

# Stromal endothelin B receptor–deficiency inhibits breast cancer growth and metastasis

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## Abstract

The endothelin (ET) axis, often deregulated in cancers, is a promising target for anticancer strategies. Whereas previous investigations have focused mostly on ET action in malignant cells, we chose a model allowing separate assessment of the effects of ETs and their receptors ET<sub>A</sub>R and ET<sub>B</sub>R in the tumor cells and the stromal compartment, which is increasingly recognized as a key player in cancer progression. In homozygous spotting lethal rats (sl/sl), a model of constitutive ET<sub>B</sub>R deficiency, we showed significant reduction of growth and metastasis of MAT B III rat mammary adenocarcinoma cells overexpressing ET<sub>A</sub>R and ET-1 but negative for ET<sub>B</sub>R. Lack of stromal ET<sub>B</sub>R expression did not influence angiogenesis. However, it was correlated with diminished infiltration by tumor-associated macrophages and with reduced production of tumor necrosis factor- $\alpha$ , both known as powerful promoters of tumor progression. These effects were almost completely abolished in transgenic sl/sl rats, wherein ET<sub>B</sub>R function is restored by expression of an intact ET<sub>B</sub>R transgene. This

shows that tumor growth and metastasis are critically dependent on ET<sub>B</sub>R function in cells of the microenvironment and suggests that successful ETR antagonist therapy should also target the stromal component of ET signaling. [Mol Cancer Ther 2009;8(8):2452–60]

## Introduction

The vasoactive peptides endothelin-1 (ET-1), ET-2, and ET-3 and their receptors ET<sub>A</sub>R and ET<sub>B</sub>R are part of an ubiquitous network, which not only regulates vascular function (1) but is also involved in cell proliferation (2), differentiation (3), migration (4), and (anti)apoptosis (5).

This network has been shown to be altered in many malignant tissues (6, 7). In breast cancer, expression of ET-1 and ET<sub>A</sub>R is correlated with the transition from normal tissue to progressively invasive lesions (8), increased tumor angiogenesis (9), and shortened survival (10). ET-1 serum levels are elevated in patients with breast and colon cancers, especially in those with lymph node or distant metastases (11, 12). The ET-1/ET<sub>A</sub>R axis plays also a critical role for ovarian carcinoma progression (13).

The role of the ET<sub>B</sub>R, however, is ambiguous. Whereas colon cancer (14), Ewing sarcoma, and neuroblastoma (15), as well as prostate cancer, are associated with down-regulation of ET<sub>B</sub>R, leading to a preponderance of ET-1/ET<sub>A</sub>R signaling (16), ET<sub>B</sub>R is up-regulated in lung cancer (17), oral squamous cancer (18), and malignant melanoma, wherein it has been identified as a marker of progression (19, 20).

Inhibition of the ET axis efficiently antagonizes tumor progression *in vitro* and in animal models. Consistent with overexpression of the respective targeted receptor(s) in the tumor cells, antagonists of ET<sub>A</sub>R or both receptors inhibit proliferation and tumor growth in colon, breast, and ovarian carcinomas (12, 21, 22) whereas selective antagonists of ET<sub>B</sub>R are successful in melanomas (3, 23). In spite of these promising findings, the results of the few available clinical studies are still unsatisfactory. Treatment of 32 patients with advanced melanomas with the dual inhibitor bosentan induced stable disease in six patients as the best achievable result (24). The ET<sub>A</sub>R inhibitor atrasentan, although effective in the reduction of surrogate markers like prostate-specific antigen and alkaline phosphatase, did not delay disease progression in men with prostate cancer (25). Combined treatment of patients with advanced non-small cell lung cancer with chemotherapy and atrasentan did not yield better results than chemotherapy alone (26).

Apparently, there are determinants, other than the expression of ETs and receptors in the tumor cells themselves, influencing the outcome *in vivo*. There is growing evidence that tumor progression does not only depend on the biological characteristics of the malignant cells but also on interaction with benign cells and components of the surrounding

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**Note:** C. Binder and T. Hagemann contributed equally to this work.

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stromal compartment (27). The ET system is an interesting candidate to influence these interactions, because ETs and their receptors are expressed by a variety of stromal cells, such as myofibroblasts, endothelial cells (28), and macrophages (29).

Macrophages are potent promoters of invasion (30). Macrophage-induced invasion of breast cancer cells through Matrigel is increased both by ET-1 and ET-2, as well as overexpression of ET<sub>A</sub>R/ET<sub>B</sub>R (31, 32). It is abolished by ET<sub>A</sub>R/ET<sub>B</sub>R inhibition. ET-2 acts as a chemoattractant for macrophages via ET<sub>B</sub>R *in vitro* and probably also *in vivo*, as ET<sub>B</sub>R-positive tumor-associated macrophages (TAM) in breast cancers often colocalize with areas of ET-2-expressing malignant cells (33). These data suggest that the ET system, especially via the ET<sub>B</sub>R, is critical for stromal reactions in tumor progression.

The spotting lethal (sl) rat represents a suitable model to study the function of the ET<sub>B</sub>R without the necessity of pharmacologic manipulations. Homozygous sl/sl rats lack functional ET<sub>B</sub>Rs due to a 301-bp deletion in the ET<sub>B</sub>R gene (34). This leads to disturbance of neural crest migration and congenital aganglionosis of the gut with development of Hirschsprung's disease, limiting the life span to maximally 4 weeks. Wild type (+/+) and heterozygous (sl/+) rats are phenotypically normal. In transgenic rescue rats, ET<sub>B</sub>R function is restored by the introduction of an intact ET<sub>B</sub>R transgene linked to the human dopamine- $\beta$ -hydroxylase promoter (35). Thus, transgenic rats of the originally ET<sub>B</sub>R-deficient phenotype (tg<sub>sl/sl</sub>) do not succumb any more to intestinal complications. The tg<sub>sl/+</sub> and tg<sub>+/+</sub> animals, expressing an endogenous and a transgenic ET<sub>B</sub>R, remain phenotypically normal.

