Technical Advance

In Vivo Imaging of Physiological Angiogenesis from Immature to Preovulatory Ovarian Follicles

Brigitte Vollmar,* Matthias W. Laschke,*
Richard Rohan,† Jochem Koenig,‡ and
Michael D. Menger*

From the Institute for Clinical and Experimental Surgery,* and
the Institute for Medical Biometrics, Epidemiology and Medical
Informatics,‡ University of Saarland, Homburg/Saar, Germany;
and the Surgical Research Laboratories,† Children's Hospital,
Harvard Medical School, Boston, Massachusetts

To develop a model for the study of physiological angiogenesis, we transplanted ovarian follicles onto striated muscle tissue and analyzed the process of microvascularization *in vivo* using repeated fluorescence microscopy. Follicles were mechanically isolated from unstimulated as well as pregnant mare's serum gonadotropin (PMSG)- or PMSG/luteinizing hormone (LH)-stimulated Syrian golden hamster ovaries and were transplanted as free grafts into dorsal skinfold chambers of untreated or synchronized hamsters. Follicles lacking thecal cell layers did not vascularize regardless whether harvested from unstimulated or PMSG-stimulated animals, but underwent granulosa cell apoptosis, as indicated *in vivo* by nuclear condensation and fragmentation of bisbenzimidazole-stained follicular tissue. In contrast, all follicles at 48 hours after PMSG treatment with a multilayered thecal shell exhibited initial signs of angiogenesis within 3 days. Vascularization was completed within 7 to 10 days, comprising a dense glomerulum-like microvascular network. Nature and extent of vascularization of follicles harvested at 72 hours after either PMSG or PMSG/LH treatment did not notably differ from each other when transplanted into the respective synchronized animals. However, follicles with PMSG/LH treatment revealed significantly larger microvessel diameters and higher capillary blood perfusion compared to follicles with sole PMSG treatment, probably reflecting the adaptation to the increased functional demand upon the LH surge. Using the unique experimental approach of ovarian follicle transplantation in the dorsal skinfold chamber of Syrian golden hamsters, we could show *in vivo* the developmental stage-dependent vascular-

ization of follicular grafts with sustained potential to meet their metabolic demand by increased blood perfusion. (Am J Pathol 2001, 159:1661-1670)

In adult tissue, angiogenesis is a characteristic of pathological conditions, such as tumor growth,^{1,2} wound healing,³⁻⁶ and inflammation.⁷ Nonpathological angiogenesis is rare and restricted to the female reproductive tissues.⁸ During the cycle of follicle development and corpus luteum formation, vascular changes are tightly regulated in that angiogenesis is turned on for brief periods and then completely inhibited. Thus, folliculogenesis offers a unique system to study not only the induction of angiogenesis, but also the maturation and regression of blood vessels.⁹

The precise control of angiogenesis in the developing ovarian follicle and corpus luteum is critical for normal reproductive function. The regulators of this physiological angiogenesis, however, are not yet completely elucidated. Preovulatory follicles in mammalian ovaries develop from the vast pool of preantral follicles. During their development preantral follicles grow through successive stages¹⁰ in which they differ in size, number of granulosa cell layers, and absence or presence of thecal cells.¹¹ Some 50 years ago, Bassett¹² described changes in the vasculature of the developing follicles and corpora lutea in rat ovaries during the estrous cycle. More recent studies have demonstrated the angiogenic potential of corpora lutea extracts,¹³ follicular fluid,¹⁴ and granulosa cell-conditioned medium.¹⁵ These angiogenic activities have later been attributed to the action of basic fibroblast growth factor,¹⁶ some heparin-binding growth factors,¹⁷ and vascular endothelial growth factor.^{18,19} Although it is widely recognized that angiogenesis is a prerequisite for ovarian and uterine function, only a few investigators have taken advantage of the physiological angiogenic processes in the female reproductive system to study

Supported by a Heisenberg Stipendium of the Deutsche Forschungsgemeinschaft, Bonn (VO 450/6-1 and 6-2 to B.V.).

Accepted for publication July 27, 2001.

Address reprint requests to Prof. Dr. Brigitte Vollmar, Institute for Clinical & Experimental Surgery, University of Saarland, 66421 Homburg/Saar, Germany. E-mail: exbvol@med-rz.uni-sb.de.

mechanisms that underlie the induction and regulation of angiogenesis. This paucity of information is related in part to the lack of simple reproducible animal models of follicular angiogenesis.

To extend our understanding of the mechanisms that regulate nonpathological vascular growth, we used the hamster dorsal skinfold chamber as the host site for ovarian follicle transplantation and systematically analyzed *in vivo* the host's angiogenic response to follicular grafts using multifluorescence microscopy.

Materials and Methods

Preparation of the Hamster Dorsal Skinfold Chamber

The chamber preparation contains one layer of striated muscle and skin and allows for intravital microscopic observation of the microcirculation in the awake animals throughout a prolonged period of time. The chamber technique and its implantation procedure have been described previously in detail.²⁰ In brief, under pentobarbital sodium anesthesia (50 mg/kg body weight *i.p.*), two symmetrical titanium frames were implanted on the extended dorsal skinfold of 8- to 10-week-old Syrian golden hamsters (body weight, 60 to 80g), so that they sandwiched the double layer of skin. One layer of skin was then removed in a circular area of ~15 mm in diameter, and the remaining layers (consisting of striated skin muscle and subcutaneous tissue) were covered with a removable coverslip incorporated into one of the titanium frames. In addition, a permanent catheter was passed from the dorsal to the ventral side of the neck and inserted into the jugular vein. After intravenous application of 0.2 ml of 5% fluorescein isothiocyanate (FITC)-labeled dextran 150,000 (Sigma, Deisenhofen, Germany), the chamber enabled for continuous observation and repetitive analysis of the microcirculation by means of intravital fluorescence microscopy in the awake animal. The animals were allowed to recover from anesthesia and surgery for at least 48 hours.

