Phosphorylation of β-catenin results in lack of β-catenin signaling in melanoma

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Abstract. The Wnt/β-catenin pathway is involved in differentiation events during embryonic development and is further described as a pathway often participating in tumor formation when aberrantly activated. Molecular studies concentrating on colorectal cancer revealed mutations of *apc*, *ctnnbi*, *btrc* and *tcf-4* genes which mimic Wnt stimulation. However, such mutations are rarely found during melanoma development. Therefore, we analyzed the β-catenin activity in this type of skin cancer. Interestingly, localization of β-catenin protein was basically cytoplasmic in melanomas *in vivo*, which was in clear contrast to findings in colon carcinoma. Congruently, the transcriptional activity of β-catenin regulating expression of β-catenin target genes was not observed in several melanoma cell lines. Further, neither LiCl nor Wnt agonist treatment led to significant activation of β-catenin signaling. This lack in functionality seems to depend on phosphorylation at threonine 41 and serine 45 of β-catenin observed in several melanoma cell lines. However, this specific endogenous phosphorylation pattern led to upregulation of other signaling pathways resulting e.g. in induction of N-cadherin expression. In summary, this study suggests a cell type-specific regulation of β-catenin function. This alternative β-catenin signaling pathway should be considered when thinking about targeting β-catenin in melanoma treatment.

Introduction

The β-catenin protein was initially discovered for its role in cell adhesion (1). As a component of adherens junctions, it promotes cell adhesion by binding to the intracellular domain of the trans-membrane protein cadherin linking it to the actin cytoskeleton through the adaptor protein α-catenin.

Additionally, the signaling function of β-catenin in the so-called canonical Wnt pathway is conferred by a soluble

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cyto-plasmic pool whose stability is tightly regulated. Strong phosphorylation of β-catenin at its N-terminal region (serine 33, serine 37, serine 45 and threonine 41) labels the molecule for proteasomal degradation. Several molecules are believed to be responsible for phosphorylation of β-catenin at the N-terminus: the serine/threonine kinase GSK-β (glycogen synthase kinase 3 β) (2), Dsh (dishevelled), APC (*Adenomatous polyposis coli*), Axin, PP2A (protein phosphatase 2A) and CKI (casein kinase 1 α and ε) (3).

Wnt signals lead to accumulation of β-catenin. Certain Wnt molecules, mainly Wnt1, Wnt2, Wnt3, Wnt3a and Wnt8 bind to Frizzled/LRP (low-density lipoprotein receptor-related protein) receptors which results in the inhibition of the APC-Axin-GSK-3β complex by activation of Dishevelled (4) and by recruitment of Axin to the plasma membrane (5). This results in an increase in hypophosphorylated β-catenin that forms active transcriptional complexes with LEF/TCF transcription factors in the nucleus (6).

At least one other Wnt signaling pathway exists often named as non-canonical Wnt signaling. The non-canonical pathway is induced by other Wnts such as Wnt5a, Wnt5b, Wnt4 and Wnt11 and can trigger intracellular calcium release (7) and activation of PKC (protein kinase C) and *CamK*II (calmodulin kinase II) (8). The involvement of Wnt5a in cell motility and invasion of melanoma cells was described recently showing an increase in activated PKC (9). This non-canonical pathway does not directly regulate β-catenin but it modulates the transcriptional activity of LEF/TCF in the nucleus.

Most oncological findings regarding the Wnt/β-catenin signaling came from the analysis of colon, breast and kidney carcinoma (10,11) where activation of the pathway has been directly implicated in disease pathogenesis. The majority of colorectal carcinomas carry inactivating mutations in the *Adenomatous polyposis coli* (APC) tumor suppressor which lead to stabilization of β-catenin (12). Additionally, mutations in the β-catenin gene *CTNNBI* were found in colon cancer leading to the constitutive activation of β-catenin/LEF/TCF-dependent canonical signaling (13). All these studies demonstrating the activation of this pathway in cancer have the idea that elevated Wnt/β-catenin signaling is oncogenic in most contexts.

Studies of β-catenin mainly have been carried out in epithelial cells of colon or breast carcinomas but rarely in cell types of other embryonic origins. In malignant melanoma, a tumor originating from melanocytes derived from the neural crest, data on β-catenin signaling are still conflicting.

CTNNBI gene mutations (2-22%) as well as APC mutations (2.5%) are rare in malignant melanoma (14-16). Hypermethylation of the APC promoter 1A seems to be more frequent (15% of cell lines and biopsy samples) but does not necessarily increase β-catenin activity (16). Indeed, β-catenin is found only in ~6-30% of human melanoma nuclei, indicating that alternative localization of β-catenin or activation of further signaling pathways could have more relevance in melanoma than the translocation of β-catenin into the nucleus (17). On the contrary, the observed presence of nuclear β-catenin in the majority of benign nevi, along with the loss of nuclear β-catenin seen in melanoma progression (18-21), support the hypothesis that activation of nuclear Wnt/β-catenin signaling is important for a controlled cellular homeostasis of the skin.

