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A Matrix Metalloproteinase-1/Protease Activated Receptor-1 signaling axis promotes melanoma invasion and metastasis

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Abstract

Hallmarks of malignant melanoma are its propensity to metastasize and its resistance to treatment, giving patients with advanced disease a poor prognosis. The transition of melanoma from non-invasive radial growth phase (RGP) to invasive and metastatically competent vertical growth phase (VGP) is a major step in tumor progression, yet the mechanisms governing this transformation are unknown. Matrix Metalloproteinase-1 (MMP-1) is highly expressed by VGP melanomas, and is thought to contribute to melanoma progression by degrading type I collagen within the skin to facilitate melanoma invasion. Protease activated receptor-1 (PAR-1) is activated by MMP-1, and is also expressed by VGP melanomas. However, the effects MMP-1 signaling through PAR-1 have not been examined in melanoma. Here, we demonstrate that an MMP-1/PAR-1 signaling axis exists in VGP melanoma, and is necessary for melanoma invasion. Introduction of MMP-1 into RGP melanoma cells induced gene expression associated with tumor progression and promoted invasion *in vitro*, and enhanced tumor growth and conferred metastatic capability *in vivo*. This study demonstrates that both the type I collagenase and PAR-1 activating functions of MMP-1 are required for melanoma progression, and suggests that MMP-1 may be a major contributor to the transformation of melanoma from non-invasive to malignant disease.

Keywords

MMP-1; PAR-1; angiogenesis; Superarray; xenograft

Introduction

Melanoma is the most rapidly increasing cancer in the United States, and the survival rate of patients with metastatic disease is <10% (Berwick *et al.*, 2009). Melanomas are classified histologically, with depth of tumor invasion being a strong prognostic indicator (Balch *et al.*, 2004). In early stage melanoma, the radial growth phase (RGP), the tumor grows laterally

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along the epidermis, is non-invasive and cured by surgical excision, with a 95% patient survival rate. RGP melanomas can progress to vertical growth phase (VGP), in which the tumor invades the dermis and subcutaneous tissue. VGP melanomas have only a 30-60% patient survival rate, with deeper invasion associated with adverse clinical outcome. VGP melanomas can also invade dermal blood and lymphatic vessels, and are therefore metastatically competent (Balch *et al.*, 2004; Breslow, 1970; Clark *et al.*, 1975; Gray-Schopfer *et al.*, 2007; Leiter *et al.*, 2004). The acquisition of the invasive VGP phenotype is therefore both biologically and clinically relevant; work to define the molecular mechanisms governing the transition of melanoma from RGP to VGP is ongoing.

To acquire the VGP phenotype, melanoma cells must degrade and remodel basement membrane and the extracellular matrix (ECM) within the skin. This matrix remodeling is mediated largely by Matrix Metalloproteinases (MMPs), which are frequently over-expressed in cancers (Fingleton, 2006). Melanoma expresses several different MMPs, depending on the stage of tumor progression. The interstitial collagenase MMP-1 is expressed specifically by VGP melanomas, where it contributes to tumor invasion and metastasis (Blackburn *et al.*, 2007; Durko *et al.*, 1998; Ntayi *et al.*, 2001), and is commonly associated with a poor clinical prognosis (Airola *et al.*, 1999; Nikkola *et al.*, 2005). Type I collagen is the major component of the dermis, and MMP-1 is thought to facilitate tumor cell invasion by degrading dermal collagen. In addition, MMP-1 proteolytically activates the G-protein coupled receptor Protease Activated Receptor-1 (PAR-1) (Boire *et al.*, 2005), suggesting that MMP-1 has a larger role in tumor progression by activating signal transduction pathways and modulating cell behavior.

PAR-1 is activated by several proteases, including thrombin, activated protein C and MMP-1, and plays important roles in normal biologic processes (Macfarlane *et al.*, 2001). PAR-1 is also an oncogene (Martin *et al.*, 2001), and is over-expressed in several types of cancers, including melanoma (Arora *et al.*, 2007). Signaling though PAR-1 facilitates tumor invasion, angiogenesis and metastasis by inducing the expression of genes associated with cell adhesion, invasion and survival (Agarwal *et al.*, 2008; Boire *et al.*, 2005; Even-Ram *et al.*, 2001; Salah *et al.*, 2007).

Like MMP-1, PAR-1 is differentially expressed in melanoma, with higher levels of found in VGP melanomas, compared to non-invasive RGP (Tellez and Bar-Eli, 2003). In patient samples, PAR-1 expression increased concomitantly with the depth of melanoma invasion, and was the best marker for poor prognosis (Depasquale and Thompson, 2008; Massi *et al.*, 2005). Further, blocking PAR-1 activation in B16 mouse melanoma prevented pulmonary metastasis (Nierodzik *et al.*, 1998), and knock-down of PAR-1 expression in a human melanoma xenograft model inhibited tumor growth and metastasis (Villares *et al.*, 2008). Thus, PAR-1 is likely a major contributor to melanoma progression.

We reported that MMP-1 expression by human VGP melanoma cells is necessary for melanoma metastasis (Blackburn *et al.*, 2007). This was attributed to the type I collagenase activity of MMP-1 and to the induction of a pro-angiogenic paracrine MMP-1/PAR-1 signaling axis in endothelial cells. Additionally, fibroblast-produced MMP-1 activated PAR-1 on breast cancer cells to promote tumor growth and invasion (Boire *et al.*, 2005).

Because both MMP-1 and PAR-1 are expressed by VGP melanoma cells, we hypothesized that activation of PAR-1 signaling by MMP-1 in VGP melanoma could induce the expression of genes to promote invasion, growth and angiogenesis. MMP-1 would then contribute to melanoma progression in two ways: by degrading dermal type I collagen to remove the physical barriers for melanoma invasion, and by activating PAR-1 on the melanoma cells to induce genes that contribute to invasion and metastasis.

Here, we demonstrate that an MMP-1/PAR-1 signaling axis exists in melanoma and promotes melanoma invasion. We also show, for the first time, that *both* the collagenase and PAR-1 activating functions of MMP-1 are required for melanoma invasion. Additionally, our study shows that MMP-1 can convert an RGP melanoma to VGP, as measured by tumor growth and metastatic ability *in vivo*. Because very few genes have been linked to the transition of melanoma from RGP to VGP, our study may contribute to understanding of mechanisms mediating the acquisition of the invasive and metastatic phenotype.

Results

An MMP-1/PAR-1 signaling axis exists in VMM12 VGP melanoma cells

The VMM12 human melanoma cell line has an invasive and metastatic phenotype typical of VGP melanoma (Blackburn *et al.*, 2007; Huntington *et al.*, 2004). VMM12 cells secrete high amounts of MMP-1 compared to both Bowes cells, a non-invasive human RGP melanoma cell line (Iida *et al.*, 2004), and normal melanocytes. VMM12 cells also produce a similar level of PAR-1 as human endothelial cells (Figure 1a).