Although primarily gut targeted, ectopic expression of the transgene outside from intestinal catecholaminergic neurons has been described (35, 36). Thus, sl rats and their transgenic counterparts were used as a subtraction/addition model to further investigate the role of stromal ET<sub>B</sub>R function for tumor progression. Tumor formation was induced by s.c. inoculation of highly invasive MAT B III rat mammary adenocarcinoma cells, which are characterized by the up-regulation of the ET-1/ET<sub>A</sub>R axis and the lack of expression of ET<sub>B</sub>R, thus providing a species-immanent tumor model, wherein ET<sub>B</sub>R is present exclusively in the stromal compartment.

## Materials and Methods

### Cell Lines, Animals, and Experimental Protocol

The 13762 MAT B III rat mammary adenocarcinoma cell line was obtained from American Type Culture Collection and cultured in RPMI 1640 + 10% fetal bovine serum. All experiments were done under endotoxin-free conditions.

Rats of the Wistar-Imamichi AR strain (sl rats) and sl transgenic rescue rats were bred as described before (36, 37). Genotyping was done using primers flanking the 301-bp deletion of the ET<sub>B</sub>R gene (37). Expression of the transgene was documented by reverse transcription-PCR (RT-PCR) as described (35).

All animal work had been approved by the local committee of Animal Care and Use. MAT B III cells, suspended in PBS at 10<sup>5</sup>/100  $\mu$ L or 10<sup>-6</sup> mol/L clazosentan (Actelion), were inoculated s.c. in the region of the right thigh of 24-h-old rats. When the first of the ET<sub>B</sub>R-deficient animals had to be euthanized due to complications of congenital aganglionosis (between days 15 and 21), the whole litter was sacrificed by decapitation in anesthesia with 2,2,2-tribromethanol (Avertin, 275 g/kg body weight, i.p.). Upon autopsy, abdomen and thoracic cavity were examined for the presence of metastases. S.c. tumors were excised and weighed; serum was collected. Tumors and organs were fixed in 4% paraformaldehyde and/or snap frozen in liquid nitrogen. Only litters of similar age and animals without signs of accidental i.v. tumor cell injection were included in the comparative analysis of tumor weight. To determine the rate of metastases, all animals were included.

### Chemotaxis Assay

BMDM were cultured for 7 d in DMEM + 100 ng/mL murine M-CSF (R&D Systems). Cell motility of 5  $\times$  10<sup>5</sup> macrophages toward CCL5, CCL2, or colony stimulating factor-1 (each 10 ng/mL, R&D Systems) was assayed using Falcon Transwells (BD Pharmingen). After incubation for 18 h, migrated cells on the lower surface were stained using Diff-Quik (Dade Behring). For each transwell, the number of migrated cells in 10 medium power fields ( $\times$ 20) was counted. All of these experiments were repeated at least thrice.

### Cloning and Transfection of Tumor Cells

Tumor cells were seeded in six-well plates, such that they were 60% to 90% confluent on the day of transfection. Small interfering RNA (siRNA) sequence for the rat ET<sub>A</sub>R (NM\_012550) was obtained from Ambion (sense siRNA strand 5'-GGACUGGUGGCUCUUUGGATT-3', antisense siRNA strand 5'-UCCAAAGAGCCACCAGUCCTT-3') and cloned into the pSilencer2.1-U6 vector system (Ambion). MAT B III cells were transfected with the pSilencer2.1-U6-RNAi plasmids for ET<sub>A</sub>R or a control plasmid containing scrambled RNA. Cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Antibiotic selection for stable cell lines started after 48 to 72 h in 4  $\mu$ g/mL puromycin (Sigma) for 30 d. Effective gene silencing was confirmed by RT-PCR and Western blot (Abcam). A mixture of all three clones was used for inoculation.

### Flow Cytometry

Excised tumors were incubated in digestion buffer (RPMI 1640 + 5% FCS + 5 mg/mL collagenase D + 0.15 mg/mL DNase). Tumors were minced, digested at 37°C for 40 min, and passed through a 19-g and 23-g needle and then through a cell strainer. Cells were pelleted at 1,500 rpm and resuspended at 50 to 100  $\times$  10<sup>5</sup>/mL in fluorescence-activated cell sorting buffer (PBS + 0.1% bovine serum albumin, 0.01% NaN<sub>3</sub>). Results were normalized to tumor weight. Cells were blocked with mouse anti-rat CD32 FcBlock (BD Bioscience Pharmingen) for 30 min on ice. Antibodies used were CD163-FITC (ED2) for macrophages, CD161-FITC (10/78), natural killer cells (all from Serotec); CD4-FITC

(OX-35) and CD25-PE (OX-39) for T reg cells, CD8a-FITC (OX-8), T cells, and granulocytes-FITC (HIS48; all from BD Bioscience Pharmingen); and respective isotype controls. Cells were analyzed on a FACScan flow cytometer using Cellquest software (Becton Dickinson). For three-way cell sorting using the MoFlo (DakoCytomation), CD8a-PE, granulocytes-biotin (BD Pharmingen), and CD163-FITC (Serotec) were applied to isolate all three cell populations from the same cell pool. TAM from tumor-bearing rats were collected by CD11b-positive selection (Miltenyibiotec). Experiments were repeated at least thrice.

#### ELISA

Quantikine ELISA kits for rat interleukin-10 (IL-10), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and rat vascular endothelial growth factor (VEGF; R&D Systems) were used according to the manufacturer's instructions. Analytic sensitivity of the assays was as follows: rat IL-10, 10 pg/mL; rat TNF- $\alpha$ , 12.5 pg/mL; rat VEGF, 3.9 pg/mL. For all ELISAs, the absorbance at 450 nm was measured and corrected at 570 nm in a plate reader (Opsys MR; Dynex Technologies). Experiments were repeated at least thrice.

