Cellular Oncology 29 (2007) 373–386
IOS Press

Expression of integrin alpha10 is induced in malignant melanoma

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Abstract. Recently, integrin alpha10 was described as a collagen type II-binding integrin expressed mainly in chondrocytes. However, by array studies we detected integrin alpha10 also to be upregulated in malignant melanoma compared to primary melanocytes. Subsequent analysis of melanoma cell lines and melanoma tumor samples confirmed this finding. Further, we demonstrated that expression of integrin alpha10 is controlled by AP-2 and Ets-1, two transcription factors known to be involved in melanoma development and progression. To investigate the functional relevance of integrin alpha10, expression was downregulated via stable antisense transfection. Proliferation assays and colony forming assays revealed no differences comparing antisense integrin alpha10 cell clones with control and wild type melanoma cells, respectively. However, antisense integrin alpha10 cell clones and Mel Im cells treated with an inhibitory antibody against integrin alpha10 showed a reduced migratory potential.

In summary, these data indicate that AP-2 and Ets-1 regulated expression of integrin alpha10 plays a role in migration of malignant melanoma cells.

Keywords: Integrin, Ets-1, AP-2, malignant melanoma, melanocytes, cancer, migration

Abbreviations

AS	antisense → downregulated expression by stable transfection with an antisense expression construct
Mock	control transfected cell clone
NHEM	normal human epidermal melanocytes
S	sense → upregulated expression by stable transfection with a sense expression construct

1. Introduction

Malignant melanoma is a highly aggressive cancer derived from melanocytes mainly in the epidermis. Some details about processes involved in tumor development are known today but the molecular cause of the disease still remains unsolved. Recent data indicated that loss of cell-cell and cell-matrix contact and changes in the cell cytoskeletal organization play an important role in early development of the disease [17,39]. Additionally, proteins known to be involved in epithelial mesenchymal transition (EMT), such as E-cadherin, MMPs, etc., and several family members of the integrin family were found to be deregulated [8,12, 34,41]. Some integrins, such as integrin alphav beta3 are known to be strongly upregulated during melanoma development and progression which correlates with a more metastatic phenotype [30].

Integrins are heterodimeric transmembrane glycoproteins that interact with extracellular matrix (ECM) molecules as well as ligands on other cells. Integrins are composed of an alpha and a beta subunit which are non-covalently linked. Both subunits consist of a large extracellular domain, a short transmembrane domain and a cytoplasmic domain [13,23,43]. The N-terminal region binds divalent cations as Ca^{2+} and Mg^{2+} with a seven-fold repeated sequence which contributes to the cation-dependent ligand binding to the integrin [22]. The beta subunit has a cysteine rich segment near the transmembrane domain and its C-terminus is necessary for the association with the actin skeleton [23,43]. To

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date, the integrin family consists of 18 different alpha and 8 different beta subunits which can associate to form 24 different integrin receptors.

Integrins play an important role as specific cell surface receptors which arrange the contact of tumor cells with ECM proteins [14,26]. This contact to the environment enables tumor cells such as melanoma cells to convert from a stationary to a migratory and invasive phase. For this transition from radial growth phase (RGP) to vertical growth phase (VGP) the onset of alphav beta3 integrin expression is a specific marker [1, 9,10,20]. In addition to the alphav integrin chain also the beta1 integrin chain may affect the metastatic potential of melanoma cells [32]. Beta1 integrins have been shown to play a role in the lymphatic dissemination of cutaneous melanoma [18].

Recently, integrin alpha10 was characterized as a member of the beta1-integrin family. It binds to collagen type II and is related to the other collagen binding integrin subunits $\alpha 1, \alpha 2$ and $\alpha 11$ with highest identity to $\alpha 11$ [6,7]. The integrin $\alpha 10\beta 1$ is mainly expressed by chondrocytes in hyaline cartilage but is also found on cells in heart valves, in the perichondrium and in some myotendinous junctions [6,7]. Analysis of the alpha10 knockout mouse demonstrates that integrin alpha10 beta1 plays a specific role in growth plate morphogenesis and function [5]. Additionally, our group could show that the expression of integrin alpha10 in chondrocytes and a chondrosarcoma cell line is regulated by the transcription factors AP-2ε and Ets-1 [45].

The present study was performed to evaluate the role of integrin alpha10 in human malignant melanoma development and progression as preliminary data indicated expression in this kind of tumor. Therefore, we screened the transcriptional profiles of integrin alpha10 in human melanoma cell lines and melanoma tissue samples, and analyzed integrin alpha10 expression in primary melanomas and melanoma metastases by immunohistochemistry. In addition, functional assays using melanoma cell clones with abrogated integrin alpha10 expression were performed to characterize the biological effects of integrin alpha10 in the pathogenesis of melanoma.

2. Material and methods

2.1. Cells and cell culture

The melanoma cell lines Mel Im, Mel Ei, Mel Wei, Mel Ho, Mel Juso, Mel Ju, SK Mel 28, SK Mel 3, and HTZ19d were described previously [38]. The cell lines Mel Ei, Mel Wei, Mel Ho, and Mel Juso were derived from primary cutaneous melanomas; Mel Im, Mel Ju, SK Mel 28, SK Mel 3 and HTZ19d were derived from metastases of malignant melanomas. Cell culture was performed as described previously [38].

Isolation and culture of normal human epidermal melanocytes (NHEM) were performed as described [38].

Microarray-based gene expression analysis using Affimetrix arrays (human HG-U133A chips (Affymetrix, Inc., Santa Clara, USA)) was performed as described in the manufacturer's protocols. For the expression analysis, the following cells and cell lines, respectively, were applied: NHEM from 5 different donors, and 27 different melanoma cell lines derived from melanomas that have been previously described and characterized [19].

2.2. Isolation of tumorous and non-tumorous human tissues

Tissue samples from primary human melanoma and melanoma metastasis obtained from patients undergoing surgical treatment were immediately snap frozen and stored at -80°C. Melanoma cells were selectively retrieved from tumor samples with PALM microlaser technology (PALM, Bernried, Germany) under microscopic control. Informed consent was obtained from all patients and the study was approved by the local Ethics Committee.

2.3. Expression analysis

Isolation of total cellular RNA from cultured cells and tissues and reverse transcription were performed as described previously [16,38]. Quantitative real time-PCR analysis was performed with specific primers for integrin alpha10, collagen type II, integrin alpha3 and integrin alpha7b (*alpha10*-forward: 5′-CAT GAG GTT CAC CGC ATC ACT-3′ and *alpha10*-reverse: 5′-AAG GCA AAG GTC ACA GTC AAG G-3′; *collagenII*-for: 5′-TCA ACA ACC AGA TTG AGA GCA TCC GC-3′ and *collagenII*-rev: 5′-GAT TGG GGT AGACG CAA GTC TCG CC-3′; *ITGA3*-for: 5′-TGC TGT ATC CCA CGG AGA TCA CCG TC-3′, *ITGA3*-rev: 5′-AAT AGG GTA GCC CAG CCA TTT ACC CG-3′, *ITGA7B*-for: 5′-GCT GTG GCT GCC CTC CAT CCC TTC-3′ and *ITGA7B-rev*: 5′-CCC TCT AGG TTA AGG CAC TTC CGG G-3′) as described [27,38].

