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

Jessica S. Blackburn1, Ingrid Liu2, Charles I. Coon1, and Constance E. Brinckerhoff1,2

1 Department of Biochemistry, Dartmouth Medical School, Hanover, NH, USA
2 Department of Medicine, Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Lebanon, NH 03756, USA

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
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 overexpressed 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 \textit{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

\textbf{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 \textit{et al.}, 2007; Huntington \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 RT² 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 $\sim$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′-GCGCATTACTCACTACATCA-3′, shPAR-1#3: 5′-GCGCATTACTCACTTCT-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, 5x10^4 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 RT² 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^ΔΔC(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 2x 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 (10^5) 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 20x 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 resuspended 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.

Acknowledgments

The authors would like to thank Shaun Coughlin (University of California, San Francisco) for his generous gift of the AP-PAR1 construct. This work was supported by NIH grants CA-77267 and AR-26599 (to C.E.B.), NIH grant T32-AI07363 (to J.S.B.) and the Prouty Pilot Grant by the Friends of the Norris Cotton Cancer Center (to C.E.B.).

References

Agarwal A, Covic L, Sevigny LM, Kaneider NC, Lazarides K, Azabdaftari G, et al. Targeting a metalloprotease-PAR1 signaling system with cell-penetrating pepducins inhibits angiogenesis,
ascites, and progression of ovarian cancer. Mol Cancer Ther. 2008; 7:2746–2757. [PubMed: 18790755]
Airola K, Karonen T, Vaalamo M, Lehti K, Lohi J, Kariniemi AL, et al. Expression of collagenase-1 and -3 and their inhibitors TIMP-1 and -3 correlates with the level of invasion in malignant melanomas. Br J Cancer. 1999; 80:733–743. [PubMed: 10360651]
Arora P, Ricks TK, Trejo J. Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer. J Cell Sci. 2007; 120:921–928. [PubMed: 17344429]
Balch C, Soong SJ, Atkins M, Buzaid A, Thompson J. An evidence-based staging system for cutaneous melanoma. CA Cancer J Clin. 2004; 54:131–149. [PubMed: 15195788]
Berwick M, Erdei E, Hay J. Melanoma Epidemiology and Public Health. Dermatologic Clinics. 2009; 27:205–214. [PubMed: 19254665]
Blackburn JS, Brinckerhoff CE. Matrix Metalloproteinase-1 and Thrombin Differentially Activate Gene Expression in Endothelial Cells via PAR-1 and Promote Angiogenesis. Am J Path. 2008; 173:1736–1746. [PubMed: 18988801]
Blackburn JS, Rhodes CH, Coon CI, Brinckerhoff CE. RNA Interference Inhibition of Matrix Metalloproteinase-1 Prevents Melanoma Metastasis by Reducing Tumor Collagenase Activity and Angiogenesis. Cancer Res. 2007; 67:10849–10858.10.1158/0008-5472.CAN-07-1791 [PubMed: 18006830]
Boire A, Covic L, Agarvai A, Jacques S, Sherifi S, Kuliopulos A. PAR1 is a matrix metalloproteinase-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell. 2005; 120:303–313. [PubMed: 15707890]
Breslow A. Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. Annuals of Surgery. 1970; 172:902–908.
Clark W, Ainsworth A, Bernardino E, Yang C, Mihm C, Reed R. The developmental biology of primary human malignant melanomas. Semin Oncol. 1975; 2:83–103. [PubMed: 790575]
Curran S, Murray GI. Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. Eur J Cancer. 2000; 36:1621–1630. [PubMed: 10959048]
Depasquale I, Thompson WD. Prognosis in human melanoma: PAR-1 expression is superior to other coagulation components and VEGF. Histopathology. 2008; 52:500–509. [PubMed: 18315603]
Durko M, Navab R, Shibata H, Brodt P. Suppression of basement membrane type IV collagen degradation and cell invasion in human melanoma cells expressing an antisense RNA for MMP-1. Biochim Biophys Acta. 1998; 1356:271–280.
Even-Ram SC, Maoz M, Pokroy E, Reich R, Katz BZ, Gutwein P, et al. Tumor Cell Invasion Is Promoted by Activation of Protease Activated Receptor-1 in Cooperation with the alpha vbeta 5 Integrin. J Biol Chem. 2001; 276:10952–10962. [PubMed: 11278329]
Fingleton B. Matrix metalloproteinases: roles in cancer and metastasis. Front Biosci. 2006; 11:479–491. [PubMed: 16146745]
Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature. 2007; 445:851–857. [PubMed: 17314971]
Haluska F, Ibrahim N. Therapeutic targets in melanoma: map kinase pathway. Current Oncology Reports. 2006; 8:400–405. [PubMed: 16901402]
Hofmann U, Houben R, Brocker E, Becker J. Role of matrix metalloproteinases in melanoma cell invasion. Biochimie. 2005; 87:307–314. [PubMed: 15781317]
Hofmann U, Westphal J, van Muijen G, Ruiter D. Matrix metalloproteinases in human melanoma. J Invest Dermatol. 2000; 115:337–344. [PubMed: 10951266]
Huntington JT, Shields JM, Der CJ, Wyatt CA, Benbow U, Slingluff CL, et al. Overexpression of collagenase 1 (MMP-1) is mediated by the ERK pathway in invasive melanoma cells. J Bio Chem. 2004; 279:33168–33176. [PubMed: 15184373]
Iida J, Wilhelmson KL, Price MA, Wilson CM, Pei D, Furcht LT, et al. Membrane Type-1 Matrix Metalloproteinase Promotes Human Melanoma Invasion and Growth. J Investig Dermatol. 2004; 122:167–176. [PubMed: 14962105]
Ishii Y, Ogura T, Tattemichi M, Fujisawa H, Otsuka F, Esumi H. Induction of matrix metalloproteinase gene transcription by nitric oxide and mechanisms of MMP-1 gene induction in human melanoma cell lines. International Journal of Cancer. 2003; 103:161–168.

