

# Effect of Tumor-Associated Mutant E-cadherin Variants With Defects in Exons 8 or 9 on Matrix Metalloproteinase 3

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Tumor progression is characterized by loss of cell adhesion and increase of invasion and metastasis. The cell adhesion molecule E-cadherin is frequently down-regulated or mutated in tumors. In addition to down-regulation of cell adhesion, degradation of the extracellular matrix by matrix metalloproteinases is necessary for tumor cell spread. To investigate a possible link between E-cadherin and matrix metalloproteinase 3 (MMP-3), we examined expression of MMP-3 in human MDA-MB-435S cells transfected with wild-type (*wt*) or three different tumor-associated mutant E-cadherin variants with alterations in exons 8 or 9, originally identified in gastric carcinoma patients. In the presence of *wt* E-cadherin, the MMP-3 protein level was decreased in cellular lysates and in the supernatant where a secreted form of the protein is detectable. Down-regulation of MMP-3 was not found in MDA-MB-435S transfectants expressing mutant E-cadherin variants which indicates that E-cadherin mutations interfere with the MMP-3 suppressing function of E-cadherin. The mechanism of regulation of MMP-3 by E-cadherin is presently not clear. We have previously found that cell motility is enhanced by expression of the mutant E-cadherin variants used in this study. Here, we found that application of the synthetic inhibitor of MMP-3 NNGH and small interfering RNA (siRNA) directed against MMP-3 reduce mutant E-cadherin-enhanced cell motility. Taken together, our results point to a functional link between MMP-3 and E-cadherin. MMP-3 is differentially regulated by expression of *wt* or mutant E-cadherin. On the other hand, MMP-3 plays a role in the enhancement of cell motility by mutant E-cadherin. Both observations may be highly relevant for tumor progression since they concern degradation of the extracellular matrix and tumor cell spread. J. Cell. Physiol. 202: 805–813, 2005. © 2004 Wiley-Liss, Inc.

The cell adhesion molecule E-cadherin is associated with catenins which link the adhesive receptor with the actin cytoskeleton (Kemler, 1993; Gumbiner, 2000). Epidemiological studies and *in vitro* investigations suggest that E-cadherin acts as a tumor and invasion suppressor: in a variety of tumors, including gastric (Shino et al., 1995) and breast carcinomas (Zschiesche et al., 1997; Bukholm et al., 1998), loss of E-cadherin and/or catenin expression have been associated with invasive tumor growth (Birchmeier and Behrens, 1994; Berx et al., 1998; Hirohashi, 1998; Van Aken et al., 2001); in human cancer cell lines, expression of E-cadherin results in a more differentiated phenotype and loss of invasiveness (Frixen et al., 1991; Vlemminckx et al., 1991; Watabe et al., 1994).

E-cadherin expression and function is controlled at multiple levels by a number of mechanisms, including transcriptional regulation, promoter methylation, gene mutation, tyrosine phosphorylation and cleavage of the extracellular domain by ectodomain shedding. For instance, E-cadherin expression is controlled by the negatively regulating transcription factors snail and sip1 (Batlle et al., 2000; Cano et al., 2000; Hemavathy et al., 2000; Comijn et al., 2001). Silencing of the E-cadherin gene by DNA hypermethylation has been found in human breast, prostate, and hepatocellular carcinomas (Graff et al., 1995; Kanai et al., 1997). Mutational inactivation of E-cadherin has been detected in breast (Kanai et al., 1994; Berx et al., 1995, 1998), gastric (Becker et al., 1994, 1999b; Oda et al., 1994; Muta

et al., 1996; Tamura et al., 1996; Berx et al., 1998; Machado et al., 1999), thyroid (Soares et al., 1997), endometrium and ovary carcinomas (Risinger et al., 1994). In addition to these somatic mutations, inactivating germline E-cadherin mutations have been described in familial gastric carcinoma patients (Gayther et al., 1998; Guilford et al., 1998; Keller et al., 1999; Richards et al., 1999; Suriano et al., 2003). The observed E-cadherin gene mutations comprise missense, splice site,

**Abbreviations:** aa, amino acids; *del 8* E-cadherin, E-cadherin with deletion of exon 8; *del 9* E-cadherin, E-cadherin with deletion of exon 9; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; *p8* E-cadherin, E-cadherin with point mutation in exon 8; h, hours; min, minutes; MMP-3, matrix metalloproteinase 3; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SD, standard deviation; siRNA, small interfering RNA; *wt* E-cadherin, wild-type E-cadherin.

Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant number: SFB 456, Project A2.

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Received 13 March 2004; Accepted 16 June 2004

DOI: 10.1002/jcp.20192

and truncation mutations. The activity of the E-cadherin/catenin complex can be regulated by tyrosine phosphorylation of  $\beta$ -catenin through pp60<sup>src</sup> (Behrens et al., 1993) or activation of c-Met or epidermal growth factor receptor EGFR (Shibamoto et al., 1994).

We and other laboratories have identified splice site E-cadherin mutations in 50% of diffuse-type gastric carcinomas (Becker et al., 1994, 1999a,b; Muta et al., 1996; Tamura et al., 1996; Machado et al., 1999). The most frequent mutations were *in frame* deletions of exons 8 or 9 which affect putative calcium binding sites within the extracellular domain of E-cadherin (Handschuh et al., 1999). Expression of these mutant E-cadherin variants in MDA-MB-435S cells resulted in decreased cellular adhesion and increased cellular motility as compared to the *wt* E-cadherin molecule and interfered with the proliferation-suppressive function of E-cadherin (Handschuh et al., 1999; Luber et al., 2000; Fuchs et al., 2002; Fricke et al., 2004). Tumor-derived E-cadherin mutations have been shown to lead to a loss of function on one site (loss of adhesive properties and proliferation-suppressive function) and a gain of function on the other site (increase in cellular motility), both of which are potentially relevant for tumor and metastasis formation.