2.4. Western blotting

Protein extracts of cells were prepared as described [38]. Protein extracts from tissues were generated by homogenizing in RIPA-buffer (Roche, Mannheim, Germany) using the PRECELLYS® 24 lyser/homogenisator (bertin technologies, Sceaux, France). Insoluble fragments were removed by centrifugation at 13000 rpm for 10 min and the supernatant lysate was immediately shock frozen and stored at −80°C. Protein isolation for cytoplasm, membrane and nuclear fractions was performed using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, EMD, Biosciences, Darmstadt, Germany) as described by the manufacturer.

For western blotting 40 µg protein lysates were loaded and separated on 8.75% SDS-PAGE gels and subsequently blotted onto a PVDF membrane. After blocking for 1 hour with 2% BSA/TBST (0.05% Tween) the membrane was incubated for 16 h at 4°C with the primary antibodies (polyclonal anti-integrin alpha10 antibody ([6], 1:2000), anti-collagen type II (Chemicon, Temecula, USA; 1:500) and anti-beta-actin (Sigma, Deisenhofen, Germany, 1:2500)). Subsequently, the membrane was washed three times in TBST, incubated for 1 h with alkaline phosphate-coupled secondary antibody (Chemicon; 1:2000) and then washed again. Finally, immunoreactions were visualized by NBT/BCIP (Sigma) staining.

2.5. Immunohistochemistry

Cryosections of primary melanoma and metastases of malignant melanomas were screened for integrin alpha10 protein expression by immunohistochemistry. The tissues were fixed and subsequently incubated with primary polyclonal anti-integrin alpha10 antibody ([6], 1:120) over night at 4°C. The secondary antibody (biotin-labeled anti-rabbit, 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 haemalaun solution (DAKO). As negative control tissues were stained with rabbit serum and IgG antibody, respectively.

2.6. Plasmid constructs

To construct the integrin alpha10 promoter 5′-deletion constructs, the human genomic region was amplified by polymerase chain reaction (PCR) with a 3′-reverse primer (5′-GAC AAG CTT GCC TGA TCG GTT TCT GTC-3′) that bound at position -3 relative to the integrin alpha10 translational start site in conjunction with different 5′-forward primers that bound at varying distances within the upstream flanking sequence (-1139: 5′-GAC GCT AGC ACC TAG CTG AGG AGT TGG-3′, -960: 5′-GAC GCTA GCG GAG CTG TGT CTT CAC AAG-3′, -350: 5′-GAC GCT AGC GAA TCC ATC TCC CAC TCC-3′). To facilitate subcloning of the amplified fragments, the reverse primer contained a *Hind*III restriction site adaptor, and the forward primers contained a *Nhe*I site. The PCR fragments and the luciferase expression vector pGL3-basic were digested separately with *Hind*III and *Nhe*I before ligation. The nomenclature used for each deletion construct (-1139, -960 and -350, respectively) indicates the number of base pairs of the upstream 5′-flanking sequence with respect to the ATG translation start codon.

2.7. Transient transfection and luciferase assay

Transfection of expression plasmids applying the Lipofectamin plus method (Invitrogen, Carlsbad, USA) and reporter gene assays were performed as described [28,38].

2.8. Stable transfection of melanoma cells with antisense alpha10

A panel of Mel Im cell clones with reduced integrin alpha10 expression was established by stable transfection with an antisense expression plasmid (base -1 to -857 cloned in antisense orientation into pCMX-PL1). Plasmids were cotransfected with pcDNA3 (Invitrogen), containing the selectable marker for neomycin resistance. Controls received pcDNA3 alone. Transfections were performed using lipofectamin plus (Invitrogen). One day after transfection, cells were placed in selection medium containing 50 µg/ml G418 (Sigma). After 25 days of selection, individual G418-resistant colonies were subcloned.

2.9. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared as described [45]. One double-stranded oligomeric binding site for Ets-1 derived from the integrin alpha10 promoter (wt Ets-1: 5′-*CGCTCAGTGTTTTCCTTGGCTTGGG*-3′) was generated corresponding to the region -183 to -158 upstream of the ATG. The fragment was end-labeled and EMSAs were performed as described [45]. For the competition studies, cold oligonucleotides of the Ets-1 wt fragment and of a mutated Ets-1 fragment (mut Ets-1: 5′-GAT*CGCTCAGTGTTGATCATGGTCTGGG* GCTCCCC-3′) containing a mutated Ets-1 binding site were added at a 400-fold molar excess.

2.10. Migration and invasion assay

Migration and invasion assays were performed using Boyden Chambers containing polycarbonate filters with 8 μm pore size (Costar, Bodenheim, Germany), essentially as described [28]. Filters were coated with gelatine (for migration assays) or Matrigel (for invasion assays to imitate the basement membrane, diluted 1:3 in H_2O; Becton Dickinson, Heidelberg, Germany), respectively. The lower compartment was filled with fibroblast-conditioned medium, used as a chemoattractant. Mel Im cells and antisense integrin alpha10 cell clones were harvested by trypsinization for 2 min, resuspended in DMEM without FCS at a density of 20000 cells/ml for the migration assay and 200000 cells/ml for the invasion assay and placed in the upper compartment of the chamber. After incubation at 37°C for 4 h, the filters were collected and the cells adhering to the lower surface fixed, stained and counted.

Additionally, migration assays were performed using monoclonal inhibitory antibodies against integrin alpha10 (mAb365, Cartela, Lund, Sweden), and IgG control antibodies. Mel Im cells were incubated with 400 ng/ml of the antibodies and incubated for 10 min before adding the cells to the upper compartment of the Boyden Chamber. Each assay was repeated at least three times.

Furthermore, migration of cells was analyzed using scratch assays ("wound-healing-assays"). Cells were seeded in high density into 6-well plates and scratched with a pipette tip in a definite array. The size of the scratch was between 0.8 and 1.5 mm. Migration into the array was measured after 24 and 48 hours. Each analysis was performed at least in triplicate.

2.11. Anchorage independent growth assay

Cells were seeded into 6-well plates in DMEM, 0.36% agar (Sigma), supplemented with 10% FCS on top of a 0.72% agar bed in similar medium. The cultures were incubated for 14 days and the colonies were measured and photographed. Colony size was measured using a Carl Zeiss microscope (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). For each cell clone the diameter of at least 20 colonies was determined and statistically analyzed.

2.12. Proliferation assays

Cell proliferation was determined using the XTT assay (Roche, Mannheim, Germany) as described [38].

2.13. Statistical analysis

Results are expressed as mean ± SD (range) or percent. Comparison between groups was made using two-way ANOVA with Bonferroni correction. A p value <0.05 was considered statistically significant and marked with a star (*: $p < 0.05$, ns: not significant). All calculations were performed using the GraphPad Prism software (GraphPad software Inc, San Diego, USA).

3. Results

3.1. Induction of integrin alpha10 transcription during melanoma development

Initially, we aimed to analyze the expression of integrin alpha10 in malignant melanoma.

