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Direct and Tumor Microenvironment Mediated Influences of 5′-Deoxy-5′-(Methylthio)Adenosine on Tumor Progression of Malignant Melanoma

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ABSTRACT

Recent studies have shown that a loss of methylthioadenosine phosphorylase (*MTAP*) gene expression exerts a tumor-promoting effect, including induction of invasiveness, enhanced cell proliferation, and resistance against cytokines. To date, the molecular mechanisms underlying these effects remain unknown. Since the loss of MTAP expression resulted in induced secretion of 5′-deoxy-5′-(methylthio)adenosine (MTA), we hypothesized that MTA might modulate the observed effects. We first determined MTA levels produced by tumor cells *in vitro* and *in situ* by means of stable isotope dilution liquid chromatography tandem mass spectrometry. Subsequently, we revealed induction of matrix metalloproteinase (MMP) and growth factor gene expression in melanoma cells accompanied by enhanced invasion and vasculogenic mimicry. In addition, MTA induced the secretion of basis fibroblast growth factor (bFGF) and MMP3 from fibroblasts and the upregulation of activator protein-1 (AP-1) activity in melanoma cells and fibroblasts. In summary, we demonstrated a tumor-supporting role of MTA. J. Cell. Biochem. 106: 210–219, 2009.

KEY WORDS: MALIGNANT MELANOMA; TUMOR MICROENVIRONMENT; METHYLTHIOADENOSINE

INTRODUCTION

Methylthioadenosine phosphorylase (MTAP, EC 24.2.28) plays a major role in polyamine metabolism and the salvage of adenine and methionine by catalyzing the phosphorylation of 5′-deoxy-5′-(methylthio)adenosine (MTA) to methylthioribose-1-phosphate and the subsequent release of adenine in the presence of orthophosphate. MTA itself is a by-product of polyamine synthesis and acts as a potent inhibitor of polyamine aminopropyltransferase and methyltransferases.

MTAP is expressed abundantly in normal cells and tissues [Olopade et al., 1995]. In contrast, many malignant cells lack MTAP activity [Hori et al., 1998; Wong et al., 1998; Christopher et al., 2002; Garcia-Castellano et al., 2002]. Tumor cells lacking this enzyme, unlike enzyme-positive cells, excrete MTA instead of metabolizing it [Savarese et al., 1983]. The reason for the frequent loss of MTAP activity became evident once the chromosomal location of *MTAP* had been determined. Starting from the centromeric end, the gene order on human chromosome 9p21 was mapped as p15—p16—MTAP—IFN-α—IFN-β [Olopade et al., 1995]. In many tumors, this

Abbreviations: AP-1, activator protein-1; bFGF, basis fibroblast growth factor; COL1A1, collagen type I α-1; COL3A1, collagen type III α-1; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; HDF, human dermal fibroblasts; MMP, matrix metalloproteinase; MTA, 5′-deoxy-5′-(methylthio)adenosine; MTAP, methylthioadenosine phosphorylase; NF-κB, nuclear factor-kappa B; RT-PCR, reverse transcriptase-polymerase chain reaction; SID-LC/MS/MS, stable isotope dilution-liquid chromatography tandem mass spectrometry; VEGF, vascular endothelial growth factor.

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region is deleted. For instance, *MTAP* gene deletions were described in melanoma, hepatocellular carcinoma, endometrial cancer, osteosarcoma, and hematological neoplasias such as lymphoblastic leukemia and non-Hodgkin lymphomas [Hori et al., 1998; Wong et al., 1998; Christopher et al., 2002; Garcia-Castellano et al., 2002; Watanabe et al., 2008].

We recently determined that loss of MTAP expression in malignant melanoma results in a higher invasive potential [Behrmann et al., 2003]. However, thus far the molecular link between loss of MTAP expression, enhanced MTA accumulation, and induction of cellular invasion has not been analyzed. In this study, we measured MTA accumulation in melanoma as a function of loss of *MTAP* gene expression and investigated the MTA-induced signaling pathways and target genes. Since MTA is known to accumulate extracellularly, we also studied the effect of MTA on fibroblasts in the microenvironment of melanoma cells.

MATERIALS AND METHODS

CELL LINES, TISSUE, AND TISSUE CULTURE

The melanoma cell lines Mel Im, Mel Ho, HTZ-19d, Mel Ei, Mel Juso, and Mel Ju were described in detail previously [Jacob et al., 1998]. Mel Im, HTZ-19d, and Mel Ju are derived from melanoma metastases, while Mel Ho, Mel Ei, and Mel Juso are derived from primary melanomas. Human dermal fibroblasts (HDF) were isolated from healthy skin. Cells were grown at 37°C/5% CO_2 in Dulbecco's modified Eagle medium (DMEM; PAN Biotech GmbH, Aidenbach, Germany), which was supplemented with penicillin (100 U/mL), streptomycin (10 μg/mL) (both from PAN Biotech), and 10% fetal calf serum (FCS, PAN Biotech). Human primary melanocytes (PromoCell, Heidelberg, Germany) derived from normal skin were cultivated in melanocyte growth medium M2 (PromoCell) under a humified atmosphere of 5% CO_2 at 37°C. Cells were used between passages 2 and 4 and not later than 2 days after trypsinization. Cells were detached for subcultivation or assay with 0.05% trypsin, 0.04% EDTA in PBS. MTA (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in water and used to treat the cells at the concentrations given for 24 h. Proliferation was determined using the XTT assay (Roche, Mannheim, Germany). Inhibition of extracellular signal-regulated kinase (ERK) signaling was achieved by treatment with UO126 (30 μM, Sigma-Aldrich Chemie).

Cryo-preserved tissue samples of malignant melanoma ($n = 5$) were used after careful histological evaluation of the samples. The areas representing the tissue of interest (*e.g.*, tumor or skin) were punched according to a protocol previously reported for tumor tissue arrays. Extraction of MTA was performed as described below after determination of the weight of the tissue samples.

