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VASCULARIZATION IN THE PATHOGENESIS AND TREATMENT OF ENDOMETRIOSIS

Thesis

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To my love

Contents

1. Summary

Endometriosis, one of the most frequent gynecological diseases nowadays, is defined as the presence of ectopic endometrial tissue in the form of endometriotic lesions outside the uterine cavity. This condition is associated with various pain symptoms and markedly affects the patients' quality of life. Despite the high prevalence of endometriosis, the knowledge of its pathogenesis is still poor. Thus, it is necessary to identify important pathogenic factors and to develop novel strategies for the treatment of this disease. For this purpose, rodent models are an important experimental approach.

Endometriotic lesions are usually induced in rodents by transplantation of isolated endometrial tissue fragments to ectopic sites. In the first part of this thesis, it was analyzed whether this approach is affected by the cellular composition of the grafts. For this purpose, endometrial tissue fragments covered with luminal epithelium (LE^+) or without luminal epithelium (LE^-) were transplanted from transgenic green fluorescent protein $(GFP)^+$ donor mice into the dorsal skinfold chamber of GFP^- wild-type recipient animals to analyze their vascularization, growth and morphology by means of repetitive intravital fluorescence microscopy, histology and immunohistochemistry during a 14-day observation period. LE^- fragments developed into typical endometriotic lesions with cyst-like dilated endometrial glands and a well vascularized endometrial stroma. In contrast, LE^+ fragments exhibited a polypoid morphology and a significantly reduced blood perfusion after engraftment, because the luminal epithelium prevented the interaction with the surrounding host tissue. This was associated with a markedly decreased growth rate of LE^+ lesions when compared to LE^- lesions. Besides, it was found that many GFP^+ microvessels grew outside the LE^- lesions and developed interconnections to the host microvasculature, indicating that inosculation is an important mechanism in the vascularization process of endometriotic lesions. These findings demonstrate that the existence of the luminal epithelium crucially affects the vascularization, growth and morphology of endometriotic lesions. Thus, it is of major importance to standardize the cellular composition of endometrial grafts in order to increase the validity and reliability of pre-clinical rodent studies in endometriosis research.

The development of endometriosis is crucially dependent on the process of angiogenesis. However, the underlying regulatory mechanisms of blood vessel development are still poorly understood. CK2 is a pleiotropic protein kinase, which is involved in the regulation of various cellular processes including angiogenesis. In the second part of this thesis, the regulatory function of CK2 in angiogenesis of endometriotic lesions was studied. For this purpose, the anti-angiogenic activity of the CK2 inhibitor quinalizarin was analyzed in a rat aortic ring assay. In addition, isolated endometrial tissue was treated with the inhibitor to

study its effect on kinase activity and on the expression of individual CK2 subunits. Moreover, endometriotic lesions were induced in dorsal skinfold chambers of quinalizarin- and vehicle-treated C57BL/6 mice to study their vascularization and morphology by means of repetitive intravital fluorescence microscopy and histology. It was found that quinalizarin dose-dependently inhibits vascular sprouting. In addition, treatment of endometrial tissue with quinalizarin reduces CK2 activity without affecting the expression of the three CK2 subunits α, α' and β. In the dorsal skinfold chamber model, quinalizarin inhibits the vascularization of endometriotic lesions, which exhibit a significantly reduced vascularized area and functional capillary density when compared to those of vehicle-treated controls. This is associated with a decreased lesion size and less endometrial glands. Thus, CK2 is a key regulator of angiogenesis in endometriotic lesions. Accordingly, inhibition of CK2 represents a novel option in the development of anti-angiogenic strategies for the treatment of the disease.

In the third part of the thesis, the effect of the tubulin-binding vascular disrupting agent (VDA) combretastatin A4 phosphate (CA4P) on the vascularization of murine endometriotic lesions was analyzed. For this purpose, endometriotic lesions were induced in BALB/c mice by syngeneic transplantation of endometrial tissue fragments into dorsal skinfold chambers. After 6 days, the animals were treated with an intraperitoneal injection of 80mg/kg CA4P or vehicle. Throughout the following 8 days, network morphology and blood perfusion of the lesions and the surrounding tissue were analyzed by intravital fluorescence microscopy. The maturation stage of the microvessels within the lesions as well as the viability and proliferating activity of endometrial glands and stroma were assessed by immunohistochemistry. At day 6, the lesions of both groups were already well vascularized, containing a mixture of α-smooth muscle actin (α-SMA)-negative, immature and α-SMA-positive, mature microvessels. Injection of CA4P rapidly induced a selective vessel collapse in the lesions without affecting the microvasculature of the surrounding tissue. This resulted in a significantly decreased functional capillary density and blood perfusion of CA4P-treated lesions after 2h when compared to vehicle-treated controls. However, the vascularization of the lesions progressively normalized until the end of the experiment. Moreover, CA4P-treated and vehicle-treated lesions exhibited comparable numbers of proliferating and apoptotic cells over time. Taken together, these findings demonstrate a selective vascular disrupting effect of CA4P on endometriotic lesions, indicating that VDAs may be suitable for the therapy of endometriosis. However, this presumes the establishment of more effective application schemes and the development of safe VDAs with improved side effect profiles.

2. Zusammenfassung

Die Endometriose ist eine der häufigsten gynäkologischen Erkrankungen, welche durch Endometrioseherde außerhalb der Uterushöhle definiert ist. Die Krankheit ist durch verschiedene Schmerzsymptomatiken gekennzeichnet und führt oft zu einer reduzierten Lebensqualität der betroffenen Patientinnen. Trotz der hohen Prävalenz ist die Pathogenese der Endometriose noch unvollständig geklärt. Es ist daher notwendig, grundlegende pathogenetische Faktoren zu identifizieren, um neue Behandlungsstrategien etablieren zu können. Hierfür werden häufig experimentelle Nagermodelle eingesetzt.

Endometrioseherde werden in Nagern durch ektope Transplantation isolierter Endometrium-Fragmente induziert. Im ersten Teil der vorliegenden Arbeit wurde untersucht, inwiefern dieser Ansatz durch die zelluläre Zusammensetzung der Transplantate beeinflusst wird. Hierzu wurden Endometrium-Fragmente mit (LE^+) oder ohne luminales Epithel (LE^-) aus transgenen *green fluorescent protein* $(GFP)^+$ Spendermäusen in die Rückenhautkammer von GFP^- Wildtyp-Empfängermäusen transplantiert, um ihre Vaskularisierung, ihr Wachstum und ihre Morphologie mit Hilfe repetitiver intravitaler Fluoreszenzmikroskopie, Histologie und Immunhistochemie über einen 14-tägigen Beobachtungszeitraum zu analysieren. LE^--Fragmente entwickelten sich zu typischen Endometrioseherden mit zystisch dilatierten Drüsen und einem gut vaskularisierten Stroma. Im Gegensatz dazu wiesen LE^+-Fragmente eine polypöse Morphologie und eine signifikant reduzierte Blutperfusion auf, da das luminale Epithel die Interaktion mit dem umgebenden Gewebe verhinderte. Dies ging mit einer deutlich verminderten Wachstumsrate der LE^+-Herde im Vergleich zu den LE^--Herden einher. Daneben wuchsen viele GFP^+ Mikrogefäße aus den LE^--Herden in die Umgebung und gingen Verbindungen mit den Gefäßen des Empfängergewebes ein. Dies weist darauf hin, dass Inoskulation ein wichtiger Mechanismus im Vaskularisierungsprozess von Endometrioseherden ist. Diese Ergebnisse zeigen, dass das luminale Epithel entscheidend die Vaskularisierung, das Wachstum und die Morphologie von Endometrioseherden beeinflusst. Entsprechend ist es wichtig, die zelluläre Zusammensetzung von Endometrium-Fragmenten zu standardisieren, um die Validität und Reliabilität prä-klinischer Nagermodelle in der Endometrioseforschung zu verbessern.

Die Enstehung einer Endometriose ist wesentlich vom Prozess der Angiogenese abhängig. Die zugrundeliegenden regulatorischen Mechanismen der Blutgefäßneubildung sind jedoch noch nicht vollständig geklärt. Die CK2 ist eine pleiotrope Proteinkinase, die an der Regulation zahlreicher zellulärer Prozesse einschließlich der Angiogenese beteiligt ist. Im zweiten Teil dieser Arbeit wurde daher die regulatorische Funktion der CK2 bei der Angiogenese in Endometrioseherden untersucht. Hierzu wurde zunächst die anti-angiogene

Wirkung des CK2 inhibitors Quinalizarin in einem Ratten-Aortenring-Assay analysiert. Daneben wurde isoliertes Endometrium mit dem Inhibitor behandelt, um seine Wirkung auf die Expression der verschiedenen CK2 Untereinheiten und auf die Kinase-Aktivität zu prüfen. Zusätzlich wurden Endometrioseherde in Rückenhautkammern von Quinalizarin- und Vehikel-behandelten C57BL/6 Mäusen induziert, um deren Vaskularisierung und Morphologie mittels intravitaler Fluoreszenzmikroskopie und Histologie zu untersuchen. Auf diese Weise konnte gezeigt werden, dass Quinalizarin dosisabhängig die Gefäßsprossung hemmt. Die Behandlung von Endometrium mit Quinalizarin reduziert die CK2 Aktivität, ohne die Expression der drei CK2 Untereinheiten α, α' and β zu beeinflussen. Im Modell der Rückenhautkammer inhibiert Quinalizarin die Vaskularisierung von Endometrioseherden, die im Vergleich zu Vehikel-behandelten Kontrollen eine signifikant reduzierte vaskularisierte Fläche und funktionelle Kapillardichte aufweisen. Dies geht mit einer verringerten Herdgröße und weniger endometrialen Drüsen einher. Folglich ist die CK2 ein wichtiger Regulator der Angiogenese in Endometrioseherden und stellt damit ein neues Target zur Entwicklung anti-angiogener Behandlungskonzepte dar.

Im dritten Teil dieser Arbeit wurde die Wirkung des Tubulin-bindenden *vascular disrupting agent* (VDA) Combretastatin A4 Phosphat (CA4P) auf die Vaskularisierung muriner Endometrioseherde untersucht. Hierzu wurden Endometrioseherde in der Rückenhautkammer von BALB/c Mäusen induziert. Nach 6 Tagen wurden die Tiere mit einer intraperitonealen Injektion von 80mg/kg CA4P oder Vehikel behandelt. Während der folgenden 8 Tage wurde die Netzwerkmorphologie und Blutperfusion der Herde und des umgebenden Gewebes mittels intravitaler Fluoreszenzmikroskopie analysiert. Der Reifegrad der Gefäße innerhalb der Herde sowie die Zellvitalität- und Proliferation der endometrialen Drüsen und des Stromas wurden mittels Immunhistochemie untersucht. Am 6. Tag waren die Herde beider Gruppen bereits gut vaskularisiert und enthielten eine Mischung aus *α-smooth muscle actin* (α-SMA)-negativen, unreifen and α-SMA-positiven, reifen Mikrogefäßen. Die Injektion von CA4P verursachte einen raschen Gefäßkollaps in den Herden, ohne die Gefäße des umgebenden Gewebes zu beeinflussen. Dies führte nach 2h im Vergleich zur Kontrolle zu einer signifikant reduzierten funktionellen Kapillardichte und Blutperfusion in den CA4P-behandelten Herden. Danach normalisierte sich jedoch die Vaskularisierung der Herde wieder bis zum Ende des Experiments. Weiterhin wiesen CA4P- und Vehikel-behandelte Herde über die Zeit eine vergleichbare Anzahl proliferierender und apoptotischer Zellen auf. Diese Ergebnisse zeigen, dass CA4P selektiv auf die Gefäße in Endometrioseherden wirkt. Entsprechend könnten VDAs zukünftig in der Therapie der Endometriose Anwendung finden. Dies setzt jedoch die Etablierung effektiverer Applikationsschemata sowie die Entwicklung verträglicherer VDAs voraus.

3. Introduction

3.1 Definition and epidemiology of endometriosis

Endometriosis is classically defined as the presence of endometrial glands and stroma outside the uterine cavity, most commonly in the pelvic peritoneum, the ovaries, and the rectovaginal septum [GALLE, 1989].

Since its first description as a clinical entity by Sampson in 1918 [SAMPSON, 1918], the pathogenesis of the disease is still a matter of discussion. In general, endometriosis occurs in three different morphological forms: 1) peritoneal endometriosis with endometriotic lesions on the surface of the pelvic peritoneum and ovaries; 2) endometrioma with ovarian cysts lined by endometrioid mucosa; 3) rectovaginal endometriosis, which is a complex solid mass of endometriotic, adipose and fibromuscular tissue, residing between the rectum and the vagina [BULUN, 2009]. The progress of the disease is variable, ranging from minimal or mild disease to highly aggressive lesions with severe invasion, infiltration and pelvic adhesions that can even affect the functions of other organs [ACIÉN and VELASCO, 2013].

Endometriosis is an estrogen-dependent disorder [GIUDICE and KAO, 2004]. Endometriotic lesions contain endometrial glands and stroma which are functionally capable of responding to exogenous, endogenous, or local hormonal stimuli [ACIÉN and VELASCO, 2013]. Accordingly, endometriosis affects women of reproductive age and normally regresses when menopause occurs due to a decrease in estrogen levels. However, recurrence is possible in postmenopausal women when endogenous hormones are present or hormones are administered for the treatment of other diseases [OXHOLM et al., 2007; BENDON and BECKER, 2012].

Endometriosis is estimated to occur in around 10% of women in reproductive age [ESKENAZI and WARNER, 1997]. Frequent clinical symptoms of the disease include chronic pelvic pain, dysmenorrhea and dyspareunia [SPACZYNSKI et al., 2003]. Moreover, the prevalence of the disease is as high as 35%-50% in women experiencing pelvic pain or infertility [MEULEMAN et al., 2009]. Endometriosis is a debilitating disorder that severely impairs the patient's quality of life [BERKLEY et al., 2005]. It is associated with frequent hospitalization, repetitive surgical treatments, expensive medical therapy and high recurrence rates [VERCELLINI et al., 2006; BERLANDA et al., 2010]. Accordingly, endometriosis places a substantial economic burden on the society. It is reported that the costs caused by endometriosis in the United States attained 69.4 billion US dollar in 2009 [SIMOENS et al., 2012].

Laparoscopic excision is considered the “gold standard” in the treatment of ovarian endometrioma. However, this is associated with recurrence rates of 11 - 32% within the first 1-5 years after excision [KOGA et al., 2013]. On the other hand, effective medications, mainly oral contraceptives and gonadotropin releasing hormone (GnRH) agonists, are well established in the treatment of endometriosis. Unfortunately, they induce numerous side effects such as impaired reproductive function and bone density loss [KAPPOU et al., 2010]. Therefore, it is of major importance to develop more promising strategies for the treatment of this frequent gynecological disease.

3.2 Theories on the pathogenesis of endometriosis

Despite substantial progress towards unraveling the pathophysiology of endometriosis during the past several decades, many questions about the etiology and pathogenesis of the disease remain unanswered. Theories on the pathogenesis of endometriosis include:

1. Implantation theory: This is the most widely accepted theory, which was initially proposed by Sampson in the 1920s [SAMPSON, 1927]. He speculated that endometriosis is caused by retrograde menstruation of endometrial fragments through the Fallopian tubes into the peritoneal cavity, where they attach to the peritoneum, followed by peritoneal invasion and the establishment of endometriotic lesions.

The implantation theory is supported by the finding that adolescent girls with congenital outflow obstruction as well as women with uterine septum and cervical stenosis exhibit a higher prevalence of endometriosis [SANFILIPPO et al., 1986; BARBIERI, 1998; NAWROTH et al., 2006]. Moreover, it is possible to induce endometriotic lesions within the peritoneal cavity by means of iatrogenic obstruction of the outflow tract in non-human primates [D'HOOGHE, 1997]. In addition, women with endometriosis have higher volumes of sloughed menstrual blood and endometrial fragments [HALME et al., 1984] and more frequent subendometrial myometrial contractile waves than healthy women [SALAMANCA et al., 1995]. Finally, the implantation theory is also supported by the typical anatomic distribution of endometriotic lesions in the peritoneal cavity [BURNEY et al., 2012]. In fact, the high occurrence of endometriotic lesions in the posterior cul de sac is explained by the accumulation of shed menstrual effluent in this area of the peritoneal cavity [DMOWSKI et al., 1984].

2. Coelomic metaplasia: This theory postulates that endometriotic lesions develop by metaplasia of the coelomic epithelium [FERGUSON et al., 1969]. It is plausible as both peritoneal and endometrial tissues share a common embryologic precursor, the coelomic cell. This theory may also explain why up to 90% of women exhibit retrograde menstruation but only a small percentage suffers from endometriosis. Moreover, endometriosis also occurs in

women in the absence of menses or in women, who congenitally lack an uterus (Rokitansky-Küster-Hauser syndrome) [BALCI et al., 2008]. However, possible factors responsible for the differentiation of cells in the peritoneal lining to endometrial cells remain poorly defined, although such transformation may be induced by endogenous stimuli or environmental factors [MERRILL et al., 1966; NISOLLE et al., 1997].

3. Embryonic Müllerian rests or Müllerianosis [BATT et al., 2013]: This theory postulates that embryonic duct remnants exhibit the capacity to differentiate into endometrial tissue under the influence of estrogen [RUSSELL, 1899]. Accordingly, epidemiological studies have shown a two-fold increased risk of endometriosis among women exposed to diethylstilbestrol in utero [MISSMER et al., 2004]. This theory is also supported by a recent report that ectopic endometrium has been found in 4 of 36 fetuses of various ages at different sites [SIGNORILE et al., 2009; 2010].

4. Lymphatic or hematogenous spread: Endometrial tissue may spread from the eutopic endometrium to distant sites through lymphatic vessels or blood vessels [HALBAN, 1924; SAMPSON, 1927]. This may explain the observation that endometriotic lesions are sometimes located in bone, lung, and brain [JUBANYIK et al., 1997]. Direct evidence for the theory of benign metastasis is derived from a report describing endometriosis within lymph nodes in 6-7% of women at lymphadenectomy [JAVERT et al., 1952]. Additionally, endometrial tissue may be distributed during surgical interventions. Accordingly, endometriotic lesions are sometimes localized in former episiotomy sites or in cesarean-section scars [BULUN, 2009; EMRE et al., 2012; GIDWANEY et al., 2012].

5. Defective immune clearance: This theory suggests that endometriosis is a multifactorial disease associated with a general inflammatory response aiming to clear the peritoneal cavity from ectopic endometriotic cells and tissue. However, this inflammatory response creates an environment that promotes the implantation and proliferation of the ectopic tissue due to defective ‘‘immunosurveillance’’ [CHRISTODOULAKOS et al., 2007]. Multiple studies have shown that the defective immunosurveillance in the peritoneal fluid of women with endometriosis includes the following factors: Reduced T-lymphocyte-mediated cytotoxicity, macrophages with increased cytokine production and reduced phagocytic activity, and compromised natural-killer cell activity [HERINGTON et al. 2011].

6. Stem/progenitor cells: This theory suggests that stem/progenitor cells originating from bone marrow or endometrium may differentiate into endometriotic lesions [SASSON et al., 2008; FIGUEIRA et al., 2011]. This view is supported by the finding that human endometrial stem cells develop into endometriotic lesions in many models [MASUDA et al., 2007; SASSON et al., 2008]. Moreover, mesenchymal stem cells have been identified in cultured endometrial cells [DU et al., 2009].

3.3 Animal models in endometriosis research

In general, non-human primate models as well as rodent models have been developed during the last decades to study the pathophysiology of endometriosis. Each animal model bears its own advantages and disadvantages [GRÜMMER, 2006; BRAUNDMEIER et al., 2009]. The major advantage of non-human primate models is that primates are able to develop endometriotic lesions spontaneously because they menstruate [YAMANAKA et al., 2012]. Additionally, the immune system, the anatomy and reproductive functions of a primate are very similar to those of humans. Accordingly, primate models are in particular suitable for investigating the pathogenesis of endometriosis. Different species of non-human primates have been used for experimental endometriosis studies including Japanese macaques, pigtailed macaques, rhesus monkeys and baboons [STORY and KENNEDY, 2004]. However, non-human primates are very expensive. Furthermore, they are limited in their use as endometriosis models because of ethical considerations. Finally, the practical handling of larger primates is not as convenient as the handling of small laboratory animals such as mice or rats.