Follicle Isolation and Transplantation

For follicle donation, 8- to 10-week-old female hamsters were intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg body weight). After laparotomy, donor ovaries were aseptically removed and placed in 30-mm-diameter Falcon plastic Petri dishes filled with 37°C warm Dulbecco's modified Eagle's medium (10% fetal calf serum, 0.1 mg/ml gentamicin), and the fluorescent vital dye bisbenzimidazole H33342 (200 μ g/ml; Sigma). After removing the surrounding tissue, the ovaries were microdissected under a stereo microscope using 27-gauge needles. According to size, the follicles were visually collected and transferred into 37°C warm bisbenzimidazole H33342-free Dulbecco's modified Eagle's medium (Figure 1A).

For follicle transplantation, the cover glass of the dorsal skinfold chamber was removed and one to three follicles

Figure 1. A: Follicles at different stages of development after mechanical microdissection in Dulbecco's modified Eagle's medium. A handpicking procedure guaranteed single connective tissue-free follicles for transplantation. **B:** Isolated follicles directly after transplantation into the hamster dorsal skinfold chamber. Scale bars: 800 μ m (**A**), 400 μ m (**B**).

of either size were placed on the striated muscle within the chamber (Figure 1B). A handpicking procedure guaranteed single connective tissue-free follicles for transplantation. In general, follicles were grouped in accordance to their initial size at the time point of harvesting and transplantation with 1) diameters <250 μ m, 2) diameters in the range of 250 to 500 μ m, and 3) diameters >500 μ m.

Donor and Recipient Animals

Two donor animals were pretreated with pregnant mare's serum gonadotropin (PMSG, Sigma) dissolved in phosphate-buffered saline (1000 U/ml). PMSG was given subcutaneously at 8 a.m. in the morning at a single dose of 2 U/10 g body weight. Follicles were harvested at either 48 hours or 72 hours after the single PMSG treatment. In parallel, skinfold chamber-equipped age-matched female hamsters ($n = 13$) were also treated by a single subcutaneous injection of PMSG (2 U/10 g body weight) and follicles were transplanted at either 48 hours or 72 hours after PMSG treatment.

One donor animal was pretreated with PMSG as described above, followed by subcutaneous application of luteinizing hormone (LH) (25 μ g/hamster; Sigma) at 8 a.m. in the morning of day 2 after application of PMSG. Follicles were harvested 24 hours after the LH application. In parallel, skinfold chamber-equipped age-matched female hamsters ($n = 7$) were also treated by PMSG followed by LH.

Follicles harvested from an animal with neither PMSG nor LH treatment were used for transplantation into nontreated skinfold chamber-equipped age-matched female hamsters ($n = 6$).

Intravital Microscopy

For *in vivo* microscopic observation, the awake animals were immobilized in a Plexiglas tube and the skinfold preparation was attached to the microscopic stage. The stage was placed on a computer-controlled microscope desk, which allowed repeated scanning of each individual follicle for intravital microscopy. Intravenous injection of 0.2 ml of 5% FITC-labeled dextran 150,000 (Sigma) guaranteed contrast enhancement by staining of the plasma. Rhodamine 6G (0.1%, 0.1 ml i.v.; Sigma) allowed for the direct *in vivo* staining of leukocytes. Intravital microscopy was performed using a modified Leitz Orthoplan microscope with a 100 W HBO mercury lamp attached to a Ploemo-Pak illuminator with blue, green, and ultraviolet filter blocks (Leitz, Wetzlar, Germany) for epi-illumination. The microscopic images were recorded by a charge-coupled device video camera (CF8/1 FMC; Kappa GmbH, Gleichen, Germany) and transferred to a video system for off-line evaluation. With the use of $\times 4$, $\times 6.3$, $\times 10$, and $\times 20$ long distance objectives (Leitz), magnifications of $\times 86$, $\times 136$, $\times 216$, and $\times 432$ were achieved on a 14-inch video screen (PVM 1444; Sony, Tokyo, Japan).

Microcirculatory Analysis

Quantitative off-line analysis of the videotapes was performed by means of a computer-assisted image analysis system (CapImage, Zeintl, Heidelberg) and included the determination of the diameter (μ m) and the size of the transplanted follicles (mm^2), the size of the growing microvascular networks (in percentage of the follicular size), the microvessel density, ie, the length of red blood cell (RBC)-perfused microvessels per observation area (cm/cm^2), and the diameters of the follicular microvessels (μ m). On ultraviolet epi-illumination the dye bisbenzimidazole is characterized by a bright blue fluorescence with only little bleaching that persists through several cell generations. The specific fluorescence/background fluorescence ratio is high enough throughout a period of 3 weeks to precisely delineate the stained follicular graft from the surrounding unaffected host tissue. The area of fully developed microvascular networks might sometimes slightly exceed the follicular tissue area with the consequence that values of the size of the growing microvascular networks are $>100\%$ of the follicular size.