We used the AP-PAR1 reporter construct to determine if MMP-1 produced by VMM12 cells cleaves PAR-1. AP-PAR1 consists of secreted alkaline phosphatase (AP) fused to the N-terminus of PAR-1 (Ludeman *et al.*, 2005). The construct is transiently transfected into cells, and when PAR-1 is cleaved, the alkaline phosphatase is released; the phosphatase activity within the media is measured to quantify PAR-1 cleavage. VMM12 VGP cells cleaved the AP-PAR1, while Bowes RGP cells, which produce little MMP-1 (Figure 1a), did not (Figure 1b). Treatment of VMM12 cells with the thrombin inhibitor hirudin had no effect on their ability to cleave AP-PAR1, indicating that thrombin is not involved in PAR1 cleavage by the VMM12 cells. An MMP inhibitor that targets MMP-1 activity blocked AP-PAR1 cleavage, and increasing concentrations of an MMP-1 neutralizing antibody led to a corresponding decrease in PAR-1 cleavage by the cells (Figure 1c). These data indicate that MMP-1 cleaves AP-PAR1 in VMM12 cells.

Because transfection with the AP-PAR1 construct results in PAR-1 over-expression in VMM12 cells, it is important to demonstrate that MMP-1 cleaves endogenous PAR-1. Since calcium flow into the cell is a hallmark of PAR-1 activation (Macfarlane *et al.*, 2001), calcium flux was measured in VMM12 cells to examine endogenous PAR-1 cleavage. Treatment of VMM12 cells with VMM12 conditioned media caused the same amount of calcium flux as 10nM thrombin, which is known to cleave PAR-1 (Macfarlane *et al.*, 2001). While treatment of the VMM12 conditioned media with the thrombin inhibitor hirudin had no effect, blocking MMP-1 activity reduced calcium flux ($p<0.001$), as did the PAR-1 inhibitor SCH79797. Combining MMP-1 neutralizing antibody and the PAR-1 inhibitor was

similar to each individual treatment, indicating that MMP-1 activates PAR-1 signaling to induce calcium flux in VMM12 cells (Figure 1d). These data indicate that an MMP-1/PAR-1 signaling axis exists in VMM12 VGP melanoma cells.

The MMP-1/PAR-1 signaling axis induces gene expression in VMM12 VGP melanoma cells

To define the role of MMP-1/PAR-1 signaling in melanoma, VMM12 cells were stably transfected with shRNAs against MMP-1 and PAR-1, or a control shRNA, MAMMX. MMP-1 expression was decreased by >90% in shMMP-1 cells, compared to shMAMMX control, while PAR-1 expression was unaffected by the MMP-1 shRNA. Likewise, PAR-1 expression was decreased by >80% in the PAR-1 shRNA line, with no decrease in MMP-1 expression (Figure 2a).

To determine if MMP-1/PAR-1 signaling affects gene expression in VMM12 cells, the shMAMMX and shMMP-1 cell lines were used in a Human Cancer Pathway RT^2 Profiler PCR array. Gene expression was up-regulated in VMM12 cells in the presence of MMP-1 (shMAMMX vs. shMMP-1, Table I); the induced genes have broad roles in tumor progression, including angiogenesis, tumor growth, inflammation and metastasis (Noted in Table I). Realtime RT-PCR validated the array, and was used to examine the expression of ANGPT1, associated with angiogenesis, the growth factor receptor FGFR2, and S100A4 and SERPINB5, which are associated with metastasis. Higher expression (p=0.002) of these 4 pro-tumorigenic genes was seen in the shMAMMX cell line compared to cells with MMP-1 knocked-down (shMMP-1, Figure 2b), or VMM12 cells with high MMP-1 but reduced PAR-1 expression (shPAR-1). Additionally, treatment of the shMMP-1 cell line with exogenous MMP-1 induced the expression of these genes, and this was blocked by the PAR-1 inhibitor SCH79797 (Figure 2c). Together, these data suggest that MMP-1 is signaling through PAR-1 to induce genes involved in tumor progression in VGP melanoma cells.

Both the collagenase and PAR-1 activating functions of MMP-1 are necessary for melanoma invasion *in vitro*

The collagenase activity of MMP-1 is thought to be particularly important for melanoma invasion, as the dermis is comprised primarily of type I collagen (Curran and Murray, 2000). However, several genes involved in tumor invasion (S100A4, SERPINB5, uPA, MMP-9) were induced in VMM12 cells via MMP-1/PAR-1 signaling (Table I). To differentiate between the collagenase and PAR-1 activating functions of MMP-1 in melanoma invasion, the MMP-1 and PAR-1 shRNA lines were used in *in vitro* invasion assays.

The collagenolytic activity of MMP-1 removes physical barriers to tumor cell movement (Hofmann *et al.*, 2000). In collagen degradation assays, VMM12 cells with knocked-down MMP-1 expression could not degrade type I collagen, compared to shMAMMX control, while the PAR-1 shRNA had no effect on collagenolysis (Figure 3a). Likewise, significantly fewer shMMP-1 cells (p<0.001) invaded through collagen coated transwells, compared to shMAMMX cells, indicating that the collagenase function of MMP-1 is necessary for invasion through type I collagen (Figure 3b,c). Interestingly, shPAR-1 cells, which have knocked-down PAR-1 expression but control levels of MMP-1, also had reduced invasion

through collagen, suggesting that PAR-1 signaling contributes to the invasive phenotype of VMM12 cells. However, when shMMP-1 cells were treated with thrombin to activate PAR-1 signaling, the cells were still unable to invade through collagen. This suggests that although PAR-1 signaling promotes pro-invasive gene expression, without the collagenase function of MMP-1, VMM12 cells cannot invade through a type I collagen barrier.

VGP melanoma cells also invade basement membrane found between the epidermal and dermal layers of skin; basement membrane surrounding the vasculature must also be breached by tumor cells for metastasis. Basement membrane is comprised partly of type IV collagen, which is not a substrate of MMP-1. However, MMP-1/PAR-1 signaling increased the expression of several genes that contribute to invasion through basement membrane, including the type IV collagenase MMP-9 (Table I). Compared to shMAMMX control, both cells with MMP-1 and PAR-1 knocked-down had reduced invasive ability ($p<0.001$) through reconstituted basement membrane (Matrigel, Figure 3b,c). However, treating shMMP-1 cells with thrombin to activate PAR-1 restored their invasiveness, suggesting that the PAR-1 activating function of MMP-1 contributes to melanoma invasion through basement membrane, while the collagenase activity of MMP-1 plays no role.

Induction of the MMP-1/PAR-1 signaling axis in Bowes RGP melanoma cells induces a VGP-like phenotype

Non-invasive radial growth phase (RGP) melanomas do not express MMP-1 (Airola *et al.*, 1999); to determine if MMP-1 is sufficient to promote an invasive VGP phenotype in RGP melanoma, the human RGP melanoma cell line Bowes was used for further experiments. Bowes cells do not express MMP-1, but do express PAR-1 (Figure 1a), suggesting that MMP-1 activation of PAR-1 signaling could occur in this RGP line.