Matrix metalloproteinases (MMPs) play an important role for invasion and metastasis of malignant cells. First, they have the ability to degrade extracellular matrix components and second, they actively cleave the ectodomain of certain transmembrane proteins. MMPs are up-regulated in the stromal components of carcinomas and can act on the surface of cancer cells, thereby promoting invasion and metastasis (Sellers and Murphy, 1981; Wilhelm et al., 1987). Expression of autoactivated stromelysin-1 (MMP-3) in mammary glands of transgenic mice led to a reactive stroma during early development (Thomasset et al., 1998). In another animal model, MMP-3 promoted mammary carcinogenesis (Sternlicht et al., 1999).

E-cadherin can be inactivated by cleavage in the extracellular domain by MMP-3 or matrilysin in a process designated ectodomain shedding (Lochter et al., 1997; Davies et al., 2001; Noe et al., 2001; Ryniers et al., 2002). Proteolytic ectodomain fragments of E-cadherin have been proposed to promote cancer cell invasion by interfering with E-cadherin function in cells containing intact E-cadherin/catenin complexes (Noe et al., 2001; Ryniers et al., 2002).

The microenvironment of the tumor host interface is important for tumor growth and metastasis (Liotta and Kohn, 2001). To determine whether there is a functional link between E-cadherin and MMP-3, we measured MMP-3 expression and secretion in MDA-MB-435S cells transfected with *wt* or mutant E-cadherin variants. The investigated E-cadherin mutations were *in frame* deletions of exons 8 (*del 8*) or 9 (*del 9*) and a point mutation in exon 8 (*p8*, D370A) which lead to alterations in the extracellular domain of E-cadherin and increase cell motility. To further investigate a functional connection between E-cadherin and MMP-3, the effect of MMP-3 inhibitors on the motility of MDA-MB-435S cells expressing mutant E-cadherin was examined.

## MATERIALS AND METHODS

### Cell cultivation

The human E-cadherin-negative cell line MDA-MB-435S (ATCC, Rockville, MD) and the wild-type and mutant E-cadherin-cDNA transfected derivatives that were established previously (Handschuh et al., 1999) were grown in

Dulbecco's modified Eagle medium (DMEM, Life Technologies, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS, PAN Biotech, Aidenbach, Germany) and penicillin-streptomycin (50 IU/ml and 50  $\mu$ g/ml; Life Technologies, Karlsruhe, Germany) at 37°C and 5% CO<sub>2</sub>. MDA-MB-435S cells were considered to be derived from a breast carcinoma until recent investigations of the gene expression profile suggested that the cells might instead be derived from a melanoma (Ross et al., 2000; Ellison et al., 2002).

### Western blot analysis

For Western blot analysis, cells were seeded at a density of  $6 \times 10^5$  cells per 10 cm tissue culture dish and lysed 24 h later with 300  $\mu$ l L-CAM buffer (140 mM NaCl, 4.7 mM KCl, 0.7 mM MgSO<sub>4</sub>, 1.2 mM CaCl<sub>2</sub>, 10 mM Hepes pH 7.4, containing 1% (v/v) Triton-X-100 (Cunningham et al., 1984)). The lysis buffer contained 2 mM phenylmethylsulfonylfluoride, 2 mM orthovanadate, 19  $\mu$ g/ml aprotinin, 20  $\mu$ g/ml leupeptin, 10 mM sodium phosphate, and 100 mM sodium fluoride. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose (Schleicher & Schuell, Dassel, Germany) membranes. Cell lysates were investigated with monoclonal antibody to MMP-3 at a dilution of 1:1,000 (#IM70, Oncogene Research Products, Cambridge, MA). Purified MMP-3 was used as control for the specificity of the antibody (#SE-153, Biomol Research Laboratories, Inc., Plymouth Meeting, PA). Monoclonal anti- $\alpha$ -tubulin antibody (#T9026, Sigma, Deisenhofen, Germany) was used to stain  $\alpha$ -tubulin as a loading control. For the detection of secreted MMP-3,  $3 \times 10^5$  non-transfected or E-cadherin (*wt* or mutant) transfected MDA-MB-435S cells were plated in 24-well plates in 500  $\mu$ l medium. After 3 h, cells were washed two times with PBS and 500  $\mu$ l DMEM (without FCS) was added. After 3 days, an aliquot of 25  $\mu$ l was removed, denatured, and analyzed by Western blot analysis as described above. For signal detection the enhanced chemoluminescence system (Amersham, Braunschweig, Germany) was used. For quantification of signals, blots were scanned and densitometric band analysis was performed using Scion Image Software from Scion Incorporation (Version Beta 4.0.2).

### RNA extraction and cDNA synthesis

Total RNA was extracted from  $3 \times 10^6$  cells in 15-cm plates after 24 h cultivation. Cells were lysed in guanidine isothiocyanate solution (GITC, #15577-018, Life Technologies), supplemented with sarcosyl solution at a final concentration of 0.5%. RNA extracted with 1 volume phenol (pH 4) and 0.2 volumes chloroform (chloroform:isoamylalcohol, 24:1, v/v) in the presence of 0.1 volume 2 M sodium acetate (pH 4). RNA was precipitated with 1 volume isopropanol, resuspended in GITC and precipitated again with isopropanol. The pellet was washed with 70% ethanol, air-dried and resuspended in 300  $\mu$ l RNase-free water. RNA concentration was measured spectrophotometrically. 2  $\mu$ g total RNA was used for reverse transcription using superscript II reverse transcriptase (Life Technologies) and oligo(dT) primer (Promega, Mannheim, Germany) according to the manufacturers' instructions. In brief, 2  $\mu$ g RNA were incubated with 1 mM dNTPs and 40 U of RNase inhibitor (Amersham Pharmacia Biotech, Freiburg, Germany) in a final volume of 20  $\mu$ l for 1 h at 42°C, followed by 5 min incubation at 95°C.