Centerline RBC velocity (V_{RBC}) in the individual microvessels was measured by frame-to-frame analysis. Volumetric blood flow (VQ) of individual microvessels was calculated from V_{RBC} and diameter (D) for each microvessel as $VQ = \pi \times (D/2)^2 \times V_{\text{RBC}}/K$, where K ($=1.3$) represents the Baker/Wayland factor,²¹ considering the parabolic velocity profile of blood in microvessels.

Rhodamine 6G-stained leukocytes were classified in accordance to their interaction with the endothelium of newly formed microvessels. Rolling cells were defined as cells moving with a velocity less than two-fifths of the centerline velocity (given as percentage of nonadherent leukocytes passing through the observed vessel segment within 20 seconds). Adherent cells were defined as cells that did not move or detach from the endothelial lining during an observation period of 20 seconds (given as number of cells per microvascular network area).

Experimental Protocol

A total of 26 follicles were harvested from the nontreated donor animal and were transplanted into the skinfold chambers of six nontreated female hamsters. A total of 14 follicles at 48 hours and 10 follicles at 72 hours after PMSG treatment were transplanted into the skinfold chambers of five and eight female synchronized hamsters. A total of nine follicles at 72 hours after PMSG/LH treatments were transplanted onto the skinfold chambers of seven female synchronized hamsters. The macroscopic appearance of the skinfold chamber preparations and the implanted grafts were documented daily. Intravital multifluorescence microscopic analysis of growth, angiogenesis, and microcirculation was performed on days 3, 5, 7, 10, and 14 after follicular graft transplantation. Measurements of vascular density and microhemodynamic parameters included only newly formed microvessels that could be clearly distinguished by their glomerulum-like arrangement from the autochthonous host striated muscle microvessels, displaying the typical parallel arrangement of the muscle capillaries.²² Vascular density was measured in five regions of interest per graft and observation time point. Microvascular diameters as well as hemodynamic parameters were determined from 10 microvessels per region of interest. At the end of the *in vivo* experiments, ie, day 14 after follicle transplantation, the animals were sacrificed with an overdose of pentobarbital, and the skinfold chamber preparations were processed for light microscopic analysis.

Histology

Follicles were harvested from ovaries of untreated and PMSG- and PMSG/LH-treated hamsters and grouped according to size, fixed in formalin (4% in phosphate-buffered saline) for 24 hours at 4°C, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin (H&E). Sections through the central plane of the follicle (ie, those containing the largest cross-sectional area) were videotaped, and cross-sectional areas of the indi-

vidual follicles were measured by computer-assisted planimetry for their staging.¹¹

Statistics

Statistical analysis was performed using a general linear mixed model with log-transformed values of the parameters vascularized area, microvessel density, microvessel diameter, red blood cell velocity, and volumetric blood flow as dependent variables. Confidence intervals were calculated by fitting a linear model to log-transformed data that in turn were retransformed into original scales (error bars in Figure 5 and 6). Accordingly, summary statistics are expressed as geometric means and 95% confidence intervals (lower/upper). Factors included into this nested design model were time with five levels, treatment with four levels, time by treatment interactions, host animal nested within treatment group, and follicle nested within host animal.

The differences between follicles of 250 to 500 μm and $>500 \mu\text{m}$ at 48 hours after PMSG as well as follicles at 72 hours after either PMSG or PMSG/LH were tested across all time points by fitting a model without the time by treatment interaction effect. *P* values give the results of *F* statistics and are not adjusted for multiplicity. Additionally, comparisons were performed at the individual time points after follicle transplantation when indicated by the comparisons across time. In that case, the interaction effect was included into the model. Calculations were performed using the restricted maximum likelihood method and the small sample correction for standard errors according to Kenward Rogers as provided by the SAS procedure mixed (SAS Institute, Cary, NC). Differences in take rate of follicles were analyzed using the Fisher exact test (SigmaStat; Jandel Corp., San Rafael, CA). The criterion for significance was taken to be $P < 0.05$.

Results

First, we analyzed growth, angiogenesis and microcirculation of follicles in dependency of their stage of development. Experiments were undertaken in nontreated animals and in animals after stimulation with PMSG for 48 hours. Follicles with diameters $<250 \mu\text{m}$ [mean geometric diameter, 100 μm (51/161, ie, lower and upper 95% confidence intervals)], which were harvested from nontreated animals, did not vascularize. Follicles with diameters $<250 \mu\text{m}$ [mean geometric diameter, 185 μm (154/242, ie, lower and upper 95% confidence intervals)], but harvested from PMSG-stimulated hamsters (48 hours) and transplanted into synchronized animals, also failed to induce neovascularization. In contrast, all follicles with diameters $>250 \mu\text{m}$ established a complete microvascular network, regardless of whether they had been harvested from stimulated ($n = 14$) or unstimulated animals ($n = 3$). This was reflected by a take rate of 100% of follicles $>250 \mu\text{m}$ in diameter, contrasting the take rate of 0% of follicles with a diameter $<250 \mu\text{m}$ ($P < 0.05$). Follicles $<250 \mu\text{m}$ were characterized by H&E histology

Figure 2. H&E-stained cross-sections of formalin-fixed isolated follicular grafts. **A:** A tri- to quadrilaminar (arrow) follicle without any trace of thecal cell layers, indicating a secondary follicle of stage 3. **B:** A follicle of stage 6, exhibiting several layers of granulosa cells (arrow) with initiation of antral cavity formation. A well-developed multilayered thecal shell is clearly seen (double arrow). Scale bars: 20 μm (A), 50 μm (B).

