Anti-matrix metalloproteinase-9 DNAzyme decreases tumor growth in the MMTV-PyMT mouse model of breast cancer

Miranda A Hallett¹, Bin Teng¹, Hisashi Hasegawa¹, Luciana P Schwab², Tiffany N Seagroves² and Tayebeh Pourmotabbed¹*

Abstract

Introduction: Despite continued improvements in diagnosis, surgical techniques, and chemotherapy, breast cancer patients are still overcome by cancer metastasis. Tumor cell proliferation, invasion and metastasis are mediated, at least in part, through degradation of basement membrane by neutral matrix metalloproteinases (MMP) produced by tumor and stromal cells. Evidence suggests that MMP-9 plays a significant role in breast tumor cell invasion and metastasis. DNAzymes or catalytic oligonucleotides are new classes of gene targeting molecules that bind and cleave a specific mRNA, resulting in decreased protein expression.

Methods: The application of anti-MMP-9 DNAzyme (AM9D) for the treatment of primary and metastatic breast cancer was evaluated in vitro and in vivo using MDA-MB-231 cells and the MMTV-PyMT transgenic breast cancer mouse model. Spontaneously developed mammary tumors in MMTV-PyMT transgenic mice were treated intratumorally with naked AM9D, once a week for 4 weeks. The stability of DNAzyme was determined in vitro and in vivo using fluorescently labeled DNAzyme.

Results: AM9D specifically inhibited expression of MMP-9 in MDA-MB-231 cells resulting in reduced invasive property of these cells by 43%. Weekly intratumoral treatment of spontaneously developed mammary tumors in MMTV-PyMT transgenic mice was sufficient to significantly reduce the rate of tumor growth and final tumor load in a dose dependent and statistically significant manner (P < 0.05). This decrease in tumor growth was correlated with decreased MMP-9 protein production within the treated tumor tissues. Tumors treated with AM9D were also less vascularized and contained more apoptotic cells compared to control and untreated tumors.

Conclusions: These results show that targeting and down regulation of MMP-9 by AM9D could prove useful as a therapy against breast carcinoma tumor growth and invasion.

Introduction

Breast cancer is one of the leading causes of cancer death in women, second only to lung cancer [1-3]. The majority of morbidity and mortality amongst cancer patients is due to metastasis of tumor cells to distant organs [2,4]. Breast cancer most commonly metastasizes to bone, lymph nodes, lung, liver, and brain [5]. Despite continued improvements in diagnosis, surgical techniques, and chemotherapy, lethality from breast cancer remains high.

Matrix metalloproteinase-9 (MMP-9) production by tumor and stromal cells is one of the most important factors for metastatic behavior of tumor cells [6-8]. MMP-9 is a member of the metzincin family of enzymes, which play an important role in normal physiological responses, including wound healing and bone formation [9]. MMP-9 becomes deregulated during tumorigenesis and is associated with pro-oncogenic events such as neo-angiogenesis, tumor cell proliferation and metastasis [10]. High level of MMP-9 expression in breast cancer is positively correlated with enhanced tumor cell invasion and metastasis [11,12] and with enhanced progression and poorer prognosis [10].
MMP-9 is conserved across several species (human, chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, and Arabidopsis thaliana). MMP-9 degrades type IV collagen, one of the most abundant collagens in the extracellular matrix (ECM) [13], which may stimulate local invasion, the first step in metastasis. In addition, MMP-9 also cleaves pro-cytokines, chemokines, and growth factors, thereby modifying their biological activity [14-16]. The downregulation of MMP-9 has been shown to increase β1-integrin expression, leading to activation of extracellular signal-regulated kinases (ERKs) and increasing apoptosis through one of two mechanisms: (1) release of cytochrome C into the cytosol and/or (2) increase in nuclear factor-kB (NF-kB) activation, followed by activation of caspase-3 [17].

Although few normal cell types express MMP-9 under normal physiological conditions, the majority of human metastatic tumor cells that have been tested consistently show elevated MMP-9 activity compared with benign control cells, including melanoma, fibrosarcoma, breast adenocarcinoma, and glioma [18-21]. In addition, tumor cells that stably express MMP-9 cDNA have been shown to have enhanced metastatic ability [22]. Thus, inhibition of MMP-9 expression could be a useful therapeutic modality to decrease the growth and invasive properties of tumor cells.

RNA-cleaving phosphodiester-linked DNA based enzymes (DNAzymes) are catalytic DNA molecules that specifically bind to and cleave targeted mRNA in a sequence-specific manner. The result is efficient degradation of the mRNA transcript, and thus, similar decreased expression levels of the encoded protein [23,24]. Catalytic oligonucleotides have emerged as novel, highly selective inhibitors or modulators of gene expression [25]. Khachigian and colleagues have reported that the DNAzymes targeting early growth response factor-1 (Egr1) mRNA inhibit neointimal formation after balloon injury to the rat carotid artery wall and reduce intimal thickening after stenting of pig coronary arteries [26]. DNAzyme targeting c-Jun causes repair of injured carotid arteries in rats [27]. Finally, a DNAzyme targeting vascular endothelial growth factor receptor 2 (VEGFR2) significantly inhibits the growth of breast tumors derived from xenografting of MDA-MB-435 cells into nude mice by inducing apoptosis [28].

