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Additional MDA-MB-231 Breast Cancer Cell Matrix Metalloproteinases Promote Invasiveness

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We are interested in two aspects of a given type of metastatic breast cancer: which potentially cancer-relevant genes are expressed and which factors determine invasiveness. Using reverse transcription real-time PCR, we detected gene expression of 26 matrix metalloproteinases (MMPs) in MDA-MB-231 breast cancer cells, including those of MMP-12, MMP-16 variant 2, MMP-19, MMP-20, MMP-21, MMP-23, MMP-24, MMP-25, MMP-25 variant 2, MMP-L1, MMP-26, MMP-27, and MMP-28, in contrast to the 13 MMPs detected until now in these cells. We found that MMP genes are expressed at widely different levels in these cells, over five orders of magnitude. After individual siRNA-induced depletions, we found that six additional species of cancer cell MMPs promote invasiveness in MDA-MB-231 cells: MMP-3, MMP-11, MMP-12, MMP-17, MMP-19, and MMP-23, thus raising the total to 12 endogenous MMPs which do so in these cells. The data support the conclusion that some cancer cell MMPs, although expressed at low levels, are needed for cancer trait in MDA-MB-231 cells, and that several endogenous MMPs play non-redundant roles in this process. The mRNA level of MMP-11, but not of other MMPs, rose substantially following individual siRNA-targeted depletion of cancer cell MMP-17 mRNA, while no MMP mRNA increased appreciably after degradation of other MMP mRNAs. This supports the conclusion that MMP-17 may be a member of an intracellular signaling pathway which downregulates MMP-11 mRNA.

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Invasion is an essential step needed several times during cancer metastasis (Cairns et al., 2003; Mareel and Leroy, 2003; Giehl et al., 2005; Christofori, 2006). Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases which digest extracellular matrix in normal processes, such as embryogenesis, reproduction and tissue remodeling, and in disease processes, such as cancer metastasis and arthritis (Egeblad and Werb, 2002; Polette et al., 2004; Deryugina and Quigley, 2006). MMPs differ by their profile of substrates, their structure and subcellular localization and, from a total of 23 known human MMPs, different subsets have been detected in various human cells (Egeblad and Werb, 2002; Martin and Matrisian, 2007; Page-McCaw et al., 2007). Some MMPs have been detected in cells from some types of cancer, some of those MMPs have been shown to promote cancer invasion, MMPs from both cancer cells and stromal cells are involved in cancer invasion, and MMPs have both cancer-promoting and cancer-suppressing roles (Egeblad and Werb, 2002; Deryugina and Quigley, 2006; López-Otín and Matrisian, 2007; Martin and Matrisian, 2007). The MMP mRNAs analyzed in this study are (their common names are in parenthesis; their accession numbers are indicated in Table 1): MMP-1 (collagenase-1); MMP-2 (gelatinase A); MMP-3 (stromelysin-1); MMP-7 (matrilysin); MMP-8 (collagenase-2); MMP-9 (gelatinase B); MMP-10 (stromelysin-2); MMP-11 (stromelysin-3); MMP-12 (metalloelastase); MMP-13 (collagenase-3); MMP-14 (MT1-MMP, MT-MMP1); MMP-15 (MT2-MMP, MT-MMP2); 6347-base long MMP-16 variant 1 mRNA (MT3-MMP, MT-MMP3; MMP-16-1 in this text; NM_005941) that generates a membrane-inserted MMP-16; 1800-base long MMP-16 variant 2 mRNA (NM_022564) which produces a secreted MMP-16 (MMP-16-2 in this text) (Matsumoto et al., 1997; Shofuda et al., 1997); MMP-17 (MT4-MMP, MT-MMP4); MMP-19 (RASI-1, MMP-18); MMP-20 (enamelysin); MMP-21; MMP-23 (femalysin); MMP-24 (MT5-MMP, MT-MMP5); MMP-25 (MT6-MMP, MT-MMP6, leukolysin); MMP-25 variant 2 (MMP-25-2 in this text) (11); MMP-L1 (matrix metallopeptidase-like 1) (12); MMP-26 (endometase, matrilysin-2); MMP-27; and MMP-28 (epilysin).

The MMPs vary by their subcellular location: (a) MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-16-2, MMP-19, MMP-20, and MMP-26 are secreted; (b) MMP-14, MMP-15, MMP-16-1, MMP-23, and MMP-24 have a transmembrane location on the cytoplasmic membrane; and (c) MMP-17 and MMP-25 are bound to the cytoplasmic membrane by glycosylphosphatidylinositol (Egeblad and Werb, 2002).