Using Affimetrix U133A, we investigated the expression of integrin alpha10 in 5 primary cultures of melanocytes, 12 cell lines derived from primary melanomas and 15 cell lines derived from melanoma metastases. In 4 of the 12 primary melanoma cell lines and 3 of the 15 metastatic cell lines we found high expression of integrin alpha10, whereas the remaining cell lines revealed expression levels similar as found in melanocytes (data not shown).

Based on this finding the expression of integrin alpha10 mRNA was analyzed in nine human melanoma cell lines and human primary melanocytes (NHEM) by quantitative PCR. Strong induction of expression was found in all melanoma cell lines compared to NHEM except of SK Mel 28 where only minor induction was seen (Fig. 1A).

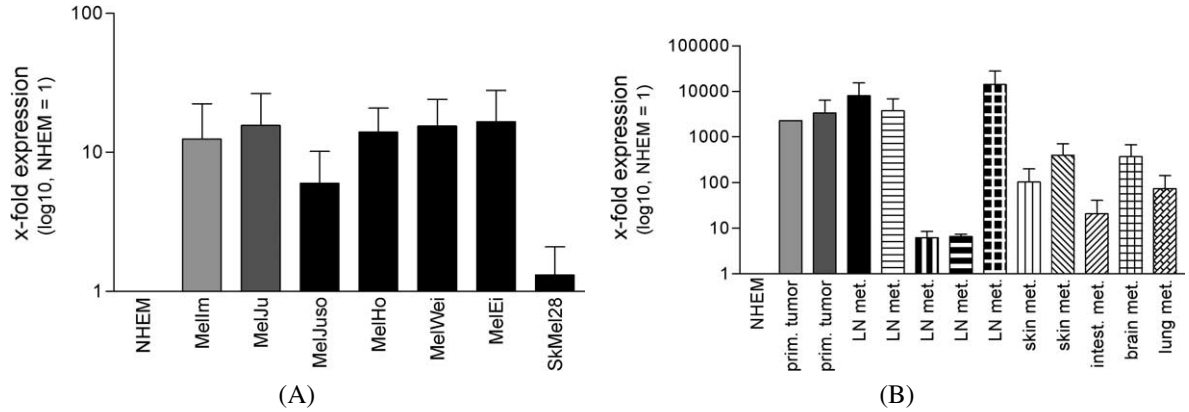


Fig. 1. Integrin alpha10 expression in melanoma cell lines compared to primary melanocytes. (A) Integrin alpha10 mRNA expression was quantified by real-time PCR. All melanoma cell lines showed strong induction of integrin alpha10 expression compared to normal human epidermal melanocytes (NHEM) except of SK Mel 28. (B) RNA was extracted from tissue samples of 2 primary melanomas (prim. tumor), 5 lymph node metastases (LN met.) and 5 distant metastases (skin met., intest. met., brain met., lung met.) by microdissection and analyzed by real-time PCR. Expression of integrin alpha10 mRNA was compared to normal human epidermal melanocytes (NHEM). Induction of expression of integrin alpha10 was found in all melanoma samples. (C) Integrin alpha10 protein expression in chondrocytes, NHEM and melanoma cell lines was analyzed by western blot analysis. In melanoma cell lines expression was induced compared to NHEM. As loading control the blot was counterstained with a ß-actin antibody. (D) Localization of integrin alpha10 expression was analyzed using cytoplasm, membrane and nuclear protein fractions isolated of Mel Im cells. Western blot analysis revealed membrane localization of integrin alpha10. (E) Immunostaining of integrin alpha10 revealed strong signals in primary melanoma compared to normal skin. (I) integrin alpha10 stained melanoma tissue (200× magnification; bv: blood vessel, mm: malignant melanoma), (II) normal skin (400× magnification), (III) negative control treated with rabbit serum (200× magnification), (IV) negative control treated with an isotype specific IgG antibody (200× magnification). (F) Integrin alpha10 protein expression in primary melanomas and melanoma metastases detected by western blot analysis. In melanoma tissues expression was induced compared to normal skin. As loading control the blot was counterstained with ß-actin antibody. (G) Analysis of collagen type II expression in melanoma cell lines and NHEM of two different donors by RT-PCR. In all melanoma cell lines examined collagen type II was detectable, whereas no expression was seen in NHEM.

Next we wanted to determine the time point of induction of integrin alpha10 expression during the course of melanoma development. RNA was isolated from 2 primary melanomas, 5 lymph node metastases and 5 distant metastases by microdissection and was screened for integrin alpha10 expression by quantitative RT-PCR. Induction of transcription of integrin alpha10 was observed in all primary melanomas, lymph node and distant metastases analyzed compared to expression levels in NHEM (Fig. 1B). Notably, *in vivo* expression of integrin alpha 10 in primary melanoma and melanoma metastases, respectively, appears higher than the expression observed in melanoma cells *in vitro*.

Furthermore, we analyzed integrin alpha10 protein expression in 7 different melanoma cell lines by western blotting using an anti-integrin alpha10 antibody (Fig. 1C). Consistent with the mRNA expression data, we found an increased integrin alpha10 expression in all but one melanoma cell lines as compared to NHEM. In addition, analysis of membrane, cytoplasmic and nuclear extracts revealed membrane localization of integrin alpha10 in melanoma cells (Fig. 1D).

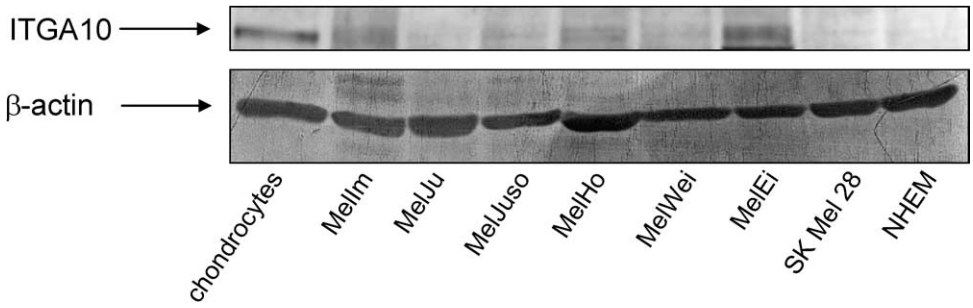
To examine integrin alpha10 protein expression *in vivo,* tissue samples of 10 patients with primary malignant melanomas were analyzed by immunohistochemistry. Representative sections are presented in Fig. 1E. In contrast to normal skin, melanoma cells showed a strong integrin alpha10 immunosignal.

Furthermore, protein extracts from primary melanomas and melanoma metastases, respectively, were analyzed by western blotting for integrin alpha10. In line with the mRNA expression data, strong expression of integrin alpha10 was observed (Fig. 1F).

As integrin alpha10 was shown to bind to collagen type II we additionally analyzed collagen type II expression in melanoma cells by RT-PCR. Notably, strong expression of its natural ligand was detected in all melanoma cells, whereas no expression was seen in NHEM of two different donors on mRNA (Fig. 1G) and protein level (data not shown).

