

Loss of E-cadherin leads to upregulation of NF κ B activity in malignant melanoma

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Malignant transformation of melanocytes frequently coincides with loss of E-cadherin expression. Here, we show that loss of E-cadherin leads to induction of nuclear factor kappa B (NF κ B) activity in melanoma cell lines. Melanoma cells show constitutively active NF κ B, whereas no activity is found in primary melanocytes. After re-expression of E-cadherin in melanoma cells, strong downregulation of NF κ B activity was found. Consistently, NF κ B activity was induced in primary human melanocytes after inhibition of E-cadherin activity by functionally blocking anti-E-cadherin antibodies. Interestingly, re-expression of E-cadherin-blocked p38 MAPK activity and the p38 MAPK inhibitors SB203580 and SB202190 almost completely prevented NF κ B activation in melanoma cells. Furthermore, cytoplasmatic β -catenin induced p38 and NF κ B activation in malignant melanoma. To our knowledge, this is the first report suggesting a correlation between E-cadherin and NF κ B activity in melanocytes and melanoma cells. In summary, we conclude that loss of E-cadherin and cytoplasmatic β -catenin induces p38-mediated NF κ B activation, potentially revealing an important mechanism of tumorigenesis in malignant melanomas.

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Introduction

The cell–cell adhesion molecule E-cadherin has been shown to execute important functions in embryogenesis and tissue architecture by forming intercellular junction complexes and establishing cell polarization (Gumbiner *et al.*, 1988). The extracellular domain of E-cadherin is involved in a molecular zipper mediating cell–cell adhesion, while the cytoplasmic tail is linked to the actin cytoskeleton via catenins (Cowin, 1994; Shapiro *et al.*, 1995). Owing to its critical function in intercellular

adhesion, E-cadherin has also been assumed to act as a tumor suppressor negatively regulating several critical steps of invasion and metastasis. Loss of E-cadherin expression during tumor development was recently observed in a variety of different tumor types including malignant melanomas (Berx *et al.*, 1995, 1996; Tamura *et al.*, 1996a, b; Silye *et al.*, 1998; Sanders *et al.*, 1999; Melki *et al.*, 2000; Poser *et al.*, 2001). Further, we have shown that inhibition of the expression of the transcriptional repressor *snail* induced re-expression of E-cadherin in Mel Im melanoma cells (Poser *et al.*, 2001). Transfection of E-cadherin cDNA into invasive carcinoma cells leads to a significant reduction of their invasive capacity *in vitro* (Frixen *et al.*, 1991; Vlemineckx *et al.*, 1991), and activation of E-cadherin expression results in growth retardation of tumor cell lines (Watabe *et al.*, 1994). Recent experiments suggest that E-cadherin is not only involved in cell–cell contact but multiple signalling pathways are activated or repressed by E-cadherin function. One of the most commonly discussed pathway is the β -catenin/LEF/T-cell factor (TCF) signalling cascade (Nollet *et al.*, 1999; Novak and Dedhar, 1999). Further, other groups could show a regulation of MAP kinases via E-cadherin (Pecce and Gutkind, 2000).

Nuclear factor kappa B (NF κ B) is a heterodimeric transcription factor that is predominantly composed of 65 and 50 kDa subunits of the Rel family. In resting cells, NF κ B is mainly retained in the cytoplasm by the inhibitor of NF κ B (I κ B) family of proteins, which mask the nuclear translocation signal of the transcription factor. Upon stimulation of cells, two defined serine residues in the NH₂ terminus of I κ B proteins are phosphorylated triggering their ubiquitination and subsequent degradation by the 26S proteasome. Hereby, the NF κ B proteins are released for translocation to the nucleus and induction of κ B-dependent genes.

NF κ B modulates multiple basic cellular functions like cell growth, differentiation, inflammatory response, and immune response. More recently, NF κ B activation has been connected with multiple aspects of oncogenesis, including the control of apoptosis, the cell cycle, differentiation, and cell migration (Baldwin, 2001). Several reports demonstrated strong and constitutive activity of NF κ B in malignant melanoma, possibly through enhanced I κ B kinase (IKK) activity and I κ B degradation (Shattuck-Brandt and Richmond, 1997;

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Yang and Richmond, 2001). The upstream signalling pathways leading to IKK activity and NF κ B activity in malignant melanoma are unknown until today.

In this study, we examined the relationship between the loss of E-cadherin expression and NF κ B signalling in malignant melanoma.

Results

One early and tremendous change in the development of malignant melanoma is the loss of E-cadherin expression. Whereas human melanocytes show expression of this cell–cell adhesion molecule, melanoma cells lose E-cadherin but show expression of N-cadherin. Nuclear extracts from malignant melanoma display strong NF κ B DNA-binding activity as compared to melanocytes. Accordingly, transient transfection assays and electrophoretic mobility shift assay (EMSA) data showed that NF κ B transcriptional and DNA-binding activity is higher in malignant melanoma cell lines than in melanocytes (Figure 1a and b).

We next examined the interplay between loss of E-cadherin and upregulation of NF κ B activity.

To induce E-cadherin expression in melanoma cells, we used two experimental models. The melanoma cell line Mel Im stably transfected with antisense snail cDNA showed induction of E-cadherin expression as previously shown by our group (Poser *et al.*, 2001). Furthermore, E-cadherin expression in the Mel Im cells was induced by transient transfection of an E-cadherin expression plasmid. Effectiveness of both transfection methods was shown previously (Poser *et al.*, 2001) and evaluated by quantitative reverse transcription (RT)–PCR and Western blots for E-cadherin revealing an E-cadherin expression of approximately 50% compared to normal human melanocytes (Figure 2a and b).