Here, we examine the effects of a novel anti-MMP9 DNAzyme (AM9D) on breast tumor growth in the mouse mammary tumor virus-driven polyoma virus middle T oncprotein transgenic (MMTV-PyMT) mouse model of breast cancer. We demonstrate for the first time that once-weekly intratumoral injection of AM9D in the absence of any carrier molecule, for four weeks, was sufficient to significantly reduce the rate of tumor growth and final tumor load in a dose-dependent and statistically significant manner (P ≤0.05). Together, the data presented here justify the further development of AM9D for its potential as an anti-tumor agent and as an ideal candidate for breast cancer therapy.

Materials and methods
DNAzyme
All DNA oligonucleotides used in these experiments were synthesised by Integrated DNA Technology (Coralville, IA, USA). DNAzymes were designed according to the specific rule of 10-23 DNAzyme [29]. The DNAzyme targeting MMP9 mRNA contains a catalytic domain of 15 highly conserved deoxynucleotides flanked by two substrate-recognition domains. The sequence of the DNAzyme targeting mRNA of mouse and human MMP-9 is 5'-GTGGTGCCAGGCTAGC TACAAC-GATTTAGGTCG-3'. In the control DNAzyme, 5'-CTAGTCACCGGCTAGCTACACAGTAAAGCTGCT-3', the catalytic sequence of DNAzyme is flanked by nine bases randomly chosen and not specific for any MMP coding sequence. In some cases, the DNAzyme was end-labeled with Alexa Fluora C5-melamide 633 or Oregon Green™ 488 C5-maleimide (Invitrogen, Carlsbad, CA, USA) using T4 Polynucleotide kinase, as suggested by the manufacturer's protocol.

Cell transfection
MDA-MB-231 human breast tumor cell lines (ATCC, Manassas, VA, USA) were plated in DMEM supplemented with 10% fetal bovine serum (FBS) and allowed to grow to 80 to 90% confluence at 37°C with 5% CO2. The cells were then serum-starved for 4 hours prior to transient transfection with Oregon Green™488-maleimide-labeled AM9D or control DNAzyme (24 μg) using Lipofectamine 2000 (Invitrogen). After 18 hours incubation at 37°C in serum-free medium, cells were collected and sorted, and the transfected cells were isolated for further analysis.

Analysis of MMP9, MMP1, MMP13, MMP14, MMP19 and MMP21 mRNA levels in transfected cells
The MMP9, MMP1, MMP13, MMP14, MMP19 and MMP21 mRNA expression levels in the DNAzyme-transfected cells were quantified by reverse transcription-polymerase chain reaction (RT-PCR) using specific MMP9 (forward primer; 5-GCAGGAATGCGGCTCT GG-3', reverse primer; 5'-CCCCTCGAAGGGATACC-3'), MMP1 (forward primer; 5'-CATTCTACTGAGATCGG-3', reverse primer; 5'-AGAAACAGAAATGAA-3'), MMP13 (forward primer; 5-GAC TTCCCAAGGAATGGTGA-3), MMP14 (forward primer; 5'-GAGCCTAGGGCATCTAGTGA-3', reverse primer; 5'-CCACCTCAATGATGATCACC -3'), MMP19 (forward
primer; 5'-GGGTCTCCTTCTTTCTACAT-3', reverse primer; 5'-CAATCCTGCAGTACTGGTCT-3'), and MMP21 (forward primer; 5'-AACAATAGGACACGC-TATGG-3', reverse primer; 5'-CATCTCTTTTCATGTCCAG-3') primers [30]. Total RNA from the transfected cells was isolated by Trizol reagent (Invitrogen) and reverse-transcribed with random hexamer primers (Promega, Madison, WI, USA) using MMLV-RT enzyme (Invitrogen, Carlsbad, CA).

Mouse or human BACT (β-actin) mRNA was also amplified as internal controls, with corresponding (human forward; 5'-CAAGAGATGCCACCGGCGGT-3', human reverse; 5'-TCTTCTGCATCTCGTCAGCA-3', mouse forward; 5'-CAGGAGATGCCACGTCCCGCA-3', mouse reverse; 5'-AAGCCTTGCGGTGCACGATG-3') primers. The PCR products were subjected to 2% agarose gel and visualized by ethidium bromide staining. Expression was quantified by an Alpha Imager 2000 documentation and analysis system (Alpha Innotech Corporation, San Leandro, CA, USA).

Analysis of MMP-9 activity by gelatin zymography
MDA-MB-231 cells were transiently transfected with AM9D or control DNAzyme in serum-free medium as stated above. Twenty-four hours post transfection-media were collected and concentrated 10-fold using Amicon Ultracell filtration units (Millipore, Co Cork, Ireland). Protein concentration of the collected media was determined by Bradford dye binding techniques (a standard Bio Rad assay) using bovine serum albumin as a standard. The MMP-9 activity in the culture media was then assessed by gelatin zymography [31].

Cell invasion assay
Cells were transfected with fluorescently labeled AM9D or control DNAzyme for 18 hours in serum-free media as above. The fluorescent positive cells were identified by flow cytometry, isolated and seeded in ECMatrix™ invasion chambers (Millipore, Billerica, MA, USA). After 24 hours incubation at 37°C with 5% CO₂, the number of cells that migrated through the ECM layer and attached to the polycarbonate membrane was quantified spectrophotometrically at 560 nm according to the manufacturer’s protocol. The assays were done in multiples and the differences in the values between groups were evaluated by analysis of variance (ANOVA). P <0.05 was considered significant.