3.2. Regulation of integrin alpha10 expression in malignant melanoma

To get insight into the transcriptional regulation of



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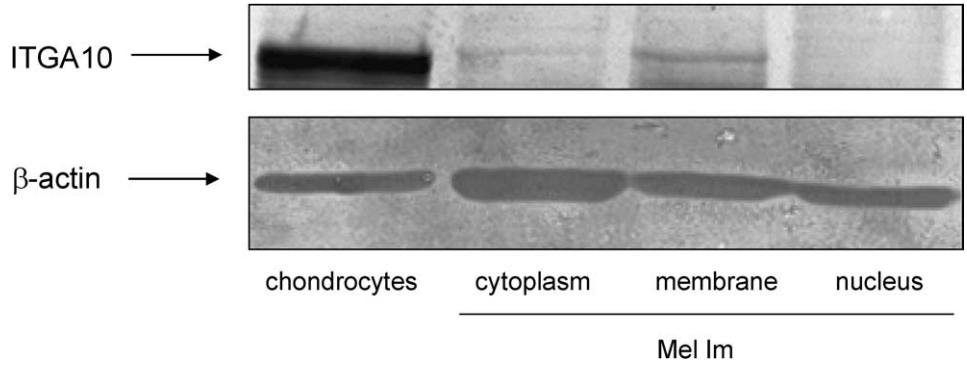

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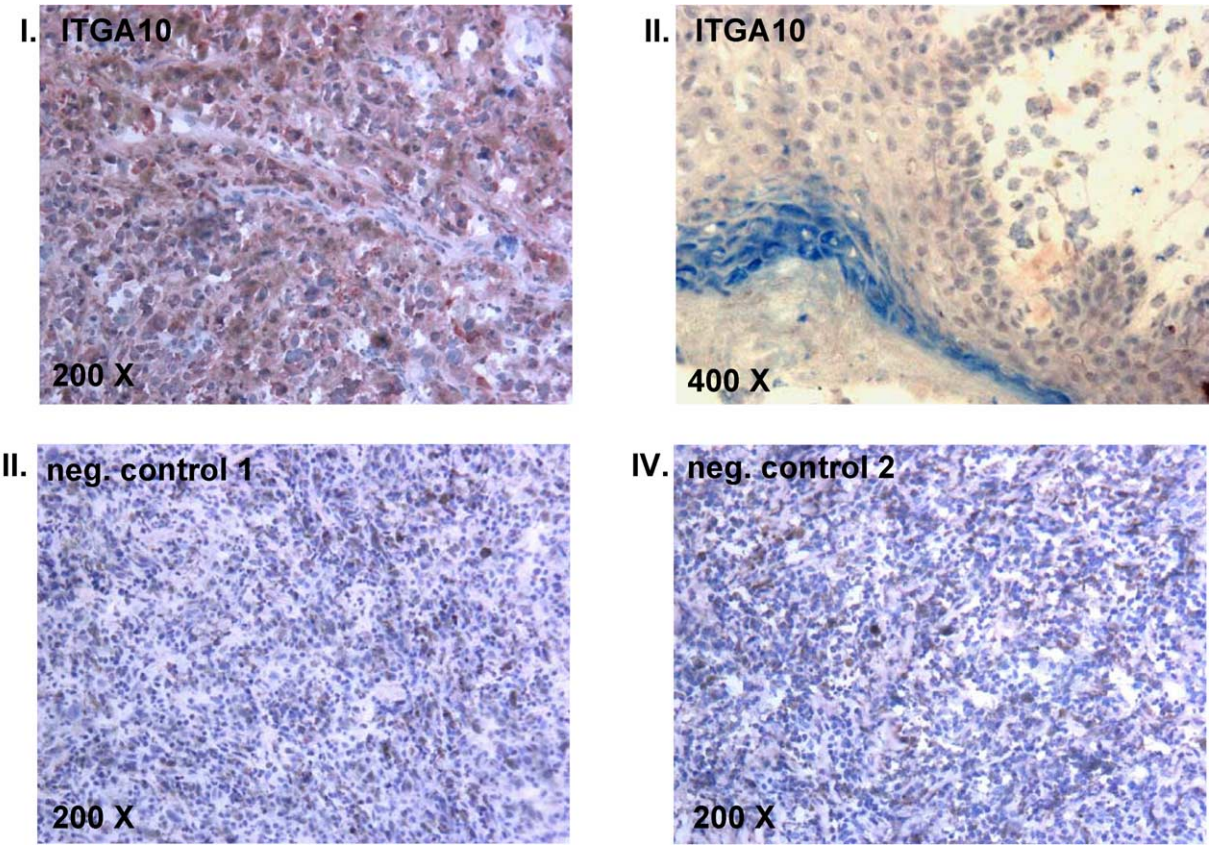

(E)

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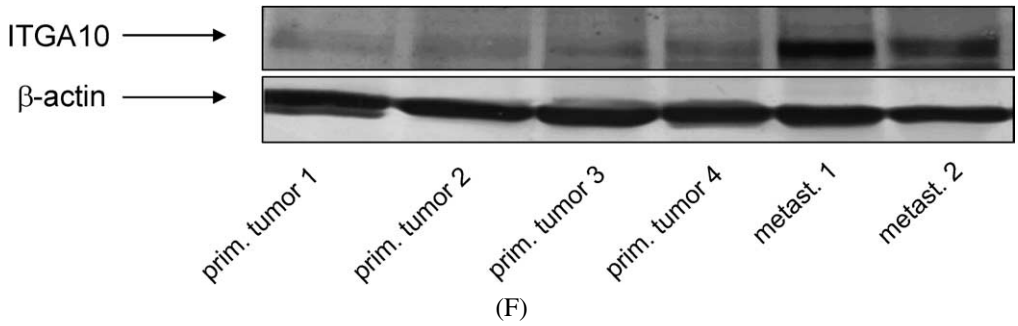

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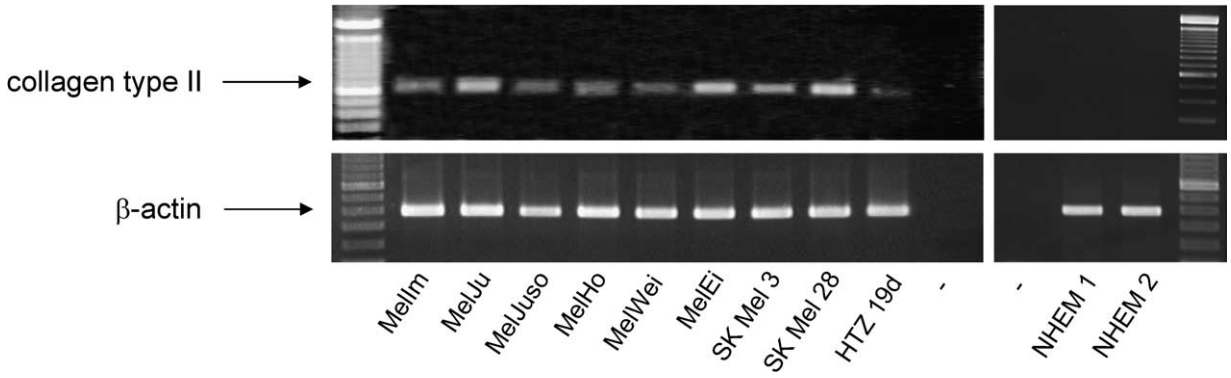


(G)

Fig. 1. (Continued).

integrin alpha10 in melanoma, we analyzed the effect of several transcription factors known to be involved in melanoma development and progression [35] on the expression of integrin alpha10. Comparing expression of melanoma cell clones with reduced snail expression [27], reduced NFkB activity [28] and induction of E-cadherin expression by transient transfection [28] did not show changes in integrin alpha10 expression (data not shown).

