratories, Hercules, CA). A full-length mouse tyrosinase cDNA, kindly provided by Dr H. Yamamoto (Tohoku University, Sendai, Japan), was labeled with [^{32}P]dCTP by the random priming method (Amersham, Piscataway, NJ). Blots were hybridized at 65°C for 16 hours, washed at high stringency, and exposed to X-Omat MR film (Kodak, Rochester, NY). For the reverse transcription-PCR analysis of MITF expression, the first-strand cDNA was generated from total RNA isolated from cultured c-kit^N or c-kit^A cells by reverse transcription using SuperScript II reverse transcriptase (Invitrogene, Carlsbad, CA) according to the manufacturer's protocol. The full-length mitf transcripts were amplified by PCR using *Taq* DNA polymerase (Invitrogene, Carlsbad, CA) and two gene-specific primers: mMITF1001 5′-ATGCTGGAAATGCTAGAATACAGTCA-3′ and mMITF1002 5′-CTAACACGCATGCTCCGTTTCTTC-3′.

Melanocyte transplantation and cryosections

The Institutional Animal Care and Use Committee of Thomas Jefferson University approved all animal studies. Either c-kit^N or c-kit^A melanocytes were transplanted intradermally into the dorsal skin of Balb/c albino mice. Melanocytes, cultured in normal conditions until 80% of confluency, were collected by trypsinization, washed with sterile saline, and counted using a hemocytometer. Either c-kit^N or c-kit^A melanocytes (1×10^6 cells per 50 μl of saline) were transplanted into mouse skin via intradermal injection. Mice were monitored visually twice a week for the presence of pigmentation. Each group of animals ($n = 3$) received c-kit^N or c-kit^A melanocytes and were euthanized 2, 4, and 12 weeks after transplantation, except for six animals that were injected with c-kit^A melanocytes and euthanized 12 weeks after transplantation. Full-thickness skin biopsies were taken and snap-frozen in OCT (optimal cutting temperature) compound (Sakura Finetek USA Inc., Torrance, CA). Skin biopsies (12 mm^2) were cut serially and 10 μm sections were fixed with ethanol:acetic acid (2:1), washed in phosphate-buffered saline, and either stained with hematoxylin and eosin or directly mounted under the coverslips in Permount compound (Fisher Scientific, Pittsburgh, PA). Sections were examined for the presence and distribution of pigmented cells by light microscopy. To determine melanocytic migration *in vivo*, each skin section was virtually divided into three layers (epidermis, upper/middle dermis, and lower dermis/connective tissue) (Figure 5a and b). The location of pigmented cells was verified on every fifth section out of 200 consecutive sections. All sections contained pigmented cells with a different pattern of distribution. The biopsy was scored positive for migration when an average 60% of the examined sections contained pigmented cells in one of the skin layers higher than the lower dermis/connective tissue.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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