### Real-time RT-PCR analysis

Quantitative RT PCR was performed essentially as described before (Rosivatz et al., 2002). Primers and probes for the detection of MMP-3 and GAPDH were purchased from Applied Biosystems (Assay on demand, MMP-3 (Hs00233962) and GAPDH (Hs99999905)). The sequences for primers and probes are under copyright of Applied Biosystems.

### cDNA array hybridization

For cDNA array hybridization, poly(A)<sup>+</sup> RNA and cDNA synthesis was performed as described elsewhere (Kingston et al., 1997). cDNA array hybridization was described previously (Bange et al., 2002). In brief, labeling of 3–5  $\mu$ l of cDNA was performed with the Megaprime kit (Amersham) in the

presence of 50  $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ]dATP. The hybridization solution contained  $5\times$  SSC, 0.5% (v/v) SDS, 100  $\mu\text{g/ml}$  baker yeast tRNA (Roche) and the radioactively labeled cDNA probe ( $2$ – $5\times 10^6$  cpm/ml). After incubation at  $68^\circ\text{C}$  for 16 h, membranes were washed under stringent conditions. Hybridization signals were quantified using a phosphorimager (Fuji BAS 2500, Fuji, Stamford, CT).

#### Cell motility assay

For time-lapse laser scanning microscopy, cells were seeded at a density of  $2\times 10^5$  cells per 3.5-cm in collagen I-coated plates with a glass bottom which were purchased from MatTek Corporation (Ashland, MA). Uncoated plates were coated for 4 h at  $37^\circ\text{C}$  with collagen I (100  $\mu\text{g/ml}$ ). Cells were cultivated in a microscope-coupled incubation chamber (Zeiss, Jena, Germany) at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ . MMP-3 inhibitor *N*-Isobutyl-*N*-(4-methoxyphenylsulfonyl) glycol hydroxamic acid (NNGH, Biomol Research Laboratories, Inc.) was used at a final concentration of 250  $\mu\text{M}$  in DMSO. NNGH is a potent inhibitor of human MMP-3 (MacPherson et al., 1997), but it also inhibits MMP-12 (Jeng et al., 1998). Cells were investigated in parallel with DMSO as a control. Phase contrast images were taken at 3-min intervals for a duration of 7 h with an Axiovert laser scanning microscope LSM 510 (Zeiss) with lens PNF  $20\times/0.4$  PH2 and a helium-neon laser at 543 nm transmission scanning mode. The percentage of motile cells and cell speed were measured as described before (Fuchs et al., 2002). In brief, the percentage of motile cells was determined by drawing the outlines of cells on an overhead transparency attached to the screen and counting those cells which had completely left the initial area within the observation time of 7 h. To calculate individual cell speeds, cells nuclei were traced using the laser scanning microscope software from Zeiss and the obtained migration paths were divided by the recording time.

#### Transfection of MDA-MB-435S cells with siRNA directed against MMP-3

siRNA was constructed directed against three different regions of MMP-3 by M. Truss. MMP-3 A 5'-aaaggacagt-ggtcctgtgt-3' which extends between amino acids 55 and 61, MMP-3 B 5'-aaccttctggcatcccga-3' which extends between amino acids 102 and 109, and MMP-3 C 5'-aagtgtgatc-caagctgtccc-3' which extends between amino acids 142 and 148. Cells were seeded at a density of  $2\times 10^5$  per well in 12-well plates and transfected 24 h later with 20  $\mu\text{M}$  siRNA. siRNA was transfected with oligofectAMINE (Invitrogen, #12252-011) as transfection reagent in Opti-MEM (Invitrogen, #31985-047). Twenty-four hour after transfection, Opti-MEM was replaced by normal medium. Supernatants and lysates were prepared 48 and 54 h later and analyzed by Western blot analysis.

#### Wound healing assay

The wound healing assay was performed essentially as described recently (Handsuh et al., 1999). In brief, cells were grown in 12-well plates at a density of  $1.5\times 10^5$  for 24 h, then cells were transfected with siRNA as described above. Twenty-four hour after transfection, Opti-MEM was replaced by normal medium. After 24–48 h, an artificial wound was introduced into the confluent cell monolayer with a yellow tip. After 24 h, cells were fixed and stained with Diff-Quick reagents (Baxter, Unterschleißheim, Germany). As a control, cells were treated in Opti-MEM without transfection reagent. In order to investigate the influence of NNGH on wound closure, cells were treated with NNGH at a concentration of 250  $\mu\text{M}$ .

## RESULTS

### Influence of E-cadherin on MMP-3 protein expression and secretion

Expression of MMP-3 was investigated in the supernatants and lysates of parental and *wt* or mutant E-cadherin expressing MDA-MB-435S cells by Western blot analysis. MMP-3 released into the medium of the investigated cell lines differed significantly (Fig. 1A). A strong reduction of MMP-3 from 6.4-fold in the