However, in melanoma cells transiently transfected with antisense Ets-1 expression plasmids [37] a strong downregulation of integrin alpha10 expression compared to NHEM was observed (Fig. 2A).

In contrast, expression of integrin alpha3 and integrin alpha7b was not affected in these cells indicating that this transcription factor specifically induces integrin alpha10 (Fig. 2B).

In line with these findings, we have already previously shown that the integrin alpha10 promoter sequence contains three Ets-1 consensus binding sites [45]. Additional, three recognition binding sites for AP-2 were found in the promoter sequence of integrin alpha10 [45].

3.3. Integrin alpha10 promoter is active in melanoma cells

To examine the integrin alpha10 gene promoter activation in melanoma cells in more detail, 1139 bp of the 5′-flanking sequence of the human integrin alpha10 gene were fused to a promoterless luciferase reporter plasmid pGL3-basic and tested for its ability to generate luciferase activity in transiently transfected cells. As expected, the construct revealed high luciferase activity in the melanoma cell line Mel Im (Fig. 3A). To identify the *cis*-acting elements mediating the human integrin alpha10 expression in melanoma, we transfected a series of nested deletion integrin alpha10 promoter-luciferase expression plasmids (-1139, -960, -350) into Mel Im cells, and subsequently, measured the luciferase activities. All three constructs generated much higher activity than the pGL3-basic luciferase vector suggesting that the integrin alpha10 promotor sequence -350 base pairs upstream of the transcriptional start site contains all regulatory elements necessary for activity in melanoma (data not shown).

To determine whether expression of Ets-1 affects the transcriptional activity of integrin alpha10 in melanoma cells, an Ets-1 expression plasmid was cotrans-

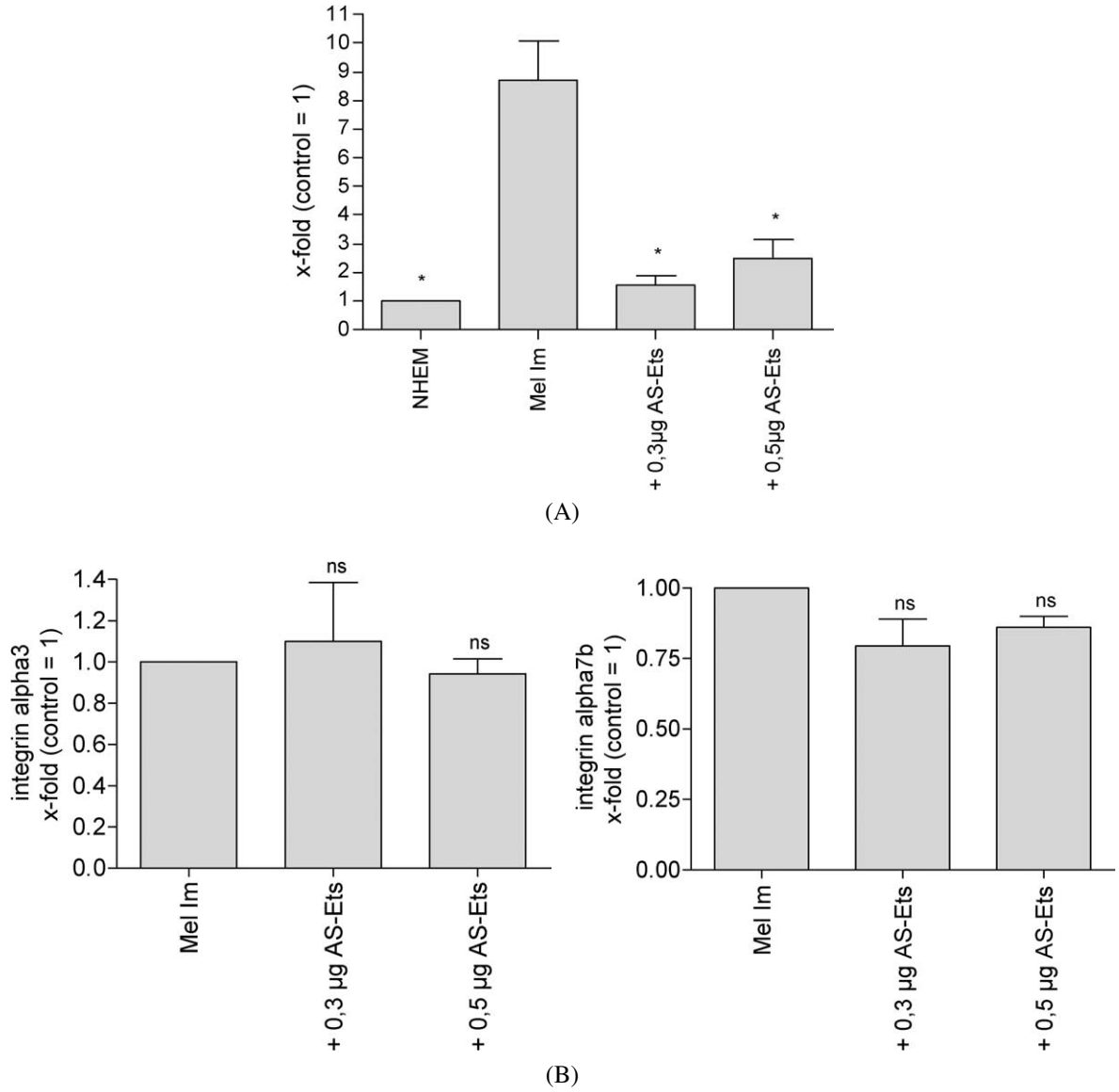
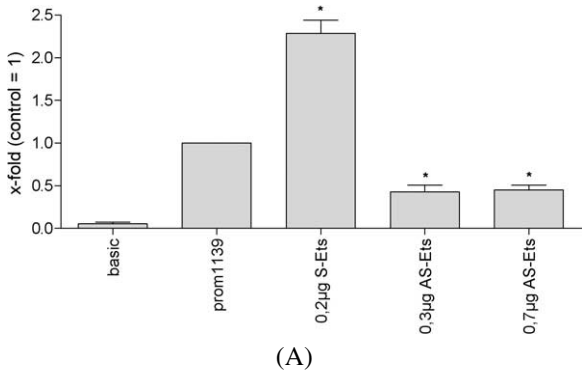


