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ANGIOGENESIS IN THE GONADAL CAPILLARY NETWORK OF THE CHICK EMBRYO

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Abstract

Seventy-one chick embryos of both sexes at the 35 Hamburger and Hamilton (H-H) developmental stage were processed for scanning electron microscopy of vascular corrosion casts and of critical point dried specimens, as well as transmission electron- and light microscopy, in order to study the angiogenic structures. The gonadal subepithelial capillary network was located at the level of the tunica albuginea under the covering epithelium. The casts showed a densely-meshed capillary network and numerous sprouting (nodular protrusions or capillary sprouts) and non-sprouting (enlarged vessels and angiogenic holes) angiogenic structures that were randomly distributed and mixed. Four types of angiogenic holes were encountered in the casts: primary (diameter < 2.5 μm), secondary (diameter > 2.5 μm), tertiary (variable diameter and circular narrowings on one side), and open angiogenic holes. We suggest that the different morphologies reflect evolution of these holes. Furthermore, the open angiogenic hole would probably either form nodular protrusions at its open ends, which tend to join with other nodular protrusions and neighboring capillaries and form new vessels; or there would be fusion with two or more neighboring open holes. Correlative critical point dried sections showed fenestrations in the capillary walls and transcapillary pillars that corresponded to the angiogenic holes found in the casts. Ultrathin sections of the vessels presented typical characteristics of growing endothelium: large nuclei with loosely textured chromatin, abundant cytoplasm rich in cell organelles and intraluminal endothelial processes.

Key Words: Angiogenesis, gonads, chick embryo, microcirculation, corrosion casting, scanning electron microscopy, transmission electron microscopy.

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Introduction

Two different mechanisms are generally thought to be involved in the development and growth of the vascular system: vasculogenesis and angiogenesis (Folkman, 1995). Vasculogenesis is the formation of a vessel by endothelial cells that have differentiated *in situ* from their mesenchymal precursors (Poole and Coffin, 1989; Risau, 1995). Angiogenesis is the formation of vessels from pre-existing vessels. Angiogenesis has been found to be stimulated by the release of angiogenic substances by the tissues to be vascularized (Folkman and Klagsbrun, 1987; Risau, 1991; Folkman and Shing, 1992; Ferrara *et al.*, 1995). Two different angiogenic processes have been described: a sprouting (Coffin and Poole, 1988; Poole and Coffin, 1989; Dieter-Lièvre and Pardanaud, 1993) and a non-sprouting angiogenic mechanism, the intussusceptive capillary growth. The latter was first described by Caduff *et al.* (1986) and consists of expansion of the capillary network by insertion of slender transcapillary tissue pillars.

Angiogenesis is an important process both in pathological and physiological situations. Thus, angiogenesis has been related to different pathological processes as tumor growth (Ausprunk and Folkman, 1977; Folkman, 1982, 1995; Folkman and Klagsbrun, 1987; Zama *et al.*, 1991; Plate *et al.*, 1994; Guidi *et al.*, 1995), inflammation and wound healing (Burger *et al.*, 1983; Arnold and West, 1991; Phillips *et al.*, 1991, 1993; Dvorak *et al.*, 1995), and rat lung fibrosis (Peao *et al.*, 1994). On the other hand, it has also been extensively studied in physiological growing tissues of adult animals: e.g., ovaries and testicles of golden hamsters (Spanel-Borowski and Mayerhofer, 1987; Spanel-Borowski *et al.*, 1987; Mayerhofer and Bartke, 1990; Forsman and McCormack, 1992), ovaries of rats (Murakami *et al.*, 1988) and rabbits (Macchiarelli *et al.*, 1991), mesentery of rats (Rhodin and Fujita, 1989).

Angiogenesis related to development has been an habitual subject of study (Poole and Coffin, 1988; Noden, 1989; Pardanaud *et al.*, 1989; DeRuiter *et al.*, 1993), especially in chick embryos (Sethi and Brookes, 1971;

Bertossi and Roncali, 1981; Bertossi *et al.*, 1987; Sorrell, 1988; DeFouw *et al.*, 1989; Ribatti *et al.*, 1991; Nico *et al.*, 1992; Smith *et al.*, 1992; Nguyen *et al.*, 1994; Henry and DeFouw, 1995).

Frequent works on chick gonadal morphogenesis can be found in the literature (Brode, 1928; Lillie, 1952; Romanoff, 1960; Witschi, 1961; De-Simone-Santoro, 1967; Rahil and Narbaitz, 1972; Stahl and Carlon, 1973; Kopp and Bertrand, 1978; Fargeix *et al.*, 1981; Popova and Scheib, 1981; Carlon *et al.*, 1983; Rodemer *et al.*, 1986; Avila *et al.*, 1989a,b; Rodemer-Lenz, 1989; Méndez-Herrera *et al.*, 1993; Civinini *et al.*, 1994; Ukeshima, 1994). In spite of this, only a few remarks on the developing gonadal vascularization of the fowl can be found in old investigations (Miller, 1903; Van Limborgh *et al.*, 1960).

The aim of this work was to study the gonadal capillary network of the chick embryo and the angiogenic structures involved in its growth by means of scanning electron microscopy (SEM) of vascular corrosion casts and critical point dried specimens, supplemented with transmission electron (TEM) and light microscopy (LM).

For this purpose, we studied the capillary network of both testicles and the left ovary at the 35 Hamburger and Hamilton (H-H) (1951) developmental stage (8.5-9 days of incubation), since the right ovary undergoes regression in most of the birds. According to classical embryological treatises (Brode, 1928; Lillie, 1952; Romanoff, 1960), sexual differentiation of chick embryos is morphologically evident in this stage (35 H-H).

Material and Methods

Seventy-one 35 H-H embryos of commercially bred chickens (*Gallus domesticus*, White Leghorn) of both sexes (thirty-four males and thirty-seven females) were used in this study. The fertilized eggs were incubated at 37.5°C and 60% relative humidity. The development of the embryos was staged according to Hamburger and Hamilton (1951).

Vascular corrosion casting

For SEM study of vascular corrosion casts, eighteen male and sixteen female embryos were injected following the technique described by Carretero *et al.* (1993). The resin used for corrosion casting was Mercox® (Mercox-Japan Vilene Co., Tokyo, Japan), supplied by Ladd Research Ind., Inc. (Williston, VT), diluted with methyl-methacrylate (Hodde and Nowell, 1980). The resulting mixture was 3:1 (Mercox®: methyl-methacrylate). A Pasteur's pipette was used as a cannula. The pipette was fire polished to a gauge similar to the vessel to be injected. Extraembryonic vessels, the umbilical artery or vein (*A. et v. umbilicales*), were chosen as the site of injection. Then, the pipette was manually inserted into the vessel and sealed with a chemical ligature (Cyanoacrylate, Loctite). After corrosion in 5% KOH and exhaustive rinsing with distilled water, the casts were processed for SEM and observed in a Hitachi (Tokyo, Japan) S-570 scanning electron microscope operated at accelerating voltages of 5-7 kV.

