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INVESTIGATIVE REPORT

Differential Expression of Thrombospondin 2 in Primary and Metastatic Malignant Melanoma

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In the present report we used oligonucleotide microarray analysis for the identification of genes characterizing the late-stage metastatic phenotype of malignant melanoma. A panel of 5,600 genes was analysed in a low aggressive and a highly aggressive (metastatic) human malignant melanoma cell line, respectively. More than 300 differentially regulated genes were identified. High metastatic potential correlated with upregulated mRNA expression in the groups of cytoskeletal proteins, apoptosis and cell cycle proteins, GTP binding proteins and oncogenes, extracellular ligands and receptors, transcription and translation factors. In contrast, most angiogenesis factors, extracellular matrix molecules, and melanoma-specific antigens were downregulated. Particular target genes were further analysed by *in situ* hybridization and immunohistochemical staining of primary malignant melanomas and melanoma metastases. Here, we show that thrombospondin 2, an extracellular matrix molecule which was differentially regulated in the microarray analysis, was strongly expressed in melanoma metastases, but not in primary tumours. The identification of thrombospondin 2 as a target molecule emphasizes the importance of cell-matrix interactions for the pathogenesis of malignant melanoma metastasis and may open future perspectives for treatment of this tumour. *Key words: immunohistochemistry; in situ hybridization; metastasis; microarray analysis.*

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Malignant melanoma is a highly aggressive tumour with dramatically increasing incidence and poor prognosis (1). At late-stage, metastatic tumour growth melanomas are often treatment resistant. Little is known about the pathogenic mechanisms leading to this late-stage metastatic tumour spread. There is a certain body of evidence derived from a series of different tumours including malignant melanoma that tumour angiogenesis plays a crucial role in tumour progression (2). Since malignant melanoma is a highly immunogenic tumour, extensive work has been undertaken in analysing immunological mechanisms, finally leading to new treatment protocols. However, the overall prognosis of melanoma patients could not be improved by all available treatment modalities up until now (3). Clinical approaches in the search for unique phenotypical tumour markers for advanced-stage metastatic melanoma, although initially promising, have failed so far (4).

The recent advances in gene chip technology provide new tools for the analysis of complex biological systems such as late-stage tumour growth, metastasis and tumour maintenance (5). Gene expression profiles have been published for a variety of different malignant tumours. Among these are rhabdomyosarcoma, renal cell carcinoma and glioblastoma (6–8). These investigations have led to the identification of cell line-specific genes and genes correlating with tumour aggressiveness. For example, high expression of cell cycle-dependent kinase (CDK)-4 has been shown to be associated with the malignant phenotype of rhabdomyosarcoma (6). Aggressive tumour growth in renal cell carcinoma has been associated with high expression of the intermediate filament, vimentin (7). In a recently published work on malignant melanoma using a high-density microarray of 1,161 genes, 78 were found to be differentially expressed (9). Among these, the chemokine, monocyte chemoattractant protein (MCP)-1, which might play a role in local immunesurveillance of malignant melanoma, has been found to be more than tenfold downregulated in the aggressive melanoma phenotype. In more recent attempts on molecular phenotyping of primary melanomas and melanoma cell lines, an invasive phenotype and candidates of tumour suppressor genes could be defined based on microarray analysis (10, 11).

In the present report, an oligonucleotide microarray technique was used to analyse two malignant melanoma cell lines of different degrees of aggressiveness; one displaying only local growth after injection into nude mice, the other showing metastatic behaviour (12). Our microarray data were confirmed by Northern blot analysis for a series of differentially expressed genes. Highly aggressive melanoma cells showed a characteristic pattern of upregulated mRNA expression for cytoskeletal proteins, apoptosis and cell cycle proteins, GTP binding proteins and oncogenes, extracellular ligands and receptors, and transcription and translation factors.

One target molecule, thrombospondin 2, was identified as showing high expression in metastases and absence of low expression in primary melanomas. Taken together, the present report provides a microarray-based characterization of the aggressive phenotype in malignant melanoma and reveals thrombospondin 2 as a target molecule which might open perspectives for the treatment of late-stage metastatic melanoma.