Oncogene. Author manuscript; available in PMC 2010 June 03.
Leiter U, Friedegund M, Shittek B, Garbe C. The natural course of cutaneous melanoma. J Surg Onc. 2004; 86:172–178.

Loffek S, Zigrino P, Angel P, Anwald B, Krieg T, Mauch C. High invasive melanoma cells induce matrix metalloproteinase-1 synthesis in fibroblasts by interleukin-1α and basic fibroblast growth factor-mediated mechanisms. Journal of Investigative Dermatology. 2005; 124:638–645. [PubMed: 15737206]

Ludeman MJ, Kataoka H, Srinivasan Y, Esmon NL, Esmon CT, Coughlin SR. PAR1 Cleavage and Signaling in Response to Activated Protein C and Thrombin. J Biol Chem. 2005; 280:13122–13128. [PubMed: 15665002]

Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-Activated Receptors. Pharmacol Rev. 2001; 53:245–282. [PubMed: 11356985]

Martin C, Mahon G, Klinger M, Kay R, Symons M, Der C, et al. The thrombin receptor, PAR-1, causes transformation by activation of Rho-mediated signaling pathways. Oncogene. 2001; 20:1953–1963. [PubMed: 11360179]

Massi D, Naldini A, Ardinghi C, Carraro F, Franchi A, Paglierani M, et al. Expression of protease-activated receptors 1 and 2 in melanocytic nevi and malignant melanoma. Human Pathology. 2005; 36:676–685. [PubMed: 16021575]

Nierodzik ML, Chen K, Takeshita K, Li JJ, Huang YQ, Feng XS, et al. Protease-Activated Receptor 1 (PAR-1) Is Required and Rate-Limiting for Thrombin-Enhanced Experimental Pulmonary Metastasis. Blood. 1998; 92:3694–3700. [PubMed: 9808563]