PROTEIN PRECIPITATION AND EXTRACTION OF MTA

Prior to protein precipitation with ice-cold methanol (600 μL; Fisher Scientific GmbH, Schwerte, Germany), 10 μL of a 224 nM aqueous solution of stable isotope labeled MTA, which had been prepared in-house following a published protocol (Robins et al., 1991), were added as internal standard to 200 μL of cell medium in an Eppendorf vial (Eppendorf AG, Wesseling-Berzdorf, Germany). The sample was vortexed, centrifuged at 9,000 × *g* for 5 min, and the supernatant was transferred to a 1.5-mL glass vial (Fisher Scientific). The protein pellet was washed twice with 200 μL of methanol (LCMS grade, Fisher Scientific) and all supernatants were combined. After drying, the residues were reconstituted in 100 μL of PURELAB Plus water (ELGA LabWater, Celle, Germany).

For cell pellet extraction, ice-cold methanol (600 μL) was added to the frozen pellets followed by the addition of internal standard as described above. For complete cell lysis, the cells were shock-frozen in liquid nitrogen and thawed on ice. The freeze/thaw cycle was performed thrice and samples were vortexed between cycles. After centrifugation the supernatant was transferred to a glass vial. The pellet was washed twice with 200 μL of methanol and all supernatants were combined. After drying, the residues were reconstituted in 100 μL of PURELAB Plus water.

Tissue punches were lysed after weighing in 50 μL water and the lysates were treated as described above for cell culture medium.

LC–ESI–MS/MS OF MTA

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was performed on an Agilent 1200 SL HPLC system (Böblingen, Germany) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems, Darmstadt, Germany). Chromatography was performed on a Waters (Eschborn, Germany) Atlantis® T3 3-μm (1.0 i.d. × 150 mm^2) reversed-phase column at 25°C. The mobile phase consisted of 0.1% acetic acid (Sigma-Aldrich) in PURELAB Plus water. MTA was eluted with a linear acetonitrile (LCMS grade, Fisher Scientific) gradient (0–100% in 10 min, 100% for 5 min, and 0% for 15 min) at a flow rate of 125 μL/min. Injection volume was 10 μL.

Quantitative determination was performed in multiple-reaction monitoring (MRM) mode using the following ion transitions: m/z 298.2 (M + H+) to m/z 136.1 (product ion) for endogenous MTA, and m/z 303.2 (M + H+) to m/z 136.1 (product ion) for the $^{13}C_5$-labeled internal MTA standard (Stevens et al., 2008).

ISOLATION AND QUANTIFICATION OF RNA

Total RNA was isolated using the RNeasy Mini Kit following the manufacturer's manual (Qiagen, Hilden, Germany). One-tenth of the RNA was used for reverse transcription as described previously [Bataille et al., 2005]. Reverse transcription was performed using Superscript II (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's manual. For quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR), 1 μL of cDNA, 10 μL of SYBR green mix (Invitrogen), and 0.5 μL of forward and reverse primers (20 μM) in a total of 20 μL were applied. The following PCR program was performed: 30 s 95°C (initial denaturation); 20°C/s temperature transition rate up to 95°C for 15 s, 3 s 68°C, 5 s 72°C, 81°C acquisition mode single, repeated for 40 times (amplification). Annealing temperatures were optimized for each primer set. PCR reactions were evaluated by melting curve analysis and 1.5% agarose gel electrophoresis. Each quantitative PCR was performed in triplicate for two separate RNA preparations.

The following primers were used for PCR: *BFGF* (MIM #134920): fwd, AGC CTA GCA ACT CTG CTG GTG ATG G, rev, ACG TAG GAG ACA CAG CGG TTC GAG; collagen type I α-1 (*COL1A1*) (MIM #120150): fwd, GGA CCT CCG GCT CCT GCT, rev: TCA CCA GGC

TCA CCA GCA GG; collagen type III α-1 (*COL3A1*) (MIM #120180): fwd, CAA GGC TGA AGG AAA CAG CA, rev, CCA ATG TCC ACA CCA AAT TCT; matrix metalloproteinase3 (*MMP3*) (MIM #185250): fwd, CAC TCA CAG ACC TGA CTC GGT TC, rev, AGG TTG GCA CTC CTT TTA GCT AC; *MMP9* (MIM #120361): fwd, GAG GTG CCG GAT GCC ATT CAC GT, rev, CCG AGC TGA CTG GAC GGT GAT G; *MMP14* (MIM #600754): fwd, GGA ACC CTG TAG CTT TGT GTC TGT C, rev: TCT CTA CCC TCA AC AAG ATT AGA TTC C; *VEGFB* (MIM #601398): fwd, TGG TGG TGC CCT TGA CTG TGG AGC, rev, CTG TCT GGC TTC ACA GCA CTG TCC.

QUANTIFICATION OF PROTEIN EXPRESSION

Vascular endothelial growth factor (VEGF) and basis fibroblast growth factor (bFGF) protein expression was measured in the cell culture supernatant after 24 h of MTA treatment using the RayBio ELISA kit human VEGF and the RayBio ELISA bFGF, respectively (RayBiotech, Norcross, GA). The ELISAs were performed according to the manufacturer's protocol.

MIGRATION AND INVASION ASSAYS

For invasion assays, Boyden chambers containing 8-μm pore size polycarbonate filters (Costar, Bodenheim, Germany) were used as described previously [Behrmann et al., 2003]. Filters were coated with either gelatin (5 mg/L) to improve cell attachment (migration) or a commercially available reconstituted basement membrane (Matrigel, diluted 1:3 in H_2O; Becton Dickinson, Heidelberg, Germany) (invasion). The lower compartment was filled with fibroblast-conditioned medium, used as a chemoattractant. Cells were harvested by trypsinization for 2 min, re-suspended in DMEM without FCS at 2×10^5 cells/mL and placed in the upper compartment of the chamber. After incubation at 37°C for 4 h, the filters were collected and the cells adhering to the lower surface were fixed, stained, and counted. Experiments were carried out in triplicate and repeated three times with consistent results.

