

Vascular Biology, Atherosclerosis and Endothelium Biology

# A Novel Noninvasive Model of Endometriosis for Monitoring the Efficacy of Antiangiogenic Therapy

Christian M. Becker,<sup>\*†‡</sup> Renee D. Wright,<sup>§</sup>  
Ronit Satchi-Fainaro,<sup>\*¶</sup> Tae Funakoshi,<sup>\*</sup>  
Judah Folkman,<sup>\*</sup> Andrew L. Kung,<sup>§</sup> and  
Robert J. D'Amato<sup>\*||</sup>

From the Vascular Biology Program, Department of Surgery,\* Children's Hospital, Harvard Medical School Boston, Massachusetts; the Department of Obstetrics and Gynecology,<sup>†</sup> Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; the Department of Obstetrics and Gynecology,<sup>‡</sup> Charité-Campus Benjamin Franklin, Berlin, Germany; the Department of Pediatric Oncology,<sup>§</sup> Dana Farber Cancer Institute and Children's Hospital, Harvard Medical School, Boston, Massachusetts; the Department of Physiology and Pharmacology,<sup>¶</sup> Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel; and the Department of Ophthalmology,<sup>||</sup> Harvard Medical School, Boston, Massachusetts

**Endometriosis, the presence of ectopic endometrial tissue, is a common disease associated with high morbidity and socioeconomic problems. Angiogenesis, the formation of new blood vessels, plays an important role in the formation and growth of endometriotic lesions. We have created a novel, noninvasive model to monitor the growth of these lesions and the associated angiogenesis *in vivo*. First, we generated luciferase-expressing transgenic mice by inserting the human ubiquitin C promoter coupled to the firefly luciferase reporter. Injection of luciferin in these mice causes full-body bioluminescence, which can be detected using a low-light CCD camera. Endometrial tissue from these transgenic mice was surgically implanted into nonluminescent recipients. Bioluminescence of lesions was noninvasively imaged after intravenous or intraperitoneal injection of luciferin. Transabdominal luminescence compared well with the location of the transgenic endometriotic lesions, and lesion size correlated with the intensity of luminescence. Systemic treatment with the angiogenesis inhibitors caplostatin and endostatin peptide mP-1 delayed and suppressed the onset and intensity of the luminescent signal. Caplostatin suppressed the growth of endometriotic lesions by 59% compared with controls. This novel, noninvasive model of endometriosis provides a means to study**

**early angiogenesis *in vivo* and to monitor endometriotic growth and the efficacy of systemic antiangiogenic therapy. (Am J Pathol 2006, 168:2074–2084; DOI: 10.2353/ajpath.2006.051133)**

Endometriosis, the presence of endometrial tissue outside the uterine cavity, is a common disorder in women. It is estimated that 10 to 15% of women of reproductive age suffer from this disease.<sup>1</sup> The condition is associated with dysmenorrhea, abdominal pain, dysuria, and dyschezia. In addition, infertility has been reported in more than 30% of women affected with the disorder.<sup>2</sup> Endometriosis is considered to be an estrogen-dependent disease because women are almost exclusively affected between menarche and menopause. Despite the high prevalence and the incapacitating symptoms, which are associated with significant interpersonal problems and socioeconomic losses, the pathogenesis of endometriosis remains unsolved.

Sampson's<sup>3</sup> description of retrograde menstruation as the driving mechanism for implantation of shed viable endometrial tissue into ectopic sites is currently widely accepted in the field. Endometriosis could be induced in women after intraperitoneal injection of their own menstrual blood.<sup>4</sup> However, there are a few inconsistencies in this theory. Menstrual blood can be found in more than 90% of women during laparoscopic surgery performed during menses.<sup>5</sup> In addition, endometriotic lesions have been found in premenarcheal girls and in rare cases in male patients.<sup>6–8</sup> Other hypotheses that have been proposed include metaplasia of coelomic epithelium,<sup>9</sup> hematological and lymphogenic spread,<sup>10</sup> and remnants of the Müllerian duct.<sup>11</sup>

---

Supported by a postdoctoral research exchange grant from the Max Kade Foundation (to C.M.B.), by a Howard Temin Award from the National Cancer Institute (to A.L.K.), by a grant from the Sidney A. Swensrud Foundation (to R.J.D.), and by a National Institutes of Health/National Cancer Institute Program Project grant P01-CA45548 (to J.F.).

A.L.K. and R.J.D. are joint senior authors.

Accepted for publication March 13, 2006.

Address reprint requests to: Robert D'Amato, Vascular Biology Program, Children's Hospital Boston, Harvard Medical School, 300 Longwood Ave., Boston, MA 02115. E-mail: robert.damato@childrens.harvard.edu.

More recently, molecular concepts have been introduced to explain the pathogenesis of endometriosis. It has been reported that impaired immunity may be responsible for the local attachment and invasion of endometrial tissue.<sup>12,13</sup> Aromatase was found to be overexpressed in endometriotic lesions, while at the same time, production of 17- $\beta$ -hydroxysteroid dehydrogenase type 2 is reduced.<sup>14,15</sup> As a result, locally elevated estradiol levels lead to a further stimulation of aromatase via increased prostaglandin E<sub>2</sub> concentrations.<sup>16,17</sup>

Despite the various hypotheses about the mechanisms responsible for the development and progression of endometriosis, it is widely accepted that angiogenesis, the formation and sprouting of new blood vessels, plays an essential role in the growth and survival of endometriotic lesions.<sup>18–23</sup> Angiogenic cytokines are elevated in the peritoneal fluid, serum, and endometriotic tissue of women suffering from the disease.<sup>19,24–29</sup> Our laboratory has found a large spectrum of angiogenic responsiveness between different strains of mice.<sup>30–32</sup> This diversity is due to polymorphisms that alter genes involved in angiogenic pathways.<sup>33</sup> We expect that this same range of genetically controlled diversity exists in humans. Thus, we speculate that endometriosis arises when retrograde menstruation occurs in a woman who also has high angiogenic responsiveness and therefore can support the implantation and growth of these lesions.

Recently, antiangiogenic therapy has been demonstrated to be efficient in suppressing the development of endometriotic lesions in rodent models.<sup>34–38</sup> We have shown that treatment with the endogenous angiogenesis inhibitor endostatin, a proteolytic fragment of collagen XVIII, or two 27-amino acid peptides of endostatin inhibit the growth of endometriotic lesions in mice without toxic side effects on reproductive function.<sup>39,40</sup> Immunohistochemical studies of blood vessels in endometriotic tissue show that new blood vessels appear in the lesion after about 1 to 2 weeks.<sup>41,42</sup> However, there has been a lack of animal models of endometriosis to continuously monitor the efficacy of therapy, particularly antiangiogenic therapy.

Recently, mouse models of endometriosis using tissue that expresses green fluorescent protein (GFP) have been described.<sup>43–45</sup> However, the use of these models is limited due to the emission spectrum of GFP and poor tissue penetration of the relatively short wavelength light. Both transgenic GFP murine tissue and human endometrium infected with adenovirus carrying the gene for GFP requires opening of the abdominal wall to visualize intraperitoneal lesions, making serial imaging impossible. To circumvent this problem, the use of bioluminescence systems was suggested.<sup>44</sup>

In our current study, we are reporting the use of endometrial tissue ubiquitously expressing luciferase in a novel model of surgically induced endometriosis. We generated transgenic mice that express firefly luciferase. In these animals, the reporter gene for luciferase is linked to the ubiquitin C (UbC) promoter, driving expression of luciferase in all cells. Systemic injection of the substrate luciferin evokes a detectable and quantifiable light signal. We then transplanted transgenic endometrial tissue from

these mice into nonluminescent recipients. By attaching luciferase-expressing tissue to the peritoneal wall of wild-type mice and injecting these animals with luciferin, we can serially monitor the initial implantation, the onset of angiogenesis, and tissue growth by noninvasive bioluminescence imaging. Finally, using this model, we can successfully monitor antiangiogenic therapy with caplostatin, a nontoxic form of TNP-470 bound to a *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer.