The expression of 13 MMPs has been detected in MDA-MB-231 breast cancer cells until now (Giambernardi et al., 1998; Grant et al., 1999; Wang et al., 1999; Kousidou et al., 2004; Bachmeier et al., 2005). In contrast, our present results show that 26 different MMP mRNAs are expressed in these cells, at widely different levels. Using a cell invasion assay, MMP-1, MMP-2, MMP-7, MMP-9, MMP-13, and MMP-14 have been reported to enhance invasiveness in MDA-MB-231 cells (Ramos-DeSimone et al., 1999; Jiang et al., 2005, 2006; Wyatt et al., 2005; Hotary et al., 2006; Merrell et al., 2006; Muñoz-Nájar et al., 2006). In contrast, using the same cell invasion assay after siRNA-induced depletions, we have made similar observations about six additional MDA-MB-231 cell MMPs: MMP-3, MMP-11, MMP-12, MMP-17, MMP-19, and MMP-23. We have also detected higher MMP-11 mRNA levels following individual siRNA-targeted depletion of cancer cell MMP-17 mRNA.

This manuscript is dedicated to the memory of Andrew J. Lonigro (1936–2007).

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TABLE 1. PCR primers

	mRNA		
Name	Accession number	Nucleotide number	Primer sequence
GAPDH	NM_002046	F917–936	5′-GCATCCTGGGCTACACTGAG-3′
		R1047–1066	5′-TGCTGTAGCCAAATTCGTTG-3′
MMP-1	NM_002421	F1813–1832	5′-GACAGAAAGAGACAGGAGAC-3′
		R1894–1913	5′-GAGTTATCCCTTGCCTATCC-3′
MMP-2	NM_004530	F2756–2777	5′-GCTGGCTGCCTTAGAACCTTTC-3′
		R2902–2925	5′-GAACCATCACTATGTGGGCTGAGA-3′
MMP-3	NM_002422	F1304–1327	5′-GAAGAGAAATTCCATGGAGCCAGG-3′
		R1382–1413	5′-AGAAATAAAAGAACCCAAATTCTTCAAAAACA-3′
MMP-7	NM_002423	F713–736	5′-GGGACATTCCTCTGATCCTAATGC-3′
		R805–838	5′-GAATTACTTCTCTTTCCATATAGTTTCTGAATGC-3′
MMP-8	NM_002424	F1499–1523	5′-CCACTTTCAGAATGTTGAAGGGAAG-3′
		R1600–1622	5′-TCACGGAGGACAGGTAGAATGGA-3′
MMP-9	NM_004994	F2002–2021	5′-GCACGACGTCTTCCAGTACC-3′
		R2111–2131	5′-GCACTGCAGGATGTCATAGGT-3′
MMP-10	NM_002425	F1444–1463	5′-ACATTGCTAGGCGAGATAGG-3′
		R1555–1574	5′-GGCTCATCTTCTTCAGTCAC-3′
MMP-11	NM_005940	F2100–2121	5′-CAACATACCTCAATCCTGTCCC-3′
		R2157–2176	5′-CAATGGCTTTGGAGGATAGC-3′
MMP-12	NM_002426	F1391–1415	5′-TTGAATATGACTTCCTACTCCAACG-3′
		R1518–1542	5′-GTGGTACACTGAGGACATAGCAAAT-3′
MMP-13	NM_002427	F1295–1314	5′-GAC TTCCCAGGAATTGGTGA-3′
		R1396–1415	5′-TGA CGCGAACAATACGGTTA-3′
MMP-14	NM_004995	F1555–1574	5′-GAGCTCAGGGCAGTGGATAG-3′
		R1707–1726	5′-GGTAGCCCGGTTCTACCTTC-3′
MMP-15	NM_002428	F2397–2416	5′-CAGGCCACACCTTCTTCTTC-3′
		R2559–2578	5′-CCAGTATTTGGTGCCCTTGT-3′
MMP-16-1	NM_005941	F5557–5581	5′-AGGTGTCAGTTCAGTGTTACTAGAG-3′
		R5691–5715	5′-AATGAGAAATGAAGCAGAAGGAGAA-3′
MMP-16-2	NM_022564	F533–555	5′-TGACAGGAAAAGTGGACAGAAAC-3′
		R659–678	5′-GATGTGCTTGTGCTGCCATT-3′
MMP-17	NM_016155	F573–592	5′-ACTCATGTACTACGCCCTCA-3′
		R649–668	5′-GAGAAGTCGATCTGGATGTC-3′
MMP-19	NM_002429	F1993–2012	5′-GGGTCCTGTTCTTCCTACAT-3′
		R2152–2171	5′-CAATCCTGCAGTACTGGTCT-3′
MMP-20	NM_004771	F1075–1094	5′-TGAGAGGGGCACTGCTTACT-3′
		R1211–1230	5′-GTCTTCTGTGGCTCCCTGAG-3′
MMP-21	NM_147191	F1111–1130	5′-AACAATAGGACACGCTATGG-3′
		R1200–1219	5′-CATCTCTTTTCCATGTCCAG-3′
MMP-23	NM_006983	F1004–1023	5′-CCAGAAGATCCTCCACAAGA-3′
		R1128–1147	5′-CAGGTGTAGGTGCCCTCATT-3′
MMP-24	NM_006690	F494–513	5′-GGCAAAAACACATCACCTAC-3′
		R627–646	5′-GGTCACTTTTGATCTCATGG-3′
MMP-25	NM_022468	F3382–3401	5′-GATCAGCATGAGGACAGAAG-3′
		R3450–3471	5′-GAAACTGACAGAGGCCCAAATC-3′
MMP-25-2	NM_022718	F2369–2388	5′-GACTCCCATCAACTCAACGC-3′
		R2542–2565	5′-AGAATAGTTCCAGAGGCAATCATT-3′
MMP-L1	NM_004142	F1845–1866	5′-CGAAACCATCATTGCCCCATCC-3′
		R1907–1926	5′-TTCCCTGTGAGGAAAGAGTC-3′
MMP-26	NM_021801	F206–225	5′-GGAATGGGACAGACCTACTT-3′
		R308–327	5′-AGTGTGCTTATTCCACTTGC-3′
MMP-27	NM_022122	F122–141	5′-TCAGGCATATCTCAACCAGT-3′
		R273–292	5′-CTGGGTGTCTTCATGATCTC-3′
MMP-28	NM_024302	F368–391	5′-GAGGCATTCCTAGAGAAGTACGGA-3′
		R440–460	5′-CTGAAACGCTCTGATGGCATC-3′