Migration of cells was assayed by scratch assays. For scratch assays ("wound-healing-assay") cells were seeded in high density into six-well plates and scratched by a pipette tip in a definite array. Migration into this array was documented and measured after 24 and 48 h. Total migration of cells after 48 h was measured using a Carl Zeiss microscope (Carl Zeiss Vision GmbH, Hallbergmoos, Germany) and set as 100%. Relative migration of treated to untreated cells was calculated. Each analysis was performed in duplicate and was repeated thrice.

COLLAGEN CONTRACTION

Collagen contraction was performed as previously described [Wixler et al., 2007]. In brief, 250 μL of fibroblast suspension (10^6 cells/mL) were added to 3 mL collagen type I solution (3 mg/mL) and placed into a 30-mm Petri dish (Greiner). Contraction of the developing collagen sponge was determined by measuring the diameter every hour over 24 h.

MATRIGEL TUBE FORMATION ASSAYS

Tube formation assays were performed as described previously [Rothhammer et al., 2007]. Growth factor reduced Matrigel™ (200 μL) (BD Biosciences, Heidelberg, Germany) was added to eight-chamber polystyrene vessel tissue culture-treated glass slides (BD Falcon, Discovery Labware, Bedford, MA, USA) and allowed to gelatinize for 20 min at 37°C. 5×10^4 melanoma cells were seeded into each chamber in DMEM/MCDB-131 medium. The cells were then treated with 100 ng/mL BMP2 or BMP4, respectively. Tube formation was assessed by phase contrast microscopy after 16 h and recorded with a digital camera (AxioCam, MR Grab, Carl Zeiss, München-Hallbergmoos, Germany).

To assay vasculogenic mimicry, 7×10^4 melanoma cells were seeded onto matrigel-coated eight-chamber polystyrene vessel tissue culture-treated glass slides. Tube formation was measured by phase contrast, recorded with a digital AxioCam camera, and an image overlay was generated using Adobe Photoshop 6.0 software.

TRANSFECTION EXPERIMENTS

Cells (2×10^5 per well) were seeded into six-well plates and transfected with 0.5 μg of activator protein-1 (AP-1) reporter constructs (Promega, Mannheim, Germany) using lipofectamine plus (melanoma cells, Invitrogen) or Amaxa transfection (fibroblasts, Amaxa GmbH, Cologne, Germany). Three hours after transfection, cells were treated for 24 h before they were lysed to measure luciferase activity. For normalization of transfection efficiency, 0.2 μg of a pRL-TK plasmid (Promega) was co-transfected and renilla luciferase activity measured by a luminometric assay (Promega). All transfection experiments were repeated thrice.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The EMSA was based on the binding of nuclear AP-1 to a ^{32}P-labeled AP-1 consensus oligonucleotide. A double-stranded oligomeric (5′-CGC TTG ATG AGT CAG CCG GAA-3′) binding site for AP-1 was purchased from Promega. The fragments were end-labeled and shifts were performed as described previously [Wenke et al., 2006].

STATISTICAL ANALYSIS

Results are expressed as mean ± SD (range) or percent. Comparison between groups was made using the Student's paired *t*-test. A *P*-value of <0.05 was considered statistically significant. All calculations were performed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

IN VITRO AND *IN VIVO* DETECTION OF MTA

Recently, we showed that expression of MTAP was strongly reduced in malignant melanoma cells [Behrmann et al., 2003]. Due to the lack of a sensitive detection method, we could not determine at that time the amounts of MTA in or secreted by melanoma cells *in vitro* or *in vivo*. Here, we employed MRM to measure MTA specifically and quantitatively by stable isotope dilution liquid chromatography tandem mass spectrometry (SID-LC-MS/MS) (Stevens et al., 2008). We could now show that MTA levels in melanoma cells are strongly increased compared to normal human epidermal melanocytes (Fig. 1A). MTA was also found in high amounts in lysates of melanoma tissue. As a matter of fact, higher levels in tissue

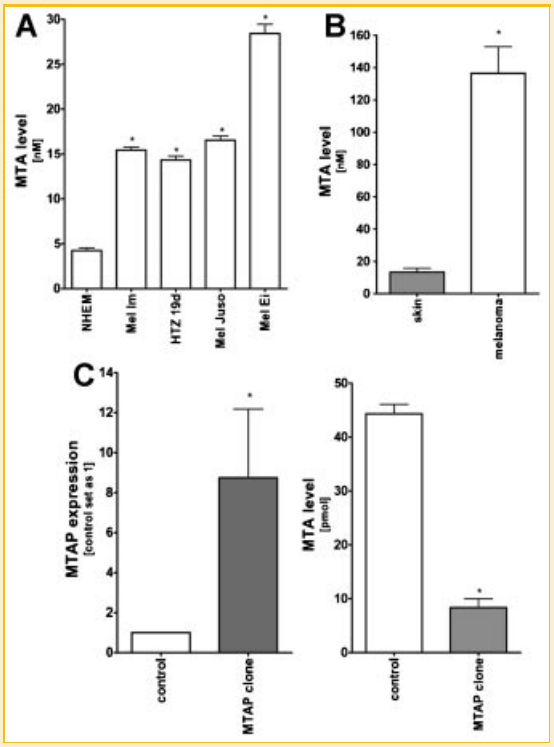

Fig. 1. Determination of MTA amounts in (A) the supernatant of melanoma cell lines (Mel Im, Mel Juso, Mel Ei, HTZ19d) and normal human melanocytes (NHEM) ($^*P < 0.05$ compared to NHEM), (B) preparations of melanoma samples (n = 5) and of normal skin (n = 3) ($^*P < 0.05$ compared to normal skin), and (C) melanoma cells stably transfected to re-express MTAP compared to wild type Mel Im cells (left: MTAP mRNA expression, right: MTA amounts) ($^*P < 0.05$ compared to control).

compared to cell culture were observed, suggesting accumulation in the tumor (Fig. 1B).