In vitro stability of DNAzyme
AM9D was incubated in PBS at 37°C, and an equal amount was removed at various time points and incubated with MMP9 mRNA at 37°C. After a 2-hour incubation the RNA samples were visualized on a 4% urea-polyacrylamide gel. For DNAzyme cellular uptake and stability, MDA-MB-231 cells were cultured on cover-glass slides. Cells were then transfected with 4 µg fluorescently labeled DNAzyme, as described above, fixed with formaldehyde at 24, 48, or 72 hours post transfection and visualized by confocal microscopy. The nucleus was visualized by 4',6-diamidino-2-phenylindole (DAPI)/anti-fade.

Animals
All animal experiments were conducted following approval by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee (IACUC). Friend virus B-type (FVB/N) female mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and crossed with PyMT-positive FVB males. The offspring were genotyped by real-time PCR on a Roche LC 480 LightCycler using the following primers and universal probe library (UPL) probe #11 (forward primer: 5' AACCCGAGTTCTCTCAACAG 3; reverse primer: 5' TCAGCAAC ACAAGGATTTTC 3') to identify MMTV-PyMT-positive females. Female mice were palpated once a week beginning at approximately 4 weeks of age and palpable tumors were measured in two dimensions (longest diameter and shortest width) with digital calipers. Tumor volume was calculated using the formula:

\[
\text{Tumor volume} = \text{Width}^2 \times \text{Length}/2
\]

When each transgenic female developed at least three palpable tumors of dimensions of 3 mm × 5 mm, which typically occurred at 8 weeks of age, each tumor was injected intratumorally with either 10 or 25 µg of AM9D or control DNAzyme suspended in PBS in a total volume of 5 µl, using a Hamilton syringe mounted with a PT2, 26G needle. Tumors identified at week 0 were injected once per week for a total of 4 weeks of therapy, and the site of intratumoral injection was varied to ensure that all areas of the tumor were exposed to the AMD9 or control DNAzyme. Palpable mammary tumors that arose after week 1 in other mammary glands of the same mice were left untreated. For each cohort, transgenic females with a combined number of at least nine tumors of comparable size were utilized (AMD9, 25 µg, n = 2 mice and 12 mammary tumors and control DNAzyme, n = 3 mice and 9 mammary tumors). An independent cohort of animals was also included in tumor endpoint volume studies, in which additional mice were treated with either control DNAzyme (25 µg, n = 3 mice and 15 mammary tumors) or AM9D (AM9D, 10 µg, n= 2 mice and 9 mammary tumors; 25 µg, n = 2 mice and 9 mammary tumors).

Tumor growth was monitored weekly by caliper measurement. All animals were euthanized one week after the last DNAzyme treatment (typically at 12 weeks of age). At necropsy, tumors were removed, final tumor dimensions were measured by calipers and the tumor...
wet weight was determined. Tumors were then either
flash frozen in liquid nitrogen, or fixed in 4% parafor-
maldehyde overnight, followed by cryoprotection in 25%
sucrose for several days. Cryoprotected tumors were
then washed with 0.1% PBS prior to embedding in opti-
mal cutting temperature (OCT) compound and prepara-
tion of 8-micron sections.

For analysis of Mmp9 mRNA expression levels in
tumors, OCT compound-embedded tumor sections
were scraped from glass slides of individual control
DNAzyme or AM9D-treated tumors to form a pool of
tumor material, and total RNA and cDNA was prepared
and analyzed by RT-PCR analysis as described above.

**Immunohistochemistry**

Mammary tumor vasculature was visualized using rat
anti-mouse CD31 antibody (1:50) (BD Biosciences, San
Jose, CA, USA) and Alexa Fluor-594 goat anti-rat IgG (H
+L) secondary antibody (Invitrogen). Stromal cells (myo-
fibroblasts) were detected using anti-α-smooth muscle
actin (α-SMA) antibody at 1:250 dilution (Sigma, St.
Louis, MO) and Alexa Fluor 488 goat anti-mouse IgG2a
(Invitrogen) secondary antibody at 1:500 dilution. MMP-
9 protein was detected using a rabbit anti-mouse MMP-9
antibody at 1:200 dilution (Santa Cruz Biotechnology,
Santa Cruz, CA) followed by Alexa Fluor-594 goat anti-
rabbit IgG antibody (1:500). Digital images were captured
using a Bio-Rad Confocal Laser Scanning Microscope,
using the Lasersharp 2000 software. Image J imaging ana-
lysis software was used for measurement of MMP-9,
CD31-immunostained endothelial area (EA), and cas-
pase-3-positive cells in the scanned immunohistochem-
istry (IHC) sections of mammary tumors. According to
Chantrain et al. [32], compared with the so-called hot
spot and the random fields methods, the EA measure-
ment method is more reproducible for quantification of
tumor vasculature.

**Statistical analysis**

All data are expressed as mean ± SD or standard error
(SE). Data were analyzed with SSPS software (SigmaStat
version 2.03) using one-way analysis of variance
(ANOVA), or Student’s t-test. Tumor growth over time
among three groups was analyzed by two-way ANOVA
using Prism software (Graphpad version 4.0b, La Jolla,
CA). In all cases, P-values <0.05 were considered statisti-
cally significant.