Treating Bowes cells with 5nM purified MMP-1 induced the expression of many genes that were induced by MMP-1 in the VMM12 VGP cells, including genes associated with angiogenesis, inflammation and invasion/metastasis (MMP-1 vs. PBS, Table I). Interestingly, there were also genes induced in Bowes cells that were not induced by MMP-1 in the VMM12 cells, including transcription factors and genes associated with cell division (Table I). Perhaps VMM12 cells, an advanced VGP melanoma, have mechanisms in place to activate the expression of these types of genes; for example, VMM12 cells have a B-RAF mutation leading to constitutive activation of MAPK signaling (Huntington *et al.*, 2004), while Bowes cells have wild-type B-RAF (data not shown). MMP-1 signaling through PAR-1 could therefore have a much greater effect in RGP melanomas.

To determine if MMP-1 expression confers a VGP-like phenotype, Bowes cells were stably transfected with a pCMV-MMP1 expression construct, which increased MMP-1 expression >100-fold, with no effect on PAR-1 expression (Figure 4a). Interestingly, MAPK signaling pathways, which are commonly activated in VGP melanomas (Haluska and Ibrahim, 2006; Ueda and Richmond, 2006), were activated in the Bowes-MMP1 cells, compared to control (Figure 4b). Treatment with either an MMP or PAR-1 inhibitor decreased the phosphorylation of MEK and p38 (Figure 4b), suggesting that MMP-1/PAR-1 signaling in Bowes RGP cells induced activation of MAPK signaling cascades.

Realtime-RT PCR demonstrated that several genes induced by treatment of Bowes cells with purified MMP-1 (Table I) were also induced by stable transfection with MMP-1 (Figure 4c). Importantly, treating Bowes-MMP1 cells with the PAR-1 inhibitor SCH79797 reduced this expression, suggesting that MMP-1/PAR-1 signaling induces the expression of genes associated with tumor progression in Bowes RGP melanoma cells. Additionally, several cell cycle genes were induced by MMP-1 in the Bowes cells (Table I), and compared to control, Bowes-MMP1 cells showed increased (p=0.0002) proliferation, which depended on activation of PAR-1 signaling (Figure 4d).

We next used a collagen degradation assay to determine if Bowes-MMP1 cells had an invasive VGP phenotype. Bowes-MMP1 cells degraded more collagen (Figure 4e), and were more invasive through type I collagen than control. The PAR-1 inhibitor reduced this invasiveness (Figure 4f), indicating that, as with the VGP cells, both the collagenase and PAR-1 activating functions of MMP-1 are important for invasion through type I collagen. However, in contrast to VMM12 cells (Figure 3c), MMP-1 expression in Bowes cells did not promote invasive ability through Matrigel (data not shown), suggesting that MMP-1/PAR-1 signaling is not sufficient to induce the expression of all genes needed for basement membrane invasion by RGP melanoma cells.

MMP-1 promotes tumor growth and metastasis of Bowes RGP melanoma cells

To determine if MMP-1 can promote the VGP phenotype *in vivo*, the cells were injected intradermally into nude mice. Tumor incidence in mice injected with Bowes-MMP1 cells was 87%, while only 38% of mice developed Bowes-pCMV control tumors. While both cell lines formed tumors 2-3 weeks after injection, the Bowes-MMP1 tumors grew significantly faster (p<0.01, Figure 5a).

MMP-1 expression also caused tumor spread into the draining lymph node in 6 out of 7 Bowes-MMP1 tumor bearing mice, compared to tumor-free lymph nodes of Bowes-pCMV injected mice. Additionally, 4 out of 7 Bowes-MMP1 tumor bearing mice had melanoma cells in their contra-lateral lymph node, and one mouse had a palpable metastasis in an auxiliary lymph node (data not shown), indicating that some Bowes-MMP1 tumors metastasized through the lymphatic system (Figure 5b). ALU PCR analyses of organs showed that lung samples from 3 mice with Bowes-MMP1 tumors were positive for human DNA, with ∼1100pg, 190pg and 150pg of human DNA found per 100ng of lung DNA (Figure 5c). In contrast, Bowes-pCMV tumor bearing mice had an average of 7pg human DNA/100ng lung DNA, which was not significantly higher than the PCR background levels found in naïve mice. These *in vivo* data suggest that MMP-1 confers aspects of the metastatic VGP phenotype in RGP cells.

MMP-1 expression can be induced in Bowes RGP cells by factors within the tumor microenvironment

While stable transfection of MMP-1 in RGP cells provides insight into the role of MMP-1 in melanoma progression, questions remain as to whether MMP-1 is expressed only after the melanoma becomes VGP, or if MMP-1 expression can be induced in RGP, where it then drives the melanoma to develop a VGP phenotype. Factors within the tumor

microenvironment can induce MMP-1 expression (Ishii *et al.*, 2003; Loffek *et al.*, 2005; Rothhammer *et al.*, 2008); we therefore treated Bowes RGP cells with factors present within the melanoma microenvironment and found that thrombin, bFGF and VEGF induced MMP-1 expression in the RGP melanoma cells (Figure 6a).

These growth factors induced MMP-1 expression only ∼5-fold, significantly less than the amount of MMP-1 produced by VGP melanomas. However, the gene expression array indicated that exogenous MMP-1 treatment induced a 24-fold increase in MMP-1 expression in Bowes cells (Table I). This was verified by realtime-RT PCR, which also showed that treating Bowes cells with the PAR-1 inhibitor reduced MMP-1 gene expression, indicating that the MMP-1/PAR-1 signaling induces a strong positive feed-back loop to promote MMP-1 gene expression in RGP melanoma cells (Figure 6b).

To determine if the slight induction of MMP-1 expression by factors within the tumor microenvironment could activate MMP-1/PAR-1 signaling and enhance MMP-1 expression in RGP cells, Bowes cells were treated with thrombin, and MMP-1 expression was examined. Thrombin induced MMP-1 expression by ∼5-fold after 24hr, and 30-fold after 48hr. Blocking MMP-1 activity with a neutralizing antibody at 24hr reduced this latter increase to only 7-fold, indicating that thrombin induction of MMP-1 in RGP cells sets up a feed-forward loop by which MMP-1 induces its own expression (Figure 6c).

Discussion

As melanoma transitions from non-invasive radial growth phase (RGP) to dermally invasive and metastatically competent vertical growth phase (VGP), patient prognosis worsens (Airola *et al.*, 1999; Tellez *et al.*, 2006). However, few genes have been identified that contribute to the RGP to VGP transition. MMP-1 is expressed specifically by VGP melanomas, where its type I collagenase activity has been linked to invasion and metastasis (Blackburn *et al.*, 2007; Hofmann *et al.*, 2005). VGP melanomas also express PAR-1, which is associated with invasion and metastasis in other types of cancer by inducing matrix remodeling, cell adhesion, angiogenesis and survival (Arora *et al.*, 2007). While paracrine MMP-1/PAR-1 signaling between tumor and stromal cells is known to promote breast cancer progression (Boire *et al.*, 2005), the concurrent expression of MMP-1 and PAR-1 in VGP melanoma led to our hypothesis that an MMP-1/PAR-1 signaling axis promotes an invasive and metastatic phenotype in melanoma.

