Variability in Melanoma Metalloproteinase Expression Profiling

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The proteolytic activities of a disintegrin and metalloproteinase (ADAM); a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and matrix metalloproteinase (MMP) families play important roles in normal and multiple pathological conditions. These metalloproteases have potential roles in the degradation of the ECM and in the processing of bioactive molecules. In the present study, RNA was isolated from multiple normal fibroblast and metastatic melanoma cell lines, as well as the isogenic normal tissue and tumor samples, and the gene expression levels of six ADAMs, eight MMPs, and four ADAMTSs were analyzed by real-time PCR. This approach allowed for detected changes in mRNA expression of the individual metalloproteinase genes to be compared between normal and metastatic states and also between tissue and cultured cells. Increased gene expression of several ADAM and MMP family members (*MMP1*, *MMP8*, *MMP15*, and *ADAM15*) occurred in melanoma tissue and was replicated in tissue cultures. In general, the level of ADAM and MMP mRNA expression was several-fold higher in cultured cells compared with the isogenic tissue from which they were derived. Passage-dependent expression patterns were observed for *MMP8* and *MMP9* in in-house-derived metastatic melanoma cell lines. This reiterates earlier suggestions that experiments using cells that have been maintained in culture should be interpreted with great care.

KEY WORDS: ADAM, MMP, tissue

INTRODUCTION

Melanoma, the malignancy of the melanocytes primarly found in the skin, is one of the most highly invasive and metastatic tumors. As a result of the incidence of melanoma doubling every 15 years,[1] it is becoming an increasingly common malignancy. According to the World Health Organization, approximately 65,000 melanoma-related deaths occur per annum, contributing 0.1% to total global mortality.[2]

Despite many experimental and clinical efforts aimed at improving the criteria for melanoma diagnosis and treatment, the most reliable prognostic factor is still the tumor thickness.[3] There is no accepted histopathological, molecular, or immunohistochemical marker that clearly defines subsets of melanoma. The production of melanoma metastases depends on the completion of a multistep process involving selection, migration, survival, and growth of a unique subpopulation of cells with very specific properties.[4] Therefore, information regarding the changes in the genomic and expression level exhibited during melanoma tumorigenesis and progression could be used for classification purposes.[5,6]

Gene expression profiles provide a snapshot of cell functions and processes at the time of sample preparation. The analysis of expression patterns of numerous genes at once in tumor cells and the comparison of the expression profile obtained with healthy cells should provide insights concerning consistent changes in gene expression that are associated with tumor cellular function.[7] Members of the adamalysin [a disintegrin and metalloproteinase (ADAM) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)] and the matrixin [matrix metalloproteinase (MMP)] subfamilies of metalloendopeptidases have all been shown to take part in processes crucial to tumorigenesis and cancer progression, such as the remodeling of the ECM, processing bioactive molecules, and ectodomain shedding.[8,9] Roughly half of the ADAMs possesses an active catalytic domain and shows a sheddase activity as well as affinity toward other substrates in the extracellular space, such as cell-adhesion molecules or components of the ECM and the plasma.[10] Two ADAMs have been shown to possess in vitro proteolytic activity against

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ECM components: ADAM9 hydrolyzes fibronectin and gelatin,[11] and ADAM10 has type IV collagenase activity.[12]

The ADAMTSs are extracellular, multidomain enzymes similar to the ADAM proteins. Their known functions include collagen processing, cleaving matrix proteoglycans, inhibition of angiogenesis, and modulating blood coagulation homeostasis.[13,14] The members of the MMP family are collectively capable of digesting all known ECM macromolecules.[15] Their role in angiogenesis, tumor growth, and metastasis has been investigated and described extensively.[15]

There are very few studies about collagenolytic metalloenzyme expression profiling in melanoma.[16–18] The first aim of the current study, therefore, was to compare the expression of metalloproteinases with previously shown or presumed collagenolytic properties in melanoma tissue and cultured cells, as well as normal tissue and fibroblasts.

In addition, there are only a few reports in the literature describing the effect of the cell-culturing process on the expression pattern changes in metalloproteinases in any system. Cell lines derived from human cancers are the most widely used resource in laboratory-based cancer research.[19] Although in vitro experiments can never reproduce the complexity of a whole organism, the simplicity of cell lines provides the ability to manipulate and analyze individual parameters specifically.[20] Although most cell-based cancer research methods use cell lines, the vast majority of these cell lines was purchased from commercial sources and thus, has already undergone numerous cell divisions [usually passage 50 (P_{50})–P_{80} and above]. The long-term culturing, as well as the inherent genetic instability[21] may contribute to phenotypic changes or subtle genomic modifications occurring during the culture process.[22] Passage-dependent expression changes in cultured melanoma cells have not yet been examined. Thus, the second aim of the current study was to evaluate the effect of the culturing process on the temporal expression of metalloproteinases.