To clearly show that the MTA level correlated negatively with MTAP expression we analyzed melanoma cells over-expressing MTAP after stable transfection generated in a recently published study [Behrmann et al., 2003]. After MTAP expression the MTA level dropped significantly (Fig. 1C).

EFFECT OF MTA ON MELANOMA CELLS

Finding high levels of MTA in the tumor led us to investigate whether MTA has an influence on tumor development or progression. As *VEGF*, *BFGF*, *MMP9*, and *MMP14* gene expression is known to correlate with melanoma progression and tumor aggressiveness, these genes were chosen for analysis. To determine the direct effect of MTA on melanoma cells, three melanoma cell lines (Mel Im, Mel Ju, Mel Ho) were treated with two doses of MTA similar to those found in melanoma tissue. By quantitative RT-PCR significant induction of *VEGF*, *BFGF*, *MMP9*, and *MMP14* gene expression was detected upon administration of MTA (Fig. 2A–D). Interestingly, no regulation of *VEGF*, *BFGF*, *MMP9*, and *MMP14* gene expression was observed in primary human melanocytes (data not shown). Induction of gene expression was confirmed at the protein level by measuring bFGF and VEGF in the supernatant of melanoma cells. Here, treatment with 0.05 mM MTA led to a 1.8- and 1.6-fold increase in VEGF and bFGF expression, respectively. Treatment with 0.1 mM MTA resulted in a respective 1.97- and 1.76-fold increase in VEGF and bFGF expression (VEGF: untreated: 661 pg/mL ± 40.5, 0.05 mM: 1197 pg/mL ± 74, 0.1 mM: 1302 pg/mL ± 269; bFGF: untreated: 4113 pg/mL ± 832, 0.05 mM: 6757 pg/mL ± 1665, 0.1 mM: 7237 pg/mL ± 598).

As a consequence of induction of gene expression in melanoma cells, an induction of invasive potential as measured in a Boyden Chamber model was observed (Fig. 3A), but not of apoptosis and proliferation (data not shown).

Due to induction of bFGF and VEGF, we were also interested in analyzing the effect of MTA on vascular mimicry of melanoma cells. Here, a significant effect on induction of vessel-like structures in the tube formation assay was found (Fig. 3B).

EFFECT OF MTA ON STROMAL FIBROBLASTS

The high levels of MTA in the tumor microenvironment let us pursue the question whether MTA also exerts an effect on stromal cells found in tumors. As shown in Figure 4A and B, expression of collagen types I and III was induced by MTA in HDF. In addition, RT-PCR revealed induction of *MMP3* (Fig. 4C), but not *MMP1* (data not shown). In agreement with the melanoma cell data, induction of *VEGF* and *BFGF* gene expression was observed (Fig. 4D and E). Functionally, proliferation and migration of fibroblasts was induced by MTA (Fig. 5A and B). Interestingly, the ability of fibroblasts to organize collagen measured in a collagen contraction assay was also increased upon MTA treatment (Fig. 5C), suggesting an induction of fibroblast activity.

SIGNALING INDUCED BY MTA

To understand the molecular mechanism of cell regulation, we determined the influence of MTA on the AP-1 and nuclear factor-kappa B (NF-κB) signaling pathways. Interestingly, in reporter gene assays we found only minor regulation of NF-κB activity by MTA at doses of 0.05 and 0.1 mM, respectively (data not shown). Conversely, AP-1 activity, which is already high in melanoma cells, was induced further (Fig. 6A). EMSA confirmed induction of AP-1 activity as evidenced by AP-1 binding to a consensus binding site (Fig. 6B).

In agreement with the results gained in melanoma cells, MTA caused an upregulation of AP-1 in fibroblasts as shown in reporter gene assays and EMSA (Fig. 6C and D). EMSA clearly indicates lack of AP-1 activity in the untreated fibroblasts and induction after MTA treatment.

To analyze whether the observed regulation of gene expression was due to induction of AP-1 activity, the effect of the ERK inhibitor (UO126) was measured in fibroblasts. Induction of collagen type III expression in dermal fibroblasts was significantly inhibited by UO126 (Fig. 6E), whereas no significant changes in *BFGF* gene expression was observed (Fig. 6F).

DISCUSSION

In previous reports we had shown that loss of MTAP expression exerted a profound impact on tumor cell activity in melanoma and

