Neurotrophins and Their Receptors Stimulate Melanoma Cell Proliferation and Migration

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Melanoma is a highly aggressive skin tumor that originates in the epidermis from melanocytes. As melanocytes share with the nervous system a common neuroectodermal origin and express all neurotrophins (NTs), we evaluated the expression and function of NTs and their receptors in melanoma. We report that primary and metastatic melanoma cell lines synthesize and secrete all NTs. Moreover, melanoma cells express the low-affinity (p75NTR) and the high-affinity tyrosine kinase NT receptors (Trk). The inhibition of Trk receptors by either K252a or Trk/Fc chimeras prevents proliferation, indicating that autocrine NTs are responsible for this effect. NT-3, NT-4, and nerve growth factor (NGF) induce cell migration, with a stronger effect on metastatic cell lines. Transfection with p75NTR small interfering RNA (p75NTRsiRNA) or treatment with K252a inhibits NT-induced melanoma cell migration, indicating that both the low- and high-affinity NT receptors mediate this effect. All melanoma cell lines express the p75NTR coreceptor sortilin by which proNGF stimulates migration in melanoma cells, but not in cells transfected with p75NTRsiRNA. These results indicate that NTs, through their receptors, play a critical role in the progression of melanoma.

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INTRODUCTION

Neurotrophins (NTs) belong to a family of structurally and functionally related molecules that promote neuronal cell survival and differentiation or cell death, depending on the cell–tissue context and the receptor involved (Chao *et al*., 2006). The NT family consists of four members: nerve growth factor (NGF), brain-derived neurotrophic growth factor, NT-3, and NT-4. NTs exert their effects by binding two classes of transmembrane receptors, the tyrosine kinase high-affinity receptor Trks (TrkA, TrkB, and TrkC) and the low-affinity neurotrophin receptor (p75NTR). TrkA, TrkB, and TrkC selectively bind NGF, brain-derived neurotrophic growth factor, and NT-3, respectively, whereas TrkB can also bind NT-4 (Dechant, 2001); p75NTR binds all NTs with equal low affinity (Chao and Bothwell, 2002). NTs perform different biological functions according to the interaction with Trk or p75NTR (Huang and Reichardt, 2003). When NTs bind to Trk, dimerization and autophosphorylation of the receptor occur, promoting survival and differentiation (Segal, 2003). On the other hand, the role of p75NTR is still controversial: in the presence of Trk, p75NTR increases high-affinity NT binding, thereby enhancing Trk's ability to promote survival (Dechant and Barde, 2002). By contrast, in the absence of Trk, p75NTR can induce apoptosis, via its own signal transduction, by interacting with a mounting number of downstream molecules (Wang *et al*., 2001). Recently, it has been shown that the proform of NT proNGF binds p75NTR, in association with its coreceptor sortilin, but not Trk (Nykjaer *et al*., 2004). More specifically, sortilin, a member of the vps-10 protein family, binds the ''pro'' region of NGF, whereas p75NTR binds mature NGF. The p75NTR–sortilin complex couples with proNGF to induce apoptosis (Kaplan and Miller, 2004).

NTs also operate in a number of non-neuronal cells (Kurihara *et al*., 2003; Rochlitzer *et al*., 2006). In the skin, a complex NT network exists in which various cells are either the target or the source of NT, thus playing autocrine and paracrine functions (Marconi *et al*., 2003, 2006). In particular, melanocytes, which share with neurons a common neuroectodermal origin, express all NTs and their receptors, except TrkC (Pincelli and Yaar, 1997; Marconi *et al*., 2006).

Melanoma, the most aggressive form of skin cancer, originates from melanocytes. The incidence and mortality rate of melanoma have increased at annual rates of 2–3% for the last 30 years (Perlis and Herlyn, 2004). Although early, thin lesions can be effectively cured by surgical excision, advanced and metastatic melanomas are generally fatal (Thompson and Uren, 2005). Most melanomas arise within

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Abbreviations: FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor; NT, neurotrophin; p75NTRsiRNA, p75NTR small interfering RNA; RT, reverse transcription; TPA, tetradecanoyl phorbol acetate

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the epidermis and then invade the basement membrane to eventually disseminate to multiple organs (Balch *et al.*, 2001). During progression, melanoma moves from the epidermal to the dermal microenvironment by virtue of various mechanisms that involve different cell types, such as keratinocytes and fibroblasts, up- and downregulation of adhesion molecule receptors, and a number of growth factors (Haass and Herlyn, 2005). Melanoma frequently metastasizes to the brain, this often being the initial site of melanoma metastases (Balch *et al.*, 2001). It has been shown that in brain-metastatic melanoma cells, NGF promotes invasion by enhancing the production of extracellular matrix-degradative enzymes (Denkins *et al.*, 2004), suggesting that NT-rich tissues such as the brain, through a potent chemotactic activity (Nicolson and Menter, 1995), support melanoma invasion and survival. Yet little is known on the role of NT and their receptors in the progression of melanoma.

We postulate that the rich NT network, which involves the vast majority of skin cells (Jerregard *et al.*, 2000; Marconi *et al.*, 2003), could play a role in melanoma development. To verify this hypothesis, we have studied the expression and function of NTs and their receptors in primary and metastatic melanoma cell lines. We provide evidence that melanoma cells express all NTs and NT receptors. Moreover, we report that NTs modulate melanoma cell proliferation and migration through their receptors and in association with sortilin.

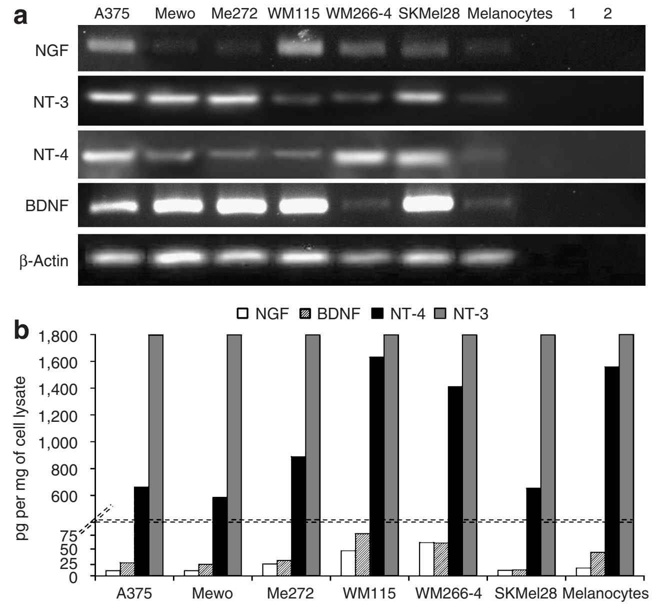

Figure 1. NT expression in melanoma cell lines. (**a**) RNA was extracted from both melanoma cell lines and normal human melanocytes. mRNA was analyzed by RT-PCR and ethidium bromide staining. β-Actin mRNA was used as an internal control. Control lanes (1, 2) represent amplification with no template and RNA without reverse transcription, respectively. (**b**) NT protein levels of both melanoma cells and melanocytes were evaluated by ELISA in a conditioned medium, as described in Materials and Methods. NT protein levels are given in pg per mg of cell lysate.