Data from our laboratory provide evidence for the importance of an alternative β-catenin signaling in the cytoplasm of melanoma cells. Cytoplasmic β-catenin activates the MAPKinase p38 and the NF-κB signaling pathway in malignant melanoma inducing N-cadherin expression (22,23).

In this study, we aimed to investigate the function of β-catenin in melanoma cells and to clarify the mechanisms leading to a different signaling function of β-catenin in melanoma compared to epithelial cells and carcinomas.

Materials and methods

Cell lines and cell culture conditions. Cell lines and culture conditions were previously described (24). The colon carcinoma cell line SW480 was generated from a primary adenocarcinoma and exhibits no β-catenin mutation. The melanoma cell line 501 mel exhibits a D32H mutation of β-catenin (25). In experiments cells were treated with 40 or 20 mM lithium chloride (Sigma-Aldrich, Steinheim, Germany)

Molecular constructs. The following constructs were generous gifts: pBAT-β-catenin from J. Behrens (Department of Experimental Medicine II, University of Erlangen, Germany), T41A and S45A from D.L. Rimm (Department of Pathology, Yale University School of Medicine, New Haven) (26).

Transfection experiments and luciferase measurements. Transient transfections experiments were described previously (27). For transient transfection the plasmids TOPflash/ FOPflash (0.5 μg/well) (Upstate, Lake Placid, NY, USA) were used. The Renilla vector was used to normalize the transfection efficiency.

Western blotting. Cells ($3x10^6$) were lysed in 200 μl RIPA-buffer (Roche, Mannheim, Germany). Cell lysate (5-40 μg) was separated on 10% SDS-PAGE gels and subsequently blotted onto a PVDF membrane. After blocking with 3% BSA/PBS the membrane was incubated for 16 h with one of the following antibodies: anti-β-catenin (1 in 3000 dilution) (Sigma), anti-hypophosphorylated β-catenin (35-50) (1 in 200 dilution) (Acris, Hiddenhausen, Germany), anti-hypophosphorylated β-catenin (27-37) (1 in 200 dilution) (upstate), anti-β-catenin-phospho 41/45 (1 in 500 dilution) (Cell Signaling), anti-β-catenin phospho S33 (1 in 500 dilution), anti-β-catenin phospho S37 (1 in 500 dilution), anti-β-catenin phospho T41 (1 in 500 dilution), anti-β-catenin phospho S45 (1 in 500 dilution),

(Abcam, Cambridge, UK), and anti-β-actin (1 in 5000 dilution) (Sigma).

Quantitative real-time PCR for Brn-2 and laminin γ2. Quantitative real-time PCR was performed on a Lightcycler (Roche, Mannheim, Germany) basically as described (27). The following PCR program was used: 30 sec 95˚C (initial denaturation); 20˚C/sec temperature transition rate up to 95˚C for 15 sec, 3 sec 60˚C, 5 sec 72˚C, 85˚C acquisition mode single, repeated for 40 times (amplification). The PCR reaction was evaluated by melting curve analysis.

Immunohistochemistry. The paraffin-embedded tissues were deparaffinized, rehydrated and subsequently incubated with primary rabbit anti-β-catenin antibody (Sigma, 1:40) and primary mouse anti-E-cadherin antibody (BD Biosciences, 1:40) over night at 4˚C. The secondary antibody (biotin-labeled anti-rabbit/anti-mouse; Dako, Hamburg, Germany) was incubated for 30 min at room temperature, followed by incubation with streptavidin-POD (Dako) for 30 min. Antibody binding was visualized using AEC-solution (Dako). Finally, the tissues were counterstained by hemalaun solution (Dako). The evaluation of the staining was performed semi-quantitatively by light microscopy.

Immunofluorescence. Cells ($5x10^5$) were grown on a chamber slide for one day and incubated with lithium chloride (20 mM) for 4 h. The cells were fixed with 4% paraformaldehyde, incubated with 0.1% Triton X-100 (Sigma) and covered with blocking solution (PBS/1% BSA) for 1 h. Thereafter, cells were incubated with β-catenin antibody (1 in 100 dilution) for 1 h. The secondary antibody was FITC-conjugated antibody (1 in 40 dilution) (Dako, Glostrup, Denmark). After mounting with Mounting Medium with DAPI (Vectashield, H-1500) images were collected by fluorescence microscopy.

Subcellular proteome extraction kit. The extraction was performed using the subcellular proteome extraction kit following the manufacturer's (Calbiochem) instructions.

Statistical analysis. Results are expressed as the mean ± SD. Comparison between groups was made using the Student's unpaired t-test. $p<0.05$ was considered statistically significant and marked with asterisks in the figures (ns, not significant). All calculations were performed using the Graph Pad Prism software (GraphPad software Inc., San Diego, CA, USA).