Nikkola J, Vihinen P, Vuoristo MS, Kellokumpu-Lehtinen P, Kahari VM, Pyrhonen S. High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. Clin Cancer Res. 2005; 11:5158–5166. [PubMed: 16033831]

Nyau C, Lorimier S, Berthier-Vergnes O, Horneck W, Bernard P. Cumulative influence of matrix metalloproteinase-1 and -2 in the migration of melanoma cells within three-dimensional type I collagen lattices. Exp Cell Res. 2001; 270:110–118. [PubMed: 11597133]

Ornstein D, Zacharski L. Treatment of cancer with anticoagulants: rationale for treatment of melanoma. International Journal of Hematology. 2001; 72:157–161. [PubMed: 11372726]

Petrella BL, Lohi J, Brinckerhoff CE. Identification of membrane type-1 matrix metalloproteinase as a target of hypoxia-inducible factor-2a in von-Hippel-Lindau renal cell carcinoma. Oncogene. 2005; 24:444:1043–1052. [PubMed: 15592504]

Rothhammer T, Braig S, Bosserhoff AK. Bone morphogenetic proteins induce expression of matrix metalloproteinases in melanoma cells and fibroblasts. European Journal of Cancer. 2008; 44:2526–2534. [PubMed: 18774289]

Salah Z, Maoz M, Pokroy E, Lotem M, Bar-Shavit R, Uziely B. Protease-Activated Receptor-1 (hPar1), A Survival Factor Eliciting Tumor Progression. Mol Cancer Res. 2007; 5:229–240. [PubMed: 17374729]

Suzuki K, Enghild J, Morodomi T, Salvesen G, Nagase H. Mechanisms of activation of tissue procollagenase by matrix metalloproteinase-3 (stromelysin). Biochemistry. 1990; 29:10261–10270. [PubMed: 2176865]

Tellez C, Bar-Eli M. Role and regulation of the thrombin receptor (PAR-1) in human melanoma. Oncogene. 2003; 22:3130–3137. [PubMed: 12789289]

Tellez CS, Davis DW, Prieto VG, Gershenson JD, Johnson MM, McCarty MF, et al. Quantitative Analysis of Melanocytic Tissue Array Reveals Inverse Correlation between Activator Protein-2[alpha] and Protease-Activated Receptor-1 Expression during Melanoma Progression. J Invest Dermatol. 2006; 127:387–393. [PubMed: 16946713]

Ueda Y, Richmond A. NF-kappa B activation in melanoma. Pigment Cell Research. 2006; 19:112–124. [PubMed: 16524427]

Villares GJ, Zigel M, Wang H, Melnikova VO, Wu H, Friedman R, et al. Targeting Melanoma Growth and Metastasis with Systemic Delivery of Liposome-Incorporated Protease-Activated Receptor-1 Small Interfering RNA. Cancer Res. 2008; 68:9078–9086. [PubMed: 18974154]
Wyatt CA, Geoghegan JC, Brinckerhoff CE. Short hairpin RNA-mediated inhibition of Matrix Metalloproteinase-1 in MDA-231 cells: Effects on matrix destruction and tumor growth. Cancer Res. 2005; 65:11101–11108. [PubMed: 16322260]
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 phosphatase 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 ≤0.002, compared to shMMP-1 gene expression, **p ≤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 ≤0.003 compared to shMMP-1 control, ##p ≤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 \textit{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. \( \dagger p<0.001 \),
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) Draining (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 $2^{\Delta\Delta C(t)}$ 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 | Gene Symbol | Gene Name                        |
|-------|-------|-------------|----------------------------------|
| 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 | TGFBI | Transforming growth factor, beta 1 |
| 2.50 | 2.06 | TGFBR1 | Transforming growth factor, beta receptor 1 |
| 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 | Gene Symbol | Gene Name                                                                 |
|---------------|-------|-------------|---------------------------------------------------------------------------|
| MMP-1 vs PBS  | 2.95  | NC          | NFKB1                       | Nuclear factor of kappa gene enhancer in B-cells 1                       |
| shMX vs shMMP-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