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Notch signaling controls sprouting angiogenesis of endometriotic lesions

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Abstract Angiogenesis is essential for the engraftment and growth of endometriotic lesions. In this study, we analyzed whether this process is regulated by Notch signaling. Endometriotic lesions were induced by endometrial tissue transplantation into dorsal skinfold chambers of C57BL/6 mice, which were treated with the γ-secretase inhibitor DAPT or vehicle. Vascularization, morphology, and proliferation of the newly developing lesions were analyzed using intravital fluorescence microscopy, histology, and immunohistochemistry over 14 days. Inhibition of Notch signaling by DAPT significantly increased the number of angiogenic sprouts within the endometrial grafts during the first days after transplantation when compared to vehicle-treated controls. This was associated with an accelerated vascularization, as indicated by a higher functional microvessel density of DAPT-treated lesions on day 6. However, inhibition of Notch signaling did not affect the morphology and proliferating activity of the lesions, as previously described for tumors. Both DAPT- and vehicle-treated lesions finally consisted of cyst-like dilated glands, which were surrounded by a well-vascularized stroma and contained comparable numbers of proliferating cell nuclear antigen-positive cells. These findings demonstrate that sprouting angiogenesis in endometriotic lesions is controlled by Notch signaling. However, inhibition of Notch signaling does not have beneficial therapeutic effects on lesion development.

Keywords Endometriosis · Angiogenesis · Notch · Tip cells · Stalk cells · γ-Secretase inhibitor

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Introduction

Endometriosis is characterized by the presence of endometriotic lesions outside the uterine cavity, which consist of endometrial tissue with stromal and glandular components [1]. It is one of the most frequent gynecological diseases, estimated to occur in ~ 10% of all women in reproductive age [2]. According to the implantation theory of Sampson [3], endometriotic lesions develop from endometrial tissue fragments, which are shed into the peritoneal cavity during retrograde menstruation.

Similar to tumors and metastases, engraftment and growth of endometriotic lesions are crucially dependent on the ingrowth of new blood vessels from the surrounding tissue for continuous oxygen and nutrient supply [4]. Hence, angiogenesis, i.e., the development of new blood vessels from preexisting ones, is not only a key event in the pathogenesis of endometriosis, but also represents a promising target for the establishment of novel treatment strategies [5–7]. In fact, numerous angiogenic growth factors have been shown to be upregulated in endometriotic lesions and in the peritoneal fluid of endometriosis patients [8, 9]. However, the molecular mechanisms regulating the formation of new microvascular networks within endometriotic lesions are still poorly understood.

The intercellular Notch signaling pathway, initially described to be crucial for organism development, is vital for fundamental processes in vascular development, such as arterial and venous differentiation, vessel maturation as well as endothelial tip and stalk cell selection during sprouting angiogenesis [10, 11]. In mammals, the Notch family consists of four single-pass, heterodimeric transmembrane proteins that serve as receptors (Notch-1 to Notch-4) for the Delta-like (Dll-1, Dll-3, and Dll-4) and Jagged (Jagged1 and Jagged2) ligands expressed on neighboring cells [12]. The

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binding of Dll-4 induces the cleavage of the Notch intracellular domain (NICD) by a disintegrin and metalloproteinase (ADAM) protease and γ-secretase. The NICD is then translocated into the nucleus, where it activates the transcription of multiple genes involved in the regulation of angiogenesis [13]. Accordingly, different γ-secretase inhibitors have been developed to block this Notch signaling cascade for the treatment of angiogenic diseases [14]. These inhibitors have been shown to reduce the blood perfusion and, in consequence, the growth of different tumors in preclinical studies [15, 16].

Based on these findings, the aim of the present study was to investigate the role of Notch signaling in angiogenesis of endometriotic lesions. For this purpose, endometriotic lesions were induced in dorsal skinfold chambers of C57BL/6 mice, which were treated with the γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-*t*-butylester) or vehicle. This approach allowed the analysis of vascularization, morphology, and proliferation of the lesions by means of intravital fluorescence microscopy, histology, and immunohistochemistry [17].

Methods

Animals

For the study, 12–16-week-old female C57BL/6 mice and C57BL/6-TgN(ACTB-EGFP)1Osb/J mice with a body weight of 22–25 g were used. The mice were housed one per cage in the conventional animal facility of the Institute for Clinical and Experimental Surgery (Saarland University, Germany) and had free access to tap water and standard pellet food (Altromin, Lage, Germany). To exclude differences between individual animals related to different sex hormone levels, estrous cycles were evaluated by cytological analysis of vaginal lavage samples. For this purpose, 15 µL of 0.9% saline was carefully pipetted into the vagina and subsequently transferred to a glass slide for examination under a phase contrast microscope (CH-2; Olympus, Hamburg, Germany). To guarantee identical cycle stages of donor and recipient mice in our endometriosis model, only mice in the diestrus stage were used for the preparation of dorsal skinfold chambers. After 48 h (corresponding to the stage of estrus), endometriotic lesions were induced in the chambers by transplanting endometrial fragments from donor mice in the estrus stage.

Dorsal skinfold chamber model of endometriosis

To investigate the effect of the γ-secretase inhibitor DAPT on angiogenesis of endometriotic lesions, we used the mouse dorsal skinfold chamber model [17, 18] (Fig. 1a). For the implantation of the dorsal skinfold chamber (Irola Industriekomponenten GmbH & Co. KG, Schonach, Germany), which has been described previously in detail [19], the mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (75 mg/kg body weight; Ursotamin®; Serumwerke Bernburg, Bernburg, Germany) and xylazine (15 mg/kg body weight; Rompun®; Bayer, Leverkusen, Germany). Thereafter, the animals could recover from anesthesia and surgical trauma after 48 h. The dorsal skinfold chamber is well tolerated by the animals, which already exhibit a normal activity as well as a normal cleaning and feeding behavior directly after implantation.

