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Bone morphogenetic proteins induce expression of metalloproteinases in melanoma cells and fibroblasts

*Tanja Rothhammer, Simone Braig, Anja-Katrin Bosserhoff**

Institute of Pathology, University of Regensburg Medical School, Franz-Josef-Strauss-Allee 11, D-93053 Regensburg, Germany

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ABSTRACT

Bone morphogenetic proteins are secreted growth factors which belong to the TGFβ super family. In recent studies, we showed that the expression of BMP-4 and -7 is induced in melanoma cells in comparison to normal melanocytes. Functional analyses revealed that BMPs are inevitable factors for migration and invasion processes of melanoma cells; however, the role of BMPs in degradation and remodelling of the extracellular matrix remained unknown. We discovered that melanoma cell clones with reduced BMP activity, generated by stable transfection with an antisense BMP-4 construct or with the BMP inhibitor chordin, showed reduced expression of MMP-1, -2, -3 and -9. Moreover, BMPs displayed paracrine effects on stromal fibroblasts. Treatment of fibroblasts with BMP-2 or -4 led to increased MMP-1, -2, -3 and -13 expression. These data show that BMPs play an important role in dissemination of tumour cells from the primary tumour, either by enhancing the matrix degrading capacity of melanoma cells themselves or by stimulating tumour surrounding fibroblasts to induce expression of matrix metalloproteinases.

1. Introduction

Bone morphogenetic proteins (BMPs) were first identified by Marshall Urist in the 1960s and named for their ability to induce ectopic bone and cartilage formation in the connective tissue of rodents.[1] Later on, it became clear that BMPs are a group of growth factors which belong to the TGFβ super family. Until today, more than 30 members of the BMP/TGFβ super family have been described in various species, where they control a broad range of biological activities such as cell proliferation, differentiation, motility and cell adhesion. Moreover, BMPs are involved in the determination of the dorsoventral body axis as well as the formation and patterning of different tissues during embryogenesis.[2–4]

BMPs activate cell signalling via two different types of transmembrane serine/threonine kinase receptors, BMP type I and type II receptors. Ligand binding leads to the formation of heteromeric receptor complexes followed by transphosphorylation and activation of the type I receptor by type II receptors. Activated type I receptors in turn initiate intracellular signalling by phosphorylation of receptor regulated Smad (Smad 1, 5, 8) proteins. Activated R-Smads form heteromeric complexes with a common partner Smad (Smad 4), translocate into the nucleus and activate transcription of BMP target genes.[5]

Recently, our group identified BMP-4 and -7 as important molecules in melanoma development and progression. We detected increased expression of BMP-4 and -7 in melanoma cell lines compared to normal epidermal melanocytes as well as strong immunohistochemical staining in tissue sections from primary and metastatic melanomas in comparison to weak or no staining in nevi.[6,7] Detailed functional analyses revealed that BMPs act as autocrine effectors on melanoma cells themselves by enhancing cell migration and invasion. We were able to show that melanoma cell clones with reduced BMP activity, due to antisense BMP-4 transfection or

* *Corresponding author:* Tel.: +49 941 944 6705; fax: +49 941 944 6602.
E-mail address: anja.bosserhoff@klinik.uni-regenburg.de (A.-K. Bosserhoff).
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stable over-expression of the BMP inhibitor chordin, displayed a reduction of their invasion capacity up to 70%.

It is known that migration and invasion processes require the degradation of extracellular matrix proteins, which can be achieved by the activation of different proteolytic enzyme systems.[8] In malignant melanoma, several proteolytic enzyme systems such as the plasminogen activator system, matrix metalloproteinases (MMPs), aspartyl proteinases and cysteine proteinases were identified to contribute to melanoma progression.[9] Especially the complex system of matrix metalloproteinases and their inhibitors (tissue inhibitor of matrix metalloproteinase, TIMP) plays an important role in this process. It has been described that the collagenases MMP-1 and -13, the gelatinases MMP-2 and -9 and stromelysin-1 (MMP-3) are expressed by melanoma cells and that this expression correlates with invasion of melanomas *in vivo*.[10–12] So far, it has not been addressed whether the expression of these MMPs in melanoma cells can be modulated by bone morphogenetic proteins.

It is known that not only tumour cells themselves secrete matrix degrading enzymes, but also tumour surrounding stroma cells. Tumour cells release a large number of growth factors and cytokines, which stimulate 'normal' stromal cells such as fibroblasts, muscle cells, endothelial cells, immune cells or inflammatory cells and lead to an activated phenotype. Activated stromal cells in turn secrete proteolytic enzymes which contribute to the degradation of the extracellular matrix and facilitate invasion of tumour cells.[13] Especially the expression of MMPs is not only restricted to tumour cells, but can also be detected in tumour surrounding stroma cells.[9,14]

In this study, we aim to investigate whether BMP induced invasion is mediated via the MMP/TIMP system in melanoma cells, as well as the paracrine role of BMPs on the tumour surrounding stromal cells.