MATERIAL AND METHODS

Cell lines and culture conditions

The two melanoma cell lines 1F6 and Mel57 were kindly provided by G. N. P. van Muijen, Department of Pathology, University of Nijmegen, Nijmegen, The Netherlands. 1F6 represents a non-metastatic human melanoma cell line, Mel57 a metastatic cell line (12, 13). The metastatic capacity has been tested in nude mice. Cell lines were maintained in RPMI 1,640 medium (Linaris, Bettingen, Germany), supplemented with 10% foetal calf serum (FCS, Linaris), 2mM L-glutamine, 100 U/ml penicillin/streptomycin and 1% non-essential amino acids.

Gene chip hybridization

Total RNA was isolated from 1F6 and Mel57 cells using the total RNeasy kit (Qiagen, Hilden, Germany). RNA concentrations were determined spectrophotometrically at 260 nm and RNA probes were labelled according to the supplier's instructions (Affymetrix, Santa Clara, CA, USA). Analysis of gene expression was carried out using the HuGeneFL Array (Affymetrix) with a capacity of 5,600 human genes. Hybridization and washing of gene chips were carried out according to the supplier's instructions. A laser scanner (Hewlett-Packard Gene Scanner™) was used for analysis of the microarray. Expression levels were calculated with commercially available software provided by Affymetrix. Data are given as fold increase of gene expression in the metastatic cell line (Mel57) compared with the gene expression in the non-metastatic cell line (1F6). A cut-off of fourfold increased/decreased expression was chosen, as has been described elsewhere (14).

Generation and cloning of cDNA probes

For Northern hybridization cDNA probes of 8 differentially expressed genes were generated by RT-PCR. For this purpose, total RNA was extracted from 1F6 and Mel57 cell lines, respectively, and mixed for probe generation. One microgram total RNA were reverse transcribed by superscript reverse transcriptase (Gibco BRL Life Technologies, Eggenstein, Germany) using hexamer priming. Specific primers (purchased from ARK Scientific Biosystems, Darmstadt, Germany) generating cDNA probes of 900–1,400 bp length were designed according to the published sequences. For PCR amplification, the following conditions were used: an initial 5-min denaturation step was followed by 10 cycles of 30 s denaturation at 94°C, 30 s annealing at 56°C, 1 min primer extension at 72°C. After that, 20 cycles of 30 s denaturation at 95°C, 30 s annealing at 60°C, and 1 min primer extension at 72°C were carried out. A terminal primer extension step of 10 min at 72°C was added. The following primers (5′ end; 3′ end) were used (gene bank accession number and generated fragment length are given in parentheses): thrombospondin 2 (L12350; 1,484 bp); 5′ CAGACCTCAAGTACGAATGC 3′; 5′GGATGAGT-

GACTATTTCCTGC 3′; 65 KD phosphoprotein (J02923; 1,252 bp); 5′ GCATCGGTGGACAAGATCTC 3′; 5′ AAGG-CTGAAGGCTCTAGAGG 3′; creatine kinase (M16364; 1,161 bp): 5′ GACGACGTCATCCAGACAG 3′; 5′ CAGC-ATCAGCAGTATCTTAGC 3′; desmoglein 2 (Z26317; 1,096 bp): 5′ TACCAGTGTGCTGCTGCAAC 3′; 5′ CTGC-CTAGAAGACACAGATC 3′; IL-11 receptor (U32324; 1,117 bp): 5′ GTCCGTGAAGCTGTTGTC 3′; 5′ GTCC-ACTGGAATCACTGAGG 3′; transforming growth factor α (X70340; 998 bp): 5′ ATGCCTCAGGATAGAACAGC 3′; 5′ TCTGTGTGAACCACTAGATC 3′; melanoma associated antigen (MAGE) 4a Antigen (U10687; 1,127 bp): 5′ CTTCACTTGCTGGAGGCAAC 3′; 5′ TTCACGGTGAAT-TCACACTTC 3′; membranetype matrix metallo-proteinase 2 (Z48482; 1,336 bp): 5′ CCACGTGTCCTGCTTTACTG 3′; 5′ GAGGCCAGCAGAATCCTTCC 3′. PCR products were separated by agarose gel electrophoresis, cut out, purified using the QIAExII gel extraction kit (Qiagen), and cloned into the pGEM-T vector (Promega, Heidelberg, Germany). All cDNA probes were sequenced using an automated capillary sequencer (Perkin Elmer, Weiterstadt, Germany). Appropriate cDNA probes were cut out from the pGEM-T vector by restriction enzyme digest and radioactively labelled for Northern hybridization.