In further experiments, the antisense snail-transfected melanoma cells showed reduction of NF κ B DNA-binding activity and transcriptional activity (Figure 3a and c). Moreover, transient E-cadherin overexpression reduced NF κ B DNA binding and transcriptional activity (Figure 3b and c). The reduction of NF κ B was shown to be dependent on the amount of E-cadherin expression by a dose curve (data not shown). Immunofluorescence staining revealed translocation of p65 from the nucleus to the cytoplasm in the Mel Im as snail clones (re-expressing E-cadherin) in comparison to Mel Im cells (Figure 3d). Additionally, Western blots show

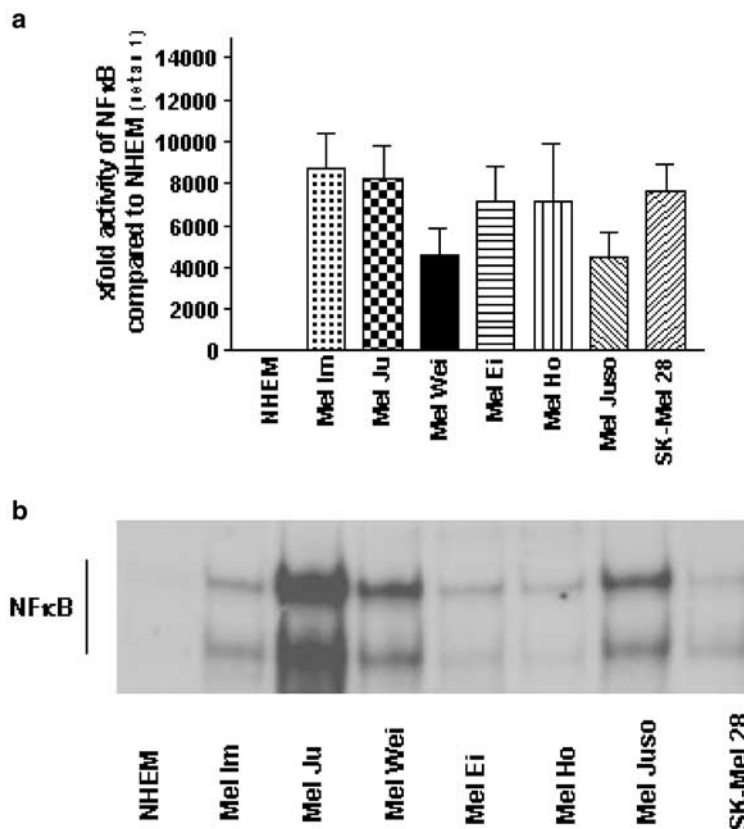


Figure 1 Reporter gene analysis and gel shift assays to analyse NF κ B activity in human primary melanocytes and melanoma cells. (a) Reporter gene assays using the NF κ B-luc construct were performed to detect NF κ B activity in normal human epidermal melanocytes (NHEM) and eight melanoma cell lines. Numbers are given as \times fold activation compared to NF κ B activity in NHEM (set as 1). (b) Nuclear NF κ B-binding activity was analysed comparing the human epidermal melanocytes (NHEM) to the seven melanoma cell lines used in (a)

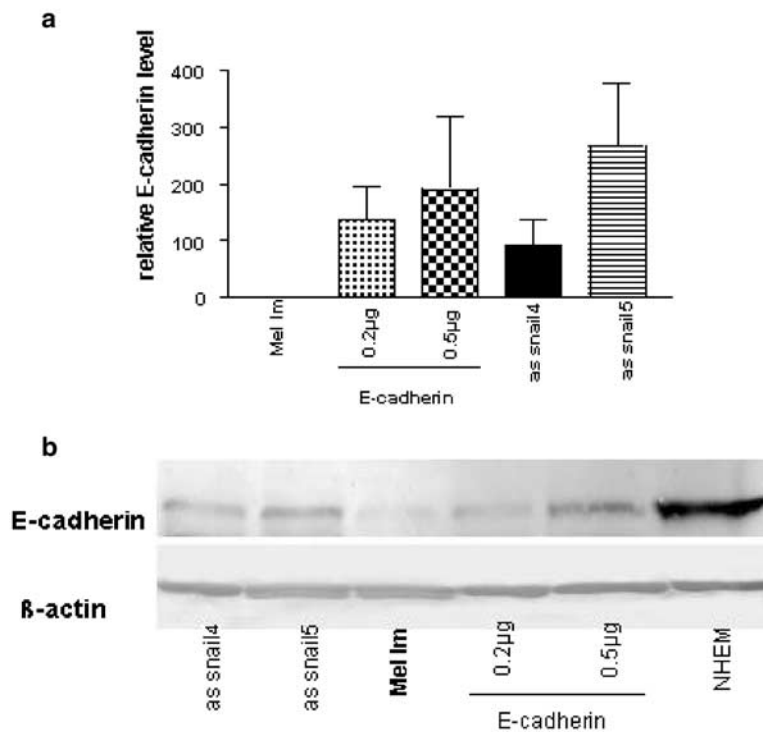


Figure 2 Real-time PCR and Western blot data for the expression level of E-cadherin in stably transfected antisense snail cell clones and transiently transfected Mel Im cells. **(a)** The transcriptional level of E-cadherin is documented with real-time PCR. **(b)** The translational level of E-cadherin protein is shown by Western blot using an antibody against E-cadherin

that the proportionate amount of p65 increases in the cytoplasm and decreases in the nucleus in the E-cadherin re-expressing cells, and the Mel Im cells show phosphorylation of p65 in the nucleus in comparison to the E-cadherin-expressing cells (Figure 3e).

Western blots analysing the amount of I κ B in the cell clones showed strong signals in the Mel Im as snail cell clones (Figure 3f).

To verify this regulatory effect, primary human melanocytes were treated with an inhibitory antibody against E-cadherin. Interestingly, NF κ B DNA-binding activity is induced in conditions where E-cadherin signalling is blocked, whereas the use of control antibodies had no effect on NF κ B DNA-binding activity (Figure 4).

Moreover, expression of the endogenous NF κ B-dependent gene interleukin 8 (IL-8) was reduced in melanoma cells transiently transfected with an E-cadherin expression plasmid, whereas expression of the NF κ B-independent gene transcriptional growth factor beta 2 (TGF β 2) remained unchanged (Figure 5).