Figure 1. Ventral view of a vascular cast showing the two mesonephroi (Ms) and ovaries of a 35 H-H female chick embryo. The gonads are located on the ventral surface of the mesonephroi on either side of the caudal vena cava (CV). Note that the left ovary (LO) is markedly bigger than the right ovary (RO). Arrows show drainage of the capillary network into the subcardinal vein (Sb). Bar = 0.5 mm.

Figure 2. Caudal view of a transverse section of a critical point dried specimen showing the disposition of the left and right ovaries ventrally to the two mesonephroi (Ms). Vessels are dilated due to perfusion fixation. Mt: metanephros; LO: left ovary; RO: right ovary; A: aorta; CV: caudal cava vein. Bar = 0.5 mm.

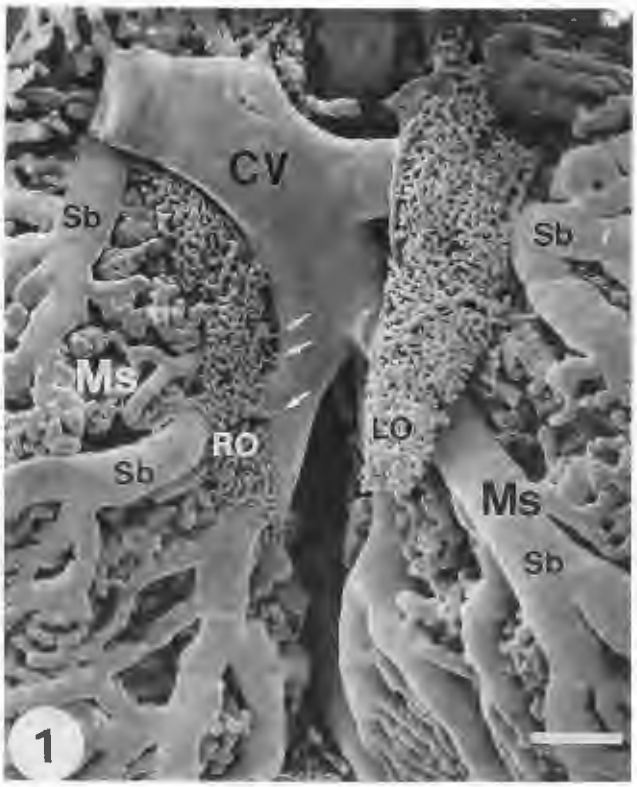
Figure 3. Transverse histological section of the left ovary showing the covering epithelium (ce), the cortical layer (c), the primary ovarian tunica albuginea (ta) and the medullar layer (me) with cavities. The subepithelial capillary network is located at the level of the primary tunica albuginea. Asterisk: subcardinal vein dilated due to perfusion fixation. Bar = 80 μm.

Figure 4. Histological section of a testicle showing the covering epithelium (ce) and the developing sexual cords (sc). The subepithelial capillary network (*) is located at the level of the definitive testicular tunica albuginea (ta), below the covering epithelium. Vessels are dilated due to perfusion fixation. Bar = 50 μm.

Figure 5. Transverse section of a vascular cast of the left ovary. The gonadal arteries (a) give rise to the superficial subepithelial capillary network (asterisk). Sb: subcardinal vein. Bar = 55 μm.