RESULTS

Expression of NT in melanoma cell lines

We have recently shown that human melanocytes synthesize and release all NTs (Marconi *et al.*, 2006). To evaluate whether malignant transformation affects the expression of NTs, we analyzed NTs in melanoma cells both at the mRNA and at the protein levels. Melanoma cells synthesize all NTs, although at different extents, similar to normal human melanocytes, as shown by reverse transcription-PCR (RT-PCR) (Figure 1a). In addition, NT-3, NT-4, brain-derived neurotrophic growth factor, and NGF proteins were detected in the lysates of all melanoma cells. NT-3 and NT-4 levels were at least sixfold more elevated than the other NTs in all cell lines, with no major difference in primary (WM115, A375) as compared to metastatic cells (Mewo, Me272, WM266-4, and SKmel28) or normal human melanocytes (Figure 1b). Discrepancies between mRNA and protein levels could be explained by the difference between produced and released protein. Alternatively, there could be some post-transcriptional modification.

Expression of NT receptors in melanoma cell lines

As most skin cells secrete NTs (Botchkarev *et al.*, 2006), we were interested in evaluating whether melanoma cells could be the target of NTs. To address this question, we investigated the expression of NT receptors in melanoma cell lines. Indeed, all investigated cell lines express p75NTR both at the mRNA and at the protein levels. p75NTR mRNA and protein were detected at various levels, but without significant difference between primary and metastatic melanoma (Figure 2a and b). TrkA, TrkB, and TrkC were expressed both at the mRNA (Figure 2c, e, and g) and at the protein levels in melanoma cells (Figure 2d, f, and h). These findings indicate that primary and metastatic melanoma could participate in the NT network not only by releasing NT but also by responding to their action. Because melanoma originates from normal melanocytes, we analyzed p75NTR and Trks in these cells. p75NTR was expressed weakly in normal melanocytes, and it increased after stimulation with tetradecanoyl phorbol acetate (TPA), a strong inducer of cell differentiation. Even after TPA treatment, it appears that p75NTR expression is lower than the one detected in most melanoma cell lines (Figure 2b). Similarly, although TrkA was expressed slightly in normal melanocytes and increased in TPA-treated cells, its level was much higher in melanoma cell lines (Figure 2d). TrkB was expressed more in melanoma cell lines than in normal melanocytes, and TPA-treated cells tend to express levels similar to those observed in A375 and Me272 cell lines (Figure 2f). Finally, TrkC was expressed weakly in normal melanocytes and was absent in differentiated melanocytes (Figure 2h). This seems to indicate that NT receptor expression increases during melanoma development.

NTs modulate melanoma cell proliferation

To verify whether NTs actually exert any function on melanoma cells, we decided to focus our attention on just three melanoma cell lines: WM115 and WM266-4, which are respectively the primary and metastatic melanoma derived from the same patient and expressing high levels of

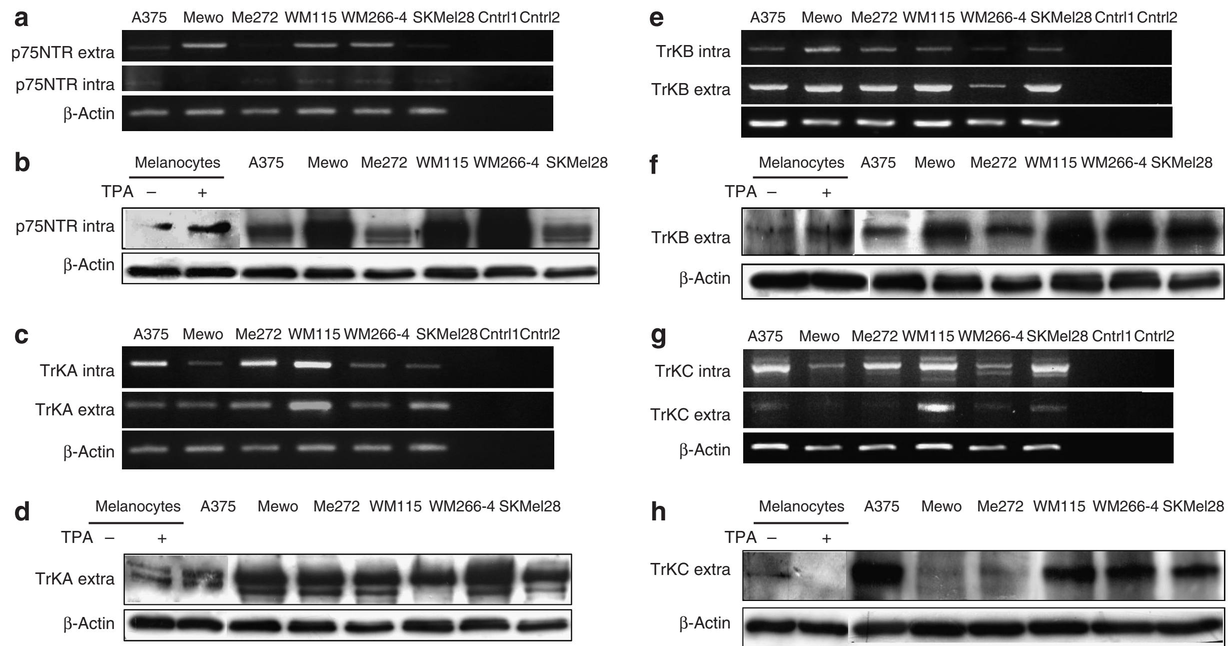

Figure 2. Expression of p75NTR and Trk receptors in melanoma cell lines. Intracellular and extracellular mRNA of (**a**) p75NTR, (**c**) TrkA, (**e**) TrkB, and (**g**) TrkC were evaluated by RT-PCR and ethidium bromide staining. β-Actin mRNA was used as an internal control. Control lanes (1, 2) represent amplification with no template and RNA without reverse transcription, respectively. (**b**) p75NTR, (**d**) TrkA, (**f**) TrkB, and (**h**) TrkC protein expression in melanoma cell lines was studied by western blotting, as described in Materials and Methods. NT receptors were also evaluated in normal melanocytes with or without the addition of TPA. β-Actin expression was used as control.