Although rodents do not menstruate and, thus, do not develop endometriosis spontaneously, they are suitable to unravel the complex interaction of different cell types and the tight regulation of distinct cytokines and growth factors, which contribute to the onset and progression of this condition [TIRADO-GONZÁLEZ et al., 2010]. In many rodent models, uteri are removed and cut into small pieces, which are then transplanted into the peritoneal cavity [SOMIGLIANA et al., 1999; HIRATA et al., 2005; FAINARU et al., 2008] or other ectopic sites. Accordingly, the heterogeneous composition of the transplanted tissue, which is composed of luminal and glandular epithelium and fibroblastic stroma may markedly affect the standardized induction of endometriotic lesions. For instance, it is well known that the luminal epithelium of the endometrium with its anti-adhesive properties acts as a physical and immunological barrier [GIPSON et al., 2008; OCHIEL et al., 2008]. Therefore, the aim of the first part of this thesis was to analyze whether the existence of this luminal epithelium in isolated endometrial tissue fragments affects their vascularization and morphological development into endometriotic lesions after transplantation to an ectopic site. For this purpose, endometrial tissue fragments with or without luminal epithelium were isolated from transgenic green fluorescent protein $(GFP)^+$ donor mice. These fragments were then transplanted into the dorsal skinfold chamber of GFP^- wild-type recipient animals and their growth and vascularization was analyzed by means of repetitive intravital fluorescence microscopy [FENG et al., 2013]. Thereby, the GFP^+/GFP^- cross-over design enabled the detailed investigation of the origin of newly formed microvascular networks inside the developing endometriotic lesions and their interaction with the surrounding host

microvasculature.

3.4 Angiogenic mechanisms in the pathogenesis of endometriosis

Although many aspects of the pathogenesis of endometriosis are still a matter of debate, there is no doubt that angiogenesis is necessary for the development and maintenance of endometriotic lesions. In fact, similar to tumors and metastases, endometriotic lesions are crucially dependent on the ingrowth of new blood vessels, supplying them with oxygen and essential nutrients [GROOTHUIS et al., 2005; MAY et al., 2008]. Accordingly, active lesions are characterized by intensive vascularization or haemorrhages during laparoscopy [MCLAREN 2000]. Morphometric studies have additionally shown that an increased vascularization also occurs in the tissue surrounding the lesions [NISOLLE et al., 1993; MCLAREN 2000]. Based on the observation that a rich vascular supply is crucial for the development and survival of endometriotic lesions, many anti-angiogenic agents have been tested to inhibit blood vessel formation and lesion growth [SOARES et al., 2012; LASCHKE and MENGER, 2012]. Thus, endometriosis belongs to the group of angiogenic diseases, which also includes cancer, psoriasis, rheumatoid arthritis and diabetic retinopathy [HEALY et al., 1998].

The development of new blood vessels is a complex process, which has been investigated in peritoneal endometriotic lesions over the past decades. Expression analyses in endometrial tissue of endometriosis patients showed an up-regulation of cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-8 as well as matrix metalloproteinases (MMPs), such as MMP-3, which may promote the growth and local neovascularization of the tissue in the peritoneal cavity [KYAMA et al., 2006]. In addition, the balance of pro- and anti-angiogenic growth factors is compromised in the peritoneal fluid of patients with endometriosis [OOSTERLYNCK et al., 1993] and peritoneal macrophages are strongly activated to produce pro-angiogenic factors [MINICI et al., 2007]. For instance, vascular endothelial growth factor (VEGF), one of the most potent angiogenic factors, has been detected in the peritoneal fluid of endometriosis patients in high concentrations, which correlated with the stage of the disease [MCLAREN et al., 1996]. Other angiogenic factors, which are crucially involved in the pathogenesis of endometriosis, include angiogenin [SUZUMORI et al., 2004], platelet-derived endothelial growth factor (PDGF) [LASCHKE et al., 2006b], and macrophage migration inhibitory factor (MIF) [VEILLAT et al., 2010].

Protein kinase CK2, an ubiquitously expressed serine/threonine kinase, is found in various intracellular compartments and on the cell surface [FAUST et al., 2000; 2001]. It is a tetramer with two catalytic subunits (α and/or α', 45 kDa or 43 kDa) and two regulatory

subunits (β, 25 kDa). In addition to the holoenzyme, the individual subunits of CK2 exist either in a free form or are associated with other cellular or viral proteins [BIBBY et al., 2005; MONTENARH, 2010]. CK2 phosphorylates more than 400 different substrates. Accordingly, it is involved in the regulation of a variety of cellular processes, including metabolism, signal transduction, proliferation, differentiation, apoptosis and tumor development [ST-DENIS et al., 2009].

Recently, protein kinase CK2 has also been shown to be crucially involved in the process of angiogenesis. In fact, inhibition of CK2 reduces endothelial cell proliferation, survival and migration as well as tube formation and sprouting in vitro and suppresses preretinal neovascularization in mouse models of proliferative oxygen-induced retinopathy [LJUBIMOV et al., 2004; KRAMEROV et al., 2008; SIDDIQUI-JAIN et al., 2010]. This indicates that CK2 inhibitors may be promising candidates for the treatment of angiogenic diseases, such as endometriosis.

Based on these findings, the aim of the second part of this thesis was to investigate the regulatory function of CK2 in angiogenesis of endometriotic lesions. For this purpose, the anti-angiogenic effects of the CK2 inhibitor quinalizarin were assessed in a rat aortic ring assay. Moreover, the effect of quinalizarin treatment on the expression of individual CK2 subunits and on the activity of CK2 in endometrial tissue was analyzed in vitro. Finally, endometriotic lesions were induced in dorsal skinfold chambers of quinalizarin- and vehicle-treated C57BL/6 mice to investigate their vascularization by means of repetitive intravital fluorescence microscopy [FENG et al., 2012].

3.5 Vascular disrupting agents for the treatment of endometriosis

Anti-angiogenic treatment represents a promising therapeutic strategy for endometriosis in particular within the early phase of the disease, when blood vessels grow into newly developing endometriotic lesions [NISOLLE et al., 1993; MCLAREN, 2000]. However, because of the complex and confusing symptoms of endometriosis, the mean interval between the onset of the disease and definitive diagnosis is usually more than 10 years [HADFIELD et al., 1996; GIUDICE, 2010]. Thus, endometriosis is most often diagnosed in an advanced stage, in which the microvasculature of the lesions is already well established and only undergoes minor remodeling processes.

To overcome this problem, older endometriotic lesions may be treated with vascular disrupting agents (VDAs) [VAN LANGENDONCKT et al., 2008]. These novel drugs have been developed for the treatment of tumors with the primary aim of targeting established tumor

microvessels, resulting in tumor ischemia and necrosis [SIEMANN et al., 2009; SPEAR et al., 2011]. VDAs can be divided into ligand directed and small molecule VDAs [HINNEN and ESKENS, 2007]. The latter ones are in a more advanced stage of clinical development and include several tubulin-binding agents. They destabilize the microtubular cytoskeleton of individual proliferating endothelial cells and increase vascular permeability and interstitial pressure inside tumors, which induces rapid vessel collapse and shutdown of intratumoral blood perfusion [HINNEN and ESKENS, 2007].

Combretastatin A4 phosphate (CA4P) is the leading compound of the tubulin-binding VDAs [CHAPLIN et al., 2006; NAGAIAH and REMICK, 2010]. It is the water-soluble prodrug of combretastatin A4, which was originally isolated from the African tree *Combretum caffrum* [WEST and PRICE, 2004]. Preclinical studies have shown that CA4P induces blood flow reduction and subsequent tumor cell death in a variety of pre-clinical models. The activity of CA4P has been linked to its ability to rapidly alter the morphology of immature endothelial cells by disrupting their tubulin cytoskeleton [YOUNG et al., 2004]. Numerous clinical studies have demonstrated that CA4P induces extensive tumor necrosis and markedly enhances tumor responses in combination with radiotherapy or chemotherapeutics [SIEMANN et al., 2009; SOSA et al., 2012]. In addition, CA4P has been successfully tested in clinical cancer trials with tolerable safety profiles [WEST and PRICE, 2004]. Of interest, CA4P has also been suggested as a compound for the treatment of conditions other than cancer, which are dependent on neovascularization, such as macular degeneration [LIM, 2006; NAGAIAH and REMICK, 2010].

Based on these findings, the aim of the third part of this thesis was to analyze the effect of a single shot administration of CA4P on the microvasculature of established endometriotic lesions in the dorsal skinfold chamber model.

4. Aims of the thesis

In the present thesis, endometriotic lesions were induced in the mouse dorsal skinfold chamber model and analyzed by means of repetitive intravital fluorescence microscopy. Using this approach, the following questions were addressed:

I. Does the luminal epithelium of transplanted endometrial tissue fragments affect their growth, vascularization and morphological development into endometriotic lesions?

II. Does the CK2 inhibitor quinalizarin affect the sprouting of microvessels in the aortic ring assay and the expression and activity of CK2 in isolated endometrial tissue?

III. Does the CK2 inhibitor quinalizarin affect the vascularization and growth of endometriotic lesions?

IV. What are the effects of the VDA CA4P on the vascularization and blood perfusion of established endometriotic lesions and the surrounding host tissue?

V. Does a single shot administration of CA4P affect cell proliferation and apoptosis within endometriotic lesions?

5. Materials and Methods

5.1 Animals

All experiments were approved by the local governmental animal care committee and were conducted in accordance with the German legislation on protection of animals and the *NIH Guidelines for the Care and Use of Laboratory Animals* (NIH Publication #85-23 Rev. 1985).

For the experiments, 14 to 16-week-old female C57BL/6-TgN(ACTB-EGFP)1Osb/J mice and corresponding C57BL/6 wild-type mice as well as BALB/c mice with a body weight of 22-25g were used (Charles River GmbH, Sulzfeld, Germany). The animals were housed one per cage within a temperature-controlled environment on a 12h/12h light-dark cycle and had free access to tap water and standard pellet food (Altromin, Lage, Germany). To exclude discrepancies between individual animals due to different sex hormone levels, estrous cycling was evaluated by cytological analysis of vaginal lavage samples. For this purpose, 15µL of 0.9% saline were carefully pipetted into the vagina and subsequently transferred on a glass slide for examination under a phase contrast microscope (CH-2; Olympus, Hamburg, Germany). Only those animals, which were in the stage of estrus, were included in the experiments.

5.2 Experimental model

5.2.1 Dorsal skinfold chamber

The mouse dorsal skinfold chamber was used for the in vivo analysis of angiogenesis and vascularization of endometriotic lesions. This chamber consists of two symmetrical titanium frames, which are implanted on the extended dorsal skinfold of the animals, so that they sandwich the double layer of skin containing cutis, subcutis and striated muscle tissue (*Fig. 1A*). The dorsal skinfold chamber model has been established in mice [CARDON et al., 1970], rats [PAPENFUSS et al., 1979], and hamsters [ENDRICH et al., 1980]. During the last two decades, this chronic model has been broadly used for the investigation of angiogenesis [VAJKOCZY et al., 1998; LAIRD et al., 2000; LASCHKE et al., 2006a, 2008a, 2011c; BINGLE et al., 2006; CZABANKA et al., 2008; STRIETH et al., 2008]. By means of intravital fluorescence microscopy, the dorsal skinfold chamber model allows for the repetitive in vivo analysis of the microcirculation over a time period of 2-3 weeks.

5.2.2 Implantation of the dorsal skinfold chamber

For the preparation of the dorsal skinfold chamber, mice were anesthetized by an intraperitoneal (i.p.) injection of ketamine (75mg/kg body weight; Pharmacia GmbH, Erlangen, Germany) and xylazine (15mg/kg body weight; Rompun, Bayer, Leverkusen, Germany).

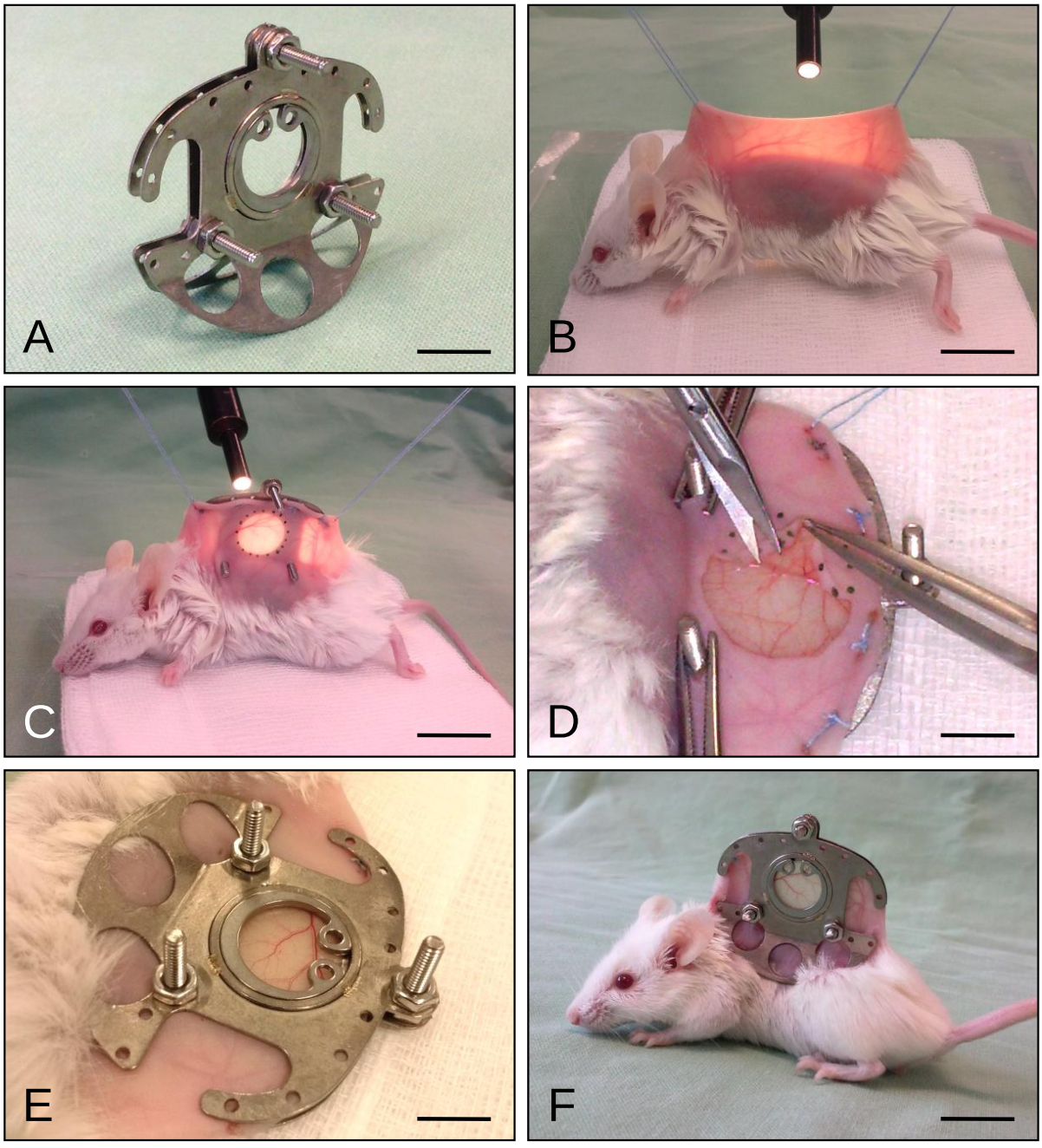

Figure 1: *A: Dorsal skinfold chamber consisting of two symmetrical titanium frames for the implantation into mice (weight ~2g). B-E: Stepwise preparation of a dorsal skinfold chamber in an anesthetized BALB/c mouse. F: The animal tolerates the chamber well. Scale bars: A = 10 mm; B, C, F = 15 mm; D = 7 mm; E = 6 mm.*

The back of the animals was then carefully shaven and chemically depilated (Asid-med Cream; ASID BONZ GmbH, Herrenberg, Germany) avoiding microinjury to the skin. Subsequently, the hair-free back was cleaned under warm water to ensure the complete removal of the depilatory cream, which otherwise may have induced inflammatory irritations affecting the quality of the chamber preparation.

In a next step, the mice were fixed in prone position and the back was exposed to medical disinfectant spray (Softasept N; B BRAUN, Melsungen, Germany). Subsequently, the extended dorsal skinfold of the animals was affixed cranially and caudally at midline with two 5-0 silk sutures in vertical position to examine the major feeding and draining blood vessels at the front and back side under transillumination (*Fig. 1B*). The first frame was then fixed to the back side of the skinfold by 5-0 silk sutures on its superior edge. The connecting screws of the frame were passed through two openings, prepared carefully to avoid injury of vascular structures, from the back to the front side at the base of the skinfold that was close to the body (*Fig. 1C*).

After marking the circular area of the later observation window under transillumination, the mouse was placed in lateral position under a stereo-microscope. Within the circular area, one layer of skin, subcutis with the panniculus carnosus muscle and two layers of the retractor muscle were completely removed by means of microsurgical instruments. Thereby, it was important to ensure that the size of the removed area (diameter: ~15mm) exceeded the diameter of the later observation window (~11mm) to avoid compression of the tissue. Moreover, special care was taken when removing the second layer of the retractor muscle, because it covered the panniculus carnosus muscle which served for the later microscopic analysis. During the entire preparation, the tissue was kept moist with 0.9% NaCl to prevent drying (*Fig. 1D*).

Finally, the second chamber frame was put on the connecting screws with a frame-to-frame distance of 400-500µm using stainless steel nuts as spacers. This distance prevented the compression of the supplying arterioles and the draining venules in the skinfold tissue. The observation window of the second frame was closed by a removable cover glass, which was fixed by means of a snap ring and provided the direct microscopic access to the chamber tissue (*Fig. 1E*). The animal was allowed to recover from anesthesia and surgical trauma for 48h after the implantation of the dorsal skinfold chamber (*Fig. 1F*). Subsequently, endometriotic lesions were induced in the observation window by transplantation of isolated endometrial tissue fragments onto the host striated muscle tissue, as described in the following section.

5.2.3 Isolation and transplantation of endometrial tissue fragments

For the isolation of endometrial tissue fragments, donor mice were anesthetized by i.p. injection of ketamine and xylazine. After midline laparotomy, the uterine horns were aseptically removed and placed in a 30mm plastic Petri dish filled with 37°C warm Dulbecco's modified Eagle medium (DMEM; 10% fetal calf serum (FCS), 100U/mL penicillin, 0.1mg/mL streptomycin; PAA Laboratories GmbH, Cölbe, Germany). Subsequently, the uterine horns were opened longitudinally by means of a micro-scissors and the endometrium was carefully excised from the underlying myometrium under a stereo-microscope (M651; Leica Microsystems, Wetzlar, Germany). Then, the endometrium was cut into small endometrial fragments of comparable size. The fragments were transferred into 37°C warm DMEM with the fluorescent dye bisbenzimide (200µg/mL; Sigma-Aldrich, Taufkirchen, Germany) for 3min, which allowed for an easy differentiation of the stained endometrium from the non-stained surrounding host tissue after transplantation into the dorsal skinfold chamber.

In a subset of experiments, transgenic C57BL/6-TgN(ACTB-EGFP)1Osb/J mice were used as donors for the isolation of endometrial tissue fragments. In these mice with an enhanced green fluorescent protein (GFP) cDNA under the control of a chicken β-actin promoter and cytomegalovirus enhancer all of the tissues, with exception of erythrocytes and hair, exhibit a green fluorescence under blue light excitation [OKABE et al., 1997].

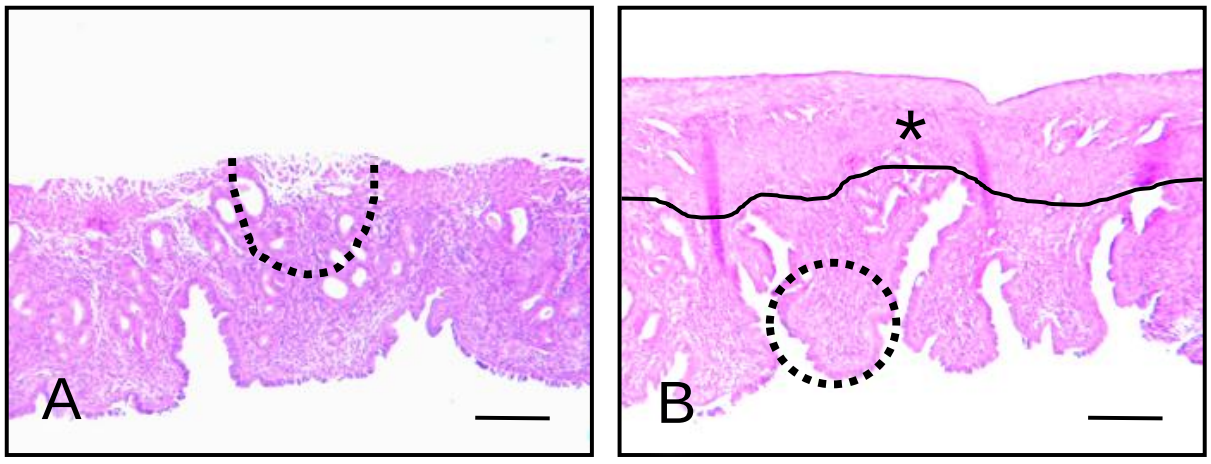

Figure 2: *A, B: HE-stained sections of longitudinally opened uterine horns from a C57BL/6-TgN(ACTB-EGFP)1Osb/J donor mouse for the isolation of a LE^- fragment (A, dotted line) and a LE^+ fragment (B, dotted line). The LE^+ fragment is excised from the luminal side of the endometrium of the intact uterine horn, which exhibits a layer of myometrium with the underlying perimetrium (B, asterisk). For the isolation of the LE^- fragment, this layer is first removed (compare A vs. B) and the fragment is then excised from the exposed basal endometrium (A). Scale bars: A, B = 170 µm.*

Accordingly, the endometrial tissue fragments and their microvessels could easily be detected by their GFP^+ signal after transplantation into the dorsal skinfold chamber of wild-

type recipient animals. To study the effect of the luminal epithelium on the development of the tissue fragments into endometriotic lesions, two different types of endometrial fragments were prepared in this subset of experiments, i.e. fragments with (LE^+) and without luminal epithelium (LE^-).