**Results**

AM9D treatment specifically reduces MMP-9 production
and suppresses the invasive behavior of breast tumor
cells in vitro

The specificity of AM9D toward Mmp9 mRNA was
demonstrated in MDA-MB-231 human breast cancer
cells. MDA-MB-231 cells express Mmp1, Mmp9,
Mmp13, Mmp14, Mmp19, and Mmp21 (Figure 1A,
lane 3). As shown in Figure 1A and 1B, contrary to con-
trol DNAzyme (lane 2), AM9D treatment (lane 1) signif-
icantly decreased the activity (Figure 1B) and the level of
Mmp9 mRNA (Figure 1A) in MDA-MB-231 cells

![Figure 1](http://breast-cancer-research.com/content/15/1/R12)

**Figure 1** Effect of AM9D treatment on metalloproteinase (MMP) expression in MDA-MB-231 cells. (A) Expression levels of MMP9, MMP1, Mmp13, Mmp14, Mmp19, and Mmp21, and BACT (β-actin) mRNA in MDA-MB-231-transfected cells. MDA-MB-231 cells were transfected with Oregon Green 488-labeled DNAzymes, control DNAzyme or mock transfection reagents as described in Materials and methods. Positively
transfected cells were identified by flow cytometry. Total RNA was isolated and Mmp9, Mmp1, Mmp13, Mmp14, Mmp19 and Mmp21, and BACT (β-
actin) mRNA were amplified by reverse-transcription (RT)-PCR and the PCR products were subjected to agarose gel and visualized by ethidium
bromide staining. Lane 1, AM9D; lane 2, control DNAzyme; lane 3, cells treated with DOTAP (N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium
propane methysulfate) transfection reagent only. (B) Gelatin zymography of culture media from transfected MDA-MB-231 cells. The cultured
media from MDA-MB-231 cells transfected with AM9D (lane 1), control DNAzyme (lane 2), or treated with DOTAP alone (lane 3) were separated
on 8% SDS polyacrylamide gel containing 1 mg/ml gelatin. (C) Histogram showing the percentage of carcinoma cells invading the ECMatrix™
matrigel matrix after treatment with AM9D compared to cells treated with control DNAzyme. Cells were transfected with Oregon Green 488
labeled-DNAzymes, sorted and cultured in a matrigel matrix invasion chamber as described in Materials and methods. *P <0.05 compared with
control (one-way analysis of variance).
without having an effect on **MMP1**, **MMP13**, **MMP14**, **MMP19** or **MMP21** mRNA levels. Although MMP-2 and -3 have also been reported to contribute to breast tumorigenesis [30], we did not detect **MMP2** or **MMP3** mRNA expression in cultured MDA-MB-231 cells. These data demonstrate that the AM9D therapy is specific as it only affects the production of MMP-9 in cells, and that reduction of **MMP9** mRNA leads to reduction in enzymatic activity, as expected.

The effect of decreased **MMP9** mRNA expression on the invasive behavior of MDA-MB-231 cells was assessed by transfecting the cells with fluorescently labeled AM9D or control DNAzyme and determining the invasive behavior of the sorted cells using the ECMatrix™ invasion chamber. As shown in Figure 1C, the mean invasion potential of MDA-MB-231 decreased by approximately 43% when transfected with AM9D compared to control DNAzyme-treated cells. These data are consistent with the reports of others demonstrating that MMP-9 is one of the key mediators of tumor cell invasion [11,12,33] and supports the idea of the DNAzyme gene-targeted approach for MMP-9 as a breast cancer therapeutic agent.

**MMP-9 is expressed in mammary tumors and the associated stroma in the MMTV-PyMT model**

The MMTV-PyMT transgenic mouse model is a widely used pre-clinical model of estrogen and progesterone receptor-negative luminal-like breast cancer with well-defined stages of progression and metastasis to lung [34,35]. More importantly, mammary adenocarcinomas exhibit changes in biomarkers similar to those observed in patients with breast cancer [34,36]. On a pure FVB/Nj strain background, all PyMT-positive females will eventually develop mammary tumors in each of their ten mammary glands, although the time of tumor onset varies among individual glands [35]. The expression patterns of various MMPs in the PyMT model [37-39] are also similar to those observed in patients diagnosed with ductal mammary adenocarcinoma [40]. Therefore, this model was chosen to ascertain the role of AM9D as a pharmacologic inhibitor of MMP-9.

To confirm the presence of MMP-9 protein in late-stage mammary carcinomas, tumors were harvested from MMTV-PyMT transgenic females at 12 weeks of age. Tumor sections were stained with antibodies to both α-SMA, a marker for stromal myofibroblasts, and MMP-9. IHC analysis demonstrated the presence of MMP-9 in the tumor epithelium, including areas highly populated with stromal fibroblasts (Figure 2). It is also likely that MMP-9 is produced by the tumor-associated macrophages that are known to be present in PyMT tumors [41,42].