p75NTR, and SKmel28, a metastatic melanoma cell line expressing low levels of p75NTR. All these melanoma cells express Trk receptors, although at different levels. We first evaluated the curve growth of the three cell lines, under basic conditions, up to 72 hours (Figure 3a). Cell growth curve demonstrated that melanoma cell lines are in a proliferative state between 24 and 72 hours after plating. Thus, successive experiments were performed at these time points. The addition of recombinant NGF, brain-derived neurotrophic growth factor, NT-3, and NT-4 to the three melanoma cell lines did not exert any significant proliferative effect when compared to control cells at 48 hours (Figure 3b). Because melanoma cells secrete all NTs, we examined proliferation after blocking endogenous NTs. K252a inhibited NT-induced Trk phosphorylation (Figure 3c) and determined a significant reduction of melanoma cell proliferation in all cell lines at 24 and 48 hours (Figure 3e). To evaluate the effect of individual NTs on cell proliferation, we inhibited each endogenous NT using recombinant human Trk/Fc chimeras, soluble Trks, which bind NTs and prevent their interaction with membrane receptors. TrkA/Fc, TrkB/Fc, and TrkC/Fc chimeras inhibited NT-induced Trk phosphorylation (Figure 3d) and significantly reduced melanoma cell proliferation when compared to control (Figure 3f). These results indicate that melanoma cells can autonomously proliferate by releasing NTs.

NTs modulate melanoma cell migration

Melanoma is notorious for its high tendency to metastasize. Melanoma cell migration and invasion are controlled in various stages of tumor progression by different growth factors and cytokines, which by autocrine and paracrine effects enable cells to grow autonomously and confer competence to metastasis (Lazar-Molnar *et al.*, 2000). To assess the role of NT on cell migration, we performed an *in vitro* Boyden/Matrigel assay. NGF, NT-3, and NT-4 induced cell migration in all melanoma cell lines (Figure 4a–c). To confirm this effect, we performed a different migration assay (scratching assay). To rule out the possibility that apparently migrated cells were indeed proliferating cells, cells were pretreated with mitomycin C, a mitosis inhibitor (Figure 4d and e). All NTs stimulated melanoma cell migration, with a stronger effect on metastatic cell lines (Figure 4a–e). To evaluate the role of NT receptors in melanoma cell migration, cells were treated with p75NTR small interfering RNA (p75NTRsiRNA). Western blotting confirmed the knockdown of p75NTR protein (Figure 5b). To obtain a more efficient p75NTRsiRNA transfection in scratching experiments (chemokinesis), we plated less cells, as compared to the previous experiment. Although mock cells were induced to migrate by NT, p75NTRsiRNA-transfected cells failed to respond to NT stimulation. Indeed, the number of migrated cells treated with p75NTRsiRNA was significantly lower than that of mock-transfected cells (Figure 5a and c). Similarly, the addition of K252a blocked melanoma cell migration (Figure 5d and e), suggesting that NTs stimulate melanoma cell migration and invasion via the cooperation of the low- and high-affinity receptors.

Expression of sortilin receptor in melanoma cell lines

As sortilin acts as a coreceptor for p75NTR and binds proNGF, independently of Trk, we evaluated the role of

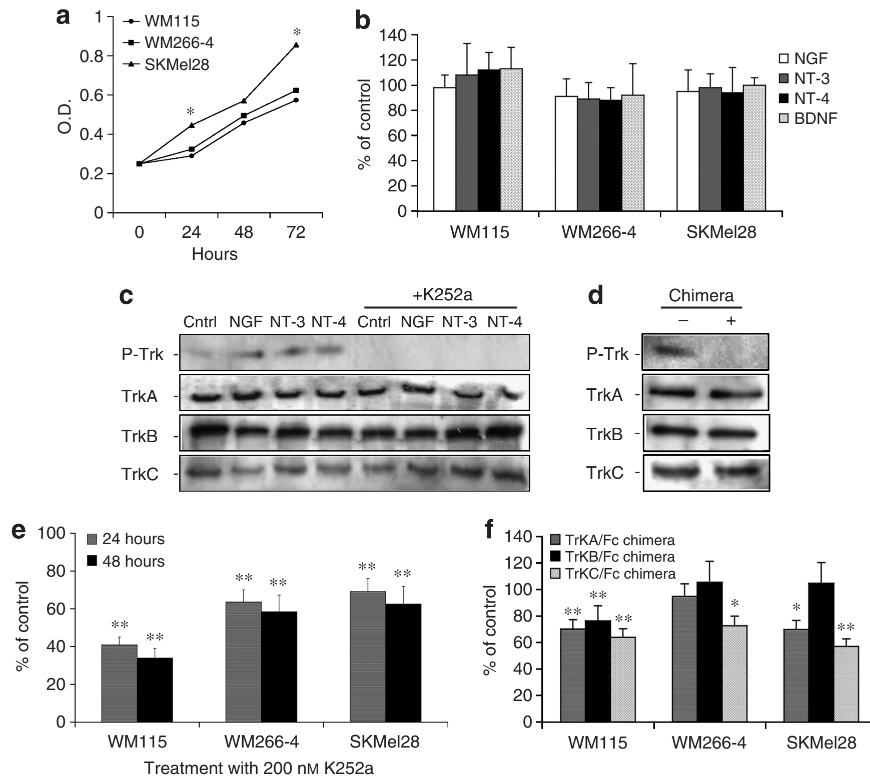

Figure 3. Endogenous NTs modulate melanoma cell proliferation. (**a**) Melanoma cell proliferation was evaluated 24, 48, and 72 hours after plating by MTT assay. (**b**) Melanoma cells were stimulated with 100 ng ml^{-1} NT for 48 hours and proliferation was evaluated by MTT assay. The results were expressed as percentage of control. Trk phosphorylation assay was assessed with or without pretreatment with either (**c**) 200 nM Trk inhibitor K252a in WM266-4 or (**d**) 1 μg ml^{-1} Trk/Fc chimeras in SKmel28 by western blotting, using an antibody against phosphorylated Trk, as described in Materials and Methods. (**e**) Melanoma cell lines were stimulated either with 200 nM K252a for 24 and 48 hours, (**f**) or with 1 μg ml^{-1} TrkA/Fc, TrkB/Fc, or TrkC/Fc chimeras for 48 hours. The proliferation was evaluated by MTT assay and the results were expressed as the percentage of control. Data are expressed as the mean ± SEM of triplicate from three different experiments. **P*<0.05; ***P*<0.01.

p75NTR–sortilin complex in melanoma cells. First, we demonstrated that all melanoma cell lines investigated in this study express sortilin mRNA, although at different levels. WM115 express low amounts of sortilin, both at the mRNA and at the protein levels, as compared to the other cell lines (Figure 6a and b). Moreover, sortilin was expressed weakly in normal melanocytes, and it increased only after stimulation with the TPA (Figure 6b).