The interplay between E-cadherin and NF κ B signalling pathway is unknown. However, it was shown that E-cadherin negatively regulates MAP kinase activity (Pece and Gutkind, 2000), a pathway that intersects with the NF κ B signalling cascade (Vanden Berghe *et al.*, 1998). Therefore, we speculated that E-cadherin may modulate NF κ B activity through the MAP kinase pathway. To verify this hypothesis, we investigated the effect of E-cadherin on the phosphorylation of four

members of the MAP kinase family (c-jun NH₂-terminal kinase (JNK), p42/44ERK1/2, p38) using the wild-type Mel Im cell line (E-cadherin negative) and two cell clones transfected with antisense snail (E-cadherin positive). Furthermore, we analysed the regulation of the protein kinase Akt.

p42ERK2 was expressed and constitutively phosphorylated in melanoma cells, whereas only weak expression and phosphorylation of p44ERK1 was found. p42ERK2 and p44ERK1 phosphorylation remained unchanged in the melanoma cell clones Mel Im as snail 4 and 5 (E-cadherin positive) as well as in Mel Im melanoma cells transiently transfected with E-cadherin (Figure 6a). Further, Western blots revealed no regulation of JNK (data not shown) which was further supported by the finding that we found no change of c-jun phosphorylation after re-expression of E-cadherin (Figure 6b) was detectable. Additionally, Figure 6c shows that the kinase Akt was not influenced by E-cadherin expression. The analysis of p38 was carried out using a quantitative enzyme-linked immunosorbent assay (ELISA) system and Western blots for phosphorylated and unphosphorylated p38. Marked downregulation of p38 phosphorylation was observed after re-expression of E-cadherin in melanoma cells with the ELISA system (Figure 6d). These data are supplemented by the Western blot for p-p38 and as the loading control p38 (Figure 6e).

To analyse the functional role of p38 MAP kinase on E-cadherin-mediated downregulation of NF κ B activity,

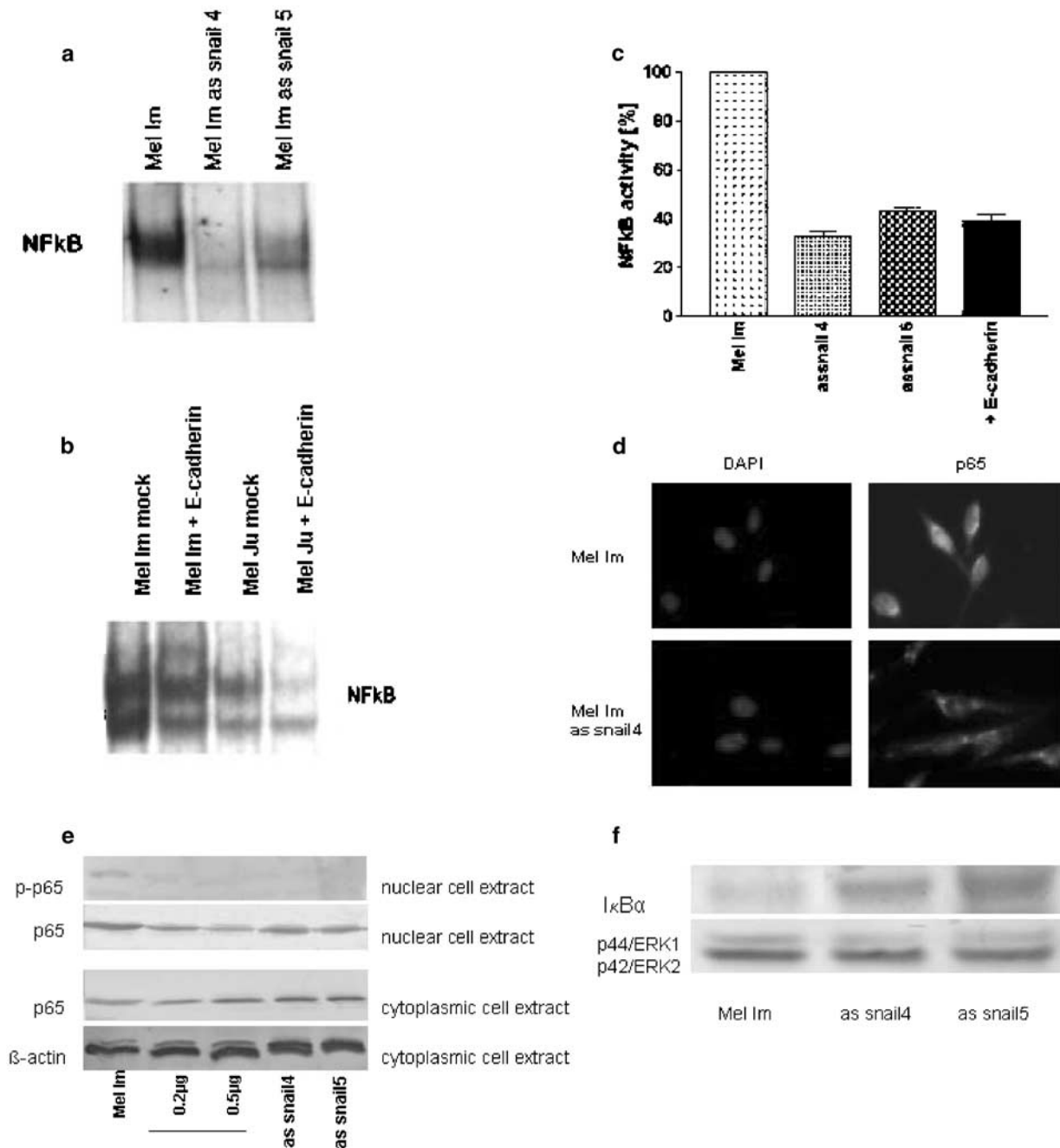


Figure 3 Reporter gene analysis, gel shift assays, Western blot, and immunofluorescence staining of p65 to analyse NF κ B activity after re-expression of E-cadherin in melanoma cells. (**a**, **b**) Nuclear NF κ B-binding activity was analysed using gel shift assays to compare the wild-type melanoma cell line Mel Im to the E-cadherin re-expressing melanoma cells. E-cadherin re-expression was induced either by stably downregulating snail expression in the melanoma cell line Mel Im (**a**, Mel Im as snail 4 and 5) or by transient transfection with an E-cadherin expression plasmid into the melanoma cell lines Mel Im and Mel Ju (**b**). (**c**) Reporter gene assays using the NF κ B-luc construct to detect NF κ B activity were performed in the melanoma cell line Mel Im in comparison to E-cadherin re-expressing cell clones generated either by stable transfection of antisense snail (Mel Im as snail 4 and 5) or transient transfection of an E-cadherin expression plasmid. Activity of NF κ B in Mel Im melanoma cells was set as 100%. (**d**) Immunofluorescence staining of p65 revealed nuclear staining in Mel Im cells, whereas in Mel Im as snail cells (with E-cadherin re-expression) p65 was located in the cytoplasm. (**e**) Western blot analysis to compare the proportional amount of nuclear and cytoplasmic p65 in the E-cadherin re-expressing as snail cells and the transiently transfected Mel Im cells. Staining of cytoplasmic β -actin ensures equal loading of the samples. (**f**) Western blot analysis of I κ B level in the melanoma cell line Mel Im compared to the E-cadherin re-expressing cell clone Mel Im as snail. Staining of p42/44 ERK ensures equal loading