Materials and Methods

Cells, RNA isolation, reverse transcription and real-time PCR

MDA-MB-231 metastatic human breast cancer cells were obtained from the U.S. National Cancer Institute and were grown in DMEM supplemented with 10% fetal bovine serum. Whole cell RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) and digested with RNase-free DNase (0.1 U/μg of RNA) in the presence of RNase inhibitor (0.5 U/μg of RNA). MMP-7, MMP-11, and MMP-23 mRNAs were reverse transcribed with avian myeloblastosis virus reverse transcriptase (14 U/μg of RNA), primed with oligo(dT), in the presence of RNase inhibitor (0.5 U/μg of RNA). Reverse transcription of other MMP mRNAs was done with Moloney murine leukemia virus reverse transcriptase (20 U/μg of RNA) and the specific reverse primers shown in Table 1. Cell mRNA levels were measured by real-time PCR of cDNA in the presence of SYBR Green I. The PCR primer pairs used are shown in Table 1. All cell MMP mRNA levels were normalized by the GAPDH mRNA level in each sample.

siRNAs and siRNA transfections

The siRNAs were designed using the Massachusetts Institute of Technology and the Integrated DNA Technologies (IDT) siRNA design tools, were synthesized chemically by IDT (Coralville, IA) and are shown in Table 2. Cell were transfected with a single siRNA in the presence of Dharmafect 2 (Dharmacon, Lafayette, CO) and then incubated for 5 days to allow for protein decay. Transfection conditions were optimized using a GAPDH siRNA (Ambion, Austin, TX). The MMP/GAPDH mRNA ratio of each cell sample transfected with a single MMP-specific siRNA was normalized by the comparable ratio of a cell sample transfected with a negative control siRNA of scrambled sequence (Ambion).