2. Materials and methods

2.1. Cell lines and tissue culture

The melanoma cell line Mel Im was described in detail previously.[6] It was derived from a metastasis of malignant melanoma. Chordin-expressing cell clones were established by stable transfection of Mel Im melanoma cells with a sense chordin expression construct (gift from Theresa E. Gratsch and K. Sue O'Shea).[15] For the generation of antisense BMP4 cell clones, a 670 bp fragment of the BMP4 coding region was cloned in antisense orientation into the pCMX-PL1 vector (coding sequence 410–1080 bp) and Mel Im cells were stably transfected. Plasmids were cotransfected with pcDNA3 (Invitrogen, NV Leek, The Netherlands) containing the selectable marker for neomycin resistance. Transfections were performed using Lipofectamine plus (Invitrogen). Human skin fibroblasts were isolated from skin as previously described.[16] Cells were grown at 37 °C/5% CO_2 in Dulbecco's modified Eagle medium DMEM; PAN Biotech GmbH, Aidenbach, Germany) supplemented with penicillin (100 U/ml), streptomycine (10 μg/ml) (both PAN Biotech GmbH) and 15% v/v foetal calf serum (FCS) (Gibco, Invitrogen, Karlsruhe, Germany).

To generate spheroids, confluent cells were trypsinised, and 7500 fibroblasts or 8000 Mel Im cells, respectively, were laid per well of 96-well plates (BD Bioscience, United States of America (USA)) covered with 1% w/v agarose in 100 μl DMEM without FCS. One day later, fresh media containing 2.5% v/v FCS was added to Mel Im cultures, followed by an incubation for 5 d. Twenty-four hours after seeding the fibroblasts, the cells were stimulated with 75 ng/ml BMP-2 and -4 in DMEM with 2.5% v/v FCS. Fibroblast cultures were incubated for 2 d and RNA was isolated as described.

2.2. Isolation and quantification of RNA

Total RNA was isolated using the RNeasy Mini Kit following the manufacturer's manual (Qiagen, Hilden, Germany). Two microliters of RNA were used for reverse transcription as described previously.[6] Reverse transcription was performed using Superscript II (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's manual. For quantitative real-time RT-PCR 1 μl cDNA preparation, 10 μl of SYBR green mix (TaKaRa Bio Europe S.A.S, Saint-Germain-en-Laye, France) and 0.5 μl of forward and reverse primer (20 μM) in a total of 20 μl were applied. The following PCR program was performed: 60 s at 95 °C, 20 °C/s temperature transition rate up to 95 °C for 15 s, 10 s annealing, 22 s at 72 °C, 10 s acquisition mode single, repeated 40 times (amplification). Annealing and melting temperatures were optimised for each primer set (Table 1). PCR reactions were evaluated by melting curve analysis and checking the PCR products on 1.5% agarose gels. Data were analysed using LightCyler software ver-

Table 1 – Primer sequences used for real-time PCR

Gene	Sequence		T_a (°C)	T_m (°C)
MMP-1	For	5′-TGGACCAAGGTCTCTGAGGGTCAA-3′	68	83
	Rev	5′-GGATGCCATCAATGTCATCCTGA-3′		
MMP-2	For	5′-GCTGGGAGCATGGCGATGGATAC-3′	68	83
	Rev	5′-GCTGGGAGCATGGCGATGGATAC-3′		
MMP-3	For	5′-GGCACAATATGGGCACTTTAAAT-3′	62	81
	Rev	5′-GTCTACACAGATACAGTCACTG-3′		
MMP-9	For	5′-CCGAGCTGACTCGACGGTGATGG-3′	69	89
	Rev	5′-GAGGTGCCGGATGCCATTCACGTC-3′		
MMP-13	For	5′-TACCAGACTTCACGATGGCATTGCTG-3′	69	89
	Rev	5′-AAAGTGGCTTTTGCCGGTGTAGGTG-3′		