For the generation of endometrial tissue fragments lacking a luminal epithelium (LE^- fragments) the myometrium with the underlying perimetrium was carefully removed from one uterine horn. Subsequently, small LE^- fragments were excised from the exposed basal endometrium (*Fig. 2A*). From the second uterine horn, endometrial tissue fragments covered with a luminal epithelium (LE^+ fragments) were isolated from the luminal side of the endometrium (*Fig. 2B*). Both fragment types exhibited a comparable initial size of 0.7-0.9mm².

For the transplantation of endometrial fragments, the animals with dorsal skinfold chambers, were anesthetized by i.p. injection of ketamine and xylazine. The cover glass of the dorsal skinfold chamber was temporarily removed and the chamber tissue was flushed with 0.9% NaCl. Subsequently, two endometrial tissue fragments were placed onto the striated muscle tissue within each chamber with a maximal distance to each other to exclude their interaction during the engraftment process (*Fig. 3*). Finally, the chamber was closed again with a new cover glass.

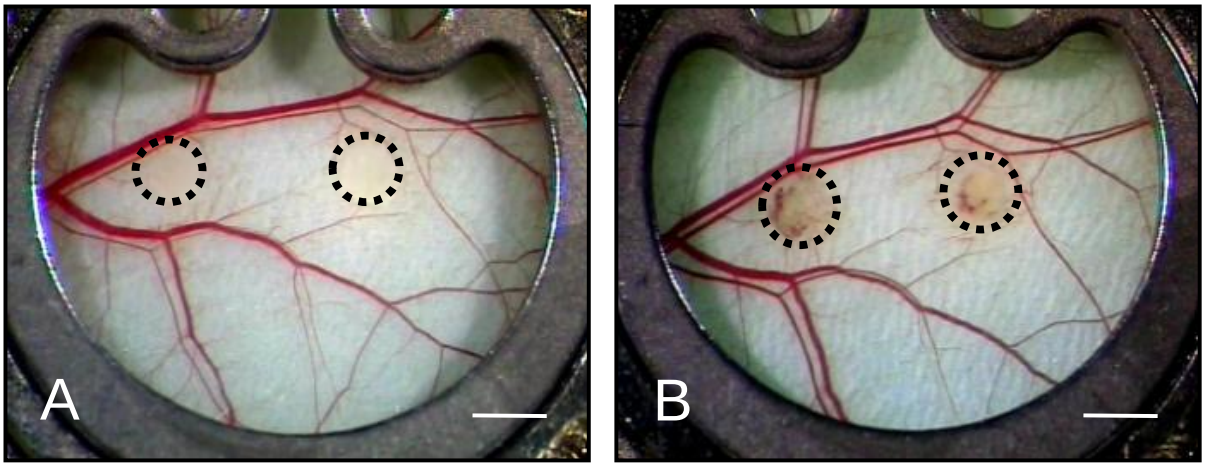

Figure 3: *A, B: Observation window of the chamber directly (A) as well as at day 6 (B) after transplantation of two endometrial tissue fragments (borders marked by dotted lines) onto the chamber tissue for the induction of endometriotic lesions. Note the reddish appearance of the grafts at day 6 (B), indicating successful engraftment with the development of a blood-perfused microvascular network. Scale bars: A, B = 1.4 mm.*

5.2.4 Intravital fluorescence microscopy

The vascularization of endometriotic lesions within the dorsal skinfold chambers was analyzed by means of intravital fluorescence microscopy. In contrast to indirect methods for the assessment of the microvasculature, such as laser Doppler flowmetry [AHN et al., 1986; TATEISHI et al., 1997], this technique ideally suits to investigate the dynamic process of angiogenesis, because it allows for the repetitive, direct visualization of the microcirculation and subsequent quantitative analyses of angiogenic and microhemodynamic parameters.

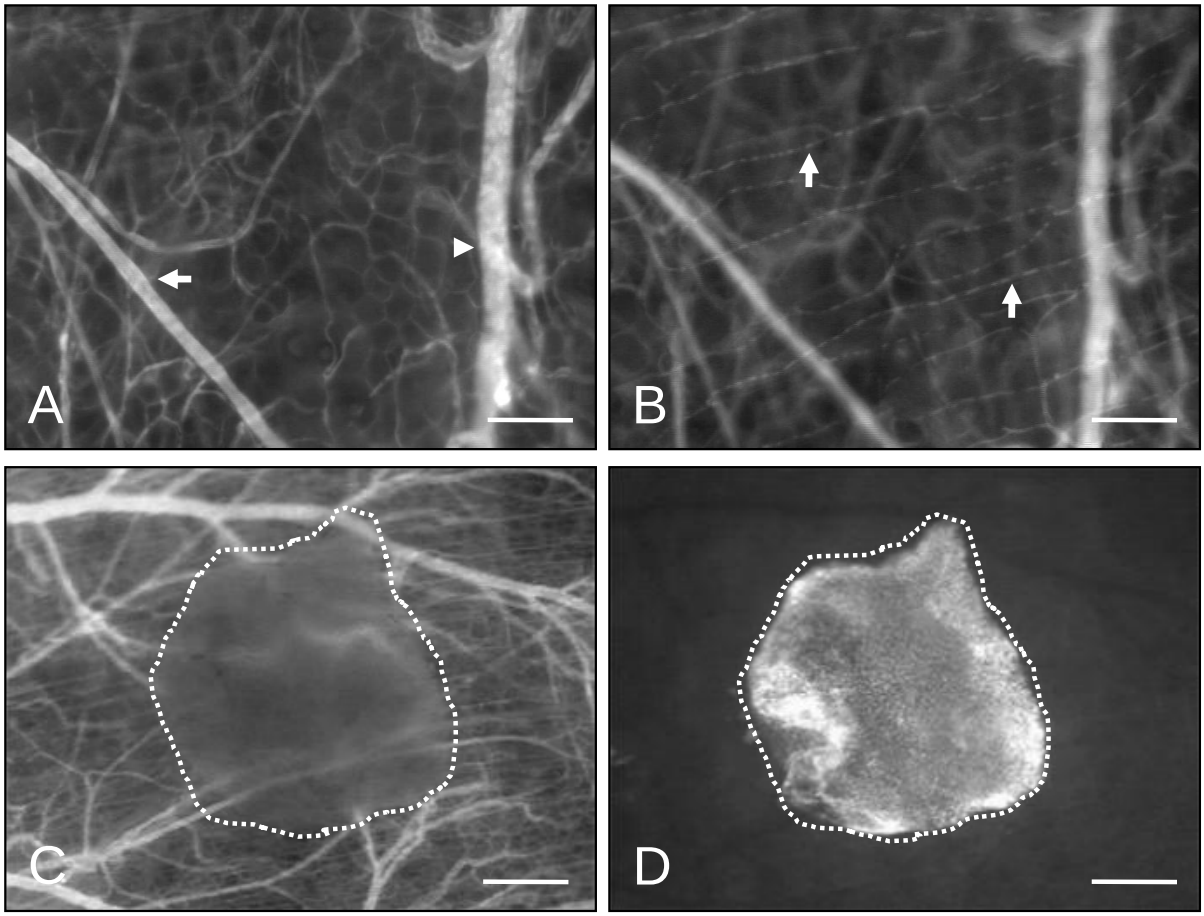

Figure 4: *Intravital fluorescent microscopic images of a mouse dorsal skinfold chamber. Blue light epi-illumination with contrast enhancement by intravascular staining of plasma with 5% FITC-labeled dextran 150,000 i.v. allows for the visualization of the microcirculation of the chamber preparation, consisting of arterioles (A, arrow), collecting venules (A, arrowhead) and parallelly arranged muscle capillaries (B, arrows). C, D: Intravital fluorescent microscopic images of an endometrial tissue fragment (borders marked by dotted line) directly after transplantation into a dorsal skinfold chamber. Because the graft is stained with the fluorescent dye bisbenzimide before transplantation, it can easily be distinguished from the non-stained surrounding host tissue of the chamber using ultraviolet light epi-illumination (D). Scale bars: A, B = 100 µm; C, D = 210 µm.*

For intravital fluorescence microscopy, the anesthetized animals were immobilized in right lateral position on a Plexiglas stage. For the visualization of microvessels by contrast

enhancement of blood plasma (*Figs. 4A-C*), 0.05mL 5% fluorescein-isothiocyanate (FITC)-labeled dextran (molecular weight: 150,000Da; Sigma-Aldrich) was injected i.v. via the retrobulbary space. Subsequently, the dorsal skinfold chamber was positioned under a Zeiss Axiotech microscope (Zeiss, Oberkochen, Germany), which was equipped with 5×, 10× and 20× long-distance objectives (Zeiss) and a 100W mercury lamp attached to an epi-illumination filter block for blue, green and ultraviolet light. The microscopic images were transferred by a charge-coupled device video camera (FK6990; Pieper, Schwerte, Germany) to a 14 inch video screen (KV-14 CT1E; Sony, Tokyo, Japan) and recorded on DVD for subsequent evaluation.

5.2.5 Microcirculatory parameters

Quantitative off-line analysis of the DVDs was performed at the end of the in vivo experiments by means of the computer-assisted image analysis system CapImage (Version 8.5; Zeintl, Heidelberg, Germany). The image analysis included the assessment of the following parameters:

1) Size of endometrial tissue fragments (% of their initial size)
2) Vascularized area (% of the fragment size at the time point of analysis)
3) Functional capillary density (cm/cm²)
4) Diameter of microvessels (µm)
5) Centerline red blood cell (RBC) velocity of microvessels (µm/s)
6) Volumetric blood flow of microvessels (pL/s)

For the determination of the size of the endometrial tissue fragments, the grafts were stained with bisbenzimide before transplantation into the observation window of the dorsal skinfold chamber. The dye bisbenzimide is characterized by a bright fluorescence with little bleaching upon ultraviolet light epi-illumination (*Fig. 4D*), which persists through several cell generations. This guaranteed that the specific fluorescence/background fluorescence ratio was adequate to precisely delineate the stained endometrial grafts from the non-stained surrounding host tissue in the dorsal skinfold chamber.

The vascularized area was defined as the area of the endometrial tissue fragments, which already exhibited blood perfused microvessels at the time point of analysis (*Fig. 5A*). This area was divided by the overall area of the fragments and is given in %. The functional capillary density of the grafts was measured by dividing the overall length of all newly formed RBC- perfused microvessels by the size of the analyzed observation area (*Fig. 5B*).

Diameters of individual microvessels within the grafts were measured perpendicular to the vessel path (*Fig. 5C*). The centerline RBC velocity (V_{RBC}) of these microvessels was then measured by the line shift method [INTAGLIETTA et al., 1970] (*Fig. 5D*). Volumetric blood flow (VQ) was calculated subsequently from V_{RBC} and diameter (d) for each microvessel as $V_Q = \pi \times (d/2)^2 \times V_{RBC}/K$, where K (= 1.3) represents the Baker/Wayland factor [BAKER and WAYLAND, 1974], considering the parabolic velocity profile of blood in microvessels. Microvessel diameters, V_{RBC} and V_Q were determined by analyzing ten microvessels per graft and observation time point. Microvessels were selected randomly in as much as those microvessels were chosen for the analysis, which crossed a vertical line drawn over the center of the video screen [*Figs. 5C and D*].

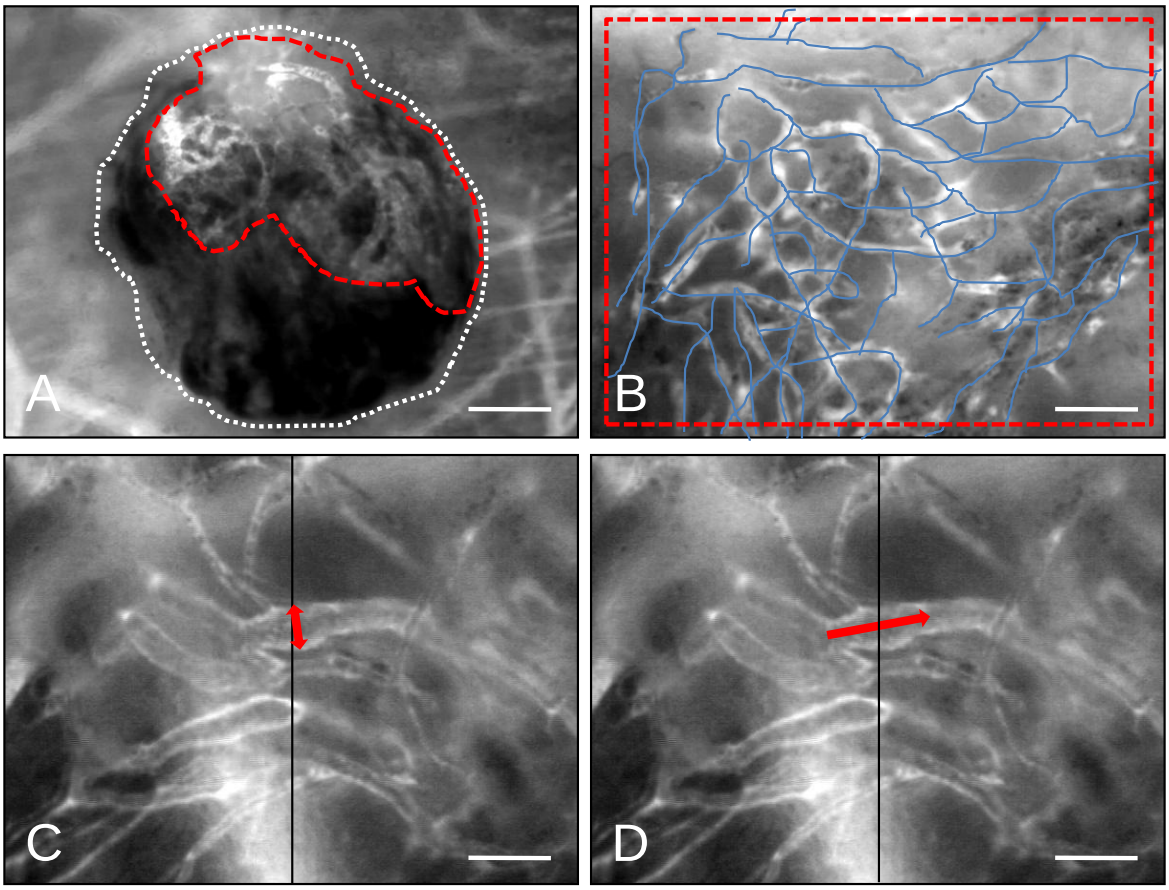

Figure 5: *Intravital fluorescence microscopy of an endometrial graft at day 6 after transplantation. The lesion border is indicated by the white dotted line and the vascularized area is indicated by the red dotted line (A).The functional capillary density of the graft is calculated by dividing the length of all the microvessels (marked in blue) by the size of the analyzed area (marked by red dotted line) (B). The diameters of microvessels, which cross a vertical line drawn over the video screen, are measured as the distance between both vessel walls (C; double arrow). The centerline RBC velocity of individual microvessels is measured by the line shift method (D, arrow). Scale bars: A = 160 µm; B = 100 µm; C, D = 80 µm.*

In a subset of experiments, the final number of microvessels was assessed, which crossed the border between the grafts and the surrounding host tissue at day 14 after transplantation. In addition, the functional capillary density as well as the diameter, V_{RBC} and VQ of arterioles and venules were measured in 4 randomly selected regions of interest in the striated muscle host tissue surrounding the endometriotic lesions.

5.3 Experimental protocol

This thesis consists of three parts, which were performed as described in the following:

1) To analyze the effect of luminal epithelium on the growth and vascularization of developing endometriotic lesions, a total of 8 LE^+ fragments and 8 LE^- fragments from 4 C57BL/6-TgN(ACTB-EGFP)1Osb/J donor mice were transplanted into the dorsal skinfold chamber of 8 C57BL/6 wild-type recipient mice (1 LE^+ fragment and 1 LE^- fragment per chamber). Intravital fluorescence microscopy was performed at days 0 (day of transplantation), 3, 6, 10 and 14 after transplantation. At the end of the in vivo experiments, i.e. day 14 after transplantation, the animals were sacrificed with an overdose of the anesthetics and the dorsal skinfold chamber preparations were processed for further histological and immunohistochemical analyses (*Fig. 6*).

2) To analyze the effects of CK2 inhibition on angiogenesis and vascularization of endometriotic lesions, a group of 10 C57BL/6 mice equipped with dorsal skinfold chambers containing 2 endometrial fragments each was daily treated with an i.p. injection of 60mg/kg body weight quinalizarin (dissolved in 50µL DMSO; Sigma-Aldrich). At this dose, other CK2 inhibitors have previously been shown to exert anti-angiogenic effects under in vivo conditions [KRAMEROV et al., 2008]. Ten vehicle-treated animals served as controls. Intravital fluorescent microscopic analyses were performed directly after endometrium transplantation (day 0) as well as at days 3, 6, 10 and 14 (*Fig. 6*).

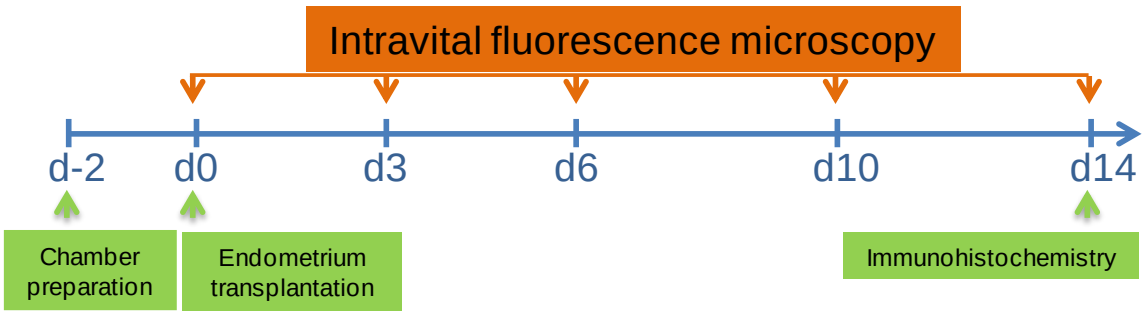

Figure 6: *Study design of part 1 and 2 of this thesis.*

3) To test the effects of CA4P on the vascularization of already established endometriotic lesions, dorsal skinfold chambers were prepared in a total of 12 BALB/c mice. In each chamber, 2 endometriotic lesions were induced by syngeneic transplantation of endometrial tissue fragments, which were harvested from 6 donor animals. At day 6, the vascularization of the lesions was first analyzed by intravital fluorescence microscopy. Only those lesions, which exhibited a well established, dense microvascular network at this time point, were included in the experiment. Six mice with 9 vascularized lesions then received a single i.p. injection of CA4P (80mg/kg body weight dissolved in 100µL aqua dest; Santa Cruz Biotechnology, Heidelberg, Germany), while the other six mice, which also exhibited 9 vascularized lesions, were treated with vehicle (100µL aqua dest i.p.) as control. Intravital fluorescent microscopic analyses of the lesions and the surrounding host tissue were performed 2h after injection (d6+2h) as well as at days 10 and 14. Thereafter, the animals were sacrificed with an overdose of the anesthetics and the dorsal skinfold chamber preparations were processed for histology and immunohistochemistry. In another set of experiments, additional CA4P-treated (n=6) and vehicle-treated control mice (n=6) with a total of 10 vascularized lesions in each group were sacrificed for histological and immunohistochemical analyses at time point d6+2h (5 lesions per group) and day 10 (5 lesions per group) (*Fig. 7*).