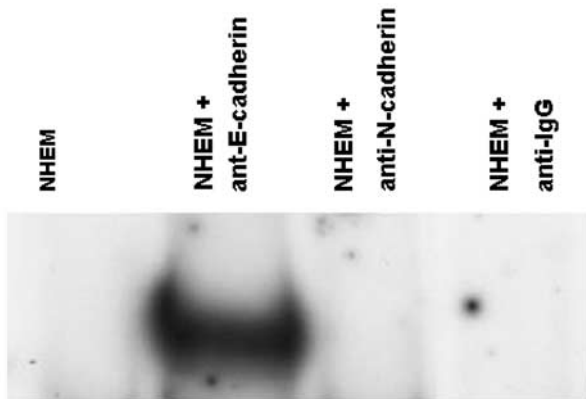


Figure 4 Gel shift assays to analyse NF κ B activity in primary human melanocytes after inhibition of E-cadherin activity. In normal human epidermal melanocytes (NHEM), no NF κ B binding to a consensus NF κ B-binding site was detectable. After treating the cells with an anti-E-cadherin antibody, which functionally blocks E-cadherin activity, induction of NF κ B was found. Anti-IgG and N-cadherin antibodies, respectively, were used as control. No NF κ B-binding activity was detectable in control treated NHEMs

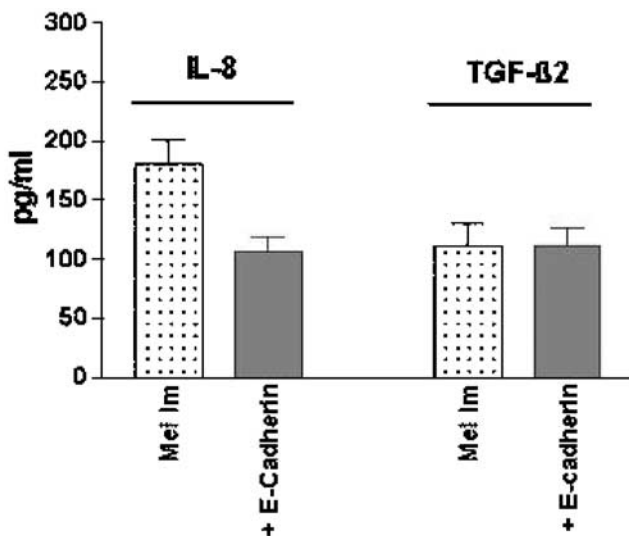


Figure 5 Expression of IL-8 and TGF β 2 in melanoma cells after re-expression of E-cadherin. The expression of IL-8 and TGF β 2 was quantified using ELISA systems. Expression was compared between Mel Im cells and the E-cadherin re-expressing Mel Im cells. A reduction of IL-8 expression was seen, whereas no change in TGF β 2 expression was found

we used inhibitors of MAP kinases. Interestingly, NF κ B transcriptional activity was strongly reduced following inhibition of p38 by SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) and SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole) in Mel Im cells (data not shown), whereas inhibitors against p42/44ERK did not show an effect (PD98059, 2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one). Further, we investigated whether MAP kinase kinase1 (MEK1) was involved

as an upstream regulating factor but found it not to be involved in p38 regulation by E-cadherin (data not shown).

As loss of E-cadherin is accompanied by free cytoplasmatic β -catenin, we analysed the localization of β -catenin in melanoma tissue by immunohistochemistry and could clearly show strong cytoplasmatic but no membrane staining (data not shown).

Also, immunofluorescence data of Mel Im cells showed cytoplasmatic and not nuclear localization of β -catenin in this cells (Figure 7a). To trigger this effect of β -catenin in the melanoma cell line Mel Im, we transiently transfected the cells with β -catenin. To check the transfection efficiency, we used the quantitative RT-PCR and immunofluorescence. Figure 8a shows the increase in cytoplasmatic fluorescence in β -catenin transiently transfected cells in comparison to nontransfected Mel Im cells. Figure 7b engage this finding with the higher expression of β -catenin after transfection. We then measured the effect of free β -catenin on p38 and NF κ B activation in melanoma cells. Transient transfection of β -catenin into the melanoma cell line Mel Im resulted in an upregulation of p38 activity (Figure 7c) and enhanced NF κ B transcriptional activity (Figure 7d). No effect on p38 and on NF κ B activity was observed after transient transfection with a γ -catenin expression plasmid. Inhibitors of p38 MAPK (SB203580, SB202190) completely blocked the β -catenin-induced NF κ B activation, suggesting that this effect is mediated by p38 MAPK.

Discussion

In this study, we have shown for the first time that strong constitutive activity of NF κ B in malignant melanoma correlates with loss of E-cadherin and induction of p38 activation.

NF κ B is a key transcription factor activated by several cellular signal transduction pathways. It is of critical importance in regulating cellular processes such as inflammation, host defence, apoptosis and also tumorigenesis. We and others have shown that NF κ B is constitutively activated in malignant melanoma as well as in other types of cancer (Huang *et al.*, 2000; Davis *et al.*, 2001; McNulty *et al.*, 2001; Suh *et al.*, 2002). Strong induction of NF κ B in malignant melanoma was previously reported by Yang and Richmond (2001) and Shattuck-Brandt and Richmond (1997). They showed constitutive I κ B kinase activity and subsequent decrease in I κ B α steady-state levels in several melanoma cell lines. However, the molecular mechanisms of constitutive NF κ B activation in melanoma and involved upstream signalling pathways remained unclear so far.