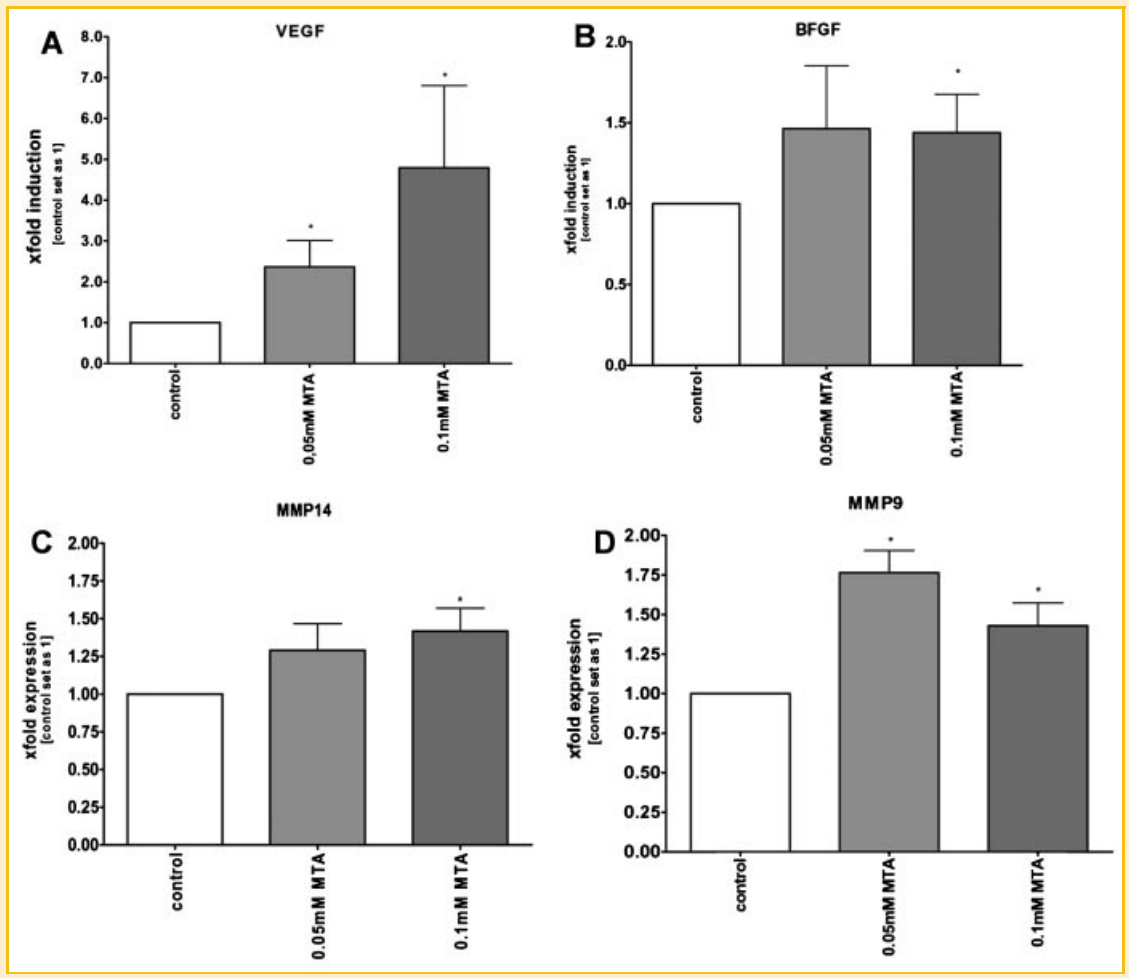

Fig. 2. Administration of MTA at doses of 0.05 and 0.1 mM, respectively, resulted in a strong induction of *VEGF*, *BFGF*, *MMP9*, and *MMP14* gene expression in Mel Im cells compared to untreated cells. Quantitative real time RT-PCR analyses of (A) *VEGF*, (B) *BFGF*, (C) *MMP14*, and (D) *MMP9* (D) gene expression, respectively, in MTA-stimulated and non-stimulated Mel Im cells. Results in Mel Ju and Mel Ho cells revealed the same effects on gene regulation. (Data are given as mean ± SD, $^{*}P < 0.05$ compared to control).

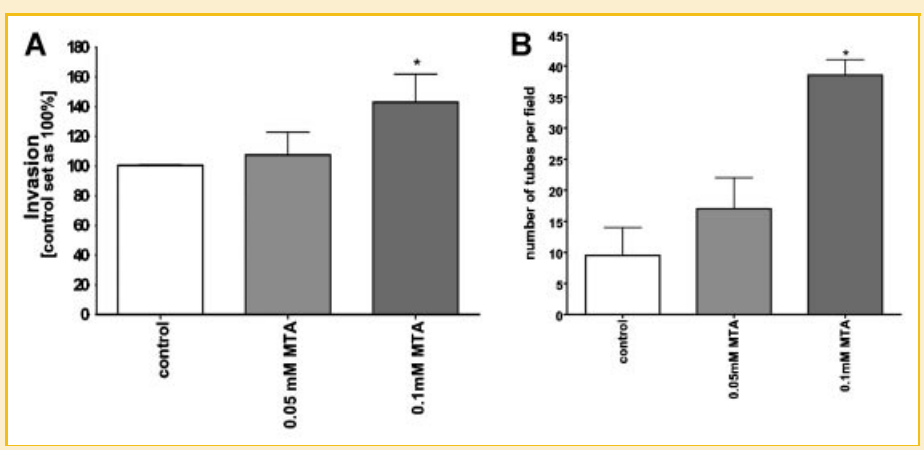

Fig. 3. Functional assays performed on MTA-stimulated Mel Im cells. (A) Boyden chamber invasion assay. Analysis of Mel Im cells stimulated with MTA at final concentrations of 0.05 and 0.1 mM, respectively, revealed an induction of their invasive potential in comparison to non-stimulated Mel Im. (B) Tube formation assay. Mel Im cells showed tube formation on matrigel, whereas the control cells built poorly tube-like structures. (Data are given as mean ± SD; $^{*}P < 0.05$).