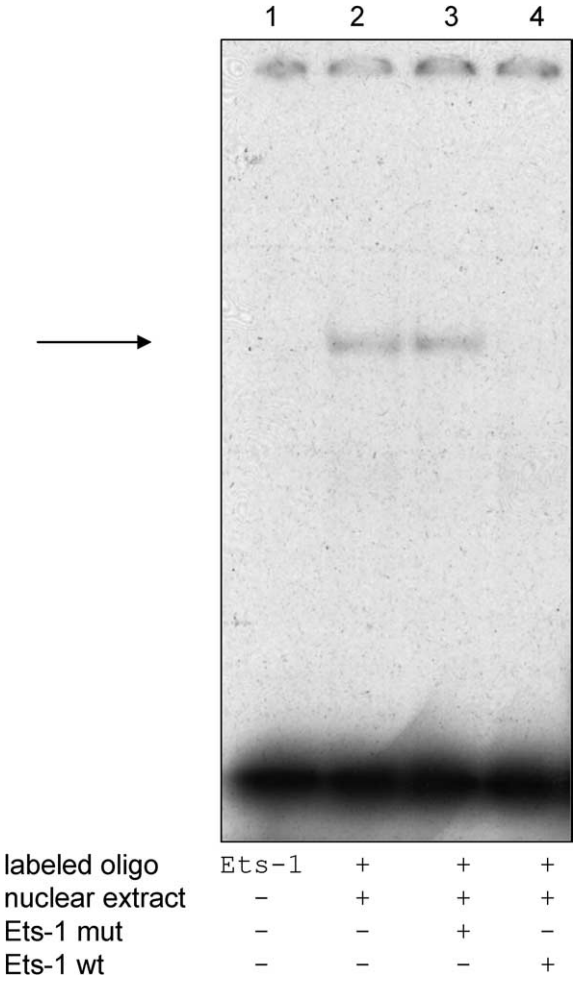
Fig. 2. Regulation of integrin alpha10 expression in antisense Ets-1 cell clones. (A) Integrin alpha10 mRNA expression in as Ets-1 transfected Mel Im cells was performed by quantitative RT-PCR. Clear downregulation of expression was found in melanoma cells with reduced Ets-1 expression (*: $p < 0.05$). (B) Expression of integrin alpha3 and integrin alpha7b mRNA in Mel Im cells transfected with as Ets-1. Expression is not regulated by Ets-1 (ns: not significant).

fected with the construct -1139. Cotransfection of Ets-1 strongly increased integrin alpha10 expression (Fig. 3A). The constructs -960 and -350 showed similar effects (data not shown). Additionally, cotransfection was performed with an antisense Ets-1 expression plasmid (AS-Ets). Here, luciferase activity of all three constructs was strongly reduced (Fig. 3A, shown for construct -1139). In comparison to the promoter construct alone, Ets-1 induced activity of the integrin alpha10 promoter up to 2.3-fold, whereas downregulation of Ets-1 led to an approximately 2-fold decrease of promoter activity.

To confirm that Ets-1 activates the expression through binding to the integrin alpha10 promoter electromobility shift assays were performed using nuclear extract from Mel Im cells and labeled oligonucleotides representing the binding site for Ets-1 (wt Ets-1) in the integrin alpha 10 promoter. A strong DNA-protein complex was observed that could be competed with unlabeled Ets-1 oligonucleotide (Fig. 3B, lane 2 and 4).



(A)



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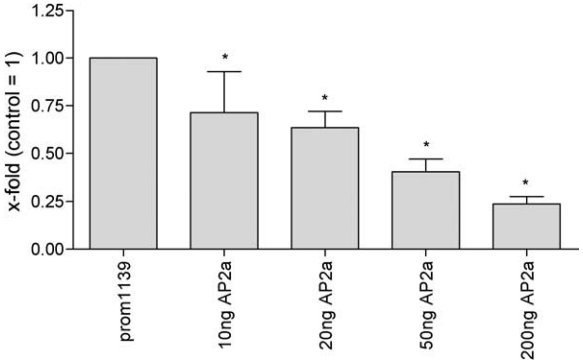


(C)

Fig. 3. Regulation of integrin alpha10 promoter activity in melanoma cells. (A) 1139 bp of the integrin alpha10 promoter region were subcloned into the pGL3-basic luciferase vector. The promoter construct -1139 was transiently transfected into melanoma cells in combination with expression plasmids for Ets-1 (S-Ets) or antisense plasmids for Ets-1 (AS-Ets), respectively. Promoter activity was analyzed, activity of pGL3-basic was used as a control. Reporter gene expression revealed promoter regulation by the transcriptional regulator Ets-1 (*: $p < 0.05$). (B) Gel mobility shift assay confirmed Ets-1 binding to the integrin alpha10 promoter. The Ets-1 binding was confirmed using oligonucleotides spanning the Ets-1 binding region (wt Ets-1) of the integrin alpha10 promoter and nuclear extract of Mel Im cells (lane 2). For competition experiments unlabeled wt Ets-1 (lane 4) and mut Ets-1 (lane 3) oligonucleotides were used. Lane 1 shows the labeled oligonucleotide without nuclear extract. (C) AP-2 downregulated integrin alpha10 promoter activity in a dose-dependent fashion (*: $p < 0.05$).

In contrast, an unlabeled fragment carrying a mutated Ets-1 binding site (mut Ets-1) did not influence the DNA-protein complex (lane 3).

Since integrin alpha10 promoter analysis has also revealed AP-2 binding sites, an AP-2 expression plasmid was co-transfected with the -1139 construct of the integrin alpha10 promoter and promoter activity was measured. Interestingly, AP-2 expression led to a dose-dependent reduction of luciferase activity of the -1139 construct (Fig. 3C). The same effects were observed when AP-2 was cotransfected with the two shorter promoter constructs (-960 and -350) (data not shown).

3.4. Functional relevance of integrin alpha10 expression

To analyze the functional role of integrin alpha10 in melanoma cells we downregulated the expression of integrin alpha10 in the melanoma cell line Mel Im by stable transfection with an integrin alpha10 antisense expression construct. Successful abrogation of integrin alpha10 expression in the cell clones was shown by quantitative RT-PCR (Fig. 4), whereas no changes of integrin alpha10 expression were seen in control transfected cell clones (mock 1 and 2). In accordance, on the protein level no expression of integrin alpha10 was detectable in the as-ITGA10 cell clones (data not shown).

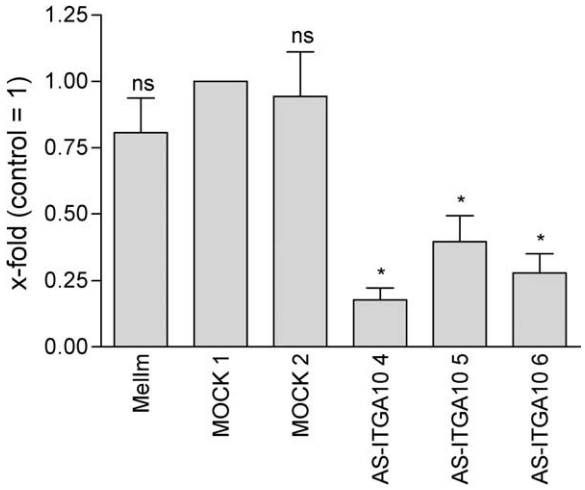


Fig. 4. Generation of melanoma cells with reduced expression of integrin alpha10. The cell line Mel Im was stably transfected with an antisense expression plasmid for integrin alpha10 and cell clones with reduced expression were generated by selection. By real-time PCR downregulation of expression of integrin alpha10 in the clones 4, 5 and 6 was confirmed whereas expression comparable to the wild type cell line Mel Im was found in the mock transfected cell clones mock 1 and 2 (*: $p < 0.05$, ns: not significant).