#### Histology and Immunohistochemistry

Tissues were fixed in buffered formalin and embedded in paraffin. Morphology was visualized by staining with H&E. To assess proliferation (%Ki67 positivity), immunohistochemistry was done using the MIB-5 monoclonal mouse anti-rat Ki67 antibody with a standard avidin-biotin complex method, diaminobenzidine, and nuclear counterstaining with hematoxylin (Dako). Ten high power fields for each sample were counted by two individual people. Macrophages were detected with the CD163 antibody and the same avidin-biotin complex method. For microvascular density quantification, tissues were stained with the rabbit polyclonal Von Willebrand factor antibody (Abcam). Computer images were used to perform manual counts of stained microvessels. Apoptosis was detected with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using a commercially available *in situ* apoptosis detection kit (Promega). The number of TUNEL-positive apoptotic cells was counted in 10 randomly selected fields ( $\times 40$ ) as a percentage of total cells.

#### Semiquantitative and Real-time RT-PCR

Semiquantitative RT-PCR with coamplification of lamin B was used for detection of ET<sub>B</sub>R expression in rats. Amplification of the endogenous (and the sequence-identical transgene) receptor yielded a 912-bp product for the wild type and a 642-bp product for the mutant form. Heterozygous animals show both forms, although, due to primer competition, they are not amplified with the same efficiency. Separate primers were used to detect exclusively the dopamine- $\beta$ -hydroxylase promoter-coupled transgene resulting in a 500-bp product. Primers and conditions have been described previously (35–37).

Multiplex real-time analysis was done using matrix metalloproteinase-2 (MMP-2), MMP-9, ET-1, ET-2, ET-3, ECE1, ECE2, ET<sub>A</sub>R, and ET<sub>B</sub>R (FAM) and 18 s rRNA (VIC) specific primers and probes with the ABI PRISM 7700 Sequence Detection System instrument and software (PE

Applied Biosystems). PCR was carried out with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 2  $\mu$ L cDNA in a 25- $\mu$ L final reaction volume. The cycling conditions were incubation at 50°C for 2 min, followed by 10 min at 95°C and 60 cycles of 15 s at 95°C, and 1 min at 60°C. Experiments were done in triplicate. Gene expression was normalized to 18S RNA by subtracting the cycle threshold (C<sub>t</sub>) value of 18S RNA from the C<sub>t</sub> value of the respective RNA of interest.

#### Statistical Analysis

Data are presented as means  $\pm$  SD. Differences in lung metastases were tested for statistical significance using the Fisher's exact test. All other data were compared with the unpaired two-tailed Student's *t* test or Mann-Whitney *U* test. *P* values of <0.05 were considered significant.