## Materials and Methods

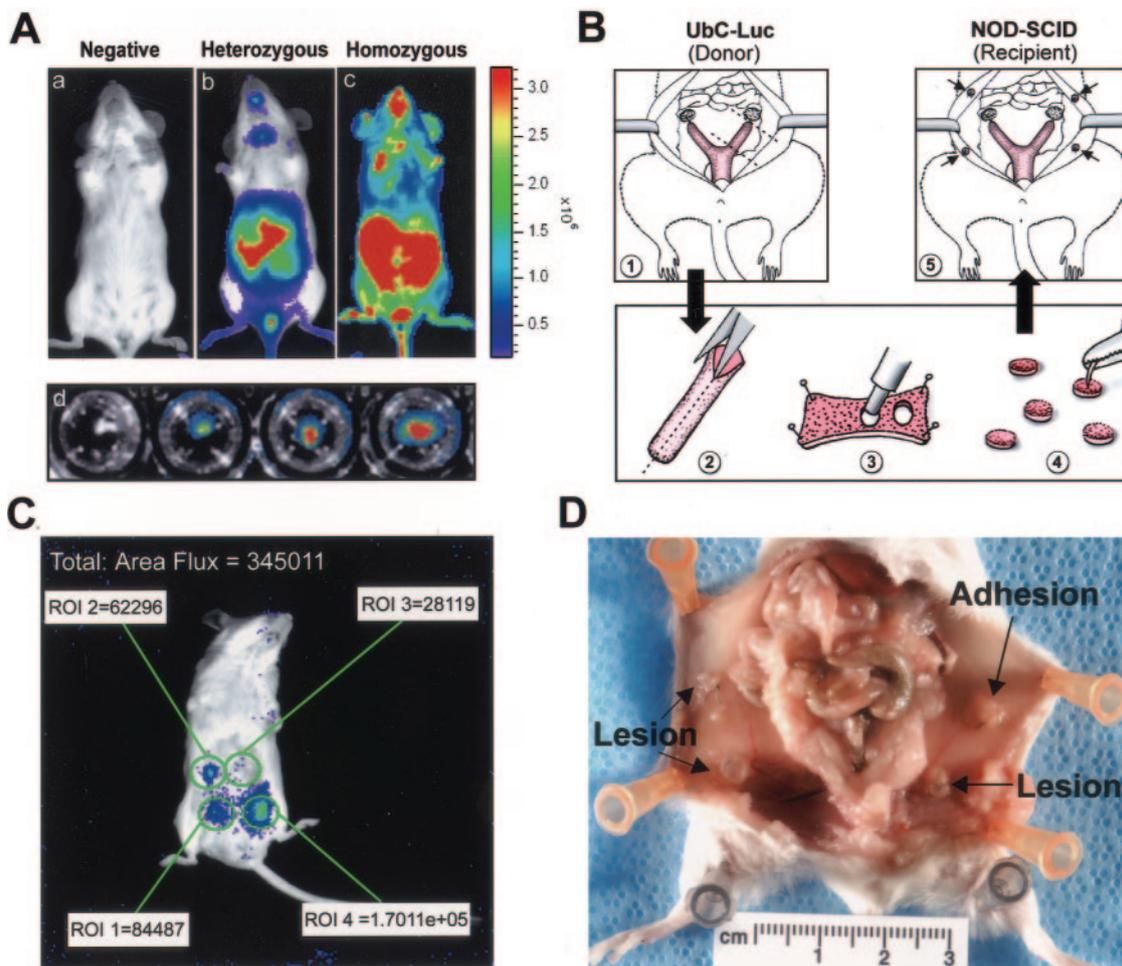
### Animal Handling and Generation of Transgenic Mice

All animal work was performed in the animal facility at Dana Farber Cancer Center (Boston, MA) in accordance with federal, local, and institutional guidelines. Eight-week-old female mice (NOD-SCID and C57BL/6-Tyr<sup>C</sup>; Jackson Laboratories, Bar Harbor, ME) and transgenic, luciferase-expressing mice were caged in groups of five with free access to chow and water and acclimated for 1 week. All surgical procedures and imaging were performed under inhalation anesthesia with isoflurane (Baxter, Deerfield, IL), and mice were observed until fully recovered.

The transgenic mice were generated as follows. The coding region for firefly luciferase from pGL3-Basic (Promega, Madison, WI) was cloned downstream of the human UbC promoter in pUB6/V5-HisA (Invitrogen). After restriction enzyme digestion, a fragment containing the UbC promoter, luciferase, and polyadenylation signal was gel purified. Transgenic mice were generated by standard pronuclear injection into C57BL/6 embryos. Transgenic founders were identified by polymerase chain reaction (PCR) of tail biopsy DNA using primers specific for the firefly luciferase gene. Offspring of positive founders were imaged using the In Vivo Imaging System (IVIS; Xenogen, Alameda, CA), and the most highly luminescent transgenic line was backcrossed to C57BL/6-Tyr<sup>C</sup> mice to achieve an albino line. Mice were subsequently propagated by intercrossing to achieve homozygous transgenic mating pairs.

### Surgical Procedure

Endometriotic lesions were surgically induced as previously described using a modified form of an established model<sup>39,40,46–48</sup> (Figure 1B). In brief, we removed both uterine horns of transgenic UbC-Luc<sup>+/+</sup> mice under anesthesia. In a Petri dish containing warmed Dulbecco's modified Eagle medium F-12 (Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco), we opened the uterine horns longitudinally with a pair of scissors. Then, using a 2-mm dermal biopsy punch (Miltex, Bethpage, NY), we removed tissue samples and subsequently sutured four of them to the peritoneal wall of anesthetized NOD-SCID or C57BL/6-Tyr<sup>C</sup> mice through a midline incision (7-0 braided silk suture; Ethicon, Somerville, NJ). To avoid



**Figure 1.** Transgenic pUb-Luc<sup>+/+</sup> mice. **A:** Imaging of a wild-type mouse (a) and mice heterozygous (b) and homozygous (c) for luciferase expression. Imaging of uterine tissue in a 96-well plate with luciferin (d) taken from a wild-type mouse (left well) and from a pUb-Luc<sup>+/+</sup> mouse (three wells to the right). Lesions were 2 mm (wild-type) and 1, 2, and 4 mm (luciferase positive) in diameter. **B:** Schematic illustration of procedure for the surgically induced endometriosis model (counterclockwise, beginning at top left). A uterine horn from a transgenic mouse expressing luciferase is removed; four 2-mm punches are taken *in vitro* and loosely attached to the peritoneal wall (one in each quadrant) of an anesthetized NOD-SCID mouse, and the abdominal of the recipient is closed. **C:** After luciferin is administered via intraperitoneal injection, luminescence can be detected using the IVIS imaging system. **D:** Same mouse as in C at necropsy. The location of the lesions corresponds with the detected luminescent signal.

overlap of luminescence, one lesion per quadrant was attached using this technique. The abdominal incision was then closed with a 5-0 braided silk suture (Ethicon) in a continuous fashion.

### Treatment with the Angiogenesis Inhibitors

TNP-470 was a generous gift from Takeda Chemical Industries Ltd. (Osaka, Japan). HPMA copolymer precursor was purchased from Polymer Laboratories (Church Stretton, UK). Caplostatin, a nontoxic form of TNP-470 conjugated to a HPMA copolymer-Gly-Phe-Leu-Gly-ethylenediamine, was synthesized as previously described.<sup>49,50</sup> Murine endostatin peptide-1 (mP-1; SynPep Corporation, Dublin, CA) is an N-terminal, 27-amino acid synthetic peptide of endostatin, a proteolytic fragment of collagen XVIII.<sup>51,52</sup> We have previously shown that the endostatin peptide mP-1 inhibits growth of endometriotic lesions.<sup>40</sup>

Starting on the day after surgery, mice received subcutaneous injections of caplostatin (30 mg/kg TNP-470-equivalent concentration) into the flank every other day or 2.8 mg/kg mP-1 via subcutaneous injection twice daily. Both compounds were dissolved in 0.9% saline. Control animals received subcutaneous saline injections twice daily. Body weight was monitored at each imaging time point.

### In Vivo Imaging

Anesthetized mice were injected with 50 mg/kg D-luciferin (Promega) intravenously via the tail vein or intraperitoneally. Mice were kept warm on a warming blanket. After 2 (intravenous) or 5 (intraperitoneal) minutes, mice were imaged using the IVIS (Xenogen) for 120 seconds at bin size 4. To quantify bioluminescence, identical circular regions of interest were positioned to encircle each luminescent lesion, and the integrated flux of photons (pho-

tons per second) within each region of interest was determined by using the Living Images software package (Xenogen).<sup>53</sup> Data were normalized to bioluminescence at the initiation of treatment for each animal.