Quantitative real-time PCR (qPCR) was used to analyze the mRNA expression levels of eight ADAMs, four ADAMTSs, and seven MMPs in isogenic melanoma tissue, primary and passaged melanoma cells, normal tissue, and passaged fibroblasts. This experimental setup allowed for detected changes in expression of the individual metalloproteinase genes to be compared between tissue and cultured cells and also between the subsequent passages in culture.

MATERIALS AND METHODS

Cell Culture

All reagents were obtained from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. The in-house fibroblast and melanoma cells were derived as follows (see also[23,24]). The metastatic melanoma and normal adjacent tissue samples were obtained from the Cooperative Human Tissue Network (CHTN; Bethesda, MD, USA). Necrotic areas, connective tissue, fatty tissue, and blood vessels were removed using sterile forceps and scissors. The samples were cut into 1 mm^3-sized pieces and incubated in 100 U/mL collagenase type I in HBSS at 37°C for 1–2 h. Cells were collected by centrifugation at 1000 *g* for 8 min and resuspended in DMEM supplemented with 10% FBS and 2.5 μg/mL Amphotericin B (Fisher BioReagents, Fair Lawn, NJ, USA). After 24 h, 50% of the media was replaced. After another 24 h, media were fully replaced with DMEM supplemented with 2% FBS, 5 ng/mL EGF, 5 μg/mL insulin, 40 μg/mL bovine pituitary extract, 50 units/ml penicillin, and 0.05 mg/ml streptomycin sulfate.[23] All cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

PCR

Nucleic acid concentrations were determined as described previously.[24] Total RNA was isolated using TRIzol reagent with in-solution DNase treatment using the RNase-Free DNase set (Qiagen, Valencia, CA, USA), followed by a clean-up using the spin columns from the PureLink Micro-to-Midi Total RNA purification system. First-strand cDNA was reverse-transcribed using SuperScript III RT with random hexamers (Fisher BioReagents). The qPCR primers were manually designed or with the aid of PerlPrimer.[25] The qPCR assays had been preoptimized with regard to $MgCl_2$ and primer concentration, as well as annealing temperature. The reactions were carried out two times, each in triplicate on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) using Platinum SYBR Green qPCR SuperMix-UDG with ROX with 200 nM each forward and reverse primers and 2 μL cDNA in a final volume of 20 μL. The PCR products were identified based on their unique melting curve and confirmed by agarose gel electrophoresis. Absolute gene expression levels were determined by the aid of qPCR standards; "copy number" refers to the absolute number of copies/5 ng extracted total RNA.

Generation and Analysis of qPCR Standards

PCR products were formed using *Tfi* DNA polymerase as per the manufacturer's recommendations and subsequently purified by using the PureLink PCR purification kit. Standards were created by transforming chemically competent JM109 cells with the pGEM-T plasmid (Promega, Madison, WI, USA) carrying a single copy of each respective target PCR product. Plasmids were purified using the PureLink HQ mini plasmid DNA purification kit. The experimental samples were calibrated to the external standard

curves generated by performing the qPCR reaction using a tenfold dilution series (10^2–10^7 copies/reaction). The amplification efficiency was close to 100% in the case of all primer pairs used. The difference in amplification efficiency between two reactions was never higher than 0.8%.

RESULTS AND DISCUSSION

Sample Distribution

A panel of 23 samples was analyzed. It included four fibroblast and eight melanoma cell lines and five normal and six melanoma tissue samples (Table 1). The normal tissue samples were all normal, adjacent samples to the melanoma tissue samples. Grouping the samples as such provides the opportunity to compare the normal samples with the melanoma samples, as well as the tissue samples with the cultured cells. As a result of the derivation of the in-house melanoma cells from individual patient samples, as well as aiming to only look at general trends, the sample pool was kept to a limited number. Samples are categorized based on pathology reports, as well as the presence or absence of two widely accepted melanoma markers, tyrosinase and MelanA (primers given in Table 2).

TABLE 1

Samples used for expression profiling

Abbreviation	Type	Origin
NAT56WF	f	CHTN, lung fibroblasts
NAT56UM	f	CHTN, lung fibroblasts
NAT58WM	f	CHTN, skeletal muscle fibroblasts
BJ	f	ATCC, foreskin fibroblasts
Mel58WM	m	CHTN
Mel56UM	m	CHTN
Mel93WF	m	CHTN
Mel80WM	m	CHTN
SK-2-MEL	m	ATCC, derived from skin metastasis
M14 clone#5	m	[a]
WM-266-4	m	ATCC, derived from skin metastasis
Hs 895.T	m	ATCC, derived from lung metastasis
tNAT56WF	NT	CHTN, lung tissue
tNAT56UM	NT	CHTN, lung tissue
tNAT58WM	NT	CHTN, skeletal muscle
tNAT46WM	NT	CHTN, skeletal muscle
tNT77WM	NT	CHTN, skeletal muscle
tMel58WM	MMT	CHTN, muscle metastasis
tMel56UM	MMT	CHTN, lung metastasis
tMel93WF	MMT	CHTN, unknown
tMel46WM	MMT	CHTN, unknown
tMel77WM	MMT	CHTN, muscle metastasis
tMel80WM	MMT	CHTN, lymph node metastasis

WM, Wistar melanoma; f, fibroblast; m, melanoma; NT, normal tissue; MMT, metastatic melanoma tissue; ATCC, American Type Culture Collection (Manassas, VA, USA).