Function of sortilin receptor in melanoma cell lines

As proNGF requires coexpression of sortilin and p75NTR to induce its biological effects (Nykjaer *et al.*, 2004), we evaluated its functions in our melanoma cell system. TUNEL assay showed that proNGF fails to affect survival in melanoma cell lines. On the other hand, K252a, as expected, induced a significant rate of apoptosis, indicating that Trk receptors are critical for melanoma cell survival (Figure 7a). Moreover, proNGF did not exert any effect on melanoma cell proliferation (Figure 7b). On the other hand, proNGF induced the migration of all melanoma cell lines, which was statistically higher than control, as shown by Boyden/Matrigel (Figure 7c) and wound-healing assays (Figure 7d and e). To confirm the role of proNGF in melanoma cell migration, cells were treated with p75NTRsiRNA. Western blotting confirmed the knockdown of p75NTR protein (Figure 7g). Although proNGF induced mock cells to migrate, it failed to have any effect on p75NTRsiRNA-transfected cells (Figure 7f and h). These findings indicate that proNGF stimulates melanoma cell migration and invasion, possibly through the p75NTR–sortilin complex.

DISCUSSION

Melanoma progresses along distinct phases. During the radial growth phase, melanoma cells grow in a horizontal way within the epidermis, whereas the vertical growth phase involves melanoma cells that invade the dermis. We report here that the skin NT network, which operates both in the dermis and in the epidermis, acts on melanoma cells by modulating proliferation and migration. First, we show that NTs, exerting their effects on melanoma cells, are not only derived from other skin cells, but are produced by the melanoma itself. Indeed, the melanoma cell lines investigated in this study release all NTs. On the other hand, although there is general agreement on the expression of p75NTR, there is still controversy on the expression of Trks in melanoma. Previous reports based on immunohistochemical methods have detected in melanoma TrkB, TrkC, but not TrkA (Innominato *et al.*, 2001). By contrast, Florenes *et al.*

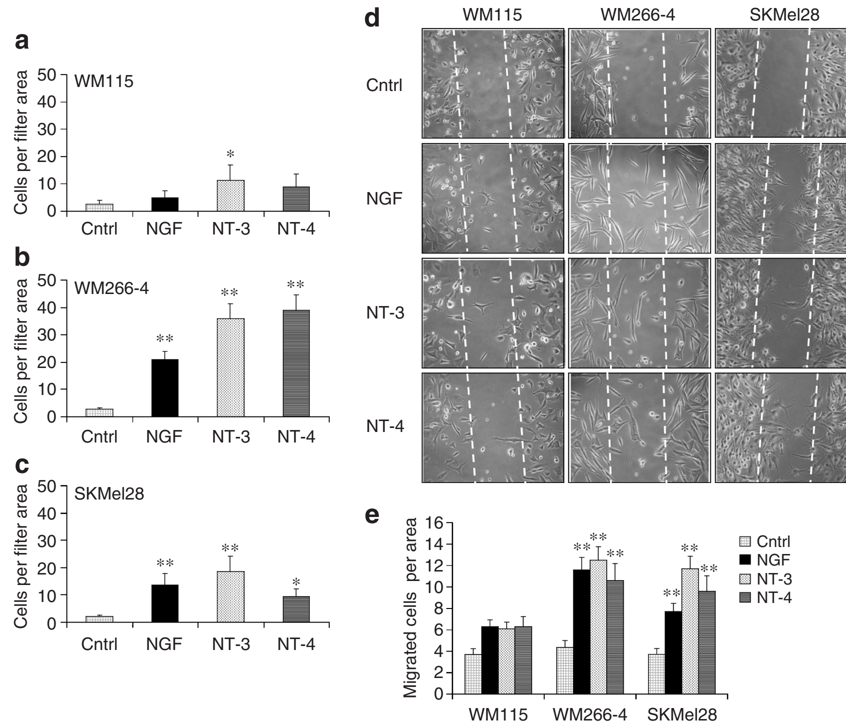

Figure 4. NTs modulate melanoma cell migration. (**a–c**) Melanoma cells plated onto 24-well Boyden/Matrigel chambers were stimulated with NT, as described in Materials and Methods. Chemotaxis assay was performed 18 hours later. Six representative fields were counted from each migration filter, and results are expressed as the number of migrated cells. Data are expressed as the mean ± SEM of triplicate from three different experiments. *$P<0.05$; **$P<0.01$. (**d**) The monolayer of melanoma cells, treated with mitomycin C, was scratched for chemokinesis assay. Cells were cultured for 48 hours with 100 ng ml^{-1} NT, and migrated cells into the scraped area were observed. (**e**) In the same experiment, six areas were counted and expressed as the mean of cell migrated per area. Data are expressed as the mean ± SEM of triplicate from three different experiments. *$P<0.05$; **$P<0.01$.

(2004) have recently detected phosphorylated TrkA in melanoma cells, suggesting that this NT receptor is active in melanoma. Consistent with this finding, we show that melanoma cell lines express all Trk receptor mRNAs. Moreover, western blotting confirms TrkA protein expression. We also demonstrate the presence of p75NTR in all melanoma cell lines, in keeping with previous works (Herrmann *et al.*, 1993). Taken together, these results suggest that autocrine NTs, along with their receptors, could contribute to melanoma progression.

To validate this premise, we have investigated the effect of NTs on melanoma cell proliferation and migration. Exogenous NTs failed to enhance melanoma cell proliferation, possibly because of the high levels of endogenous NTs. On the other hand, K252a and soluble Trk receptors markedly reduced cell proliferation, indicating that endogenous NTs are mitogenic for melanoma cells. This is, to our knowledge, the first work on the effect of autocrine NT on melanoma cell proliferation. Other works have investigated the role of NT receptors on melanoma survival and brain invasiveness, pointing to the predominant role of p75NTR (Marchetti *et al.*, 2004). However, these reports draw their conclusions without taking into account the presence of Trks and the well-known interactions between the high- and the low-affinity NT receptors. The available data demonstrate that when p75NTR signals alone, the net effect is cell death, whereas survival signals are induced only when p75NTR complexes with Trk (Huang and Reichardt, 2003). Therefore, considering our finding of all Trks being expressed in melanoma cells, it is more conceivable that NTs mediate proliferation, through the high-affinity receptor complex Trk–p75NTR (Kuruvilla *et al.*, 2004).

The expression of p75NTR in melanoma has been associated with enhanced brain metastasis, independently of Trk. We wanted to investigate the role of NTs and their receptors on melanoma invasiveness. Using a migration assay, we demonstrated that all NTs stimulate melanoma cell migration, which is significantly higher in metastatic melanoma than in primary melanoma cell lines. This seems to correlate with the work of Marchetti *et al.* (2004), who have emphasized the role of NT in metastatic melanoma. Indeed, blocking p75NTR inhibits NT-induced melanoma migration, highlighting the role of the low-affinity NT receptor. However, our results strongly indicate that Trk receptors are also responsible for melanoma cell migration and proliferation, as K252a and soluble Trk receptors prevent these effects, in keeping with the expression of all Trks in melanoma.