Critical point dried specimens

Eight male and eleven female embryos were processed for critical point drying. The embryos were perfused through the umbilical artery with 2-3 ml of 0.1 M phosphate buffered 2% paraformaldehyde and 0.5% glutaraldehyde (pH = 7.2). The two mesonephroi and gonads were dissected out and post-fixed by immersion in the above mentioned solution. The samples were washed 2-4 times in 0.2 M cacodylate buffer (pH = 7.2) at 4°C, immersed in liquid nitrogen and freeze-sectioned transversely with a razor blade. The specimens were then dehydrated in graded ethyl-alcohols and critical point dried.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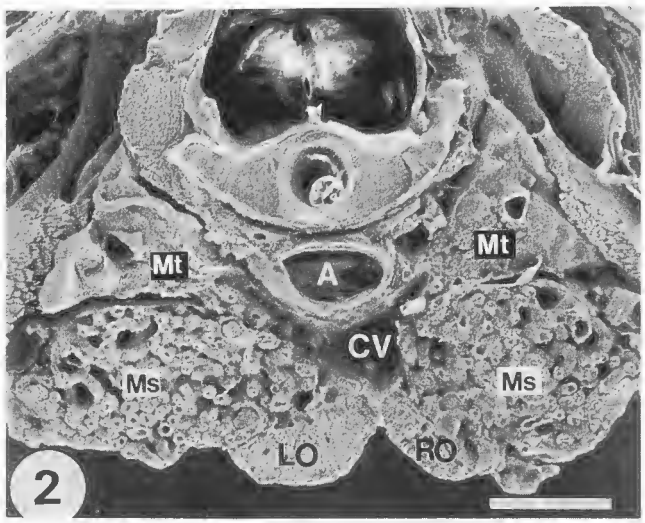
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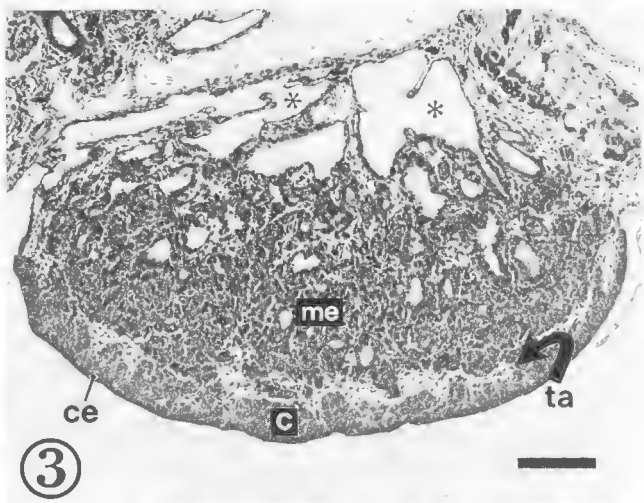
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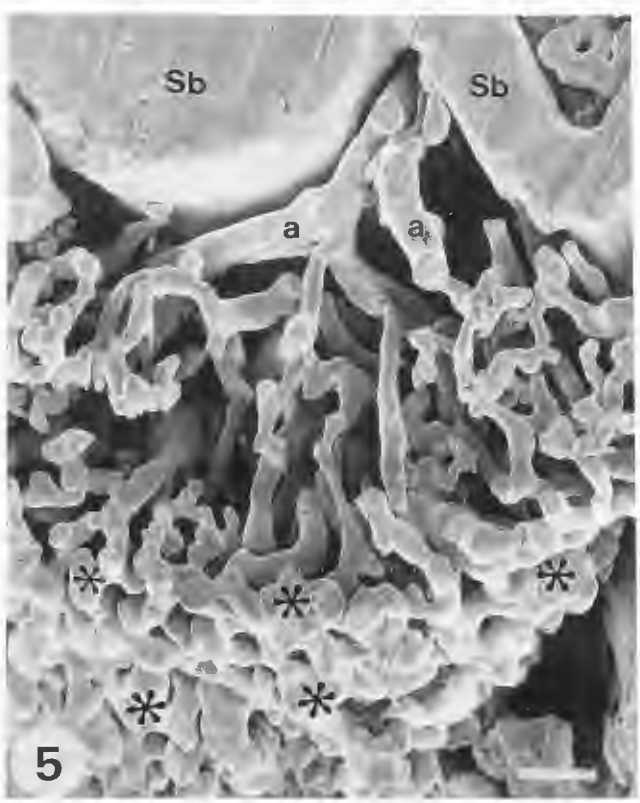
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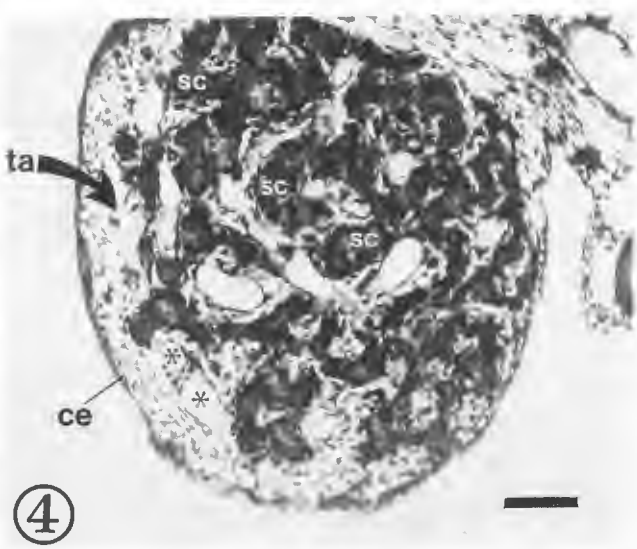
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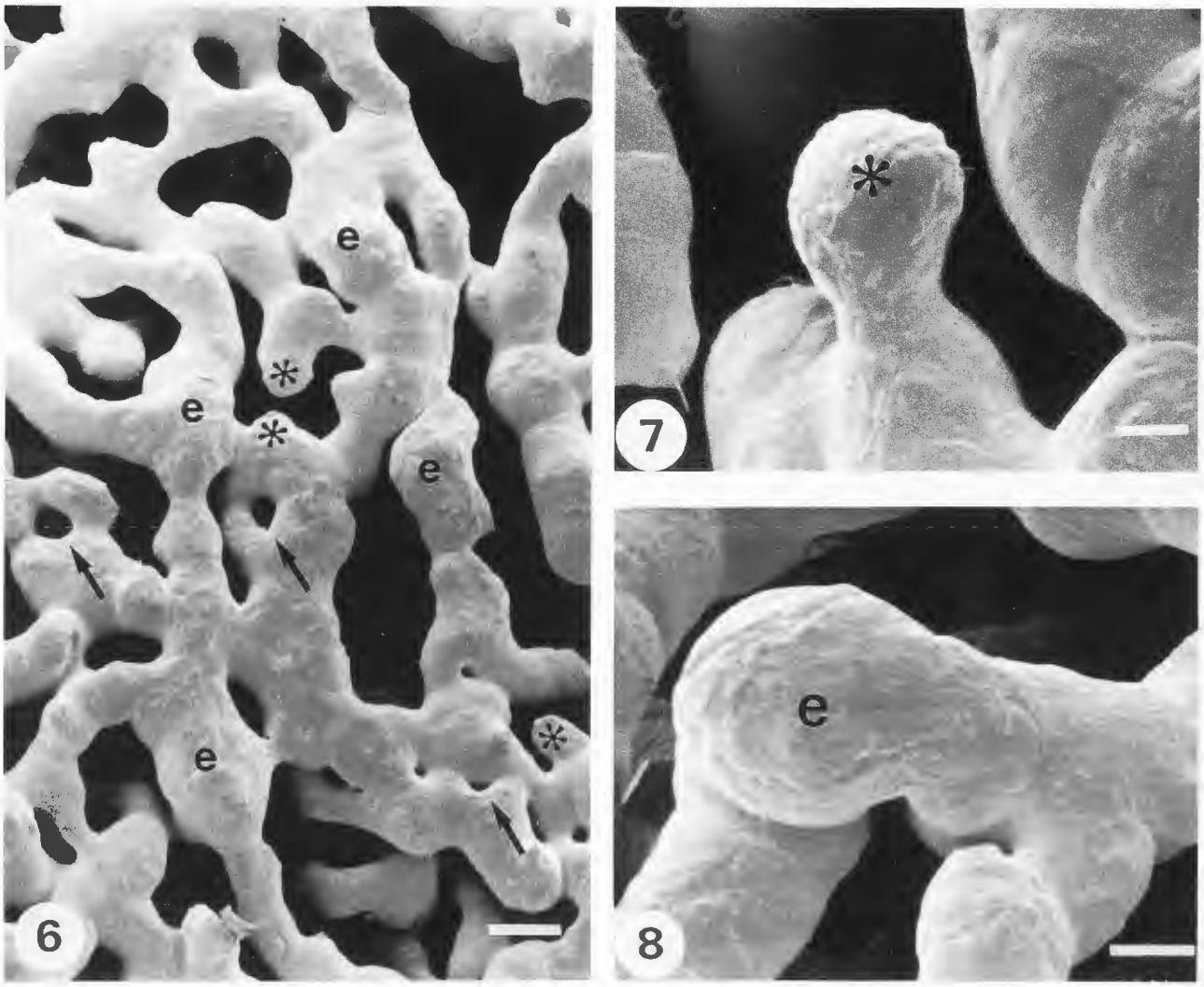