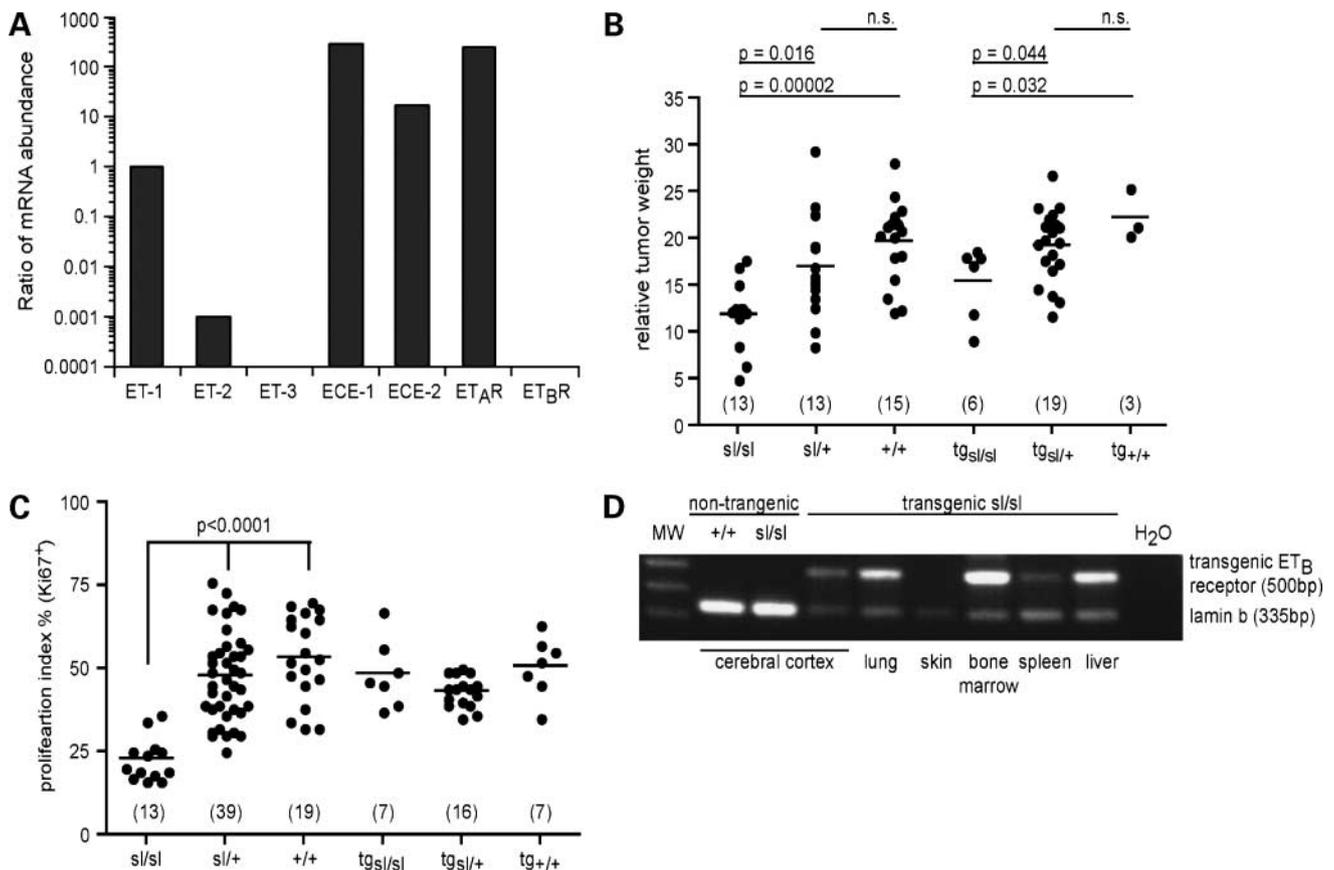
## Results

### Stromal ET<sub>B</sub>R Deficiency Reduces Local Tumor Growth

First, syngeneic MAT B III cells were characterized by RT-PCR, showing strong expression of ET-1, ECE-1/ECE-2, ET<sub>A</sub>R; weak expression of ET-2; and lack of ET-3 and ET<sub>B</sub>R expression (Fig. 1A). We then asked whether stromal ET<sub>B</sub>R expression would influence the growth of MAT B III cells *in vivo* in the sl rat model. After s.c. inoculation of the syngeneic-invasive tumor cells, all rats developed a local tumor at the injection site. Absolute and relative weights (normalized to body weight) of these tumors were significantly lower in ET<sub>B</sub>R-deficient sl/sl rats than in heterozygous and wild-type animals (Fig. 1B). Upon H&E staining (not shown), tumors in the genetic subgroups did not differ from each other. Cells with the typical signs of apoptosis were rarely detectable in H&E-stained sections of any genotype. This was confirmed by TUNEL staining, showing no difference between the three populations (Table 1). However, proliferation rate was significantly lower in tumors of sl/sl rats, as shown by Ki67 staining (Figs. 1C and 2A and B).

There was no sign of lower blood supply as a possible cause of reduced tumor growth, as areas of necrosis were generally rare and not increased in sl/sl animals. Measurement of microvessel density did not yield significant differences (Table 1). Equally, VEGF serum concentrations in the three cohorts were similar (Table 2). IL-10 serum levels were determined by ELISA. IL-10 was detectable in all samples without any significant differences (Table 2).

If ET<sub>B</sub>R expression by stromal cells is essential for tumor progression, then differences in tumor growth across the genetic subpopulations should disappear upon reconstitution of ET<sub>B</sub>R deficiency in transgenic rescue rats. In fact, lower Ki67 expression of tumors in ET<sub>B</sub>R-deficient sl/sl rats compared with their heterozygous and wild-type littermates could be completely rescued by reexpression of ET<sub>B</sub>R in the respective transgenic rats (Figs. 1C and 2C). However, there was still a marginally significant difference between transgenic sl/sl rats and their heterozygous and wild-type counterparts with regard to tumor weight (Fig. 1B). As the dopamine- $\beta$ -hydroxylase-linked transgene is originally



**Figure 1.** Expression of the ET system and local tumor growth. **A**, RNA expression profile of ETs, receptors, and related proteins in MAT B III cells (real-time RT-PCR, one representative of three independent experiments). **B**, ET<sub>B</sub>R deficiency reduces tumor growth. Relative weight of the MAT B III-induced tumors at the inoculation site (normalized to body weight) in the three genotypes of si rats and their transgenic counterparts. Only litters of similar age (four in both groups) were analyzed. The animal numbers per genotype are given in brackets. **C**, ET<sub>B</sub>R-deficiency reduces proliferation. Proliferation index (percentage of tumor cell nuclei staining positive for Ki67) in the local tumors. Numbers (in brackets) are higher as for tumor weight, as all inoculated animals were included (six litters). **D**, RNA expression of transgenic ET<sub>B</sub>R in various tissues of transgenic rescue si rats (RT-PCR, lamin b expression used as control). Primers recognize exclusively the transgenic receptor, not the endogenous form.

targeted to the intestine — although having been described also in lung, muscle, and other tissues (35) — we investigated whether differential transgene expression may account for these discrepancies. In fact, transgenic ET<sub>B</sub>R-RNA was not ubiquitously expressed in our animals. It was detectable in the cerebral cortex, bone marrow, spleen, liver, and lung but was absent in the skin and s.c. compartment (Fig. 1D).

### The ET-1/ET<sub>A</sub>R Loop Regulates Tumor Cell Proliferation and Apoptosis Independent of Stromal ET<sub>B</sub>R Function

As overexpression of ET-1 and ET<sub>A</sub>R is known to induce an autocrine loop to promote tumor cell growth and invasion, we evaluated the functional contribution of the ET<sub>A</sub>R in this context. For transient inhibition, the

**Table 1.** Apoptosis, proliferation, and microvessel density in tumors derived from MAT B III and MAT B III ET<sub>A</sub>R siRNA cells (means ± SD)

	MAT B III			MAT B III-ET <sub>A</sub> R siRNA		
	s/si (n = 17)	s/si+ (n = 39)	+/+ (n = 19)	s/si (n = 4)	s/si+ (n = 24)	+/+ (n = 10)
TUNEL positivity (%)	23.1 ± 8.7*	23.0 ± 9.9	20.0 ± 8.7	48.4 ± 9.4*	40.9 ± 11.9	42.6 ± 8.1
Ki67 positivity (%)	22.8 ± 6.2 <sup>†</sup>	47.7 ± 13.3 <sup>†</sup>	53.3 ± 12.9 <sup>†</sup>	22.5 ± 5.9	24.6 ± 4.8	22.8 ± 7.1
Microvessel density (vessels/mm <sup>2</sup> )	35.0 ± 14	38.0 ± 16	41.0 ± 22	n.p.	n.p.	n.p.

Abbreviation: n.p., not performed.