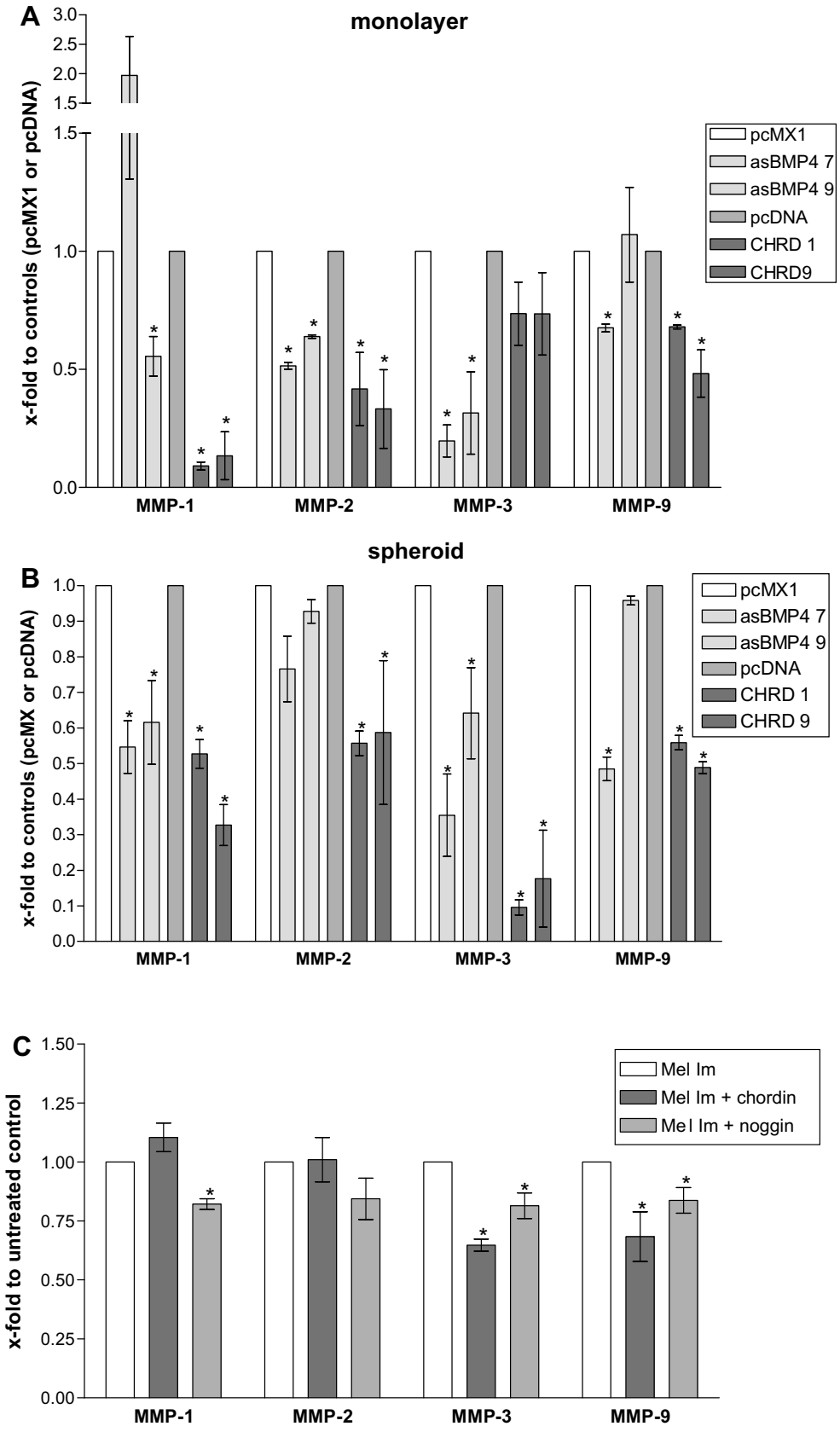

Fig. 1 – Expression of MMP-1, -2, -3 and -9 in antisense BMP-4 and chordin over-expressing melanoma cell clones. Quantitative RT-PCR analysis showed MMP-1, -2, -3 and -9 expression levels in stably transfected antisense BMP-4 and chordin over-expressing cell clones grown in monolayer (A) as well as in spheroid cultures (B). Bars indicate SD (*$P < 0.05$). (C) Quantitative real-time RT-PCR analyses of MMP-1, -2, -3 and -9 in melanoma cells incubated with 500 ng/ml of the BMP inhibitors chordin or noggin for 48 h. Bars indicate SD (*$P < 0.05$).

sion 3.52 (Roche, Ellwangen, Germany). Each quantitative PCR was performed in triplicate for two sets of RNA preparations.

2.3. Quantification of MMP-1 and -3 protein expression

The expression of MMP-1 and -3 was analysed by assaying cell culture supernatants of BMP-treated fibroblasts using enzyme-linked immunosorbent assays (ELISA). For the ELISA, 7×10^6 fibroblasts were cultured in 3 ml DMEM + 15% v/v FCS in T25 culture flasks (BD Falcon, Franklin Lakes, USA) and treated with 100 ng/ml BMP-2 or -4 (R&D Systems GmbH, Wiesbaden – Nordenstadt, Germany), respectively, for 24 h. For the MMP-1 ELISA (GE Healthcare, Buckinghamshire, United Kingdom) 200 μl of the conditioned cell culture supernatants and for the MMP-3 ELISA (R&D Systems) 100 μl were assayed. Cell culture supernatants of at least three different experiments were analysed.

2.4. Conditioned media

Equal amounts (1×10^6) of the antisense BMP-4, the chordin over-expressing and the control transfected melanoma cell clones were seeded into T75 culture flasks in 10 ml of DMEM with FCS and cultured for 48 h. After 48 h the cell culture supernatant was removed and used for the cultivation of fibroblasts for another 24 h. Fibroblasts were harvested after 24 h of cultivation in conditioned media, the RNA was extracted and the expression of MMPs analysed using quantitative real-time RT-PCR.