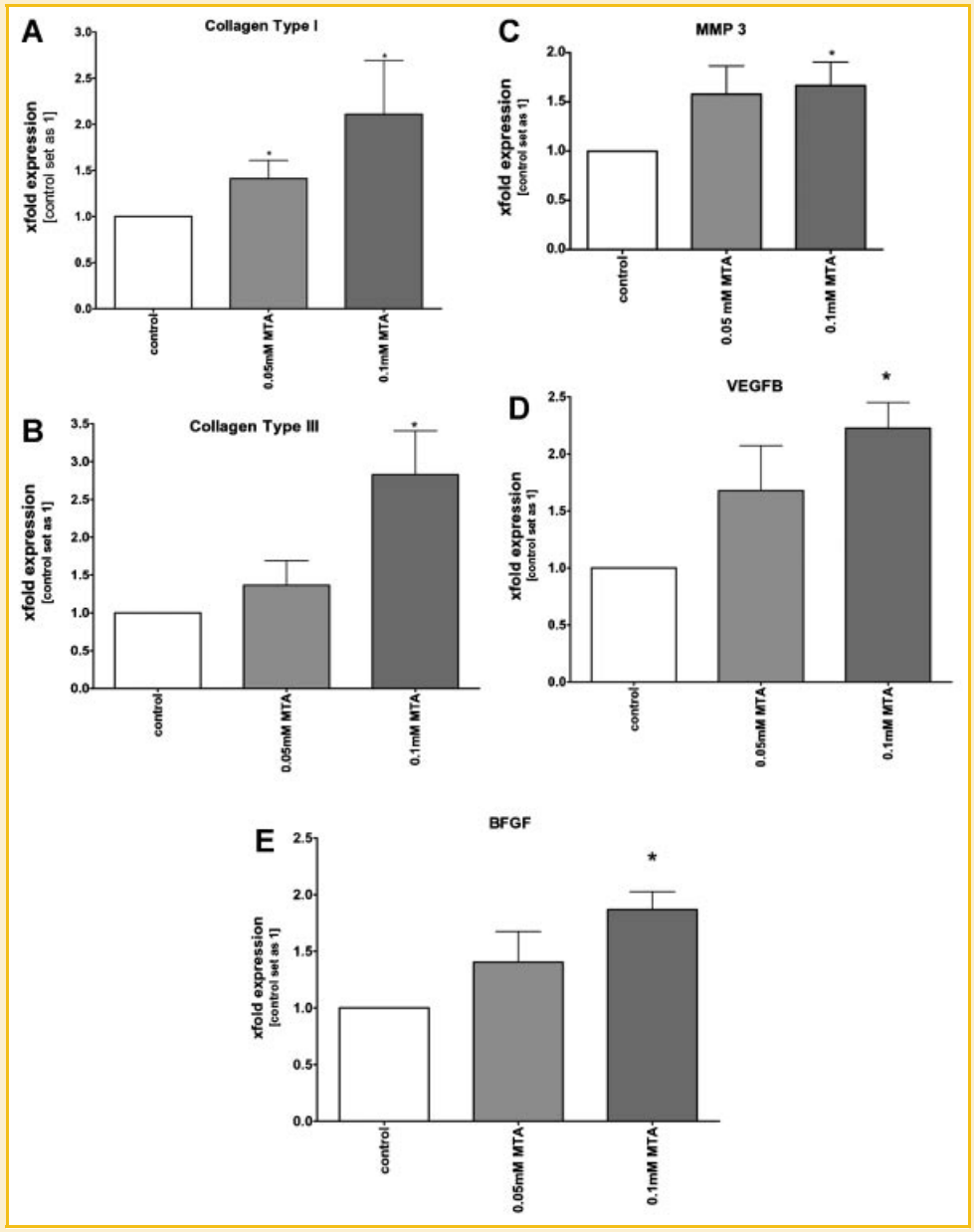

Fig. 4. Quantitative real time RT-PCR revealed an induction of (A) *COL1A1*, (B) *COL3A1*, (C) *MMP3*, (D) *VEGF*, and (E) *BFGF* gene expression in HDF stimulated with MTA at final concentrations of 0.05 and 0.1 mM, respectively, in comparison to untreated HDF. (Data are given as mean ± SD; $^{*}P < 0.05$).

hepatocellular carcinoma [Behrmann et al., 2003; Hellerbrand et al., 2006]. However, the molecular basis for this finding remained unsolved. Here we could show that the MTA levels in cell culture medium and *in vivo* correlated directly with intracellular MTAP levels. In addition, it appears that the observed effects of experimental re-expression of MTAP, such as reduced invasiveness, have been mediated by reduced MTA levels and, therefore, one may hypothesize that MTA itself has a tumor-promoting effect.

In our analysis, we demonstrated an induction of the gene expression of the metalloproteinases *MMP9* and *MMP14* as well as the growth factors *VEGF* and *BFGF* upon treatment of melanoma cells but not of melanocytes with MTA. These genes play a significant role in tumor development and progression of malignant melanoma. Deregulation of *BFGF* gene expression is a known early event in melanoma development and a main driver of tumor cell invasion [Reed et al., 1994; Ahmed et al., 1997] in addition to *MMP9* and *MMP14* [Hofmann et al., 2000; Shellman et al., 2006]. *VEGF*, on the other hand, does not only play a role in tumor invasion, but induces the so-called vascular mimicry of melanoma cells [Salven et al., 1997; Maniotis et al., 1999]. By this mechanism melanoma cells gain the ability to form a tubular network in the tumor that supports blood flow and supplies nutrients and oxygen. In agreement with the data on regulation of gene expression, MTA was able to induce melanoma cell invasion and vasculogenesis. More importantly, effects of MTA were not only observed in the