as tri- to quadrilaminar follicles without any trace of theca cell layers (Figure 2A), indicating secondary follicles of stage 3 to 5 according to the classification of Roy and Greenwald.¹¹ Follicles with a diameter $>250 \mu\text{m}$ exhibited several layers of granulosa cells with beginning antrum formation and a well-developed multilayered thecal shell (Figure 2B), reflecting stages of follicular development >6 .¹¹

In case of follicle vascularization, initial angiogenesis was characterized by sinusoidal sacculations, capillary budding, and sprout formation. Throughout the following days protrusion of sprouts was observed, leading to interconnection of individual sprouts with a growing microvascular network (Figure 3). Finally, follicular grafts presented with a complete glomerulum-like microvascular network (Figure 3). The development of sprouts originated in $>80\%$ from the host striated muscle capillaries. Sprouts also developed from postcapillary venules of the host tissue, but only rarely from arterioles (Figure 4A). Blood flow from the follicular grafts was consistently drained by a microvascular system, which consisted of

Figure 3. Intravital fluorescence microscopic images of a follicular graft (borders are indicated by **double arrows**) directly after transplantation (**A** and **B**) as well as at day 5 (**C**) and day 10 (**D**) after transplantation into the hamster dorsal skinfold chamber. In contrast to the initial lack of nutritive capillaries within the freshly transplanted graft (**A** and **B**), on day 5 after transplantation (**C**) the newly formed microvessels begin to create a network of capillaries although substantial parts of the follicular graft still lack vascularization (**asterisks**). At day 10 after transplantation (**D**) the follicular graft exhibits a complete and fully developed glomerulum-like microvasculature (**asterisk**) that is supplied by feeding arterioles (**arrows**) and drained by a postcapillary venule (**arrowhead**). **A**, **C**, and **D**: Blue light epi-illumination with contrast enhancement by 5% FITC-labeled dextran 150,000 i.v. **B**: Ultraviolet epi-illumination of bisbenzimidazole-stained follicular tissue. Scale bars, 100 μm .

former capillary vascular segments of the host striated muscle (Figure 4B).

Although no signs of angiogenesis and new vessel formation could be observed in follicles of a diameter $<250 \mu\text{m}$ throughout the entire observation period of 14 days, quantitative analysis of larger follicles revealed a progressive increase of the area of vascularization with increasing microvessel density from day 3 to day 5, which then remained constant throughout the remainder of the experiment (Figure 5, A and B). Discrimination of these grafts according to size demonstrated that follicles with diameters between 250 and $500 \mu\text{m}$ showed almost comparable areas of vascularization (Figure 5A), but a tendency toward higher average values of microvessel density (Figure 5B) on days 3 and 5 compared with the $>500 \mu\text{m}$ -follicles. Capillary red blood cell velocity comparably increased ~ 10 -fold from day 3 to day 10 in both the smaller and larger follicles (Table 1). Follicular capillary diameters were found significantly reduced from day 3 to day 10, amounting to $\sim 8 \mu\text{m}$ at day 14 (Table 1). Up to day 10, capillary diameters in larger follicles were markedly wider than those in smaller follicles (Table 1).

Calculation of individual capillary blood perfusion in follicular grafts revealed a progressive increase of blood flow until vascularization was completed (days 7 to 10), which, however, was followed by a fall of 40 to 50% at day 14. In addition, capillaries of larger follicles showed markedly higher blood perfusion on days 5 to 14 when compared with those of smaller follicles (Table 1).

In a second set of experiments, we compared the vascularization of follicles that were harvested 72 hours after either PMSG or PMSG/LH treatment and transplanted into synchronized animals. The area of vascularization ranged between 60 and 90% (days 7 to 14) without significant differences between the two cohorts of follicles (Figure 6A). Concomitantly, microvessel density increased twofold from day 3 to day 5, and then remained constant at $\sim 250 \text{ cm/cm}^2$ in both the PMSG and the PMSG/LH treatment group (Figure 6B). Capillary red blood cell velocity increased significantly from $<100 \mu\text{m}/\text{second}$ at day 3 to 236 to $309 \mu\text{m}/\text{second}$ at day 10 in both PMSG- and PMSG/LH-treated follicles (Table 2), similarly as observed in follicles after 48 hours of PMSG treatment. In parallel, follicular capillary diameters de-

Figure 4. Intravital fluorescence microscopic images of the microvasculature of follicular grafts at day 14 after transplantation into hamster dorsal skinfold chambers. **A:** High magnification reveals the interaction of the newly formed microvessels with the microvasculature of the host tissue, demonstrating an arteriole (white arrows) that serves as vascular supply and multiple intercapillary anastomoses (black arrows) between the follicular capillaries (asterisk) and the striated muscle capillaries (arrowheads). **B:** Blood from the follicular graft is almost completely drained by a postcapillary vessel (arrowheads), which may function as a venule, but represents a former striated muscle capillary, as clearly indicated by the parallel arrangement with the other striated muscle capillaries (arrows). Blue light epi-illumination with contrast enhancement by 5% FITC-labeled dextran 150,000 i.v. Scale bars: 100 μm (A), 150 μm (B).

creased significantly during the first 10 days after transplantation (Table 2). Capillary blood perfusion markedly increased between day 3 and day 7 in the PMSG/LH-treated follicles. Intergroup comparison indicated significantly wider capillaries and higher perfusion values throughout the entire observation period in the PMSG/LH-treated follicles compared to those harvested and transplanted after sole PMSG treatment (Table 2).