2.5. Tissue extracts

To isolate proteins from primary melanoma tumour tissue, tumour environment and normal skin, cryoconserved tissue was used. By staining of a representative section with H&E, a pathologist characterised tumour and normal tissue. The tumour tissue TB71 was derived from a primary tumour, whereas the tissue called TB82 was isolated from a skin metastasis. The corresponding normal skin tissues were used as control. Hundred milligrams of healthy skin tissue, tissue of melanoma tumour environment or melanoma tissue, respectively, were disrupted in 500 μl sterile water using the Precellys 24 (Bertin Technologies, Saint-Quentin-en-Yvelines Cedex, France) extraction device with 2.8 mm diameter ceramic beads. The settings were as follows: 6800 rpm, 3 cycles with 30 s run time and 30 s waiting time. After disruption, the cell debris was removed by centrifugation at 10,000 rpm, and the protein concentration of the supernatant was analysed using the bicinchoninic acid protein assay reagent (Pierce Biotechnology, Rockford, IL, USA).

2.6. Collagen digestion

Rat tail collagen type I (38.5 μg) and 25 μg tissue extracts were incubated at 25 °C for 48 h. For activation of MMPs, 0.002 mM aminophenylmercuric acetate was added to the reaction mix. As a positive control, 1 μl of collagenase (0.002 μg/ml) was added to 38.5 μg of collagen type I and incubated for 2 h at 25 °C. After digestion the collagen fragments were separated on 10% polyacrylamide gels and stained with coomassie blue solution (0.25 g coomassie brilliant blue R250, 45 ml methanol, 45 ml H_2O, 10 ml acetic acid 100% for 100 ml of coomassie blue solution) for 30 min. Afterwards the gels were destained in 7% acetic acid for 4 h and dried using the DryEase Mini-Gel drying system (Invitrogen, Carlsbad, USA).

2.7. Analyses of band density

Analyses of the band density of collagen degradation products were carried out using the gel analysis tool of the ImageJ software freely available from the NIH web site (http://rsb.info.nih.gov/ij/).

2.8. Statistical analysis

Results are expressed as mean ± SD (range) or percent. Comparisons between groups were made using the Student's unpaired *t*-test. $P < 0.05$ was considered statistically significant. All calculations were done using the GraphPad Prism software (GraphPad software, Inc, San Diego, CA).

3. Results

3.1. Expression of matrix degrading enzymes in melanoma cells with reduced BMP activity

Recent analyses by our group revealed that melanoma cell clones with reduced BMP activity, due to stable transfection with either antisense BMP-4 constructs or the BMP inhibitor chordin, showed reduced invasion capacities.[6] However, until now it was not clear how the invasion process is influenced by BMPs. We, therefore, analysed the expression of members of the MMP system in our antisense BMP-4 and chordin over-expressing cell clones. We revealed that in chordin over-expressing cell clones, the reduction of BMP activity led to a reduced expression of matrix metalloproteinases-1, -2 and -9 in monolayer cell culture, regulation of MMP-3 did not reach significance (Fig. 1A). In antisense BMP-4 cell clones, all MMPs were found to be regulated, except of MMP-1 expression in asBMP-4 clone 7, showing an increase in the expression level and MMP-9 expression in clone 9, exhibiting no alterations in the expression. To consider the well-known influence of the microenvironment on melanoma cells[17] and to approach the *in vivo*, conditions we investigated the MMP expression in three-dimensional cell cultures (Fig. 1B). Moreover, additionally formed cell–cell- and cell–matrix-contacts in the three-dimensional model system may influence MMP expression. In spheroid cultures, both the asBMP-4 clones 7 and 9 showed a reduction in the expression of MMP-1. In contrast, the expression of MMP-2 and -3 was marginally lower in spheroid cultures than in monolayer cultures. The chordin over-expressing cell clones showed a more distinct reduction in MMP-3 expression in three-dimensional cultures than in monolayer cultures. In contrast, the decrease of the MMP-1 expression in chordin over-expressing cell clones was more obvious in the monolayer cultures. Additionally, treatment of the parental melanoma cell line Mel Im with the BMP inhibitors chordin and noggin showed reduced expression of MMP-3 and -9 (Fig. 1C).

3.2. MMP expression in fibroblasts after BMP treatment

It is known that apart from melanoma cells also activated tumour surrounding stroma cells as fibroblasts secrete matrix degrading enzymes. The activation of stroma cells is generally achieved by paracrine stimulation with cytokines or growth factors which are released by the tumour cells. We, therefore, analysed paracrine effects of BMPs on fibroblasts with special focus on matrix degrading enzyme systems.

Dermal fibroblasts were cultured either as monolayer or as spheroid cultures and treated with 100 ng/ml BMP-2 or -4, respectively, for 24 h. Quantitative real-time RT-PCR analyses revealed an induction of MMP-2, -3 and -13 expression in BMP-treated fibroblasts in monolayer and of MMP-3 and MMP-13 in spheroid cultures, whereas expression of MMP-9 stayed unchanged and MMP-2 showed a small but not significant increase (Fig. 2A and B). BMP treatment leads to an induction of MMP-1 expression in monolayer cultures, but has no influence on MMP-1 expression in spheroid cultures. Because of the low overall protein expression of MMP-13 detected in ELISA, merely the expression of MMP-1 and -3 was investigated in additional studies and an increase in MMP-1 and -3 protein expression in cell culture supernatants from BMP-treated fibroblasts cultured in monolayer could be shown (Fig. 3).