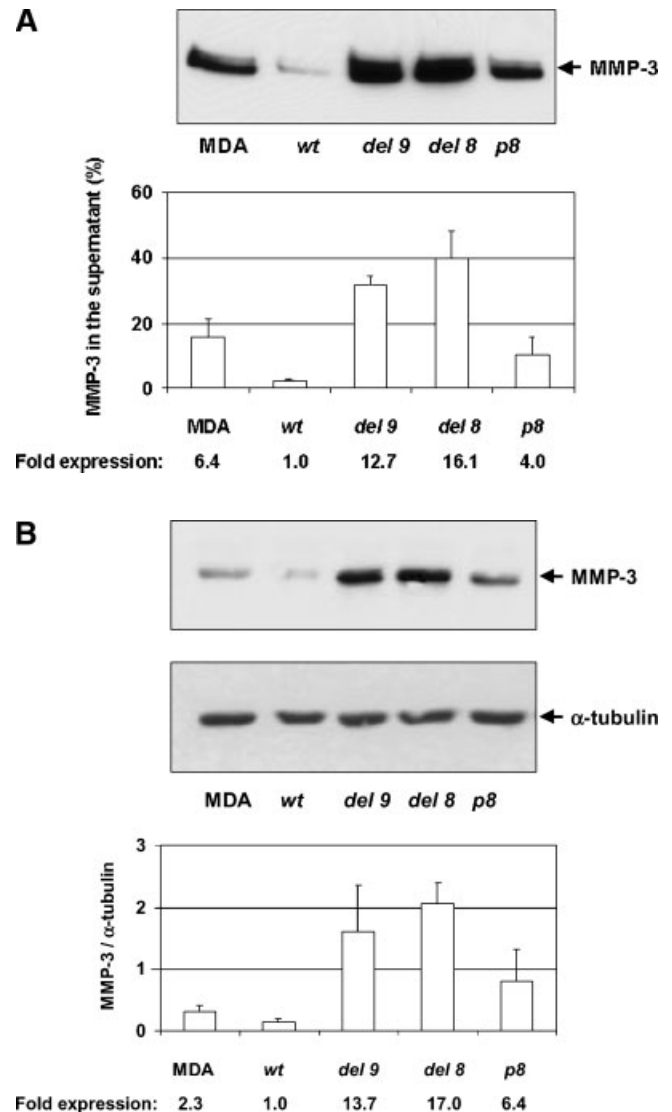


Fig. 1. MMP-3 levels in supernatants and lysates of MDA-MB-435S E-cadherin transfectants. **A:** MMP-3 released into the medium of parental (MDA), *wt* and mutant (*del 9*, *del 8*, *p8*) E-cadherin expressing MDA-MB-435S cells was determined by Western blot analysis using a monoclonal antibody against MMP-3 at a dilution of 1:1,000. Shown is the mean of three independent experiments  $\pm$  standard deviation (SD). **B:** Parental, *wt* and mutant (*del 9*, *del 8*, *p8*) E-cadherin expressing MDA-MB-435S cells were lysed and Western blots were performed using a monoclonal antibody against MMP-3. Western blots were stripped and rehybridized using monoclonal anti- $\alpha$ -tubulin antibody. Shown is the mean of three independent experiments  $\pm$  SD. Band intensities were quantified by densitometric analysis.

supernatant of parental cells to 1.0-fold in *wt* E-cadherin expressing cells was detectable. In contrast, in the presence of *del 8* and *del 9* E-cadherin mutations, the MMP-3 down-regulating function of E-cadherin was impaired and instead up-regulation of MMP-3 was observed. The amount of MMP-3 detected in the supernatant of mutant E-cadherin expressing cells was 12.7-fold (*del 9*), 16.1-fold (*del 8*), or 4.0-fold (*p8*) elevated as compared to *wt* E-cadherin expressing cells. In cellular lysates, the amount of MMP-3 was 2.3-fold (parental cells), 13.7-fold (*del 9*), 17.0-fold (*del 8*), or 6.4-fold (*p8*) elevated in the indicated cell lines as compared to *wt* E-cadherin expressing cells (Fig. 1B).

### Influence of E-cadherin on MMP-3 mRNA expression

To further investigate how E-cadherin influences MMP-3 expression, we determined whether the effect of *wt* and mutant E-cadherin expression on the MMP-3 protein level was reflected on the MMP-3 mRNA level. cDNA-macroarray analysis revealed differential expression of MMP-3 in parental MDA-MB-435S cells as compared to transfectants expressing *wt* or mutant E-cadherin variants (*del 8*, *del 9* and *p8* E-cadherin) (Fig. 2A). MMP-3 levels were 0.2-fold (parental), 0.7-fold (*del 9*), 0.8-fold (*del 8*), and 0.2-fold (*p8*) as compared to 1.0-fold in *wt* E-cadherin expressing cells.

MMP-3 mRNA expression was also investigated by quantitative real time PCR analysis (Fig. 2B). mRNA levels were 0.5-fold (parental), 1.6-fold (*del 9*), 2.9-fold (*del 8*), and 0.8-fold (*p8*) altered as compared to 1.0-fold in *wt* E-cadherin expressing cells.

### Influence of the MMP-3 inhibitor NNGH on cellular morphology and motility

MMP inhibitors have been shown to up-regulate cadherin function and to restore normal cell-cell contact

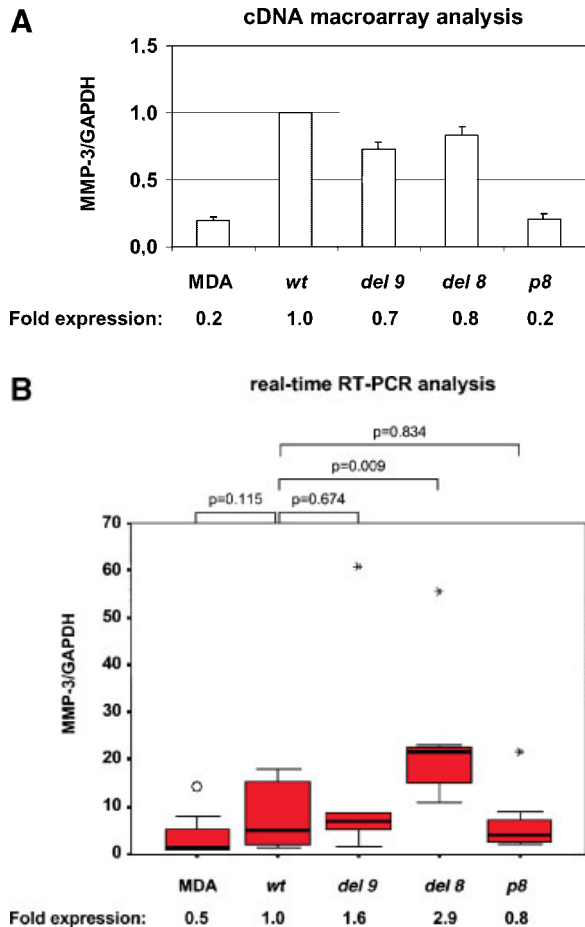


Fig. 2. mRNA expression levels of MMP-3. mRNA expression levels were analyzed in parental (MDA), *wt* or mutant E-cadherin expressing MDA-MB-435S cells by cDNA-macroarray analysis (A) or real-time RT-PCR analysis (B). A: Shown is the mean of signal intensities of the different MMP-3 clones on the cDNA-macroarray  $\pm$  SD. B: Eight real time PCR experiments with six different RNA preparations were performed. A boxplot is shown, the thick line indicates the median, the bars show the maximum and the minimum values. Statistical analysis was performed applying the Mann-Whitney test, 2-tailed. *P*-values < 0.05 were significant. Means were used to calculate fold expression values.