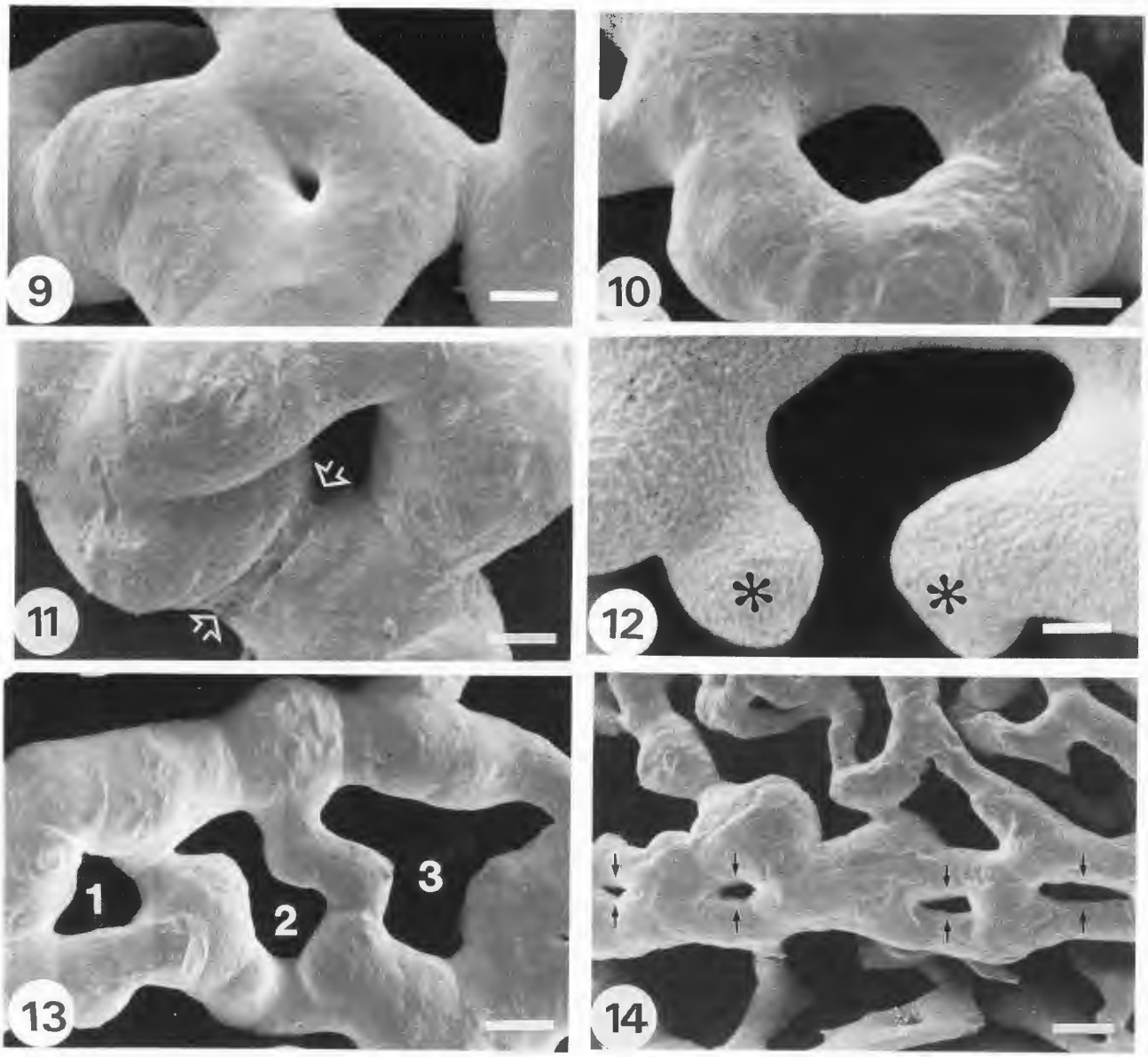
Figures 6-8. Cast gonadal subepithelial capillary network of the left ovary. **Figure 6.** Overview; note numerous angiogenic structures such as nodular protrusions (*), enlarged vessels (e), and angiogenic holes (arrows) of different types. Bar = 25 μm. **Figure 7.** Finger-like nodular protrusion (*) in the capillary network. Bar = 7 μm. **Figure 8.** Enlarged capillary (e) in the left ovary. Bar = 10 μm.

Two additional embryos (one male and one female) were perfused with the fixative, washed with physiological saline (38°C), dissected, frozen in 2-methylbutane for 20 seconds and transferred into liquid nitrogen. Subsequently, they were embedded in O.C.T Compound 4583 (Tissue-tek®, Miles, Elkhart, IN) and horizontally serially sectioned in a cryostat (2800-Frigocut-E, Reichert-Jung, Vienna, Austria). The slides, approximately 40 μm thick, were then processed for critical point drying.

After critical point drying, the samples were mounted on stubs, coated with gold with a sputter coater, and observed in a Hitachi S-570 scanning electron microscope at an acceleration voltage of 15-20 kV.

Transmission electron microscopy

Four chick embryos (two males and two females) were used for analysis by TEM. The samples were dissected out and immersed in the fixative for 2 hours, then rinsed with 0.2 M cacodylate buffer, postfixed in 0.1 M cacodylate buffer supplemented with 1% osmium tetroxide (pH = 7.4) for 2 hours, and again rinsed with the buffer. The specimens were dehydrated in a series of graded acetone solutions and embedded in Spurr resin (TAAB, Aldermaston, Berk., U.K.). Ultrathin sections were stained with alkaline lead citrate and observed in a Hitachi H-7000 transmission electron microscope.



Figures 9-14. Capillary network of the left ovary. **Figure 9.** Primary angiogenic hole. Bar = 8 μm. **Figure 10.** Secondary angiogenic hole. Bar = 8 μm. **Figure 11.** Tertiary angiogenic hole in a cast; note the circular narrowing on one side (arrows). Bar = 8 μm. **Figure 12.** Open angiogenic hole in a cast; note the nodular protrusions (*) arising from the open ends of the hole. Bar = 8 μm. **Figure 13.** Several open angiogenic holes could be seen associated in a cast: 1: secondary angiogenic hole; 2: the shape of the angiogenic hole may reflect association of two open angiogenic holes; 3: the shape of the angiogenic hole may reflect association of three open angiogenic holes. Bar = 15 μm. **Figure 14.** Angiogenic holes (arrows) located in a single vessel. Bar = 20 μm.

Light microscopy

Correlative analyses were made with twelve embryos (five males and seven females) processed for routine LM. For this purpose, the embryos were perfused with the mentioned fixative, postfixed by immersion in 10% buffered formaldehyde, dehydrated in graded ethyl-alcohols and embedded in paraffin. Finally, the samples were serially sectioned and stained with hematoxylin-eosin.

Measurements were made directly on micrographs, covering a constant area of the gonadal subepithelial capillary network of approximately 180 mm^2, by means of a measuring magnifier, x10 (Peak, Electron Microscopy Sciences, Washington, D.C.). Approximate length

of the gonads was measured on micrographs of critical point dried specimens. Vascular density of the gonadal subepithelial capillary network was estimated by means of a stereologic test grid (Weibel, 1973).

The nomenclature used conforms to Nomina Anatomica Avium (Baumel *et al.*, 1979) and to NEV (1994).