3.3. MMP expression in fibroblasts after treatment with melanoma cell culture supernatants

To analyse direct effects of BMPs secreted by melanoma cells, we cultured dermal fibroblasts in conditioned cell culture media from the antisense BMP-4, the chordin transfected or control transfected melanoma cell clones. We found that fibroblasts cultured in cell culture supernatants from the

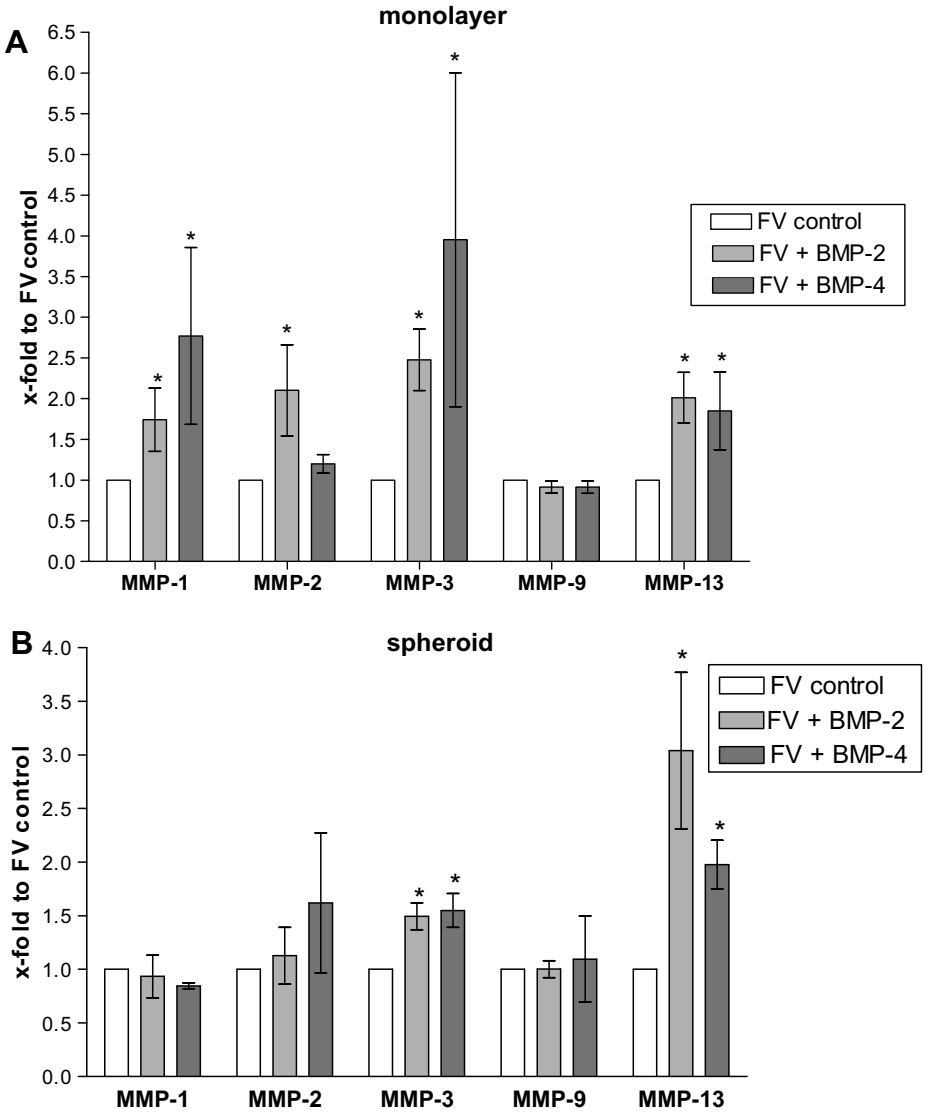

Fig. 2 – MMP expression in fibroblasts after BMP treatment. Quantitative real-time RT-PCR analyses of fibroblasts (FV) treated with 100 ng/ml of BMP-2 and -4, respectively, and cultured either in monolayer (A) or in spheroid cultures (B), showed increased expression of MMP-1, -2, -3 and -13. The expression of MMP-9 was not altered. Bars indicate SD (*$P < 0.05$).