Our data demonstrate that an MMP-1/PAR-1 signaling axis exists in VGP melanoma. This signaling induced the expression of 20 cancer specific genes, with known functions in angiogenesis, tumor growth, inflammation, invasion and metastasis (Table I). Thrombin, which is frequently found in the melanoma microenvironment (Ornstein and Zacharski, 2001), also activates PAR-1 to induce the expression of genes involved with melanoma progression. Interestingly, while there was some overlap between the genes induced by MMP-1 and thrombin, such as IL-8 and uPA (Tellez and Bar-Eli, 2003), thrombin and MMP-1 may differentially induce gene expression in melanoma cells via PAR-1, as MMP-1 did not induce the expression of MMP-2 or integrins, which are induced by thrombin (Tellez and Bar-Eli, 2003). MMP-1, however, induced the expression of several growth factors

(FGFR2 and IGF1) and genes linked to metastasis (SERPINB5 and S100A4), which have not been associated with thrombin/PAR-1 signaling. This is in agreement with our previous work (Blackburn and Brinckerhoff, 2008), which demonstrated that activation of PAR-1 by MMP-1 and thrombin can have separate, and additive, effects.

The collagenase activity of MMP-1 is important for melanoma progression, yet data presented here indicate that MMP-1 activation of PAR-1 is also critical for melanoma invasion through the ECM (Figure 3). For example, MMP-1 activation of PAR-1 signaling induced invasion through basement membrane, while the collagenase function of MMP-1 had no role this process. Conversely, while MMP-1/PAR-1 signaling was necessary for invasion of VMM12 cells through type I collagen, cells could not physically move through the collagen barrier without MMP-1 collagenolytic activity (Figure 3c). These findings provide a new model for MMP-1 in melanoma progression, where MMP-1 activation of PAR-1 signaling induces pro-invasive gene expression in the tumor cells, and the collagenase function of MMP-1 remodels the collagen-rich dermis. Both actions of MMP-1 would be necessary for the acquisition of the VGP phenotype.

Currently, it is unclear if MMP-1 expression is a consequence of the biochemical changes which lead to VGP, or whether MMP-1 itself directly contributes to the conversion of RGP melanoma to VGP. Factors in the tumor microenvironment induce MMP-1 expression in melanoma (Figure 6a (Ishii *et al.*, 2003; Loffek *et al.*, 2005; Rothhammer *et al.*, 2008)), and MMP-1 also strongly induced MMP-1 expression in Bowes RGP cells (Figure 6b). This suggests a feed-forward mechanism: a slight induction of MMP-1 by factors within the microenvironment induces MMP-1/PAR-1 signaling, leading to increased expression of MMP-1 by the RGP cells (Figure 6c). MMP-1/PAR-1 signaling did not contribute to MMP-1 expression in VMM12 cells (Figure 2a), perhaps because VMM12 cells have an activating B-RAF mutation that is largely responsible for MMP-1 expression in these cells (Huntington *et al.*, 2004). Thus, the induction of MMP-1/PAR-1 signaling by exogenous factors may provide a mechanism by which a less advanced RGP melanoma increases MMP-1 expression to facilitate tumor progression.

MMP-1 induced several aspects of the VGP phenotype in RGP cells, including the expression of pro-tumorigenic genes (Table I), invasion through type I collagen *in vitro* and increased tumor growth *in vivo*. Importantly, MMP-1 expression conferred metastatic capability in Bowes tumors (Figure 5), suggesting that MMP-1 may be sufficient to induce a metastatic phenotype in melanoma. However, not every MMP-1 expressing tumor was metastatic, and it is unclear whether MMP-1 expression itself is sufficient to induce metastasis, or if the metastatic tumors gained additional mutations. The latter scenario seems likely, as MMP-1 expression in the RGP cells did not permit invasion through basement membrane, which is essential for tumor cells to enter the vasculature and to extravasate at the site of metastasis (Curran and Murray, 2000). However, none of the Bowes parental or Bowes-pCMV control tumors metastasized, demonstrating that MMP-1 is central to the acquisition of the metastatic phenotype in melanoma.

In conclusion, these data demonstrate that an MMP-1/PAR-1 signaling axis exists in melanoma, and that the combined PAR-1 activating and collagenolytic functions of MMP-1

are necessary for tumor cell invasion. Additionally, we found that MMP-1 expression is sufficient to promote aspects of a metastatic phenotype in non-invasive melanoma cells, suggesting that MMP-1 plays an important role in the transition of melanoma from benign to malignant disease.

Materials and methods

Cell culture, conditioned media and activation of MMP-1

VMM12 cells were cultured as described (Huntington *et al.*, 2004). Bowes cells were from ATCC (Manassas, VA, USA) and cultured according to manufacturer's directions. For serum-free conditions, cells were cultured in media supplemented with 0.2% lactalbumin hydrolysate. For conditioned media, 5×10^6 were plated in 10cm dishes, and after 24hr, media were switched to 4mL serum-free media. For all experiments, purified MMP-1 or MMP-1 in conditioned media was activated using 10μg/mL trypsin for 1hr at 37°C. A 4-fold molar excess of soybean trypsin inhibitor (SBTI, Sigma, St. Louis, MO, USA), was added to neutralize the trypsin (Suzuki *et al.*, 1990). Controls were similarly treated with trypsin/SBTI.

Reagents

Purified MMP-1 was from Abcam (Cambridge, MA, USA). Purified human α-thrombin was from Hematological Technologies (Essex Junction, VT, USA). The PAR-1 antagonist SCH79797 was from Tocris (Ellisville, MO, USA). The MMP inhibitors II and V were from Calbiochem (Gibstown, NJ, USA). The thrombin inhibitor hirudin was from Sigma. TNF, VEGF and bFGF were from BD Biosciences (San Jose, CA, USA). The following antibodies were from Cell Signaling (Danvers, MA, USA): mouse anti-phospho-MEK, mouse anti-phospho-p38, mouse anti-human actin. Mouse anti-human PAR-1 was from Beckman-Coulter (Miami, FL, USA), rabbit anti-human MMP-1 and MMP-1 neutralizing antibody were from Calbiochem, and mouse anti-FLAG from Abcam.

Exogenous MMP-1 expression

The Tag2B-CMV and Tag2B-CMV-MMP1 expression plasmid were described (Wyatt *et al.*, 2005). Bowes cells were transfected using Lipofectamine 2000, according to manufacturer's directions (Invitrogen, Carlsbad, CA, USA), and stable transfectants were selected with 1mg/ml G418. Clones were examined for MMP-1 expression by realtime-RT PCR (see below), and Bowes-pCMV-MMP1 clones with >1000-fold increase in MMP-1 expression compared to the parental Bowes line were pooled. Bowes-pCMV clones with no significant change in MMP-1 expression compared to the parental line were pooled.