Northern blot analysis

Northern hybridization was carried out as recently described (15). In short, the purified cDNA fragments were labelled to high specific activity with [^{32}P] dATP using the "ready prime" labelling kit (Amersham Pharmacia, Braunschweig, Germany). cDNA probes of the following genes were used for Northern hybridization: desmoglein 2, thrombospondin 2, IL-11 receptor, creatine kinase, 65 kD phosphoprotein, transforming growth factor (TGF)-α, membrane-type matrix metalloproteinase (MT-MMP)-2, and MAGE 4a. Hybridization was done in the prehybridization solution containing 5×10^6 cpm/ml of the labelled probe. After hybridization for 16 h at 65°C and 6 h at 60°C, membranes were washed twice with $2 \times$ SSPE/0.1% sodium dodecyl sulphate (SDS) at room temperature, once with $1 \times$ SSPE/0.1% SDS at 60°C and once again with $0.2 \times$ SSPE/0.1% SDS at room temperature. Membranes were exposed to Hyperfilm™ (Amersham Pharmacia) with intensifying screens at -80°C for 3 days. mRNA loading differences were corrected by the amount of GAPDH mRNA.

In situ hybridization

Formalin-fixed sections (5 μm thick) of 6 primary melanomas (Clark Level II–IV) and 6 cutaneous melanoma metastases from different patients were analysed for mRNA expression *in situ*. cDNA probes cloned into pGEM-T vector (see above) of the following genes were used: desmoglein 2, thrombospondin 2, and creatine kinase. All three tested genes displayed a highly differential expression pattern in the microarray analysis. *In vitro* transcriptions of sense and antisense probes and hybridization were performed, as described elsewhere (15). The non-hybridized RNA probe was removed by incubation with high stringent washing solutions (50% formamide, $2 \times$ saline-sodium citrate, and 5 mM EDTA at 54–57°C) under constant stirring. For autoradiography, slides were dipped in NTB-2 Kodak solution (1:2 in 800 mM ammonium acetate) and exposed for 5 weeks at 4°C.

Immunohistochemistry

Formalin-fixed paraffin-embedded sections (5 μm thick) of 20 primary melanomas (Clark Levels II–IV) and 20 cutaneous melanoma metastases, all from different patients, were analysed by immunohistochemistry using a moAb against desmoglein 2 (Progen, Heidelberg, Germany). Cryostat sections of 5 primary melanomas and 5 cutaneous melanoma metastases were analysed using a moAb against thrombospondin 2 (Chemicon, Temecula, CA, USA). Sections were stained using the APAAP method according to the manufacturer's specifications (Dako, Hamburg, Germany). Neofuchsin was used as a chromogene. Counterstaining was performed with haematoxylin.

RESULTS

RNA profiling of melanoma cell lines 1F6 and Mel57

The HuGeneFL Array (Affymetrix) was used to analyse the two melanoma cell lines Mel57 and 1F6 representing different stages of tumour aggressiveness. Gene expression was compared in both cell lines, using the gene expression in the low aggressive cell line 1F6 as baseline. Genes were grouped into different categories according to their major functions: angiogenesis/cytokines, actin/tubulin/myosin/migration, apoptosis/cell cycle, coagulation, GTP binding/tumour suppressor genes/protooncogenes, immunoglobulin and MHC genes, kinases/phosphate, ligands and receptors, melanoma-specific genes, redoxmolecules, transcription/translation factors, metabolism, and miscellaneous. All genes displaying an increase or decrease of expression of more than fourfold were regarded as significantly up- or downregulated.