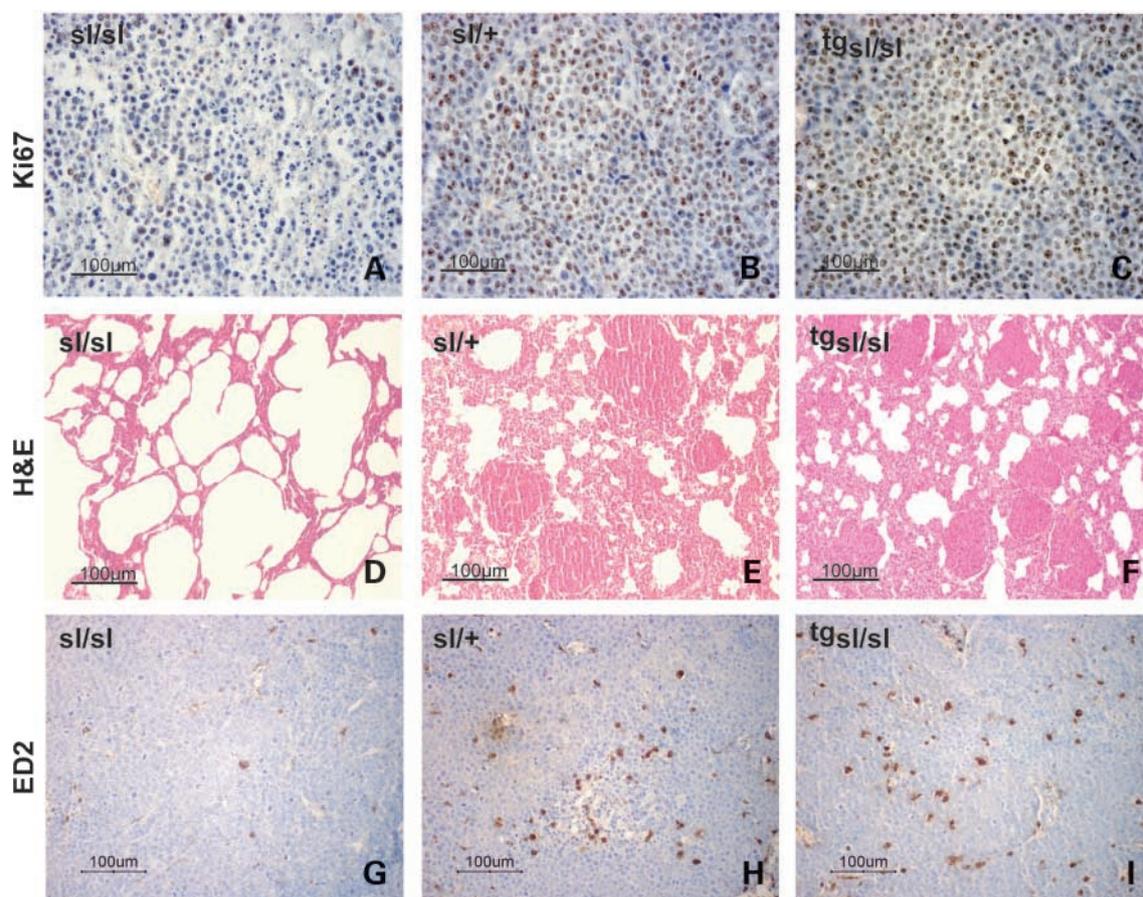
\*P values for all subgroups of MAT B III versus respective MAT B III-ET<sub>A</sub>R siRNA, <0.001.

<sup>†</sup>P values for s/si versus s/si+ and +/+, <0.0001.

inoculated MAT B III cell suspension additionally contained the ET<sub>A</sub>R antagonist clazosentan. For permanent silencing, we used MAT B III cells, wherein ET<sub>A</sub>R expression had been abolished by stable transfection of ET<sub>A</sub>R siRNA (Fig. 3A). Transient ET<sub>A</sub>R inhibition had no effect on tumor growth, presumably due to rapid regrowth of the inoculated cells after expiry of the clazosentan effect (data not shown). MAT B III ET<sub>A</sub>R siRNA cells, in contrast, proliferated only slowly *in vitro* and consequently produced smaller tumors *in vivo* (doubling time 57 versus 33 hours). This was accompanied by a generally lower expression of Ki67 and a higher amount of apoptosis (Table 1). However, there was no evidence that the autocrine ET-1/ET<sub>A</sub>R loop in the tumor cells was influenced by the microenvironment. Proliferation and apoptosis of MAT B III-ET<sub>A</sub>R siRNA cells *in vivo* did not differ between the genetic subgroups (Table 1), and the generally lower weight of MAT B III-ET<sub>A</sub>R siRNA-induced tumors was independent of whether ET<sub>B</sub>R was present or absent in the stromal compartment (Fig. 3B).

### Stromal ET<sub>B</sub>R Deficiency Reduces Metastatic Spread

Next, we were interested in whether ET<sub>B</sub>R function in the tumor stroma would influence tumor dissemination. MAT B III cells are highly metastatic. Metastases were found predominantly in the lungs. Other organs were not involved, except in two sl/+ animals with peritoneal spread. Despite their massive tumor load, these animals had only very small local tumors, suggesting accidental intravascular tumor cell injection. They were, therefore, excluded from evaluation. Metastatic involvement of the lungs was shown predominantly in sl/+ and +/+ animals (Fig. 4A). Thirteen of 21 heterozygous and 14 of 27 wild-type rats had pulmonary metastases in contrast to only 1 of 14 homozygous animals ( $P = 0.006$ , Fisher's exact test). Whereas metastatic disease in sl/+ and +/+ lungs was usually disseminated, metastases in sl/sl lungs, if there were any, could only be found upon evaluation of multiple serial sections (Fig. 2D and E). These effects were completely abolished by restoration of ET<sub>B</sub>R function. All of the tg<sub>sl/sl</sub> animals, expressing the transgene, had metastatic disease in the lungs (Figs. 4A and 2F).