Results

Location of the gonadal subepithelial capillary network

At the 35 H-H developmental stage, the gonads appeared as small bean-shaped bodies located on the ventral surface of the two mesonephroi on either side of the caudal vena cava (*Vena cava caudalis*) (Figs. 1 and 2). Both testicles exhibited a similar size (approximately 1.65 mm long), whereas the left ovary (approximately 1.75 mm long) was markedly bigger than the right one (approximately 1.00 mm long) (Figs. 1 and 2). On transversal sections, the left ovary displayed a multilayered covering epithelium (2-3 layers thick), a cortical layer and a medullar layer with some cavities (Fig. 3). The cortical and medullar layer were separated by the primary ovarian tunica albuginea. On the other hand, both testicles showed similar characteristics; a thin covering epithelium (1-2 layers thick), together with developing sexual cords and interstitial tissue could be observed (Fig. 4). The definitive testicular tunica albuginea was located under the covering epithelium (Fig. 4).

The gonadal subepithelial capillary network covering the surface of the gonads could be easily distinguished in the casts (Figs. 1 and 5). The gonadal arteries (*Aa. gonadales*), branches from secondary and tertiary mesonephric arteries, were connected to the gonadal subepithelial capillary network (Fig. 5). The mesonephric arteries are branches of the aorta {for further details on mesonephric vascularization, see Carretero *et al.* (1995)}. The gonadal subepithelial capillary network drained into the caudal vena cava and the subcardinal veins at different points (Fig. 1). The gonadal subepithelial capillary network was located at the level of the tunica albuginea (Fig. 4).

Morphology of the gonadal subepithelial capillary network

The casts showed that the capillary network of the gonads was densely-meshed (Fig. 6) and exhibited numerous angiogenic structures that could be classified into two different types: sprouting (nodular protrusions or capillary sprouts) and non-sprouting (enlarged vessels and angiogenic holes). Both types of angiogenic structures were found randomly distributed and mixed throughout the gonadal capillary network (Fig. 6).

Some differences could be noticed between the capillary network of the testicles and that of the left ovary. The subepithelial capillary network of the left ovary exhibited a mean density of 78.03 % $\pm$ 3.84 (the number of samples measured, n, was eight). Moreover, the mean number of angiogenic holes was 22.88 $\pm$ 6.71 (n = 9) and that of nodular protrusions was 20.44 $\pm$ 7.43 (n = 9). The capillary network of the testicles was less dense-meshed (66.04 % $\pm$ 5.14; n = 11), and presented a lower number of angiogenic holes (2.84 $\pm$ 2.82; n = 13) and nodular protrusions (15.53 $\pm$ 5.56; n = 13) when compared to that of the left ovary. Analysis with the Student's t-test showed that differences between means of the left ovary and testicles were statistically significant ($p < 0.005$) in all cases.

Nodular protrusions were seen as globular or finger-like outgrowings (Fig. 7), usually short, that seemed to contact either with each other or neighboring capillaries (Fig. 6). They were found all over the capillary network, both related and non-related, to angiogenic holes.

Many areas of the capillary network showed dilatations (30-40 μm of diameter) which could be located all along the capillary or at circumscribed points (Fig. 8). We called these structures enlarged vessels.

Angiogenic holes were mostly observed in dilated capillaries, exhibiting various morphologies (Fig. 6), which allowed their classification into primary, secondary, tertiary and open angiogenic holes. These different types of holes were usually grouped and mixed.

Primary angiogenic holes were seen as tiny transcapillary holes whose largest diameter was less than 2.5 μm (Fig. 9).

Secondary angiogenic holes were those holes with diameters larger than 2.5 μm (Fig. 10). These were the most common holes observed in the casts.

Tertiary angiogenic holes had a variable diameter. They were similar to the secondary angiogenic holes, except that the former presented narrowings, sometimes circular, at one point (Fig. 11).

Open angiogenic holes were defined as incomplete holes on one side, thus, the vascular ring around the hole showed a discontinuity (Fig. 12). Several open angiogenic holes sometimes could be observed associated forming wider holes (Fig. 13).

Angiogenic holes were also found serially located in a single vessel of larger diameter (Fig. 14).

Transcapillary pillars could be found in critical point dried sections of the gonadal capillary network which could be correlated with the angiogenic holes found in the vascular casts (Fig. 15). The luminal surface of the capillaries sometimes presented areas with fenestrations (Fig. 16). Two kinds of fenestrations could be observed: large fenestrations (240-1950 nm) and small fenestrations (approximately 90 nm) (Fig. 16). Some of the large fenestrations contained smaller fenestrations inside (Fig. 16).