It has been shown in neuronal cells that when p75NTR signals independently of Trk, it requires sortilin as a coreceptor. p75NTR–sortilin complex bound to proNGF induces apoptosis (Nykjaer *et al.*, 2004). In this study, although all melanoma cells express sortilin, unexpectedly

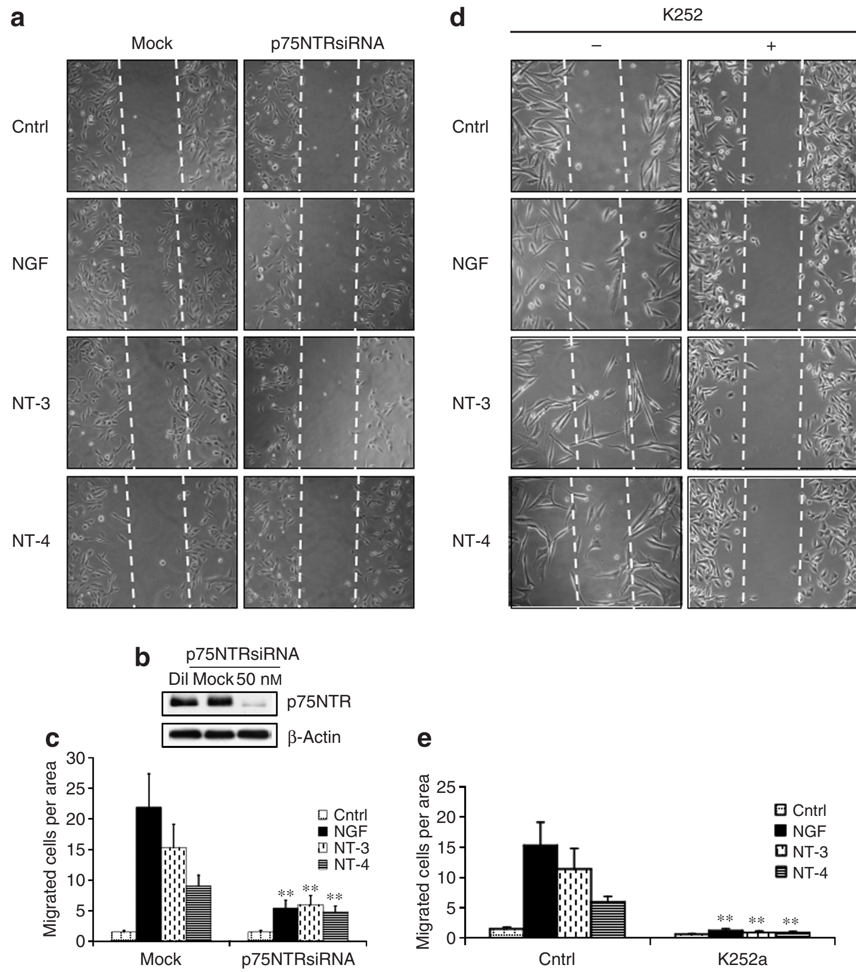

Figure 5. NTs modulate melanoma cell migration through Trk–p75NTR complex. (**b**) WM266-4 melanoma cells were transfected with 50 nM p75NTRsiRNA, and transfection was controlled by western blotting. As a control, mock cells and cells treated with diluent alone were used. (**a**, **c**) Twenty-four hours after transfection, the cells were scratched and treated with 100 ng ml^{-1} NT for 48 hours. Migration was calculated as follows: (**c**) in the same experiment, six areas were counted and expressed as the mean of cell migrated per area. Data are expressed as the mean ± SEM of triplicate from three different experiments. *$P<0.05$; **$P<0.01$. (**d**) WM266-4 melanoma cells were treated with 200 nM K252a for 48 hours during the chemokinesis assay. Migrated cells into the scraped area were observed. (**e**) In the same experiment, six areas were counted and expressed as the mean of cell migrated per area. Data are expressed as the mean ± SEM of triplicate from three different experiments. **$P<0.01$.

proNGF does not affect melanoma cell viability. Because p75NTR in association with Trk plays a role in melanoma cell migration, we investigated if p75NTR–sortilin complex, independently of Trk, could mediate this effect. Interestingly, proNGF strongly stimulates melanoma cell migration, suggesting that p75NTR mediates melanoma invasiveness, most likely in association with sortilin. This finding is in good agreement with the work of Shonukan *et al.* (2003), who have postulated that p75NTR mediates proNT- or NT-induced melanoma migration by interacting with the actin-bundling protein fascin. Although Shonukan's paper fails to address the role of sortilin, the notion that proNGF binds to the p75NTR–sortilin complex only makes it very likely that sortilin is involved in melanoma cell migration.

In conclusion, we propose that melanoma development takes advantage of a special microenvironment provided by the skin NT network, which also includes melanoma cells. Indeed, melanoma cells are the target of both paracrine and autocrine NT effects, which favor survival and invasiveness of this tumor.

MATERIALS AND METHODS

Melanoma cell cultures

A375, Mewo, and Me272 cells were cultured in RPMI 1640 (Biochrom AG, Berlin, Germany) with 200 mM L-glutamine, 1% penicillin–streptomycin, and 10% fetal bovine serum (FBS). WM115, WM266-4, and SKmel28 (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Biochrom AG) supplemented with 200 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1% penicillin–streptomycin, and 10% FBS. Normal human melanocytes were obtained from the foreskin (Marconi *et al.*, 2006) and were cultured in Medium 154 containing FBS, basic fibroblast growth factor, transferrin, hydrocortisone, bovine pituitary extract, heparin, insulin, penicillin, streptomycin, and amphotericin B (Cascade Biologics, Portland, OR). Institutional approval for experiments was not necessary.

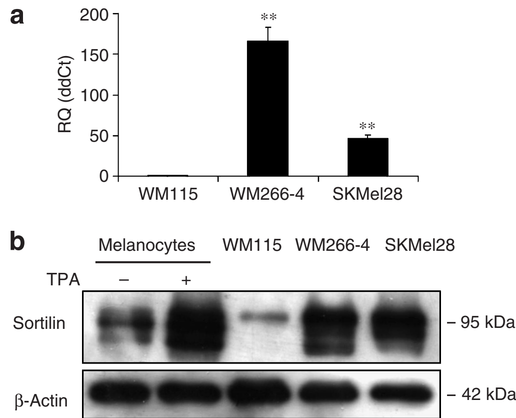

Figure 6. Melanoma cell lines express sortilin receptor. (**a**) Sortilin mRNA levels were measured by real-time PCR quantitative analysis. VIC-actin was used as an internal control for relative quantification, and WM115 sortilin expression was used as calibrator. Double-sided Student's *t*-test was performed between samples and calibrator. Data are expressed as the mean ± SEM of triplicate from three different experiments. $^{**}P<0.01$. (**b**) Sortilin protein both in melanoma cell lines and melanocytes was evaluated by western blotting (see Materials and Methods). β-Actin expression was used as control.