Results

The current study was designed to elucidate the role of β-catenin in melanoma cells. Several studies suggest an important role of β-catenin in malignant melanoma (18) however, classical target genes of β-catenin such as c-myc or laminin γ2 were only rarely found to be induced in melanomas.

Endogenous status of β-catenin expression in malignant melanoma. To examine β-catenin expression *in vivo*, we stained tissue samples from patients with primary malignant melanomas. Representative sections are presented in Fig. 1A. The distribution of β-catenin in healthy skin was basically restricted
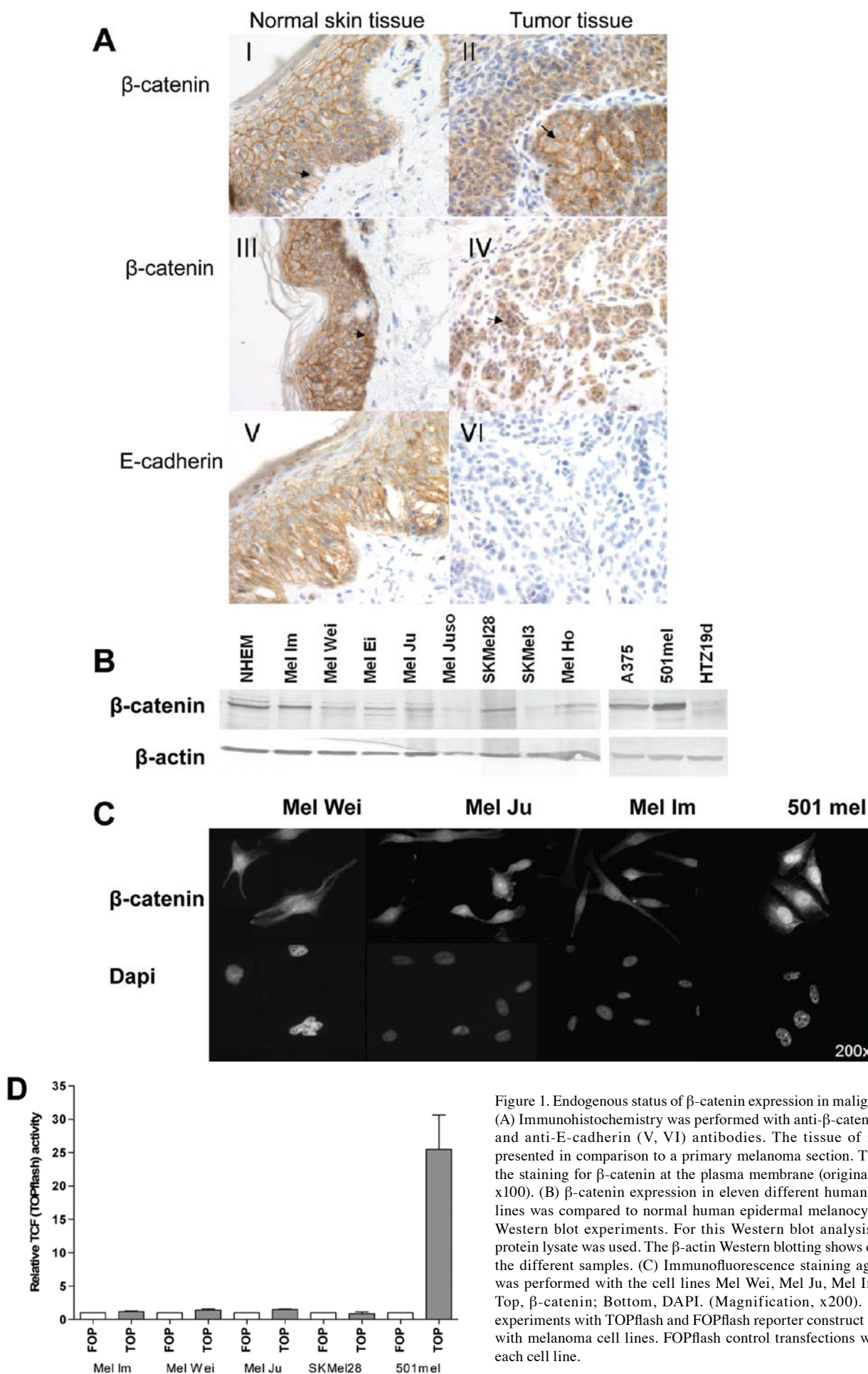
Figure 1. Endogenous status of β-catenin expression in malignant melanoma. (A) Immunohistochemistry was performed with anti-β-catenin (I, II, III, IV) and anti-E-cadherin (V, VI) antibodies. The tissue of normal skin is presented in comparison to a primary melanoma section. The arrow shows the staining for β-catenin at the plasma membrane (original magnification, x100). (B) β-catenin expression in eleven different human melanoma cell lines was compared to normal human epidermal melanocytes (NHEM) in Western blot experiments. For this Western blot analysis 10 μg overall protein lysate was used. The β-actin Western blotting shows equal loading of the different samples. (C) Immunofluorescence staining against β-catenin was performed with the cell lines Mel Wei, Mel Ju, Mel Im and 501 mel. Top, β-catenin; Bottom, DAPI. (Magnification, x200). (D) Luciferase experiments with TOPflash and FOPflash reporter construct were performed with melanoma cell lines. FOPflash control transfections were set as 1 for each cell line.