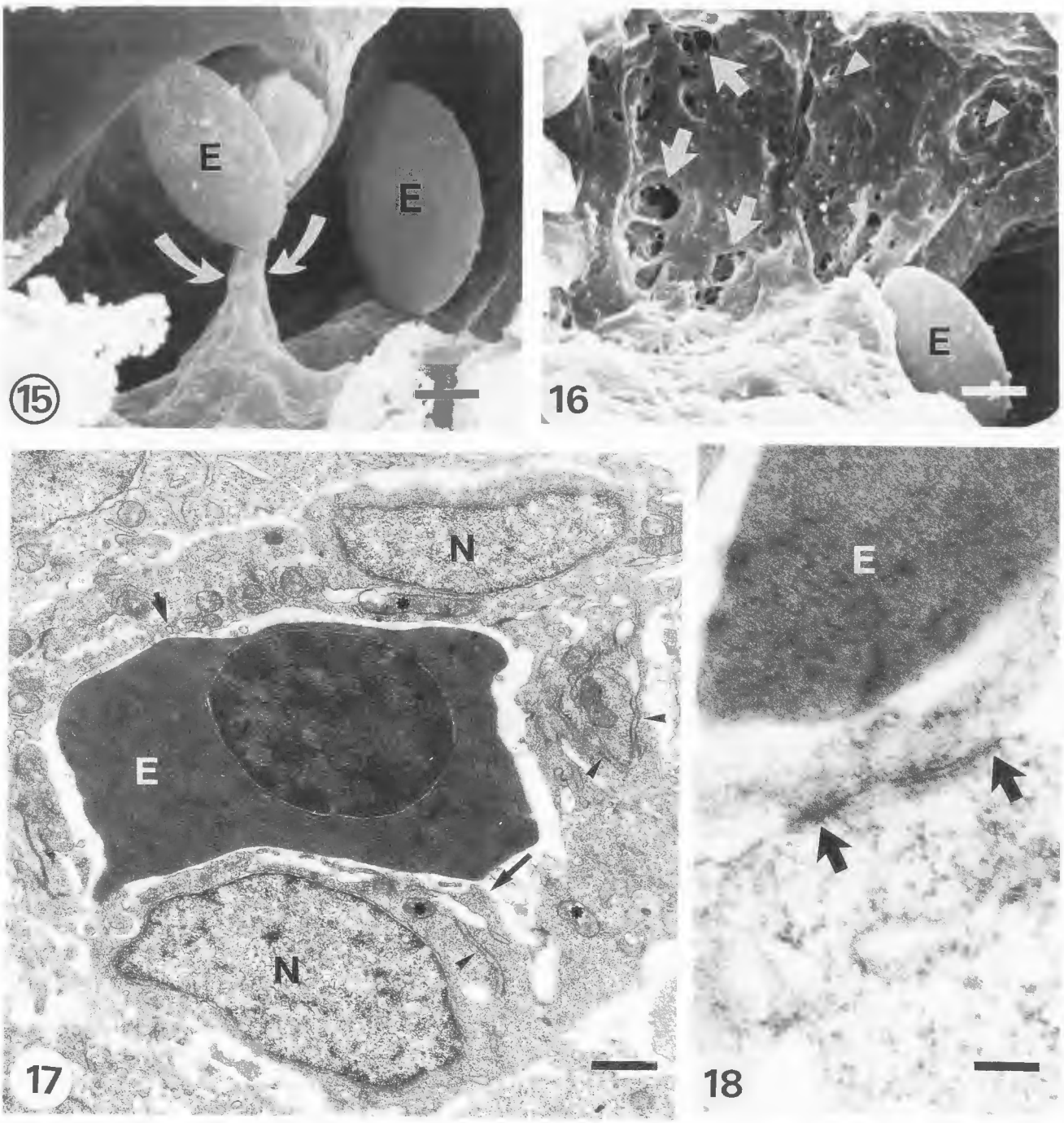


Figure 15. Slender transcapillary pillar (arrows) in a critical point dried section of a testicle. This image may correlate to those found in the casts of primary angiogenic holes. E: erythrocyte. Bar = 2.5 μm.

Figure 16. Intraluminal aspect of the endothelium showing large fenestrations (arrows) and small fenestrations (arrowheads) in a critical point dried section of a vessel of the left ovary. Note the presence of small fenestrations inside some of the large fenestrations. E: erythrocyte. Bar = 3 μm.

Figure 17. Transmission electron microscopical section of a vessel in the left ovary. The endothelial nucleus (N) protrudes into the lumen and shows loosely textured chromatin. The cytoplasm is rich in organelles: mitochondria (asterisks), rough endoplasmic reticulum (arrowheads) and pinocytic vesicle (short arrow). Some projections into the lumen of the vessel can be observed (long arrow). E: nucleated erythrocyte in the lumen of the vessel. Bar = 860 nm.

Figure 18. Tight junction-like unions between two adjacent endothelial cells. E: erythrocyte. Bar = 123 nm.

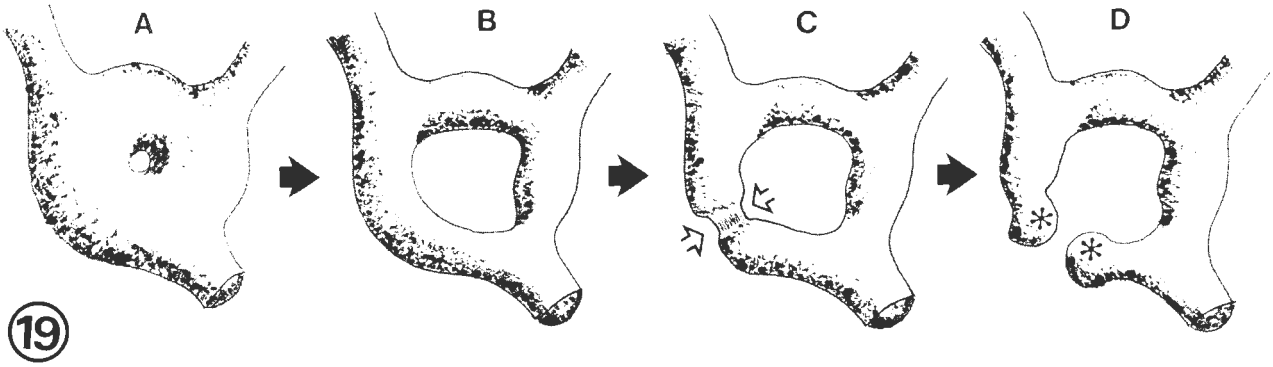


Figure 19. The development suggested for the angiogenic holes is schematically represented. Primary angiogenic holes (**A**) are the first to appear. Their diameter subsequently increases above 2.5 μm, being then called secondary angiogenic holes (**B**). Some of these holes develop a narrowing (open arrows) on one side, tertiary angiogenic holes (**C**). This narrowing is the first sign of discontinuity that would lead to an open angiogenic hole (**D**). The latter may give rise to nodular protrusions (*) at its open ends.

Transmission electron microscopy sections of vessels in the testicles and the left ovary presented typical immature-proliferating endothelial cells (Fig. 17). Activated endothelial cells showed large, round nuclei with loosely textured chromatin, protruding into the lumen. They exhibited an abundant cytoplasm rich in mitochondria, rough endoplasmic reticulum and pinocytic vesicles (Fig. 17). Sometimes, endothelial processes with cell organelles could be found protruding into the lumen (Fig. 17). Endothelial cell junctions were mostly tight junction-like (*Zonula occludens*) (Fig. 18). Vessels lacked a completely formed basal lamina.

Discussion

Vasculogenesis and angiogenesis are the two mechanisms involved in blood vessel formation. Vasculogenesis seems to be restricted to early developmental periods, whereas angiogenesis can occur during the entire life span (Risau, 1991). Although angiogenesis has been related to several pathological processes, it might be best studied during embryonic development because the vascular pattern forms in the absence of pathological mechanisms. Chick embryos were chosen for this study since they have been largely used as a model for angiogenesis (Sethi and Brookes, 1971; Bertossi and Roncali, 1981; Bertossi *et al.*, 1987; Sorrell, 1988; DeFouw *et al.*, 1989; Pardanaud *et al.*, 1989; Ribatti *et al.*, 1991; Nico *et al.*, 1992; Smith *et al.*, 1992; Nguyen *et al.*, 1994; Carretero *et al.*, 1995; Henry and DeFouw, 1995). Furthermore, corrosion casting is becoming more habitual to study the chick embryo vascular system (Dollinger and Armstrong, 1974; Brigham and Rosenquist, 1982; Fuchs and Lindenbaum, 1988; Bockman *et al.*, 1989; Burton and Palmer, 1989; Ditrich and Splechtna, 1989; DeRuiter *et al.*, 1991; Carretero *et al.*, 1993, 1995; Kondo *et al.*, 1993).

According to Romanoff (1960), both testicles and the left ovary exhibit active proliferation of the sexual parenchyma and interstitium by days 8 and 9 of incubation (35 H-H) in chick embryos. The right ovary, however, does not undergo proliferation and was, therefore, excluded from this study.

No former works could be found on architecture and/or development of the vascular system in chick gonads. Miller (1903) stated that the gonadal veins (*Vv. gonadales*) drained into the caudal vena cava and that they originated from the subcardinal veins, but he studied neither their distribution nor their capillary network. Van Limborgh *et al.* (1960) roughly studied the gonadal capillary network in duck embryos.