**DNAzyme is stable in vitro and in vivo and is present in mammary tumors for at least 14 days post single intratumoral injection**

Prior to testing AM9D for its effect on mammary tumor growth, the *in vivo* stability and cellular uptake of naked DNAzyme molecules was examined by intratumorally injecting tumor-bearing MMTV-PyMT transgenic female mice with fluorescently labeled AM9D in PBS. The animals were then sacrificed at 7, 10, and 14 days (Figure 3A, a-c) post AM9D injection, and mammary tumors were harvested, sectioned, and viewed under a fluorescent microscope. As shown in Figure 3A, fluorescently-labeled oligonucleotides could be easily detected in a diffuse pattern within the tumor for up to 14 days (Figure 3A, c). Moreover, AM9D could also be detected in adjacent, non-injected mammary tumors of the same

---

**Figure 2** Immunohistochemical staining for metalloproteinase (MMP)-9 and α-smooth muscle actin (α-SMA) in mammary tumor sections. Tumors were resected from mice and double stained with antibodies to α-SMA to detect stromal cells (A) and MMP-9 (B). When channels were merged (C), these data show that MMP-9 was present in both stromal and tumor cells. Magnification 200×; scale bar is equivalent to 100 µm.
mouse (Figure 3A, d), indicating a wider distribution pattern than might be expected from intratumoral injection. Therefore, the DNAzymes are stable in vivo and can efficiently distribute within the injected tumor and to an adjacent non-injected tumor.

To further examine the stability of the DNAzyme in solution and in vitro, DNAzyme prepared in PBS was incubated for up to 14 days at 37°C. Aliquots were removed at different time intervals and the amount and activity of DNAzyme remaining over time was determined by applying the DNAzyme to a 6% urea-polyacrylamide gel electrophoresis of cleaved MMP9 RNA by AM9D. AM9D was incubated in PBS at 37°C for 14 days; an equal amount was removed at days 1, 3, 5, 7, 10, and 14 (1D to 14D, respectively) and incubated with MMP9 RNA substrate at 37°C for 2 hours. The products were then visualized on a 4% urea-polyacrylamide gel. Lane 1, RNA substrate alone; lane 2, AM9D without prior incubation at 37°C (0) cleaved RNA substrate into two fragments. AM9D incubated at 37°C for 1, 3, 5, 7, 10, and 14 days, lanes 3 to 8 respectively, did not lose its catalytic activity toward RNA substrate. (C) Stability of AM9D in vivo. The MDA-MB-231 cells were transfected with Oregon Green fluorescently labeled AM9D for 72 hours, and fixed and analyzed for the uptake and stability of AM9D molecule in the cells by fluorescent microscopy (400× magnification). (a) The nucleus is stained with 4′,6-diamidino-2-phenylindole (DAPI) and (b) AM9D is shown in green. (c) The overlap of AM9D with DAPI staining indicates that AM9D is present in both the cell cytosol and nuclei, as shown by the arrow.

AM9D treatment reduces final tumor load in the MMTV-PyMT tumor model

The efficacy of AM9D to reduce breast tumor volume in MMTV-PyMT transgenic mice was tested by directly injecting two concentrations (10 or 25 µg) of AM9D or control DNAzyme into mammary tumors of transgenic females bearing at least three tumors per mouse, each at an early palpable size (approximately 3 mm × 5 mm), once a week for 4 weeks. Tumor palpations were performed weekly to determine changes in tumor volume over time. The growth rate of AM9D-treated tumors (n = 12) was slower than both control DNAzyme-treated tumors (n = 9) and untreated tumors (n = 7) (Figure 4A). This resulted in a significant reduction in the final tumor volume of AM9D-treated compared to control DNAzyme-treated (P <0.001) and untreated (P <0.01) tumors at age 12 weeks (Figure 4A). In fact, administration of AM9D at 10 µg was sufficient to reduce the size of the tumor by 39.5% (n = 9, P ≤0.01) compared to control
(n = 24), which increased to 50% when 25 µg of AM9D was utilized (n = 21, P ≤ 0.01) (Figure 4B).

IHC analysis of the mammary tumors (Figure 4C) confirmed that AM9D treatment successfully downregulated MMP-9 protein expression. As shown in Figure 4C (b), AM9D treatment reduced mean MMP-9 expression by 66 ± 11% as compared to the control DNAzyme treatment (Figure 4C, a). This was further confirmed by the observation that the Mmp9 mRNA levels were 77% lower in AM9D-treated tumors compared with those tumors treated with control DNAzyme (Figure 4D). Taken together, these data show that AM9D efficiently decreases MMP-9 expression in tumors, resulting in the observed anti-tumor effects.

AM9D treatment suppresses angiogenesis and stimulates apoptosis in mammary tumors

MMP-9 has been shown to play a role in tumor progression through increase of bioavailability of VEGF and other factors that promote angiogenesis [43]. To determine the mechanism of tumor volume reduction by AM9D, the tumor slices were stained for CD-31 and for activated caspase-3 to assess the effect of AM9D on angiogenesis and apoptosis, respectively. As shown in Figure 5A and 5B, AM9D treatment significantly reduced the number of blood vessels in the tumor as demonstrated by the lack of robust CD-31 immunostaining in the AM9D-treated group (Figure 5A, c) versus untreated (Figure 5A, a) or the control DNAzyme-treated (Figure 5A, b) groups. Moreover, our data also indicate that AM9D potently induces apoptosis in the tumors, as only AM9D-treated tumors contained a large number of caspase-3-positive cells, as shown in Figure 5B (b). Quantitative analysis (Figure 5C) indicated that the number of CD31-positive cells was reduced 5-fold and that the intensity of the apoptotic cells increased 83-fold in tumors treated with AM9D compared to controls, respectively. These data suggest that the simultaneous