[a]Obtained as a generous gift from Dr. Barbara M. Mueller (La Jolla Institute for Molecular Medicine, La Jolla, CA, USA).

Expression of ADAMTS Family Members

Four ADAMTS genes were chosen for the present study (Table 2; Fig. 1). ADAMTS13 was included, as it was cloned originally from a metastatic melanoma cell line.[26] This protease, however, proved to be undetectable on the mRNA level in all of the samples. All three procollagen N-propeptidases (*ADAMTS2*, *ADAMTS3*, *and ADAMTS14*) showed very low baseline expression. *ADAMTS2* showed the highest overall expression in the studied samples; however, this was still on the order of only 10^3–10^4 copies/5 ng RNA. The fibroblast samples expressed the highest copy numbers for these targets. The expression of the *ADAMTS* genes in all other samples was between 0 and 10^2 copies/5 ng RNA.

Expression of ADAM Family Members

Eight ADAM family members were examined herein (Table 2; Fig. 2A). *ADAM8*, *ADAM28,* and *ADAM33* showed equally low baseline expression in melanoma and normal tissues, which all but disappeared in cultured cells. The one exception was *ADAM33*, where expression was increased over tenfold in fibroblasts. ADAM33 causes rapid induction of endothelial cell differentiation in vitro and angiogenesis ex vivo and in vivo.[27] This proliferative property might be one induced based on microenvironmental effects (see below).

Three of the ADAMs (*ADAM10*, *ADAM12*, and *ADAM15*) showed more than a twofold increase in melanoma tissue compared with the normal tissue samples. *ADAM10* was decreased slightly in the cultured melanoma cells compared with fibroblasts. This ADAM was over tenfold increased in the cell lines compared with the tissue samples. ADAM10 is involved in the shedding of many membrane-bound proteins, such as TNF-α, ErbB ligands betacellulin (BTC), and EGF, Notch, and E-cadherin.[28–30] Consequently, changes in ADAM10 catalytic activity are implicated in carcinogenesis. Excess ADAM10 activity may promote cell growth in cancer proliferation assays, because of enhanced production of soluble, mature forms of BTC and EGF.[31]

ADAM12 was threefold decreased in the cultured melanoma cells compared with fibroblasts. However, this ADAM was approximately tenfold increased in the cell lines compared with the tissue samples, especially in the fibroblasts. ADAM12 functions as a sheddase, adhesion molecule, and ECM-degrading proteinase and is involved in cancer progression. Its cysteine-rich domain is known to support tumor cell adhesion through syndecans, which

TABLE 2

Primers used in this study

Target	Sequence
HTYR	F-TGACCCAATATGAATCTGGT
	R-GGACTAGCAAATCCTTCCAG
MelanA	F-CTCATTAAGGAAGGTGTCCTG
	R-GGGTAACCATAGATGAAGTGAG
ADAM08	F-ATCATGGCAGGCAGCATT
	R-CCAAAAAGCTCTCCAGGTAGG
ADAM09	F-GCGGGATTAATGTGTTTGGA
	R-TGCTCCACAGGAACAATCTCT
ADAM10	F-CAGAACTATGGGTCTCATGTACCTC
	R-CTCTGTTCCAGAATCATGTGG
ADAM12	F-GTCAAATGGCGGTTGAGAAA
	R-AACACCATGGGAAATGGGTA
ADAM15	F-TCATTCTCTGGGCCTACGG
	R-TCCATGTTCACACCTCCTGA
ADAM17	F-CAAAGGAAGCTGACCTGGTT
	R-TATTTCCCTCCCTGGTCCTC
ADAM28	F-CCTTATTCTGTTGGCGTTGTTCA
	R-CCAAAGTTGTGGCCCATTTC
ADAM33	F-ACCGAAACTTGAACCACACC
	R-GTCCTGAGAAGCTGGTCCAC
MMP1	F-GACTTAGTCCAGAAATACCTG
	R-CAAAGAATTCCTGCATTTGC
MMP2	F-GAATACCATCGAGACCATGC
	R-AGCCAATGATCCTGTATGTG
MMP3	F-CTGTTGATTCTGCTGTTGAG
	R-AAGTCTCCATGTTCTCTAACTG
MMP8	F-CCAGCAAGAACATTTCTTCC
	R-CAGTTAAGCCATTTATTGCCTC
MMP9	F-GGTAAGGAGTACTCGACCTG
	R-ACAAACTGTATCCTTGGTCC
MMP13	F-AAATTATGGAGGAGATGCCC
	R-AACAAGTTGTAGCCTTTGGA
MMP14	F-TACCGACAAGATTGATGCTG
	R-AACGGTAGTACTTGTTTCCAC
MMP15	F-CATGGAAACAACCTCTTCCT
	R-CTTGAAGTTGTCAACGTCCT
ADAMTS2	F-TATGGAAAGTCCATGAGCCT
	R-GATCGTGGTATTCATCGTGG
ADAMTS3	F-ATATGCAAAGTCCATCAGCC
	R-GTTCAGAGTGGTTGAGATCAG
ADAMTS13	F-AACCTTCTACAGAGAATGTGAC
	R-GGAGCCACATTAATGAAGAG
ADAMTS14	F-GGAGGAAATCACCAGAACTC
	R-TCTTGTTAATGGGATGTAGGG