to the membrane of keratinocytes and melanocytes (I, III) where E-cadherin (V) is localized. The melanoma samples showed β-catenin immunostaining in the cytoplasm and at the membrane (II, IV) however, no obvious β-catenin expression was detectable in the nucleus of tumor cells. As already known, melanomas showed loss of E-cadherin expression (VI). Our findings were confirmed by images on the homepage of the human protein atlas (www.proteinatlas.org) where a small tissue microarray of 12 malignant melanoma tissues showed staining of β-catenin in the cytoplasm and at the membrane in 12 of 12 melanoma cases. Only 2 of the 12 melanoma tissues present additional nuclear staining.

The endogenous levels of β-catenin in eleven different melanoma cell lines (Mel Wei, Mel Ei, Mel Juso, A375 derived from primary melanomas; Mel Im, Mel Ju, SKMel28, SKMel3, HTZ19d, 501 mel and Mel Ho derived from mela-noma metastasis) were determined compared to normal human epidermal melanocytes (NHEM) in Western blot analysis. Here all cells, beside the cell line 501 mel, A375 and melanocytes, showed relatively low amounts of β-catenin (Fig. 1B).

The cell line 501 mel was described in detail in the publication of Rubinfeld *et al* (28) as a cell line with strong β-catenin protein expression leading to activated Wnt/β-catenin signaling. As 501 mel is described to exhibit transcriptional active β-catenin we chose this cell line as positive control for the canonical Wnt signaling pathway in the further analysis.

Next, the sub-cellular distribution of β-catenin was deter-mined by immunofluorescence staining. Anti-β-catenin staining of melanoma cell lines Mel Wei, Mel Ju and Mel Im revealed apparently similar amounts of β-catenin in nucleus and cytoplasm (Fig. 1C). The melanoma cell line 501 mel showed elevated β-catenin accumulation in the nucleus (Fig. 1C).

Next to protein localization the activity of the Wnt/β-catenin pathway is an important read-out. The transcriptional activity of β-catenin was analyzed using the TCF luciferase reporter constructs termed TOPflash and the mutant control FOPflash. The transfection efficiency was normalized by co-transfection of a renilla (pRLTK) construct. Only minor LEF/TCF activity was revealed in melanoma cell lines Mel Im, Mel Wei, Mel Ju and SKMel28 whereas strong activity was observed in the melanoma cell line 501 mel (Fig. 1D).

We revealed that in melanoma *in vivo* β-catenin is localized predominantly in the cytoplasm of the cells. The analyzed melanoma cell lines Mel Wei, Mel Ju and Mel Im displayed an equal distribution of β-catenin in cytoplasm and nucleus which is different to the melanoma cell line 501 mel which showed accumulated β-catenin protein levels in the nucleus. These findings, supported by the lack of transcriptional activity in several melanoma cell lines, were very surprising with respect to the current literature.

Analysis of transcriptional activity of β-catenin. Fig. 1 shows heterogeneity of the analyzed melanoma cell lines regarding β-catenin protein amount, cellular β-catenin locali-zation and transcriptional activity.

Next, stabilization and translocation of β-catenin into the nucleus was induced by treatment with lithium chloride (LiCl). LiCl is an inhibitor of GSK-3β activity and is widely used to mimic Wnt signaling. The Western blots in Fig. 2A show

relatively low levels of β-catenin in the untreated (control; lanes 1 and 3) melanoma cell lines Mel Im, Mel Wei and Mel Ju in cytoplasm and nucleus. Particularly, the analysis of the nuclear protein extract (control; lane 3) revealed low amounts of nuclear β-catenin in Mel Im, Mel Wei and Mel Ju. The positive control 501 mel displayed higher protein amounts of β-catenin in general (control; lanes 1 and 3) and enhanced accumulation of β-catenin in the nucleus (control; lane 3) in special. LiCl treatment (40 mM) led to a nuclear and cytoplasmic increase of β-catenin in all melanoma cell lines used in the study after 4 h incubation time, as shown by Western blot (LiCl; lanes 2 and 4; Fig. 2A). Additionally, we determined the nuclear translocation of β-catenin after LiCl treatment with immunofluorescence analysis in several melanoma cell lines (data not shown). These Western blots and immunofluo-rescence data indicated that the mechanism of nuclear import of β-catenin is functional in melanoma cells but remains low under physiological conditions.