Subsequently, the uterine horns of four C57BL/6 mice were isolated and placed in a 30-mm-diameter plastic Petri dish, containing 37 °C Dulbecco's Modified Eagle's Medium (DMEM; 10% fetal calf serum (FCS), 100 U/mL penicillin, 0.1 mg/mL streptomycin; PPA, Cölbe, Germany). The uterine horns were opened longitudinally under a stereomicroscope (M651; Leica Microsystems, Wetzlar, Germany) and fixed horizontally with the endometrium at the bottom on a sterile cork plate by means of four 26G needles. The perimetrium on top as well as the underlying myometrium was carefully removed by means of microsurgical instruments. Circular endometrial fragments of comparable size (diameter: ~ 1 mm) were then dissected from the exposed basal endometrium and stained for 30 s with the fluorescent dye bisbenzimide (200 µg/mL; Sigma-Aldrich, Taufkirchen, Germany) [20]. Subsequently, two endometrial fragments were transplanted into the striated muscle tissue within each dorsal skinfold chamber (Fig. 1b, c) to analyze their vascularization by means of intravital fluorescence microscopy over 14 days. Throughout this observation period, a group of eight animals was daily treated with an i.p. injection of 10 mg/kg DAPT (Sigma-Aldrich). The used dosage of DAPT has formerly been shown to effectively inhibit Notch signaling [21]. Eight vehicle-treated (10% ethanol in corn oil) mice served as controls.

In a second set of experiments, green fluorescent protein (GFP)-positive endometrial fragments were isolated from the uterine horns of four C57BL/6-TgN(ACTB-EGFP)1Osb/J mice and transplanted into dorsal skinfold chambers of nine DAPT-treated and nine vehicle-treated C57BL/6 mice as described above. After 3, 6, and 14 days, mice ($n = 3$ per group and observation time point) were euthanized with an overdose of the anesthetics, and the endometriotic lesions with the surrounding tissue were analyzed by immunohistochemistry.

Intravital fluorescence microscopy

Intravital fluorescence microscopy was performed under ketamine–xylazine anesthesia directly (d0) as well as on days 3, 6, 10, and 14 after endometrium transplantation.

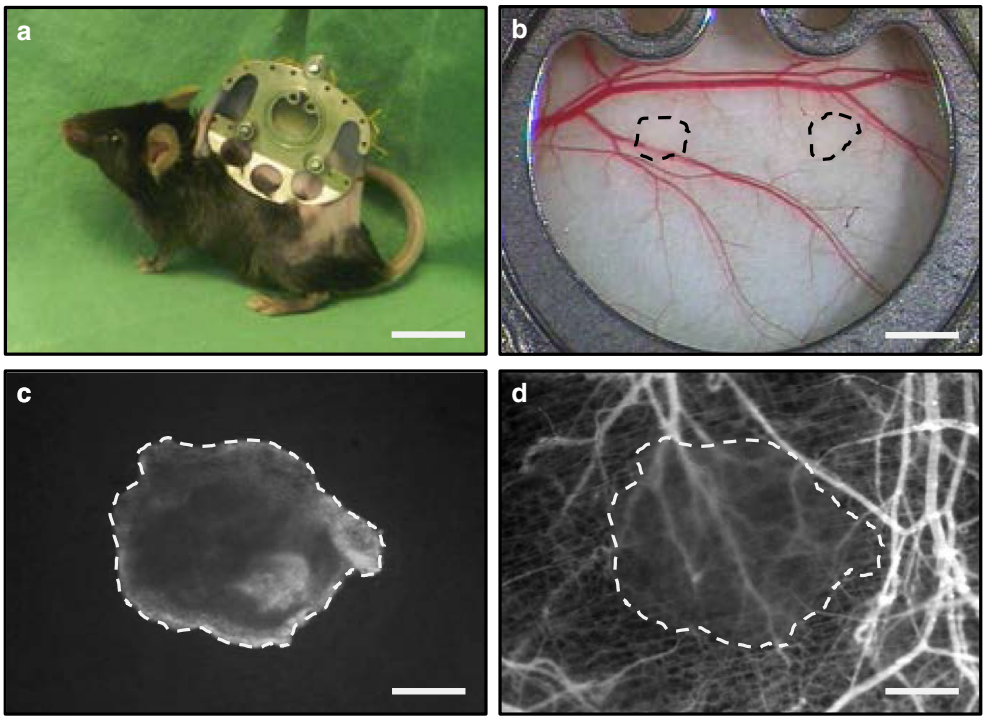

Fig. 1 **a** C57BL/6 mouse with a dorsal skinfold chamber. **b** Observation window of the dorsal skinfold chamber directly after transplantation of two endometrial fragments (borders marked by broken line) onto the host striated muscle tissue. **c, d** Intravital fluorescence microscopy in ultraviolet light epi-illumination of a bisbenzimide-stained endometrial fragment (borders marked by broken line) directly after transplantation (**c**). Bisbenzimide labels the chromatin of individual cell nuclei. Hence, areas with a high cell density or overlapping tissue layers appear particularly bright. The surrounding host microvasculature of the dorsal skinfold chamber is visualized in blue light epi-illumination by i.v. injection of 5% FITC-labeled dextran for contrast enhancement by staining of the blood plasma (**d**). Scale bars: $a = 16$ mm; $b = 2$ mm; $c, d = 340$ µm

For this purpose, 0.1 mL 5% fluorescein isothiocyanate (FITC)-labeled dextran (150,000 Da; Sigma-Aldrich) was injected into the retrobulbar venous plexus to enhance the blood vessel contrast by staining of the intravascular blood plasma (Fig. 1d).