in fibroblasts (Ho et al., 2001). In order to investigate the influence of MMP-3 inhibition on cellular morphology and motility, MDA-MB-435S transfectants were seeded on collagen I-coated glass dishes and treated with the MMP inhibitor *N*-Isobutyl-*N*-(4-methoxyphenyl-sulfonyl) glycol hydroxamic acid (NNGH) (MacPherson et al., 1997). As described previously, *wt* E-cadherin transfectants revealed epithelial morphology and formed colonies, while MDA-MB-435S cells expressing *del 8* E-cadherin formed lamellipodia (Fig. 3) (Handschuh et al., 1999). In the presence of NNGH, *del 8* E-cadherin transfectants developed less lamellipodial protrusions and more sites of cell-cell contact than untreated cells. NNGH had only minor effects on cellular morphology of *wt* E-cadherin transfectants.

We have demonstrated recently that *del 8* E-cadherin expressing cells are more motile than *wt* E-cadherin expressing cells (Handschuh et al., 1999; Fuchs et al., 2002). When NNGH was added to these cells, the migration distances remained essentially unchanged in *wt* E-cadherin expressing cells, while the path length of *del 8* E-cadherin transfectants were reduced (Fig. 4A). The percentage of motile cells, which defines those cells which move out of the initial space within the observation time of 7 h, was significantly elevated in *del 8* (57.8%) versus *wt* E-cadherin (17.8%) expressing cells (*P* = 0.046, Fig. 4B). After addition of NNGH, the motility of *del 8* E-cadherin transfectants was reduced to 17.8% (*P* = 0.043). In contrast, NNGH did not significantly influence the percentage of motile *wt* E-cadherin transfectants (*P* = 0.105, 28.9% with NNGH).

Speed ranges and median cell speeds were weakly affected by NNGH in *wt* E-cadherin transfectants (*P* = 0.634, Fig. 4B, speed ranges: 2.9–35.1 without and 2.0–61.6  $\mu$ m/h with NNGH, medians: 12.4 without and 14.9  $\mu$ m/h with NNGH). In contrast, cell speeds were

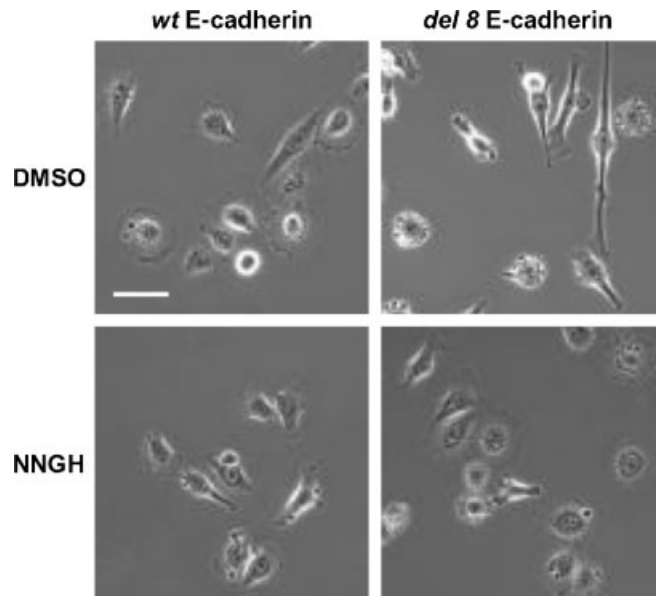


Fig. 3. Influence of NNGH on cellular morphology. *wt* and *del 8* E-cadherin expressing MDA-MB-435S cells were plated on collagen-I coated dishes with a glass bottom in the presence or absence of MMP-3 inhibitor NNGH and phase contrast images were taken. *wt* E-cadherin transfectants showed epithelial morphology and colonies, while *del 8* E-cadherin expressing cells formed lamellipodia, the typical motility-associated protrusions. NNGH had only minor effects on the morphology of *wt* E-cadherin expressing cells while *del 8* E-cadherin transfectants showed less lamellipodia upon NNGH treatment. Bar represents 20  $\mu$ m.