Beside the assessment of microarchitecture and microhemodynamics of the newly developed follicular networks, *in vivo* staining of leukocytes by intravenous injection of rhodamine 6G allowed us to analyze the interaction of leukocytes with the microvascular endothelial lining. Interestingly, almost all of the leukocytes (>98%) passed the follicular microvascular networks without transient tethering or rolling interaction. Moreover, firm adherence of leukocytes within the follicular networks

Figure 5. Quantification of vascularized area (%) (A) and microvessel density (cm/cm^2) (B) of follicles after free transplantation into hamster dorsal skinfold chambers, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Follicles with diameters of 250 to 500 μm (filled squares) and diameters >500 μm (open squares) were harvested from hamsters at 48 hours after PMSG treatment and transplanted into synchronized animals. Follicles exhibiting diameters <250 μm , which were harvested from unstimulated animals or from animals at 48 hours after PMSG treatment and transplanted into unstimulated or synchronized animals, did not vascularize and are not displayed because of log-scaling of the y axis. Values are given as geometric means and 95% confidence intervals, which are based on a linear mixed model of log-transformed data. ^a, $P < 0.05$ versus day 3; ^b, $P < 0.05$ versus days 3 and 5; [#], $P < 0.05$ versus follicles with diameters of 250 to 500 μm .

was only rarely observed (Figure 7A). There were no signs of inflammation within follicular tissue and host tissue in direct proximity of the grafts, as indicated by the absence of macromolecular leakage (Figure 7B).

Epi-illumination of the bisbenzimidazole-stained follicular tissue revealed a homogeneous nuclear staining after transplantation and subsequent vascularization without condensation and fragmentation of chromatin (Figure 8, A and B). These apoptotic indices, however, were apparent in the case of insufficient follicular vascularization (Figure 8, C and D), but in particular in the case of lack of vascularization, as observed in follicles with diameters <250 μm (Figure 8, E and F).

Discussion

The hamster is thought to be the ideal model for the study of follicular angiogenesis, because the hamster's 4-day estrous cycle is extremely regular and maintained also in the face of excessively elevated concentrations of estro-

Table 1. Microvessel Diameter, Capillary Red Blood Cell Velocity, and Capillary Volumetric Blood Flow in Newly Developed Microvascular Networks of Follicular Grafts Upon Harvesting from Hamsters at 48 Hours after PMSG Treatment and Transplantation into Dorsal Skinfold Chambers of Synchronized Animals

	Day 3	Day 5	Day 7	Day 10	Day 14
Follicles with diameters of 250 to 500 μm (mean diameter 353 μm (305/404))					
Microvessel diameter (μm)	14.0 (12.4/15.8)	11.6 (10.3/13.1)*	10.2 (9.0/11.5) [†]	8.5 (7.6/9.6) [‡]	8.0 (7.1/9.1) [‡]
V_{RBC} ($\mu\text{m}/\text{sec}$)	28.5 (18.9/42.8)	91.6 (60.9/137.8)*	123.9 (82.4/186.4)*	220.7 (146.7/332.0) [‡]	142.2 (94.5/214.0)*
Volumetric blood flow (pL/sec)	3.4 (2.2/5.2)	7.4 (4.8/11.4)*	7.7 (5.0/11.9)*	9.6 (6.2/14.9)*	5.5 (3.6/8.5)
Follicles with diameters >500 μm (mean diameter 750 μm (607/970))					
Microvessel diameter (μm)	16.0 (14.1/18.1)	15.1 (13.3/17.1) [¶]	11.8 (10.4/13.4) [†]	10.6 (9.4/12.1) ^{¶¶}	8.1 (7.1/9.2) [§]
V_{RBC} ($\mu\text{m}/\text{sec}$)	23.4 (15.3/35.9)	103.6 (67.6/158.6)*	167.1 (109.1/255.9)*	240.0 (156.7/367.5) [†]	213.2 (139.3/326.5) [†]
Volumetric blood flow (pL/sec)	3.6 (2.2/5.8)	14.1 (8.8/22.7) ^{¶¶}	14.0 (8.7/22.5) ^{¶¶}	16.2 (10.1/26.1)*	8.3 (5.2/13.4)*

Values are given as geometric means with lower and upper 95% confidence intervals (number/number), which are based on a linear mixed model of log-transformed data.

* $P < 0.05$ versus day 3.

[†] $P < 0.05$ versus days 3 and 5.

[‡] $P < 0.05$ versus days 3, 5, and 7.

[§] $P < 0.05$ versus days 3, 5, 7, and 10.