TABLE 2. siRNAs

mRNA		siRNA	
Name	Nucleotide number	Number	Sequence
MMP-1	305–323	siRNA 1	5′-rArCrCrArGrArUrGrCrUrGrArArArCrCrCrUrGrUrU -3′ 5′-rCrArGrGrGrUrUrUrCrArGrCrArUrCrUrGrGrUrUrU -3′
MMP-1	1196–1215	siRNA 2	5′-rGrCrArUrArUrCrGrArUrGrCrUrGrCrUrCrUrUdTdT -3′ 5′-rArArGrArGrCrArGrCrArUrCrGrArUrArUrGrCdTdT -3′
MMP-3	66–85		5′-rArUrGrArArGrArGrUrCrUrUrCrCrArArUrCrCdT-3′ 5′-rGrGrArUrUrGrGrArArGrArCrUrCrUrUrCrArUdTdT-3′
MMP-7	368–386	siRNA 1	5′-rArGrUrGrGrUrCrArCrCrUrArCrArGrGrArUrCdTdT-3′ 5′-rGrArUrCrCrUrGrUrArGrGrUrGrArCrCrArCrUdTdT-3′
MMP-7	448–466	siRNA 2	5′-rArCrArUrGrUrGrGrGrGrCrArArArGrArGrArUdTdT-3′ 5′-rArUrCrUrCrUrUrUrGrCrCrCrCrArCrArUrGrUdTdT-3′
MMP-11	484–502	siRNA 1	5′-rCrArUrCrArUrGrArUrCrGrArCrUrUrCrGrCrCdTdT-3′ 5′-rGrGrCrGrArArGrUrCrGrArUrCrArUrGrArUrGdTdT-3′
MMP-11	1225–1244	siRNA 2	5′-rCrUrUrCrUrUrCrCrGrArGrGrCrArGrGrGrArCdTdT-3′ 5′-rGrUrCrCrCrUrGrCrCrUrCrGrGrArArGrArArGdTdT-3′
MMP-12	1133–1153		5′-rArUrUrArUrCrCrCrArArGrArGrCrArUrArCrAdTdT-3′ 5′-rUrGrUrArUrGrCrUrCrUrUrGrGrGrArUrArArUdTdT-3′
MMP13	609–627		5′-rArUrUrArUrGrGrArGrGrArGrArUrGrCrCrCrAdTdT-3′ 5′-rUrGrGrGrCrArUrCrUrCrCrUrCrCrArUrArArUdTdT-3′
MMP-14	764–782		5′-rUrCrArUrGrArUrCrUrUrCrUrUrUrGrCrCrGrAdTdT-3′ 5′-rUrCrGrGrCrArArArGrArArGrArUrCrArUrGrAdTdT-3′
MMP-17	766–786		5′-rGrGrCrCrUrCrGrUrCrArUrCrGrUrCrArArArGrUrG-3′ 5′-rCrUrUrUrGrArCrGrArUrGrArCrGrArGrGrCrCrUrU-3′
MMP-19	961–979	siRNA 1	5′-rArGrArArCrCrCrArGrUrCrCrCrArUrGrCrCrAdTdT-3′ 5′-rUrGrGrCrArUrGrGrGrArCrUrGrGrGrUrCrUdTdT-3′
MMP-19	1273–1291	siRNA 2	5′-rCrUrArUrUrGrGrCrCrUrCrUrCrArArCrCrArAdTdT-3′ 5′-rUrUrGrGrUrUrGrArGrArGrGrCrCrArArUrArGdTdT-3′
MMP-23	323–341		5′-rCrArGrGrArUrCrCrUrCrUrCrCrUrUrCrCrCrGdTdT-3′ 5′-rCrGrGrGrArArGrGrArGrArGrGrArUrCrCrUrGdTdT-3′

Cell invasion assay

Boyden chambers with an 8-μm pore size, 30-mm^2 area membrane (Falcon inserts 353097, BD, Franklin Lakes, NJ) were coated with 15–25 μg (usually 20 μg) of a preparation of extracellular matrix (Matrigel, BD). About 2×10^5 cells in serum-free medium were placed in the upper chamber. Medium supplemented with 10% fetal bovine serum was added to the bottom chamber. Loaded cells were incubated for about 24 h, cells were scraped off the upper side of the membrane, and the cells attached to the bottom side of the membrane were fixed, stained with hematoxylin and counted. The same number of cells was seeded in each transwell. Of the cells which invaded through Matrigel, the number of cells that had been transfected with an MMP-specific siRNA was corrected relative to the number of cells which had been transfected with a negative control siRNA of scrambled sequence (Ambion). Matrigel concentration was adjusted so that a maximum percentage of the loaded MDA-MB-231 cells, and a negligible percent of the applied NIH-3T3 cells, appeared on the under side of the membrane.