To compare the intensity of the luminescent signal with the actual size of the lesions, a set of experiments was performed during which mice were imaged weekly and a group of mice was euthanized ( $n = 5/\text{week}$ ). At necropsy, endometriotic lesions were measured in two perpendicular diameters ( $D_1$  and  $D_2$ ) with a caliper, and cross-sectional lesion area (CSA) was calculated using the formula for an ellipse ( $D_1 \times D_2 \times \pi/4$ ) as previously described.<sup>39,40,47</sup>

### Histology

In a separate set of experiments, mice were treated with caplostatin or vehicle for 5 weeks. Mice were imaged at various time points. At necropsy, lesions were measured as described above. Subsequently, the lesions were excised including a peritoneal rim, fixed in zinc fixative (BD Biosciences Pharmingen, San Diego, CA) for 24 hours at room temperature, and then embedded in paraffin. Sections ( $5 \mu\text{m}$ ) were first stained with hematoxylin and eosin to evaluate tissue viability and quality. Microvessel density was determined by immunocytochemical staining using a horseradish peroxidase detection system (DAKO, Carpinteria, CA) with rat anti-mouse CD31 antibody (monoclonal, dilution, 1:250; BD Biosciences Pharmingen) according to the manufacturer's instructions. The slides were counterstained with hematoxylin. At low magnification ( $\times 40$ ), regions with the highest vessel density ("hot spots") were scanned as described by Weidner et al.<sup>54</sup> These regions were then counted at a  $\times 400$  magnification in a blinded fashion. Three different fields were randomly chosen and each counted twice. The average score was determined and used as the final value.<sup>55</sup>

### Estrous Cycling

Two weeks after the initiation of treatment with caplostatin ( $n = 4$ ) or vehicle ( $n = 5$ ), we began daily vaginal smears of mice to determine whether the drug had any obvious adverse effects on the hormonal cycle. The same technique was used as described earlier.<sup>39,40</sup> In brief, autoclaved blunt wooden toothpicks were carefully inserted through the vaginal orifice after being wetted in 0.9% saline. The superficial cell layers of the vaginal wall were carefully scraped, and the cells were immediately transferred into a drop of Evans Blue (Sigma, St. Louis, MO) in 0.9% saline on a histology slide. A glass coverslip was laid on top of the drop, and the cells were examined under a microscope (Carl Zeiss, Thornwood, NY) at  $\times 100$  magnification. Each slide was analyzed by two independent investigators who were blinded to the form of treatment and the previous data for each mouse. The procedure was performed on 11 consecutive days.

### Statistical Analysis

For statistical analysis, GraphPad InStat and Sigma Stat statistics software packages were used. The CSA average of all seven lesions was taken as the animal mean, and the SD and SEM for all mice per group were calculated. Repeated-measures analysis did not show significance between within-animal lesions.

The least square means of the CSA for established lesions (lesions  $>0 \text{ mm}^2$ ) were compared among groups using one-way analysis of variance. When the overall analysis of variance indicated a significant  $F$ -test, pair wise comparisons among treatments with control mice were conducted using post hoc Dunnett test. Two-tailed values of  $P < 0.05$  were considered statistically significant. Student's  $t$ -test and Wilcoxon rank sum test were used to compare CD31 counts.

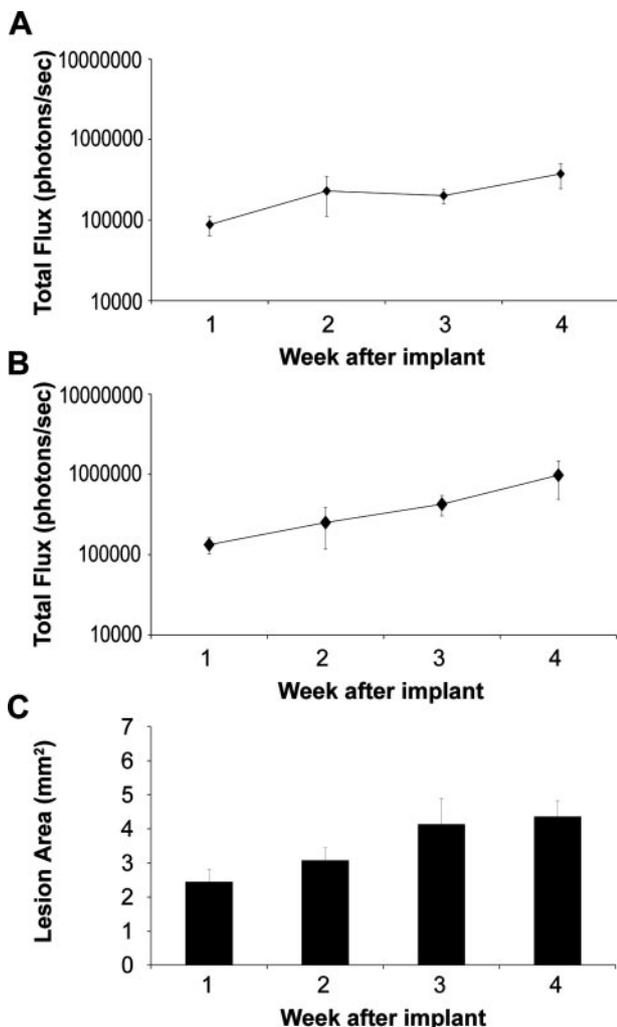
### Results

#### *Transgenic UbC-Luc<sup>+/+</sup> Mice Ubiquitously Express Luciferase*

Transgenic mice were generated as described. Because black fur attenuates light transmission, albino mice were generated by crossing transgenic founders to C57BL/6-Tyr<sup>C</sup> albino mice. As expected, luminescence was ubiquitous, and the degree of luminescence correlated with the zygosity of the animal for the transgenic cassette (Figure 1A). The transgenic line was maintained with homozygosity at the Tyr<sup>C</sup> and UbC-Luc transgenic loci. To show that uterine tissue of homozygous mice expressed the enzyme, we incubated endometrial fragments from a transgenic mouse with D-luciferin in a 96-well dish (Figure 1A). Luminescence was clearly detectable from these lesions.

#### *Transgenic Endometriotic Lesions Show Transabdominal Luminescence*

Because black- or agouti-colored hair results in  $\sim 10$ -fold reduction in transmitted luminescence (data not shown), we used albino mice as transplant recipients. C57BL/6-Tyr<sup>C</sup> and NOD-SCID albino mice were implanted with endometriotic lesions from C57BL/6-UbC-Luc<sup>+/+</sup> transgenic mice (Figure 1B). Transplanted recipients were imaged 2 weeks after surgery and before euthanasia at 4 weeks. After intraperitoneal injection of luciferin, luminescent spots could clearly be seen through the abdominal wall using the IVIS imaging system. These areas corresponded well with the location of the implanted lesions (Figure 1, C and D). Interestingly, if a lesion was enclosed in an adhesion (for example by peritoneal fat) and thereby was not in contact with luciferin, no signal was seen. Therefore, mice were thoroughly scanned postmortem for the presence of lesions in the peritoneal cavity.



**Figure 2.** Intensity of luminescence corresponds with the size of the lesions. Luminescence was measured by the IVIS imaging systems as photons per second for all four lesions ( $n = 5$  mice/time point). Luminescence was measured in 5 mice/time point (1 to 4 weeks after implantation of transgenic luciferase lesions). **A:** Mean signal intensity after intraperitoneal injection of luciferin. **B:** Mean signal intensity after intravenous injection of luciferin. The intravenous injection was performed after no signal could be detected from the previous intraperitoneal injection. **C:** At necropsy, endometriotic lesions were measured in two perpendicular diameters ( $D_1$  and  $D_2$ ) with a caliper, and CSA was calculated using the formula for an ellipse ( $D_1 \times D_2 \times \pi/4$ ) as previously described.<sup>39,40,47</sup> Bars indicate the SEM.

### Luminescence Corresponds with Lesion Size

We were then interested to see whether luminescence increases over time. Mice were imaged once weekly after intraperitoneal injection of luciferin. Mean luminescence of luciferase-expressing lesions increased steadily over time (Figure 2A). A similar incline in luminescence intensity was seen when mice were injected intravenously (Figure 2B). Groups of mice ( $n = 5$ ) were euthanized after each imaging time point. Mean lesion area as measured with a caliper also increased over time, correlating well with the intensity of the luminescence (Figure 2C).