To analyze primary and early effects of LiCl on the tran-scriptional activity of β-catenin we again used the TCF luciferase reporter. Although enhanced nuclear β-catenin was found after LiCl treatment for 4 h (40 mM), significant stimulation of the TOPflash reporter construct was not measurable in Mel Im, Mel Wei, Mel Ju and SKMel28 (Fig. 2B). As a control, the melanoma cell line 501 mel showed high transcriptional activa-tion of TCF activity in general which was significantly enhanced after stimulation with LiCl. Only a long-term incubation with LiCl for 24 h led to a slight induction of TCF luciferase activity in the cell lines Mel Im, Mel Wei, Mel Ju and SKMel28 (Fig. 2B). However, again the positive control 501 mel exhibits higher transcriptional activity of β-catenin compared to the other melanoma cell lines. Taken together, these results indicate that short-term stimulation of Wnt signaling did not result in significant activation of β-catenin transcriptional acti-vity in every melanoma cell line although a shift of β-catenin into the nucleus after treatment with LiCl was visible.

Phosphorylation of endogenous β-catenin. β-catenin signaling can be constitutively activated by truncation or mutation of serine and threonine residues in exon 3. As a result, an N-terminally hypophosphorylated form of β-catenin accumulates, translocates to the nucleus, and activates LEF/TCF transcriptional activity. Therefore, we became interested in the phosphorylation status of β-catenin in the melanoma cell lines.

Analysis with an antibody detecting hypophosphorylated β-catenin at amino acids (aa) 27-37 showed these aa, including the regulative important serine 33 and serine 37, to be hypophos-phorylated in melanoma cells whereas the use of an antibody detecting hypophosphorylated β-catenin at position aa35-50 revealed that this region, including threonine 41 and serine 45, is phosphorylated in several melanoma cell lines (Fig. 3A). The control cell lines SW480 and 501 mel showed the expres-sion of hypophosphorylated β-catenin at the specific aa. We confirmed the results by using antibodies detecting the specific phosphorylation of serine 45, threonine 41, serine 37 and serine 33. The cell lines 501 mel, SKMel28 and Mel Im were analyzed for their phosphorylation status of β-catenin in cytoplasmic extracts (Fig. 3B). Serine 33 and serine 37 were not phosphorylated in all cell lines compared to a positive
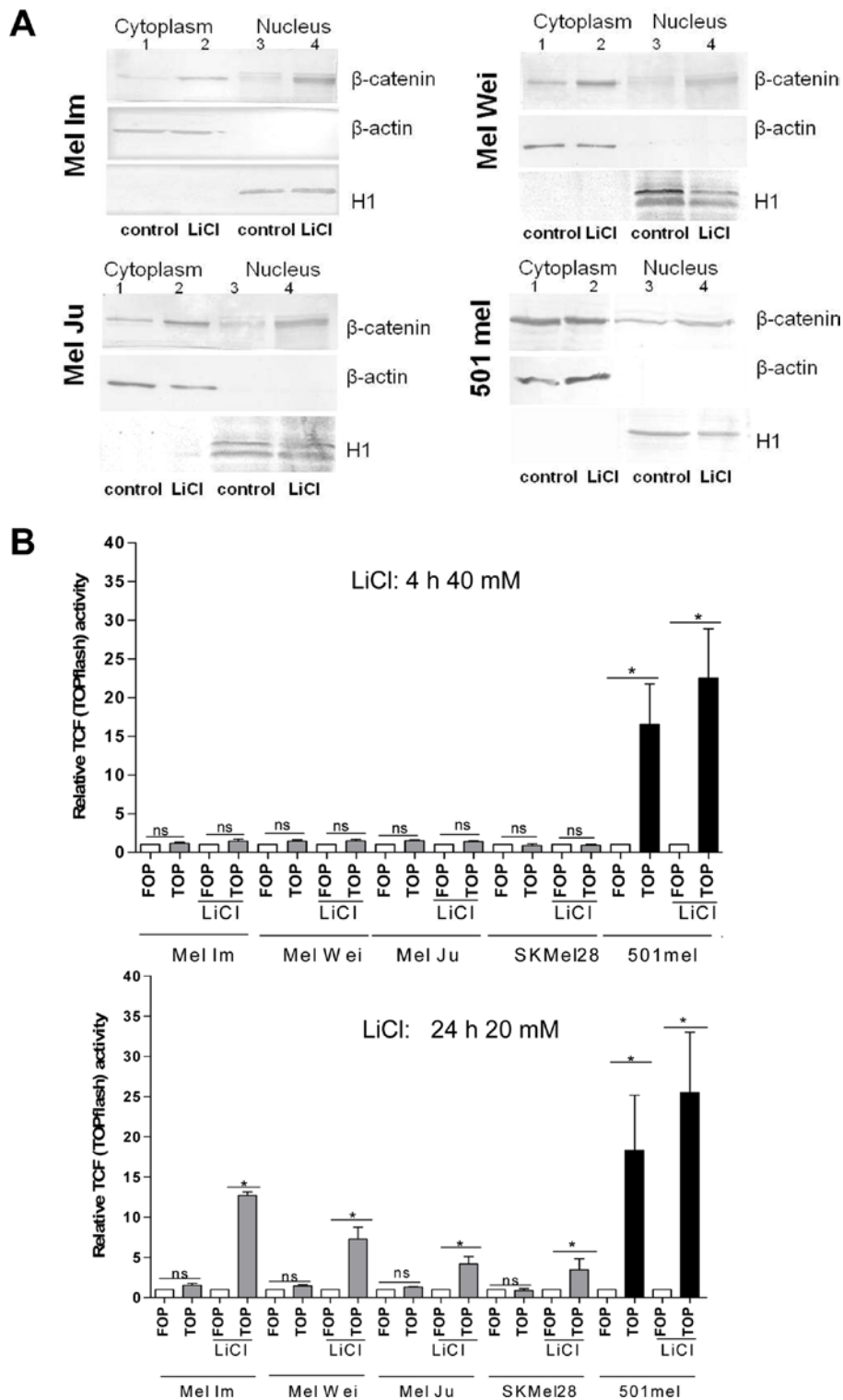
Figure 2. Localization of β-catenin in malignant melanoma. Analysis of transcriptional activation of β-catenin. (A) Cytoplasmic and nuclear protein extracts of Mel Im, Mel Wei, Mel Ju and 501 mel cells [either untreated or treated with lithium chloride (LiCl)] were analyzed by Western blots using an anti-β-catenin antibody. β-actin and H1 (histone H1) staining are loading and quality controls for the cytoplasmic and nuclear extracts. (B) Reporter gene analyses with TOPflash and FOPflash reporter construct were performed. FOPflash control transfections were set as 1 for each cell line. TOPflash activity was analyzed after incubation of the cell lines Mel Im, Mel Wei, Mel Ju, SKMel28 and 501 mel with lithium chloride (LiCl) for 4 and 24 h, respectively. ($^*p < 0.05$; ns, not significant).