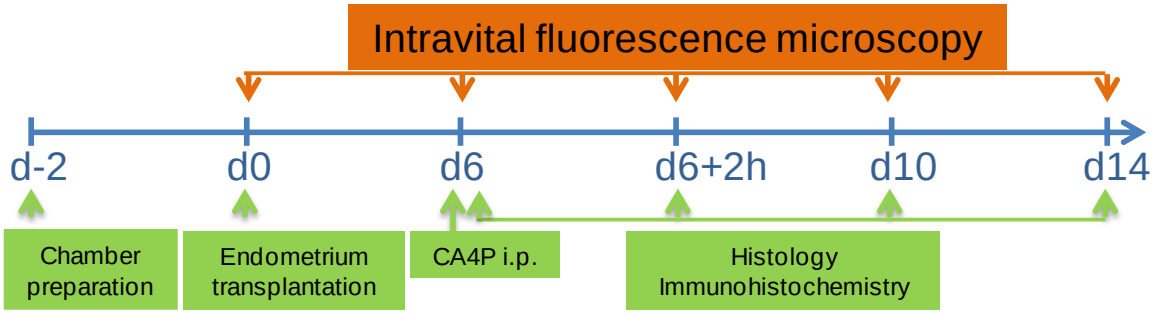

Figure 7: *Study design of part 3 of this thesis.*

5.4 Histology and immunohistochemistry

5.4.1 Histology

For light microscopy, specimens of the dorsal skinfold chamber preparations were fixed at 4°C in formalin for 24h and then embedded in paraffin. Two-µm-thick sections were cut and stained with hematoxylin and eosin (HE) according to standard procedures.

5.4.2 Immunohistochemistry

For immunohistochemical detection of GFP^+ microvessels within and around the transplanted endometrial tissue fragments, paraffin-embedded 2µm-thick sections were stained with a monoclonal rat-anti-mouse antibody against CD31 (1:30; 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 Cy3 antibody (1:50; Dianova GmbH) and a biotin-labeled donkey-anti-goat antibody (1:15; Jackson ImmunoResearch, Baltimore, MD, USA), which was detected by fluorescein labeled-streptavidin (1:50; Vector Labs, Burlingame, CA, USA), were used. For this purpose, the sections were placed in Coplin jars with 0.05% citraconic anhydride solution (pH 7.4) for 1h at 98°C and then incubated overnight at 4°C with the first antibody. This was followed by the incubation with the appropriate secondary antibody at 37°C for 2h.

To study the expression of the different CK2 subunits α, α' and β in the endometrium by means of immunofluorescence microscopy, the uterine horns of 3 C57BL/6 mice were embedded in Tissue TEK (R. Jung GmbH, Nussloch, Germany), snap frozen, and stored at - 80C°. Subsequently, 2-µm-thick sections were cut on a cryostat (Leica, Nussloch, Germany) and stained with the rabbit antibody #26 against CK2α (1:50; [FAUST et al., 1999]), the rabbit antibody #30 against CK2α' (1:50; [FAUST et al., 1999]) and the rabbit antibody #32 against CK2β (1:1400; [FAUST et al., 1999]) as primary antibodies. A goat-anti-rabbit Cy3 antibody (1:400; Dianova GmbH) served as secondary antibody.

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA)-positive cells and apoptotic cleaved-caspase-3-positive cells within endometriotic lesions was performed by a mouse monoclonal anti-PCNA antibody (1:200; Dako Deutschland GmbH, Hamburg, Germany) and a rabbit polyclonal anti-cleaved caspase-3 antibody (1:100; New England Biolabs GmbH, Frankfurt, Germany) as primary antibodies. This was followed by a biotin-labeled goat anti-mouse antibody (1:100; Abcam, Cambridge, UK) and a biotin-labeled goat anti-rabbit antibody (ready-to-use, Abcam), which served as secondary antibodies. Subsequently, the tissue sections were incubated with avidin-peroxidase (1:50; Sigma-Aldrich). 3,3' diaminobenzidine was used as chromogen. The sections were counterstained with hemalaun and the fraction of PCNA-positive and cleaved caspase-3-positive glandular and stromal cells (in % of the total glandular and stromal cell number), were assessed by light microscopy (BX60; Olympus, Hamburg, Germany).

Additional sections of endometriotic lesions from day 6 were stained with a monoclonal rat-anti-mouse antibody against the endothelial cell marker CD31 (1:30; Dianova GmbH) and a mouse-anti-mouse antibody against α-smooth muscle actin (α-SMA) (1:50; Sigma-Aldrich).

A goat-anti-rat Cy3 antibody (1:50; Dianova GmbH) and a goat-anti-mouse Alexa 488 antibody (1:200; Invitrogen, Darmstadt, Germany) served as secondary antibodies.

For all immunofluorescent microscopic analyses, cell nuclei were stained with Hoechst 33342 (1:500; Sigma-Aldrich). The immunofluorescence sections were examined using a BZ-8000 fluorescence microscopic system (Keyence, Osaka, Japan).

5.5 In vitro experiments

5.5.1 Aortic ring assay

To analyze in vitro the anti-angiogenic activity of different doses of the CK2 inhibitor quinalizarin, an aortic ring assay was performed [EHRMANTRAUT et al., 2010]. For this purpose, aortic rings of 3 male Sprague Dawley rats (250-300g body weight) were embedded in 200µL Matrigel (BD Matrigel™ Matrix; BD Biosciences, Heidelberg, Germany) in 48-well tissue culture grade plates and were allowed to polymerize for 20min at 37°C in a humidified atmosphere containing 5% CO_2. Subsequently, the wells were overlaid with 800µL of DMEM (10% FCS, 100U/mL penicillin, 0.1mg/mL streptomycin; PAA) supplemented with 10µM, 25µM and 50µM quinalizarin (Labotest, Niederschöna, Germany) or vehicle (DMSO; Sigma-Aldrich), respectively. The rings were maintained at 37°C and 5% CO_2 for 6 days with medium change at day 3. All assays were done in sextuplicate. Vascular sprouting from each ring was examined by phase-contrast microscopy (BZ-8000; Keyence) and quantitatively analyzed by means of the software package CapImage (version 8.5; Zeintl). The analyses included the determination of the area (mm^2) and the maximal length (µm) of the outer aortic vessel sprouting.

5.5.2 Western blot analyses of isolated endometrium

To investigate the action of quinalizarin on the expression of CK2 subunits and on the activity of CK2 in endometrial tissue, the uterine horns of 8 C57BL/6 mice were placed in 30-mm-diameter plastic Petri dishes, containing 37°C DMEM (10% FCS, 1U/mL penicillin, 0.1mg/mL streptomycin; PAA). The uterine horns were opened longitudinally and small endometrial fragments (~2-3mm^3) were carefully dissected from the uterine muscle under a stereo microscope (M651; Leica). The pooled fragments of two uterine horns were then transferred in 6-well tissue culture grade plates, containing 1mL DMEM (10% FCS, 1U/mL penicillin, 0.1mg/mL streptomycin; PAA), which was supplemented with 25µM quinalizarin or vehicle (DMSO), and cultivated at 37°C and 5% CO_2 for 24h. Subsequently, the endometrial fragments were snap frozen and stored at -80°C.

For protein extraction, the tissue samples were crushed in a mortar and then suspended in 200µL of Ripa buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 0.5% sodium desoxycholate, 1% TritonX100, 0.1% sodium dodecylsulfate), containing the protease inhibitor cocktail Complete™ (Roche Diagnostics, Mannheim, Germany), and incubated for 1h at 4°C. The cell extract was centrifuged at 13,000 x g for 10min. The supernatant was used for the determination of the protein content by a modified Bradford method with the BioRad dye reagent (BioRad, Munich Germany) and subsequently subjected to Western blot analyses and kinase assays.

For Western blot analyses, proteins dissolved in SDS buffer (130mM Tris-HCl, pH 6.8, 0.02% bromophenol blue (w/v), 10% β-mercaptoethanol, 20% glycerol (v/v), and 4% SDS) were separated on a 12.5% SDS-polyacrylamide gel in electrophoresis buffer (25mM Tris/HCl, pH 8.8, 192mM glycine and 3.5mM SDS). The proteins were then transferred onto a PVDF Western blotting membrane (Roche Diagnostics) in a buffer containing 20mM Tris/HCl, 150mM glycine, pH 8.3 by tank blotting. The membrane was blocked with blocking buffer (PBS, pH 7.4, 0.1% Tween20, 5% skimmed milk) for 1h and subsequently incubated with the monoclonal antibody 1A5 against CK2α (1:100; [SCHUSTER et al., 2001]), with the rabbit antibody #30 against CK2α' (1:1000; [FAUST et al., 1999]), the monoclonal antibody 6D5 against CK2β (1:50; [NASTAINCZYK et al., 1995]) and as a loading control with the monoclonal antibody Clone DM 1A against tubulin (1:1000; Sigma-Aldrich). After washing the membrane with washing buffer (PBS, pH 7.4, 0.1% Tween20), the membrane was incubated with a peroxidase-conjugated goat-anti-rabbit antibody (1:30.000; Dianova GmbH) or a goat-anti-mouse antibody (1:10.000; Dianova GmbH), which served as secondary antibodies. Proteins were visualized by the ECL Lumi-Light blotting detection reagent (Roche Diagnostics) according to the manufacturer's instructions.

5.5.3 CK2 kinase activity assay

To study the CK2 kinase activity in isolated endometrium in vitro, an aliquot of the cell extract containing 50µg of total protein was mixed with 20µL kinase buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 10mM $MgCl_2$, 1mM dithiothreitol (DTT)). After addition of 30µL of CK2 mix (25mM Tris-HCl, pH 8.5, 150mM NaCl, 5mM $MgCl_2$, 1mM DTT, 50µM ATP, 0.19mM (final conc.) CK2 specific substrate peptide with the sequence RRRDDDSDDD, 10µCi / 500µL [^{32}PγATP]) the reaction mix was incubated at 37°C for 5min. The reaction was stopped on ice. The sample was pipetted onto Whatman-P81cation-exchange paper and washed 3 times with 85mM phosphoric acid for 5min, followed by washing with ethanol. The dried filter paper was counted for Čerenkov radiation in a scintillation counter (Liquid Scintillation Analyzer 190S AB/LA; Canberra-Packard GmbH, Dreieich, Germany).

5.6 Statistical analysis

After testing the data for normal distribution and equal variance, differences between two groups were analyzed by the unpaired Student's t-test and differences between multiple groups were analyzed by ANOVA followed by the Student-Newman-Keuls post hoc test. To test for time effects in the individual groups, ANOVA for repeated measures was applied. This was followed by the Student-Newman-Keuls test, including the correction of the alpha error according to Bonferroni probabilities to compensate for multiple comparisons (SigmaStat; Jandel Corporation, San Rafael, CA, USA). All values are expressed as mean ± SEM. Statistical significance was accepted for a value of $P<0.05$.

6. Results

6.1 Effect of luminal epithelium on the development of endometriotic lesions

6.1.1 Angiogenesis and network morphology of developing endometriotic lesions

Directly after transplantation into the dorsal skinfold chamber of C57BL/6 wild-type mice, the endometrial tissue fragments from transgenic C57BL/6-TgN(ACTB-EGFP)1Osb/J donor mice could easily be detected due to their GFP signal (*Figs. 8A-D*). LE^- fragments typically exhibited an irregular shape with a frayed border (*Figs. 8A and C*), whereas LE^+ fragments were rounded and sharply separated from the surrounding host tissue by their luminal epithelium (*Fig. 8B*), which could be clearly visualized in higher magnification (*Fig. 8D*).

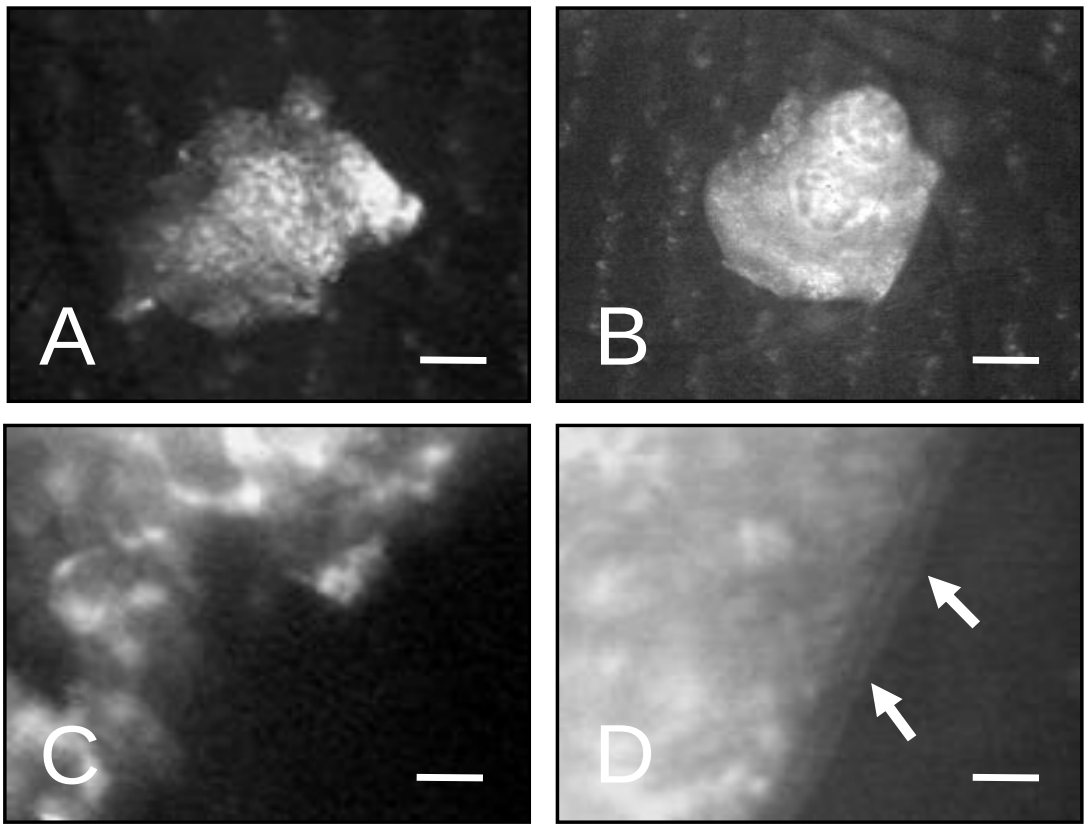

Figure 8: *A-D: Intravital fluorescent microscopic images of a LE^- fragment (A, C) and a LE^+ fragment (B, D) directly after transplantation into the dorsal skinfold chamber. The fragments can be easily detected in blue light epi-illumination due to their GFP signal. The luminal epithelium of the LE^+ tissue is clearly visible in higher magnification (D, arrows). Scale bars: A, B = 230 µm; C, D = 20 µm.*

During the further time course of the experiment, both fragment types vascularized and finally exhibited dense microvascular networks with a comparable functional capillary density of ~300cm/cm² at day 14 (*Figs. 9A-C*). However, the vascularization process was accelerated in the group of LE^- fragments, as indicated by a significantly higher functional capillary density at day 3 when compared to that of LE^+ fragments (*Fig. 9C*). Moreover, the final morphology of the microvascular networks markedly differed between LE^- and LE^+ fragments. Microvascular networks of LE^- fragments developed many interconnections to the host microvasculature of the chamber tissue (*Figs. 9A and D*), whereas only a few microvessels could pierce into the LE^+ fragments. This was restricted to areas, which were not covered with luminal epithelium (*Figs. 9B and D*).

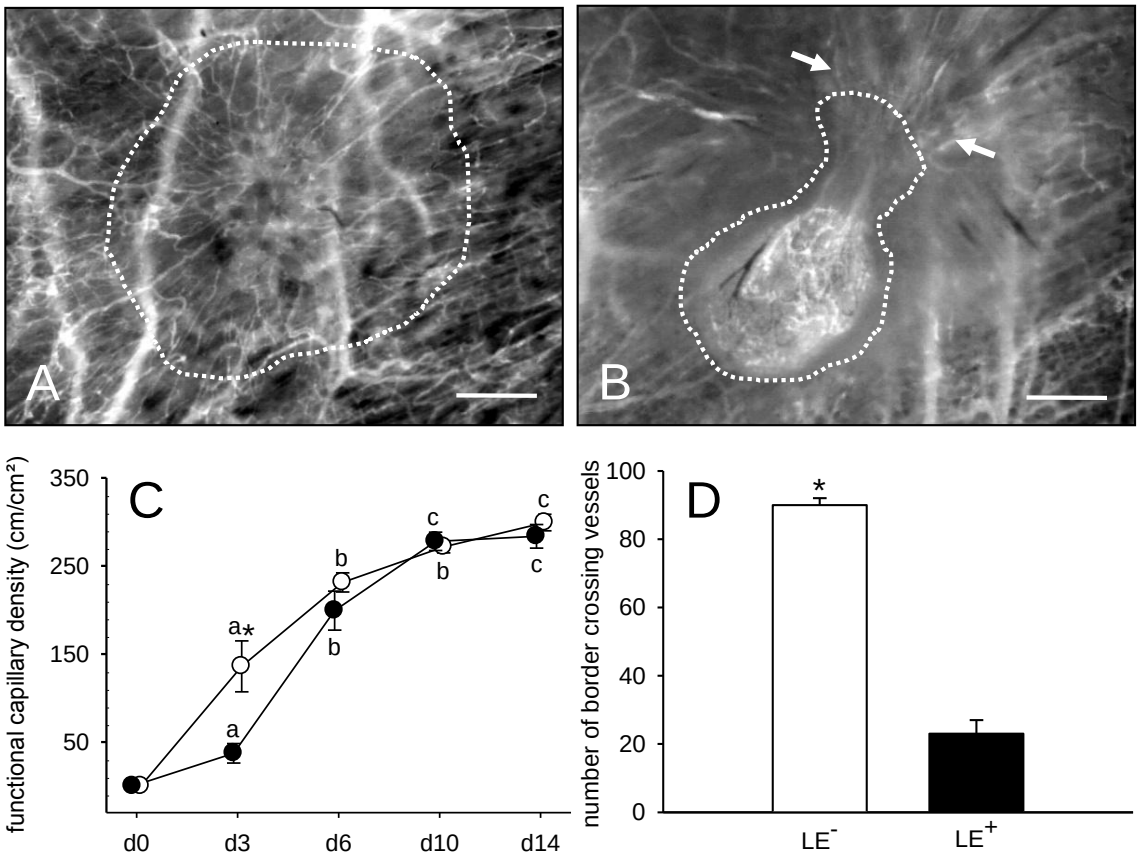

Figure 9: *A, B: Intravital fluorescent microscopic images of a vascularized LE^- fragment (A, border marked by dotted line) and a LE^+ fragment (B, border marked by dotted line) at day 14 after transplantation into the dorsal skinfold chamber of a C57BL/6 wild-type mouse. The microvascular network of the LE^- fragment exhibits many interconnections to the host microvasculature of the chamber tissue (A), whereas only a few microvessels pierce into the LE^+ fragment (B, arrows). Blue light epi-illumination with contrast enhancement by intravascular staining of plasma with 5% FITC-labeled dextran 150,000 i.v.. Scale bars: 300 µm. C, D: Functional capillary density (cm/cm²) and number of border crossing vessels (at day 14) of LE^- fragments (white circles and bar, n = 8) and LE^+ fragments (black circles and bar, n = 8) after transplantation into dorsal skinfold chambers of C57BL/6 wild-type mice, as assessed by intravital fluorescence microscopy and computer-assisted off-line analysis. Means ± SEM. [a]P<0.05 vs. day 0 within each individual group; [b]P<0.05 vs. days 0 and 3 within each individual group; [c]P<0.05 vs. days 0, 3 and 6 within each individual group; *P<0.05 vs. LE^+ fragments.*

By grafting GFP^+ endometrial tissue fragments into GFP^- recipient animals, it was further demonstrated that both LE^- and LE^+ fragments still exhibited GFP^+ microvessels at day 14 after transplantation. In the group of LE^- fragments, many GFP^+ microvessels grew outside the grafts into the surrounding host tissue, where they developed interconnections to the GFP^- microvessels of the chamber tissue (*Figs. 10A and C*). In contrast, the GFP^+ microvessels could not pass the luminal epithelium in the group of LE^+ fragments (*Figs. 10B and D*).