Interestingly, we found that no signal was seen when luciferin was immediately injected intravenously on the day after surgery before angiogenesis could occur (Figure 3A). However, luminescence emerged when mice

were injected intravenously 3 days after surgery and increased thereafter. On the other hand, luminescence was clearly detected immediately after surgery when the same mice were injected with the substrate intraperitoneally, providing high local concentrations of luciferin to the lesions (Figure 3B).

### Monitoring of Antiangiogenic Treatment

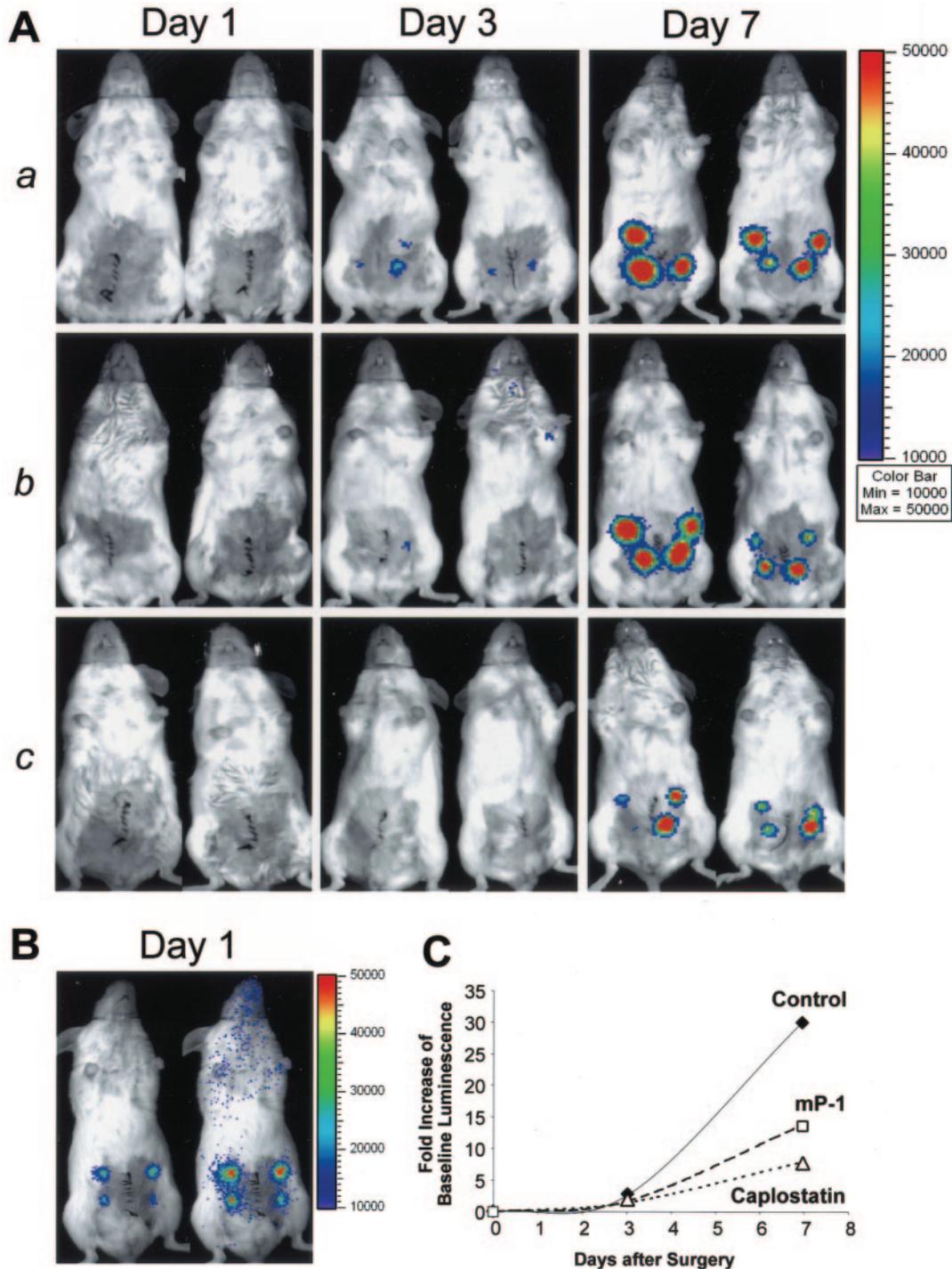
This noninvasive model of endometriosis may be used to monitor lesion growth during treatment with angiogenesis inhibitors. Therefore, in a first series of experiments, we treated mice with the angiogenesis inhibitors caplostatin and the endostatin peptide mP-1. In all groups, no signal was detected after intravenous injection of luciferin (Figure 3A) 1 day after surgery. As previously mentioned, luminescence after intravenous injection of luciferin occurred on day 3 after surgery and increased strongly on day 7. Treatment with the angiogenesis inhibitors mP-1 and caplostatin delayed the appearance and decreased the intensity of the signal on days 3 and 7 (Figure 3, A and C). This suggests that treatment with these angiogenesis inhibitors may inhibit early events underlying blood vessel sprouting.

### Caplostatin Inhibits Growth of Endometriotic Lesions

We then investigated whether caplostatin would inhibit endometriotic growth. We have previously shown the inhibitory effect of the 27-amino acid endostatin peptide mP-1.<sup>40</sup> To noninvasively monitor lesion growth, we used the luciferase model. Intravenous injections of luciferin at various time points showed an increase in luminescence over time (Figure 4A). As predicted from our previous experiment, repeated caplostatin treatment over a period of 5 weeks significantly reduced the signal intensity of the transgenic luciferase-positive lesions (Figure 4A). After 35 days, measurement of the endometriotic lesions showed that antiangiogenic therapy with caplostatin had suppressed lesion growth by 59% (control: 4.23 mm<sup>2</sup>, SEM 0.65 versus 1.73 mm<sup>2</sup>, SEM 0.18,  $P < 0.001$ ) (Figure 4B).

To prove that the inhibitory effect of caplostatin was not due to unspecific toxicity, eg, due to potentially unconjugated TNP-470 molecules, we measured the body weight of all mice at different time points. After an initial loss of body weight due to the surgery, all mice kept their weight throughout the treatment (Figure 4C). Caplostatin was previously shown to have no effect on body weight when injected to normal mice (ie, no surgery).<sup>50</sup>

Free TNP-470 has been shown to suppress estrogen levels and cause detrimental effects in reproduction. To exclude that the inhibitory effect on the endometriotic lesions was due to suppression of the hormonal cycle, we performed daily vaginal smears in the middle of the study for 11 consecutive days. There was no difference in the number of estrus cycles of mice treated with caplostatin compared with control animals (Figure 4D).