Transfection of melanoma cell line

About 70,000 cells per well were plated on six-well plates in a penicillin/streptomycin-free melanoma medium. After 24 hours, cells were transfected with 50 nM p75NTRsiRNA (Dharmacon Inc., Lafayette, CO) combined with Lipofectamin 2000 and Opti-MEM (both from Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. As controls, water (diluent) or water containing Lipofectamin 2000 and Opti-MEM (mock) was used. WM266-4 or WM115 melanoma cell lines were transfected twice and p75NTR protein level was detected by western blotting, as described below. For chemokinesis assay, cells were washed 24 hours after the second transfection with serum-free medium and then treated with serum-free medium containing 100 ng ml^{-1} NT, 2.5 ng ml^{-1} proNGF, or diluent, respectively. Migration was checked 48 hours after treatment.

ELISA

Melanoma cells were cultured in complete DMEM or RPMI 1640 culture medium, and melanocytes were cultured in complete Medium 154. At subconfluency, fresh medium for each cell type was substituted with the same medium containing 0.1% BSA without FBS. After 48 hours, the conditioned medium was collected and NT

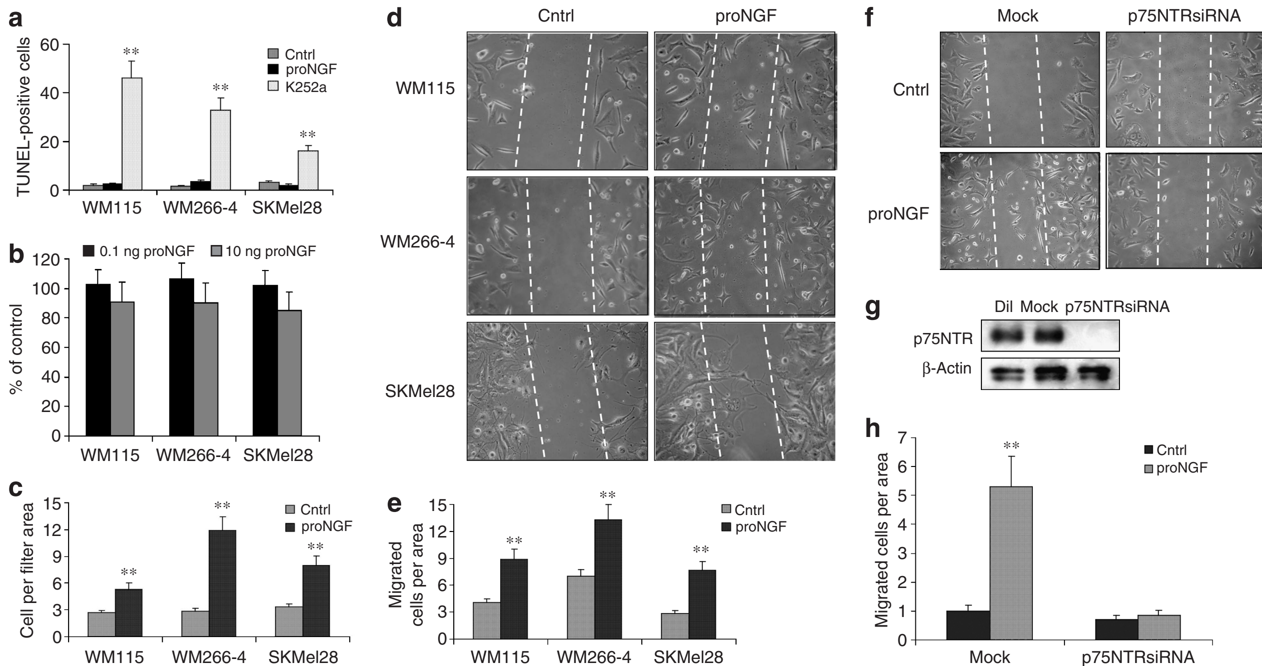

Figure 7. proNGF modulates melanoma cell migration. (**a**) Melanoma cells were treated with 10 ng ml^{-1} proNGF or diluent alone for 96 hours, and TUNEL assay was used to detect apoptosis. K252a-treated cells were used as positive control. (**b**) Melanoma cells were stimulated with 0.1 or 10 ng ml^{-1} proNGF for 72 hours. Proliferation was evaluated by MTT assay, and results were expressed as the percentage of control. (**c**) Melanoma cells plated onto 24-well Boyden/Matrigel chambers were stimulated with proNGF, as described in Materials and Methods. Chemotaxis assay was performed 18 hours later. Six representative fields were counted from each migration filter, and results are expressed as the number of migrated cells. Data are expressed as the mean ± SEM of triplicate from three different experiments. $^{*}P<0.05$; $^{**}P<0.01$. (**d**) Melanoma cells were scratched and treated with 2.5 ng ml^{-1} proNGF. After 48 hours, migrated cells into the scraped area were evaluated as follows: (**e**) six areas were counted and expressed as the mean of migrated cell per area. Data are expressed as the mean ± SEM of triplicate from three different experiments. $^{*}P<0.05$; $^{**}P<0.01$. (**g**) WM115 melanoma cells were transfected with 50 nM p75NTRsiRNA, and transfection was controlled by western blotting. As a control, mock cells and cells treated with diluent alone were used. (**f**, **h**) Twenty-four hours after transfection, the cells were scratched and treated with 2.5 ng ml^{-1} proNGF for 48 hours. Migration was calculated as follows: (**h**) six areas were counted and expressed as the mean of cell migrated per area. Data are expressed as the mean ± SEM of triplicate from three different experiments. $^{**}P<0.01$.

quantization was performed by a two-site enzyme immunoassay (Quantikine; Promega, Madison, WI) according to the manufacturer's instructions. The sample concentration was determined by absorbance at 540 nm against recombinant human NT standard protein. NT protein levels are given in pg per mg of cell lysate and results are expressed as the mean ± SEM of triplicate from three different experiments.