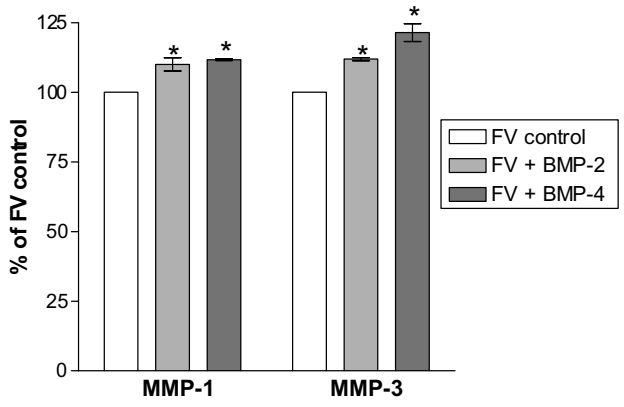

Fig. 3 – MMP protein expression of BMP-treated fibroblasts Fibroblasts (FV) were treated with 100 ng/ml BMP-2 or -4 for 24 h. MMP-1 and -3 protein expression was analysed by ELISA ($n = 3$). Bars indicate SD (*$P < 0.05$). Mean of MMP-1 expression of untreated fibroblast was 8.5 ng/ml (SD: 2.4), MMP-3 expression 14.3 ng/ml (SD: 4.6).

parental cell line or control transfected melanoma cells showed higher MMP-3 expression than fibroblasts cultured with media from cell clones with reduced BMP activity. A reduced induction of MMP-3 expression could not be observed in fibroblasts exposed to asBMP-4 clone 7 supernatant. The incubation with conditioned media from the cell clones leads to no significant reduction of MMP-1 expression (Fig. 4).

3.4. *Collagen degrading capacity of different tissue extracts*

To investigate whether there are differences in MMP expression between tumour tissue, the tumour microenvironment and healthy skin tissue, we analysed the collagen degrading capacity of different tissue extracts (Fig. 5). We incubated different tissue lysates with or without adding collagen and determined the proteolytic activity. The definite cleavage fragments of collagen were analysed and the band densities are shown in fig. 6. We were able to detect an increase in collagen degrading activity from normal skin to the tumour microenvironment and the highest activity in tumour tissue extracts (Fig. 6).

In addition, we treated Mel Im cells with the general MMP-inhibitor GM6001 (Calbiochem, San Diego, USA) and performed a Boyden chamber assay. We observed that the inhibition of MMPs by GM6001 leads to a reduction of the invasive potential of about 40% (data not shown).

4. Discussion

Malignant melanoma is a disease that accounts for only 4% of all skin cancers but for the majority of skin cancer-associated deaths.[17] Cancer progression is characterised by highly invasive and metastatic tumour cells and by the lack of effective treatment of metastatic disease. Invasion and metastasis are multi-step processes that require loss of growth control, cell migration and the degradation of the extracellular matrix. Especially the proteolysis and degradation of extracellular matrix structures are essential for tumour cell dissemination.

Previous studies showed that BMPs are involved in the regulation of invasion of malignant melanoma.[6,7] However, the molecular background remained unclear. In this study, we were able to show that BMPs regulate the expression of matrix metalloproteinases (MMPs) and thereby contribute to the degradation of extracellular matrix components and invasion of malignant melanoma.

MMPs play a crucial role in degradation of collagens and thereby contributing to the invasion and migration of melanoma. Inhibition of MMPs by the general inhibitor GM6001 leads to a strong reduction of the invasive potential of the melanoma cells used in this study.

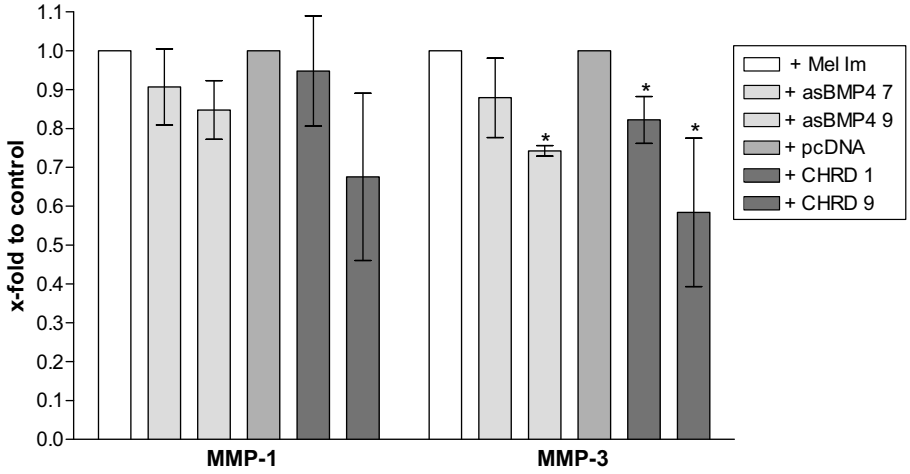

Fig. 4 – MMP expression of fibroblasts cultured with conditioned media. AsBMP-4 and chordin over-expressing cell clones were cultured for 48 h, the supernatant was used for the incubation of fibroblasts (FV) for 24 h. MMP-1 and -3 expression were analysed using quantitative real-time PCR and compared to conditioned medium from the parental cell line and control transfected cells. Bars indicate SD (*$P < 0.05$).