[¶] $P < 0.05$ versus follicles with diameters of 250 to 500 μm .

gens induced by superovulation, which would disrupt the estrous cycle of the rat.²³ Moreover, the hamster is suitable for the use of the skinfold chamber preparation that allows intravital microscopic observations of follicular transplants in conscious animals throughout a prolonged period of time. The availability of different fluorescent markers for *ex vivo* and *in vivo* staining extended the possibilities of intravital microscopy from purely morpho-

logical analysis to the study of complex physiological and cellular events.²² The systematic *in vivo* analysis of dynamic processes during follicular graft vascularization, such as capillary sprouting, capillary network development, microvascular blood perfusion, cellular communication (leukocyte-endothelial cell interaction), and change of permeability (edema formation) may provide new insights into the process of physiological angiogenesis of the female reproductive system, markedly extending the present knowledge achieved from studies using vascular corrosion cast techniques.^{24,25}

Herein, we demonstrate the vascularization of mature preovulatory, but not immature preantral follicles, including a distinct temporal and spatial pattern of blood vessel growth and regression. Interestingly, the host's capillaries and venules, but not arterioles, showed the potential to protrude vascular sprouts. *In vitro* studies have indicated that the contact of vascular smooth muscle cells inhibits the stabilization of endothelial capillary-like structures,²⁶ which might be the reason that vascular sprouting from arterioles was rarely observed, and if so, only from the very terminal arteriolar vessels. Moreover, endothelial cell-pericyte contact is thought to play a permissive role in controlling angiogenesis and determining blood vessel maturation *in vivo* in normal adult tissue,^{27,28} supposedly also including the female reproductive system. This implies that retraction of pericytes with subsequent capillary-like tube formation, as demonstrated in electron microscopic studies,²⁹ might take place in capillaries, but not in arterioles. Ultrastructural analyses showed that early capillary sprout formation in the bovine corpus luteum is usually preceded by pericyte migration at the tips of the sprouts. These pericytes serve as guiding structures aiding outgrowth of endothelial cells in the bovine corpus luteum.³⁰

Beside luteal follicular tissue, nonluteal follicular tissue, namely the theca or follicular wall, also possesses angiogenic activity as demonstrated by the stimulation of endothelial cell proliferation and migratory activity by nonluteal ovarian tissue extracts.³¹ These findings coincide with vascular endothelial growth factor immunohistochemistry data in that both luteinized granulosa cells and thecal cells are the predominant sites of angiogenic activity within the follicles.³² In support of this, we show now

Figure 6. Quantification of vascularized area (%) (A) and microvessel density (cm/cm^2) (B) of follicles after free transplantation into hamster dorsal skinfold chambers, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Follicles with diameters >500 μm were harvested from hamsters at 72 hours after either PMSG (open circles) or PMSG/LH treatment (open triangles) and transplanted into synchronized animals. Values are given as geometric means and 95% confidence intervals, which are based on a linear mixed model of log-transformed data. a , $P < 0.05$ versus day 3.

Table 2. Microvessel Diameter, Capillary Red Blood Cell Velocity, and Capillary Volumetric Blood Flow in Newly Developed Microvascular Networks of Follicular Grafts (Diameters $>500\ \mu\text{m}$) upon Harvesting from Hamsters at 72 Hours after either PMSG or PMSG/LH Treatment and Transplantation into Dorsal Skinfold Chambers of Synchronized Animals

	Day 3	Day 5	Day 7	Day 10	Day 14
Follicles at 72 hours after PMSG treatment (mean diameter $762\ \mu\text{m}$ (690/898))					
Microvessel diameter (μm)	15.5 (13.7/17.5)	10.6 (9.4/11.9)*	9.3 (8.2/10.5)*	8.0 (7.0/9.0)†	7.1 (6.3/8.0)‡
V_{RBC} ($\mu\text{m}/\text{sec}$)	74.6 (48.8/114.0)	103.8 (68.0/158.7)	226.0 (147.9/345.4)†	236.0 (154.5/360.6)†	122.8 (80.4/187.6)§
Volumetric blood flow (pL/sec)	10.8 (6.7/17.3)	7.0 (4.3/11.2)	11.7 (7.3/18.9)	9.0 (5.6/14.5)	3.7 (2.3/6.0)§
Follicles at 72 hours after PMSG/LH treatment (mean diameter $961\ \mu\text{m}$ (863/1130))					
Microvessel diameter (μm)	19.6 (16.9/22.6)¶	14.7 (12.7/17.0)*¶	12.5 (10.8/14.4)¶	10.4 (9.0/12.0)¶	9.6 (8.3/11.1)¶
V_{RBC} ($\mu\text{m}/\text{sec}$)	39.0 (24.3/62.5)¶	188.5 (117.4/302.7)*	277.2 (172.6/445.1)*	308.7 (192.2/495.7)*	171.2 (106.6/275.0)*
Volumetric blood flow (pL/sec)	9.0 (5.3/15.3)	24.6 (14.4/41.8)*¶	26.0 (15.3/44.3)¶	20.2 (11.9/34.3)*¶	9.5 (5.6/16.2)¶

Values are given as geometric means with lower and upper 95% confidence intervals (number/number), which are based on a linear mixed model of log-transformed data.

* $P < 0.05$ versus day 3.

† $P < 0.05$ versus days 3 and 5.

‡ $P < 0.05$ versus days 3, 5, and 7.

§ $P < 0.05$ versus days 7 and 10.