control provided (breast carcinoma tissue, SW626 cells). However, threonine 41 and serine 45 were phosphorylated in SKMel 28 and Mel Im in comparison to the control 501 mel.

In conclusion, we revealed marked differences in phosphorylation of threonine 41 and serine 45 comparing β-catenin

in melanoma cell lines (Fig. 3C). We speculated that only β-catenin of 501 mel is hypophosphorylated and has LEF/TCF transcriptional activity.

It is suggested that hypophosphorylated β-catenin at Ser33, Ser37 and Thr41 is more transcriptionally active than forms
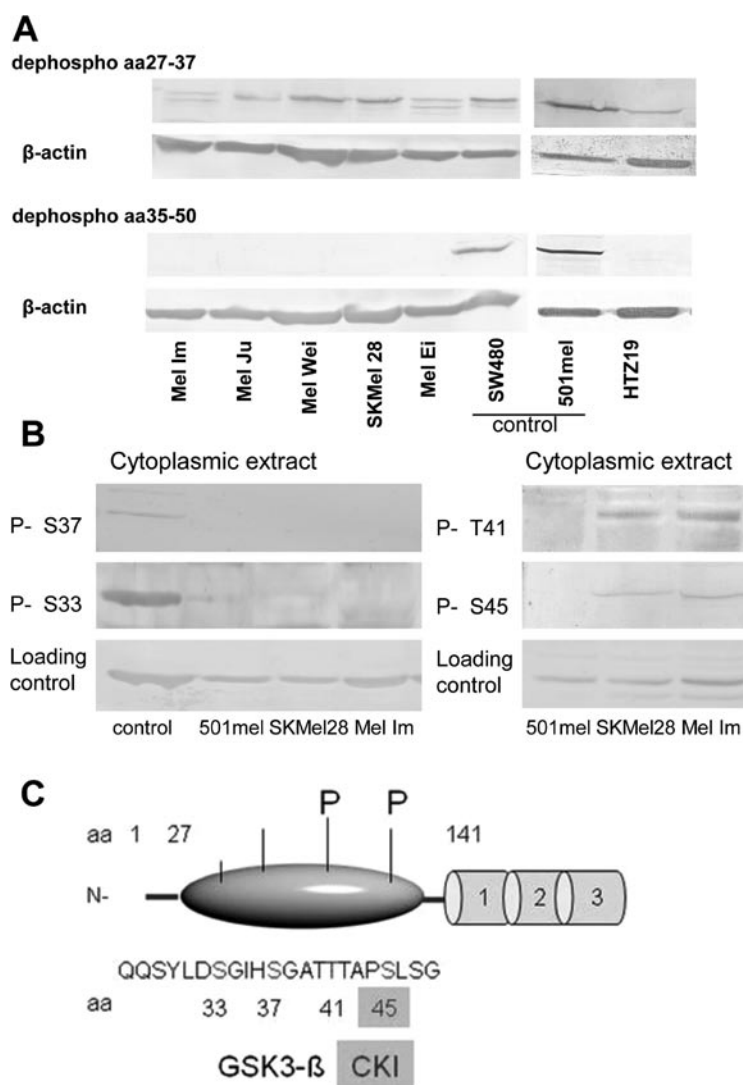
Figure 3. Analyses of the phosphorylation status of β-catenin. (A) Top, Western blot with anti-hypophosphorylated β-catenin antibody (amino acids 27-37). Bottom, Western blot with anti-hypophosphorylated β-catenin antibody (amino acids 35-50). Lysates of nine melanoma cell lines (Mel Im, Mel Ju, Mel Wei, SKMel28, Mel Ei, 501 mel, HTZ19d, Mel Juso and SKMel3) and the colon carcinoma cell line SW480 were analyzed. The positive controls for hypophosphorylated β-catenin were the cell lines 501 mel and SW480. Absence of signals in the melanoma cell lines suggests phosphorylation at the positions aa41 and aa45. (B) Western blots with anti-phosphorylated β-catenin antibody (amino acids serine 33, serine 37, threonine 41 and serine 45) were performed. Cytoplasmic extracts of three melanoma cell lines (Mel Im, SKMel28 and 501 mel) are shown. The positive control for phosphorylated β-catenin was protein lysate from the breast cancer cell line SW626 and the kidney cell line 293T. (C) Schematic overview of β-catenin N-terminal phosphorylation status of SKMel28 and Mel Im. P, phosphorylation; aa, amino acids.