The expression pattern shows that the highly aggressive cell line (Mel57) displayed a dramatic upregulation of genes from cytoskeletal proteins, apoptosis and cell cycle proteins, GTP binding proteins and oncogenes, extracellular ligands and receptors, transcription and translation factors. Among these the most striking findings were observed in the actin/myosin/migration group, showing strong upregulation for creatine kinase (262.7-fold), desmoglein 2 (61.4-fold), semaphorin F (52.8-fold), BL 34B cell activation gene (52.2-fold) and neurofilament F (45.8-fold), all involved in cytoskeletal organization. Among the GTP binding proteins, helix-loop-helix protein 6088 (GS08), and putative oncogene, displayed the strongest upregulation (46.9-fold). Ocular albinism type 1 gene (187.2-fold), LDL receptor (58.4-fold) and IL-11 receptor (43-fold) were highly upregulated within the ligands and receptors group. Most transcription factors displayed strong upregulation. Among these are neuronal-specific factors, e.g. neuronal PAS2 (57.7-fold), and the more common transcription factors such as interferon-stimulated gene factor 3 (50.8-fold) and erythroblastosis virus oncogen homolog 1 (24.8-fold). Interestingly, the majority of angiogenesis factors and related molecules were downregulated, e.g. platelet-derived growth factor (PDGF)

and vascular permeability factor. However, one striking observation was the strong upregulation of the angiogenesis factor, cyr-61 (239.4-fold).

Strong upregulation of thrombospondin 2 (51.2-fold) was observed in the group of extracellular matrix molecules. However, most of the molecules involved in extracellular matrix organization were downregulated. The majority of these genes were members of the family of collagens (e.g. − 152.8-fold downregulation for collagen alpha 3 type IX). The majority of melanoma-specific genes were also downregulated, especially the family of MAGE/BAGE/GAGE proteins.

Northern blot analysis

A set of differentially regulated genes, showing a more than tenfold increased or decreased expression in the microarray analysis, was selected for Northern blot hybridization (Fig. 1). It could be shown that the data from Northern blot analyses paralleled the microarray expression pattern. Three genes (desmoglein 2, thrombospondin 2, and IL-11 receptor) were only expressed in the highly aggressive cell line Mel57 but absent or
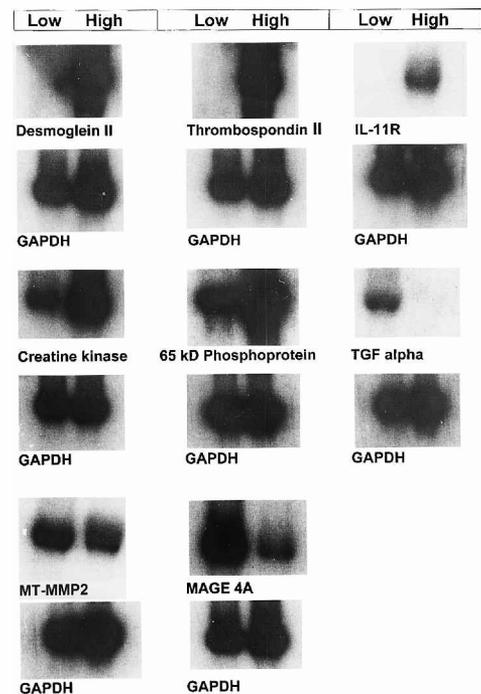

Fig. 1. Northern blot analysis of differentially expressed genes in a highly aggressive and a low aggressive melanoma cell line, respectively. cDNA probes of different lengths (900–1400 bp) were generated and used for Northern hybridization of mRNA extracted from two malignant melanoma cell lines (high and low aggressiveness). The fold induction of gene expression (derived from the microarray analysis) was 61.4 for desmoglein 2, 51.2 for thrombospondin 2, 43.0 for IL-11R, 262.7 for creatine kinase, 22.2 for 65 KD phosphoprotein, − 10.6 for TGF-alpha, − 31.5 for MT-MMP 2, and − 12.8 for melanoma associated antigen (MAGE) 4A, respectively. Equal loading of RNA was confirmed by Northern analysis of the housekeeping gene GAPDH.

weakly expressed in the low aggressive 1F6 cells. A further two (65 kD phosphoprotein, creatine kinase) were strongly induced in Mel57 cells but significantly expressed in both cell lines. In contrast, three genes showed significantly enhanced expression in the low aggressive cell line 1F6 compared with the highly aggressive cell line Mel57 (TGF-α; membrane-type matrix metalloproteinase 2, and MAGE 4a).