HTYR, Human tyrosinase; F, forward; R, reverse.

trigger signaling events and lead to β1 integrin-dependent cell spreading.[32,33] ADAM12 also cleaves various ECM molecules including gelatin, type IV collagen, and fibronectin, suggesting a potential role for this enzyme in ECM digestion in cancer invasion and metastasis.[34] ADAM12 is also up-regulated in cancers of stomach, liver, and colon, and ADAM12 levels in urine correlate with breast cancer progression.[34]

ADAM9 showed the highest overall expression in all sample types, with slightly higher copy numbers in the normal tissue samples and the fibroblasts. It also showed the greatest increase in cultured cells compared with the tissue samples from which they were derived. *ADAM15* was more than twofold increased in melanoma tissue, as well as in the melanoma cells, compared with the normal tissue samples and fibroblasts. In a previous study, the expression of ADAM9 was analyzed in melanoma in vivo and in melanoma cell lines in vitro.[17] The protein expression appeared to be restricted to the melanoma cells within the invading front. mRNA analysis showed ADAM9 expression in varying amounts in all cell lines, independent of their invasive and metastatic properties. ADAM9 is up-regulated in breast, pancreatic, and stomach cancers.[35] It has been suggested that ADAM9 plays a role in tumorigenesis, invasion, and metastasis through modulation of growth factor activity and integrin function.

ADAM15 was increased fivefold in the cultured cells compared with the tissue samples. The data about the role of ADAM15 in cancers are inconsistent. Expression of ADAM15 is up-regulated in various cancers of the breast, prostate, stomach, and lung, and treatment of carcinoma cell

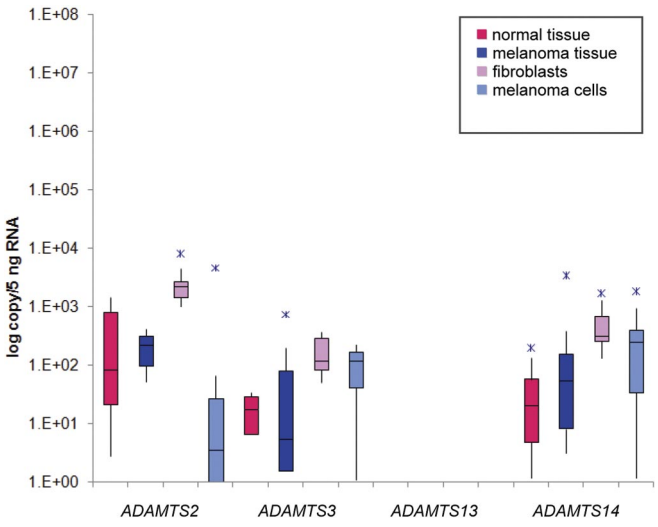

FIGURE 1

Expression of *ADAMTS2*, *ADAMTS3*, *ADAMTS13*, and *ADAMTS14* in normal and melanoma tissue samples, fibroblasts, and melanoma cells.