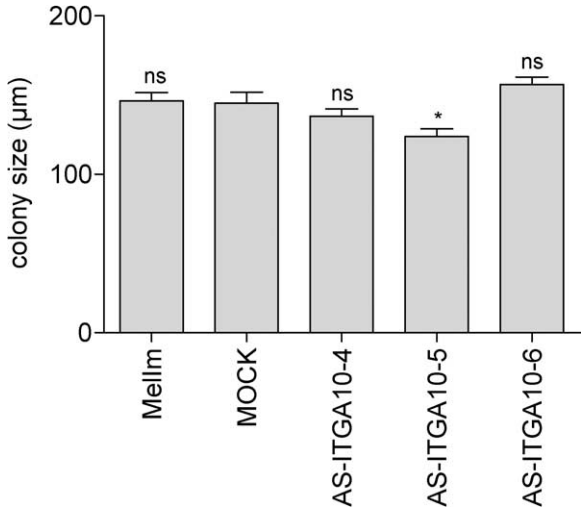


Fig. 5. Effect of reduction of integrin alpha10 on anchorage-independent growth. Changes in anchorage-independent growth of the cell clones expressing less integrin alpha10 were analyzed in comparison to non transfected Mel Im cells and the mock transfected clone 2. Only marginal changes were found after downregulation of expression of integrin alpha10 (*: $p < 0.05$, ns: not significant).

Moreover, attachment on collagen type II coated surfaces was reduced in the as-ITGA10 cell clones as compared to mock-transfected and control cells, further confirming the effective downregulation of integrin alpha10 expression in the as-ITGA10 cell clones (data not shown).

To get insight into the functional role of integrin alpha10 on the progression of melanoma, we performed several functional assays comparing the as-ITGA10 cell clones with mock-transfected and control cells, respectively.

Initially, we analyzed the proliferation of the antisense integrin alpha10 cell clones and found no differences to controls (data not shown).

Furthermore, we performed colony formation assays to evaluate the ability for anchorage independent growth. Here, only one clone (clone 5) revealed marginal differences compared to controls, while in the other two clones colony formation was not significantly affected (Fig. 5).

Interestingly, migration assays using gelatine coated filters in the Boyden Chamber system showed a significant reduction of the migratory potential of all three cell clones with less integrin alpha10 compared to the mock transfected cell clone (Fig. 6A).

To verify this finding, the assay was repeated with untransfected Mel Im cells in the presence of inhibitory antibodies against integrin alpha10 and unspecific control antibodies. In line with the findings seen in the as-integrin alpha10 clones, the inhibitory antibodies significantly blocked the migratory potential of Mel Im cells (Fig. 6B).

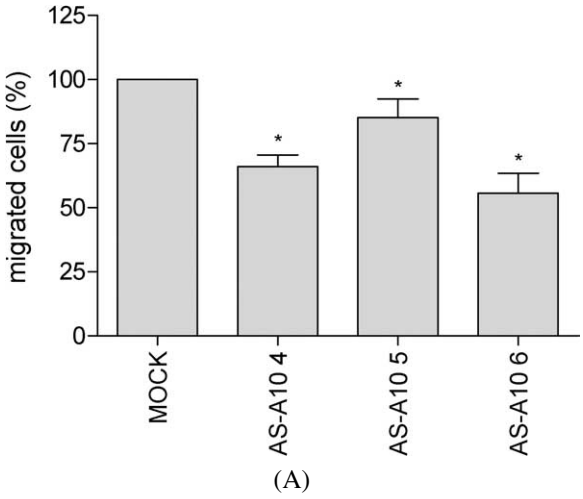
To further evaluate the effect of integrin alpha10 on undirected migration, wound healing assays (scratch assays) were performed. The as-integrin alpha10 cell clones showed no significant different effects in undirected migration compared to the control and to the Mel Im cells (data not shown).

Analysing the effect of integrin alpha10 on invasion, further assays with the Boyden Chamber system to test the invasiveness of cells through Matrigel, imitating the basement membrane, were performed. The as-integrin alpha10 cell clones showed no significant changes in invasive potential compared to the mock transfected cell clone and wild type Mel Im cells (Fig. 6C).

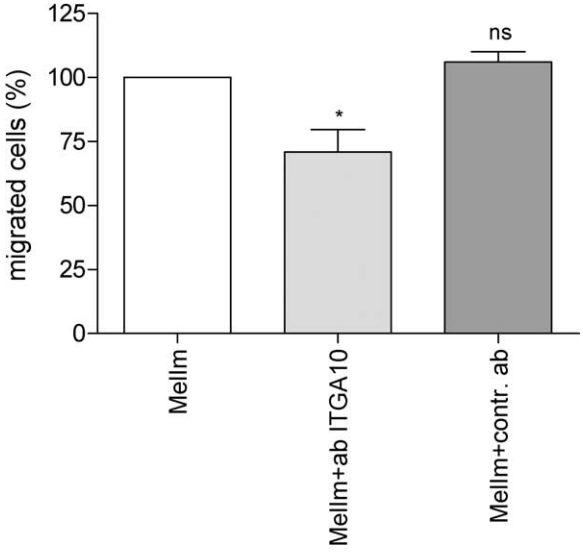
Taken together, integrin alpha10 expression does not effect cell proliferation or anchorage-independent growth, but decreases cell migration directed to a chemoattractant.

4. Discussion

In this study we investigated the transcription profile of integrin alpha10 in malignant melanoma. We initi-



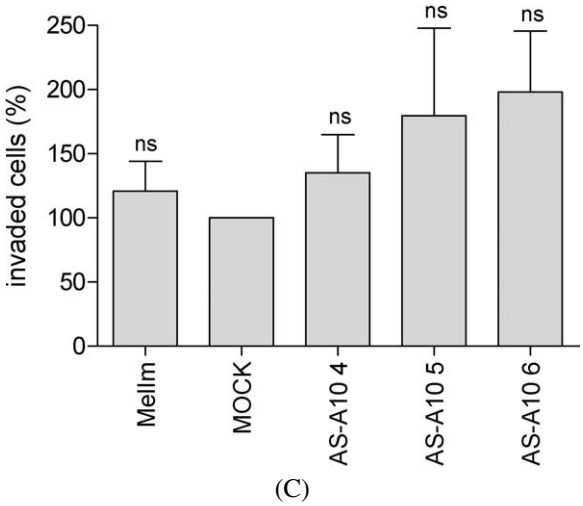
(A)



(B)

Fig. 6. Effect of reduction of integrin alpha10 on migration and invasion. Analysis of the cell clones expressing less integrin alpha10 in Boyden Chamber assays revealed a reduction of the migratory potential in the as cell clones in comparison to Mel Im cells and mock control (A). The same effect was shown using an inhibitory antibody against integrin alpha10 while control antibody did not affect the migratory potential (B). There were no significant changes of the invasive potential of the as integrin alpha10 cell clones compared to Mel Im cells and the mock control (C). Assays were performed in triplicate (*: $p < 0.05$, ns: not significant).

ated this study to explore the hypothesis that integrin alpha10 plays a role in the pathogenesis of melanoma since array data suggested an upregulation of integrin alpha10 expression. Specifically, we were interested to analyze whether integrin alpha10 transcription is altered during melanoma development and progression



(C)

Fig. 6. (Continued).

and whether loss of integrin alpha10 expression correlates with tumor characteristics.