phosphorylated at these sites. To assess the possibility that the defined phosphorylation pattern in melanoma is regulating the activity we used wild-type β-catenin, T41A and S45A β-catenin mutant constructs. A comparison of TOPflash activity after transfection of all constructs revealed lack of activity of wild-type and S45A β-catenin, interestingly, T41A resulted in an induction (Fig. 4A). The efficiency of transient transfection of these constructs was examined using quantitative real-time PCR (data not shown).

Having found transcriptional activity of the T41A mutant, we consequently examined mRNA expression of known target genes of β-catenin/LEF/TCF after transfection of wild-type β-catenin, T41A and S45A into Mel Im cells. As example, we observed induction in Brn-2 expression after expression of T41A in the Mel Im cell line (Fig. 4B). We performed further quantitative real-time PCR for the target genes MMP1,

MMP3, MMP9, BMP2 Osteopontin, MITF and Conductin (data not shown) and confirmed the result shown in Fig. 4B. This further supports that partial phosphorylation of endogeneous β-catenin is the reason for transcriptional inactivity of endogenous β-catenin in malignant melanoma cells. Western blot results showed the phosphorylation of threonine 41 and serine 45 of endogenous β-catenin in several analyzed melanoma cell lines.

Discussion

Melanoma cells prefer the β-catenin-independent non-canonical signaling pathway. The involvement of canonical Wnt signaling in melanocyte embryogenesis and development is of great importance. Wnt signals (mainly Wnt1 and Wnt3a) have been implicated in the induction, migration and differentiation
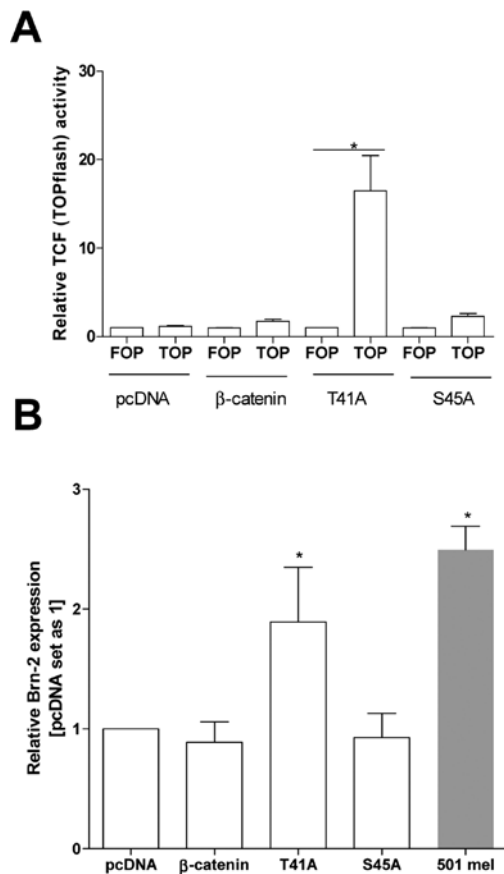
Figure 4. Influence of the mutant forms of β-catenin in malignant melanoma. (A) Luciferase experiments with TOPflash and FOPflash reporter construct were performed with the melanoma cell line Mel Im to analyze the influence of wild-type β-catenin, T41A and S45A mutant construct of β-catenin on LEF/TCF regulation. FOPflash results were set as 1 and the transfection efficiency was proofed with a renilla pRLTK construct. (*p<0.05; ns, not significant). (B) Quantitative real-time PCR of β-catenin target gene Brn-2 after transfection of wild-type β-catenin, T41A and S45A variants of β-catenin. The expression status is in comparison to the positive control 501 mel.