In situ *hybridization of primary melanomas and melanoma metastases*

Three candidate genes, creatine kinase, desmoglein 2, and thrombospondin 2, showing pronounced differences in the expression pattern in the microarray, and Northern blot analyses were tested for *in situ* mRNA expression in 6 primary melanomas and 6 melanoma metastases. For thrombospondin 2, a linear epidermal staining was observed within the basal layer of the epidermis (Fig. 2A, B). Only a few isolated cells were positive within the connective tissue surrounding the tumour. However, a strong staining was detected in all melanoma metastases throughout the tumour (Fig. 2C, D). Creatine kinase was not detected either

in primary melanomas or in metastases. Desmoglein 2 mRNA expression could not be detected in all primary melanomas tested (data not shown). Within the metastases, the mRNA expression pattern for desmoglein 2 varied. Staining was strongest at the margins and considerably weaker in the centre of the tumour (data not shown). Taken together, thrombospondin 2 shows a stage-dependent expression in primary and metastatic melanoma and may thus be involved in the process of tumour progression.

Immunohistochemistry of primary melanomas and *melanoma metastases*

To extend our studies, immunohistochemical staining of primary melanomas and melanoma metastases were undertaken for thrombospondin 2 and desmoglein 2. Immunohistochemical staining for thrombospondin 2 was carried out on frozen sections of 5 primary melanomas and 5 melanoma metastases. Immuno-histochemical staining for desmoglein 2 was done on paraffin-embedded tissues of 20 primary melanomas and 20 melanoma metastases. Primary melanomas were negative for thrombospondin 2 (Fig. 3A). However, a
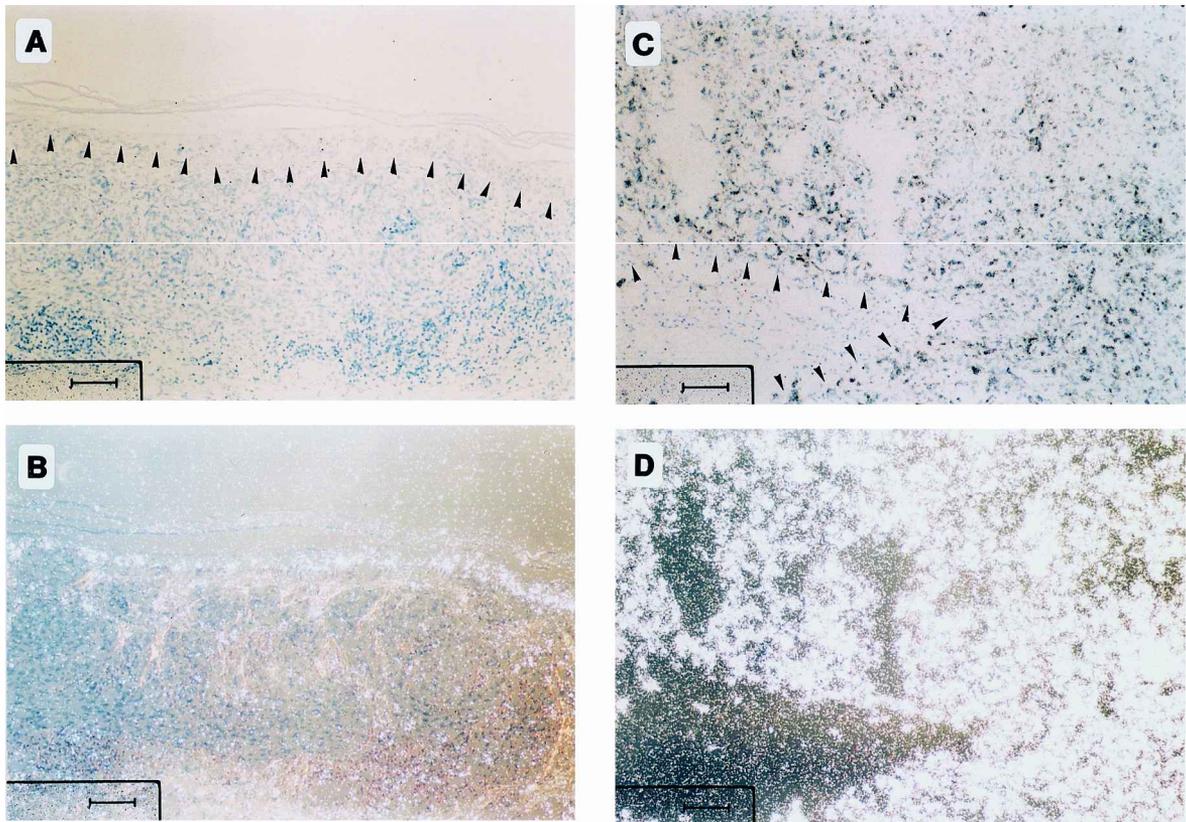