Results

It is important to identify as many as possible of the determinants of invasiveness of a given type of metastatic human breast cancer because they are potential targets for therapy. As a model of a given type of metastatic breast cancer, we chose to work with MDA-MB-231 cells because they are well characterized to be breast, human, metastatic, invasive, estrogen receptor-negative, progesterone receptor-negative cancer cells which do not overexpress HER2 (Neve et al., 2006). Of the 23 known human MMPs, 13 were found to be expressed in MDA-MB-231 cells (Giambernardi et al., 1998; Grant et al., 1999; Wang et al., 1999; Kousidou et al., 2004; Bachmeier et al., 2005). The models were that either 13 or more different MMP genes might be expressed in MDA-MB-231 cells. To test these models, this was measured at the mRNA level rather than the protein level because reverse transcription real-time PCR is about 10 orders of magnitude more sensitive than immunoblotting (Bakalova et al., 2005; Pérez-Ruiz et al., 2007). These experiments were done with non-confluent cells, because MDA-MB-231 cell density affects MMP expression levels (Bachmeier et al., 2005). We designed and used PCR primer pairs specific for either only the membrane-bound MMP-16-1 (NM_005941) or only the secreted MMP-16-2 (NM_022564). MDA-MB-231 cells expressed 26 different mRNAs, including those of MMP-12, MMP-16-2, MMP-19, MMP-20, MMP-21, MMP-23, MMP-24, MMP-25, MMP-25-2, MMP-L1, MMP-26, MMP-27, and MMP-28 (Fig. 1). One possibility is that MMP genes whose expression is higher might be more likely to promote invasiveness, but the relative levels of various MMP mRNAs in MDA-MB-231 cells are unknown. In MDA-MB-231 cells, MMP genes were expressed at widely different levels, over five orders of magnitude (Fig. 1). Using three primer pairs, each one specific either for MMP-25 (NM_022468) or what were known as MMP-25-2 (NM_022718) or MMP-L1 (NM_004142), three RNAs were detected, present are different levels (Fig. 1).

Toward testing the role of endogenous MMPs in invasiveness, first we designed siRNAs for them and singly transfected MDA-MB-231 cells with one of each of these siRNAs. The mRNAs of MMP-1, MMP-7, MMP-11 mRNA, and MMP-19 were degraded, each individually targeted by one out of two different siRNAs, and the mRNA levels of MMP-3, MMP-12, MMP-13, MMP-14, MMP-17, and MMP-23 were decreased, each separately induced by one siRNA (Fig. 2).

Depending on the type of MMP and whether it is from a cancer cell or a stromal cell, some MMPs either promote or suppress cancer progression (Egeblad and Werb, 2002; Martin and Matrisian, 2007). Therefore, for each type of expressed MMP, it is necessary to test whether it affects invasiveness, and if so, whether the effect is positive or negative. Six different MMPs were found to control MDA-MB-231 cell invasion (Ramos-DeSimone et al., 1999; Jiang et al., 2005, 2006; Wyatt et al., 2005; Hotary et al., 2006; Merrell et al., 2006;

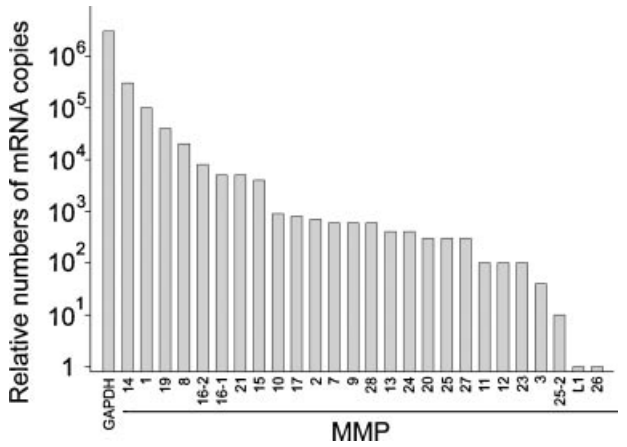

Fig. 1. Approximate relative numbers of mRNA copies of various MMPs in MDA-MB-231 cells. Levels of mRNA were measured by reverse transcription real-time PCR and normalized by total cell RNA.

Muñoz-Nájar et al., 2006). The models were that either six or more different MMP genes might determine the invasiveness of MDA-MB-231 cells. To test these models, we used the Matrigel cell invasion assay because it has become the standard method to monitor cell invasiveness. After siRNA transfection, we incubated cells for 5 days. After mRNA depletion, the level of any human protein would have decreased appreciably by then because the half-lives of most human proteins are much shorter than that. MMP-1, MMP-3, MMP-7, MMP-11, MMP-12, MMP-13, MMP-14 and MMP-19 have been found to regulate invasion in studies spread among various types of cancers (Ramos-DeSimone et al., 1999; Farina et al., 2002; Andarawewa et al., 2003; Folgueras et al., 2004; Jiang et al., 2005, 2006; Sadowski et al., 2005; Wyatt et al., 2005; Hotary et al., 2006; Jost et al., 2006; Merrell et al., 2006; Muñoz-Nájar et al., 2006; Sarkar et al., 2006). We tested these and two other MMPs next because we are interested in the integrated view of what happens in the type of metastatic breast cancer represented by MDA–MB-231 cells.