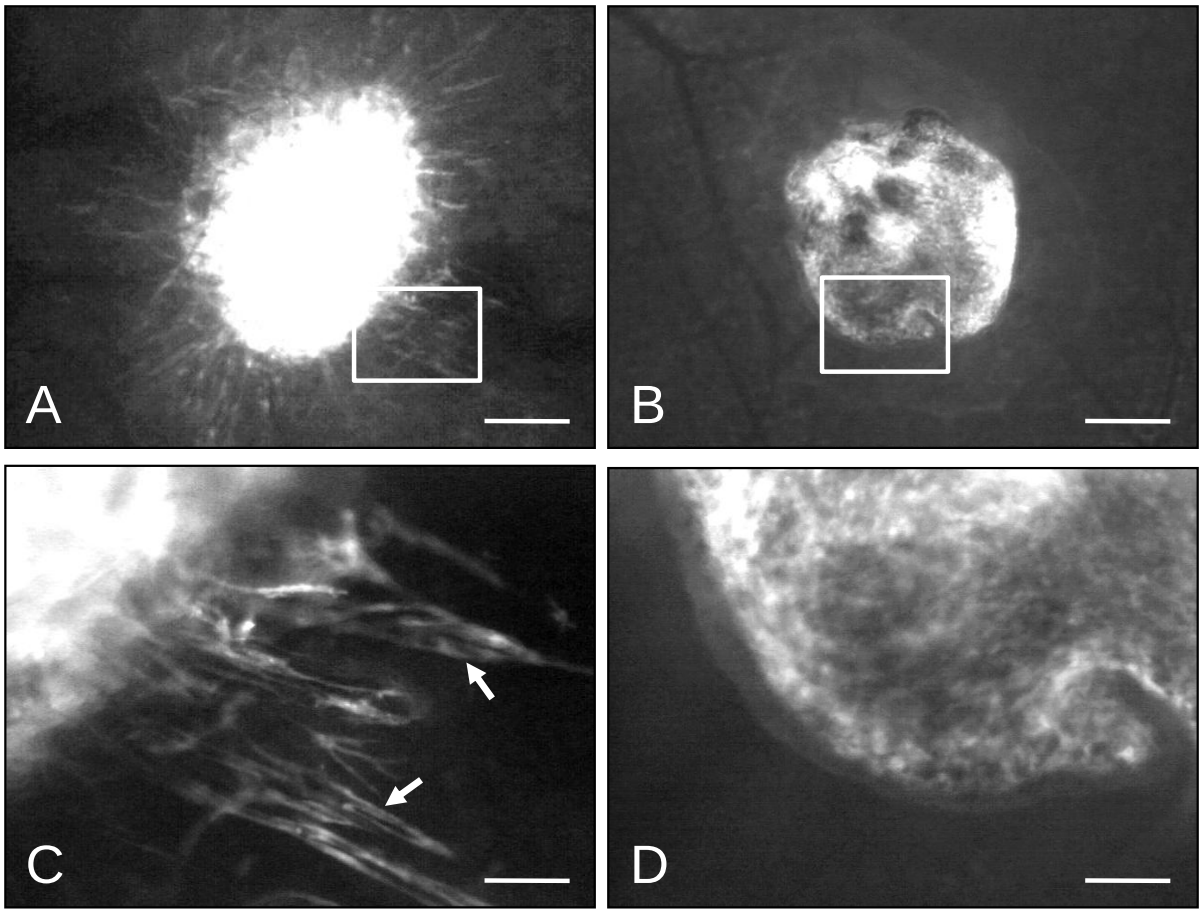

Figure 10: *Intravital fluorescent microscopic images of a LE^- fragment (A and C = higher magnification of window in A) and a LE^+ fragment (B and D = higher magnification of window in B) at day 6 after transplantation into the dorsal skinfold chamber of a C57BL/6 wild-type mouse. The detection of GFP in blue light epi-illumination reveals that many GFP^+ microvessels (C, arrows) grow outside the LE^- fragment, whereas these microvessels cannot pass the luminal epithelium of the LE^+ fragment (D). Scale bars: A, B = 300 µm; C, D = 90 µm.*

The differing network morphology between the two groups was associated with marked differences in microhemodynamic parameters. Microvessels of LE^- fragments exhibited a diameter of 13µm at day 3, which slightly decreased to 11µm at day 14 (*Fig. 11A*). In the group of LE^+ fragments, the microvascular diameters rapidly declined from 12µm at day 3 to 8µm at day 6 and then remained constant until the end of the experiments (*Fig. 11A*).

Moreover, the centerline RBC velocity of LE^- fragments progressively increased from 140µm/s at day 3 to 350µm/s at day 14 and was significantly higher when compared to that of LE^+ fragments (day 3: 50µm/s; day 14: 180µm/s) (*Fig. 11B*). Accordingly, calculated values of volumetric blood flow were also markedly elevated in the group of LE^- fragments throughout the observation period when compared to those of LE^+ fragments (*Fig. 11C*). Finally, the growth of LE^+ fragments was markedly affected by the luminal epithelium. They exhibited a significantly smaller final size at day 14 in compared to LE^- fragments (*Fig. 11D*).

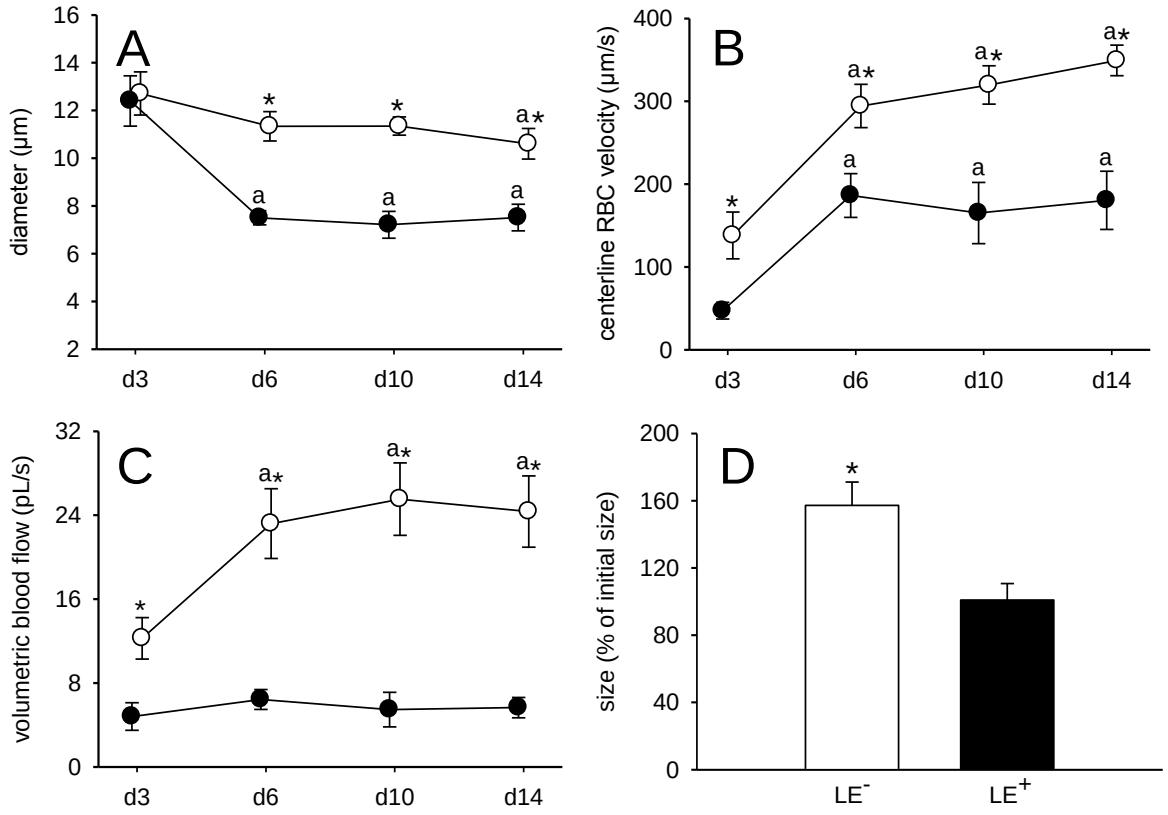

Figure 11: *Diameter (µm) (A), centerline RBC velocity (µm/s) (B) and volumetric blood flow (pL/s) (C) of microvessels in LE^- fragments (white circles, n = 8) and LE^+ fragments (black circles, n = 8) after transplantation into the dorsal skinfold chamber of C57BL/6 wild-type mice, as assessed by intravital fluorescence microscopy and computer-assisted off-line analysis. D: Size (% of initial size) of LE^- fragments (white bar, n = 8) and LE^+ fragments (black bar, n = 8) at day 14 after transplantation into the dorsal skinfold chamber of C57BL/6 wild-type mice, as assessed by intravital fluorescence microscopy and computer-assisted off-line analysis. Means ± SEM. ^{a}P<0.05 vs. day 3 within each individual group; *P<0.05 vs. LE^+ fragments.*

6.1.2 Histomorphology and blood vessel origin of endometriotic lesions

Histological examination of HE-stained sections of LE^- fragments at day 14 after transplantation into the dorsal skinfold chamber revealed that they had developed into typical endometriotic lesions with cyst-like dilated endometrial glands, which were surrounded by a well vascularized stroma (*Fig. 12A*).

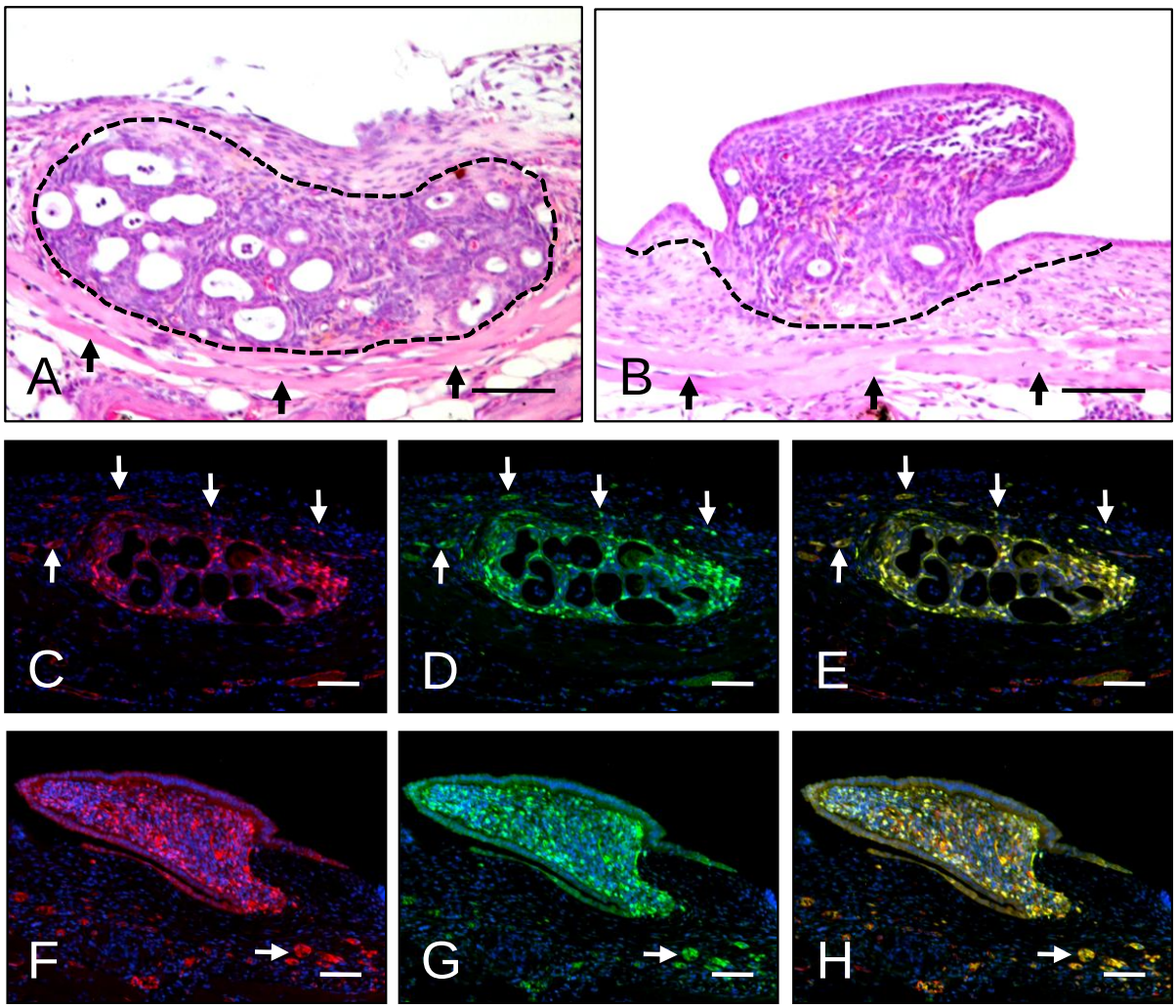

Figure 12: *A, B: HE-stained sections of a LE^- fragment (A, border marked by broken line) and a LE^+ fragment (B, border marked by broken line) at day 14 after transplantation onto the host striated muscle tissue (arrows) of the dorsal skinfold chamber. The LE^- fragment has developed into a typical endometriotic lesion with cyst-like dilated endometrial glands, which are surrounded by a well vascularized stroma. In contrast, the lesion originating from the LE^+ fragment exhibits a polypoid morphology with less glands and a coverage of luminal epithelium. Scale bars: 70 µm. C-H: Immuno-histochemical analysis of the origin of microvessels within and around a LE^- fragment (C-E) and a LE^+ fragment (F-H) at day 14 after transplantation into the dorsal skinfold chamber of a C57BL/6 wild-type mouse. Histological sections were stained with Hoechst 33342 to identify cell nuclei (C-H, blue), an antibody against CD31 for the detection of endothelial cells (C, F, red) and an antibody against GFP (D, G, green). E and H display merges of (C, D) and (F, G), respectively. In contrast to the polypoid lesion originating from the LE^+ fragment, the endometriotic lesion originating from the LE^- fragment is surrounded by many GFP^+ microvessels (arrows). Scale bars: 75 µm.*

In contrast, lesions from LE^+ fragments exhibited a polypoid morphology with less glands and a coverage of luminal epithelium (*Fig. 12B*). More detailed immunohistochemical analyses of both lesion types confirmed the intravital microscopic findings. Endometriotic lesions originating from LE^- fragments were surrounded by many GFP^+ microvessels, which grew out of the lesions into the GFP^- host tissue of the dorsal skinfold chamber (*Figs. 12C-E*). In contrast, polypoid lesions originating from LE^- fragments only exhibited the outgrowth of a few GFP^+ microvessels in areas lacking the barrier of the luminal epithelium (*Figs. 12F-H*).

6.2 Regulatory function of CK2 in angiogenesis of endometriotic lesions

6.2.1 Anti-angiogenic activity of quinalizarin

In a first set of in vitro experiments, the anti-angiogenic activity of the CK2 inhibitor quinalizarin was studied by means of an aortic ring assay. In the vehicle-treated control group, cultivation of rat aortic rings stimulated the outgrowth of tubular, vessel-like structures from the aortic wall into the surrounding Matrigel. After 6 days, this resulted in the formation of a dense network of newly developed vascular sprouts with a sprout area of ~9mm² and a maximal sprout length of ~1400µm (*Figs. 13A, E and F*). Treatment with quinalizarin dose-dependently inhibited this sprout formation (*Figs. 13B-F*). There was even a complete suppression of vessel sprouting in the group of aortic rings, which were cultivated in DMEM supplemented with 50µM quinalizarin (*Figs. 13D, E and F*), indicating a strong anti-angiogenic activity of the CK2 inhibitor.

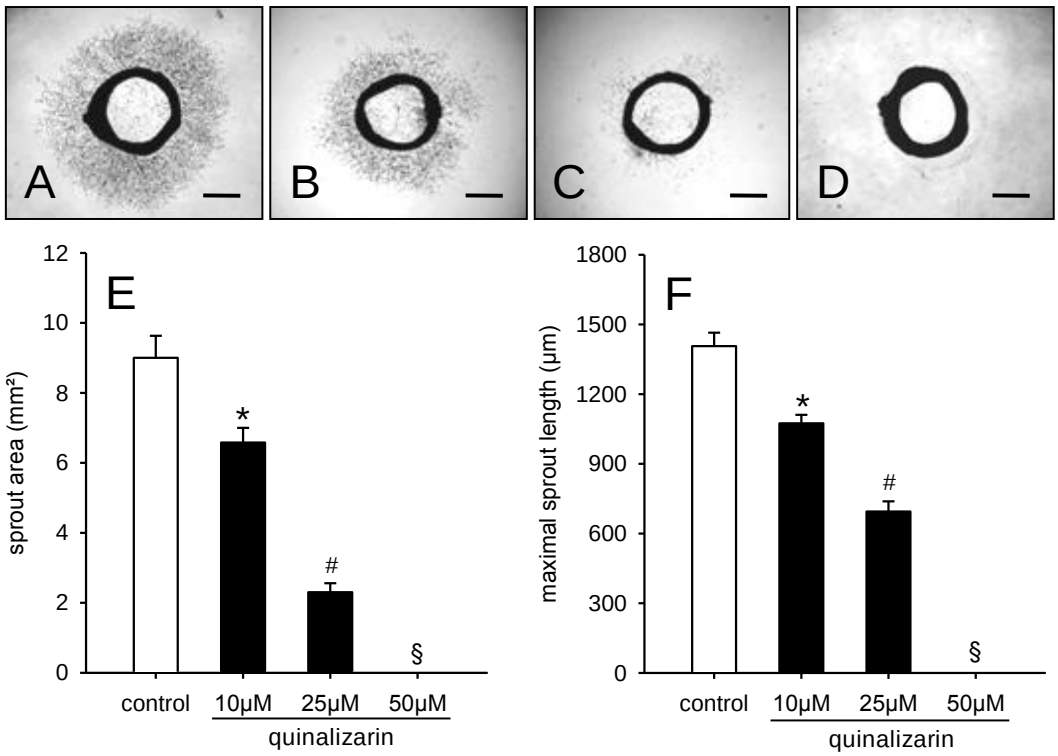

Figure 13: *A-D: Representative images of rat aortic rings with vascular sprouting upon 6 days of incubation in DMEM supplemented with vehicle (DMSO, A) or 10 µM (B), 25 µM (C) and 50 µM (D) quinalizarin. Scale bars: 830 µm. E, F: Sprout area (mm²) (E) and maximal sprout length (µm) (F) of the outer aortic endothelial cell sprouting at day 6 after incubation of aortic rings, as assessed by transillumination phase-contrast microscopy and computer-assisted image analysis. The rings were incubated in DMEM supplemented with vehicle (control, white bar) or 10 µM, 25 µM and 50 µM quinalizarin (black bars). Means ± SEM. *P<0.05 vs. control; #P<0.05 vs. control and 10 µM quinalizarin; §P<0.05 vs. control, 10 µM and 25 µM quinalizarin.*

6.2.2 Expression of CK2 subunits in uterine endometrium

Protein kinase CK2 is a tetramer, which is composed of two catalytic α- and/or α'-subunits and two regulatory β-subunits.

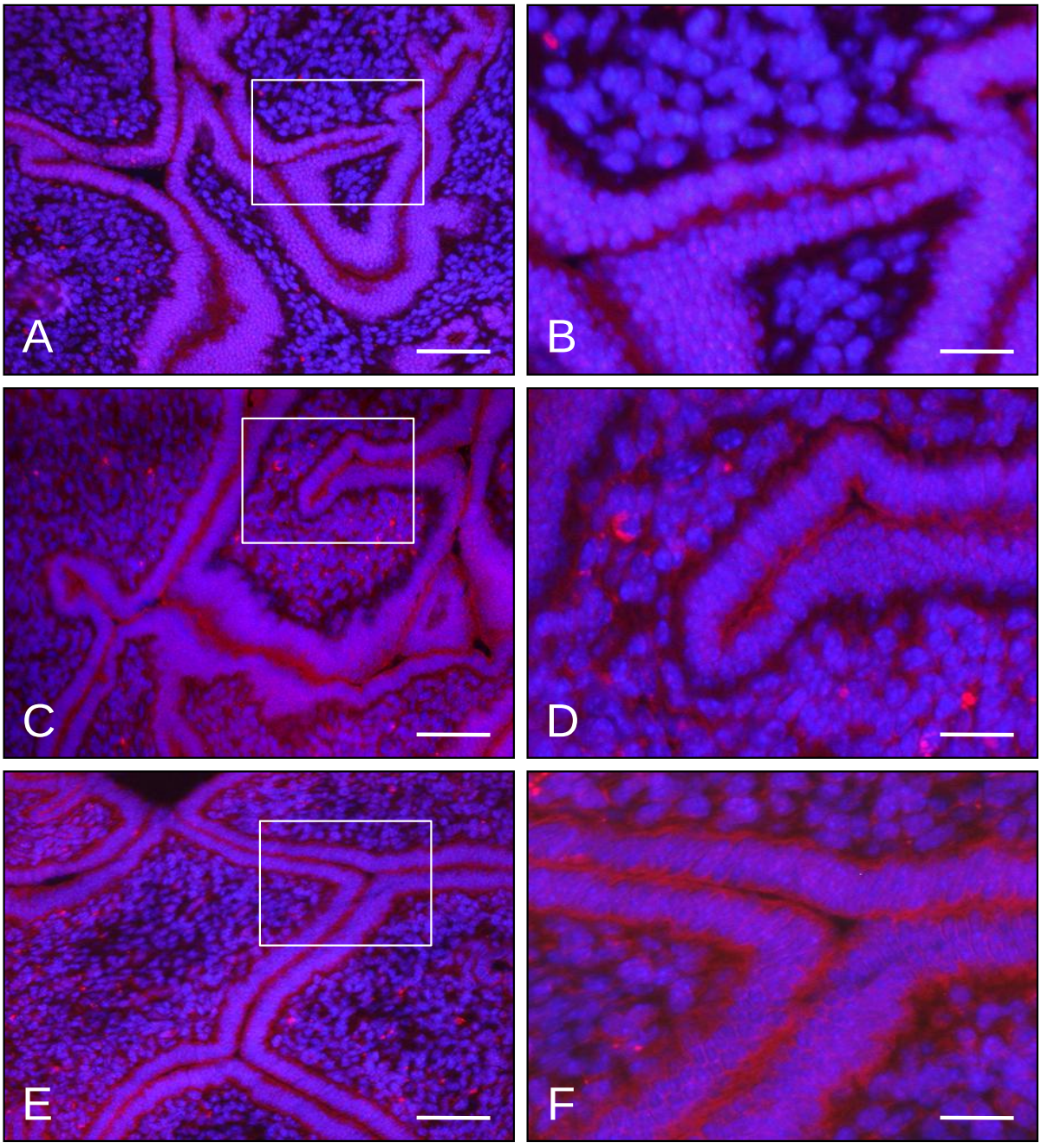

Figure 14: *A-F: Representative immunofluorescent microscopic images of the expression of different CK2 subunits in uterine horns of C57BL/6 mice. Histological sections were stained with an antibody against CK2α (A, B, red), CK2α' (C, D, red) and CK2β (E, F, red) as well as Hoechst 33342 to identify cell nuclei (A-F, blue). B, D and F display higher magnifications of the regions of interest (marked by closed line) in A, C and E. Scale bars: A, C, E = 65 µm; B, D, F = 20 µm.*

Immunofluorescent microscopic analyses of uterine horns from C57BL/6 donor mice revealed that all three subunits are expressed in the endometrium (*Fig. 14*). Of interest, CK2α is mainly located in the glandular epithelium (*Figs. 14A and B*), whereas the other two subunits CK2α' and CK2β are expressed in both glandular and stromal cells of the endometrium (*Figs. 14C-F*).