**Figure 2.** Morphologic characterization of MAT B III-induced local tumors and metastases. **A–C**, immunostaining of local tumors for Ki67, showing a reduced amount of proliferation in sl/sl (**A**) compared with sl/+ animals (**B**) and the rescue effect of the transgene in tg<sub>sl/sl</sub> (**C**). **D–F**, H&E staining of lung sections from sl/sl rats (**D**), showing normal tissue, and sl/+ rats (**E**) with multiple metastases, similar to the tg<sub>sl/sl</sub> animals (**F**). **G–I**, immunostaining of the local tumors for the rat macrophage antigen ED-2, showing lower numbers of infiltrating TAM in sl/sl animals (**G**) than in sl/+ (**H**) and tg<sub>sl/sl</sub> rats (**I**). Sections of tumors and metastases from +/+ animals are not shown separately, because they did not differ from the sl/+ samples.

**Table 2. Cytokine serum concentrations (pg/mL, means  $\pm$  SD)**

	sl/sl ( <i>n</i> = 12)	sl/+ ( <i>n</i> = 34)	+/+ ( <i>n</i> = 19)
TNF- $\alpha$	12.39 $\pm$ 5.04*	28.6 $\pm$ 23.84*	23.31 $\pm$ 20.24*
VEGF	451.2 $\pm$ 35.03	454.0 $\pm$ 24.13	466.8 $\pm$ 32.01
IL-10	55.1 $\pm$ 18.21	62.33 $\pm$ 23.15	57.38 $\pm$ 14.26

\**P* values for sl/sl versus sl/+ and +/+, <0.05.

Transient ET<sub>A</sub>R inhibition by clazosentan did not influence metastasis formation. The effect of permanent silencing could not be analyzed, because tumors derived from MAT B III ET<sub>A</sub>R siRNA cells grew so slowly that sl/sl rats in these litters had to be euthanized because of progression of Hirschsprung's disease before development of metastases in any of the genetic subgroups.

### Tumors in ET<sub>B</sub>R-Deficient Rats Contain Fewer Tumor-Associated Macrophages

MAT B III cells express ET-1 and ET-2, which are both chemoattractants for macrophages. Because we have shown that this is mediated via ET<sub>B</sub>R (33), we asked whether tumors in ET<sub>B</sub>R-deficient rats would contain lower amounts of infiltrating TAM. Measurement of ET<sub>B</sub>R mRNA expression in peripheral blood monocytes, the origin of the TAM, confirmed expression of the correct ET<sub>B</sub>R transcript in wild-type and heterozygous monocytes (Fig. 4B). As shown by immunohistochemistry with the macrophage marker CD 163, significantly fewer TAM were detectable in tumors from sl/sl than from heterozygous or wild-type animals (Figs. 4C and 2G and H). Equally, quantitative analysis of the composition of the whole tumor leukocyte infiltrate by flow cytometry yielded a significantly lower content of TAM in sl/sl than in sl/+ or +/+ tumors, whereas there were no differences in the amount of granulocytes, T cells, and natural killer cells (Table 3). Again, this effect was completely counteracted by the ET<sub>B</sub>R transgene in tg<sub>sl/sl</sub> animals (Figs. 4C and 2I). Surprisingly, this was not caused by reexpression of a functional ET<sub>B</sub>R in the monocytes themselves, as the transgene was not detectable in these cells (Fig. 4B).

The reduced number of TAM in sl/sl animals was not due to a constitutive lack of monocytoid cells. Peripheral blood counts and cytopins of peritoneal lavages yielded comparable amounts of monocytes and peritoneal macrophages in all genetic subgroups (not shown). Equally, we could not show a general chemotaxis defect in sl/sl macrophages. Bone marrow-derived monocytoid cells of all genetic subpopulations were subjected to migration assays toward colony stimulating factor-1 and the chemokines CCL-2 and CCL-5 (Fig. 4D). Migration rates were identical.

Recently, we have shown that macrophage-induced invasion of malignant cells is critically dependent on macrophage-derived TNF- $\alpha$  (11). Moreover, secretion of TNF- $\alpha$  is inducible by ET-1 and vice versa (38, 39). We therefore measured the serum concentrations of TNF- $\alpha$  in tumor-bearing rats (Table 2). TNF- $\alpha$  concentrations were generally low but still significantly lower in sl/sl than in heterozy-

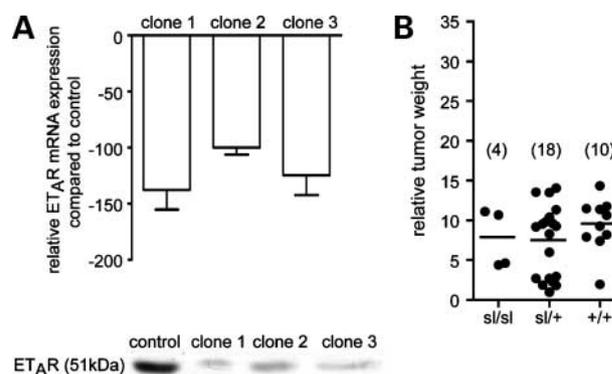
gous and wild-type animals. We also showed earlier that TNF- $\alpha$  was responsible for MMP up-regulation (11). Therefore, we analyzed the mRNA expression of MMP-2 and MMP-9 in 10<sup>6</sup> fluorescence-activated cell sorted TAM from tumor-bearing animals. MMP-2 and MMP-9 expression was significantly lower in TAM from sl/sl rats compared with TAM from sl/+ or +/+ rats (*n* = 10; Fig. 4E).