MMP-3 suppressed invasion in MDA-MB-231 cells, when a peptide inhibitor was used (Farina et al., 2002), and MMP-11 inhibited invasion in a mouse breast cancer in experiments that could not distinguish MMPs from stromal cells versus cancer cells (Andarawewa et al., 2003; Folgueras et al., 2004). In contrast, individual depletions of cancer cell MMP-3 mRNA and MMP-11 mRNA, induced by one or two different siRNAs, respectively, all three suppressed MDA-MB-213 cell invasion (Fig. 3). MMP-19 behaved either as an invasion enhancer (Sadowski et al., 2005) or an invasion inhibitor (Jost et al., 2006) in keratinocytes under different experimental conditions. In contrast, MMP-19 mRNA depletions in MDA-MB-231 cells, triggered separately by two different siRNAs, both inhibited MDA-MB-231 cell invasion (Fig. 3). MMP-17 overexpression did not affect MDA-MB-231 cell invasion (Chabottaux et al., 2006). However, siRNA-induced MMP-7 mRNA depletion inhibited MDA-MB-231 cell invasion (Fig. 3).

We are not aware of any evidence of a role of MMP-23 in the invasion of any cancer or even the detection of MMP-23 in any cancer. MMP-23 gene expression was about three orders of magnitude lower than that of the highest MMP, MMP-14, in MDA-MB-231 cells (Fig. 1). Single siRNA-induced MMP-23 mRNA depletion inhibited MDA-MB-231 cell invasion (Fig. 3).

MMP-12 enhances glioma invasion (Sarkar et al., 2006). Individual siRNA-triggered MMP-12 mRNA depletion inhibited MDA-MB-231 cell invasion (Fig. 3). MMP-7 enhanced invasion in cancers other than MDA-MB-231 (Folgueras et al., 2004). MMP-7 mRNA depletions, triggered separately by two different siRNAs, both inhibited MDA-MB-231 cell invasion (Fig. 3). MMP-13 was found to promote MDA-MB-231 cell invasion, based on blockage with an antibody (Merrell et al., 2006). Single siRNA-triggered MMP-13 mRNA depletion inhibited MDA-MB-231 cell invasion (Fig. 3). Our data support the conclusion that MDA-MB-231 cancer cell MMP-7, MMP-12 and MMP-13 promote invasiveness.

MDA-MB-231 cell invasion decreased either after MMP-1 depletion generated by an shRNA to MMP-1 mRNA nucleotides 305–323 (Wyatt et al., 2005), or after MMP-14 depletion produced either by an siRNA to MMP-14 mRNA nucleotide positions 755–774 or 462–482 or by a ribozyme (Hotary et al., 2006; Muñoz-Nájar et al., 2006). To minimize the chances of siRNA off-target effects, using individual siRNAs to more than one site in the target mRNA is recommended. MDA-MB-231 cell invasion was also inhibited after MMP-1 mRNA depletions, triggered separately by two different siRNAs to MMP-1 mRNA positions 305–323 and 1,196–1,215,

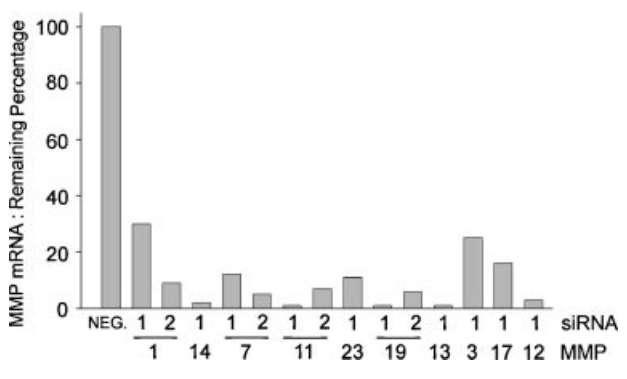

Fig. 2. Remaining levels of MMP mRNAs in MDA-MB-231 cells, after transfection with one siRNA specific to the indicated MMP mRNA, relative to a non-specific negative control siRNA (NEG.), measured by reverse transcription real-time PCR and normalized by GAPDH mRNA levels.