6.2.3 CK2 expression and activity in quinalizarin-treated endometrial tissue

In another set of experiments, isolated endometrial fragments were cultivated for 24h in DMEM supplemented with 25µM quinalizarin. As shown by Western blot analysis, this did not affect the expression of different CK2 subunits within the endometrial tissue in comparison to vehicle-treated controls (*Fig. 15A*). However, treatment with quinalizarin reduced the activity of protein kinase CK2 to 68% of the activity measured in vehicle-treated control tissue (*Fig. 15B*).

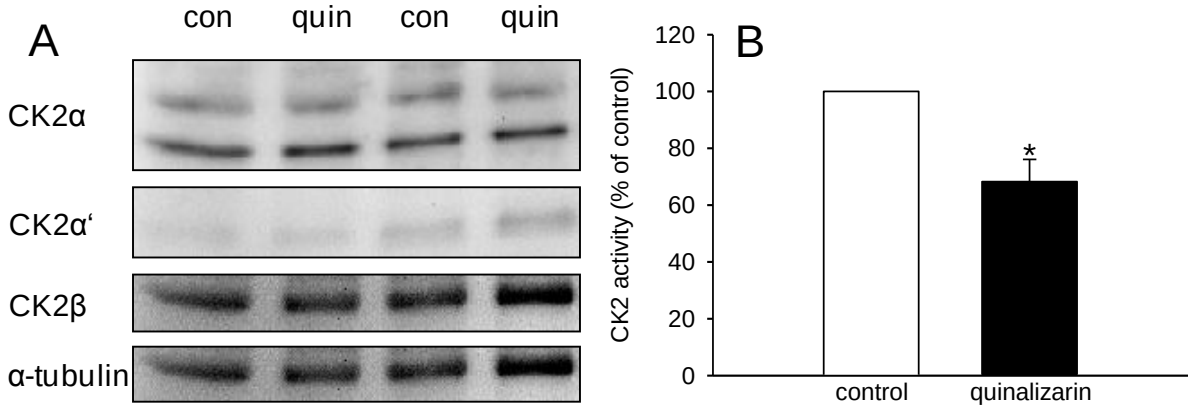

Figure 15: *A: Representative Western blots of CK2α, CK2α' and CK2β protein expression of endometrial fragments, which were cultured for 24h in DMEM supplemented with vehicle (con) or 25µM quinalizarin (quin). The antibody Clone DM 1A against α-tubulin served as loading control. B: CK2 activity (% of control) of endometrial fragments, which were cultivated for 24h in DMEM supplemented with vehicle (con) or 25µM quinalizarin (quin), as assessed by the CK2 kinase assay. Means ± SEM. *P<0.05 vs. con.*

6.2.4 Vascularization of quinalizarin-treated endometriotic lesions

The anti-angiogenic effect of the CK2 inhibitor quinalizarin on vascularization of endometriotic lesions was analyzed in the dorsal skinfold chamber model. By means of intravital fluorescence microscopy, newly formed microvessels could be observed in engrafting endometriotic lesions of control mice at day 3 after transplantation of endometrial tissue fragments (*Figs. 16A, E and F*).

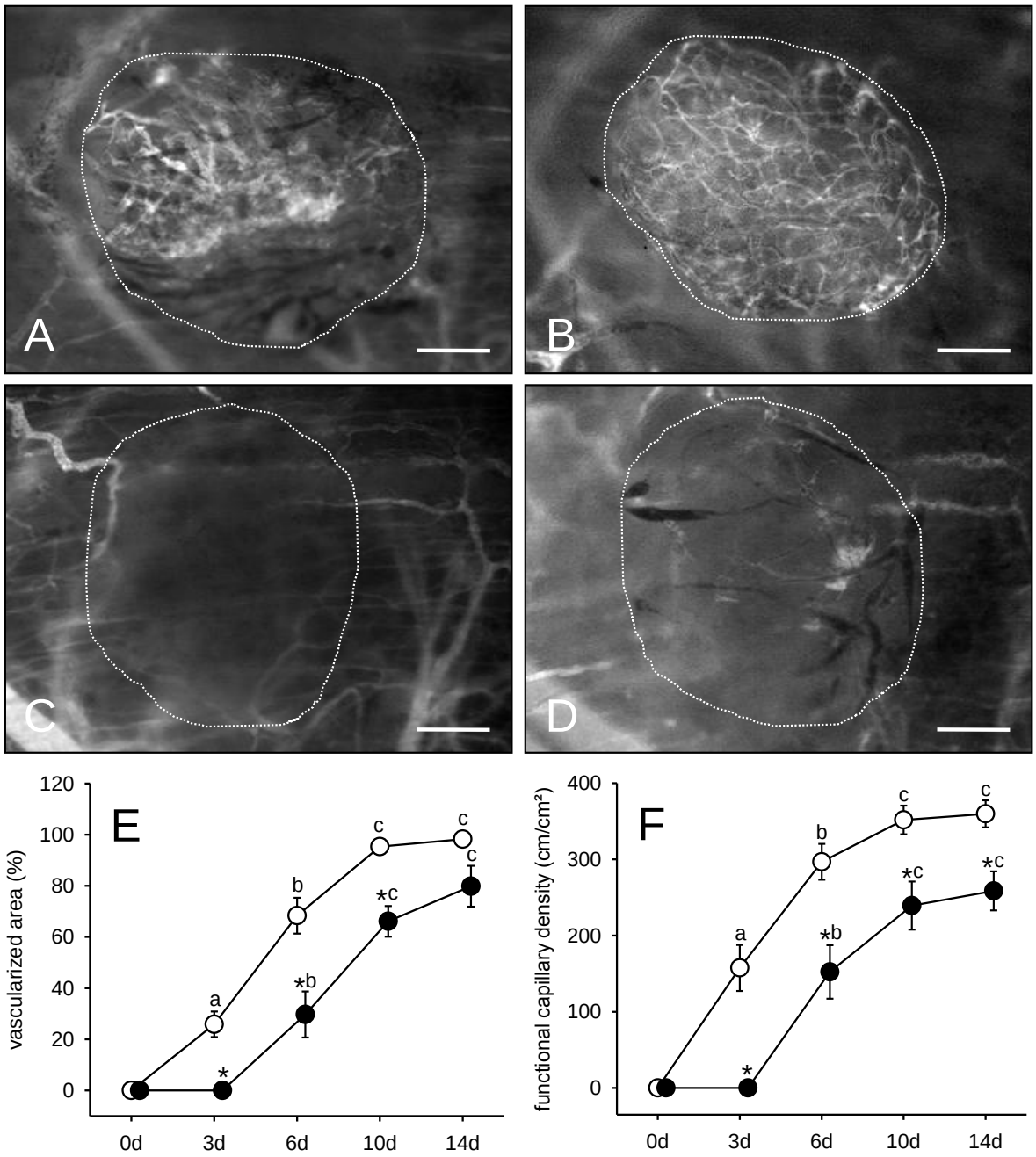

Figure 16: *Intravital fluorescent microscopic images of endometriotic lesions (borders marked by dotted line) at day 3 (A, C) and day 6 (B, D) after transplantation of endometrial fragments into the dorsal skinfold chamber of a vehicle-treated control mouse (A, B) and a quinalizarin-treated animal (C, D). Note that the newly developing microvascular network of the endometriotic lesion in the quinalizarin-treated animal exhibits a markedly reduced functional capillary density when compared to that of the control mouse. Blue light epi-illumination with contrast enhancement by 5% FITC-labeled dextran 150,000 i.v.. Scale bars: 150 µm. E, F: Vascularized area (%) (E) and functional capillary density (cm/cm²) (F) of endometriotic lesions in dorsal skinfold chambers of vehicle-treated control mice (white circles) and quinalizarin-treated animals (black circles), as assessed by intravital fluorescence microscopy and computer-assisted off-line analysis. Means ± SEM. [a]P<0.05 vs. day 0 within each individual group; [b]P<0.05 vs. days 0 and 3 within each individual group; [c]P<0.05 vs. days 0, 3 and 6 within each individual group; *P<0.05 vs. control.*

Throughout the further observation period, an increasing number of microvessels developed within the lesions (*Fig. 16B*), finally resulting in the formation of glomerulum-like microvascular networks with a functional capillary density of ~360cm/cm² at day 14 (*Figs. 16E and F*). In contrast, CK2 inhibition strongly suppressed this angiogenic process. In fact, endometriotic lesions of quinalizarin-treated animals exhibited a complete lack of vascularization at day 3 after endometrium transplantation (*Figs. 16C, E and F*). Although new microvessels developed in this group during the following days (*Fig. 16D*), the lesions exhibited a significantly reduced vascularized area and functional capillary density until the end of the experiments when compared to those of the control group (*Figs. 16E and F*).

The analysis of microhemodynamic parameters did not show marked differences between quinalizarin-treated and vehicle-treated animals. In both groups, newly formed microvessels exhibited diameters of 11-12µm and centerline RBC velocities of 182-243µm/s between days 6 and 14 (*Tab. 1*). Accordingly, calculated values of volumetric blood flow ranged between 16-17pL/s (*Tab. 1*).

Table 1: *Diameter (µm), centerline RBC velocity (µm/s) and volumetric blood flow (pL/s) of microvessels in endometriotic lesions within dorsal skinfold chambers of vehicle-treated control mice and quinalizarin-treated animals, as assessed by intravital fluorescence microscopy and computer-assisted off-line analysis.*

	Day 3	Day 6	Day 10	Day 14
Microvessel diameter (µm)				
Control	12.8±1.3	11.0±0.8	10.6±0.7	12.1±0.9
Quinalizarin	-	11.6±0.9	10.7±0.7	11.0±0.8
Centerline RBC velocity (µm/s)				
Control	80.3±18.4	182.2±17.8[a]	220.0±33.1[a]	208.9±26.7[a]
Quinalizarin	-	215.0±42.3	237.9±34.9	243.1±33.5
Volumetric blood flow (pL/s)				
Control	9.4±4.4	15.6±4.0	16.8±4.5	16.8±3.1
Quinalizarin	-	15.3±3.1	17.2±3.4	17.1±2.8

Means ±SEM; [a]P < 0.05 vs. day 3 within each individual group.

6.2.5 Histomorphology of quinalizarin-treated endometriotic lesions

Histological analyses of the dorsal skinfold chamber preparations at day 14 showed that in both groups the transplantation of isolated endometrium onto the host striated muscle tissue had resulted in typical endometriotic lesions. These lesions were composed of endometrial glands, which were surrounded by a vascularized endometrial stroma (*Fig. 17*). However, the lesions of quinalizarin-treated animals exhibited a reduced lesion size and less glandular components when compared to those of control mice. This indicates that inhibition of CK2 suppresses the normal development of endometriotic lesions.

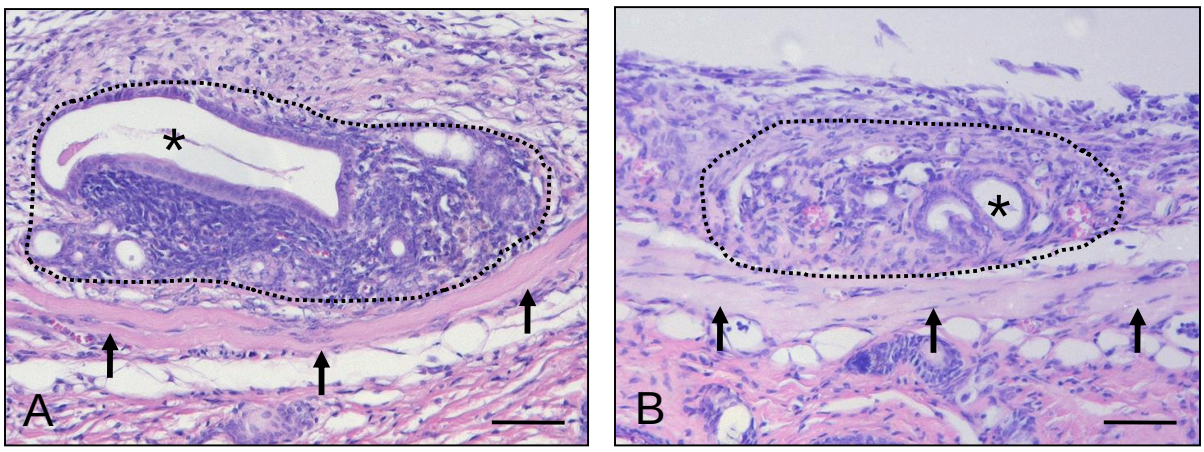

Figure 17: *HE-stained cross sections of endometriotic lesions at day 14 after endometrium transplantation onto the striated muscle tissue (arrows) within the dorsal skinfold chamber of a vehicle-treated control mouse (A) and a quinalizarin-treated animal (B). Both endometriotic lesions are characterized by cyst-like dilated endometrial glands (asterisks), which are surrounded by a vascularized endometrial stroma. However, the lesion of the quinalizarin-treated animal exhibits a reduced lesion size and less glandular components when compared to that of the control mouse. Scale bars: 60 µm.*

6.3 Vascular disrupting effects of CA4P on endometriotic lesions

6.3.1 Vascularization of endometriotic lesions

Directly after transplantation into the dorsal skinfold chamber, endometrial tissue fragments did not exhibit an own blood supply and, thus, were solely dependent on oxygen diffusion from the microvasculature of the surrounding host tissue (*Fig. 18A*). During the following days, they induced angiogenesis at the transplantation site, resulting in the rapid ingrowth of newly formed microvessels into the grafts. Accordingly, most of the fragments had already developed to well vascularized endometriotic lesions at day 6, which presented with dense, blood-perfused microvascular networks during intravital fluorescence microscopy (*Fig. 18B*).

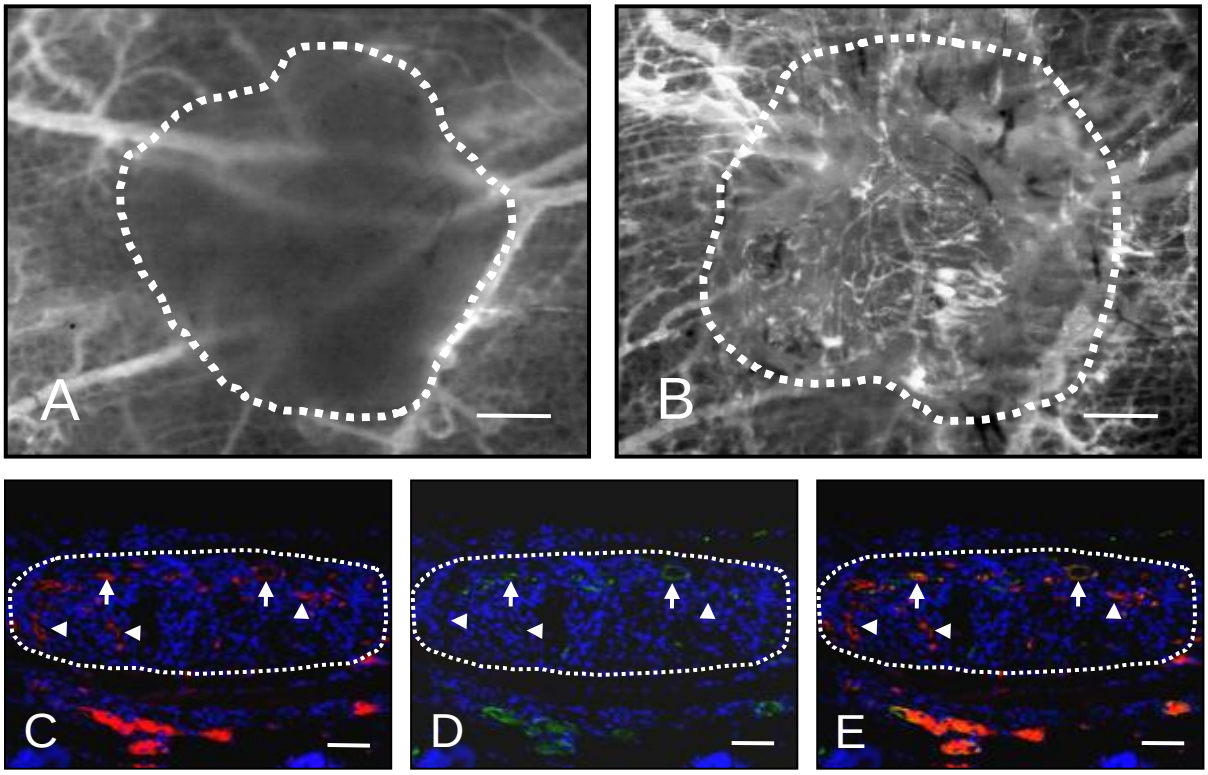

Figure 18: *A, B: Intravital fluorescent microscopic images of an endometrial fragment (border marked by dotted line) directly (A) as well as at day 6 (B) after transplantation into the dorsal skinfold chamber of a BALB/c mouse. Blue light epi-illumination with contrast enhancement by intravascular staining of plasma with 5% FITC-labeled dextran 150,000 i.v.. Scale bars: A, B = 240 µm. C-D: Immunohisto-chemical characterization of microvessels within an endometriotic lesion (borders marked by dotted line) at day 6 after transplantation of an endometrial fragment onto the host striated muscle tissue of a dorsal skinfold chamber. Histological sections were stained with Hoechst to identify cell nuclei (C, D, blue), an antibody against CD31 for the detection of endothelial cells (C, red) and an antibody against α-SMA (D, green). E displays merge of C and D. Note that the lesion contains a mixture of α-SMA-positive mature microvessels (arrows) and α-SMA-negative immature microvessels (arrowheads). Scale bars: C-E = 55 µm.*

More detailed immunohistochemical analyses of these networks revealed that some of their microvessels were already covered with a layer of stabilizing α-SMA-positive perivascular cells (*Figs. 18C-E*). However, the networks also contained many α-SMA-negative microvessels (*Figs. 18C-E*). These findings indicate a heterogeneous maturation stage of the microvasculature of the lesions at this observation time point.

6.3.2 CA4P action on the microvasculature of endometriotic lesions

At day 6, endometriotic lesions of both experimental groups exhibited a comparable vascularized area and functional capillary density before injection of CA4P and vehicle, respectively (*Figs. 19A and C; 20A and B*). Treatment with CA4P rapidly induced a vessel collapse in the lesions, resulting in a significantly reduced vascularized area and functional capillary density after 2h when compared to vehicle-treated controls (*Figs. 19B and D; 20A and B*).