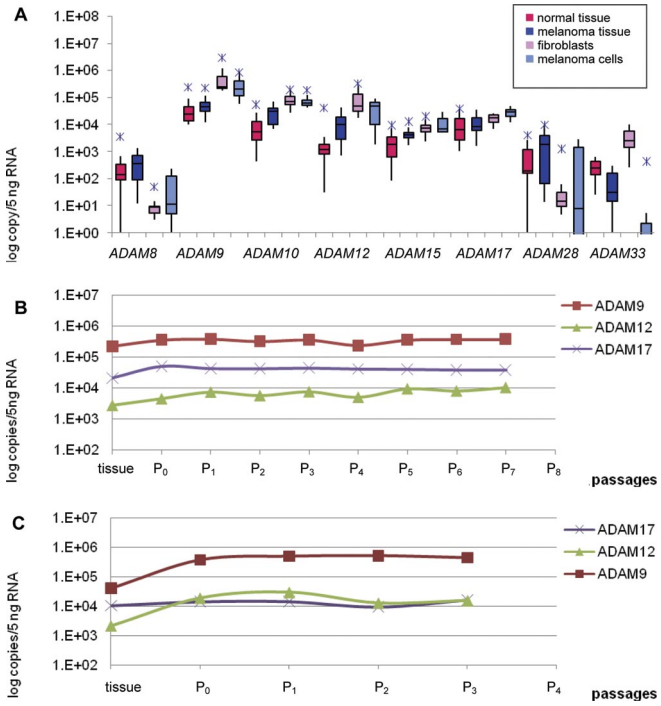

FIGURE 2

ADAM expression profiling. (*A*) Expression of *ADAM8*, *ADAM9*, *ADAM10*, *ADAM12*, *ADAM15*, *ADAM17*, *ADAM28*, and *ADAM33* in normal and melanoma tissue samples, fibroblasts, and melanoma cells. (*B*) *ADAM9*, *ADAM12*, and *ADAM17* expression in Mel80WM cells as a function of passage number. (*C*) *ADAM9*, *ADAM12*, and *ADAM17* expression in Mel65WM cells as a function of passage number. SD are small so that error bars are not visible when the *y* axis is logarithmic.

lines with anti-ADAM15 antibodies reduces cell proliferation.[36,37] However, the recombinant disintegrin domain of human ADAM15 is reported to be a potent intrinsic inhibitor of angiogenesis, tumor growth, and metastasis.[38]

ADAM17 showed the same expression for normal and melanoma tissues. It was increased, however, in melanoma cells compared with the melanoma tissue and the fibroblasts. ADAM17 is overexpressed in cancers of the breast, ovary, kidney, colon, and prostate.[35] It was identified first as the proteinase responsible for the shedding of the important proinflammatory cytokine, TNF-α,[39] hence earning the alternative name TACE. ADAM17 was also demonstrated to be the TGF-α sheddase and has since been reported to shed several additional, important cell surface proteins, including the EGFR ligands amphiregulin and heparin-binding (HB)-EGF, l-selectin, TNFRII, collagen XVII, and growth hormone receptor.[40] The receptor tyrosine kinase EGFR and its downstream signaling pathway are key regulators of cell proliferation, and it is deregulated frequently in cancer. EGFR can be activated by several ligands processed by ADAM17, including amphiregulin, epigen, epiregulin, HB-EGF, and TGF-α, as well by substrates of ADAM10, including EGF and BTC.[41,42] Overexpression of these ligands is a common event in many tumors and correlates frequently with poor prognosis.[43]

Expression of MMP Family Members

MMP8, *MMP9*, and *MMP13* showed undetectable to very low expression (10^2 copies/5 ng DNA) in all samples studied (Fig. 3A). One exception was *MMP8*, which was clearly up-regulated in the cultured melanoma cells; it showed tenfold up-regulation compared with the melanoma tissue and 50- to 100-fold up-regulation compared with the fibroblasts. MMP8 is expressed in neural crest and adult melanoma cells but not in melanocytes.[44] Several DNA microarray analyses demonstrated up-regulation of *MMP8* in melanoma.[18] WT MMP8 inhibits melanoma growth in vitro and tumor formation in vivo,[45] indicating