MMP-1 and PAR-1 knockdown

pSuper-H1-MAMMX and pSuper-H1-MMP1 shRNA expression plasmids were described (Blackburn *et al.*, 2007). PAR-1 shRNAs were designed using the Block-IT shRNA algorithm (Invitrogen), and cloned into the psiRNA-H1 expression vector (Invivogen, San Diego, CA, USA), according to manufacturer's directions. The PAR-1 shRNAs targeted the following sequences: shPAR1#1: 5′-GCCTCCCACTAAACATCA-3′, shPAR-1#3: 5′-GCGCATTACTCATTCCTT-3′, shPAR-1#4: 5′-CCAAGGGAATATTGCCAA-3′.

VMM12 cells were transfected with the PAR-1 shRNA constructs using Lipofectamine 2000, and stable transfectants were selected using 500μg/mL zeocin (Invivogen). Clones with >80% knock-down of PAR-1 expression, measured by realtime RT-PCR (below), were pooled.

PAR-1 cleavage assays

For the AP-PAR1 assay, VMM12 cells were co-tranfected with AP-PAR1 (Ludeman *et al.*, 2005) and pCMV-eGFP (Wyatt *et al.*, 2005) expression constructs using Lipofectamine 2000. After 24hr, 5×10^4 cells were plated in 24-well dishes in regular growth media for an additional 24hr. Cells were washed with PBS and treated with VMM12 conditioned serum-free media, or non-conditioned serum-free media (control). After 1hr, media were collected and used in the Attophos Alkaline Phosphatase Assay kit (Promega, Madison, WI, USA) to determine the amount of AP-PAR-1 that had been cleaved. Data were normalized to the GFP fluorescence in each well. For calcium flux assays, VMM12 cells (10^4) were plated in 96-well dishes in regular growth media for 24hr, then treated with Fluro-4-NW dye (Invitrogen) according to manufacturer's directions. Cells were treated for 1hr with serum-free media conditioned for 24hr by the VMM12 cells, or non-conditioned serum-free media as a negative control. VMM12 cells were also treated with 10nM thrombin in serum-free media as a positive control. All media were treated with trypsin/SBTI as described above.

Gene expression analysis

Cells (10^5) were plated in 6-well dishes. Bowes cells were treated with either 5nM MMP-1 or PBS for 24hr in media containing only 1% FBS, and other cell lines were treated for 24hr with media containing 1% FBS that had been previously conditioned for 24hr by the same cell line, and had MMPs activated. RNA was harvested using the RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA), and 5μg total RNA was used in the Human Cancer Pathway RT^2 Profiler PCR array (SA Biosciences, Frederick, MD, USA), following the manufacturer's protocol. For other experiments, realtime RT-PCR was performed as described (Blackburn *et al.*, 2007), using the primer sets listed in Supplementary Table I. All data were analyzed using the $2^{\Delta\Delta C(t)}$ method, normalized to GAPDH.

Immunoblotting

Cells (10^5) were plated in 6-well plates in regular growth media for 24hr, then media were switched to 1mL serum-free media. Cells were lysed in 2× Laemmeli buffer, and proteins were precipitated from the media using 10% trichloroacetic acid. Western blots were performed as described (Petrella *et al.*, 2005). All antibodies were used at a 1:1000 dilution.

Proliferation assay

Cells (10^4) were plated in 12-well dishes in media containing 1% FBS that had been conditioned by the same cell line for 24hr, and had MMPs activated. Every 48hr, cells were harvested, stained with trypan blue, and viable cells were counted. For some experiments, cells were treated with 50μM SCH79797 or DMSO.

Collagen degradation and invasion assays

Collagen degradation assays were performed as described (Wyatt *et al.*, 2005), using 10^5 cells in 500μl buffered type I collagen (Organogenesis, Boston, MA, USA), supplemented with 20μL/mL 0.05% trypsin (Mediatech, Manassas, VA, USA) to activate MMP-1. Media released due to collagen degradation were collected after 48hr and weighed. Invasion assays were performed as described (Petrella *et al.*, 2005), using fluroblock transwells (BD Biosciences) coated with 1mg/mL type I collagen or 1mg/mL Matrigel (BD Biosciences). Cells were plated in the upper well in serum-free media conditioned by the same cell line, with MMPs activated, and media containing 10% FBS was used in the lower well as a chemoattractant. For some experiments, 5nM thrombin was added to the upper chamber. After 24hr, invaded cells were stained with CalceinAM (BD Biosciences). Fluorescent cells were counted in 3 fields per transwell at 20× magnification, and micrographs taken. Images were converted to grey scale and colors inverted.

Tumor growth and analysis of metastasis

Cells were stained with trypan blue, viable cells were counted using a hemocytometer, and then 10^7 live cells were resuspendend in 500μL PBS. Female nude mice (strain *nu/nu*, Charles River, Wilmington, MA, USA) were injected intradermally (10^6 cells, 50μL) into the right flank, 8 mice per group. Tumors were measured weekly with calipers. When tumors reached 10mm diameter, or after 12 weeks, mice were sacrificed, and the draining and contralateral lymph nodes and the right lung were fixed, sectioned and stained with anti-human MART1 to visualize metastases (Department of Research Pathology, Dartmouth-Hitchcock Medical Center). To quantify the human DNA in the lung due to metastases, DNA was prepared from the left lung of each mouse, and PCR for human ALU sequences was performed (Blackburn *et al.*, 2007). Animal studies were approved by the Institutional Animal Care and Use Committee at Dartmouth College.

Statistical analysis

All experiments were done in triplicate, at least 3 separate times. All numerical values represent the mean ± SD. Statistical significance was calculated using the Student's t-test and was assigned to values <0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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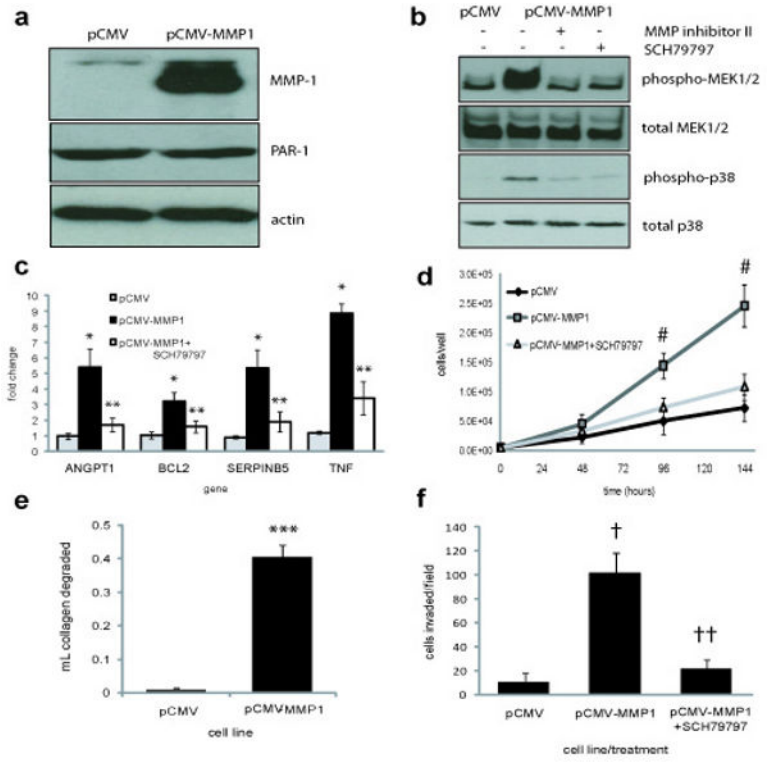

Figure 1.