Fig. 2. In situ hybridization of thrombospondin 2. Cryostat sections from primary melanomas and cutaneous melanoma metastases all from different patients were investigated for *in situ* mRNA expression of thrombospondin 2. Thrombospondin 2 displayed a highly differential expression pattern in the microarray analysis. Thrombospondin 2 expression in a primary melanoma (*A/B*) and a melanoma metastasis (*C/D*) ((*A/C*) bright field illumination, (*B/D*) dark field illumination). Arrowheads in (*A*) demarcate the dermo-epidermal border. Note positive staining of *in situ* hybridization signals along this border (*B*). Arrowheads in (*C*) demarcate an area of connective tissue, showing a negative staining pattern, located at the lower left part of the metastasis. Bars in (*A–D*) = 100 µm.
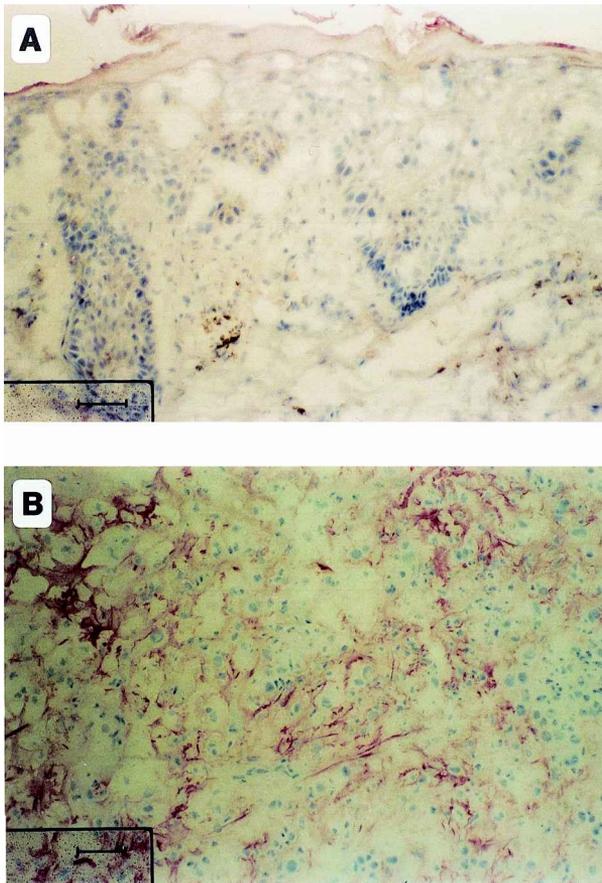
Fig. 3. Immunohistochemical staining for thrombospondin 2. Sections from primary malignant melanomas (A) and melanoma metastasis (B) were stained using the APAAP method according to the manufacturer's specifications (Dako). Neofuchsin was used as a chromogene and haematoxylin was used for counterstaining. Bars in (A/B) = 50 µm.

strong staining pattern for thrombospondin 2 was observed in all 5 tested melanoma metastases (Fig. 3*B*). For desmoglein 2, the majority of primary tumours (12 out of 20) were negative (data not shown). However, desmoglein 2 was found to be focally expressed within isolated tumour areas in 8 out of 20 primary melanomas. In contrast, desmoglein 2 was expressed in all metastases (20 out of 20) in a strong expression pattern (data not shown). Isolated faint staining close to the basal membrane zone of the epidermal layer was observed in two of the primary melanomas (data not shown). Taken together, the presented immunohistochemical data support a role of thrombospondin 2 in metastatic melanoma.

DISCUSSION

Late-stage melanoma displaying systemic metastasis is often devastating for the patient, with a mean survival time of 6 months. Thus, it is of prime importance to identify molecules contributing to this late-stage metastatic phenotype. Here we analysed the late-stage meta-

static phenotype by using microarray analysis of two human melanoma cell lines that differed in their metastatic behaviour. More than 300 genes among 5,600 tested were differentially regulated. Differentially expressed genes belonged to 14 different functional groups and partly showed dramatic up- or down-regulation, making a case for their role in the pathogenesis of melanoma metastasis.