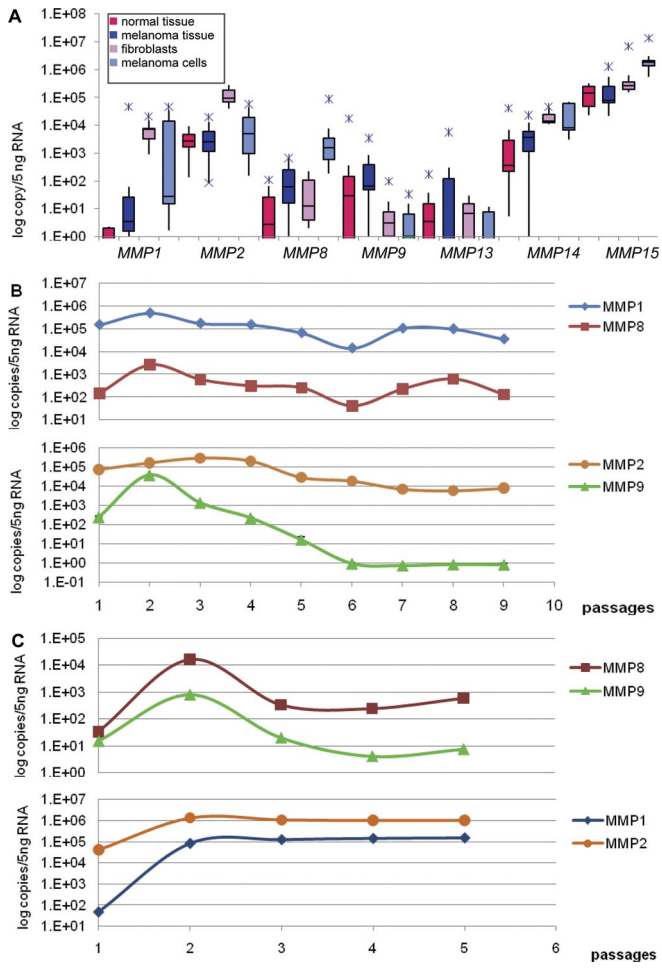

FIGURE 3

MMP expression profiling. (*A*) Expression of *MMP1*, *MMP2*, *MMP8*, *MMP9*, *MMP13*, *MMP14*, and *MMP15* in normal and melanoma tissue samples, fibroblasts, and melanoma cells. (*B*) *MMP1*, *MMP2*, *MMP8*, and *MMP9* expression in Mel80WM cells. (*C*) *MMP1*, *MMP2*, *MMP8*, and *MMP9* expression in Mel65WM cells. SD are small so that error bars are not visible when the *y* axis is logarithmic.

an inhibitory effect of MMP8 on tumorigenesis and metastasis. However, *MMP8* is often mutated in melanoma, and the mutant MMP8 proteins have reduced enzyme activity and inhibitory effects on tumorigenesis.[45] The low expression of *MMP13* observed here was not surprising, as *MMP13* induction in tumors occurs in the stroma rather than the tumor cells themselves.[46]

MMP9 showed an overall low baseline expression in the tissue samples, highly variable copy numbers ($0–10^4$ copies/5 ng RNA) in the normal tissue types, and $10^3–10^2$ copies/5 ng RNA in the melanoma samples. *MMP9* was undetectable or showed very low expression in cultured cells. MMP9 production is not associated with the clinical outcome of melanoma.[47]

MMP1 mRNA was mostly undetectable in normal tissue samples, and the melanoma samples had very low baseline expression (10–100 copies/5 ng RNA; Fig. 3A). In cultured cells, however, *MMP1* was up-regulated significantly. The expression levels of the mRNA were between 10^3 and 10^4 copies/5 ng RNA in the case of the fibroblasts and highly variable ($10^2–3\times10^4$ copies/5 ng RNA) in the case of the melanoma cells. Clinical studies have shown the expression of MMP1 to be positively correlated with the malignancy and poor outcome of melanoma.[48] MMP1 is produced by invasive but not noninvasive melanomas.[49] There is evidence suggesting that MMP1 produced from melanoma cells plays a role in tumor progression by degrading matrix proteins and generating active growth factors such as TGF-β in vivo.[49,50] *MMP1* has been shown to be up-regulated in fibroblasts that are cocultured in fibrillar collagen with melanoma cells.[16]

MMP2 showed high basal expression ($10^3–10^4$ copies/5 ng RNA) in both tissue types, as well as the melanoma cells (Fig. 3A), presumably indicating constitutive expression levels. In fibroblasts, however, *MMP2* was increased 100-fold compared with melanoma cells. MMP2 is believed to play an important role in skin and uveal melanoma progression. A significant correlation was found between MMP2 expression and clinical outcome in sinonasal and oral malignant melanoma. Greater overall survival was seen in patients with low MMP2 expression.[51] The MMP2 gene is expressed constitutively in many cells; therefore, MMP2 is seen in many tumors and at several stages of tumor progression.[52–56] Overexpression of MMP2 is associated with tumor progression in human melanoma, and its expression is even more pronounced in invasive melanomas.[47,57–60] MMP2 was shown to be present by immunohistochemistry in malignant melanoma lesions but not in benign and atypical nevi.[61] The overexpression of MMP2 has also been linked to hematogenous metastasis and to impaired survival in male melanoma patients, suggesting a difference according to sex.[58] MMP2 shows multiple functions as an extracellular/cell surface enzyme and is broadly recognized for its matrix-degrading ability and involvement in cell motility.[62] A great variety of nonmatrix MMP2 physiological targets has also been identified, including bFGF, FGFR1, and the CX_3CL1 chemokine fractalkine.[63–65]

MMP14 showed equal baseline expression in both tissue types, $10^2–10^4$ copies/5 ng RNA (Fig. 3A), again presumably showing constitutive expression levels. In cultured cells, *MMP14* expression was increased eight- to tenfold in fibroblasts and melanoma cells. MMP14/membrane type 1 (MT1)-MMP is usually up-regulated in carcinoma cells.[66] Expression levels of MT1-MMP correlate with the malignant nature of cells. Weakly tumorigenic and poorly invasive human breast carcinoma cell lines do not express MT1-MMP or MMP2, but invasive and metastatic ones do.[67] A similar correlation was reported in human cervical cancer cell lines.[68] Greater overall survival was seen in patients with low MT1-MMP in sinonasal and oral malignant melanomas.[51] However, fibroblast-derived MT1-MMP promotes the invasion and growth of head and neck squamous cell carcinomas.[69] MT1-MMP specifically cleaves native type I and type III collagens[70] and a variety of cell surface-bound biomolecules[71] and activates pro-MMP2 and pro-MMP13.[72,73] As MMP2 has type IV collagenase activity, and stromal MMP13 is important in melanoma tumor growth and organ-specific metastasis,[46] MT1-MMP is believed to be one of the key enzymes for invasion of the basement membrane in the metastatic process, as well as in migration, as it confers invasive activity to the cells.