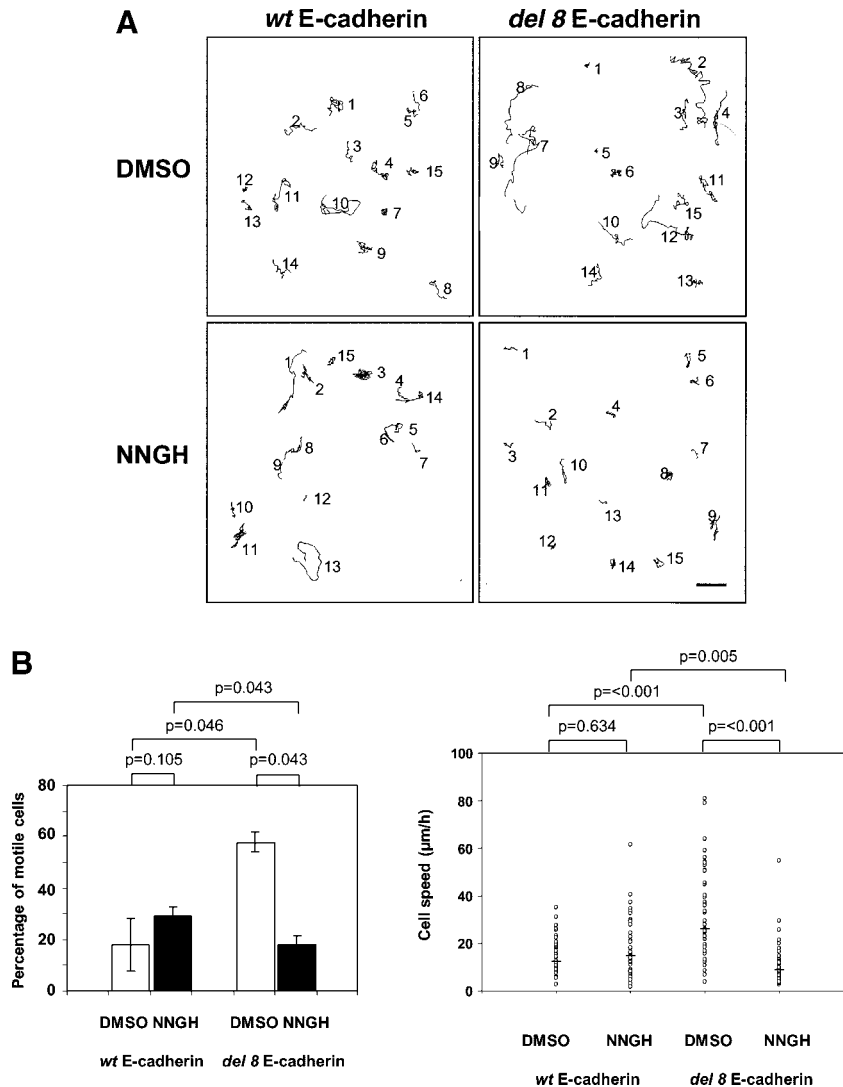


Fig. 4. Influence of NNGH on mutant E-cadherin-enhanced cell motility. Transfected MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin were plated on collagen I-coated dishes with a glass bottom and traced for 7 h using a laser scanning microscope. Phase contrast images were acquired in 3-min intervals. **A**: Cell nuclei were traced semiautomatically applying the Zeiss LSM software. Cell paths of 15 randomly chosen cells within a microscopic field ( $460 \times 460 \mu\text{m}$ ) are shown. One representative of three independent experiments is shown. Bar represents  $50 \mu\text{m}$ . **B**: Left part; the percentage of motile

cells in the presence of DMSO as control or the MMP inhibitor NNGH was determined by counting cells of a microscopic field which moved completely out of the initial space within the observation time. Shown is the mean of three independent experiments. Right part: Each column shows the ranges of cell speeds from a total of 45 cells. Black horizontal bars represent the median. Statistical analysis was performed applying the Mann-Whitney test, 2-tailed.  $P$ -values  $< 0.05$  were significant.

significantly decreased by NNGH in *del 8* E-cadherin expressing cells ( $P = < 0.001$ , Fig. 4B, speed ranges:  $4.0$ – $80.9$  without and  $2.6$ – $54.6 \mu\text{m/h}$  with NNGH, medians:  $26.0$  without and  $9.0 \mu\text{m/h}$  with NNGH).

#### Inhibition of MMP-3 expression by application of siRNA

NNGH is not specific for MMP-3, but also inhibits other MMPs, for instance MMP-12 (MacPherson et al., 1997; Jeng et al., 1998). Therefore, small interfering RNA (siRNA) specific for MMP-3 was designed, extending between amino acids 55–61 (siRNA A), 102–109 (siRNA B), and 142–148 (siRNA C) (Fig. 5A). *del 8* E-cadherin expressing MDA-MB-435S cells were transfected with siRNA A–C and the MMP-3 protein level was examined by Western blot analysis in cellular lysates and in the supernatant (Fig. 5B). We found that expression of siRNA A had the strongest inhibiting effect and significantly reduced the amount of secreted

MMP-3 in the supernatant (Fig. 5C) and in cellular lysates (Fig. 5D) below 20% of the original level at 48 and 54 h after transfection. MMP-3 expression was similarly reduced in parental, *wt*, *del 9*, and *p8* E-cadherin expressing cells by siRNA A (data not shown).

#### Effect of siRNA against MMP-3 on cell migration in a wound healing assay

In order to determine if the migrative behavior of *del 8* E-cadherin expressing MDA-MB-435S cells could be altered by siRNA against MMP-3, cells were transfected with siRNA A and wound closure was observed in a wound healing assay. In this test, cells are grown to confluency and then an artificial wound is created. Cells which enter the wound are regarded as motile. We found that siRNA A reduced the number of *del 8* E-cadherin expressing MDA-MB-435S cells which had grown into the space within the observation time, although to a lesser extent than NNGH (Fig. 6).