PAR-1 cleavage by MMP-1 occurs in VMM12 VGP melanoma cells.

(**a**) Western blot analysis of MMP-1 protein production by normal melanocytes, Bowes RGP melanoma cells, and VMM12 VGP melanoma cells, and analysis PAR-1 protein expression by normal endothelial cells, Bowes and VMM12 cells. PAR-1 blots were re-probed for actin, as a loading control. MMP-1 band is 54kD, PAR-1 is 61kD, actin is 43kD. (**b**) VMM12 cells were transfected with AP-PAR1, and treated with media conditioned for 24hr by either Bowes or VMM12 cells. The amount of alkaline phosphatase in the media, due to PAR-1 cleavage, was measured after 1hr. (**c**) VMM12 conditioned media (CM) were treated with either DMSO, 0.05U/mL hirudin (thrombin inhibitor), 5μM MMP inhibitor II, which blocks activity of MMP-1,-3,-7,-9 or 5μM MMP inhibitor V, which blocks MMP-2,-3,-8,-9,-12,-13 activity. MMP-1 neutralizing antibody or anti-FLAG (IgG control) were added at the indicated concentration to VMM12 CM. Media were used to treat AP-PAR1 transfected VMM12 cells for 1hr. Alkaline phophatase activity was measured to quantify PAR-1 cleavage. *p=0.02 and **p<0.001, compared to anti-FLAG IgG treatment, ***p<0.001, compared to DMSO treatment. (**d**) Calcium flux in VMM12 cells was measured using Fluro-4-NW dye. Cells were loaded with dye, and then treated for 1hr with 10nM thrombin in serum-free media (positive control) or VMM12 conditioned media (CM). Calcium flow into the cells was measured by quantifying the fluorescence in each well. CM were also treated with DMSO, 1μg/mL anti-FLAG, 0.05 hirudin, 1μg/mL anti-MMP-1, or VMM12 cells were treated with 50nM SCH79797. Because data were not significantly different between VMM12 CM, DMSO and anti-FLAG treatments, results were pooled as "VMM12 CM" to simplify the graph. For all experiments, MMPs in the CM were activated

as described, and data are representative of at least 3 individual experiments. #$p<0.001$, compared to VMM12 CM.

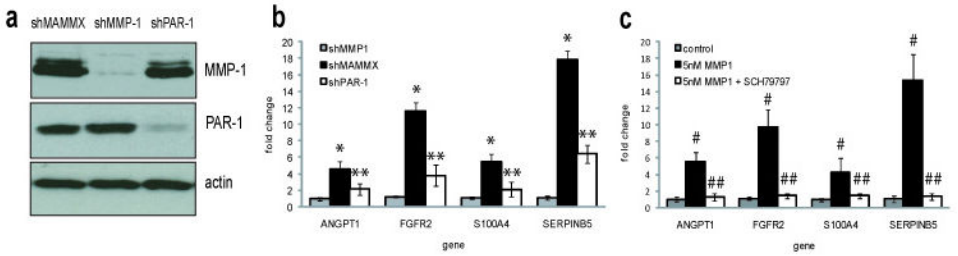

Figure 2.

MMP-1 induces gene expression in VMM12 cells via PAR-1 activation.

(**a**) Western blot analysis of MMP-1 and PAR-1 protein production by VMM12 cells stably transfected with scrambled control shRNA (shMAMMX), MMP-1 shRNAs (shMMP-1) and PAR-1 shRNAs (shPAR-1). PAR-1 blots were re-probed for actin, as a loading control. MMP-1 band is 54kD, PAR-1 is 61kD, actin is 43kD. (**b**) shMAMMX, shMMP-1 and shPAR-1 cells were treated with media conditioned by the same cell line for 24hr, with MMPs activated as described. Gene expression was measured by realtime RT-PCR. *$p \leq 0.002$, compared to shMMP-1 gene expression, **$p \leq 0.025$, compared to shMAMMX gene expression. (**c**) shMMP-1 cells were treated with either DMSO (control), 5nM activated MMP-1 or 5nM MMP-1+50nM SCH79797. After 24hr, cells were harvested and gene expression measured by realtime-RT PCR. #$p \leq 0.003$ compared to shMMP-1 control, ##$p \leq 0.005$, compared to treatment with 5nM MMP-1. For all, data were normalized to GAPDH, and were analyzed by the $2^{\Delta\Delta C(t)}$ method, and are representative of 3 experiments.

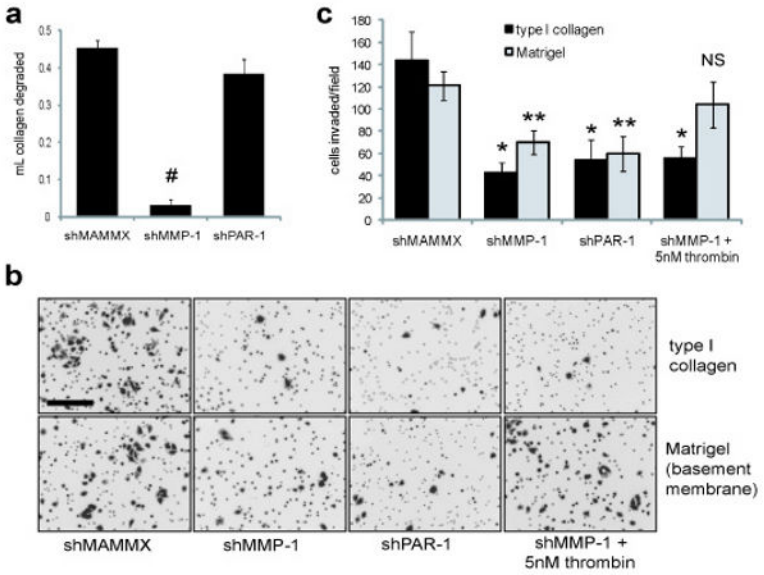

Figure 3.

Both the collagenase and PAR-1 activating functions of MMP-1 are required for melanoma cell invasion.