MMP15 showed the highest overall expression in all of the samples used in this study ($10^5–10^7$ copies/5 ng RNA; Fig. 3A). This gene was increased threefold in melanoma tissue compared with the normal tissue types and increased fivefold in melanoma cells compared with the fibroblasts. When comparing *MMP15* expression in the tissue samples and the cultured cells, this gene was increased tenfold in culture. Elevated expression of MMP15/MT2-MMP has been reported in glioblastomas, and it has also been reported to correlate with invasiveness.[74] Ovarian,[75] urothelial,[76] and breast[77,78] carcinomas also showed elevated levels of MT2-MMP. In a previous study, the expression of MT2-MMP was not detected in benign lesions, and in melanoma cells, MT2-MMP was moderately or strongly detected.[79] It was also found to be colocalized with MMP2.[79] The function of MT2-MMP is not as well described as that of MT1-MMP and is just beginning to be addressed. Studies using a cell line derived from a MT1-MMP knockout mouse have shown the important contribution of MT2-MMP to cell invasion of fibrin matrices.[80] MT2-MMP shows activity against laminin.[70] MT2-MMP

also activates MMP2 to the fully active form in a pathway that is TIMP-2-independent.[81]

Taken together, the data presented in the current study clearly demonstrate that increased gene expression of several ADAM and MMP protein family members (such as *MMP1*, *MMP8*, *MMP15*, or *ADAM15*) has occurred in melanoma tissue and was replicated in tissue cultures. The variable expression of *MMP1*, *MMP2*, and *ADAM8* in the cultured melanoma cells highlights the heterogeneity of the metastatic melanoma samples, presumably indicating the presence of the different subtypes of melanoma in our sample pool. The expression of metalloproteinases can be regulated by microenvironmental effects,[16,17,59,82,83] which may be recapitulated by the tumor tissue samples.

We found an overall tendency for several metalloproteinase targets to be as much as 100-fold up-regulated in culture, in fibroblasts, and in cultured melanoma cells. When establishing the in-house-derived cell lines, the tumor tissue is digested, and the cells are essentially set free of the extracellular structures. This may parallel the metastatic process by which tumor cells are breaking away from the primary tumor and forming the micrometastases or metastatic tumors. The success of the formation of the metastatic tumor depends on the survival of these cells. Metastasizing cells need to be able to invade the local tissue, break through the basal lamina, invade the capillary system, survive the harsh conditions of the circulation, extravasate, and proliferate at the distant site. Studies in animals show that typically far less than one in every 1000 malignant tumor cells that enter the bloodstream will survive to produce a tumor at a new site.[84] To be able to potentially do all of this while adapting to the rapidly changing environment, elevated expression levels of numerous genes key in the metastatic process may be required. The primary cells in cell culture have to adapt to the drastically different environment of the tissue culture dish: attach and proliferate. Once these cells are established and capable of surviving the culturing and the passaging process, high copy numbers are no longer needed to produce the presumably elevated protein levels. This may reflect the decline in the gene expression levels of several MMPs from the first passage onward.

Alternatively, our study may simply reflect the artificial effects of cell culturing on metalloproteinase gene expression. In the culturing process, cells are transferred from a physiological tissue context to a monolayer culture. Structures, such as the ECM, are no longer present, leading to changes in the patterns of engagement of ligand–receptor interactions, which may modulate cell behaviors. In the tumor tissue, the stromal content might be as high as 90% (in the case of desmoplastic tumors, such as many carcinomas of the breast, stomach, and pancreas),[85] and tumor–stroma interactions have been well documented to play a significant role in tumor development and progression.[86] In cell culture, however, the population is homogenous; therefore, these heterotypic cell–cell interactions are not present. Lastly, some of these changes in expression patterns may reflect cells adapting to harshly different, new conditions. Environmental changes, such as pH or temperature imbalances, might impact changes in gene expression, morphology, and cell behavior. This reiterates the suggestion that experiments using cells that have been maintained in culture should be interpreted with great care.

Temporal Gene Expression of Metalloproteinases during Cell Culturing

Two in-house-derived metastatic melanoma cell lines (Mel80WM and Mel65WM) were used to investigate the effect of the culturing process on metalloproteinase gene expression compared with isogenic tissue. Mel80WM cells were used up until P_7, and Mel65WM cells were investigated from P_0 to P_3.