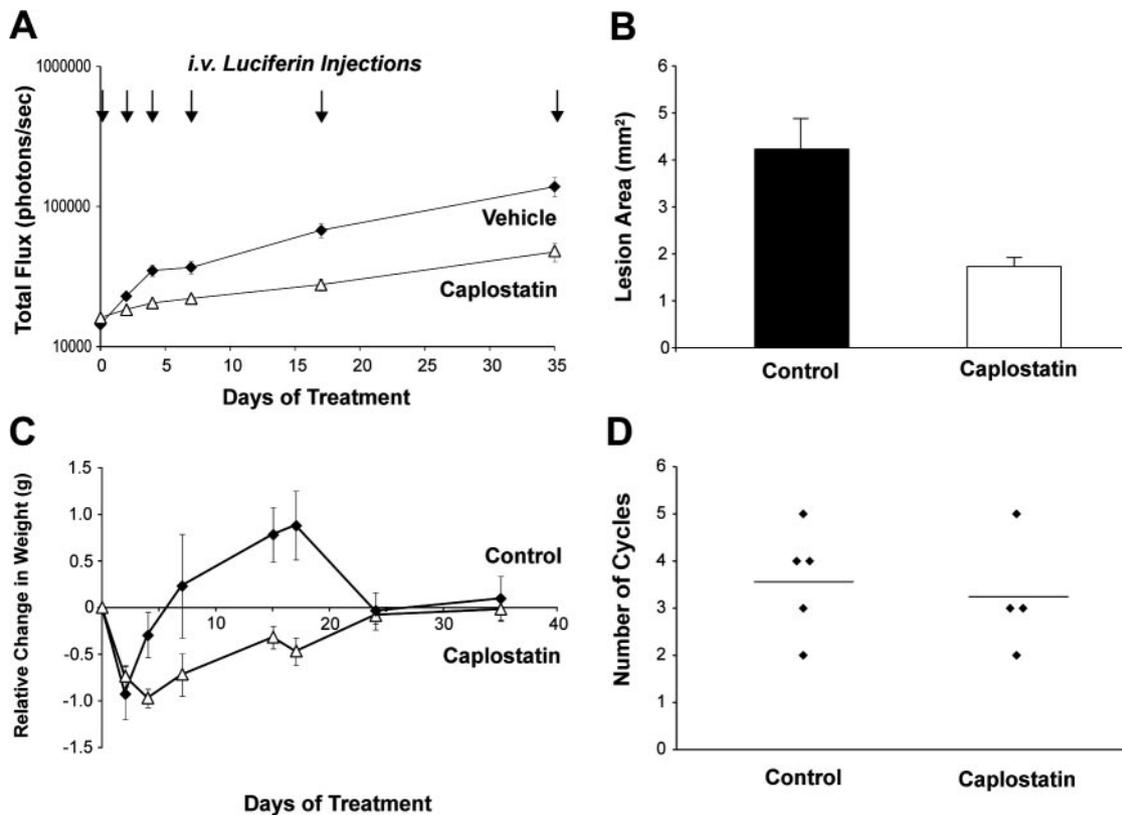


**Figure 3.** Angiogenesis inhibitors delay the onset and suppress the intensity of the luminescent signal. **A:** IVIS imaging after intravenous injection of luciferin on days 1, 3, and 7 after the surgical procedure. Mice treated with the angiogenesis inhibitors endostatin peptide mP-1 (2.8 mg/kg twice daily) (**b**) or caplostatin (30 mg/kg every other day) (**c**) had a delay in the onset of detectable luminescent signal compared with saline-treated mice (**a**). No signal was seen in all groups on day 1 after surgery if luciferin was injected intravenously. All pictures were taken with the same threshold of sensitivity. **B:** Luminescence was detected in mice already on day 1 after surgery if luciferin was injected intraperitoneally. **C:** Line graph showing the fold increase in baseline luminescence in the three groups (control, ◆; mP-1, △; and caplostatin, □).

*Caplostatin Decreases Microvessel Density in Endometriotic Lesions*

After 5 weeks of caplostatin treatment, morphology of normal endometrium was preserved in the trans-

planted lesions (Figure 5, A and B). To determine whether the detected inhibitory effect of caplostatin treatment on endometriotic growth was at least in part due to inhibition of angiogenesis, we counted microvessel density in endometriotic lesions after 5



**Figure 4.** Treatment with caplostatin inhibits the growth of endometriotic lesions. **A:** Mean luminescence of transplanted transgenic luciferase-positive endometriotic lesions after intravenous injection of luciferin (arrows indicate the day of each injection). Systemic caplostatin treatment decreased the intensity of the luminescent signal over a period of 35 days ( $n = 6/\text{group}$ ). **B:** At necropsy, lesions were measured with a caliper. Caplostatin significantly reduced lesion area after 5 weeks of treatment, reflecting the luminescence results. **C:** Relative change in body weight in both groups (error bars indicate the SEM). **D:** The stage of the estrous cycle was determined each day by vaginal smear as previously described.<sup>39,40</sup> Evan's Blue staining revealed under the microscope a stage-specific morphology (magnification,  $\times 200$ ). Number of full cycles individual mice went through over a period of 11 days started 2 weeks after the initiation of therapy (horizontal bar indicates the mean).

weeks of treatment. Microvessel densities in representative endometriotic lesions are shown in Figure 5, C and D. Lesions treated with caplostatin had mean vessel counts of 36.6 (SEM 2.4) per high-power field versus 52.4 (SEM 4.3) in lesions of vehicle-treated mice (Figure 5E). The data indicate that caplostatin inhibits growth of endometriotic lesions by significantly reducing microvessel density ( $P < 0.003$ ).

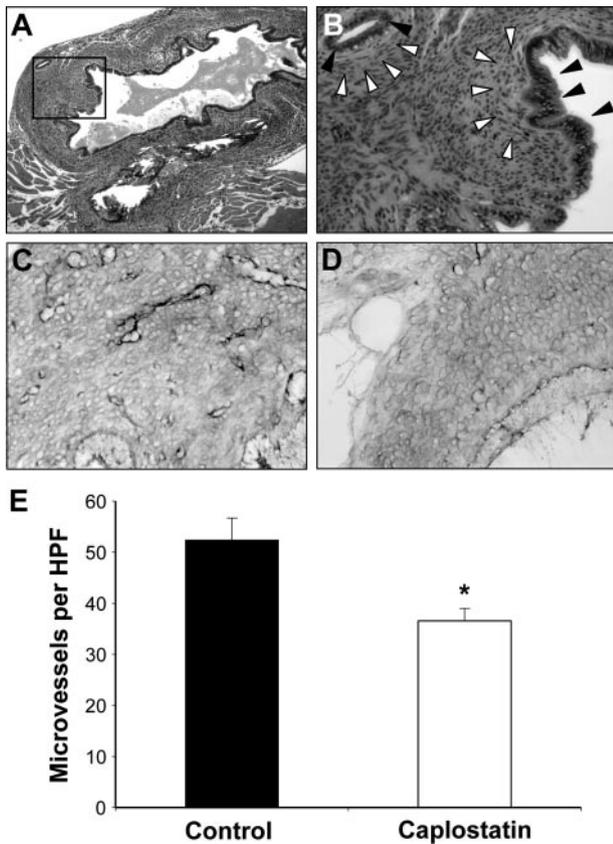
### Discussion

Endometriosis is a highly prevalent disease associated with substantial clinical symptoms and socioeconomic effects. However, the pathogenesis of endometriosis still remains unclear. As a consequence, current medical treatment designed to suppress local and systemic estradiol levels is often associated with significant side effects and high recurrence rates. In addition, infertility, another clinical characteristic of endometriosis, is not decreased with medical therapy, and therapeutic suppression of estrogen production further prolongs the time during which fertilization cannot occur.

Therefore, it is of paramount importance to study new mechanisms involved in this disease in the expectation that this may eventually lead to new therapeutic approaches. Animal models are necessary to achieve this

goal. In this study, we describe the generation of transgenic mice ubiquitously expressing luciferase. These mice were used to develop a new mouse model of endometriosis that can be used to noninvasively monitor lesion growth, the onset of angiogenesis and the response to antiangiogenic therapy.

Transgenic mice ubiquitously expressing the gene for luciferase were created by linking the gene to the ubiquitin C promoter. Mice containing both alleles of the luciferase gene produced a stronger luminescent signal after injection with luciferase than heterozygous animals. Using these homozygous animals, our goal was then to create a novel noninvasive model of endometriosis. Therefore, we transplanted transgenic luciferase<sup>+/+</sup> endometrial tissue into wild-type mice. Using an *in vivo* imaging system, we demonstrated that transplantation of transgenic, luciferase-expressing endometrial tissue could noninvasively be detected through the abdominal wall. In addition, we showed that the intensity of the luminescent signal correlates well with the lesion size. Hence, lesions can be monitored noninvasively over a period of at least 4 weeks. This could considerably reduce the number of animals necessary for a study, because it will become obsolete to sacrifice cohorts of animals for measurements of lesions at different time points.



**Figure 5.** Caplostatin treatment reduces microvessel density in endometriotic lesions. **A:** Formalin-fixed, paraffin-embedded lesions retained morphology of normal endometrium (magnification,  $\times 100$ ; hematoxylin and eosin staining). **B:** Both epithelial glands (**black arrowheads**) and stromal cells (**white arrowheads**) are clearly visible in a representative endometriotic lesion after 5 weeks of systemic caplostatin treatment (magnification,  $\times 400$ ; hematoxylin and eosin staining). **C** and **D:** Immunohistochemical staining of zinc-fixed, paraffin-embedded sections of endometriotic lesions ( $n = 5$  mice). Vessels were counted twice in three areas in every slide stained with CD31 and averaged. Detection with a biotinylated rabbit anti-rat antibody, horseradish peroxidase, and a 3,3'-diaminobenzidine chromogen (magnification,  $\times 400$ ); counterstaining with hematoxylin. Endometriotic lesion from a mouse treated for 5 weeks with saline (**C**) or caplostatin (**D**). **E:** Using the method of Weidner et al,<sup>54</sup> microvessel density was determined in endometriotic lesions after 5 weeks of systemic saline (control) or caplostatin treatment. Caplostatin treatment significantly ( $P < 0.003$ ) decreased microvessel density in endometriotic lesions compared with lesions from control mice.