of melanocytes from the neural crest. Interestingly, these two Wnts were not detected during migration or invasion events of melanoma cells. More importantly, Chien et al (18) claimed that Wnt3a promotes the induction of markers for melanocytic differentiation in melanoma cells leading to decreased proliferation and upregulation of melanocytic genes (18). In accordance with these findings, changes of molecules regulated by the canonical β-catenin signaling are discussed controversially for malignant melanoma and mutations of molecules of the Wnt/β-catenin signaling pathway are rare. Mutations of APC (Adenomatous polyposi coli) were found in only 3-7% of melanomas (15,16,29) and of β-catenin (CTNNB1) in only 2-22% (14,15,28). In comparison, APC mutations in colorectal cancer were found with an 80% frequency.

To clarify the role of β-catenin signaling in malignant melanoma we performed detailed analyses. We could clearly demonstrate that β-catenin is mainly distributed in the cytoplasm of melanoma cells in vivo and under culture conditions. Studies of Maelandsmo et al and Pecina-Slaus et al support this finding (21,30). In only 5.6 or 6.4%, respectively, of the analyzed samples nuclear localization of β-catenin was observed by immunohistochemistry. Kielhorn et al have also shown

that 86% of totally 157 melanoma cases retained normal membranous β-catenin staining. Additionally, a publication of Kageshita et al showed that loss of β-catenin expression is associated with disease progression in malignant melanoma (20) supporting our finding that transcriptional activity of β-catenin is reduced in melanoma.

With further experiments we tried to elucidate β-catenin modification and β-catenin regulation in melanoma cell lines. β-catenin degradation is initiated upon amino-terminal serine/threonine phosphorylation. GSK-3β phosphorylates the amino acids serine 33, 37 and threonine at position 41 (31,32). The phosphorylation of the N-terminal serine at position 45 is accomplished by CKI. β-catenin phosphorylation at these four phospho-S/T residues is critical for β-catenin recognition by the F-box protein β-Trcp, which is the specificity component of a ubiquitination apparatus (33,34). The significance of S33, S37, T41 and S45 phosphorylation in degradation is underlined by the observation that mutations at these S/T residues frequently occur in human colorectal cancer and several other malignancies (29,35,36).

We analyzed the phosphorylation of these amino acids in several malignant melanoma cells in comparison to the melanoma cell line 501 mel (described as having an active Wnt/β-catenin pathway) and determined remarkable phosphorylation at the positions T41 and S45. The occurrence of two phosphorylated and two hypophosphorylated amino acids at the N-terminal region can lead to a non-degradable but also non-nuclear active β-catenin. That partial phosphorylation of β-catenin does not lead automatically to degradation of the protein was also shown by Kielhorn et al (37). The group found phospho-β-catenin in the nucleus of melanomas in vivo.

Surprisingly, the transfection of the T41A hypophosphorylated variant activates the transcriptional activity of the TOPflash promoter system and expression of classical β-catenin target genes. We assume that T41 phosphorylation in several melanoma cell lines, like SKMel28 and Mel Im, prevents transcriptional activity of β-catenin. To summarize, incomplete phosphorylation as found in melanoma protects β-catenin from degradation but the transcriptional activity is not completely attained.

In general, the discrepancy in β-catenin function when comparing melanoma cells to epithelial cell types can be due to the embryological origin of the cells. Nuclear function of β-catenin and activation of the canonical β-catenin pathway was predominantly described for epithelial cell types (38). As melanocytes are derived from the neural crest, cellular determinations can be clearly different. Classical cellular processes such as EMT (epithelial mesenchymal transition) are commonly described using epithelial cells. Certain changes such as upregulation of snail and down-regulation of E-cadherin can be similar in melanoma cells, however, other changes as shown for β-catenin can differ (38-40).

The question remains whether the cytoplasmic β-catenin is 'useless' for malignant melanoma cells or whether it entails an alternative signaling pathway. Our group has recently shown that the cytoplasmic β-catenin has alternative signaling functions beside transcriptional activation of LEF/TCF. We analyzed this alternative pathway because the overall results shown confirmed no classical nuclear function of β-catenin in malignant melanoma. We have previously shown that

β-catenin can activate the MAPkinase p38 and NF-κB in the cytoplasm of melanoma cell lines (22). This effect was inducible through the transfection of a β-catenin expression construct which increased the whole β-catenin protein amount in the cytoplasm of the cells (22).

However, further investigations are necessary to clarify the mechanisms of modification of β-catenin in malignant melanoma which on the one hand regulates the cytoplasmic signaling cascade of β-catenin and on the other hand averts the canonical signaling in malignant melanoma. In summary, we have demonstrated a new signaling function of β-catenin which could be regulated by phosphorylation.

Acknowledgements

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