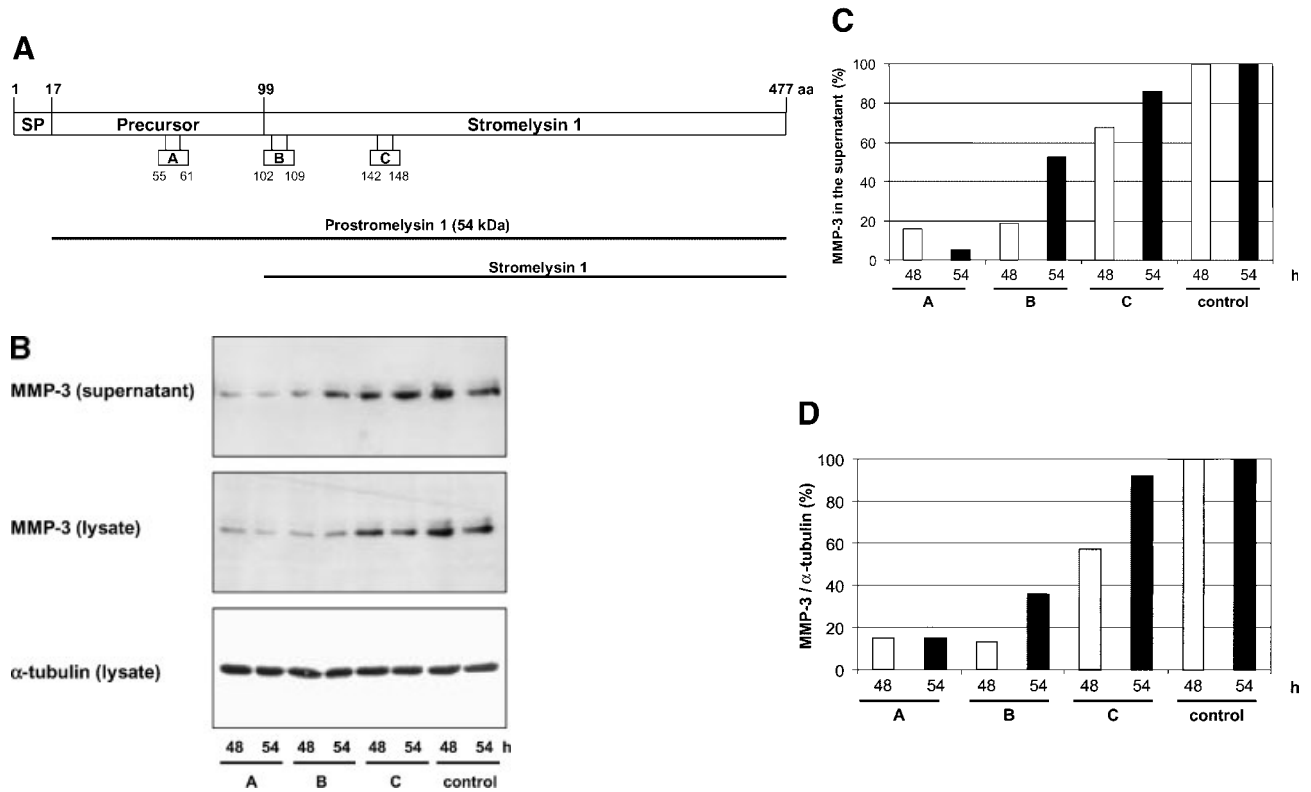


Fig. 5. Influence of siRNA against MMP-3 on MMP-3 expression. **A:** Schematic drawing showing the location of siRNA A-C within stromelysin 1. aa: amino acid. **B:** *del 8* E-cadherin expressing MDA-MB-435S cells were seeded in 12-well plates. After 24 h, cells were transfected with 20  $\mu$ M siRNA in transfection reagent Oligofect AMIN<sup>TM</sup> in Opti-MEM. After 24 h, medium was changed and cells were cultivated in normal medium with 10% FCS. Supernatants and

cellular lysates were harvested 48 or 54 h after transfection. Western blots of supernatants and cellular lysates were performed as described in legend to Figure 1. Densitometric analysis was performed to determine band intensities and the quantitative results of the amount of MMP-3 in the supernatant (**C**) and in the cellular lysates after normalization to  $\alpha$ -tubulin are shown (**D**).

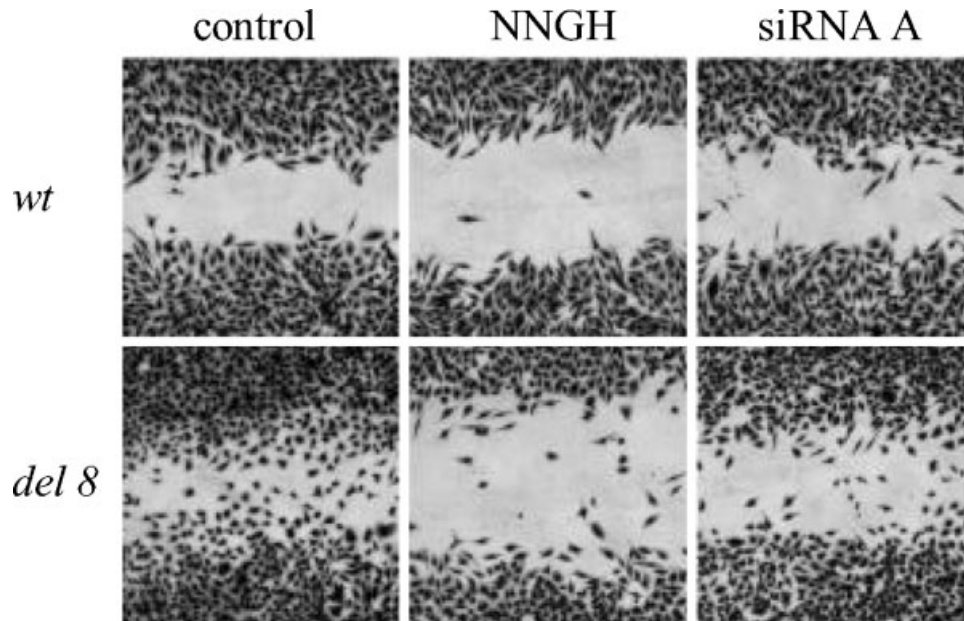


Fig. 6. Influence of siRNA on cell migration in a wound healing assay. *wt* and *del 8* E-cadherin expressing MDA-MB-435S cells were seeded at a density of  $1.5 \times 10^5$  cells per well in 12-well plates and were grown to confluency overnight. Then, transfection with siRNA A was performed. Twenty-four hour after transfection, an artificial wound was created. After 24 h, cells were fixed and stained with DiffQuick reagent. Shown is one representative of three independent experiments.