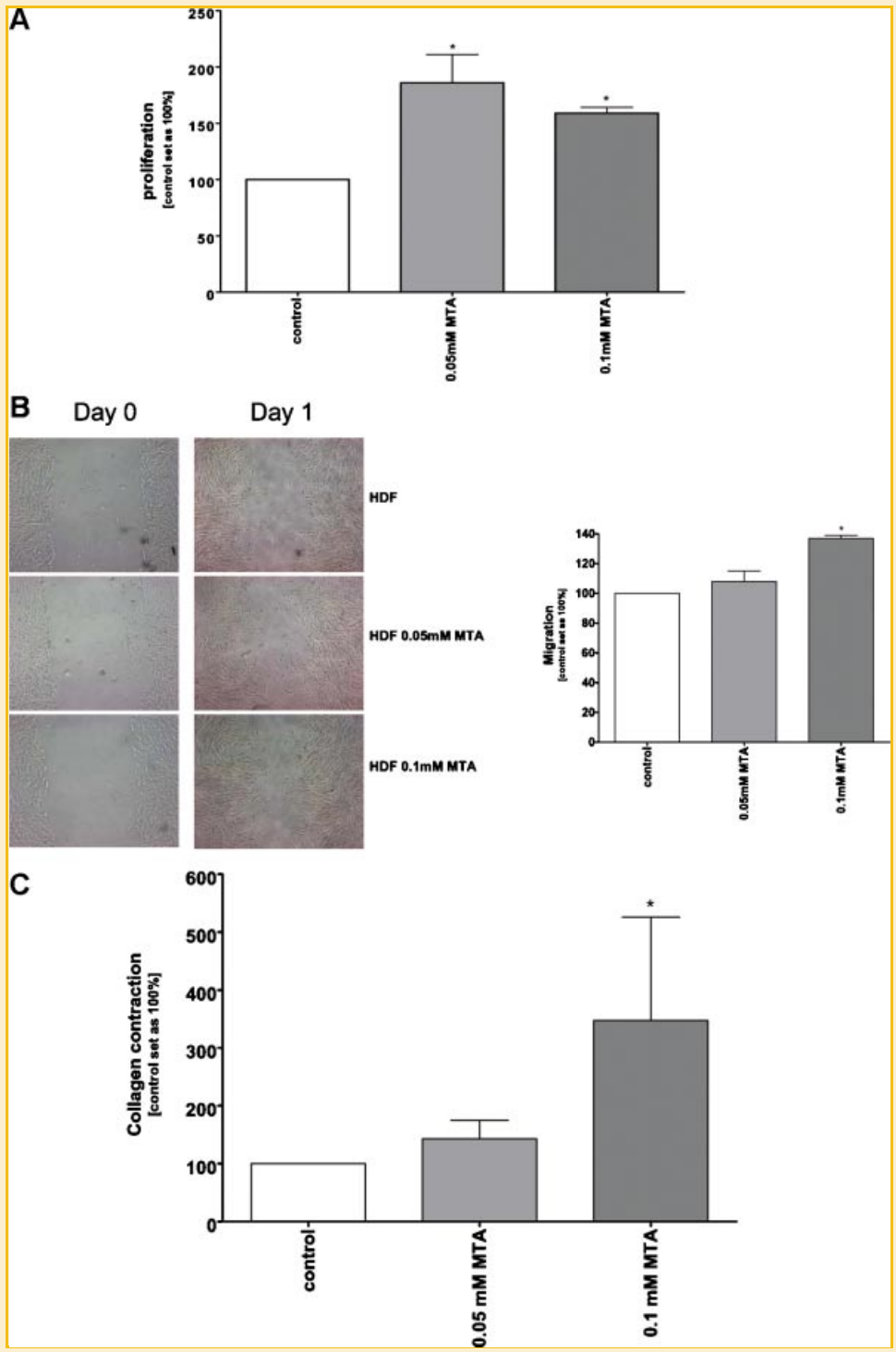

Fig. 5. Functional assays of HDF stimulated with MTA at final concentrations 0.05 and 0.1 mM, respectively. (A) Proliferation assay. MTA-stimulated HDF showed an increase in proliferation compared to control cells (set as 100%). (B) Scratch assay. Migration capacity of MTA-stimulated HDF was calculated relatively to untreated HDF (set as 100%). (C) Collagen contraction assay. MTA-stimulated HDF showed a higher capability of organizing collagen fibers in comparison to untreated HDF (control set as 100%). (Data are given as mean ± SD; $^{*}P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tumor cells, but also in fibroblasts that constitute an important cellular component of the tumor microenvironment. Here, fibroblast activation accompanied by induction of gene expression of collagen types I and III as well as *MMP3* was found. In addition, the fibroblasts' ability to contract and organize collagen was enhanced. *BFGF* gene expression was also upregulated. Finally, there are studies that have revealed an anti-inflammatory activity on macrophages and lymphocytes, thus, further supporting