RT-PCR

Total RNA was extracted from cultures using TRI Reagent method performed as described by Sigma (Sigma-Aldrich, St Louis, MO). One microgram of total RNA extracted was reverse transcribed and amplified as described (Pincelli *et al.*, 1994) with a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT). To perform a semiquantitative evaluation of mRNA, β-actin was used as a housekeeping gene. The relative intensity of bands on autoradiograms was quantified by scanning laser densitometry. The linearity of PCR was obtained by plotting value from densitometric analysis in each band versus the cDNA concentration. Nucleotide sequences of the oligomers used (MWG Biotech, Ebersberg, Germany) are listed in Table S1. The PCR was carried out at least three times for each sample. No reverse-transcribed mRNA and buffer without template were used as controls.

Western blot analysis

Cells were harvested on ice in p75NTR buffer (150 mM NaCl, 15 mM $MgCl_2$, 1 mM EGTA, 50 mM Hepes, 10% glycerol, 1% Triton X-100, pH 7.5, and protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin) or Trk and sortilin buffer (50 mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, pH 7.4, and protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin) (Sigma-Aldrich). Western blotting was performed as previously described (Marconi *et al.*, 2004). Membranes were incubated overnight at 4 °C with primary anti-human p75NTR mouse mAb (1:1,000; Upstate, Lake Placid, NY), anti-human neurotensin receptor 3/sortilin mouse mAb (1:250; BD Biosciences, San Jose, CA), anti-human TrkB rabbit polyclonal antibody (1:750; Upstate), anti-human TrkC goat polyclonal antibody (1:750; Upstate), anti-human TrkA rabbit polyclonal antibody (1:1,000; Upstate), and anti-human β-actin mAb (1:5,000; Sigma-Aldrich). Membranes were then incubated for 45 minutes at room temperature with the following peroxidase-conjugated secondary antibodies: goat anti-mouse (1:3,000; Bio-Rad, Hercules, CA) for p75NTR and β-actin; goat anti-rabbit (1:3,000; Bio-Rad) for TrkA and TrkB; and donkey anti-goat (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for TrkC. Membranes were washed and developed using the ECL chemiluminescent detection system (Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, England).

Phosphorylation detection

A total of 500,000 melanoma cells were seeded in a p35 plate with fresh 0.5% FBS melanoma medium for 24 hours before stimulation. Two hours before stimulation, fresh 0.5% FBS melanoma medium was replaced. To inhibit Trk phosphorylation, 200 nM k252a or 1 μg ml^{-1} TrkA/Fc, TrkB/Fc, and TrkC/Fc were added for 1 hour, and then the medium was replaced with fresh 0.5% FBS melanoma medium containing 100 nM NT. After 5 minutes, cell lysate was obtained with 1 × SDS sample buffer (62.5 mM Tris-Hcl (pH 6.8 at 25 °C), 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue), sonicated for 15 seconds, heated at 100 °C for 5 minutes, microcentrifuged for 5 minutes, and 20 μl was loaded onto 7% SDS-PAGE gel. Western blotting was performed as previously described. Nitrocellulose membrane was blotted with Phospho-TrkA antibody that recognizes all phosphorylated Trks (1:1,000; Cell Signaling Technology, Danvers, MA). Membranes were then incubated with the secondary goat anti-rabbit (1:3,000; Bio-Rad). Membranes were developed using the ECL chemiluminescent detection system (Amersham Biosciences UK Limited).

Real-time PCR

Total cellular RNA was extracted from the three melanoma cell lines using the TRI Reagent method performed according to the manufacturer's instructions (Sigma-Aldrich). Quantitative real-time PCR was performed with an ABI 7500 (Applied Biosystems, Foster City, CA) for sortilin. As an internal control, housekeeping gene β-actin mRNA expression was measured in a separate tube. RNAse-free H_2O was used as a negative control. A 1 μg portion of RNA was subjected to retro-transcription and amplification in a 50 μl reaction mixture using the One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems). Both sortilin and β-actin real-time PCR were performed using Pre-Developed TaqMan Assay Reagents (sortilin MGB probe was FAM™ dye labeled and β-actin MGB probe was VIC™ dye labeled, Applied Biosystems). Thermal cycling conditions for One-Step RT-PCR were as follows: initial reverse transcription at 48 °C for 30 minutes, then DNA polymerase activation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minute. Data from each sample were compared with WM115 sortilin expression, as calibrators, using the Sequence Detection Software Version 1.2.3 according to the relative quantification ($\Delta\Delta C_t$) study method (Applied Biosystems). Results were obtained as the mean from three independent experiments. Double-sided Student's *t*-test was performed between samples and calibrator.

MTT assay

For cell growth curve, cells were plated in a 96-well tissue culture plate (5,000 cells per well) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed at 0, 24, 48, and 72 hours after plating. For proliferation experiments, cells were plated in a 96-well tissue culture plate and treated 24 hours after seeding with 100 ng ml^{-1} human recombinant NT (Sigma-Aldrich) or 200 nM K252a (Merck Biosciences, Darmstadt, Germany) or 1 μg ml^{-1} recombinant human TrkA/Fc, TrkB/Fc, and TrkC/Fc chimeras (R&D Systems, Minneapolis, MN) for 48 hours. Cells were also treated with 10 ng ml^{-1} proNGF (a kind gift from Barbara L Hempstead, New York, NY) for 72 hours. Cell proliferation was assessed following incubation with MTT (Sigma-Aldrich) solution at 37 °C for 4 hours. The formazan dye produced after DMSO solubilization was evaluated by a multiwell scanning spectrophotometer at 540 nm. The results are expressed as optical density units or as viability percentage compared to control. Results are calculated as the mean ± SD of three different experiments. Student's *t*-test was performed for comparison of the means.

TUNEL staining

A total of 8000 melanoma cells per well were plated onto four-well permanox chamber slides. Twenty-four hours after seeding, 10 ng ml^{-1} proNGF or 200 nM K252a was added. Medium alone was used as control. Ninety-six hours later, cells were fixed in paraformaldehyde (4% in phosphate-buffered saline) and permeabilized with Triton X-100 (0.1%) and sodium citrate (0.1%) at 4 °C. The slides were stained with the *In Situ* Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland) as recommended by the manufacturer. Approximately 100 cells were evaluated in randomly selected high-power fields, and the percentage of TUNEL-positive cells was counted. Negative control was obtained by replacing the primary incubation with a nucleotide mixture without TdT. Fluorescent specimens were analyzed by confocal scanning laser microscopy (Leica TCS4D) in conjunction with a conventional optical microscope (Leica DM IRBE).