## Discussion

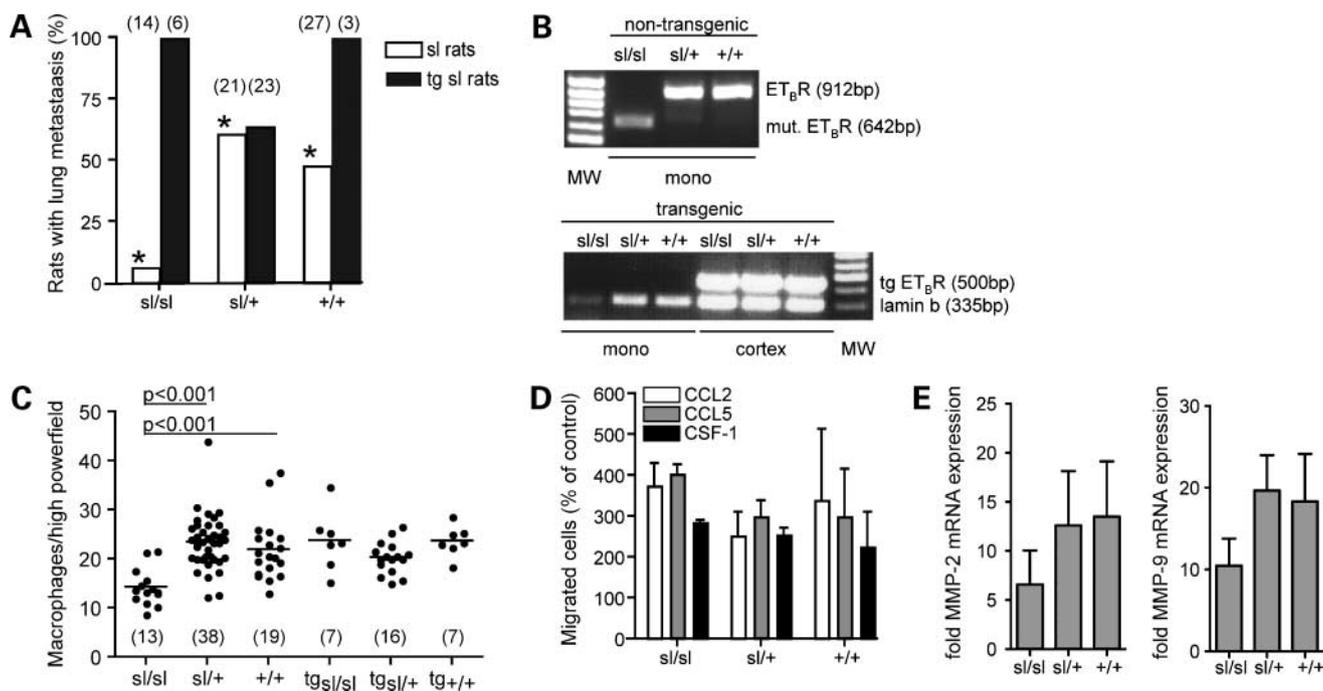
Members of the ET network are often deregulated in cancer cells. As they are also produced by the tumor microenvironment, they are very likely to be involved in interactions between malignant and benign tumor components that influence tumor progression and metastasis.

To better dissect these effects, we chose an animal model wherein ET<sub>B</sub>R expression is modulated exclusively in the tumor stroma whereas the tumor cells are ET<sub>B</sub>R-negative and show overexpression of ET<sub>A</sub>R and ET-1. There we could show that the stromal ET<sub>B</sub>R plays a critical role for malignant progression. Growth and especially metastasis of MAT B III rat mammary adenocarcinoma cells were greatly reduced in homozygous sl/sl rats, constitutively lacking a functional ET<sub>B</sub>R, compared with their littermates with normal ET<sub>B</sub>R expression.

As ET<sub>B</sub>R inhibition can trigger apoptosis (5), we speculated that smaller tumors might be caused by diminished survival. However, although cell death could be induced by down-regulation of tumor cell ET<sub>A</sub>R via siRNA, thus confirming their dependency on an autocrine ET-1/ET<sub>A</sub>R loop for survival, this was not influenced by the presence or absence of stromal ET<sub>B</sub>R. The amount of apoptotic cells did not differ between the genetic subgroups, irrespective of whether ET<sub>A</sub>R was overexpressed or down-regulated.



**Figure 3.** ET<sub>A</sub>R knockdown in MAT B III cells. **A**, expression of ET<sub>A</sub>R RNA (real-time RT-PCR, relative expression compared with the controls) and ET<sub>A</sub>R protein (Western blot) after stable transfection with the pSilencer 2.1-U6-RNAi plasmid for ET<sub>A</sub>R. **B**, ET<sub>A</sub>R-deficient cells induce smaller tumors independent of the genotype of the stromal compartment. Relative weight of local tumors (normalized to body weight) induced MAT B III ET<sub>A</sub>R siRNA cells (mixture of all three clones) in the three sl genotypes. Four litters of similar age were analyzed; numbers per genotype are given in brackets.



**Figure 4.**  $ET_B$ R deficiency, metastasis formation, and infiltrating TAM. **A**,  $ET_B$ R deficiency reduces lung metastases. Animals with lung metastases in the three genotypes of sl rats and their transgenic counterparts; \*,  $P = 0.001$  and  $P = 0.004$ , Fisher's exact test. **B**, endogenous  $ET_B$ R mRNA expression in peripheral blood monocytes of nontransgenic rats (RT-PCR) showing the deletion transcript in sl/si cells, the wild type in +/+ cells, and both forms in heterozygous cells. The transgene is expressed in the cerebral cortex of all transgenic animals but is absent in the monocytes (RT-PCR with lamin expression used as control). **C**, tumors in sl/si rats contain fewer TAM. Rate of TAM per high power field (means  $\pm$  SD) in the three genetic subgroups of sl and tg sl rats; numbers of analyzed animals (from six litters) are given in brackets. **D**,  $ET_B$ R deficiency does not induce a general chemotaxis defect. Chemotaxis assays of bone marrow–derived macrophages of the various genetic subgroups using the indicated chemoattractants (means  $\pm$  SD). **E**, total RNA was isolated from CD11b+ selected tumor infiltrating macrophages for real-time PCR analysis of MMP-2 and MMP-9 expression. Data are represented as fold induction of mRNA expression compared with wild-type macrophages,  $n = 10$ .