Microscopy was performed by means of a Zeiss Axiotech microscope (Zeiss, Oberkochen, Germany) with a 100 W mercury lamp attached to an epi-illumination filter block for blue, green, and ultraviolet light. The microscopic images were recorded by a charge-coupled device (CCD) video camera (FK-6990 IQS, Pieper GmbH, Schwerte, Germany) and transferred to a DVD video system (LQ-MS 800, Panasonic, Osaka, Japan) for subsequent off-line evaluation. By means of 5 ×, 10 ×, and 20 × long-distance objectives (Zeiss), magnifications of × 115, × 230, and × 460 were achieved on a 14-inch video screen (KV-14CT1E; Sony, Tokyo, Japan).

Off-line analysis of the microscopic images was done in a blinded manner by means of the computer-assisted image analysis system CapImage (Zeintl, Heidelberg, Germany). Analysis included the assessment of the number of vessel sprouts in each developing endometriotic lesion (in mm^{-2}), the vascularized lesion area (area with clearly distinguishable microvascular networks in % of the total lesion area), and the functional microvessel density (FMD), i.e., the length of red blood cell (RBC)-perfused microvessels (in cm/cm^2). Moreover, we assessed the diameter (in µm) and the centerline RBC velocity (in µm/s) of 10 newly formed microvessels in two randomly chosen observation areas within each endometriotic lesion.

After the last intravital fluorescence microscopy, the mice were euthanized with an overdose of the anesthetics, and the endometriotic lesions with the surrounding tissue were processed for further histological and immunohistochemical analyses.

Histology and immunohistochemistry

For light microscopy, formalin-fixed specimens of endometriotic lesions were embedded in paraffin. Subsequently, 3-µm-thick sections were cut and stained with hematoxylin and eosin (HE) according to standard procedures.

Proliferating cells within endometriotic lesions were detected by means of a mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody as primary antibody (1:100; Dako Cytomation, Hamburg, Germany). The tissue

sections were then incubated with the corresponding secondary antibody. 3,3′-Diaminobenzidine tetrahydrochloride was used as chromogen. The sections were counterstained with 1% methyl green and examined by light microscopy (BX60; Olympus, Hamburg, Germany). Numbers of PCNA-positive endometrial stromal and glandular cells (in % of the total cell number) were assessed in eight lesions per group.

For the immunohistochemical detection of GFP-positive and GFP-negative microvessels within endometriotic lesions, sections were stained with a monoclonal rat anti-mouse antibody against CD31 (1:100; dianova GmbH, Hamburg, Germany) to detect endothelial cells and with a goat anti-GFP antibody (1:200; Biomol, Hamburg, Germany) to enhance GFP fluorescence. As secondary antibodies, a goat anti-rat Alexa555 antibody (1:50; Thermo Fisher Scientific GmbH, Dreieich, Germany) and a biotin-labeled donkey anti-goat antibody (1:100; Thermo Fisher Scientific GmbH), which was detected by Alexa488-labeled-streptavidin (1:50; Thermo Fisher Scientific GmbH), were used. The sections were placed in Coplin jars with 0.05% citraconic anhydride solution (pH 7.4) for 1 h at 98 °C and then incubated overnight at 4 °C with the primary antibody, followed by the appropriate secondary antibody at 37 °C for 1 h. Cell nuclei were stained with Hoechst 33342 (1:500; Sigma-Aldrich) to merge the images exactly. For the quantitative analysis of the density of all CD31-positive vessels (given in mm^{-2}) and the fraction of GFP/CD31-positive vessels (given in mm^{-2}) within the lesions, the sections were examined with a BX60 microscope (Olympus).

Statistics

Differences between the two experimental groups were assessed by the unpaired Student's *t*-test. To test for time effects within each experimental group, ANOVA for repeated measurements was applied. This was followed by the Student–Newman–Keuls post hoc test including the correction of the α-error according to Bonferroni probabilities to compensate for multiple comparisons (SigmaStat; Jandel Corporation, San Rafael, CA, USA). Data are given as mean ± SEM. Statistical significance was accepted for a value of $P < 0.05$.

Results

Microvascular network formation in endometriotic lesions

For the induction of endometriotic lesions, isolated endometrial fragments from C57BL/6 donor animals were transplanted into the striated muscle tissue within the dorsal skinfold chamber of DAPT-treated and vehicle-treated mice. These endometrial fragments exhibited a comparable initial size of 0.97 ± 0.05 mm^2 and 0.96 ± 0.03 mm^2. Throughout the observation period of 14 days, intravital fluorescence microscopy allowed the high-resolution imaging and quantification of microvascular network formation within the grafts (Fig. 2a–f). This vascularization process was characterized by the early onset of blood perfusion in a few large microvessels of the grafts on day 3 after transplantation (Fig. 2e, f). Angiogenic sprouts developed from these microvessels as well as from capillaries and postcapillary venules of the surrounding host tissue and progressively grew toward each other (Fig. 3a–d). In both groups, the number of these sprouts peaked between days 6 and 10 (Fig. 3e). Noteworthy, the number of sprouts was markedly higher in endometrial grafts of DAPT-treated mice when compared to those of vehicle-treated controls (Fig. 3e). Accordingly, DAPT-treated grafts also exhibited a significantly increased vascularized area and FMD on day 6 (Fig. 2a–f).