![Figure 4 Effect of AM9D on the rate of tumor growth, final mean tumor volume, and metalloproteinase (MMP)-9 expression.](http://breast-cancer-research.com/content/15/1/R12/figures/4)
Figure 5 Angiogenesis and apoptosis immunohistochemical staining of tumors

(A) Staining for CD31 (red) to detect the vasculature in (a) untreated mammary tumors and mammary tumors harvested from mice treated with (c) 25 µg of AM9D or (b) control DNAzyme. Untreated tumors (a) and tumors treated with control DNAzyme (b) contained more blood vessels than tumors treated with AM9D (c). Images are shown at 200x magnification. Scale bar is equivalent to 100 µm.

(B) Immunohistochemical staining for caspase-3 to indicate apoptosis in mammary tumors harvested from mice treated for 4 weeks with (a) control DNAzyme or (b) 25 µg AM9D. Very few caspase-3-positive cells (green) were detected in tumors treated with control DNAzyme (a) compared to AM9D-treated (b) tumors, where large regions stained positive for caspase-3 (green). Scale bar is equivalent to 100 µm.

(C) Histogram showing the mean CD31-immunostained endothelial area and mean apoptotic area in mammary tumors following treatment with AM9D as compared to untreated tumors and control DNAzyme-treated tumors. *P <0.05 compared with control (one-way analysis of variance and Student’s t-test).
anti-angiogenic and pro-apoptotic effect of AM9D delays tumor growth over time, and decreases tumor volume at our study endpoint.

Discussion
In this study, we showed for the first time, that the down-regulation of MMP-9 in mammary tumors by a novel anti-MMP-9 DNAzyme molecule results in a significant reduction in final tumor volume in the MMTV-PyMT transgenic mouse model of breast cancer. Downregulation of MMP-9 by AM9D was accompanied by a decrease in MMP-9 expression, decreased angiogenesis and increased apoptosis. Moreover, these effects were accomplished by intratumoral injection of naked DNAzyme without the use of any carriers. AM9D treatment also reduced the invasive potential of cultured MDA-MB-231 cells in vitro (Figure 1C). Together, these data indicate that specific inhibition of MMP-9 expression by DNAzyme has potential as a novel therapeutic modality to decrease the growth and invasion of carcinoma cells in the clinical setting.

It is known that MMP-9 plays a key role in angiogenesis by releasing VEGF [43] and that its downregulation induces apoptosis by stimulating the ERK pathway [17]. Martin et al. [44] have demonstrated that tumors developed in MMTV-PyMT MMP-9 wild-type mice are larger in size and are more highly vascular compared to those tumors that developed in MMTV-PyMT MMP-9 null mice. Thus, these data suggest that AM9D treatment affects tumor growth via different pathways, as downregulation of MMP-9 by AM9D inhibited angiogenesis and induced apoptosis (Figure 5) as demonstrated by lack of CD31 staining and the enhanced presence of caspase-3 in AM9D-treated tumors.

Our results are consistent with those of Almholt et al. [40] in which the broad-spectrum MMP inhibitor, Galardin/GM6001, significantly reduced primary mammary tumor growth and lung metastasis in the MMTV-PyMT model. However, contrary to broad-spectrum MMP inhibitors, including GM6001, AM9D treatment specifically downregulates MMP-9 without affecting the expression of other members of the MMP family. As demonstrated by the extent of cytotoxicity of broad-spectrum MMP inhibitors in prior clinical trials [45-47], total inhibition of MMP is not practical. Various MMPs can exert both pro-tumorigenic and anti-tumorigenic properties [48], and some MMPs are critical for normal physiological processes, such as bone growth and remodeling, ovulation, and wound healing [49]. Further, in comparison with GM6001 [40], the intratumoral injection of AM9D not only reduced the required frequency of therapy, but was also equally efficient in reducing final tumor size. Once weekly, intratumoral injections of 25 µg AM9D (1.25 mg/Kg) was sufficient to reduce the size of these spontaneously developed tumors by 50% as compared to the 51% tumor reduction observed following daily administration of 100 mg/Kg of GM6001. Thus, the high degree of specificity of AM9D for targeting MMP-9, its in vivo stability, and the lack of any observed in vivo toxicity (Hallett M, Dalal P, Sweatman T, Pourmotabbed T: Naked Anti-Matrix Metalloproteinase-9 DNAzyme Administered Systemically Distributes to All Organs of Healthy and MMTV-PyMT Transgenic Mice and Is Safe; manuscript in review) should enhance the clinical response of solid tumors, including breast tumors, to AM9D treatment, while evading the serious side effects experienced with systemic therapy based on broad-spectrum MMP inhibitors.

The MMTV-PyMT transgenic model limited our ability to assess the efficacy of AM9D on treating spontaneous lung metastasis in vivo because not all tumors in each animal grow synchronously, and thus, not all tumors were intratumorally treated with therapy. Therefore, it was not feasible to determine the origin of metastatic cells (from treated or untreated tumors). The efficacy of AM9D in inhibiting lung metastasis is under investigation using a mouse model of metastasis.