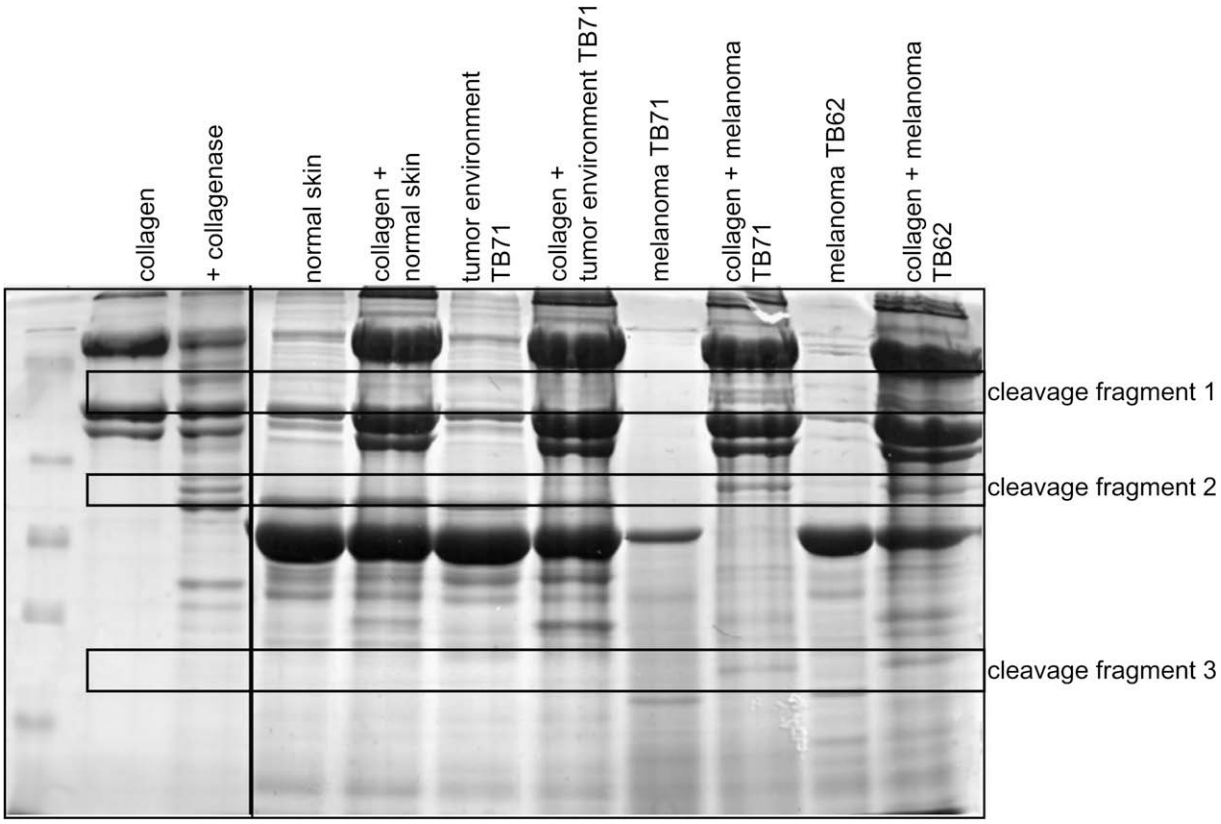

Fig. 5 – Collagen degrading capacity of tissue extracts. Tissue lysates were incubated with or without collagen. After activating MMPs, the specific collagen degradation products were determined by separation on polyacrylamide gels. Tissue extracts from melanoma tissue showed strong degradation of type I collagen, whereas extracts from the tumour environment and from normal skin showed less degradation.