In an additional set of experiments, isolated endometrial fragments from transgenic C57BL/6-TgN(ACTB-EGFP)1Osb/J donor animals were transplanted into the dorsal skinfold chamber of DAPT-treated and vehicle-treated C57BL/6 wild-type mice. This experimental approach allowed the immunohistochemical differentiation between preexisting GFP/CD31-positive microvessels originating from the endometrial fragments and ingrowing GFP-negative microvessels of the surrounding host tissue at different time points after transplantation (Fig. 4a–f). The immunohistochemical analyses revealed that the density of GFP/CD31-positive microvessels within the developing endometriotic lesions was comparable in the two groups over time (Fig. 4h). However, GFP-negative microvessels were particularly detected within lesions of DAPT-treated mice (Fig. 4a–f). Accordingly, DAPT-treated lesions also exhibited a significantly higher overall density of CD31-positive microvessels on day 3 when compared to lesions of vehicle-treated animals (Fig. 4g).

Taken together, these findings indicate an accelerated vascularization of DAPT-treated endometrial fragments with an enhanced early ingrowth of microvessels from the surrounding host microvasculature when compared to vehicle-treated controls.

Microhemodynamics in endometriotic lesions

Blood-perfused microvessels within DAPT-treated and vehicle-treated endometrial grafts exhibited initial diameters of ~ 11–15 µm at day 3 (Table 1). During the following days, these diameters progressively decreased to ~ 10 µm at day 14 without significant differences between the two groups. However, centerline RBC velocities were markedly higher in microvessels of DAPT-treated grafts during the first 6 days

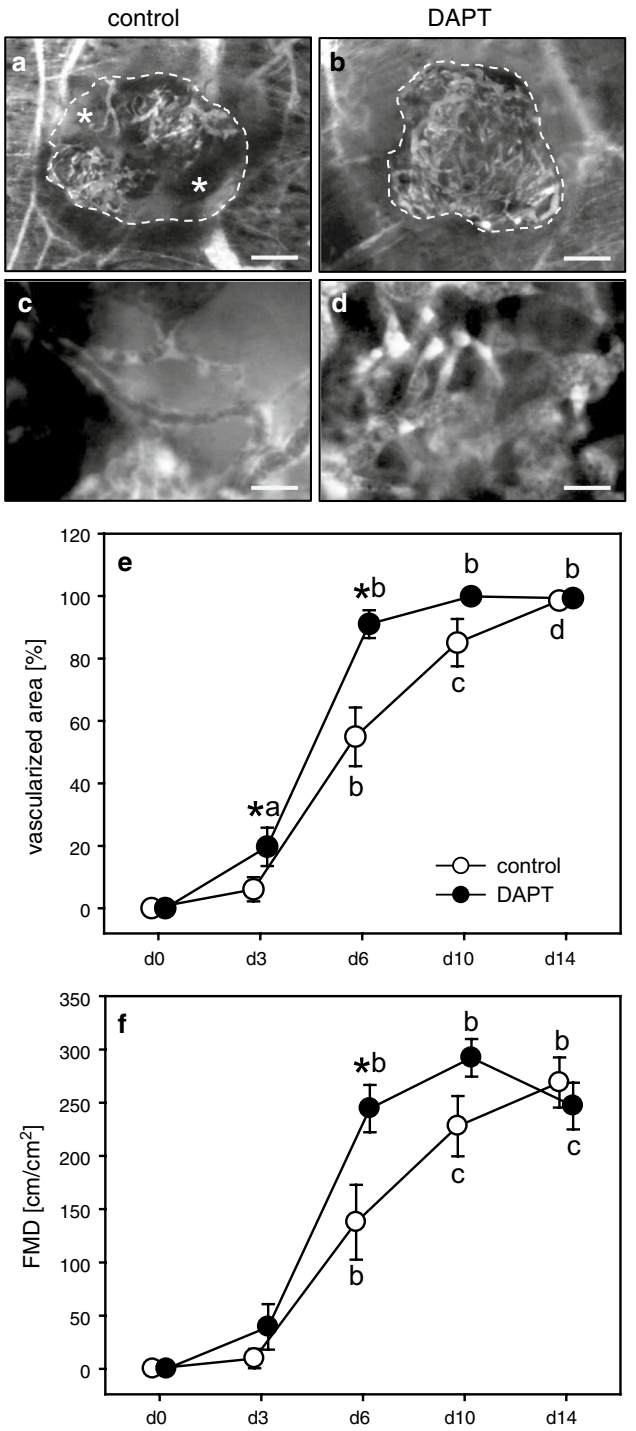

Fig. 2 a–d Intravital fluorescence microscopy (blue light epi-illumination, contrast enhancement by 5% FITC-labeled dextran) of endometriotic lesions (**a**, **b**, borders marked by broken lines) on day 6 (**a**–**d**) after transplantation of endometrial fragments into the dorsal skinfold chamber of DAPT-treated mice (**b**, **d**) and vehicle-treated control animals (**a**, **c**). Note that the vehicle-treated lesions still exhibit non-vascularized areas (**a**, asterisks) and a reduced microvessel density (**c**) when compared to DAPT-treated lesions (**b**, **d**). Scale bars: *a*, *b* = 320 µm; *c*, *d* = 90 µm. **e, f** Vascularized area (**e**, %) and FMD (**f**, cm/cm^2) of endometriotic lesions in dorsal skinfold chambers of DAPT-treated mice (black circles; $n = 8$) and vehicle-treated control animals (white circles; $n = 8$), as assessed by intravital fluorescence microscopy and quantitative image analysis. Mean ± SEM. * $P < 0.05$ versus control; [a] $P < 0.05$ versus day 0; [b] $P < 0.05$ versus days 0 and 3; [c] $P < 0.05$ versus days 0, 3, and 6; [d] $P < 0.05$ versus days 0, 3, 6, and 10

after transplantation when compared to vehicle-treated controls (Table 1).