Recently, two models of endometriosis have been introduced where endometrial tissue was infected with GFP-expressing adenovirus.<sup>44,45</sup> Fortin et al<sup>44</sup> monitored subcutaneous GFP fluorescent lesions, whereas Hirata et al<sup>45</sup> used a GFP lighting system at necropsy to identify lesions. However, because of the emission spectrum of GFP, it is currently not possible to reliably detect intra-peritoneal endometriotic lesions. In addition, due to its transient nature, adenoviral infection of cells may cause fading fluorescence over time. One way to circumvent such obstacles could be the use of GFP-expressing lentivirus<sup>56</sup> or the transplantation of GFP-positive endometrial tissue from transgenic animals into wild-type mice. However, in our own experience we found that this approach does not provide satisfactory results to reliably monitor lesion growth in a noninvasive fashion (data not shown). Therefore, we believe that the luciferase model

may be better suited for testing the efficacy of new drugs for the treatment of endometriosis.

For any animal model, it is desirable to resemble the human condition as closely as possible. Unfortunately, neither mice nor rats develop spontaneous disease. Very recently, however, a mouse model has been described where local overexpression of the oncogene K-ras leads to extensive endometriotic lesions.<sup>57</sup> This elegant genetic model of endometriosis is of great interest because it is the first "spontaneous" mouse model of endometriosis. Yet, it has some limitations such as a long latency until the development of lesions (8 months) and only a 50% penetrance.<sup>58</sup> Unless these obstacles can be overcome, despite the advantages of being a spontaneous model, its widespread use will be difficult because of high costs for animals and drugs. However, based on the results with our luciferase model presented here, it may be interesting to incorporate *in vivo* imaging into the K-ras model. By breeding the genetically engineered K-ras mice to existing reporter mice in which luciferase is turned on by cre-mediated recombination, ie, lox-STOP-lox-luciferase, location and bulk of endometriosis lesions could be followed noninvasively.<sup>59,60</sup> This would make such a resulting model attractive for drug testing because animals with established disease could be identified early and divided into treatment groups.

Underlying the establishment of the luciferase model described in this study is a modification of a rat and a mouse model.<sup>46,48</sup> The advantage of the transplantation model is its high reproducibility, the well-defined and rather short time frame and the possibility to measure lesion growth.<sup>39,40,47</sup> In addition, bioimaging is now widely used for many applications such as oncology, gene expression, and immunology.<sup>61-63</sup> Implementation of this system should therefore be facilitated by the technical ease of the described procedures. We are aware of a possible downside of the transplantation model, which is that the suture disrupts the mesentherial layer, thereby exposing the basement membrane of the peritoneum. Assuming that Sampson's<sup>3</sup> theory of retrograde menstruation and the attachment, invasion, and growth of endometrium is correct, it is conceivable that we are altering the "natural process" of endometriosis. However, our results, which demonstrated the first signs of angiogenesis about 3 days after transplantation correlate well with immunohistological studies from endometriotic lesions that were not attached surgically to the peritoneal wall.<sup>35,41,42,64</sup> Others have transplanted human endometrial xenografts in mice without attaching it to the abdominal wall.<sup>36,65</sup> Although this model mimics the adhesion of the lesions, we believe it is associated with other problems. For example, lesions may be missed because they often get stuck in the postsurgical adhesions that form. In addition, this model does not allow for exact measurement of lesion growth.

Our model described herein also allows the establishment of syngeneic lesions.<sup>3</sup> Specifically, endometrial tissue harvested from C57BL/6-UbC-Luc<sup>+/+</sup> transgenic mice can be transplanted into co-isogenic C57BL/6-Tyr<sup>C</sup> recipient mice. However, we found that about 2 weeks after surgery, the intensity of the luminescence signal

reached a plateau phase and decreased thereafter (data not shown). We believe that this phenomenon may be due to an immune reaction to luciferase in the transplanted tissue. When we repeated the experiments with NOD-SCID mice, the signal increased steadily over the time of surveillance (up to 5 weeks). Thus, for long-term experiments, SCID mice would be preferred.

Angiogenesis is now widely accepted to play a pivotal role in endometriosis.<sup>23,66,67</sup> Antiangiogenic therapy has therefore been suggested as a novel therapeutic approach.<sup>18,34–36,68,69</sup> We have recently shown that both the endogenous angiogenesis inhibitor endostatin, a proteolytic fragment of collagen XVIII, and two synthetic peptides of endostatin inhibited growth of endometriotic lesions in a mouse model.<sup>39,40</sup> In our current work, we generated a novel, noninvasive model to study angiogenesis *in vivo*. We were able to monitor angiogenesis in the endometriotic, luciferase-expressing lesions by injecting intravenous luciferin, the substrate for the enzymatic reaction. No luminescence was seen immediately after the surgery with intravenous luciferin, as expected, because no connection between the host's blood stream and the transgenic lesions existed. The first luminescence was noted after 3 to 4 days, at which time point first blood vessels may have sprouted from the peritoneal vessels into the transplanted lesions, supplying the luciferase-containing cells with luciferin. These findings correlate closely with the immunohistochemical findings of Eggermont et al.<sup>41</sup> They and others describe the occurrence of earliest mouse blood vessels in human endometrium that had been inserted into the murine abdominal cavity 3 days after surgery. In our study, we systemically treated mice with the angiogenesis inhibitors mP-1 and caplostatin. MP-1 is a 27-amino acid fragment of the endogenous angiogenesis inhibitor endostatin.<sup>52</sup> In a recent study, we were able to significantly inhibit growth of endometriotic lesions in a similar, nonlabeled mouse model with mP-1.<sup>40</sup> Caplostatin is a nontoxic form of the highly potent angiogenesis inhibitor TNP-470.<sup>70</sup> It has been shown to inhibit angiogenesis in various tumor models and during liver regeneration.<sup>50</sup> In the current study, we show that treatment with either of these angiogenesis inhibitors delays the appearance of luminescence of the transgenic lesions, indicating that the model can be used to noninvasively monitor blood vessel sprouting.

An early event in angiogenesis consists of the extravasation of plasma and proteins through leaky blood vessels leading to the deposition of a fibrin-rich gel, which then promotes angiogenesis.<sup>71</sup> This process is probably induced by hypoxia in the transplanted lesions, which leads to the expression of vascular endothelial growth factor (VEGF), one of the most potent and well-studied pro-angiogenic cytokines. VEGF was originally described as vascular permeability factor (VPF) because of its strong permeabilizing effect on vessels.<sup>72–74</sup> VEGF/VPF plays an important role in endometriosis in human patients, and anti-VEGF therapy was successful in inhibiting establishment of endometriotic lesions in a mouse model.<sup>26,35</sup> It is also possible that the inhibitory effect of caplostatin and the endostatin peptide mP-1 can in part be explained by an inhibitory effect on permeability. In

fact, both caplostatin and mP-1 have recently been shown to suppress VEGF-mediated hyperpermeability in the Miles assay.<sup>49,52</sup>

We have previously shown that antiangiogenic therapy with endostatin inhibited the growth of fresh but not established endometriotic lesions.<sup>39</sup> Therefore, we began treatment in this study immediately after surgery. It is likely that there are two waves of angiogenesis occurring in this model. Initially, VEGF is likely to be up-regulated due to local hypoxia in the transplanted lesions that may prevail until a blood supply from the host is established. We clearly demonstrate that treatment with caplostatin or the endostatin peptide mP-1 delays the first wave of angiogenesis as shown by reduced luminescence. The inhibition of luminescence was detectable throughout the experiment (5 weeks). Microvessel density was significantly reduced by 30% at that time. Despite the fact that this may be the result of the initial delay in vessel growth during the 1st week, we believe that caplostatin inhibits angiogenesis even in established lesions. It should be noted that caplostatin does have an inhibitory effect in established mouse and human tumors.<sup>50</sup>

Four weeks of treatment with caplostatin inhibited the growth of endometriotic lesions by 59%. The difference in luminescence intensity between vehicle and caplostatin-treated mice continuously differed over time and was decreased by 66% at the end of the experiment. We were able to show that caplostatin treatment also decreased microvessel density in endometriotic lesions, also suggesting an antiangiogenic mechanism. In tumors, antiangiogenic drugs work through increasing apoptosis in tumor tissue indirectly via their inhibitory effect on the proliferating, oxygen- and nutrient-supplying endothelial cells.<sup>75</sup> It is possible that the demonstrated suppressive effect of caplostatin is due to a combination of antiangiogenic and permeability-inhibiting mechanism.<sup>49</sup> All mice initially lost weight after the surgical procedure. Caplostatin-treated mice weighed approximately 1 g less than control mice (data not shown) at the time of the procedure as a matter of chance. It is possible that these smaller mice were more vulnerable to the surgical procedure and therefore needed more time to recuperate. After 2 to 3 weeks, the weight differences were equalized. We have previously shown that higher doses of caplostatin did not have a negative effect on weight in mice.<sup>50</sup> We do not believe that the small difference in initial weight had any influence on the delayed angiogenic response in the caplostatin group because luminescence remained lower in this group throughout the study and immunohistochemical staining of the lesions showed a reduced microvessel count.