The ADAM family members, *ADAM9*, *ADAM12*, and *ADAM17*, as well as four collagenolytic MMPs (*MMP1*, *MMP2*, *MMP8*, and *MMP9*) were targeted. For both cell lines, the three ADAMs showed less than one logarithmic unit difference between the highest and the lowest observed value (Fig. 2B and C). When comparing these results with those observed previously (Fig. 2A), *ADAM12* and *ADAM17* are consistent in that they are up-regulated in culture less than tenfold. *ADAM9*, however, showed more significant up-regulation previously in culture compared with the melanoma tissue samples (Fig. 2A) than what was observed in the Mel80WM or the Mel65WM cells.

MMP1, *MMP2*, *MMP8*, and *MMP9* showed significant up-regulation in the early passages in both melanoma cell lines (Fig. 3B and C). *MMP1* showed a spike in both cell lines; however, this was not as significant in the Mel80WM cells (3.3-fold) as in the Mel65WM cells (32-fold up-regulated). Although *MMP1* continued to show varied expression levels in the Mel80WM cells, it stayed stable in the Mel65WM cells. In the MMP summaries (Fig. 3A), *MMP1* showed variable expression (between 10^1 and 10^4 copies of mRNA) in the melanoma cells. One reason for this earlier result could have been the variable expression of the *MMP1* gene as observed in Mel80WM. Depending on at what passage the examined cell line is, the copy number was variable compared with the tissue sample.

MMP2 showed slow, passage-wise up-regulation in Mel80WM culture, peaked at fivefold higher at P_3, and then slowly down-regulated to over tenfold less than in tissue (Fig. 3B). In contrast, the expression of *MMP2* was up-regulated 1000-fold at P_2 in the Mel65WM cells (Fig.

3C), and it remained at that level for all subsequent passages. This MMP also showed variable expression in the summarized expressions (500–50,000 copies/5 ng RNA depending on the cell line; Fig. 3A).

The gene expression pattern of *MMP8* mirrored that of *MMP1* in the Mel80WM cells, showing variable copy numbers throughout the passages (Fig. 3B). In Mel65WM, the *MMP8* copy numbers were at least tenfold higher than in the isogenic tissue sample (Fig. 3C). This MMP was up-regulated in the expression summaries (ten- to 100-fold depending on the cell line; Fig. 3A), which proved to be somewhat consistent with the passage-dependent observations.

MMP9 was consistently 100-fold up-regulated in both melanoma cell lines compared with the isogenic tissue samples (Fig. 3B and C). In the subsequent passages, the expression of *MMP9* started decreasing steadily and disappeared by P_4 (for Mel65WM) or P_6 (for Mel80WM). The expression of *MMP9* was also down-regulated in the summarized expression patterns (Fig. 3A).

It has been noted previously that long-term culturing of cancer cells may lead to phenotypic and genotypic changes.[22] Commercially available, established cell lines are usually purchased at P_{50}–P_{80} and above. When pairing the high number of doublings with the inherent genetic instability of immortal or transformed cells,[21] the phenotypic and genotypic changes observed are not surprising. Qualitative and quantitative changes in MMP expression patterns were demonstrated previously in primary and passaged cultures of rabbit corneal fibroblasts.[87] MMP9 secretion in HUVECs was shown to be passage-dependent.[88] This secretion was restricted to very early passage cells, decreased rapidly as the cells were maintained in culture, and was not detected by the second passage.[88] The present study, however, emphasizes the significance of radical changes in gene expression in primary cells and at the earliest passages.

SUMMARY

Despite the complexity of metalloproteinase regulation, five major levels of endogenous control can be recognized: gene transcription, mRNA stability, translation, proenzyme activation, and inhibition of enzymatic activity.[89–92] Collectively, these mechanisms should confine degradative activity to those sites and situations where it is biologically necessary. However, tumor cells have developed multiple strategies to escape the checkpoints controlling metalloenzyme mRNA expression and proteolytic activity, acquiring new properties that lead to tumor growth and invasion. The present study has found a number of metalloproteinases whose expression differs in melanoma compared with normal tissue and cell lines.

Multiple, coordinated gene expression involving metalloproteinases may be critical for initiation of metastasis. For example, breast cancer metastasis to the lung uses epiregulin (a ligand for EGFR), COX2, MMP1, and MMP2.[93] Also, the enzyme/inhibitor balance has been shown to favor the enzyme in breast cancer tumor tissue compared with adjacent, nontumor tissue,[94] and thus, consideration of the levels of natural metalloproteinase inhibitors (such as TIMPs and reversion-inducing, cysteine-rich protein with Kazal motifs) is important. We have presently identified several metalloproteinases up-regulated in melanoma tissue, which can contribute to ECM degradation and facilitate tumor progression.

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