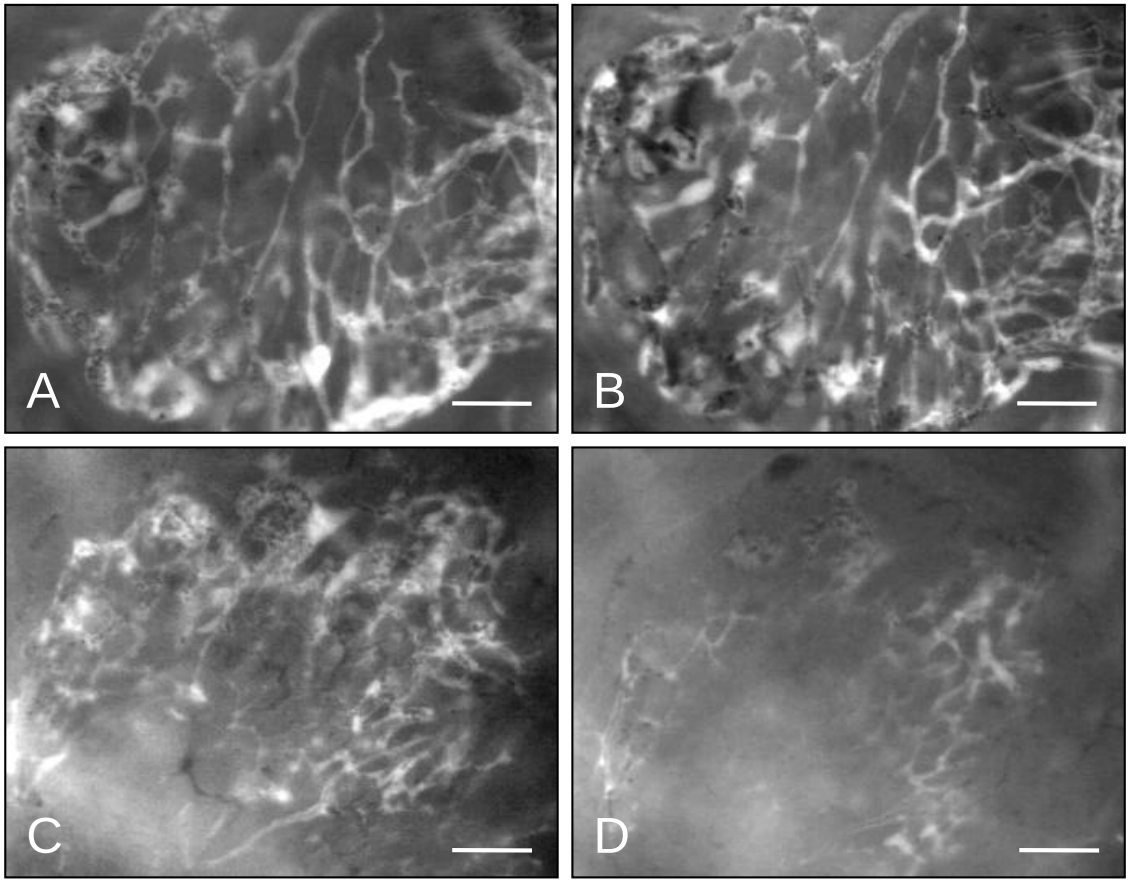

Figure 19: *A-D: Intravital fluorescent microscopic images of vascularized endometriotic lesions within dorsal skinfold chambers of BALB/c mice at day 6 before (A, C) as well as 2h after injection of vehicle (B) or CA4P (D). Note that the injection of the vehicle does not induce any changes of the lesion microvasculature (compare B vs. A). In contrast, CA4P treatment results in a massive collapse of the microvessels (compare D vs. C). Blue light epi-illumination with contrast enhancement by intravascular staining of plasma with 5% FITC-labeled dextran 150,000 i.v.. Scale bars: 90 µm.*

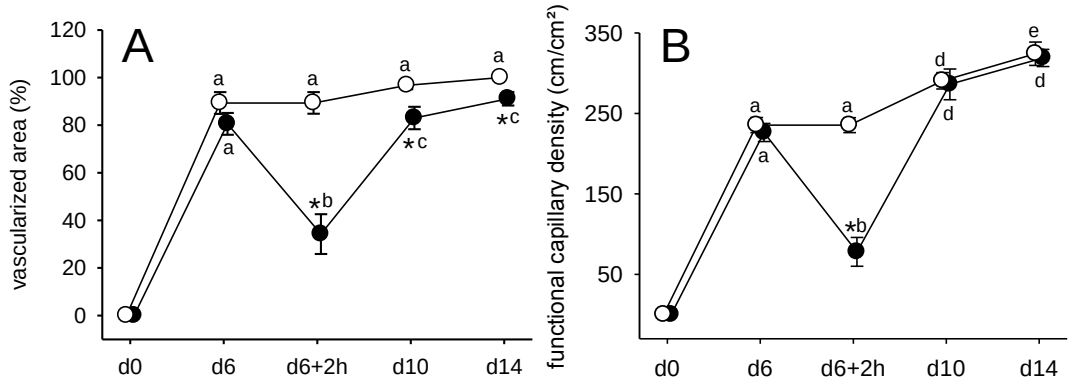

Figure 20: *A, B: Vascularized area (%) (A) and functional capillary density (cm/cm²) (B) of vehicle-treated endometriotic lesions (white circles, control, n=9) and CA4P-treated endometriotic lesions (black circles, n=9), as assessed by intravital fluorescence microscopy and computer-assisted image analysis. CA4P and vehicle were injected after the first measurement at day 6 (d6), which was followed by measurements after 2h (d6+2h), 4 days (d10) and 8 days (d14). Means ± SEM. [a]P < 0.05 vs. d0 within each individual group; [b]P < 0.05 vs. d0 and d6 within each individual group; [c]P < 0.05 vs. d0 and d6+2h within each individual group; [d]P < 0.05 vs. d0, d6 and d6+2h within each individual group; [e]P < 0.05 vs. d0, d6, d6+2h and d10 within each individual group; *P < 0.05 vs. control.*

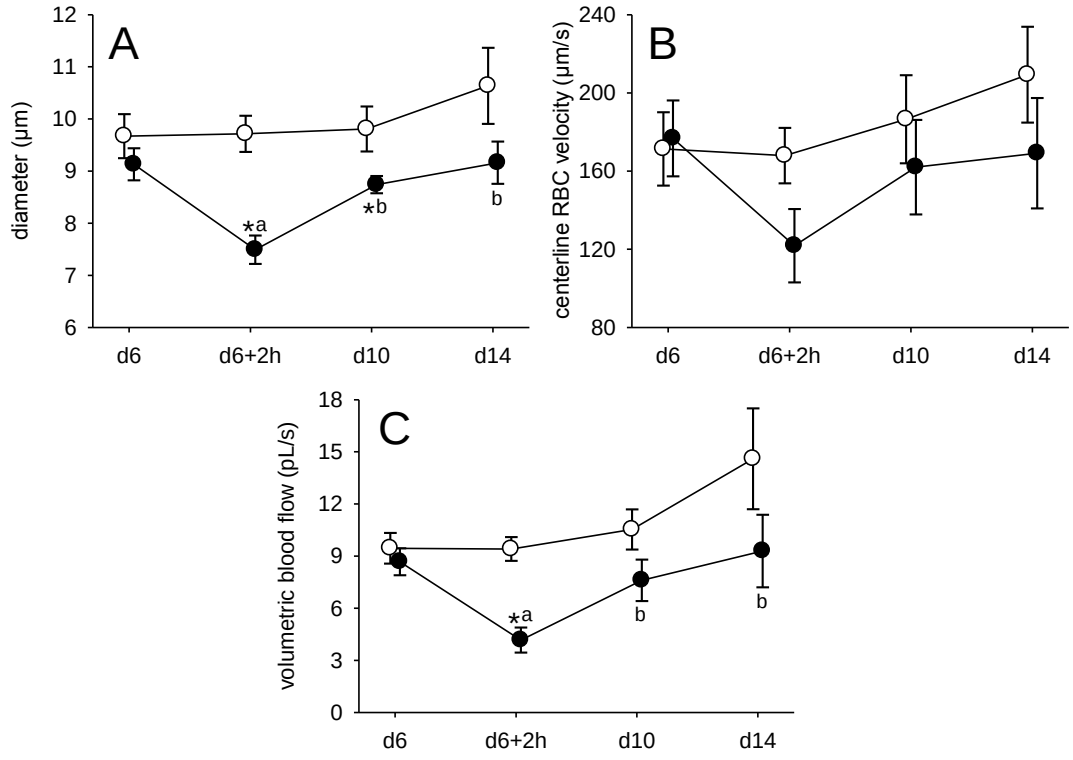

Figure 21: *A-C: Diameter (µm) (A), centerline RBC velocity (µm/s) (B) and volumetric blood flow (pL/s) (C) of microvessels in vehicle-treated endometriotic lesions (white circles, control, n=9) and CA4P-treated endometriotic lesions (black circles, n=9), as assessed by intravital fluorescence microscopy and computer-assisted image analysis. CA4P and vehicle were injected after the first measurement at day 6 (d6), which was followed by measurements after 2h (d6+2h), 4 days (d10) and 8 days (d14). Means ± SEM. [a]P < 0.05 vs. d6 within each individual group; [b]P < 0.05 vs. d6+2h within each individual group; *P < 0.05 vs. control.*

This was associated with a marked reduction of the diameter, centerline RBC velocity and volumetric blood flow of individual microvessels within the lesions (*Figs. 21A-C*). However, this CA4P effect was only short-lived. Throughout the following observation time points, i.e. 4 and 8 days after injection of the compound, parameters of network morphology (*Figs. 20A and B*) and microhemodynamics (*Figs. 21A-C*) progressively normalized to values similar to those measured in the control group.

6.3.3 CA4P action on the microvasculature of the host tissue

In contrast to the microvasculature of endometriotic lesions, the surrounding physiological microvasculature of the chamber tissue was not affected by the injection of CA4P.

Table 2: *Functional striated muscle capillary density (cm/cm^2) as well as diameter (µm), centerline RBC velocity (µm/s) and volumetric blood flow (pL/s) of arterioles and venules in randomly selected regions of interest in the dorsal skinfold chamber of CA4P-treated and vehicle-treated control mice, as assessed by intravital fluorescence microscopy and computer-assisted image analysis.*

	Day 3	Day 6	Day 10	Day 14
Functional capillary density (cm/cm^2)				
Control	262 ± 12	265 ± 13	265 ± 11	255 ± 17
CA4P	242 ± 16	235 ± 12	237 ± 15	222 ± 15
Arteriolar diameter (µm)				
Control	25.8 ± 3.0	25.7 ± 3.1	24.5 ± 2.3	26.4 ± 2.0
CA4P	20.9 ± 1.6	21.4 ± 1.3	20.0 ± 1.6	20.5 ± 1.6*
Arteriolar centerline RBC velocity (µm/s)				
Control	1403 ± 123	1426 ± 146	1217 ± 181	1338 ± 161
CA4P	1690 ± 147	1469 ± 130	1721 ± 193	1646 ± 123
Arteriolar volumetric blood flow (pL/s)				
Control	579 ± 127	583 ± 138	421 ± 59	553 ± 74
CA4P	458 ± 78	393 ± 32	414 ± 59	409 ± 39
Venular diameter (µm)				
Control	31.3 ± 1.1	31.4 ± 1.1	29.2 ± 0.9	32.4 ± 1.3
CA4P	30.8 ± 0.8	32.5 ± 1.2	30.2 ± 0.8	29.8 ± 1.2
Venular centerline RBC velocity (µm/s)				
Control	316 ± 20	339 ± 36	320 ± 41	287 ± 24
CA4P	338 ± 18	278 ± 20	301 ± 24	348 ± 42
Venular volumetric blood flow (pL/s)				
Control	191 ± 22	203 ± 26	168 ± 26	181 ± 14
CA4P	192 ± 9	176 ± 12	164 ± 10	182 ± 17

*Means ± SEM; *P < 0.05 vs. control at corresponding time points.*

Striated muscle tissue of CA4P-treated chambers exhibited a constant functional capillary density and constant arteriolar and venular microhemodynamic parameters over time (*Tab. 2*). In addition, there were no relevant differences when compared to the striated muscle of vehicle-treated control chambers (*Tab. 2*).

6.3.4 CA4P action on proliferation and cell death in endometriotic lesions

To further analyze the effect of CA4P treatment on cell survival and proliferation, endometriotic lesions were additionally examined by means of histology and immunohistochemistry. By this, it was found that the single injection of CA4P did not affect the histomorphology of the lesions. In both groups, the lesions were characterized by cyst-like dilated glands, which were surrounded by a vascularized stroma (*Figs. 22A and B*). They did not exhibit any signs of necrotic cell death.

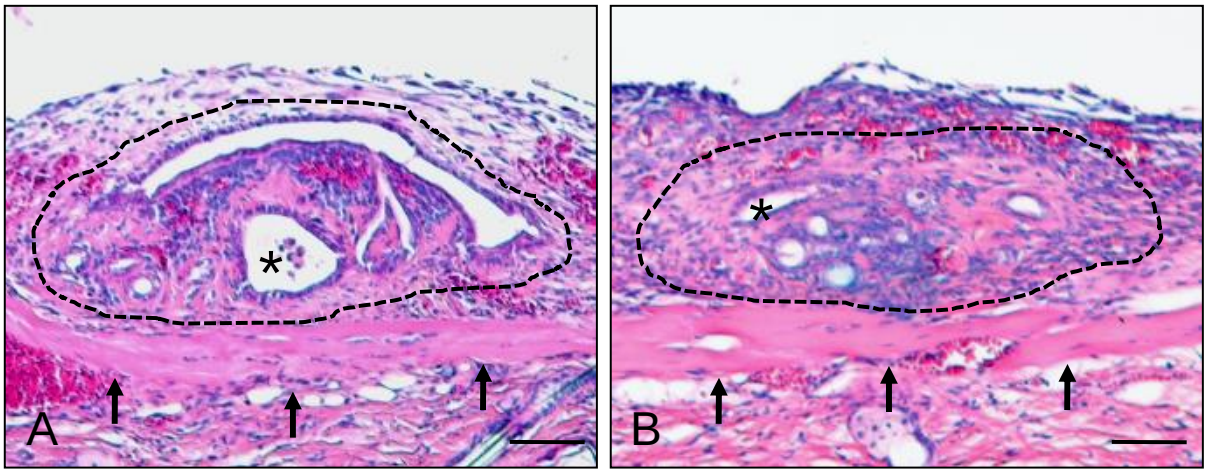

Figure 22: *A, B: HE-stained cross sections of endometriotic lesions (borders marked by broken lines) at day 14 after transplantation of endometrial fragments onto the striated muscle tissue (arrows) within the dorsal skinfold chamber of a vehicle-treated (A) and a CA4P-treated BALB/c mouse (B). Both lesions exhibit a comparable histomorphology with cyst-like dilated endometrial glands (asterisks), which are surrounded by a vascularized stroma. Scale bars: 60 µm.*

Moreover, CA4P-treated and vehicle-treated lesions presented with a comparably high number of proliferating stromal and glandular cells over time (*Figs. 23A, B and E*). Finally, there were also some cleaved caspase-3-positive apoptotic cells within the stromal and glandular fraction of the lesions, which, however, did not markedly differ in number between the two groups (*Figs. 22C, D and F*).

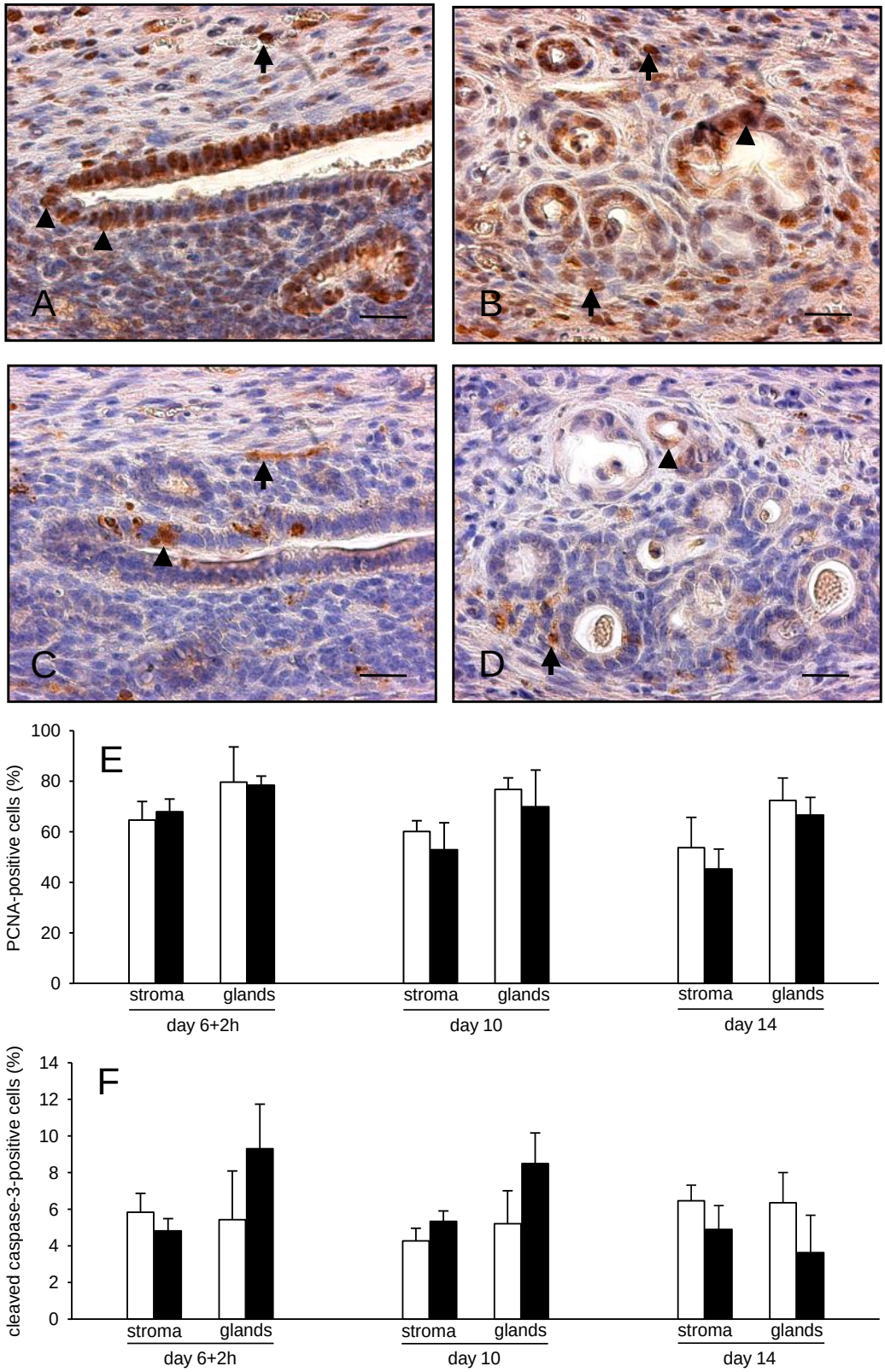

Figure 23: *A-D: Immunohistochemical detection of PCNA (A, B) and cleaved caspase-3 (C, D) in the glandular epithelium (arrowheads) and the stroma (arrows) of endometriotic lesions at day 10 after transplantation of endometrial tissue fragments within the dorsal skinfold chamber of a vehicle-treated (A, C) and a CA4P-treated BALB/c mouse (B, D). Scale bars: 25 µm. E, F: PCNA-positive cells (%) (E) and cleaved caspase-3-positive cells (%) (F) in the stroma and glands of vehicle-treated endometriotic lesions (white bars, control, n=5-9) and CA4P-treated endometriotic lesions (black bars, n=5-9) 2h after injection (day 6+2h) as well as at day 10 and day 14. Means ± SEM.*

6.4 Summary of the results

In the present thesis, the mouse dorsal skinfold chamber model was used to analyze in vivo the vascularization of endometriotic lesions under different conditions. The major findings of this thesis are:

i) After transplantation into the dorsal skinfold chamber, LE^- fragments develop into typical endometriotic lesions with cyst-like dilated endometrial glands and a well vascularized endometrial stroma. In contrast, LE^+ fragments exhibit a polypoid morphology and a significantly reduced blood perfusion after engraftment, because the luminal epithelium prevents the interaction of the grafts with the surrounding host tissue. This results in a reduced growth rate of LE^+ lesions when compared to LE^- lesions. Besides, many GFP^+ microvessels grow outside the LE^- lesions and develop interconnections to the host microvasculature, indicating that inosculation is an important mechanism in the vascularization process of these endometriotic lesions.

ii) The CK2 inhibitor quinalizarin inhibits dose-dependently vascular sprouting in the aortic ring assay. In addition, treatment of endometrial tissue with quinalizarin reduces CK2 activity without affecting the expression of the three CK2 subunits α, α' and β. In the dorsal skinfold chamber model, quinalizarin inhibits the vascularization of endometriotic lesions, which exhibit a significantly lower vascularized area and functional capillary density when compared to that of vehicle-treated controls. This is associated with a reduced lesion size and less endometrial glands.

iii) Injection of the vascular disrupting agent CA4P rapidly induces a selective vessel collapse in endometriotic lesions without affecting the microvasculature of the surrounding host tissue of the dorsal skinfold chamber. This results in a significantly decreased functional capillary density and blood perfusion of CA4P-treated lesions after 2h when compared to vehicle-treated controls. However, the vascularization of the lesions progressively normalizes during the first 8 days after the injection of the compound. Moreover, CA4P-treated and vehicle-treated lesions exhibit comparable numbers of proliferating and apoptotic cells over time.