In summary, despite recent advances in the field, there still exists only a limited amount of knowledge about the underlying cause and pathophysiology of endometriosis. Part of this may be due to a lack of sufficient and adequate animal models.<sup>76</sup> Angiogenesis has been shown to play a role in endometriosis both in human disease and in animal models. We show that growth of endometriotic lesions and early angiogenesis can be monitored noninvasively using a model of luciferase transgenic mice. We believe that this model will facilitate testing the efficacy of

drugs, which may eventually lead to the development of urgently needed new therapeutic approaches for women suffering from this disease. Caplostatin shows promise in this model, supporting a basis for clinical trials of endometriosis in humans.

### Acknowledgments

We thank David A. Sampson for technical assistance and discussions and Kashi Javaherian for his assistance in planning experiments. We also thank Kristin Gullage for help with the figures.

### References

- Cramer DW, Missmer SA: The epidemiology of endometriosis. *Ann NY Acad Sci* 2002, 955:11–22; discussion 34–16, 396–406
- The Practice Committee of the American Society for Reproductive Medicine: Endometriosis and infertility. *Fertil Steril* 2004, 81:1441–1446
- Sampson J: Peritoneal endometriosis due to the menstrual dissemination of endothelial tissue into the peritoneal cavity. *Am J Obstet Gynecol* 1927, 14:422–469
- Ridley JH, Edwards IK: Experimental endometriosis in the human. *Am J Obstet Gynecol* 1958, 76:783–789; discussion 789–790
- Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM: Retrograde menstruation in healthy women and in patients with endometriosis. *Obstet Gynecol* 1984, 64:151–154
- Marsh EE, Laufer MR: Endometriosis in premenarcheal girls who do not have an associated obstructive anomaly. *Fertil Steril* 2005, 83:758–760
- Oliker AJ, Harris AE: Endometriosis of the bladder in a male patient. *J Urol* 1971, 106:858–859
- Pinkert TC, Catlow CE, Straus R: Endometriosis of the urinary bladder in a man with prostatic carcinoma. *Cancer* 1979, 43:1562–1567
- Meyer R: Über eine adenomatöse Wucherung der Serosa in einer Bauchnarbe. *Zeitschr Geburtsh Gynakol* 1903, 49:32–41
- Halban J: Hysteroadenosis mestatica. *Wien Klin Wochensh* 1924, 37:1205–1228
- Russell WW: Aberrant portions of the Müllerian duct found in an ovary: ovarian cysts of Müllerian origin. *Bull Johns Hopkins Hospital* 1899, 10:8–10
- Dmowski WP, Steele RW, Baker GF: Deficient cellular immunity in endometriosis. *Am J Obstet Gynecol* 1981, 141:377–383
- Oosterlynck DJ, Cornillie FJ, Waer M, Vandeputte M, Koninckx PR: Women with endometriosis show a defect in natural killer activity resulting in a decreased cytotoxicity to autologous endometrium. *Fertil Steril* 1991, 56:45–51
- Noble LS, Simpson ER, Johns A, Bulun SE: Aromatase expression in endometriosis. *J Clin Endocrinol Metab* 1996, 81:174–179
- Zeitoun K, Takayama K, Sasano H, Suzuki T, Moghrabi N, Andersson S, Johns A, Meng L, Putman M, Carr B, Bulun SE: Deficient 17beta-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize 17beta-estradiol. *J Clin Endocrinol Metab* 1998, 83:4474–4480
- Bulun SE, Zeitoun KM, Takayama K, Sasano H: Estrogen biosynthesis in endometriosis: molecular basis and clinical relevance. *J Mol Endocrinol* 2000, 25:35–42
- Noble LS, Takayama K, Zeitoun KM, Putman JM, Johns DA, Hinshelwood MM, Agarwal VR, Zhao Y, Carr BR, Bulun SE: Prostaglandin E2 stimulates aromatase expression in endometriosis-derived stromal cells. *J Clin Endocrinol Metab* 1997, 82:600–606
- Becker CM, Bartley J, Mechsner S, Ebert AD: Angiogenesis and endometriosis. *Zentralbl Gynakol* 2004, 126:252–258
- Donnez J, Smoes P, Gillerot S, Casanas-Roux F, Nisolle M: Vascular endothelial growth factor (VEGF) in endometriosis. *Hum Reprod* 1998, 13:1686–1690
- Ferriani RA, Charnock-Jones DS, Prentice A, Thomas EJ, Smith SK: Immunohistochemical localization of acidic and basic fibroblast growth factors in normal human endometrium and endometriosis and the detection of their mRNA by polymerase chain reaction. *Hum Reprod* 1993, 8:11–16
- Oosterlynck DJ, Meuleman C, Sobis H, Vandeputte M, Koninckx PR: Angiogenic activity of peritoneal fluid from women with endometriosis. *Fertil Steril* 1993, 59:778–782
- Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB, Taylor RN: Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J Clin Endocrinol Metab* 1996, 81:3112–3118
- Taylor RN, Lebovic DI, Mueller MD: Angiogenic factors in endometriosis. *Ann NY Acad Sci* 2002, 955:89–100; discussion 118, 396–406
- Arici A, Tazuke SI, Attar E, Kliman HJ, Olive DL: Interleukin-8 concentration in peritoneal fluid of patients with endometriosis and modulation of interleukin-8 expression in human mesothelial cells. *Mol Hum Reprod* 1996, 2:40–45
- Fujimoto J, Sakaguchi H, Hirose R, Tamaya T: Expression of platelet-derived endothelial cell growth factor (PD-ECGF) related to angiogenesis in ovarian endometriosis. *J Clin Endocrinol Metab* 1999, 84:359–362
- McLaren J, Prentice A, Charnock-Jones DS, Smith SK: Vascular endothelial growth factor (VEGF) concentrations are elevated in peritoneal fluid of women with endometriosis. *Hum Reprod* 1996, 11:220–223
- Osuga Y, Tsutsumi O, Okagaki R, Takai Y, Fujimoto A, Suenaga A, Maruyama M, Momoeda M, Yano T, Taketani Y: Hepatocyte growth factor concentrations are elevated in peritoneal fluid of women with endometriosis. *Hum Reprod* 1999, 14:1611–1613
- Steff AM, Gagne D, Page M, Rioux A, Hugo P, Gosselin D: Serum concentrations of insulin-like growth factor-1, soluble tumor necrosis factor receptor-1 and angiogenin in endometriosis patients. *Am J Reprod Immunol* 2004, 51:166–173
- Suzumori N, Zhao XX, Suzumori K: Elevated angiogenin levels in the peritoneal fluid of women with endometriosis correlate with the extent of the disorder. *Fertil Steril* 2004, 82:93–96
- Rogers MS, Rohan RM, Birsner AE, D'Amato RJ: Genetic loci that control vascular endothelial growth factor-induced angiogenesis. *FASEB J* 2003, 17:2112–2114
- Rogers MS, Rohan RM, Birsner AE, D'Amato RJ: Genetic loci that control the angiogenic response to basic fibroblast growth factor. *FASEB J* 2004, 18:1050–1059
- Rohan RM, Fernandez A, Udagawa T, Yuan J, D'Amato RJ: Genetic heterogeneity of angiogenesis in mice. *FASEB J* 2000, 14:871–876
- Rogers MS, D'Amato RJ: The effect of genetic diversity on angiogenesis. *Exp Cell Res* 2005, 312:561–574
- Dabrosin C, Gyorfy S, Margetts P, Ross C, Gaudie J: Therapeutic effect of angiostatin gene transfer in a murine model of endometriosis. *Am J Pathol* 2002, 161:909–918
- Hull ML, Charnock-Jones DS, Chan CL, Bruner-Tran KL, Osteen KG, Tom BD, Fan TP, Smith SK: Antiangiogenic agents are effective inhibitors of endometriosis. *J Clin Endocrinol Metab* 2003, 88:2889–2899
- Nap AW, Griffioen AW, Dunselman GA, Bouma-Ter Steege JC, Thijsen VL, Evers JL, Groothuis PG: Antiangiogenesis therapy for endometriosis. *J Clin Endocrinol Metab* 2004, 89:1089–1095
- Somigliana E, Viganò P, Rossi G, Carinelli S, Vignali M, Panina-Bordignon P: Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. *Hum Reprod* 1999, 14:2944–2950
- Yagyu T, Kobayashi H, Matsuzaki H, Wakahara K, Kondo T, Kurita N, Sekino H, Inagaki K, Suzuki M, Kanayama N, Terao T: Thalidomide inhibits tumor necrosis factor-alpha-induced interleukin-8 expression in endometriotic stromal cells, possibly through suppression of nuclear factor-kappaB activation. *J Clin Endocrinol Metab* 2005, 90:3017–3021
- Becker CM, Sampson DA, Rupnick MA, Rohan RM, Efstathiou JA, Short SM, Taylor GA, Folkman J, D'Amato RJ: Endostatin inhibits the growth of endometriotic lesions but does not affect fertility. *Fertil Steril* 2005, 84(Suppl 2):1144–1155
- Becker CM, Sampson DA, Short SM, Javaherian K, Folkman J, D'Amato RJ: Short synthetic endostatin peptides inhibit endothelial migration in vitro and endometriosis in a mouse model. *Fertil Steril* 2006, 85:71–77