¶ $P < 0.05$ versus days 5, 7, and 10.

|| $P < 0.05$ versus follicles at 72 hours after PMSG treatment.

for the first time by direct *in vivo* analysis that preovulatory follicles with diameters $>250\ \mu\text{m}$, ie, follicles of stage 6 to 10¹¹ with prominent theca cell layers do have the capac-

ity to elicit neovascularization by the host when transplanted onto the striated muscle tissue of synchronized animals. No vascular response, however, could be observed in follicular implants lacking thecal cells, regardless of whether transplanted into unstimulated or superovulated hosts. The latter observation may directly reflect the natural situation of small preantral follicles that have no special vascular supply of their own. Our observation of developmental stage-dependent neovascularization of follicles extends the previous findings of Gospodarowicz and Thakral,¹³ who demonstrated with the use of the rabbit cornea the capability of ovarian corpora lutea, but not of PMSG-stimulated follicles, to induce neovascularization. However, these authors failed to specify the follicular grafts, whereas the present study now offers proof that the developmental stage of the follicles, in particular the presence of thecal cells rather than the hormone balance of the recipient, is the determinant for graft vascularization. Moreover, the present result of comparable vascularization of large preovulatory follicular grafts transplanted into synchronized animals after either PMSG or PMSG/LH treatment is in line with the results from *in vivo* and *in vitro* assays^{31,33} that the thecal cell layer is the major source of vascular endothelial growth factor production and thus determines vascular growth and development of the follicle.³⁴ In support of this, thecal production of angiogenic factors seems to be independent of preovulatory stage or follicular status.⁸

Beside specific angiogenic factors, such as vascular endothelial growth factor,³⁴ angiogenin,³⁵ and angiopoietin-1 and -2,⁹ hypoxic stress with release of hypoxia-induced factor should be considered as a regulator of angiogenesis and the driving force for new vessel formation.³⁶ On the other hand, tissue mass may act as a critical component overtasking the host tissue's capacity to respond on the angiogenic stimuli. Comparing angiogenesis of the cohort of smaller (diameter range of 250 to 500 μm) and the cohort of larger follicles (diameter $>500\ \mu\text{m}$) transplanted at 48 hours after PMSG treatment, it seems that the smaller follicles have a more favorable relation between their tissue mass and their capacity to induce vascularization, as indicated by the tendency of development of microvascular networks with a slightly

Figure 7. Intravital fluorescence microscopic images of a follicular microvascular network at day 14 after transplantation. Using green light epi-illumination with visualization of rhodamine 6G-stained white blood cells (**A**), signs of inflammatory reactions of the host tissue could not be found, as indicated by only few leukocytes (arrows, **A**) interacting with the endothelium of follicular capillaries (asterisk, **B**) and draining venules (arrowheads, **B**). Moreover, blue light epi-illumination with contrast enhancement using 5% FITC-labeled dextran 150,000 i.v. revealed absence of macromolecular leakage and edema formation (**B**). Scale bars, 200 μm .

Figure 8. Intravital fluorescence microscopic images of follicles at day 7 after transplantation into hamster dorsal skinfold chambers. Note the dense microvascular network of the follicle (**A**, **asterisk**) with absence of cells displaying apoptotic chromatin condensation (**B**), whereas the follicle with only peripheral vascularization (**C**, **asterisk**) exhibits a small fraction of apoptotic cells (**D**, **arrow**). Lack of vascularization (**asterisk**) of a small follicle ($<250\ \mu\text{m}$, follicular border is outlined, **E**) is associated with a marked incidence of cell apoptosis (**F**, **arrows**), supposedly indicating atretic regression of the graft. Intravital fluorescence microscopy with blue light epi-illumination and contrast enhancement using 5% FITC-labeled dextran 150,000 i.v. (**A**, **C**, **E**) and ultraviolet epi-illumination of bisbenzimidazole-stained follicles (**B**, **D**, **F**; **asterisks** indicate follicular oocytes). Scale bars: $50\ \mu\text{m}$ (**A–D**), $30\ \mu\text{m}$ (**E** and **F**).

higher capillary density. However, our results also show that the higher metabolic demand, which larger follicles are expected to have because of their tissue mass, is met by a higher individual capillary blood perfusion because of wider capillaries and hence lower resistance to blood flow. Therefore, increased capillary blood perfusion in follicular grafts might best be interpreted as an adapta-

tion to the increased functional demand of the graft for oxygen and nutrients. This view ideally fits with the observation of increased perfusion of PMSG/LH-stimulated follicles compared to those after sole PMSG treatment, because PMSG/LH-stimulated follicles may consist mainly of granulosa cells converting to luteal cells with a high metabolic demand, whereas follicles after sole

PMSG treatment compromise a higher fraction of metabolically less active granulosa cells.

Although the present study did not primarily focus on follicular regression and atresia, we observed some apoptotic cells within the follicular graft during the regression of the microvascular network. Moreover, in case of lack of vascularization, apoptosis of granulosa cells seems to determine the fate of the follicular graft, as has been proposed as a concept of maintenance of total cell mass and homeostasis of the ovary.³⁷

By adding new data on the sparse knowledge on vascular development of reproductive tissue, we conclude, that ovarian follicle transplantation in the skinfold chamber of Syrian golden hamsters offers an useful experimental approach, not only to focus on follicular physiology, but also on overall aspects of physiological angiogenesis.