Morphology and cell proliferation of endometriotic lesions

Histological analyses of the dorsal skinfold chamber preparations on day 14 revealed that the endometrial grafts of the two groups had developed into endometriotic lesions of typical appearance. They contained cyst-like dilated endometrial glands, which were surrounded by a vascularized endometrial stroma (Fig. 5a, c). Moreover, the lesions of the two groups contained comparable numbers of PCNA-positive cells in the stroma and glands (Fig. 5b, d, e). This indicates that they did not differ in terms of their proliferating activity.

Discussion

Angiogenesis is an essential process in the pathogenesis of endometriosis. Particularly in the initial phase of development, endometriotic lesions are characterized by a high angiogenic activity, because the formation of new microvessels is a major prerequisite for their engraftment and long-term persistence in the peritoneal cavity [4, 7]. Driven by hypoxia, shed endometrial fragments produce high amounts of pro-angiogenic growth factors [22]. This stimulates sprouting angiogenesis within the ectopic tissue and the surrounding host environment [4]. In the present study, we show that this process is controlled by Notch signaling. Treatment of engrafting murine endometriotic lesions with the γ-secretase inhibitor DAPT enhanced sprout formation in their newly developing microvascular networks and, thus, accelerated their vascularization when compared to vehicle-treated controls.

For our analyses, we induced endometriotic lesions by endometrial tissue transplantation into mouse dorsal skinfold chambers. This experimental approach has the disadvantage that the development of endometriotic lesions is not examined in the physiological environment of the peritoneal cavity, which exhibits among other characteristics also a different host microvasculature. Hence, the effects of Notch inhibitors on the vascularization of endometriotic lesions may differ between these two sites of tissue engraftment. On the other hand, the chamber provides continuous access to the lesions and, thus, ideal conditions for the repetitive

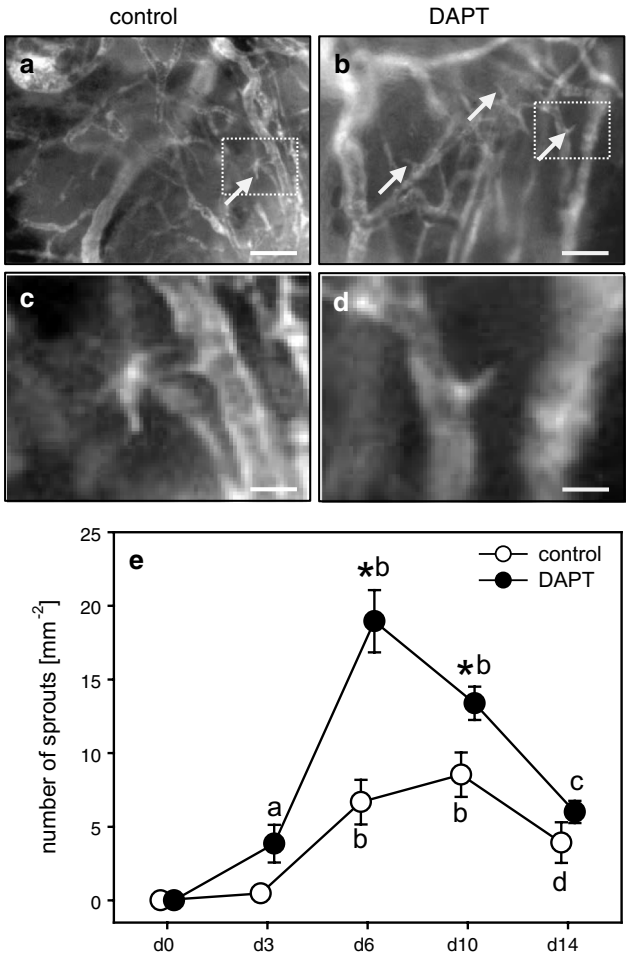

Fig. 3 **a–d** Intravital fluorescence microscopy (blue light epi-illumination, contrast enhancement by 5% FITC-labeled dextran) of the newly developing microvascular networks within endometriotic lesions on day 6 (**a–d**) after transplantation of endometrial fragments into the dorsal skinfold chamber of a DAPT-treated mouse (**b**, **d**) and vehicle-treated control animal (**a**, **c**). Note that the microvascular network of the DAPT-treated lesion exhibits more angiogenic sprouts (**b**, arrows) when compared to that of the vehicle-treated control (**a**, arrow). **c** and **d** display higher magnifications of the inserts in **a** and **b**. Scale bars: *a*, *b* = 290 µm; *c*, *d* = 55 µm. **e** Number of sprouts (mm^{-2}) of endometriotic lesions in dorsal skinfold chambers of DAPT-treated mice (black circles; $n = 8$) and vehicle-treated control animals (white circles; $n = 8$), as assessed by intravital fluorescence microscopy and quantitative image analysis. Mean ± SEM. * $P < 0.05$ vs. control; [a] $P < 0.05$ versus day 0; [b] $P < 0.05$ versus days 0 and 3; [c] $P < 0.05$ versus days 0, 6 and 10; [d] $P < 0.05$ versus day 10

high-resolution imaging of growing angiogenic sprouts and microvascular network formation by means of intravital fluorescence microscopy [6, 23, 24]. Because Notch signaling is well known to regulate particularly these cellular processes, the dorsal skinfold chamber was the most suitable endometriosis model for this preclinical study. Moreover, the regulatory function of Notch during angiogenesis is well preserved in different types of benign and malignant tissues. Therefore, we feel that the findings of our study are very likely transferable to more common sites of endometriosis.