In this study, we demonstrated that NF κ B activity is associated with the loss of E-cadherin in malignant melanoma. The experiments presented revealed that re-expression of E-cadherin in melanoma cells either by transient transfection of E-cadherin or stable by

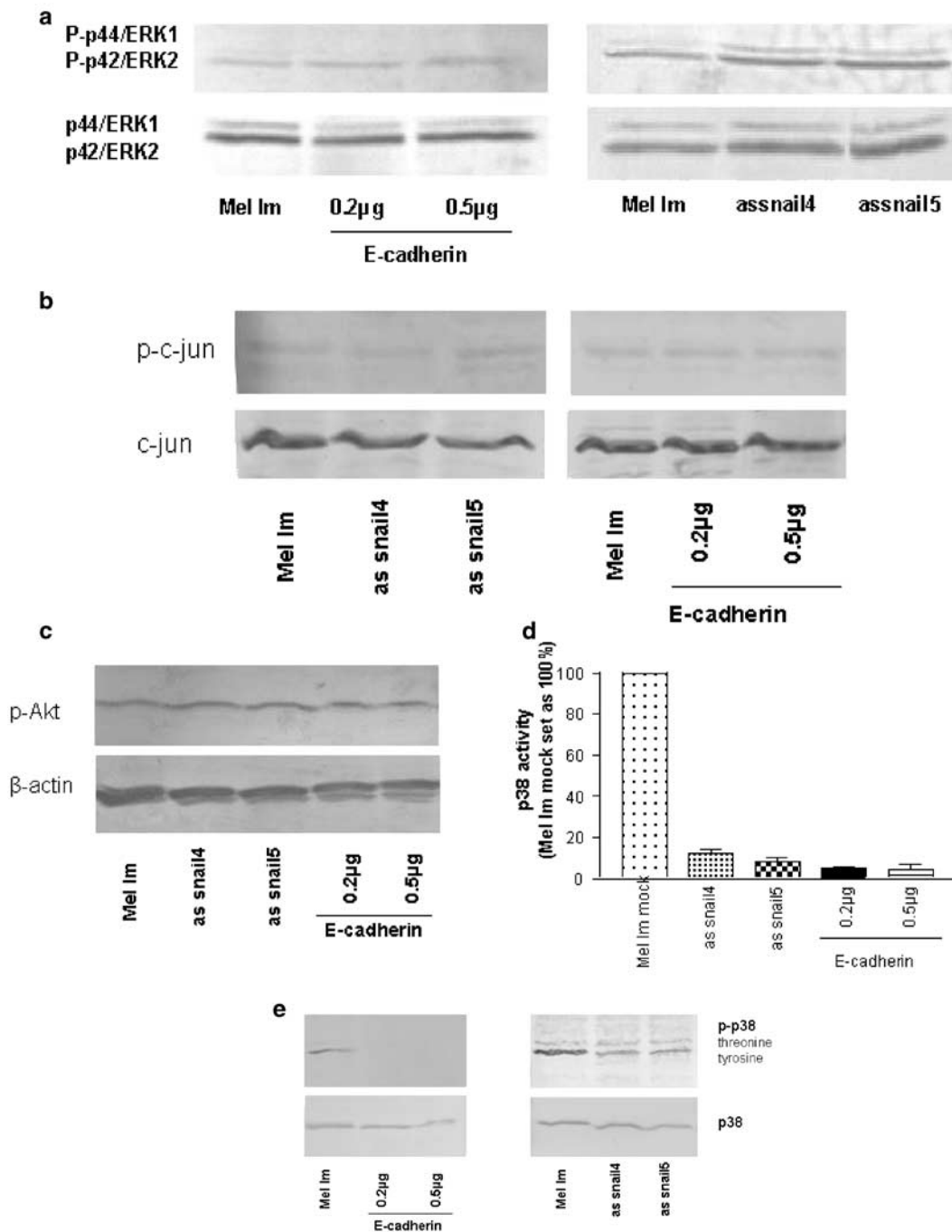


Figure 6 Analysis of MAP kinase activity in melanoma cells before and after re-expression of E-cadherin. ERK1/2 and c-jun expression and activity in the wild-type Mel Im cell line was compared to the two stably antisense snail-transfected cell clones (as snail 4 and 5, right panel) that show re-expression of E-cadherin and to Mel Im cells transiently transfected with E-cadherin (using 0.2 or 0.5 μ g plasmid DNA, left panel). **(a)** ERK2 was detected phosphorylated in melanoma cells, whereas only weak expression and phosphorylation of ERK1 was found. Upregulation of E-cadherin in the melanoma cells led to no changes in ERK1/2 phosphorylation. **(b)** The phosphorylation of c-jun was not changed comparing E-cadherin- and no E-cadherin (Mel Im)-expressing cells in Western blots for phosphorylated c-jun. The Western blot for unphosphorylated c-jun shows the equal loading of each lane. **(c)** The phosphorylation of Akt was not changed comparing E-cadherin- and no E-cadherin-expressing cells in a Western blot detecting phosphorylated Akt. β -Actin shows the equal loading of the samples. **(d)** The analysis of p38 was carried out using an ELISA system. Downregulation of p38 phosphorylation was observed after re-expression of E-cadherin in melanoma cells (as snail 4 and 5) and E-cadherin transiently transfected cells compared to the mock-transfected Mel Im cells (set as 100%). Equality in loading was assured by adjusting total protein amount. **(e)** Western blots were performed in addition to the ELISA analysis. Using an antibody against P-p38, downregulation of the phosphorylation of p38 could be shown. The Western blot of unphosphorylated p38 shows the equal loading of each lane