(**a**) VMM12 shRNA lines were used in a type I collagen degradation assay. Cells were embedded in type I collagen, and after 48hr, the media released from the collagen gel were weighed to determine the amount of collagen that had been degraded. #$p<0.001$, compared to collagen degradation by shMAMMX cells. (**b**) VMM12 shRNA lines were used in invasion assays. Cells were plated on fluroblock transwells coated with either 1mg/mL type I collagen or 1mg/mL Matrigel, as described in Materials and Methods. The lower chamber was filled with media containing 10% FBS, as a chemoattractant. For some experiments, the shMMP-1 cells were treated with 5nM thrombin in the upper chamber to activate PAR-1. After 24hr, invaded cells were stained with CalceinAM dye. Micrographs shown are representative of at least 3 experiments. Scale bar = 100μm. (**c**) Quantification of invaded cells from (**b**), with 3 fields counted per well. Data are representative of 4 individual experiments. *$p<0.001$, compared to invasion through type I collagen by shMAMMX cells, **$p<0.001$, compared to invasion through Matrigel by shMAMMX cells, NS, not significant compared to shMAMMX.

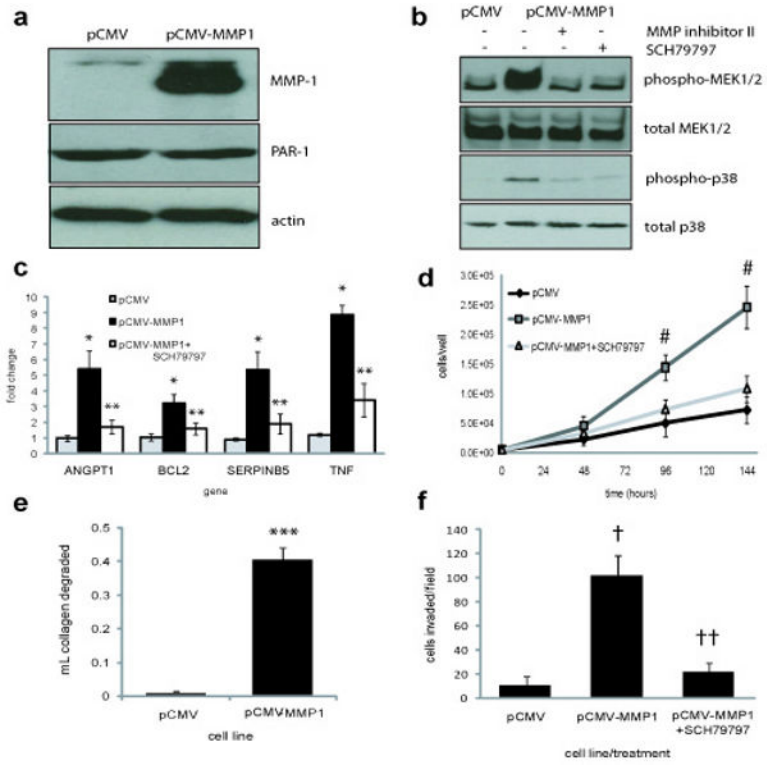

Figure 4.

MMP-1 expression in Bowes RGP cells induces some aspects of the VGP phenotype *in vitro*, via PAR-1 activation.

(**a**) Bowes cells were stably transfected with pCMV (empty vector control) or pCMV-MMP1. MMP-1 and PAR-1 protein levels were measured by western blot. PAR-1 blots were re-probed for actin, as a loading control. MMP-1 band is 54kD, PAR-1 is 61kD, actin is 43kD. (**b**) Bowe-pCMV and Bowes-pCMV-MMP1 cells were serum-starved for 2hr, then treated for 15′ with media from the same cell line, with MMPs activated as described. Media were treated with either DMSO (-), 5μM MMP inhibitor II, or cells were pre-treated with 50nM SCH79797, as indicated. The phosphorylation status of MEK1/2 and p38 were examined by western blot of the cell lysates. Blots were re-probed with antibodies against the corresponding total protein. MEK1/2 band size is 44kD, p38 is 38kD. (**c**) Realtime RT-PCR was used to measure the expression of selected genes in cells treated with media conditioned by the same cell line, with MMPs activated. Cells were treated with either DMSO or 50nM SCH79797. Data are normalized to GAPDH expression and were analyzed using the $2^{-\Delta\Delta C(t)}$ method. *$p \leq 0.002$, compared to pCMV gene expression, **$p \leq 0.015$, compared to Bowes-MMP1 gene expression. (**d**) Cells were plated in media conditioned by the same cell line, with MMPs activated, and viable cells were counted after 48, 96, and 144hr. Cells were treated with either DMSO or 50μM SCH79797. #$p<0.001$, compared to pCMV-MMP1+SCH79797. (**e**) Cells were used in a type I collagen degradation assay. Media released due to collagen degradation were quantified after 48hr. ***$p<0.001$, compared to pCMV transfected cells. (**f**) Cells were plated in type I collagen invasion assays as described. Cells were treated with either DMSO or 50nM SCH79797. †$p<0.001$,

compared to Bowes-pCMV, ††p<0.001, compared to Bowes-pCMV-MMP1. All data shown are representative of 4 individual experiments.

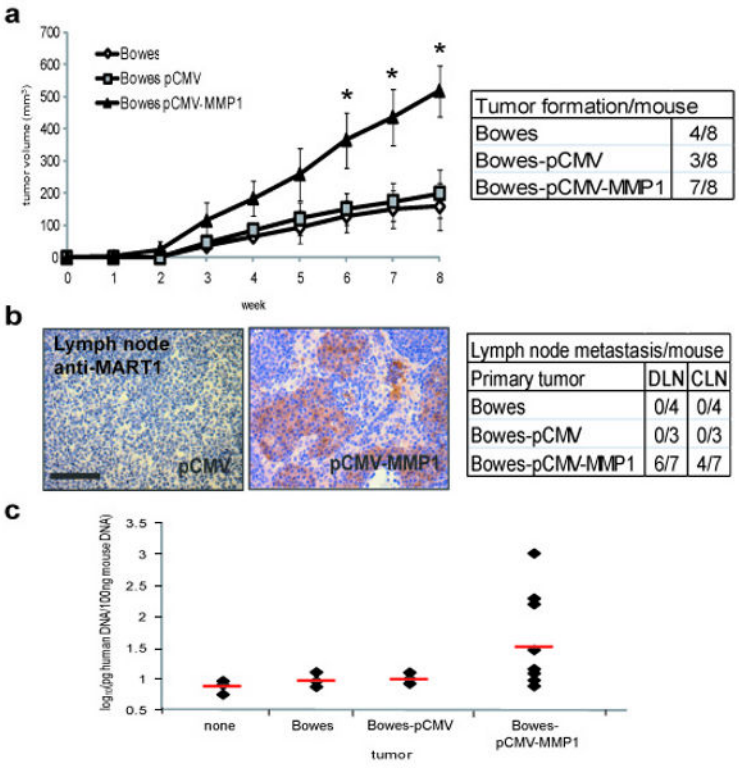

Figure 5.