Filter assay

The migration assay was performed with a 24-well BioCoat Matrigel Invasion Chambers migration system (BD Biosciences). A total of 10^5 cells per insert (8-μm pore size) were seeded and cultured for 24 hours at 37 °C. A 500 μl portion of DMEM containing L-glutamine and penicillin–streptomycin was added to the cells in the upper compartment. A 600 μl portion of DMEM containing L-glutamine and penicillin–streptomycin with or without 100 ng ml^{-1} NT or 2.5 ng ml^{-1} proNGF was added in the lower compartment; water was used as control. After 18 hours, cells were fixed in 95% ethanol for 30 seconds. As migrated cells move from the upper side to the lower side of the filter, the filter was cut after fixing and cells were removed from the upper side. Filters were washed with water, stained with toluidine blue for 7 minutes, and rinsed. Filters were placed on a glass slide, and from each filter six fields were counted using an optical microscope and the average of the migrated cells was calculated. The results are calculated as the mean ± SD of three different experiments. Student's *t*-test was performed for comparison of the means.

Scratching assay

A total of 50,000 cells were plated on six-well tissue culture plates and then treated with 10 μg ml^{-1} mitomycin C for 2 hours. Twenty-four hours later, the cells were washed three times in serum-free medium and three lines for each well were drawn along the cell monolayer with a sterile plastic tip. Plates were washed twice with serum-free medium to remove all detached cells and incubated in serum-free medium with 100 ng ml^{-1} NT (NGF, NT-3, or NT-4), 50 nM p75NTRsiRNA, 200 nM K252a, and 2.5 ng ml^{-1} proNGF. Water (diluent) or water containing Lipofectamin 2000 and Opti-MEM (mock) were used as controls. Cells were monitored at 48 and 72 hours from stimulation. The result of each experiment was expressed as the mean of migrated cells from six different areas. The final results are expressed as the mean ± SD of three different experiments. Student's *t*-test was performed for comparison of the means.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Reverse transcription-PCR primers.

REFERENCES

Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG *et al.* (2001) Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol* 19:3635–48

Botchkarev VA, Yaar M, Peters EM, Raychaudhuri SP, Botchkareva NV, Marconi A *et al.* (2006) Neurotrophins in skin biology and pathology. *J Invest Dermatol* 126:1719–27

Chao MV, Bothwell M (2002) Neurotrophins: to cleave or not to cleave. *Neuron* 33:9–12

Chao MV, Rajagopal R, Lee FS (2006) Neurotrophin signalling in health and disease. *Clin Sci (Lond)* 110:167–73

Dechant G (2001) Molecular interactions between neurotrophin receptors. *Cell Tissue Res* 305:229–38

Dechant G, Barde YA (2002) The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat Neurosci* 5:1131–6

Denkins Y, Reiland J, Roy M, Sinnappah-Kang ND, Galjour J, Murry BP *et al.* (2004) Brain metastases in melanoma: roles of neurotrophins. *Neuro-oncology* 6:154–65

Florenes VA, Maelandsmo GM, Holm R, Reich R, Lazarovici P, Davidson B (2004) Expression of activated TrkA protein in melanocytic tumors: relationship to cell proliferation and clinical outcome. *Am J Clin Pathol* 122:412–20

Haass NK, Herlyn M (2005) Normal human melanocyte homeostasis as a paradigm for understanding melanoma. *J Investig Dermatol Symp Proc* 10:153–63

Herrmann JL, Menter DG, Hamada J, Marchetti D, Nakajima M, Nicolson GL (1993) Mediation of NGF-stimulated extracellular matrix invasion by the human melanoma low-affinity p75 neurotrophin receptor: melanoma p75 functions independently of trkA. *Mol Biol Cell* 4:1205–16

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem* 72:609–42

Innominato PF, Libbrecht L, van den Oord JJ (2001) Expression of neurotrophins and their receptors in pigment cell lesions of the skin. *J Pathol* 194:95–100

Jerregard H, Akerud P, Arenas E, Hildebrand C (2000) Fibroblast-like cells from rat plantar skin and neurotrophin-transfected 3T3 fibroblasts influence neurite growth from rat sensory neurons *in vitro*. *J Neurocytol* 29:653–63

Kaplan DR, Miller FD (2004) Neurobiology: a move to sort life from death. *Nature* 427:798–9

Kurihara H, Shinohara H, Yoshino H, Takeda K, Shiba H (2003) Neurotrophins in cultured cells from periodontal tissues. *J Periodontol* 74:76–84

Kuruvilla R, Zwefel LS, Glebova NO, Lonze BE, Valdez G, Ye H *et al.* (2004) A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. *Cell* 118:243–55

Lazar-Molnar E, Hegyesi H, Toth S, Falus A (2000) Autocrine and paracrine regulation by cytokines and growth factors in melanoma. *Cytokine* 12:547–54

Marchetti D, Aucoin R, Blust J, Murry B, Greiter-Wilke A (2004) p75 neurotrophin receptor functions as a survival receptor in brain-metastatic melanoma cells. *J Cell Biochem* 91:206–15

Marconi A, Atzei P, Panza C, Fila C, Tiberio R, Truzzi F *et al.* (2004) FLICE/caspase-8 activation triggers anoikis induced by β1-integrin blockade in human keratinocytes. *J Cell Sci* 117:5815–23

Marconi A, Panza MC, Bonnet-Duquennoy M, Lazou K, Kurfurst R, Truzzi F *et al.* (2006) Expression and function of neurotrophins and their receptors in human melanocytes. *Int J Cosmet Sci* 28:1–7

Marconi A, Terracina M, Fila C, Franchi J, Bonté F, Romagnoli G *et al.* (2003) Expression and function of neurotrophins and their receptors in cultured human keratinocytes. *J Invest Dermatol* 121:1515–21

Nicolson GL, Menter DG (1995) Trophic factors and central nervous system metastasis. *Cancer Metastasis Rev* 14:303–21

Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS *et al.* (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature* 427:843–8

Perlis C, Herlyn M (2004) Recent advances in melanoma biology. *Oncologist* 9:182–7

Pincelli C, Fantini F, Giannetti A (1994) Nerve growth factor and the skin. *Int J Dermatol* 33:308–12

Pincelli C, Yaar M (1997) Nerve growth factor: its significance in cutaneous biology. *J Investig Dermatol Symp Proc* 2:31–6

Rochlitzer S, Nassestein C, Braun A (2006) The contribution of neurotrophins to the pathogenesis of allergic asthma. *Biochem Soc Trans* 34:594–9

Segal RA (2003) Selectivity in neurotrophin signaling: theme and variations. *Annu Rev Neurosci* 26:299–330

Shonukan O, Bagayogo I, McCrea P, Chao M, Hempstead B (2003) Neurotrophin-induced melanoma cell migration is mediated through the actin-bundling protein fascin. *Oncogene* 22:3616–23

Thompson JF, Uren RF (2005) Lymphatic mapping in management of patients with primary cutaneous melanoma. *Lancet Oncol* 6:877–85

Wang X, Bauer JH, Li Y, Shao Z, Zetoune FS, Cattaneo E *et al.* (2001) Characterization of a p75(NTR) apoptotic signaling pathway using a novel cellular model. *J Biol Chem* 276:33812–20