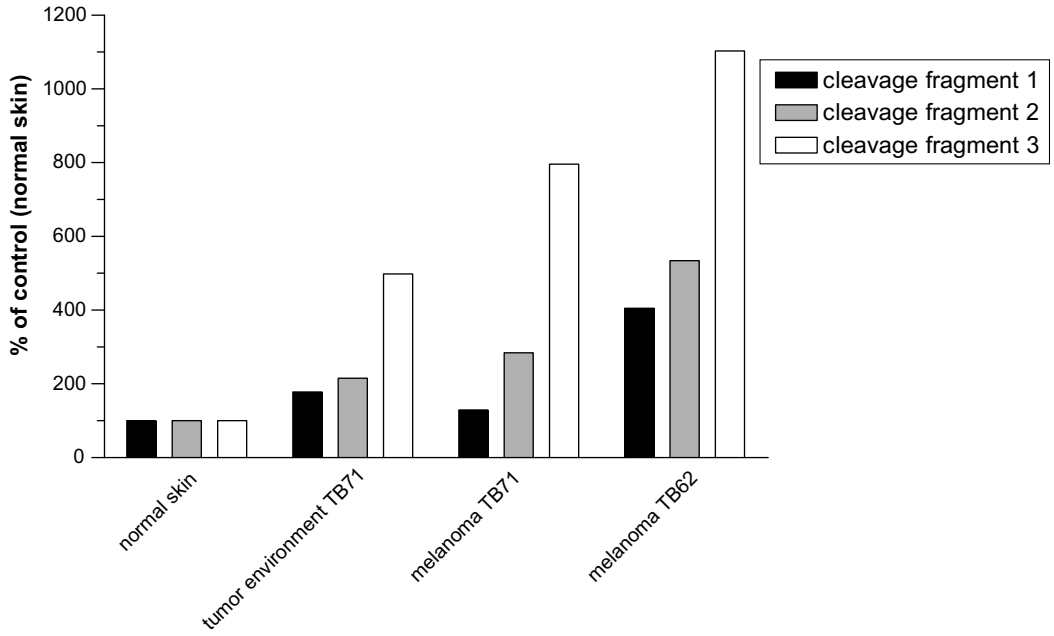

Fig. 6 – Band density analyses of collagen degradation products. Analysis of the band densities of specific cleavage fragments 1, 2 and 3 shown in Fig. 5.

The transcription of MMPs is influenced by many factors including hormones, growth factors, oncogenes and cytokines. Mitogen-activated protein kinases (MAPKs) mediate these cell signals from the membrane to the nucleus and induce phosphorylation and translocation of transcription factors belonging to the Jun and Fos families.[18,19] They interact with the activator protein-1 (AP-1) binding site in several MMP promoters[20] and activate the gene expression. It was shown that transcription factors of the ETS family bind on PEA3 elements in MMP promoters[19] to form complexes with other transcription factors, e.g. AP-1 and function as co-activators.[21]

BMPs may regulate the expression of MMPs in a direct or indirect manner. It is known that apart from the Smad signalling pathway, BMP signalling occurs via the MAPK pathway activating extracellular regulated kinase (ERK) 1/2, c-Jun N-terminal kinases (JNK) and p38. Members of the Fos/Jun family are upregulated and interact with AP-1 binding sites,[22] which may result in activation of MMPs. Whether the Smad or MAPK pathway is involved in the regulation of MMP expression by BMP needs to be investigated in further studies.

In monolayer cell cultures, we found a reduction of MMP-1, -2, -3 and -9 expression in antisense BMP-4 and chordin over-expressing cell clones, which shows that BMPs are a key regulator of MMP expression.

Because chordin acts as a general inhibitor of the BMP signalling, the higher reduction of MMP-1 expression in chordin over-expressing cell clones than antisense BMP-4 cell clones indicates that MMP-1 expression is regulated rather by other BMPs than by BMP-4. However, in antisense BMP-4 cell clones, the MMP-3 expression was reduced to a larger extent than chordin over-expressing cell clones. These findings suggest that MMPs are regulated individually by particular BMPs.

The reduction of MMP-1, -2, -3 and -9 expression by antisense BMP-4 and chordin over-expressing cell clones could also be observed in three-dimensional cultures, yet with slight differences compared to monolayer cultures. The asBMP-4 cell clones showed no significant reduction in MMP-2 expression. The cell–cell contacts in spheroids as well as further regulation mechanisms may influence the expression of MMPs and could be a possible explanation for the different expression patterns of MMPs in spheroids compared to the cells grown in monolayer cultures.

Some studies analysing osteoblast differentiation showed that the expression of MMP-2 or -13, respectively, could be induced by treatment with recombinant BMP-2 in cell culture. Moreover, the expression of the tissue inhibitor of matrix metalloproteinases 1 (TIMP-1) was strongly reduced after BMP-2 treatment.[23,24]

By treatment of fibroblasts with BMP-2 and -4 we could show an increase in MMP-2, -3 and -13 expression both in monolayer and three-dimensional melanoma cell cultures. Also in this set of experiments, there are differences in monolayer and three-dimensional cultures. In monolayer cultures treatment with BMP-2 and -4 leads to an increase of MMP-1 expression, whereas in spheroid cultures no change concerning the expression of MMP-1 could be observed. Also MMP-1 and -3 protein expression are increased in BMP-treated fibroblasts, which could be determined by ELISA. However, the increase is small potentially due to basal BMP expression of fibroblasts. Incubation of fibroblasts in conditioned medium from antisense BMP-4 and chordin over-expressing cell clones showed partly a reduced expression of MMP-3 compared to the medium from control transfected cells, whereas changes in MMP-1 were not significant. Loffek and colleagues showed an induction of proMMP-1 synthesis in co-cultured melanoma cells and fibroblasts.[14]

The collagen degrading capacity is much stronger in melanoma tissue than in the tumour environment. Tumour environment shows a higher activity in contrast to the corresponding normal skin tissue. These data reinforce the previous findings of increased expression of several MMPs in tumour progression.[9]

In general, by reducing the activity of BMPs by antisense BMP-4 and chordin over-expressing cell clones, the MMP expression could not be completely inhibited, which indicates that apart from BMPs additional mechanisms are involved in the regulation of MMPs.

In conclusion, we could show that BMPs are involved in regulation of MMPs. Whether there are additional ways of regulating invasion of malignant cells by BMPs need to be elucidated in further studies.

Overall this study supports the important role of BMPs in melanoma progression by regulating gene expression not only in melanoma cells but also in tumour-associated cells.

Conflict of interest statement

None declared.

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