In contrast to cell survival, proliferation was significantly influenced by stromal  $ET_B$ R function, its complete lack resulting in lower proliferation rates in the respective tumors. This seems surprising because pharmacologic  $ET_B$ R inhibitors, although reported to reduce proliferation of cancer cells *in vitro* (18) and tumor growth in nude mice (23, 40), usually do so on condition that the targeted tumor cells overexpress  $ET_B$ R. In our model, the tumor cells are  $ET_B$ R negative. Hence,  $ET_B$ Rs on stromal cells obviously interact with the malignant cells and modulate their biological behavior.

As the ET axis is known for its role in the vascular system, it would seem that stromal endothelial cells are the most

likely candidates to influence tumor progression.  $ET_B$ R overexpression in primary breast cancers was associated with increased neoangiogenesis (9), whereas inhibition by specific or dual antagonists diminished vascularization (23, 41). Paradoxically, injection of the  $ET_B$ R inhibitor BQ788 in melanoma xenografts resulted in elevated vessel numbers (5). We found no significant difference in VEGF production and microvessel density between the genetic subgroups, consistent with the observation that pharmacologic inhibition does not necessarily yield the same effects as constitutive deficiency (42).

In many tissues, binding of ETs to  $ET_A$ R induces vasoconstriction whereas activation of  $ET_B$ R induces the opposite

**Table 3.** Flow cytometric analysis of the whole tumor leukocyte infiltrate

	Percentage of cells per tumor infiltrate (means $\pm$ SD*)			Significance ( $P$ ) sl/si versus sl/+ and +/+
	sl/si	sl/+	+/+	
CD 163(ED2)+ macrophages	9.56 $\pm$ 7.21	22.08 $\pm$ 11.27	19.11 $\pm$ 12.43	<0.001
CD4+ T cells	3.53 $\pm$ 1.46	3.33 $\pm$ 1.85	3.57 $\pm$ 1.74	>0.05
CD8+ T cells	6.34 $\pm$ 3.95	8.04 $\pm$ 2.2	7.34 $\pm$ 2.7	>0.05
CD161a+ NK cells	3.45 $\pm$ 2.23	3.32 $\pm$ 2.81	4.07 $\pm$ 3.65	>0.05
Granulocytes	15.21 $\pm$ 3.75	16.23 $\pm$ 8.2	18.42 $\pm$ 0.4	>0.05

\*Normalized to tumor weight; Student's  $t$  test.

effect (43). As vascular resistance is elevated in sl/sl rats (44), predominance of ET<sub>A</sub>R in ET<sub>B</sub>R deficiency would be expected to shift the vasomotor balance toward vasoconstriction and reduce intratumoral blood flow (45). However, areas of necrosis as a potential sign of insufficient perfusion were not increased in tumors of ET<sub>B</sub>R-deficient rats.

TAM, an essential part of the stromal immune infiltrate, are powerful promoters of tumor progression. We and others have shown that TAM induce matrix metalloprotease-mediated invasion and metastasis, which is dependent on TNF- $\alpha$  (11, 46, 47). Consistent with these findings, serum concentrations of TNF- $\alpha$  and the amount of infiltrating TAM were significantly lower in ET<sub>B</sub>R-deficient animals. Neither constitutively lower numbers of monocyte cells nor a general chemotaxis defect could explain these results.

In correspondence to the lower amount of TAM, metastasis to the lungs was almost completely abolished in ET<sub>B</sub>R-deficient rats. As a proof of principle, transgenic overexpression of a functional ET<sub>B</sub>R counteracted these effects. Transgenic rescue rats did not show any difference between the genetic subgroups with regard to lung metastasis and TAM infiltration. The effect on local tumor growth, however, was only partially neutralized, as tumors were still smaller in sl/sl transgenic animals. Because the transgene was detectable in the lungs but absent in the skin, differing tissue-specific expression levels most probably account for the discordant effect on tumor growth and dissemination. The gene dose has been shown to be crucial for ET<sub>B</sub>R function (35, 44). Inoculated tumor cells may have grown slowly as long as they were confined to the still ET<sub>B</sub>R-deficient cutis/subcutis compartment. With increasing recruitment of transgene-positive stromal cells and contact with positive neighboring tissues, the inhibitory action of the nonfunctional ET<sub>B</sub>R on proliferation and invasion was effectively antagonized.

The surprising finding that TAM infiltration is restored in transgenic animals, although the transgene is not expressed in these cells, points to a role of other cells in the microenvironment, which may influence TNF- $\alpha$  production and/or macrophage chemoattraction. Interestingly, it has recently been shown that endothelial ET-1/ET<sub>B</sub>R signaling blocks T-cell adhesion and infiltration into tumors in the ID8 ovarian cancer model in mice (48). This was dependent on intercellular adhesion molecule-1 and TNF- $\alpha$ . In our model, there was no difference in tumor infiltration by lymphocytes.

In conclusion, whereas malignant cell survival in our model depends on autocrine stimulation via the ET-1/ET<sub>A</sub>R loop, growth of the whole tumor and metastasis formation is regulated by ET<sub>B</sub>R function in the stromal compartment. Lack of functional ET<sub>B</sub>R in the TAM seems essential; however, contribution of other stromal cells to this effect has to be postulated. This shows that tumor progression can be modulated not only by ET signaling within the malignant cells but also by exchange of signals between different cell types within the tumor tissue. It may also explain the contradictory results of pharmacologic ET receptor antagonists *in vivo*. Considering the potential use of ET<sub>B</sub>R inhibitors as

clinical anticancer agents, further evaluation of their effects on stromal cells is warranted.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Molecular Cancer Therapeutics

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