Hypoxia-induced release of vascular endothelial growth factor (VEGF) is a fundamental stimulus for sprouting angiogenesis [25–27]. The cellular organization of sprouts is controlled by Notch, which is closely coupled to the VEGF pathway. The binding of VEGF-A to VEGF receptor (R)-2 on a microvascular endothelial cell promotes its expression of the membrane-bound Notch ligand Dll4 [28]. Consequently, the cell becomes a non-proliferative tip cell, which determines the growth direction of the nascent sprout [27]. Dll4 on the tip cell interacts with Notch1 receptors on adjacent endothelial cells, which become proliferating stalk cells, resulting in the elongation of the sprout [29].

The well-balanced signaling between individual endothelial cells helps to restrict tip cell formation in response to VEGF, thereby establishing the adequate ratio between tip and stalk cells required for correct sprouting and branching patterns [10]. The inhibition of Notch signaling, in turn, has been shown to increase the number of tip cells and sprouts in the microvasculature of tumors [15, 16, 30, 31]. However, this hypersprouting did not promote tumor growth but was associated with an impaired development of functional blood-perfused microvascular networks, resulting in reduced tumor sizes. These interesting findings suggest that inhibition of Notch signaling may be also beneficial for the treatment of endometriosis. Nonetheless, it should be considered that Notch inhibition may also markedly affect the physiological function of the reproductive organs and induce teratogenic side effects. Hence, it still has to be clarified, whether there are possible indications of Notch inhibitors in future endometriosis therapy.

In line with tumor studies, we also detected significantly more sprouts in DAPT-treated endometrial grafts when compared to vehicle-treated controls (Fig. 6), proving the regulatory function of Notch signaling in angiogenesis of endometriotic lesions. However, this did neither affect their morphological development nor their proliferative activity. This may be explained by the fact that besides sprouting angiogenesis other mechanisms essentially contribute to the vascularization of endometriotic lesions. In fact, the lesions originate from shed endometrial fragments, which already contain fully developed microvessels. In the present study, these preexisting microvessels could be detected by their GFP signal after transplantation of isolated endometrial fragments from transgenic C57BL/6-TgN(ACTB-EGFP)1Osb/J donor animals into the dorsal skinfold chamber of DAPT-treated and vehicle-treated C57BL/6 wild-type mice. Of interest, the lesions of both groups contained a comparable number of GFP-positive microvessels. However, we found that GFP-negative microvessels growing into the lesions from the surrounding host microvasculature were particularly detected in DAPT-treated mice, resulting in