41. Eggermont J, Donnez J, Casanas-Roux F, Scholtes H, Van Langendonck A: Time course of pelvic endometriotic lesion revascularization in a nude mouse model. *Fertil Steril* 2005, 84:492–499
42. Grummer R, Schwarzer F, Balczyk K, Hess-Stumpff H, Regidor PA, Schindler AE, Winterhager E: Peritoneal endometriosis: validation of an in-vivo model. *Hum Reprod* 2001, 16:1736–1743
43. Fortin M, Lepine M, Merlen Y, Thibeault I, Rancourt C, Gosselin D, Hugo P, Steff AM: Quantitative assessment of human endometriotic tissue maintenance and regression in a noninvasive mouse model of endometriosis. *Mol Ther* 2004, 9:540–547
44. Fortin M, Lepine M, Page M, Osteen K, Massie B, Hugo P, Steff AM: An improved mouse model for endometriosis allows noninvasive assessment of lesion implantation and development. *Fertil Steril* 2003, 80(Suppl 2):832–838
45. Hirata T, Osuga Y, Yoshino O, Hirota Y, Harada M, Takemura Y, Morimoto C, Koga K, Yano T, Tsutsumi O, Taketani Y: Development of an experimental model of endometriosis using mice that ubiquitously express green fluorescent protein. *Hum Reprod* 2005, 20:2092–2096
46. Cummings AM, Metcalf JL: Induction of endometriosis in mice: a new model sensitive to estrogen. *Reprod Toxicol* 1995, 9:233–238
47. Efsthathiou JA, Sampson DA, Levine Z, Rohan RM, Zurakowski D, Folkman J, D'Amato RJ, Rupnick MA: Nonsteroidal antiinflammatory drugs differentially suppress endometriosis in a murine model. *Fertil Steril* 2005, 83:171–181
48. Vernon MW, Wilson EA: Studies on the surgical induction of endometriosis in the rat. *Fertil Steril* 1985, 44:684–694
49. Satchi-Fainaro R, Mamluk R, Wang L, Short SM, Nagy JA, Feng D, Dvorak AM, Dvorak HF, Puder M, Mukhopadhyay D, Folkman J: Inhibition of vessel permeability by TNP-470 and its polymer conjugate, caplostatin. *Cancer Cell* 2005, 7:251–261
50. Satchi-Fainaro R, Puder M, Davies JW, Tran HT, Sampson DA, Greene AK, Corfas G, Folkman J: Targeting angiogenesis with a conjugate of HPMA copolymer and TNP-470. *Nat Med* 2004, 10:255–261
51. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997, 88:277–285
52. Tijn Tham Sjin RM, Satchi-Fainaro R, Birsner AE, Ramanujam VM, Folkman J, Javaherian K: A 27 amino acid synthetic peptide corresponding to the N-terminal zinc binding domain of endostatin is responsible for its anti-tumor activity. *Cancer Res* 2005, 65:3656–3663
53. Rubin JB, Kung AL, Klein RS, Chan JA, Sun Y, Schmidt K, Kieran MW, Luster AD, Segal RA: A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. *Proc Natl Acad Sci USA* 2003, 100:13513–13518
54. Weidner N, Semple JP, Welch WR, Folkman J: Tumor angiogenesis and metastasis: correlation in invasive breast carcinoma. *N Engl J Med* 1991, 324:1–8
55. Becker CM, Farnebo FA, Iordanescu I, Behonick DJ, Shih MC, Dunning P, Christofferson R, Mulligan RC, Taylor GA, Kuo CJ, Zetter BR: Gene therapy of prostate cancer with the soluble vascular endothelial growth factor receptor Flk1. *Cancer Biol Ther* 2002, 1:548–553
56. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D: Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 2002, 295:868–872
57. Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, Jacks T: Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. *Nat Med* 2005, 11:63–70
58. Boyd J: Mouse models of gynecologic pathology. *N Engl J Med* 2005, 352:2240–2242
59. Lyons SK, Meuwissen R, Krimpenfort P, Berns A: The generation of a conditional reporter that enables bioluminescence imaging of Cre/loxP-dependent tumorigenesis in mice. *Cancer Res* 2003, 63:7042–7046
60. Safran M, Kim WY, Kung AL, Horner JW, DePinho RA, Kaelin WG Jr: Mouse reporter strain for noninvasive bioluminescent imaging of cells that have undergone Cre-mediated recombination. *Mol Imaging* 2003, 2:297–302
61. Beilhack A, Schulz S, Baker J, Beilhack GF, Wieland CB, Herman EI, Baker EM, Cao YA, Contag CH, Negrin RS: In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. *Blood* 2005, 106:1113–1122
62. Kung AL, Zabudoff SD, France DS, Freedman SJ, Tanner EA, Vieira A, Cornell-Kennon S, Lee J, Wang B, Wang J, Memmert K, Naegeli HU, Petersen F, Eck MJ, Bair KW, Wood AW, Livingston DM: Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway. *Cancer Cell* 2004, 6:33–43
63. Nogawa M, Yuasa T, Kimura S, Kuroda J, Sato K, Segawa H, Yokota A, Maekawa T: Monitoring luciferase-labeled cancer cell growth and metastasis in different in vivo models. *Cancer Lett* 2005, 217:243–253
64. Bruner-Tran KL, Webster-Clair D, Osteen KG: Experimental endometriosis: the nude mouse as a xenographic host. *Ann NY Acad Sci* 2002, 955:328–339; discussion 340–322, 396–406
65. Zamah NM, Dodson MG, Stephens LC, Buttram VC Jr, Besch PK, Kaufman RH: Transplantation of normal and ectopic human endometrial tissue into athymic nude mice. *Am J Obstet Gynecol* 1984, 149:591–597
66. Healy DL, Rogers PA, Hii L, Wingfield M: Angiogenesis: a new theory for endometriosis. *Hum Reprod Update* 1998, 4:736–740
67. Smith SK: Angiogenesis. *Semin Reprod Endocrinol* 1997, 15:221–227
68. Olive DL, Lindheim SR, Pritts EA: New medical treatments for endometriosis. *Best Pract Res Clin Obstet Gynaecol* 2004, 18:319–328
69. Taylor RN, Mueller MD: Anti-angiogenic treatment of endometriosis: biochemical aspects. *Gynecol Obstet Invest* 2004, 57:54–56
70. Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J: Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature* 1990, 348:555–557
71. Feng D, Nagy JA, Dvorak AM, Dvorak HF: Different pathways of macromolecule extravasation from hyperpermeable tumor vessels. *Microvasc Res* 2000, 59:24–37
72. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT: Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 1989, 246:1309–1312
73. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989, 246:1306–1309
74. Senger DR, Perruzzi CA, Feder J, Dvorak HF: A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res* 1986, 46:5629–5632
75. Folkman J: Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971, 285:1182–1186
76. Story L, Kennedy S: Animal studies in endometriosis: a review. *ILAR J* 2004, 45:132–138