Conclusions
Our results indicate that the downregulation of MMP9 mRNA and protein expression with naked anti-MMP-9 DNAzyme is sufficient to reduce mammary tumor burden. We also describe that tumor size reduction is a result of decreased MMP-9 expression, decreased angiogenesis, and increased apoptotic cells in tumors treated with AM9D. These findings suggest specific targeting and downregulation of MMP-9 by AM9D could prove useful as a therapy against breast carcinoma tumor growth and invasion.

Abbreviations
ANOVA: analysis of variance; α-SMA: alpha smooth muscle actin; AM9D: anti-MMP-9 DNAzyme; bp: base pairs; DAPI: 4',6-diamidino-2-phenylindole; DMEM: Dulbecco’s modified Eagles medium; DNAzyme: catalytic oligodeoxynucleotide; EA: endothelial area; ECM: extracellular matrix; Egr-1: early growth response factor-1; ERK: extracellular signal-regulated kinase; FBS: fetal bovine serum; FVB: Friend virus B-type; IHC: immunohistochemistry; MMP: matrix metalloproteinase; MMTV-PyMT: mouse mammary tumor virus-polyoma virus middle T; NF-κB: nuclear factor-κB; OCT: optimal cutting temperature; PBS: phosphate-buffered saline; RT-PCR: reverse transcription-polymerase chain reaction; SE: standard error; VEGF: vascular endothelial growth factor receptor 2.

Authors’ contributions
MH performed in vivo and in vitro research, participated in the design and coordination of the study, analysis and interpretation of all results, and drafting of the manuscript. BT performed in vitro research and immunohistochemistry. HH participated in the design, execution, acquisition, analysis and interpretation of in vitro research. LS provided training for the animal model and wrote the paper. All authors have read and approved the manuscript for publication. TS provided MMTV-PyMT transgenic mice, financial support, and wrote the paper. TP designed research, performed in vivo and in vitro research, analyzed and interpreted all the data, wrote the paper, and provided financial support.
Competing interests

Tayebah Pourmotabbed has applied for a patent entitled, Inhibition of tumour growth and invasion by anti-matrix metalloproteinase DNAzyme, US Divisional Patent Application Serial number 12/390,628. We have no other competing interests to declare.

Acknowledgements

We thank Dr. Kent Hunter for generously providing the MMTV-PyMT+ mice that had been previously backcrossed to the FVB/Nj strain background. We also would like to thank Drs. Yi Lu, RK Rao, Trevor Sweatman, and David Armbruster for their constructive criticism and input. Finally, we would like to thank Pooja Dalal for her assistance. This work was partially supported by USPHS GRANT CA-107183 (TP) and USPHS F31CA144572 (MH).

Author details

1Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, 19 S. Manassas St, Memphis TN 38163, USA. 2Center for Cancer Research, Department of Pathology, University of Tennessee Health Science Center, 19 S. Manassas St., Memphis TN 38163, USA.

Received: 22 May 2012 Revised: 12 January 2013 Accepted: 8 February 2013 Published: 13 February 2013