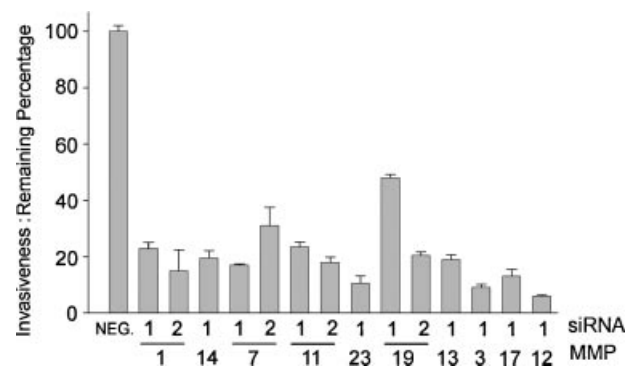

Fig. 3. Remaining invasiveness of MDA-MB-231 cells, after transfection with one siRNA specific to the indicated MMP mRNA, relative to a non-specific negative control siRNA of scrambled sequence (NEG.), measured by a cellular invasion assay.

and by MMP-14 mRNA depletion, induced by an siRNA to MMP-14 mRNA bases 764–782 (Fig. 3). Our MMP-1 and MMP-14 results support the conclusion that these are not off-target non-specific effects.

We asked next whether the siRNA-induced degradation of the targeted mRNA of one MMP may be accompanied by a change in the cell level of the mRNA of another MMP. Single siRNA-induced depletion of endogenous MMP-17 mRNA resulted in substantially higher mRNA levels of MMP-11, but not of other MMPs; other MMP mRNAs did not rise appreciably after individual siRNA-triggered decreases of other cancer cell MMP mRNAs (Fig. 4 and data not shown).

Discussion

To our knowledge, the present work provides the first evidence of the following. (1) Six additional types of cancer cell MMPs, MMP-3, MMP-11, MMP-12, MMP-17, MMP-19, and MMP-23 each individually enhances invasiveness in MDA-MB-231 cells, based on single siRNA-induced depletions, raising the total to 12 different MMPs which do so in these cells. In contrast: (a) MMP-3 was reported to suppress invasion in these cells, based on a peptide inhibitor (Farina et al., 2002); and (b) MMP-11 inhibited invasion in a mouse breast cancer in experiments that could not distinguish stromal cell MMPs from those of cancer cells (Andarawewa et al., 2003; Folgueras et al., 2004). (2) Some cancer cell MMPs which are expressed at very low levels are needed for this cancer trait in MDA-MB-231 cells. (3) Cancer cell MMP-17 is part of an apparently intracellular signaling pathway which downregulates the MMP-11 mRNA level in MDA-MB-231 cells. (4) Detection of 26 different MMP mRNAs in MDA-MB-231 cells, which are expressed at widely different levels in these cells, over five orders of magnitude. In contrast, the current number of human MMPs, cumulative from various types of cells, had been 23 (Martin and Matrisian, 2007; Page-McCaw et al., 2007). (5) MMP-25 (NM_022468), MMP-25-2 (NM_022718) and MMP-L1 (NM_004142) are expressed, as three different transcripts, in MDA-MB-231 cells. In contrast, the latter two mRNAs are not posted now by the National Center for Biotechnology Information because of insufficient evidence. (6) Expression of MMP-16-2, MMP-21, MMP- 23, MMP-24, MMP-25-2, MMP-L1, and MMP-27 mRNAs in any cancer cell, thus detecting 26 species rather than the current 19 in these cells. (7) Expression of MMP-12, MMP-20, MMP-25, and MMP-28 mRNAs in any breast cancer cell, detecting 26 species rather than the current 15 in these cells. (8) Expression of MMP-19 and MMP-26 mRNAs in MDA-MB-231 breast cancer cells, detecting 26 species rather than the current 13 in these cells.

There are many publications connecting various MMPs, including MMP-14, to invasion (Egeblad and Werb, 2002; Polette et al., 2004; Deryugina and Quigley, 2006; Martin and Matrisian, 2007). In contrast, there are two publications linking one cellular MMP, MMP-14, to cell migration, but the possibility that the connection was to both cell migration and invasion cannot be ruled out because a cell invasion assay was not included then (Koshikawa et al., 2000; Kajita et al., 2001). In reports on MMPs and invasion, it has become standard practice to do Matrigel invasion assays without testing cell migration separately (e.g., Ramos-DeSimone et al., 1999; Farina et al., 2002; Jiang et al., 2005, 2006; Wyatt et al., 2005; Hotary et al., 2006; Merrell et al., 2006; Muñoz-Nájar et al., 2006). It makes sense that MMPs, which are proteinases either secreted or located on the cell surface, would participate in invasion. It seems less likely that in MDA-MB-231 cells, one of the MMPs in Figure 3 would be needed only for cell locomotion, which involves pushing cellular structures, such as the cytoskeleton, at the advancing front of the cell and pulling them at the retreating back of the cell (Giehl et al., 2005). In addition, there is previous evidence that MMP-1, MMP-3, MMP-7, MMP-11, MMP-12, MMP-13, MMP-14, and MMP-19 participate in cancer invasion, and no evidence that MMP-1, MMP-3, MMP-7, MMP-11, MMP-12, MMP-13, or MMP-19 play a role in migration per se (Egeblad and Werb, 2002; López-Otín and Matrisian, 2007; Martin and Matrisian, 2007). We suggest that the invasion assay results in Figure 3 reflect effects on cell invasiveness, rather than effects only on cell migration. Regardless, both cell invasion and migration are important because both are essential for metastasis.