We found that integrin alpha10 transcription was induced in all melanoma cell lines examined compared to normal human epidermal melanocytes. mRNA expression data were confirmed in all but two melanoma cell lines (Mel Ju and Mel Wei) on the protein level, potentially due to post-transcriptional regulation. Furthermore, high integrin alpha10 expression was found *in situ* in primary melanoma and melanoma metastasis. Interestingly, integrin alpha10 expression in melanoma *in situ* appears higher than in melanoma cell lines in culture. It may be speculated, that interaction with the microenvironment further enhances integrin alpha10 in melanoma cells *in vivo*.

Here, we found that expression of integrin alpha10 is controlled by the transcription factors AP-2 and Ets-1, both known to play an important role in the progression of melanoma.

The *ets-1* gene was first characterized as the cellular proto-oncogene of the retroviral *v-ets* oncogene of the avian leukaemia retrovirus E26 [29,44]. *Ets-1* is the prototype of the *ets* gene family of transcription factors. This family contains a growing number of transcriptional activators and inhibitors; their activity is regulated by phosphorylation and protein-protein interactions (for reviews see [11,35,40]). The members of the Ets family are characterized by a conserved Ets domain, which binds to double-stranded DNA containing a GGAA/T core sequence. Some *ets* genes are involved in chromosomal translocations leading to the production of chimeric fusion proteins that are associated with various leukemias and soft tissue cancers.

Several lines of evidence suggest that Ets-1 plays an important role in differentiation, proliferation, angiogenesis, apoptosis, and in tumor vascularization and invasion [15,37]. Several Ets-1 target genes as urokinase type of plasminogen activator (uPA), various matrix-metalloproteinases (MMPs), BMP4 and integrin ß3 are associated with an invasive phenotype. Ets-1 promotes angiogenesis by inducing the expression of integrin ß3 and of several proteases necessary for early steps of new blood vessel formation [33,46]. Consequently, Ets-1 inhibition by an antisense strategy or a dominant negative molecule was shown to inhibit angiogenesis [31,36,46]. Further, during embryogenesis Ets-1 mRNA expression is transiently induced in epithelial structures during the dispersion of somites into the mesenchymal sclerotome and during emigration of neural crest cells from which mature melanocytes derive [42]. Recently, our group suggested a role for Ets-1 in melanoma development [37]. In previous studies in chondrocytes we identified binding sites for Ets-1 in the integrin alpha10 promoter and could demonstrate a role for Ets-1 in the regulation of the integrin alpha10 expression [45]. In line with these findings we could show now in melanoma cells that Ets-1 binds to the integrin alpha10 promoter and positively regulates the integrin alpha10 expression in malignant melanoma. This Ets-1 regulation is specific for integrin alpha10 as integrin alpha3 and integrin alpha7b two integrins with increased expression in melanoma development [27] were not regulated by Ets-1.

The transcription factor activator protein-2 (AP-2) also has been shown to affect melanoma progression. Loss of AP-2 is associated with the transition of melanoma cells from radial growth phase to vertical growth phase [4]. Further, it has been shown that non-metastatic melanoma cell lines expressed high levels of AP-2, whereas highly metastatic melanoma cell lines did not express AP-2 [24]. These observations have been supported by several studies analyzing melanoma specimens obtained from patients [3,25]. Therefore, loss of AP-2 can be considered as an important molecular event in melanoma progression, which results in deregulation of AP-2 target genes involved in tumor growth and metastasis. For example, loss of AP-2 expression in metastatic melanoma cells resulted in overexpression of the melanoma cell adhesion molecule (MCAM/MUC18) [24] and BMP4 [38], and in downregulation of c-KIT, a tyrosine kinase receptor [21].

With regards to integrin alpha10 expression we could demonstrate the dual functional role of AP-2 both as a transcriptional activator and as a repressor of gene transcription. On the one hand, we could demonstrate in a previous study that AP-2 increased integrin alpha10 expression in chondrocytes [45]. On the other hand, in this study we found that AP-2 suppressed integrin alpha10 in melanoma cells. Potentially, different AP-2 binding co-factors are responsible for the different AP-2 effects observed in malignant melanoma cells and chondrocytes.

In addition to the transcriptional regulation, we studied the functional role of integrin alpha10 in melanoma cells. Functional assays revealed that integrin alpha10 expression does not seem to affect cell proliferation, anchorage independent growth and the invasive potential of malignant melanoma cells. The lack of a distinct effect on these functional parameters could be explained by a redundancy of integrin expression. A similar redundant integrin expression was described concerning collagen-binding beta1 integrins expressed on chondrocytes [5]. Alpha10 integrin-deficient growth plates in mice showed less severe abnormalities compared to the beta1-null growth plates. In the alpha10 integrin-deficient animals this effect is speculated to be due to redundancy. As the primary candidate for such a redundant collagen-binding integrin alpha1 beta1 integrin is predicted [5]. Possibly, the expression of integrin alpha10 in malignant melanoma is redundant equivalent to the findings in chondrocytes.

However and interestingly, we found that integrin alpha10 affects directional migration of melanoma cells. Repression of integrin alpha10 expression or application of an inhibitory antibody against integrin alpha10 inhibited the migration of melanoma cells *in vitro*. It has been shown previously that integrin alphav beta3 promotes chemotactic mobility in human melanoma [2]. In that study blocking of alphav beta3 with an antibody leads to an abolished chemotaxis. Possibly, integrin alpha10 has a similar function as alphav beta3 and interacts with ligands that influence directed migration. Active movement of cancer cells is considered to be essential for cellular distribution of malignant melanoma cells. We found upregulation of integrin alpha10 expression to be an early event in tumor development already seen in primary tumors. We speculate that upregulation of integrin alpha 10 *in vivo* supports local dissemination of the tumor e.g. in the dermis. Thus, upregulation of integrin alpha10 in malignant melanoma may contribute to development and progression of the tumor.

In summary, the development of malignant melanoma is associated with deregulated cell-matrix contacts to the surrounding microenvironment as an early event

in tumor development. Taken together with the results presented in this study integrin alpha10 is one molecule of a pattern of proteins activated during human cancer progression leading to the migratory phenotype of tumor cells. Further investigations for example using a mouse model are warranted to understand the underlying mechanisms induced by activation of integrin alpha10 expression.

Acknowledgement

We are indebted to Dr. J. Johnson (University of Munich, Germany) for providing melanoma cell lines and to Sibylla Lodermeier and Susanne Wallner for excellent technical assistance. This work was supported by grants from the DFG and the Deutsche Krebshilfe to A.B.

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