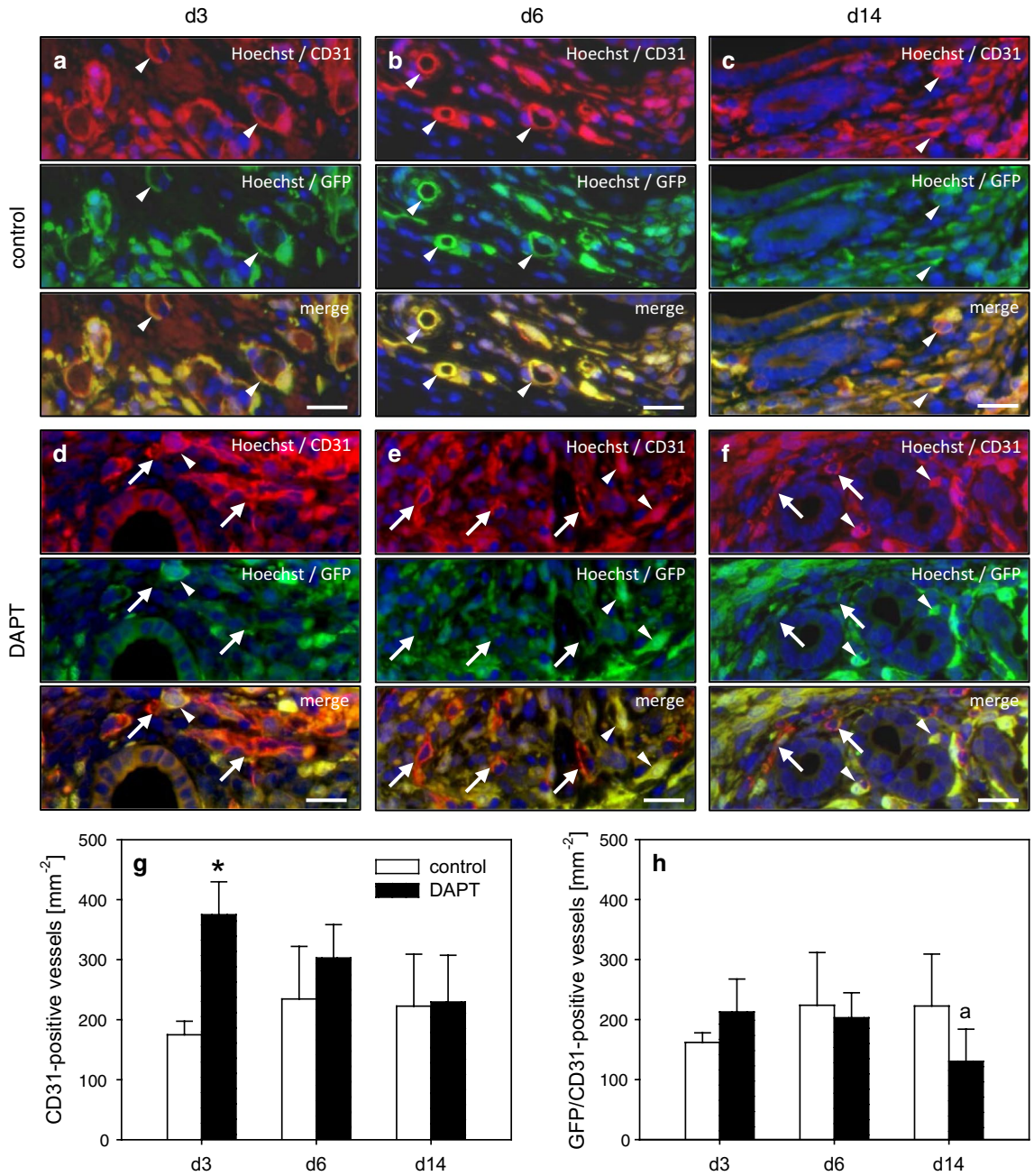

Fig. 4 **a–f** Immunohistochemical sections of endometriotic lesions on day 3 (**a**, **d**), 6 (**b**, **e**), and 14 (**c**, **f**) after transplantation of endometrial fragments into the dorsal skinfold chamber of DAPT-treated mice (**d–f**) and vehicle-treated control animals (**a–c**). The sections were stained with Hoechst 33342 to identify cell nuclei (**a–f**, blue), an antibody against CD31 for the detection of endothelial cells (**a–f**, upper panel, red) and an antibody against GFP (**a–f**, middle panel, green). Lower panels in **a–f** display merges of upper and middle panels. GFP/CD31-positive microvessels (**a–f**, arrowheads) can be detected within the lesions of both groups, whereas GFP-negative microvessels (**d–f**, arrows) are particularly found within lesions of DAPT-treated mice. Scale bars: 21 µm. **g, h** CD31-positive vessels (**g**, mm^{-2}) and GFP/CD31-positive vessels (**h**, mm^{-2}) of endometriotic lesions in dorsal skinfold chambers of DAPT-treated mice (black bars; $n = 3$) and vehicle-treated control animals (white bars; $n = 3$) on day 3, 6, and 14, as assessed by immunohistochemical analysis. Mean ± SEM. * $P < 0.05$ versus control; [a] $P < 0.05$ versus day 3 and 6

a significantly higher microvessel density on day 3 when compared to vehicle-treated animals (Fig. 6). This enhanced early ingrowth may have increased the probability of an interconnection of host microvessels with the preexisting GFP-positive microvessels of the lesions, also referred to as inosculation [20]. In line with this view, an accelerated onset of blood perfusion was detected in the lesions of DAPT-treated mice (Fig. 6).

Table 1 Diameter (μm) and centerline RBC velocity (μm/s) of microvessels within endometriotic lesions in dorsal skinfold chambers of DAPT-treated ($n = 8$) and vehicle-treated control mice ($n = 8$), as assessed by intravital fluorescence microscopy and quantitative image analysis

	d3	d6	d10	d14
Diameter (μm)				
Control	11.1 ± 2.2	14.3 ± 1.4	11.6 ± 0.5	10.6 ± 0.3
DAPT	15.1 ± 0.8	15.5 ± 0.9	12.0 ± 0.4[a]	10.3 ± 0.3[b]
Centerline RBC velocity (μm/s)				
Control	31.7 ± 17.7	107.4 ± 19.6	243.7 ± 46.3	221.7 ± 34.4
DAPT	134.1 ± 56.3	237.3 ± 37.4*	253.0 ± 30.2	224.4 ± 33.1

Mean ± SEM

* $P < 0.05$ versus control; [a] $P < 0.05$ versus day 3 and 6; [b] $P < 0.05$ versus day 3, 6, and 10