In the present study, the gonadal capillary network has been so-called "subepithelial" due to its location at the level of the tunica albuginea under the covering epithelium in both the left ovary and the testicles.

Differences in the structure of the capillary network of the testicles and that of the left ovary found in the casts may reflect a higher and faster development of the left ovarian capillary network than that of the testicles in agreement with the findings of Mittwoch *et al.* (1971).

Scarce leakages of the corrosion casting medium found in some angiogenic areas of the gonadal capillary network were considered as a result of increasing vascular permeability. In this sense, leakage of blood through the fragmentary endothelium of the capillary sprouts is common (Schoefl, 1963; Warren and Shubik, 1966; Eddy and Casarett, 1973; Grunt *et al.*, 1986; Lametschwandtner *et al.*, 1990) and is followed by extravasal coagulation, so fibrin threads surround the growing sprouts (Grunt *et al.*, 1986). In addition, fibrin and its degradation products are effective in inducing angio-

genesis (Thompson and Campbell, 1982). Furthermore, casting medium leakage is in good accordance with the finding of some areas with large fenestrations in critical point dried samples of the gonadal capillary network.

Angiogenic structures found in the casts of the capillary network of the testicles and the left ovary were classified into sprouting (nodular protrusions or capillary sprouts) and non-sprouting (enlarged vessels and angiogenic holes).

Nodular protrusions or capillary sprouts found in casts have been extensively reported in the literature in both physiological and pathological situations. They have been found in the corpus luteum of golden hamsters (Spanel-Borowski and Mayerhofer, 1987; Spanel-Borowski *et al.*, 1987; Forsman and McCormack, 1992) and rabbits (Macchiarelli *et al.*, 1991), the rat ovarian follicle (Murakami *et al.*, 1988), the metaphysis of osteopetrotic rats (Aharinejad *et al.*, 1995), wound healing (Phillips *et al.*, 1991), and tumor growth (Grunt *et al.*, 1986; Bugajski *et al.*, 1989; Zama *et al.*, 1991). However, little data could be found on these angiogenic structures in corrosion casts of chick embryos (Carretero *et al.*, 1995). Fusion of these outgrowths with each other or with neighboring capillaries can form anastomoses and, therefore, expand the capillary network.

Many authors found enlarged or dilated capillaries in physiological, as well as pathological states, and related them to endothelial proliferation. Spanel-Borowski *et al.* (1987) and Forsman and McCormack (1992) found enlarged capillaries in the developing follicular vasculature of rabbits and hamsters, respectively. Moreover, it has been a common finding in studies of the vasculature of tumors (Grunt *et al.*, 1986; Bugajski *et al.*, 1989; Zama *et al.*, 1991). On the other hand, Aharinejad and Böck (1992, 1994) studied dilatations in capillaries of rat exocrine pancreas and showed that grooves or furrows that defined these dilatations corresponded to pericyte processes that reinforced the capillary wall. Thus, they related these dilatations of the capillaries to areas without pericytes that were easily dilated during casting medium perfusion. We think that enlarged capillaries found in the capillary network of both testicles and the left ovary may be mostly a result of endothelial proliferation since enlarged capillaries were not always related to grooves or furrows.

The last vascular cast structures described in the present study were the angiogenic holes. These structures were first described by Caduff *et al.* (1986) in vascular casts of the postnatal rat lung, being defined as intussusceptional or intussusceptive capillary growth. Further studies (Burri and Tarek, 1990; Patan *et al.*, 1992, 1993, 1996), showed the ultrastructure of these angiogenic elements and demonstrated that they were not artifacts, but an alternative to capillary sprouting, being found in capillary networks of many developing tissues of young animals and embryos. All these works dealt with the process of formation of the angiogenic holes but gave no clues on their further evolution.

In the present study, four different types of angiogenic holes, coincident in time, were found in the casts of the testicles and left ovarian capillary network. From these observations, a serial evolution of these holes is suggested by us (Fig. 19). Primary angiogenic holes of less than 2.5 μm of diameter were the holes encountered by the above mentioned authors and would be the first to appear (Fig. 19A). Then, they would increase their diameter and develop a narrowing on one side (secondary and tertiary angiogenic holes) (Figs. 19B and 19C). The vascular ring surrounding the hole would subsequently open at the narrowing to form the open angiogenic hole (Fig. 19D). Secondary angiogenic holes (diameters larger than 2.5 μm) were suggested by Burri and Tarek (1990) and were only seen by Patan *et al.* (1992) in the capillary network of the rat submandibular gland. The two other types of holes found in this study were not previously described. The formation of a narrowing on one side of the hole (tertiary angiogenic hole) would be the first sign of discontinuity of the vascular ring surrounding the hole that would lead to an open angiogenic hole. We suggest two different fates for these open angiogenic holes: (1) Formation of nodular protrusions at their open ends (Fig. 19D). These nodular protrusions would merge others or neighboring capillaries and, therefore, there would be formation of new vessels by sprouting. (2) Fusion of two or more neighboring open angiogenic holes. This fusion would create either wider holes (see Fig. 13) or splitting of a vessel into two when fusion of angiogenic holes takes place in holes located in a single vessel of larger diameter (see Fig. 14). These two angiogenic mechanisms may interact and represent a way of expansion and reorganization of the capillary network.

Angiogenic holes had been previously demonstrated by corrosion casting (Caduff *et al.*, 1986; Patan *et al.*, 1992), *in vivo* microscopy (Patan *et al.*, 1993) and transmission electron microscopy of serial sections (Burri and Tarek, 1990; Patan *et al.*, 1993, 1996). This is, however, the first time that slender transcapillary pillars have been shown three-dimensionally by means of SEM of critical point dried sections of the gonads.

Correlative TEM showed that vessels in the testicles and the left ovary at this stage exhibited typical characteristics of proliferating endothelium, thus, large nuclei with loosely textured chromatin, abundant cytoplasm rich in organelles, intraluminal endothelial processes, etc. Similar characteristics have been found in angiogenic vessels in the chick embryo adenohypophysis (Bertossi and Roncali, 1981) and the thyroid and suprarenal

glands (Bertossi *et al.*, 1987).

Patan *et al.* (1993) suggested that the presence of slender endothelial processes projecting into the lumen of the capillaries could facilitate a first transcapillary interendothelial contact, necessary for intussusceptive capillary growth. Some of the intraluminal projections found in our TEM sections may have such a function.

No arterial angiogenic buds were found in the capillary network of the left ovary, suggesting a capillary or venous origin of the capillary network. This has been a common finding in tumors (Kligerman and Henel, 1961; Grunt *et al.*, 1986; Bugajski *et al.*, 1989). This may be explained by the findings of Reinhold and Van Den Berg-Blok (1984), since they found that the angiogenic substances produced by tumor cells are secreted into the interstitial space and may, therefore, first reach the venous side of the vascular tree.