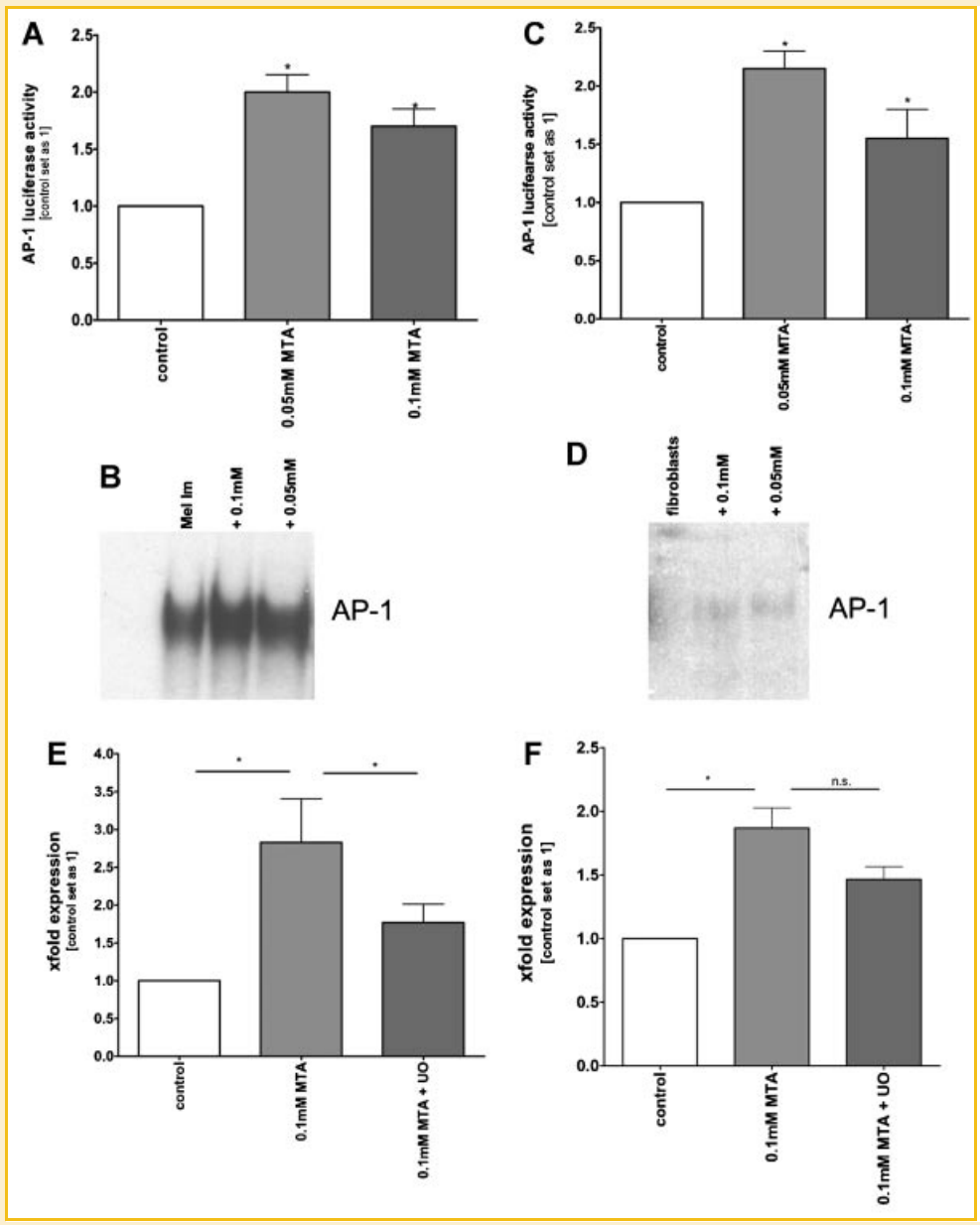

Fig. 6. Signaling induced by MTA in Mel Im and HDF cells. (A) Reporter gene assay in Mel Im cells. The AP-1 reporter gene assay revealed in MTA-stimulated Mel Im cells (0.05 and 0.1 mM) an increase in AP-1 activity in comparison to control cells (set as 1). (B) The gel mobility shift assay confirmed an induction of AP-1 activity in MTA stimulated Mel Im cells. Nuclear extracts of untreated (control) and MTA-stimulated Mel Im cells were used. (C) Reporter gene assay in HDF. AP-1 reporter gene assay revealed in MTA-stimulated HDF (0.05 and 0.1 mM) an increase in AP-1 activity compared to untreated cells (set as 1). (D) The gel mobility shift assay confirmed an induction of AP-1 activity in MTA-stimulated HDF. Nuclear extracts of untreated (control) and MTA-stimulated HDF were used. (E) Quantitative real-time RT-PCR revealed an inhibition of induction of *COL3A1* gene expression in HDF stimulated with MTA at a final concentration of 0.1 mM in comparison to untreated HDF after treatment with UO126 (30 μM). (F) Quantitative real-time RT-PCR revealed no significant inhibition of induction of *BFGF* gene expression in HDF stimulated with MTA at a final concentration of 0.1 mM after treatment with UO126 (30 μM). (Data are given as mean ± SD; $^{*}P < 0.05$).

a tumor-promoting effect of MTA [Di Padova et al., 1985; Cerri et al., 1993; Hevia et al., 2004].

Several groups have reported pro-apoptotic effects of MTA on hepatoma cells, and inhibitory effects on proliferation and invasion of various cell lines as well as differentiation and growth factor receptor activation [Shafman et al., 1984; de Ferra et al., 1984; Riscoe et al., 1987; Maher, 1993; Ansorena et al., 2002; Avila et al., 2004]. Remarkably, in most studies higher, pharmacological doses were administered, whereas the MTA levels achieved here mirrored endogenous tumor levels. In addition, the therapeutic effect of MTA seems to be dependent on the cell type and the MTAP level in the cells [Basu et al., 2007].

Recently, MTA was shown to modulate NF-κB activity and was speculated to have anti-inflammatory function in liver [Hevia et al.,

2004]. However, in melanoma no significant effects of MTA were observed hinting to a cell type specific role of MTA.

Little is known about the molecular mechanisms by which MTA exerts the described effects on cells. Here, uptake or recognition of MTA by a receptor in addition to induction of signaling pathways is largely unidentified. In cells with the ability to process MTA by expression of MTAP, a rapid uptake and immediate catabolism was observed that could be abrogated by the administration of 5'-methylthiotubercidin, which is a known inhibitor of not only the degradation of 5'-methylthioadenosine by 5'-MTAP, but also of the aminopropyltransferases responsible for the synthesis of spermine and spermidine [Eloranta et al., 1982]. Recent studies revealed an elevation of cAMP after treatment of lymphocytes with MTA [Christa et al., 1983]. Another study could show, that MTA is an agonist of the adenosine A1 receptor, but it exerts an antagonistic effect on adenosine A2 receptor [Munshi et al., 1988]. In this study, we could show that activity of the transcriptional regulator AP-1 is enhanced after MTA treatment, potentially leading to induced gene expression of MMPs and growth factors known to be regulated by AP-1 [Chakraborti et al., 2003; Hess et al., 2004]. Follow-up studies have to reveal whether AP-1 is activated via cAMP or whether different mechanisms lead to this enhancement of activity either by extracellular or intracellular MTA. Interestingly, blockage of AP-1 activity resulted in reduction of MTA-induced collagen type III gene expression in fibroblasts, but no effect on MTA-induced expression of *BFGF* was observed. This suggested that in addition to AP-1 other pathways were induced by MTA.

In summary, we demonstrated the impact of increased MTA secretion on several cellular functions of melanoma cells including invasion and vascular mimicry. In addition to the tumor cells, fibroblasts were also stimulated by MTA to develop a tumor-supportive phenotype. Our work questions a potential therapeutic role of MTA as speculated by others. It rather suggests that MTA may serve as a potential biomarker for cancer diagnosis and progression.

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