There was appreciable inhibition in the MDA-MB-231 cell invasion assay cell after individual siRNA-induced degradation of endogenous MMP-3, MMP-11, MMP-12, MMP-17, MMP-19, and MMP-23 mRNA, in addition to MMP-1, MMP-7, MMP-13, and MMP-14 mRNA. These results support the conclusion that some cancer cell MMPs which are expressed at very low levels, are needed for this cancer trait in MDA-MB-231 cells, and that various cancer cell MMPs play non-redundant roles in promoting this process. Each MMP has a different profile of substrates (Egeblad and Werb, 2002). If each substrate needed to be digested for invasion to occur, then each of the corresponding MMPs would be required for invasion.

Single siRNA-targeted depletion of cancer cell MMP-17 mRNA led to an substantial elevation of the mRNA level of MMP-11, but not of other MMPs, in an apparently compensatory effect. Other MMP mRNAs did not increase appreciably after individual siRNA-induced decreases of other endogenous MMP mRNAs. This supports the conclusion that MMP-17 is part of a signaling pathway which downregulates

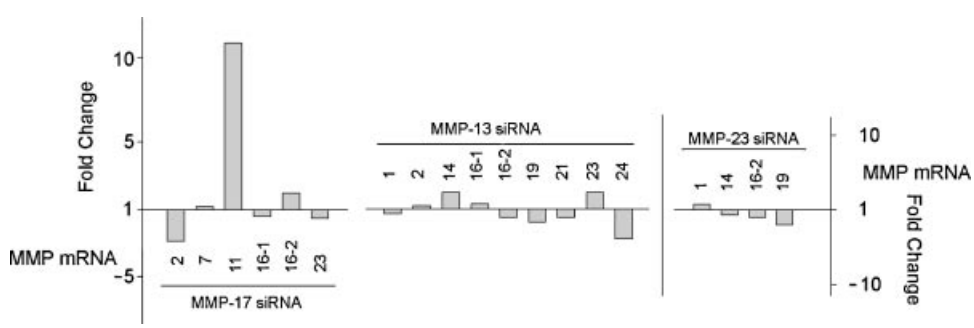

Fig. 4. Effect of transfection of MDA-MB-231 cells with one siRNA to either MMP-17, MMP-13 or MMP-23 on the levels of the indicated MMP mRNAs, relative to a non-specific negative control siRNA, measured by reverse transcription real-time PCR and normalized by GAPDH mRNA levels.

MMP-11 mRNA. Mice deficient in MMP-7, MMP-3, MMP-2 or MMP-8 showed increased expression of (a) MMP-3 and MMP-10, (b) MMP-7 and MMP-10, (c) MMP-9 or (d) MMP-13, respectively (Rudolph-Owen et al., 1997; Esparza et al., 2004; Hartenstein et al., 2006). It is not possible to know whether these apparent pathways links were between different cells or within a cell, because these experiments were done in animals. The link between MMP-17 and MMP-11 is apparently within a cell because the experiments were done with a cell line.

Matrix metallopeptidase-like 1 (NM_004142; MMP-L1) was originally reported as a 1934-base long mRNA, but was later suppressed at the National Center for Biotechnology Information (NCBI) because of insufficient support for the protein. MMP-25-2 (NM_022718) was first posted as a 3105-base long mRNA, but was subsequently suppressed at NCBI because of insufficient evidence for the transcript. In contrast, our data support the conclusion that MMP-25 (NM_022468, 3565-base long mRNA), MMP-25-2 and MMP-L1 are expressed, as three different transcripts, in MDA-MB-231 cells.

Many other factors have been reported to be involved, or to be candidates to participate, in the promotion or suppression of cancer invasiveness, in studies spread among many different types of cancer cells. In view of the complexity suggested by the present study, it seems important to identify the factors that enhance or inhibit the invasiveness of a given type of metastatic cancer cell, and the cell signaling pathways that connect them in that cell.

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