References

1. American Cancer Society. Breast Cancer Facts and Figures 2007-2008. American Cancer Society, Inc. Atlanta, GA, 2009 [http://www.cancer.org].
2. Weigel B, Peterse JL, van ’t Veer LJ. Breast cancer metastasis: markers and models. Nat Rev Cancer 2005, 5:591-602.
3. Phadke PA, Vaidya KS, Nash KT, Hurst DR, Welch DR. Matrix metalloproteinases and the development of cancer. Chem Biol 1996, 3:989-904.
4. Kanai Y, Hayakawa T, Okada Y. Matrix metalloproteinase-9 (92 kDa gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation with cellular invasion. J Cell Biol 1997, 139:1861-1872.
5. Noel A, Jost M, Maquoi E. Matrix metalloproteinases and their biological function in human gliomas. Int J Dev Neurosci 1999, 17:495-502.
6. Chintala SK, Tomm JC, Rao JS. Matrix metalloproteinases and their role in breast cancer model provides a reliable model for human diseases. Breast Cancer Res 2006, 8:R12-23.
7. Goodyer SG, Yousif GI, El-Naggar AK. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. J Histochem Cytochem 2007, 55:151-158.
8. Sun LQ, Wray NP, Khachigian LM. Catalytic nucleic acid enzymes: from lab to applications. Pharmacol Rev 2000, 52:325-347.
9. Hegeduš J, Cho H, Xie X, Elcner GI. Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. J Cell Physiol 2008, 216:480-488.
10. Bu CH, Pourmotabbed T. Mechanism of activation of human neutrophil gelatinase B: Discriminating between the role of Ca in activation and catalysis. J Biol Chem 1995, 270:18563-18569.
11. Griseau N, Gaudry G, Mornon JP, Bismuth C. Catalytic nucleic acids: from lab to applications. Proc Natl Acad Sci 2007, 104:16331-16336.
12. Zhang L, Gasper WL, Stass SA, Ioffe OB, Davis MA, Missson AJ. Angiogenic inhibition mediated by a DNAzyme that targets vascular endothelial growth factor receptor 2. Cancer Res 2002, 62:5463-5469.
13. Sun LQ. Sun LQ, Caia M, Saravolac RG, Prasad A, Gerlach WL. Catalytic nucleic acids: from lab to applications. Pharmacol Rev 2000, 52:325-347.
14. Hegeduš J, Cho H, Xie X, Elcner GI. Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. J Cell Physiol 2008, 216:480-488.
15. Bu CH, Pourmotabbed T. Mechanism of activation of human neutrophil gelatinase B: Discriminating between the role of Ca in activation and catalysis. J Biol Chem 1995, 270:18563-18569.
16. Chintala SK, Tomm JC, Rao JS. Matrix metalloproteinases and their role in breast cancer model provides a reliable model for human diseases. J Histochem Cytochem 2007, 55:151-158.
17. Goodyer SG, Yousif GI, El-Naggar AK. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. J Histochem Cytochem 2007, 55:151-158.
18. Griseau N, Gaudry G, Mornon JP, Bismuth C. Catalytic nucleic acids: from lab to applications. Proc Natl Acad Sci 2007, 104:16331-16336.
19. Sun LQ. Sun LQ, Caia M, Saravolac RG, Prasad A, Gerlach WL. Catalytic nucleic acids: from lab to applications. Pharmacol Rev 2000, 52:325-347.
20. Hegeduš J, Cho H, Xie X, Elcner GI. Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. J Cell Physiol 2008, 216:480-488.
21. Bu CH, Pourmotabbed T. Mechanism of activation of human neutrophil gelatinase B: Discriminating between the role of Ca in activation and catalysis. J Biol Chem 1995, 270:18563-18569.
22. Chintala SK, Tomm JC, Rao JS. Matrix metalloproteinases and their role in breast cancer model provides a reliable model for human diseases. J Histochem Cytochem 2007, 55:151-158.
23. Griseau N, Gaudry G, Mornon JP, Bismuth C. Catalytic nucleic acids: from lab to applications. Proc Natl Acad Sci 2007, 104:16331-16336.
24. Sun LQ. Sun LQ, Caia M, Saravolac RG, Prasad A, Gerlach WL. Catalytic nucleic acids: from lab to applications. Pharmacol Rev 2000, 52:325-347.
25. Hegeduš J, Cho H, Xie X, Elcner GI. Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. J Cell Physiol 2008, 216:480-488.
26. Bu CH, Pourmotabbed T. Mechanism of activation of human neutrophil gelatinase B: Discriminating between the role of Ca in activation and catalysis. J Biol Chem 1995, 270:18563-18569.
27. Chintala SK, Tomm JC, Rao JS. Matrix metalloproteinases and their role in breast cancer model provides a reliable model for human diseases. J Histochem Cytochem 2007, 55:151-158.
28. Griseau N, Gaudry G, Mornon JP, Bismuth C. Catalytic nucleic acids: from lab to applications. Proc Natl Acad Sci 2007, 104:16331-16336.
29. Sun LQ. Sun LQ, Caia M, Saravolac RG, Prasad A, Gerlach WL. Catalytic nucleic acids: from lab to applications. Pharmacol Rev 2000, 52:325-347.
30. Hegeduš J, Cho H, Xie X, Elcner GI. Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. J Cell Physiol 2008, 216:480-488.
39. Almholt K, Green KA, Juncker-Jensen A, Nielsen BS, Lund LR, Romer J: Extracellular proteolysis in transgenic mouse models of breast cancer. J Mammary Gland Biol Neoplasia 2007, 12:83-97.

40. Almholt K, Juncker-Jensen A, Laerum OD, Dana K, Johnsen M, Lund LR, Romer J: Metastasis is strongly reduced by the matrix metalloproteinase inhibitor Galardin in the MMTV-PyMT transgenic breast cancer model. Mol Cancer Ther 2008, 7:2758-2767.

41. Lin EY, Nguyen AV, Russell RG, Pollard JW: Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 2001, 193:727-740.

42. Lin EY, Gouon-Evans V, Nguyen AV, Pollard JW: The macrophage growth factor CSF-1 in mammary gland development and tumor progression. J Mammary Gland Biol Neoplasia 2002, 7:147-162.

43. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D: Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000, 2:737-744.

44. Lin EY, Carter KJ, Jean-Philippe SR, Chang M, Mobashery S, Thiollay S, Lynch CC, Matrisian LM, Fingleton B: Effect of ablation or inhibition of stromal matrix metalloproteinase-9 on lung metastasis in a breast cancer model is dependent on genetic background. Cancer Res 2008, 68:6251-6259.

45. Smyth E: The trouble with inhibitors. Signalling Scissors: New Perspectives on Proteases, Horizon Symposium, Brescia, Italy, Nature Publishing Group 2003, 1-4[http://www.nature.com/horizon/proteases/background/pdf/inhibitors.pdf].

46. Wojtowicz-Praga SM, Dickson RB, Hawkins MJ: Matrix metalloproteinase inhibitors. Invest New Drugs 1997, 15:61-75.

47. Martin MD, Matrisian LM: The other side of MMPs: protective roles in tumor progression. Cancer Metastasis Rev 2007, 26:717-724.

48. Martin MD, Matrisian LM: Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 2002, 295:2387-2392.

doi:10.1186/bcr3385

Cite this article as: Hallett et al: Anti-matrix metalloproteinase-9 DNAszyme decreases tumor growth in the MMTV-PyMT mouse model of breast cancer. Breast Cancer Research 2013 15:R12.