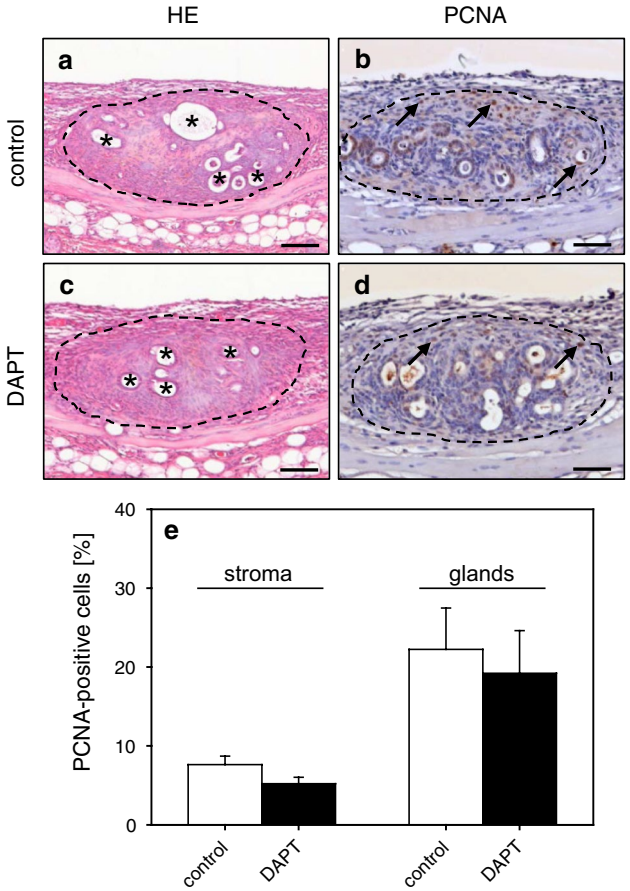

Fig. 5 **a–d** Histological and immunohistochemical sections of endometriotic lesions (borders marked by broken lines) on day 14 after transplantation of endometrial fragments into the dorsal skinfold chamber of DAPT-treated mice (**c**, **d**) and vehicle-treated control animals (**a**, **b**). HE-stained sections (**a**, **c**) show that the endometriotic lesions of the two groups are characterized by cyst-like dilated endometrial glands (asterisks), which are surrounded by an endometrial stroma. Immunohistochemical detection of PCNA reveals that the lesions of the two groups contain comparable numbers of proliferating stromal and glandular cells (**b**, **d**, arrows). Scale bars: *a*, *c* = 170 μm; *b*, *d* = 120 μm. **e** PCNA-positive stromal and glandular cells (%) of endometriotic lesions in dorsal skinfold chambers of DAPT-treated mice (black bars; $n = 8$) and vehicle-treated control animals (white bars; $n = 8$), as assessed by immunohistochemical analysis. Mean ± SEM

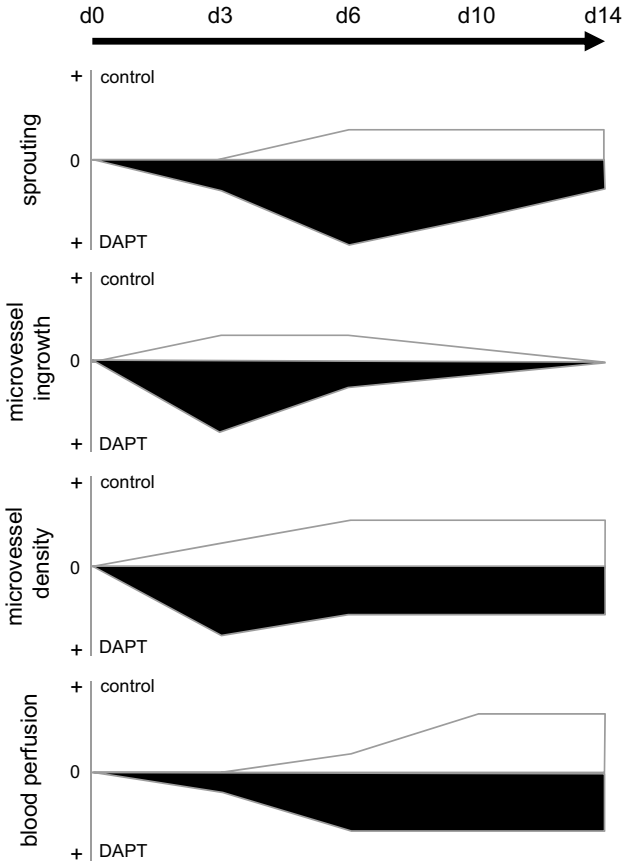

Fig. 6 Schematic illustration summarizing and comparing sprouting, microvessel ingrowth, microvessel density, and blood perfusion (extent is indicated on a scale between 0 and +) during the vascularization process of endometriotic lesions from day 0 to day 14 after transplantation of endometrial fragments into the dorsal skinfold chamber of DAPT-treated mice (black curve) and vehicle-treated control animals (white curve)

The vascularization of endometriotic lesions is further dependent on vasculogenesis, which is defined as the incorporation of circulating endothelial progenitor cells from the bone marrow into the microvascular endothelium of newly developing microvessels [32–35]. Hence, inosculation and

vasculogenesis may have compensated the potential adverse effects of Notch inhibition on the development of functional microvascular networks in the lesions. Finally, it should be noted that, in contrast to malignant tumors, endometriotic lesions are benign in nature. Thus, they are not subject to uncontrolled and unlimited sprouting angiogenesis of immature microvessels, but rather on the development of a mature, stabilized microvasculature [36], which is adjusted to the needs for oxygen and nutrients of the ectopic endometrial tissue. Correspondingly, we only observed an accelerated vascularization of DAPT-treated endometriotic lesions during the first 6 days after induction, whereas their final microvascular networks on day 14 exhibited a microvessel density, which was comparable to that of vehicle-treated controls.

Taken together, our novel findings demonstrate that sprouting angiogenesis in endometriotic lesions is regulated by Notch signaling. However, in our experimental setting, inhibition of Notch did not have beneficial therapeutic effects on lesion development, as previously reported for tumors. This may be due to the fact that the establishment of a blood supply in endometriotic lesions underlies different modes of vascularization, including sprouting angiogenesis, inosculation, and vasculogenesis. Hence, it would be interesting to clarify in future studies whether the combined targeting of these processes may be an effective therapeutic strategy for the treatment of endometriosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

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