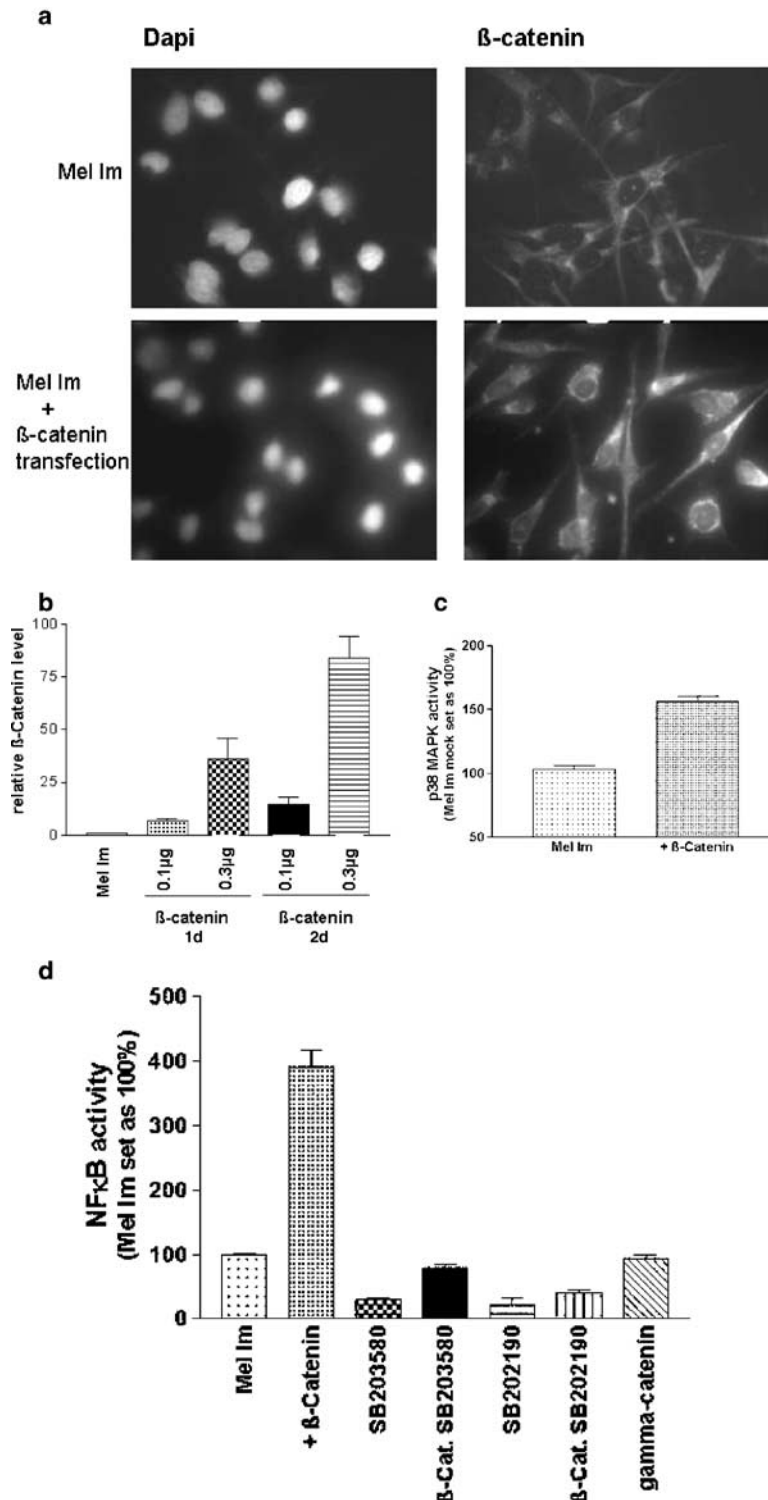


Figure 7 Influence of β -catenin on p38 MAPK and NF κ B activity. (a) Immunofluorescence staining of β -catenin revealed cytoplasmic staining in Mel Im cells. After transfection of β -catenin, the immunofluorescence staining in the cytoplasm of the transfected cells is stronger than the control Mel Im cells. (b) Upregulation of β -catenin mRNA expression in the transiently transfected Mel Im cells was analysed by real-time PCR after 24 and 48 h. (c) Regulation of p38 activity by β -catenin was analysed using transient transfection of a β -catenin expression plasmid into Mel Im melanoma cells followed by a p38 MAPK ELISA. The control Mel Im cells were mock transfected. An upregulation of p38 MAPK by β -catenin in comparison to the control was shown. The p38 activity in the mock-transfected control was set as 100%. (d) NF κ B reporter gene assays using the NF κ B-luc construct to detect NF κ B activity were performed in the melanoma cell line Mel Im. The cells were either mock transfected or transiently transfected with β -catenin or γ -catenin expression vectors. An upregulation of NF κ B activity by β -catenin in comparison to the control was shown, whereas γ -catenin had no effect

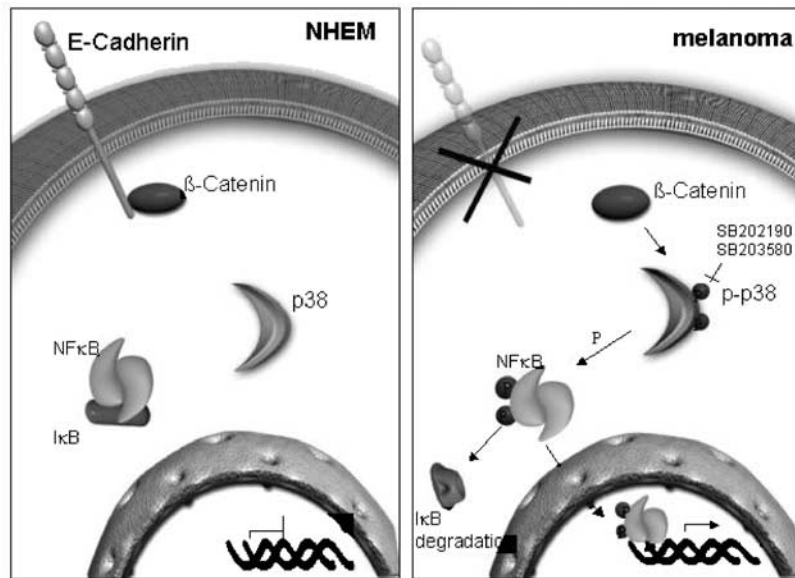


Figure 8 Model: Loss of E-cadherin stimulates the transactivation potential of NF κ B by targeting β -catenin and p38 in melanoma cells. We propose that loss of E-cadherin expression leads to enhanced free cytoplasmic β -catenin resulting in activation of p38 MAPK. p38 MAPK is inducing NF κ B binding and transcriptional activity

inhibiting snail expression (Poser *et al.*, 2001) led to downregulation of NF κ B DNA-binding activity and NF κ B transcriptional activity shown in Figure 3a–c. Furthermore, our data indicate the enhanced shift of p65 into the cytoplasm in E-cadherin-expressing cells. The non-E-cadherin-expressing Mel Im cells exhibit a higher amount of phosphorylated p65 in the nucleus. Figure 3f and 5 present enhanced I κ B level and reduced expression of NF κ B target genes after re-expression of E-cadherin. With the expression of E-cadherin, the amount of the inhibitory protein (I κ B) of NF κ B increases, the translocation of p65 into the nucleus is prevented and the therefore target genes will not be stimulated by NF κ B.