Further analysis of the *in vitro* microarray data showed that these analyses may identify target genes for *in vivo* gene expression in melanoma metastases. Desmoglein 2 was found to be differentially regulated in the microarray analysis (61.4-fold upregulation in the highly aggressive cell line compared with the low aggressive cell line). Accordingly, all tested melanoma metastases showed desmoglein 2 expression as determined by *in situ* hybridization and immunohistochemistry. The majority of primary melanomas were negative for desmoglein 2 expression. However, focal staining was observed in 8 out of 20 primary tumours. Similar findings, at least with regard to primary melanomas, have recently been described (16). This argues for a stage-dependent expression of desmoglein 2 in malignant melanoma. Desmoglein 2 is a desmosomal plaque protein and belongs to the family of cadherins, transmembrane cell adhesion molecules that mediate cell–cell contact and cell–matrix interaction (17). In accordance with the presented findings, it has recently been shown that metastatic lesions of breast carcinomas re-express members of the family of cadherins and catenins after initial down-regulation (18). Close cell–cell contact might therefore be of importance for tumour growth and maintenance after metastasis. Indeed, cadherins may promote survival of tumour cells after metastasis via inhibition of apoptosis (19). Although desmoglein 2 might be of functional importance for tumour progression, it may not serve as a phenotypical marker for melanoma metastases.

Thrombospondin 2 was 51.2-fold up-regulated in the presented microarray analysis of the melanoma cell lines. This is the most important finding since further *in vivo* analyses revealed a remarkable difference in the expression pattern in primary melanomas and that of metastases. There was a strong thrombospondin 2 expression throughout all metastases. In contrast, in primary tumours thrombospondin 2 expression was restricted to basal epiderminal keratinocytes at the dermo-epidermal border. Thrombospondin 1 and 2 play a central role in extracellular matrix organization and angiogenesis. However, the functional importance of thrombospondins for tumour growth is still a matter of debate. There is experimental evidence showing that thrombospondin 1 may exert anti-angiogenic effects and therefore acts as an inhibitor of tumour metastasis (20). Conversely, it had been shown that thrombospondins might be involved in tumour progression in breast

carcinoma and gallbladder cancer (21, 22). The anti-angiogenic capacity of thrombospondin 2 is less clearcut (23). Recent data demonstrate that oncogenically transformed cells down-regulate thrombospondin 2 (24). Thus, down-regulation of thrombospondin 2 might be an initial step during tumour development and re-expression a feature of metastatic spread of tumour cells. Since thrombospondin 2 interferes negatively with the activity of matrix metalloproteinase 2 (MMP2; 25, 26) down-regulation of thrombospondin 2 might promote the activity of these tissue remodelling proteins, supporting initial tumour growth. In line with this, it had been shown that tumours of experimentally induced squamous cell carcinomas grew more rapidly in thrombospondin 2-deficient mice concomitant with enhanced angiogenesis (27). Interestingly, however, tumours in control mice showed strong thrombospondin 2 expression during late-stage growth. Thus, thrombospondin 2 does not necessarily interfere negatively with enhanced tumour growth. A possible explanation for this obvious controversy can be found in a recent paper about thrombospondin 1 expression in a mouse glioma tumour model (28). Here, it was shown that high thrombospondin 1 expression of progressive tumours did not inhibit enhanced tumour growth and evidence was provided that late-stage tumours may overcome the anti-angiogenic capacity of this molecule. The latter data may explain our findings of strong and constant expression of thrombospondin 2 in melanoma metastases. From a functional point of view, thrombospondin 2 expression might promote tumour growth and maintenance of metastases via its role in extracellular matrix organization and cell–matrix interaction. In a recently published microarray analysis of malignant melanoma, the role of RhoC, a member of the Rho family of GTPases, has been emphasized for melanoma metastasis (29). Similar findings had been reported for RhoA, another member of this family in rat hepatoma cells (30). Among their various functions, Rho GTPases are involved in cytoskeleton organization and formation of focal adhesion complexes mediating cell–matrix interactions.

Taken together, our findings suggest that thrombospondin 2 and to a lesser degree desmoglein 2 might serve as target molecules in melanoma metastasis. Cell–matrix interactions and cell–cell contacts might both play a role in tumour progression in malignant melanoma. These target molecules may be of importance for future therapeutic approaches.

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