MMP-1 expression in Bowes RGP cells promotes tumor growth and metastasis.

(**a**) Bowes, Bowes-pCMV and Bowes-pCMV-MMP1 cells were injected intradermally into nude mice (10^6 cells/injection). Tumor incidence was noted (table) and tumors were measured weekly with calipers. *p<0.01, compared to Bowes-pCMV. (**b**) Draning (DLN) and contralateral (CLN) lymph nodes from tumor bearing mice were stained with anti-human MART-1. Micrographs are representative of DLN from each group. Scale bar=100μm. Lymph nodes positive for MART-1 staining were quantified (table). (**c**) ALU PCR was performed as described to quantify the amount of human DNA in the lungs of tumor bearing mice. Naïve mice were used as a negative control. Each point represents a sample from one mouse. Horizontal lines are the average for each group. Note that the data are in log scale.

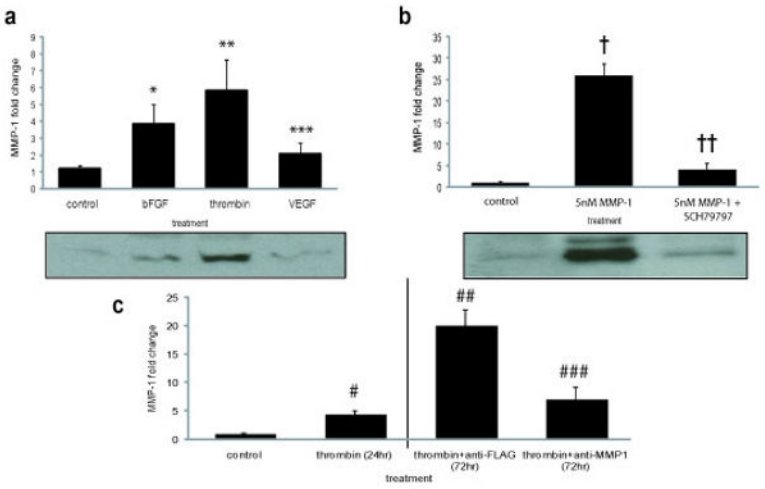

Figure 6.

Factors in the tumor microenvironment may induce MMP-1 expression in Bowes RGP melanoma cells, and MMP-1 strongly induces MMP-1 expression via PAR-1.

(**a**) Bowes cells were treated for 24hr in serum-free media with 5nM thrombin, 25ng/mL bFGF, 10ng/mL VEGF. MMP-1 expression was measured by realtime-RT PCR. Data were normalized to GAPDH, and analyzed by the $2^{\Delta\Delta C(t)}$ method. **p*=0.015, ***p*=0.002, ****p*=0.042, compared to control. The corresponding western blot is also shown, with an exposure time of 5 minutes. The MMP-1 band size is 54kD. (**b**) Bowes cells were treated with DMSO, 5nM MMP-1 or 5nM MMP-1+50nM SCH79797. After 24hr, MMP-1 expression was measured by realtime-RT PCR. Data were normalized to GAPDH expression and analyzed using the $2^{(-\Delta\Delta Ct)}$ method. †*p*<0.001 compared to control, ††*p*<0.001 compared to 5nM MMP-1. Media were also collected and used for western blot to measure MMP-1 protein (30 sec exposure). (**c**) Bowes cells were treated with 5nM thrombin for 24hr in media containing 1% FBS. Media were collected and MMPs activated, then treated with 1μg/mL MMP-1 neutralizing antibody or anti-FLAG IgG control, as indicated. Media were added back to cells for an additional 48hr, for 72hr total treatment. MMP-1 expression was examined using realtime RT-PCR. #*p*=0.02 compared to control, ##*p*<0.001, and ###*p*=0.05, compared to treatment with thrombin for 24hr.

Table I

MMP-1 induces gene expression in melanoma cells

Bowes	VMM12		
MMP-1 vs PBS	shMX vs shMMP-1	Gene Symbol	Gene Name
Angiogenesis			
3.81	3.27	ANGPT1	Angiopoietin 1
3.58	3.53	IL8	Interleukin 8
2.35	6.28	MMP9	Matrix metalloproteinase 9
2.27	3.29	TEK	TEK tyrosine kinase
Cell Division/Apoptosis			
4.08	NC	BAD	BCL2-antagonist of cell death
3.61	NC	BAX	BCL2-associated X protein
2.20	NC	BCL2	B-cell CLL/lymphoma 2
2.55	NC	BRCA1	Breast cancer 1, early onset
2.89	NC	CCNE1	Cyclin E1
2.23	NC	CDK2	Cyclin-dependent kinase 2
2.30	NC	CDK4	Cyclin-dependent kinase 4
3.68	2.39	CDKN1A	Cyclin-dependent kinase inhibitor 1A
2.62	2.27	CDKN2A	Cyclin-dependent kinase inhibitor 2A
Growth Factors			
2.33	2.95	ERBB2	V-erb-b2
NC	12.38	FGFR2	Fibroblast growth factor receptor 2
3.76	8.82	IGF1	Insulin-like growth factor 1
NC	2.35	PDGFA	Platelet-derived growth factor alpha
3.23	NC	TGFB1	Transforming growth factor, beta 1
2.50	2.06	TGFBR1	Transforming growth factor, beta receptor I
Inflammation			
NC	2.50	IFNA1	Interferon, alpha 1
8.75	NC	IFNB1	Interferon, beta 1, fibroblast
3.58	3.53	IL8	Interleukin 8
7.41	2.33	TNF	Tumor necrosis factor
Transcription Factors			
2.27	2.25	E2F1	E2F transcription factor 1
2.46	NC	FOS	V-fos FBJ murine osteosarcoma oncogene homolog
2.30	2.17	JUN	Jun oncogene
2.32	2.72	MAP2K1	Mitogen-activated protein kinase kinase 1
2.97	2.13	MYC	V-myc myelocytomatosis viral oncogene homolog

Bowes	VMM12		
MMP-1 vs PBS	**shMX vs shMMP-1**	**Gene Symbol**	**Gene Name**
2.95	NC	NFKB1	Nuclear factor of kappa gene enhancer in B-cells 1
2.22	NC	NFKBIA	NFKB alpha
Metastasis Associated			
2.38	NC	MDM2	Mdm2, transformed 3T3 cell double minute 2
23.92	66.26	MMP1	Matrix metalloproteinase 1
2.35	6.28	MMP9	Matrix metalloproteinase 9
2.60	NC	MTA1	Metastasis associated 1
2.51	NC	MTA2	Metastasis associated 1 family, member 2
NC	4.14	PLAU	Plasminogen activator, urokinase
2.00	2.17	S100A4	S100 calcium binding protein A4
3.81	17.88	SERPINB5	Serpin peptidase inhibitor, clade B, member 5
2.14	2.75	SERPINE1	Serpin peptidase inhibitor, clade E, member 1

Values=fold increase in gene expression, NC=no change compared to control