Normal human epithelial melanocytes (NHEM) show no NF κ B activity but strong E-cadherin expression. Consistently, we could show that treatment of primary human melanocytes with functionally blocking anti-E-cadherin antibodies led to induction of NF κ B activity. This gives the hint for the exceptional role of E-cadherin for the normal human melanocytes and the developing of consequences if E-cadherin expression is lost in melanoma cells.

Previously, other reports demonstrated a correlation between E-cadherin and regulation of MAP kinases. Pece and Gutkind (2000) analysed the activity of MAP kinases p42/p44 in keratinocytes after formation of adherent junctions and revealed remarkable increase in the state of activation. However, in this study, only an unspecific MAPK activity assay was used and consequently no information on the kind of MAPK could be gained. Here, to clarify the regulation of NF κ B by E-cadherin in more detail, we started to analyse the influence of E-cadherin on different MAP kinases. Our assays demonstrated strong activity of the MAP

kinases in the melanoma cell lines. After retransfection of E-cadherin, our data revealed marked downregulation of p38 MAPK activity in melanoma cells, whereas erk1, erk2, and JNK activity remained unchanged. In accordance to these results, the use of inhibitors of p38 MAPK activity resulted in an almost complete inhibition of NF κ B activity and enhanced I κ B level.

A possible interaction between p38 MAPK and NF κ B was first suggested by Maulik *et al.* (1998). They presented evidence that the p38 inhibitor SB203580 prevents NF κ B nuclear translocation during ischemic adaptation. Similarly, tumor necrosis factor (TNF)-induced NF κ B activity is blocked by p38 inhibitors (Beyaert *et al.*, 1996; Carpentier *et al.*, 1998). Carter *et al.* (1999) presented that this regulation is in part dependent on the modulation of the activation of transcription factor IID (TFIID). Further, activation of NF κ B can occur due to phosphorylation of the p65 subunit of NF κ B. This alternative mechanisms of NF κ B activation was discussed by several groups (Vanden Berghe *et al.*, 1998; Madrid *et al.*, 2001). It has been shown that the cytokines TNF and interleukin-1 β lead to phosphorylation of p65 through pathways distinct from induced nuclear translocation. Evidence has been presented that p38 MAP kinase is involved in p65 phosphorylation (Wesselborg *et al.*, 1997; Bergmann *et al.*, 1998). A study performed by Wang and Richmond (2001) revealed that NF κ B is not regulated by the extracellular signal-regulated protein kinase (ERK) cascade in malignant melanoma, further supporting our data. These authors also demonstrated the importance of p38 MAPK in activating NF κ B, but, in contrast to our experiments, MEKK1 was involved in the analysed MGSA/GRO- α -induced signalling of E-cadherin.

The importance of p38 activity for invasion of malignant melanoma cells was determined by Denkert *et al.* (2002) This study revealed that p38 MAPK inhibition resulted in reduced invasive potential and downregulation of matrix-metalloproteinase 2 (MMP2) expression.

The strong and constitutive activity of the MAPK pathways has been reported previously for malignant melanoma (Englaro *et al.*, 1998; Kortylewski *et al.*, 2001; Denkert *et al.*, 2002). Here, we further investigated the mechanism leading to p38 MAPK activation in malignant melanoma. In our study, we have shown that β -catenin is involved in p38 MAPK activation. Transient transfection of β -catenin resulted in an enhanced p38 activity, which correlates with the enhancement of NF κ B activity. Owing to the loss of E-cadherin, enhanced amounts of free β -catenin can be found in the cytoplasm (Novak and Dedhar, 1999; Orsulic *et al.*, 1999). Further, Li *et al.* (2001) recently described stabilization of β -catenin in melanoma cells by N-cadherin. They suggest the stabilization to be controlled by activation of Akt/PBD through N-cadherin-mediated intercellular interaction.

It has been suggested by several reports that the switch from E-cadherin to N-cadherin expression in malignant melanoma has a main impact on tumor development and progression. For example, Li *et al.* (2001) demonstrated that N-cadherin promotes survival and migration of melanoma cells, processes otherwise being repressed and strictly controlled via E-cadherin.

Striking, NF κ B activation is connected to multiple aspects of oncogenesis, including the control of apoptosis and migration and NF κ B activity is elevated in malignant melanoma.

To our knowledge, this is the first report showing a correlation between E-cadherin expression and NF κ B activity (Figure 8). In summary, we conclude that loss of E-cadherin and cytoplasmatic β -catenin induces p38-mediated NF κ B activation. These findings potentially reveal an important mechanism of tumor genesis in malignant melanomas.

Materials and methods

Cell lines and cell culture conditions

The melanoma cell line Mel Im has been described in detail previously (Jacob *et al.*, 1998). It was derived from a metastasis of malignant melanomas. Further, melanoma cell lines Mel Ju, Mel Wei, Mel Juso, Mel Ho, Mel Ei, HTZ-19d, and SK-Mel 28 were used (Jacob *et al.*, 1998). For tissue culture, the cells were maintained in DMEM supplemented with penicillin (400 U/ml), streptomycin (50 μ g/ml), L-glutamine (300 μ g/ml), and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and splitted 1:5 every 3 days.

Human primary melanocytes derived from normal skin were cultivated in melanocyte medium MGM-3 (Gibco, Eggenstein, Germany) under a humidified atmosphere of 5% CO₂ at 37°C. Cells were used between passages 6 and 10 and not later than 3 days after trypsinization. Cells were detached for subcultivation or assay with 0.05% trypsin, 0.04% EDTA in phosphate-buffered saline (PBS).