References

- Folkman J: Tumor angiogenesis. *Adv Cancer Res* 1985, 43:174–203
- Folkman J: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995, 1:27–31
- Greenburg GB, Hunt TK: Proliferative response in vitro of vascular endothelial and smooth muscle cells exposed to wound fluids and macrophages. *J Cell Physiol* 1978, 97:353–360
- Polverini PJ, Cotran PS, Gimbrone MA, Unanue ER: Activated macrophages induce vascular proliferation. *Nature* 1977, 269:804–806
- Banda MJ, Knighton DR, Hunt TK, Werb Z: Isolation of a nonmitogenic angiogenic factor from wound fluid. *Proc Natl Acad Sci USA* 1982, 79:7773–7777
- Dvorak HF: Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986, 315:1650–1659
- Jones MK, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ, Tarnawski AS: Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs. Insight into mechanisms and implications for cancer growth and ulcer healing. *Nat Med* 1999, 5:1418–1423
- Reynolds LP, Killilea SD, Redmer DA: Angiogenesis in the female reproductive system. *FASEB J* 1992, 6:886–892
- Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG: Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. *Lab Invest* 1998, 78:1385–1394
- Tonetta SA, diZerega GS: Intraovarian regulation of follicular maturation. *Endocr Rev* 1989, 10:205–229
- Roy SK, Greenwald GS: An enzymatic method for dissociation of intact follicles from the hamster ovary: histological and quantitative aspects. *Biol Reprod* 1985, 32:203–215
- Bassett DL: The changes in the vascular pattern of the ovary of the albino rat during the estrous cycle. *Am J Anat* 1943, 73:251–291
- Gospodarowicz D, Thakral KK: Production of a corpus luteum angiogenic factor responsible for proliferation of capillaries and neovascularization of the corpus luteum. *Proc Natl Acad Sci USA* 1978, 75:847–851
- Frederick JL, Shimanuki T, diZerega GS: Initiation of angiogenesis by human follicular fluid. *Science* 1984, 224:389–390
- Rone JD, Goodman AL: Preliminary characterization of angiogenic activity in media conditioned by cells from luteinized rat ovaries. *Endocrinology* 1990, 127:2821–2828
- Neufeld G, Ferrara N, Schweigerer L, Mitchell R, Gospodarowicz D: Bovine granulosa cells produce basic fibroblast growth factor. *Endocrinology* 1987, 121:597–603
- Grazul-Bilska AT, Redmer DA, Reynolds LP: Production of heparin-binding angiogenic factor(s) by bovine corpora lutea during pregnancy. *J Anim Sci* 1992, 70:254–262
- Kamat BR, Brown LF, Manseau EJ, Senger DR, Dvorak HF: Expression of vascular permeability factor/vascular endothelial growth factor by human granulosa and theca lutein cells: role in corpus luteum development. *Am J Pathol* 1995, 146:157–165
- Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E: Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest* 1993, 91:2235–2243
- Endrich B, Asaishi K, Goetz A, Messmer K: Technical report—a new chamber technique for microvascular studies in unanesthetized hamsters. *Res Exp Med* 1980, 177:125–134
- Baker M, Wayland H: On-line volume flow rate and velocity profile measurement for blood in microvessels. *Microvasc Res* 1974, 7:131–143
- Menger MD, Lehr HA: Scope and perspectives of intravital microscopy—bridge over from in vitro to in vivo. *Immunol Today* 1993, 14:519–522
- Greenwald GS: Follicular selection in the cyclic hamster: a 1960 model. *Hum Reprod* 1995, 10:2225–2227
- Macchiarelli G, Nottola SA, Vizza E, Kikuta A, Murakami T, Motta PM: Ovarian microvasculature in normal and hCG stimulated rabbits. A study of vascular corrosion casts with particular regard to the interstitium. *J Submicrosc Cytol Pathol* 1991, 23:391–395
- Forsman AD, McCormack JT: Microcorrosion casts of hamster luteal and follicular vasculature throughout the estrous cycle. *Anat Rec* 1992, 233:515–520
- Nehls V, Drenckhahn D: A microcarrier-based cocultivation system for the investigation of factors and cells involved in angiogenesis in three-dimensional fibrin matrices in vitro. *Histochem Cell Biol* 1995, 104:459–466
- Hirschi KK, D'Amore PA: Pericytes in the microvasculature. *Cardiovasc Res* 1996, 32:687–698
- Hirschi KK, Rohovsky SA, D'Amore: PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol* 1998, 141:805–814
- Egginton S, Hudlicka O, Brown MD, Graciotti L, Granata AL: In vivo pericyte-endothelial cell interaction during angiogenesis in adult cardiac and skeletal muscle. *Microvasc Res* 1996, 51:213–228
- Amselgruber WM, Schafer M, Sinowatz F: Angiogenesis in the bovine corpus luteum: an immunohistochemical and ultrastructural study. *Anat Histol Embryol* 1999, 28:157–166
- Makris A, Ryan KJ, Yasumizu T, Hill CL, Zetter BR: The nonluteal porcine ovary as a source of angiogenic activity. *Endocrinology* 1984, 115:1672–1677
- Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N, Zeleznik AJ: Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. *Endocrinology* 1992, 131:254–260
- Koos R, LeMaire WJ: Factors that may regulate the growth and regression of blood vessels in the ovary. *Semin Reprod Endocrinol* 1983, 1:295–307
- Gordon JD, Mesiano S, Zaloudek CJ, Jaffe RB: Vascular endothelial growth factor localization in human ovary and fallopian tubes: possible role in reproductive function and ovarian cyst formation. *J Clin Endocrinol Metab* 1996, 81:353–359
- Lee HS, Lee I, Kang TC, Jeong GB, Chang SI: Angiogenin is involved in morphological changes and angiogenesis in the ovary. *Biochem Biophys Res Commun* 1999, 257:182–186
- Neeman M, Abramovitch R, Schiffenbauer YS, Tempel C: Regulation of angiogenesis by hypoxic stress: from solid tumours to the ovarian follicle. *Int J Exp Pathol* 1997, 78:57–70
- Williams GT, Smith CA: Molecular regulation of apoptosis: genetic controls of cell death. *Cell* 1993, 74:777–779