## DISCUSSION

In the present study, we found that expression of *wt* E-cadherin in MDA-MB-435S cells leads to down-regulation of MMP-3 protein expression and secretion which is in accordance with a recent publication (Nawrocki-Raby et al., 2003). The mechanism of MMP-3 down-regulation by *wt* E-cadherin is presently unknown. In contrast, MDA-MB-435S transfectants expressing mutant *p8, del 8*, or *del 9* E-cadherin showed elevated levels of MMP-3 as compared to *wt* transfectants. We observed a strong dependence of MMP-3 protein and RNA levels on cell density (Laux et al., in preparation and our unpublished observations). A further hint for a functional link between E-cadherin and MMP-3 derives from our result that the MMP-3 inhibitor NNGH and siRNA against MMP-3 down-regulate cell motility stimulated by mutant E-cadherin. Our previous data suggest that the motility-increasing activity of mutant E-cadherin can be blocked by inhibitors of phosphatidylinositol (PI) 3-kinase and epidermal growth factor receptor (EGFR) (Fuchs et al., 2002). In the present study, we found that beside PI 3-kinase and EGFR, also MMP-3 seems to be involved in mutant E-cadherin-enhanced cell motility. We propose a model, whereby E-cadherin down-regulates MMP-3 to prevent tumor invasion. This invasion suppressor function seems to be impaired by E-cadherin mutations. Taken together, the functional link between E-cadherin and MMP-3 may be important for tumor invasion and metastasis.

Several studies suggest a relation between E-cadherin and MMP expression. It has been shown that E-cadherin suppresses expression of MMP-9 (Munshi et al., 2002). On the other side, down-regulation of E-cadherin stimulates secretion of MMP-9 in murine skin carcinoma cell lines (Llorens et al., 1998). Expression of E-cadherin resulted in down-regulation of MMP-2 in prostate cancer cell lines (Luo et al., 1999) and of MT1-MMP in squamous cancer cells (Ara et al., 2000). The mechanism which regulates the functional interaction between E-cadherin and MMPs is unclear. Beside its function in cell adhesion, E-cadherin-associated  $\beta$ -catenin plays a role as signalling molecule.  $\beta$ -catenin binds to transcription factors of the T-cell factor/lymphoid-enhancer factor (TCF/LEF) family and regulates among other genes *c-myc* and cyclin D1, MMP-7, gastrin, and ITF-2 (He et al., 1998; Crawford et al., 1999; Shtutman et al., 1999; Tetsu and McCormick, 1999; Koh et al., 2000; Kolligs et al., 2002). Potential TCF4 binding sites were identified in the promoters of several MMPs, including MMP-1, MMP-3, and MMP-7 (Brabletz et al., 1999). MMP-3 could possibly be a target of the  $\beta$ -catenin/TCF4 pathway. However, our present data suggest that regulation of MMP-3 by E-cadherin or E-cadherin-associated proteins takes place on the protein level and not or not only on the mRNA level in the MDA-MB-435S cell line.

Beside the data that E-cadherin regulates expression of MMPs, MMPs reciprocally have been found to influence E-cadherin activity. The function of transmembrane molecules like E-cadherin can be influenced or inhibited by loss of one of their domains. MMPs have, in addition to their ability to degrade matrix proteins and to enhance cell motility, the capacity to cleave ectodomains of certain transmembrane proteins. Several groups have shown that MMP-3 and MMP-7 (matrilysin) cleave the extracellular domain of E-cadherin, thereby producing an 80 kDa soluble E-

cadherin fragment (Lochter et al., 1997; Noe et al., 2001). The remaining membrane-bound part of E-cadherin is subsequently proteolytically degraded (Ito et al., 1999). Such E-cadherin cleavage results in the translocation of  $\beta$ -catenin from the cell membrane to the cytoplasm, where it accumulates (Lochter et al., 1997). Lack of the membrane-bound fragment causes invasion and leads to loss of E-cadherin-mediated cell adhesion (Noe et al., 2001). In the present study, we found a high amount of secreted MMP-3 in mutant E-cadherin expressing cells and the question if this is important for motility enhancement needs to be resolved. Also, whether MMP-3 can cleave the mutant E-cadherin variants investigated in this study remains to be demonstrated. Responses of MMP-3 found in mammary epithelium are proliferation and branching in ductal cells and apoptosis in alveolar cells (Sympson et al., 1994; Boudreau et al., 1995; Witty et al., 1995). We observed enhanced cell proliferation in the presence of E-cadherin mutations (Fricke et al., 2004). The question whether enhanced secretion of MMP-3 and increased cell proliferation of mutant E-cadherin expressing MDA-MB-435S cells are correlated remains to be solved.

siRNA can be used to specifically down-regulate gene expression by RNA interference. After formation of an RNA induced silencing complex (RISC), mRNA is degraded (McManus and Sharp, 2002). In the present study, we demonstrate that MMP-3 expression can be down-regulated by siRNA and cell migration shown by a wound healing assay is reduced concomitantly. To our knowledge, this is the first study that siRNA can be used to interfere with MMP-3 expression. siRNA directed against the region encoding prostromelysin 1 was most effective. Recently, a ribozyme has been constructed which was effective against stromelysin (Jarvis et al., 2000). MMP-9 silencing by RNA interference was recently shown to trigger induction of E-cadherin mediated cell adhesion and to reduce cell motility (Sanceau et al., 2003).

Taken together, in further experiments, the questions whether  $\beta$ -catenin is involved in the up-regulation of MMP-3 expression by mutant E-cadherin in MDA-MB-435S and whether the cleaved E-cadherin fragment influences cell motility have to be clarified. In addition, the question if MMP-3 in the supernatant is in its active confirmation, needs to be clarified. The microenvironment of the tumor host interface is important for tumor growth (Liotta and Kohn, 2001). Secretion of MMPs by stromal cells influences tumor cell growth. But also tumor cells seem to have found ways of modulating the amount of MMP: E-cadherin not only up-regulates cell adhesion, but also down-regulates MMP-3. However, E-cadherin mutations which show multiple effects on cell adhesion (Handschuh et al., 1999), motility (Handschuh et al., 1999; Fuchs et al., 2002), proliferation (Fricke et al., 2004), and tumorigenicity (Kremer et al., 2003), have achieved a novel function: the up-regulation of the proteolytic functions of tumor cells by enhanced secretion of MMP-3.

## ACKNOWLEDGMENTS

We thank M. Truss for the design of siRNA.

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