An inhibitory mouse monoclonal E-cadherin antibody (clone M106, 0.2 μ g/ μ l, Takara, Shiga, Japan) was used in a concentration of 1 μ g/ml to analyse the effect of E-cadherin inhibition on primary human melanocytes. The cells were incubated for 24 h.

SB203580 and SB202190 (Calbiochem, San Diego, USA) were used as specific inhibitors for p38 MAPK, and PD98059 as a specific inhibitor of p42/44 ERK. Cells were treated for 24 h with a concentration of 10 and 20 μ M (for SB203580 and SB202190) or 1 and 10 μ M (for PD98059).

Transfection experiments and luciferase measurements

For transient transfections 2 \times 10⁵ cells were seeded into each well of a six-well plate and transfected with 0.5 μ g plasmid DNA using the lipofectamine plus method (Gibco) according to the manufacturer's instructions. The cells were lysed 24 h after transfection and the luciferase activity in the lysate was quantified by a luminometric assay (Promega Corp., Madison, USA). Transfection efficiency was normalized according to renilla luciferase activity by cotransfecting 0.1 μ g of the plasmid pRL-TK (Promega). All transfections were repeated at least four times. For transient transfection, the plasmids NF κ B-luc (Promega), pBAT-E-cadherin (generous gift from Gabriele Handschuh (Institute of Pathology, LMU Munich, Germany)), and β - γ -catenin expression vectors (generous gift from Eric Fearon, University of Michigan Medical School, USA) were used.

Further, a panel of Mel Im cell clones was established by stable transfection with an antisense Snail expression plasmid (Poser *et al.*, 2001) under the control of a CMV promoter and cotransfected with the neo-selectable pcDNA3 plasmid (Invitrogen, Groningen, Holland). Transfection was performed using lipofectamine plus (Gibco) according to the manufacturer's instructions. Transfected cells were cultured under selective conditions using G418 (Sigma) in a concentration of 50 μ g/ml. Controls received pcDNA3 alone. After 25 days of selection, individual G418-resistant colonies were subcloned. E-cadherin expression levels of these clones were determined by Western blot analysis of the cell lysates.

Western blotting

Cells (3 \times 10⁶) were lysed in 200 μ l RIPA buffer (Roche, Mannheim, Germany) and incubated for 15 min at 4°C. Insoluble fragments were removed by centrifugation at 13000 r.p.m. for 10 min and the supernatant lysate was immediately shock frozen and stored at -80°C. In total, 7-40 μ g of RIPA complete cell lysate or nuclear and cytoplasmatic protein fraction were loaded per lane and separated on SDS-PAGE gradient gels (Invitrogen) and subsequently blotted onto a PVDF membrane. After blocking for 1 h with 3% BSA/PBS, the membrane was incubated for 16 h with one of the following antibodies: anti-Erk1/2, anti-P-ERK1/2, anti-p38, anti-P-SAPK/JNK, anti-I κ B ζ , anti-p-Akt (all from Cell signaling, Beverly, USA), anti-c-jun, and anti-P-c-jun (both from Upstate, Waltham, USA), anti E-cadherin (BD Transduction Laboratories), anti β -actin (Sigma), respectively. We used an alternative blocking method with 5% nonfat dry milk in TBS for the antibodies: anti p65 (Rockland) anti P-p65 (Cell signaling), and anti-P-p38 (Biosource). After three washing steps with PBS or TBS, respectively, the membrane was incubated for 1 h with an alkaline phosphate-coupled secondary anti-mouse or anti rabbit IgG antibody (AP303A, Chemicon, Hofheim, Germany) and then washed again. Finally, immunoreactions were visualized by NBT/BCIP (Sigma) staining.

Immunofluorescence

p65 immunofluorescence analysis was performed as described previously (Hellerbrand *et al.*, 1998). Briefly, 4×10^5 cells were seeded on four-well culture slides (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and fixed after growing for 24 h with ice-cold acetone. Subsequently, slides were washed in PBS and unspecific binding was blocked using 5% BSA/PBS. Then slides were incubated with an anti p65 antibody (1:50; Rockland, Gilbertsville, PA, USA) and anti- β -catenin antibody (Santa Cruz Biotechnology) in 5% BSA/PBS, washed three times with PBS and incubated with FITC-anti-rabbit (1:400; DAKO, Glostrup, Denmark), washed again and finally sealed with VectaShield mounting medium (Vector Laboratories, Burlingame, CA, USA) including 1 μ g/ml DAPI (Sigma). Slides were viewed using a Zeiss Axioplan epifluorescence microscope (Zeiss, Jena, Germany) and images were taken with a cooled charge-coupled device camera (Photometrics, Tucson, AZ, USA).

p38-ELISA

P-p38 MAPK was measured quantitatively using a phospho ELISA p38 MAPK (pTPY180/182) kit (BioSource International, USA). The ELISA was performed as described by the manufacturer.

IL-8 and TGF- β 2 ELISA

IL-8 and TGF- β 2 expression were analysed in the supernatant of melanoma cells using ELISAs from Promocell (Heidelberg, Germany) and R&D Systems (Minneapolis, USA), respectively. The assays were performed as described by the manufacturers.

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Gel shift experiments

A double-stranded oligomeric binding site for NF κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3', Promega) was phospholabelled and used for gel mobility-shift assays. Nuclear extracts were prepared from primary melanocytes and Mel Im cells and gel shifts were performed as described previously (Bosserhoff *et al.*, 1996).

Abbreviations

ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated protein kinase; I κ B (IkappaB), inhibitor of NF κ B; IL-8, interleukin 8; JNK, c-jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; Mekk1, MAP kinase kinase1; MMP2, matrix-metalloproteinase 2; NF κ B, nuclear factor kappa B; NHEM, normal human epidermal melanocytes; PBS, phosphate-buffered saline; PD98059, 2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one; PKB, protein kinase B; RT, reverse transcription; SAPK, stress-activated protein kinase; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; TCF, T-cell factor; TGF β 2, transcriptional growth factor beta 2.

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