7. Discussion

7.1 Discussion of materials and methods

Endometriosis studies are widely performed in rodent models, because they bear the advantages of low costs and easy handling as well as the potential for genetic manipulation and, thus, generation of transgenic mouse strains [WILKOSZ et al., 2011; DAFTARY et al., 2013]. Endometriotic lesions can be easily induced in rodents by transplanting endometrial tissue fragments into the peritoneal cavity [BECKER et al., 2006; SCHWAGER et al., 2011]. Although rodent models of peritoneal endometriosis have been shown to be a useful tool to elucidate the efficacy of various anti-angiogenic therapies for the treatment of this disease, they exhibit the disadvantage that the vascularization of endometriotic lesions cannot be visualized non-invasively in vivo. In fact, using these models, the vascularization of lesions is usually assessed by means of histology or immunohistochemistry of isolated specimens at a single time point after killing the animals.

To overcome this problem, the dorsal skinfold chamber model was used in the present thesis. This model bears the unique advantage that the formation of new microvascular networks in endometriotic lesions and their microhemodynamic characteristics can be studied non-invasively using the combination of high-resolution multi-fluorescence microscopy and subsequent computer-assisted off-line analysis [LASCHKE and MENGER, 2007b]. Accordingly, this model maximizes the data, which can be obtained from one animal and, thus, also reduces the number of animals required for a study. In addition, it allows the study of dynamic microcirculatory parameters. In fact, numerous parameters can be assessed in the dorsal skinfold chamber model of endometriosis. They include lesion growth, vascularized area, functional capillary density, microvessel diameter, microvascular perfusion, vascular permeability, leukocyte-endothelial cell interaction as well as apoptotic cell death [LASCHKE et al., 2005a; LASCHKE and MENGER, 2007a].

In the present intravital microscopic setting, it was possible to record the microscopic images on DVD and to analyze them quantitatively at the end of the in vivo experiments, which reduced the stress for the animals, because the observation time of each microscopy could be shortened to a minimum. Directly before the microscopy started, the fluorescent dye FITC-labeled dextran was injected intravenously via the retrobulbary vascular plexus. For this purpose, it is necessary to use a dextran with a high molecular weight (150 kDa) to guarantee that the dye only extravasates in cases of disturbed endothelial integrity. Under the given conditions, this allowed for contrast enhancement of individual microvessels by intravascular staining of the blood plasma.

The dorsal skinfold chamber model in its current form is based on several different chamber techniques, which have been introduced into research to investigate the microcirculation and the vascularization of tumor implants [SANDISON, 1928; ALGIRE, 1943; ARFORS et al., 1970]. The early chamber models exhibited the disadvantage that new granulation tissue formed during the process of wound healing, which did not allow the analysis of a physiological tissue microcirculation. Accordingly, further developments of the chamber technique followed the basic principle of providing a chronic access to exposed tissues, which guaranteed that these tissues were not influenced by the chamber itself and the implantation procedure. Originally, the chambers consisted of two aluminium frames, covered with a thin layer of Teflon S to reduce their weight, thermal conductivity and to increase their biological inertness [ENDRICH et al., 1980]. Nowadays, the chambers are made of titanium, which also exhibits these material properties and additionally provides improved stability [MENGER et al., 2002].

The dorsal skinfold chamber model is a useful experimental approach not only to study the process of angiogenesis but also to develop novel anti-angiogenic strategies for the treatment of angiogenic diseases. In 2005, this model has been introduced for the first time in the field of endometriosis research [LASCHKE et al., 2005b]. Since then, it has been used in numerous studies to test the anti-angiogenic effects of different compounds on the vascularization of newly developing endometriotic lesions. These include growth factor inhibitors [LASCHKE et al., 2006b], COX-2-inhibitors [LASCHKE et al., 2007a], immunosuppressants [LASCHKE et al., 2006a] and phytochemicals [LASCHKE et al., 2008b]. In these studies, the skinfold chamber model has been proven to be a suitable approach to analyze the vascularization process in endometriotic lesions under highly standardized conditions.

7.2 Discussion of the results

7.2.1 Effect of luminal epithelium on the development of endometriotic lesions

Because rodents do not develop spontaneous endometriosis, endometriotic lesions are usually induced for research purposes in these species by transplanting endometrial tissue fragments to ectopic sites [GRÜMMER et al., 2006]. The results of the present thesis demonstrate that this approach is crucially dependent on the cellular composition of the grafts. In fact, it was found that the vascularization, morphology and growth of endometriotic lesions originating from LE^+ fragments markedly differ from those of LE^- fragments. While LE^- fragments develop into typical endometriotic lesions with cyst-like dilated endometrial glands

and a well vascularized endometrial stroma, lesions originating from LE^+ fragments exhibit a polypoid morphology and a reduced blood perfusion after engraftment. This is associated with a decreased growth rate when compared to LE^- lesions.

These novel findings are in line with the results of a previous study, in which uterine tissue samples were fixed with the luminal epithelium of the endometrium to the peritoneum of the abdominal cavity [KÖRBEL et al., 2010]. As assessed by high-resolution ultrasound imaging, these grafts presented with lower volumes throughout a 4-week observation period when compared to samples, which were fixed with the perimetrium to the peritoneum. In addition, they exhibited decreased numbers of proliferating stromal and glandular cells. This can be explained by the natural barrier function of the luminal epithelium [GIPSON et al., 2008; OCHIEL et al., 2008], which prevented the interaction of the grafts with the surrounding host tissue. Accordingly, it was found in the present thesis that only the areas of LE^+ fragments, which were not covered with luminal epithelium, engrafted well at the transplantation site.

Using the technique of intravital fluorescence microscopy it could be shown that LE^- lesions exhibit an accelerated vascularization when compared to LE^+ lesions, which is associated with a markedly improved blood perfusion. This may be due to the fact that the microvascular networks of LE^- lesions develop many interconnections to the surrounding host microvasculature. In contrast, microvessels could not pass the luminal epithelium of LE^+ lesions. Accordingly, these lesions were only supplied by a few microvessels at the base of their polypoid structure. In line with these findings, LE^+ lesions also exhibited a markedly decreased growth throughout the observation period of 14 days when compared to LE^- lesions. This further supports the concept that the development of endometriotic lesions is crucially dependent on vascularization [GROOTHUIS et al., 2005].

The transplantation of GFP^+ endometrial tissue fragments into the dorsal skinfold chamber of GFP^- wild-type recipient mice allowed for studying the origin of microvessels within and around newly developing endometriotic lesions. Of interest, it was found that both LE^- and LE^+ lesions still exhibited GFP^+ microvessels at day 14 after transplantation. This proves that these vessels, which originated from the GFP^+ donor mice, were able to survive the whole isolation, transplantation and engraftment process. In addition, many GFP^+ microvessels grew outside the LE^- lesions into the surrounding host tissue, where they developed interconnections to the host microvasculature, which is termed as external inosculation [LASCHKE et al., 2009]. Accordingly, it can be concluded that besides angiogenesis and vasculogenesis [TAYLOR et al., 2009; LASCHKE et al., 2011a], the inosculation of pre-existing microvessels within endometrial tissue fragments is an additional important mechanism, which contributes to the vascularization of endometriotic lesions.

Finally, it should be mentioned that endometriotic lesions with a polypoid growth pattern have also been observed under clinical conditions [MOSTOUFIZADEH and SCULLY, 1980].

However, the literature on polypoid endometriosis is limited to a small number of case reports [PARKER et al., 2004]. Moreover, the etiopathogenesis of this rare type of endometriosis is completely unknown, although postmenopausal age and hyperestrinism have been proposed to be important risk factors [PARKER et al., 2004]. Based on the observations of the present thesis, it may be speculated that spread endometrial tissue fragments with a luminal epithelium contribute to the formation of polypoid lesions. On the other hand, polypoid lesions in endometriosis patients often exhibit hyperplastic or metaplastic glands and sometimes also cytologic atypia [PARKER et al., 2004], which indicates that their pathogenesis is much more complex and associated with marked cellular abnormalities.

Taken together, the results of this first part of this thesis demonstrate that the existence of luminal epithelium in transplanted endometrial tissue fragments affects their vascularization, growth and morphological development into endometriotic lesions in mice. Thus, it is of major importance to standardize endometrial grafts in order to increase the validity and reliability of pre-clinical animal studies in endometriosis research. In addition, these novel findings indicate that various growth patterns of endometriotic lesions may be crucially determined by the initial cellular composition of the ectopic endometrial tissue.

7.2.2 Regulatory function of CK2 in angiogenesis of endometriotic lesions

CK2 is a pleiotropic serine/threonine protein kinase, which is involved in various fundamental cellular processes [LITCHFIELD, 2003]. CK2 has also been shown to be a key signaling molecule in angiogenesis, mediating endothelial cell proliferation, migration and tube formation [LJUBIMOV et al., 2004]. Accordingly, small molecule or antisense drugs based on CK2 inhibition have been proposed to be promising future therapeutics for angiogenic diseases [KRAMEROV et al., 2008]. In line with this, the results of the present thesis demonstrate for the first time that inhibition of CK2 suppresses vascularization of developing endometriotic lesions.

For the inhibition of CK2, quinalizarin was used in the vitro and vivo experiments. To date, the selectivity of this cell-permeable compound towards CK2 is higher than that of any other commercially available CK2 inhibitor, such as emodin, 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB) or 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT) [COZZA et al., 2009]. However, the anti-angiogenic potential of quinalizarin has not been tested so far. Therefore, a rat aortic ring assay with different doses of this CK2 inhibitor was performed. By this, it was found that quinalizarin effectively suppresses vascular sprout formation in vitro in a dose range of 10-50µM.

By means of immunohistochemistry, the expression of CK2α, CK2α' and CK2β in the endometrium of C57BL/6 mice was studied. Of interest, all three subunits could be detected in the endometrial tissue with the strongest expression in the glandular epithelium. Considering the fact that CK2 has been shown to regulate intracellular transport processes and secretory function of different cell types, such as pancreatic β-cells [MENG et al., 2010] or adrenocortical carcinoma cells [LAWNICKA et al., 2010], this indicates that CK2 may also be crucially involved in uterine secretory activity. Moreover, it could be shown by means of a CK2 activity assay that quinalizarin is suitable to inhibit the activity of CK2 without affecting its expression in the endometrium.

Blood vessel formation in endometriotic lesions is a complex process, which is driven by multiple angiogenic growth factors. In a former study, it was found that only the combined inhibition of vascular endothelial growth factor (VEGF), platelet-derived endothelial growth factor (PDGF) and fibroblast growth factor (FGF), but not inhibition of VEGF alone, effectively suppresses angiogenesis and vessel maturation in endometriotic lesions [LASCHKE et al., 2006b]. Thus, the selective inhibition of one growth factor is not sufficient to affect endometriotic lesions, because this type of treatment is overwhelmed by an increased or redundant activity of other angiogenic factors. To overcome this problem, the use of pleiotropic agents, such as CK2 inhibitors, represents a promising alternative. In fact, CK2 participates in several major pathways activated by different angiogenic growth factors, including Ras-Raf-MEK-ERK, p38 MAPK, PKC and PI3 kinase-Akt [LJUBIMOV et al., 2004]. Accordingly, it was found that CK2 inhibition by quinalizarin significantly delays and suppresses the vascularization of newly developing endometriotic lesions, which was associated with a reduction of lesion sizes. Further long-term studies have to clarify now, whether the application of CK2 inhibitors may also be successful in the therapy of older lesions during chronic endometriosis.

Besides angiogenesis and inosculation, vascularization of endometriotic lesions also involves vasculogenesis, which is defined as de novo formation of microvessels from circulating endothelial progenitor cells [BECKER et al., 2011; LASCHKE et al., 2011a; 2011b]. Although not further analyzed in the present experiments, the suppression of this process may have been an additional mechanism, contributing to the reduced functional capillary density of endometriotic lesions in quinalizarin-treated animals. In fact, it was recently reported that incorporation of bone marrow-derived hematopoietic stem cells into sites of neovascularization is decreased by inhibition of CK2 [KRAMEROV et al., 2008]. This indicates that vasculogenesis is another biological process, which underlies the regulatory function of CK2.

In summary, the results of this second part of the thesis demonstrate for the first time that protein kinase CK2 is a key regulator of angiogenesis in developing endometriotic

lesions. Accordingly, CK2 may represent a novel target in the establishment of future anti-angiogenic strategies for the treatment of endometriosis. This, however, requires the development of highly specific CK2 inhibitors, suppressing angiogenesis in pathological conditions without inducing significant side effects.

7.2.3 Vascular disrupting effects of CA4P on endometriotic lesions

The application of VDAs represents a novel, promising strategy in cancer therapy. In contrast to anti-angiogenic compounds, VDAs bear the major advantage that they target already established microvascular networks in tumors. Hence, they may be particularly suitable in advanced disease stages. Although it is well known that this type of treatment is currently still associated with significant side effects [HASANI and LEIGHL, 2011; SUBBIAH et al., 2011; HOLLEBECQUE et al., 2012], which are certainly not acceptable in the treatment of benign diseases such as endometriosis, the third part of this thesis was performed to basically clarify in a proof-of-principle study whether a vascular disrupting approach may be also effective in case of endometriotic lesions.

CA4P destabilizes the tubulin cytoskeleton in endothelial cells, resulting in vessel collapse. Interestingly, this effect is most pronounced in tumor tissue, which has been explained by the fact that tumors usually exhibit a highly angiogenic phenotype with many proliferating endothelial cells and the continuous formation of fragile, immature microvessels [TOZER et al., 2002; WEST and PRICE, 2004]. The endothelial cells of these microvessels have a less developed actin cytoskeleton when compared to mature, quiescent endothelial cells, which can maintain their cell shape despite tubulin depolymerization [CHAPLIN and DOUGHERTY, 1999]. Moreover, immature microvessels are not covered by stabilizing perivascular cells and, thus, are much more susceptible to vessel collapse. For these reasons, it was necessary in the present work to analyze firstly the maturation stage of microvessels in the murine endometriotic lesions at day 6 after induction. By immunohistochemical staining of α-SMA, a mixture of mature and immature microvessels within the lesions was found, which exactly reflects the conditions in peritoneal lesions of endometriosis patients. There, early red lesions exhibit a high fraction of immature microvessels [MATSUZAKI et al., 2001], which makes them an attractive target for anti-angiogenic therapy [NAP et al., 2004]. But even older black lesions have been shown to still contain several immature microvessels [MATSUZAKI et al., 2001], which may be due to continuous remodeling processes within the microvasculature of these lesions.

By means of intravital fluorescence microscopy it was shown that rapidly after administration of CA4P the functional capillary density of endometriotic lesions was significantly reduced when compared to vehicle-treated controls. This was not associated with negative side effects on the physiological microvasculature of the surrounding host

tissue, which indicates a similar selective vascular disrupting effect of CA4P on endometriotic lesions as observed in tumor studies. However, in contrast to tumors, this short-term shutdown of blood perfusion did neither affect the viability nor the proliferating activity of the lesions throughout the observation period of 8 days following the treatment. It may be speculated that this is due to a high resistance of the endometrial cells to hypoxia. In line with this hypothesis, numerous experimental endometriosis studies have reported that transplanted endometrial tissue samples are able to survive the initial hypoxic period, during which they lack an own blood supply, before they develop into typical endometriotic lesions with functional endometrial glands and stroma [BECKER et al., 2008; CAPOBIANCO et al., 2011; LASCHKE et al., 2011b; WANG et al., 2013]. On the other hand, VDAs are known to increase the vascular permeability in tumors [REYES-ALDASORO et al., 2008]. Because tumors already exhibit a high interstitial fluid pressure [BOUCHER et al., 1990], this markedly contributes to tissue injury, whereas it may be less harmful in endometriotic lesions.

There may be several possibilities to improve the therapeutic efficacy of VDAs in the treatment of endometriosis. Firstly, CA4P exhibits a vascular disrupting activity over a broad dose range far below the maximum tolerated dose [HINNEN and ESKENS, 2007; HE et al., 2011]. In addition, it was found that the amount of administered CA4P positively correlates with the recovery of blood flow [TOZER et al. 2001]. Microvessels of P22 rat carcinosarcoma showed a partial recovery of blood flow after treatment with 30mg/kg CA4P, which was not the case at a dose of 100mg/kg [TOZER et al., 2001]. Accordingly, doses of CA4P higher than 80mg/kg as given in the present experiments may also induce a long-term perfusion failure of endometriotic lesions. Secondly, repeated daily or twice daily doses of CA4P may exert a significantly stronger anti-tumor effect than could ever be achieved by any single dose of the drug [TOZER et al., 2002]. This indicates that it is worthwhile to test different application schemes of VDAs in further experimental endometriosis studies. Finally, VDAs are capable of increasing the efficacy of other anti-cancer therapies such as radiotherapy or chemotherapy [SIEMANN et al., 2009]. A similar concept may also be promising for the multimodal management of endometriosis, for instance, to improve the efficacy of anti-angiogenic or anti-inflammatory compounds.

Taken together, the present proof-of-principle study demonstrates for the first time a short-term selective vascular disrupting effect of CA4P on murine endometriotic lesions without affecting the surrounding microvasculature of the host tissue. This indicates that VDAs are a novel class of drugs, which may be suitable to target endometriotic lesions. However, for this purpose safer VDAs have to be developed first, which exhibit improved side effect profiles, justifying their application in the treatment of a benign disease such as endometriosis.

7.2.4 Conclusions and clinical perspectives

The progression of endometriosis is crucially dependent on the process of angiogenesis. Based on this fact, different factors determining the angiogenic process in endometriotic lesions were analyzed in the three parts of this thesis. They included the composition of transplanted endometrial tissue fragments as well as the effect of the regulatory function of CK2 and the vascular disrupting agent CA4P on lesion vascularization. For the experiments, the dorsal skinfold chamber model was used. This model bears the advantage of repetitive and direct in vivo observation of the process of angiogenesis and vascularization within endometriotic lesions by means of intravital fluorescence microscopy.

In the first part of the present thesis it was found that the luminal epithelium in transplanted endometrial tissue fragments crucially affects their vascularization, growth and morphological development into endometrotic lesions. This finding is of major importance because it clearly demonstrates that the composition of endometrial grafts has to be standardized in order to increase the reliability of pre-clinical rodent studies in future endometriosis research.

The second part of this thesis demonstrates for the first time that the vascularization and size of developing endometriotic lesions are significantly decreased by inhibiting the protein kinase CK2. Accordingly, CK2 plays a crucial role in the regulation of angiogenesis in endometriotic lesions. This finding also indicates that CK2 may be a promising candidate for the development of novel treatment strategies for the future therapy of endometriosis.

In the third part of this thesis, treatment of established endometriotic lesions with CA4P showed a short-term selective vascular disrupting effect on the lesions without affecting the surrounding physiological microvasculature of the host tissue. Therefore, VDAs represent a novel class of drugs, which may be further developed to target endometriotic lesions.

8. References

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10. Curriculum vitae

Name	Dilu Feng
Date of Birth	30.09.1982
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School Education

1989 – 1995	Primary School: Chongqing, China
1995 – 1998	Middle School: Chongqing, China
1998 – 2002	High School: Chongqing, China

Study of Medicine

9/2002 – 7/2007	Bachelor Degree in Forensic Medicine, Tongji Medical College, Hua Zhong University of Science and Technology (HUST), Wuhan, China. Internship in Xiaogan Center Hospital, Xiaogan, China; Becoming familiar with various kinds of diseases in each main department.
9/2007 – 7/2010	Master Degree in Gynecological Oncology. Union Hospital, HUST, Wuhan, China; Course: Experiment Techniques of Immunology, Medical Molecular Biology, Basic Immunology, Histochemistry, SPSS, Emergency medicine, Statistics. Residency in Union Hospital, Wuhan, China; Work in Gynecological Oncology team: communication with patients, primary diagnosis, writing case files and rout handing as a surgery assistant.
9/2010 – present	MD/PhD program, Institute for Clinical and Experimental Surgery, University of Saarland, Homburg/Saar, Germany

Medical Research

9/2008 - 5/2010	Gynecological laboratory in Union Hospital, Wuhan, China; Research topic: ''Dendritic cell vaccine targeting for endometriosis by inducing CTL''; Experimental techniques training: Cell culture, PCR, Western blot, FCM, Transfection.

9/2010 – present	Institute for Clinical and Experimental Surgery, University of Saarland, Homburg/Saar, Germany; Main research focus on endometriosis.

Scholarships and Awards

11/2005	Grade A scholarship
11/2006	Scholarship for excellent extracurricular activities member
6/2007	Excellent graduate
9/2007	National scholarship for graduate student
11/2008	Scholarship for excellent student union leader
	Outstanding student
10/2009	Scholarship for excellent student union leader
5/2010	Excellent graduate
6/2010	National scholarship for study abroad

11. Publications

Original articles

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