Finally, many resemblances have been found in the present work between angiogenic structures in the testicles and left ovary of the chick embryo and those previously found in tumors. In this sense, Zama *et al.* (1991) already found similarities between angiogenic structures in rat glioma and those found in the neonatal rat cerebellar cortex. Further studies are to be done to elucidate common angiogenic factors and pathways that may shed light on the still widely unknown embryonic and tumoral angiogenic mechanisms.

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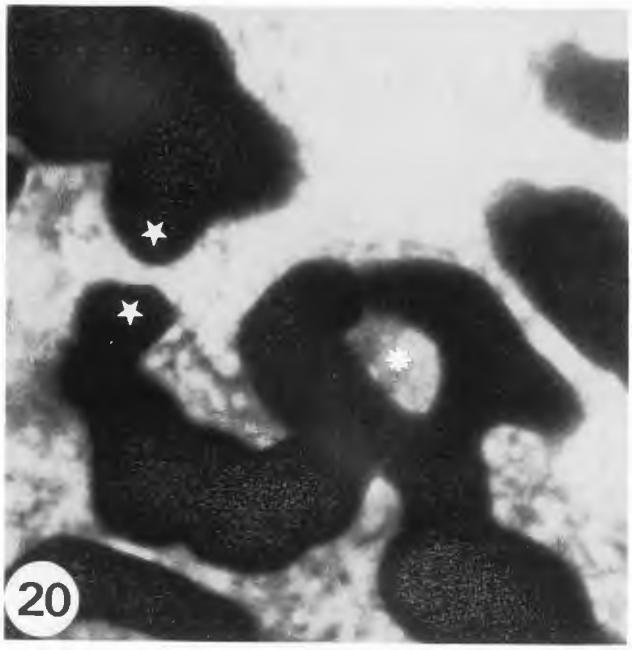

Figure 20. Detail of the subepithelial capillary network of the left ovary in the CLSM. Capillaries are filled with Mercox® stained with Acridine orange (color green in original print); the surrounding tissue has been counterstained with light green (color red in original print). Note the tissue pillar (*) surrounded by the corresponding vascular ring of a secondary angiogenic hole. Some structures compatible with nodular protrusions (stars) can also be observed. No incomplete filling of the vessels can be found. Bar = 19 μm.

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Discussion with Reviewers

R.H. Christofferson: "What is in a name...?" Blind endings in casts can be caused by several different mechanisms, and are considered to be artefacts by skeptics. I am a bit worried about the absence of endothelial

cell nuclear imprints on the sprouts, and the lack of erythrocyte imprints, since erythrocytes are trapped in sprouts. Those rounded blebs may be resin projecting into wash-out medium. What evidence do you have that the structures in, e.g., Figures 6, 7 and 12 are vascular sprouts and not blind endings due to incomplete filling?
Authors: This and other questions from reviewers led us to use confocal laser scanning microscopy (CLSM) to study the gonadal subepithelial capillary network filled with fluorescent Mercox®. Briefly, a fluorescent dye (Acridine orange) was added to the mixture to be injected (Castenholz, 1995). After injection, the surrounding tissue was fixed with 10% buffered formaldehyde and counterstained with eosine or light green. The samples were then mounted and studied in a Leica TCS-4D CLSM (Leica Mikroskopie, Wetzlar, Germany) equipped with a krypton-argon laser. Blind endings (nodular protrusions) found in the gonadal subepithelial capillary network were not artefacts due to incomplete filling but sprouting angiogenic structures. Figure 20 shows some blind endings compatible with nodular protrusions that present no empty spaces in the interior of the vessel. No trapped erythrocytes could be found in the blind endings since erythrocytes of the chick embryo are easily distinguished due to their autofluorescent nature in the CLSM (unpublished findings). Moreover, Mercox® always filled the interior of the vessel, and no leakage could be observed. Furthermore, the existence of tissue pillars surrounded by the vascular ring of angiogenic holes supports the finding of angiogenic holes in our casts and tissue pillars in critical point dried specimens.

R.H. Christofferson: Vascular growth coincides with vascular remodelling. Figure 14 may be interpreted as fusion of two adjacent vessels, or as angiogenic holes. What rationale do you have for your interpretation of "splitting of the vessel into two"?
Authors: Embryos of earlier stages did not show two vessels located in this area but only one. Therefore, splitting of the vessel into two is more likely to occur. *A priori*, angiogenic holes can increase or decrease their diameter. In the case of the vessel of Figure 14, decreasing of the diameter of the angiogenic holes would reflect fusion of two adjacent vessels whereas increasing of the diameter would lead to splitting of the vessel into two. In order to demonstrate what happens and support our hypothesis about the evolution of the angiogenic holes, Table 1 presents data on the mean number and mean diameter of the angiogenic holes found in the subepithelial capillary network of the left ovary of 35 H-H and 40 H-H chick embryos. As it can be observed, the number of angiogenic holes decreases in the stage 40 H-H. Holes are, therefore, a temporal angiogenic structure that disappears with maturation of the capillary network. This disappearance could be due to either occlusion or opening of the angiogenic holes as said before. However, the fact that the diameter of the holes increases as development progresses (Table 1) suggests that angiogenic holes tend to open as commented by the authors. These findings are in agreement with the works of Burri and Tarek (1990) and Patan *et al.* (1992, 1993). These authors noticed that angiogenic holes do not tend to diminish their diameter but to increase it.

R.H. Christofferson: Have you investigated the possibility of vasculogenesis taking place in parallel and in cooperation with angiogenesis in the chick gonads? That is, can there be capillary beds that are formed but yet not (completely) perfused at the 35 H-H stage? Can you design an experiment that could answer this question?
Authors: Recently, some authors have suggested that both vasculogenesis and angiogenesis are complementary during vascular development (DeRuiter *et al.*, 1993). Both processes have been described taking place in parallel in several systems of the chick embryo (Poelmann *et al.*, 1990; Feinberg and Noden, 1991; Wilms *et al.*, 1991). If a part of the gonadal subepithelial capillary network of the chick embryo developed parallel by vasculogenesis and was not connected with the general gonadal circulation, vascular corrosion casting would not be useful to demonstrate its existence. It would be, therefore, necessary to reveal the presence of endothelial cells by means of immunohistochemical methods. Unfortunately, only specific antibodies for endothelial and hemopoietic cells of quail embryo are available: QH1 (Pardanaud *et al.*, 1987) and MB1 (Péault *et al.*, 1983). However, it may be possible to extrapolate results from quail to chick embryos since they are highly related zoologically. The authors propose the following experiment: injection of quail embryos with fluorescent Mercox®, and fluorescent immunolabelling of endothelial cells with QH1 antibody. Observation of these samples in the CLSM would show the existence of endothelial cells not related with Mercox®. It would be therefore possible to suspect about the existence of a developing capillary bed not connected with the general gonadal circulation with a vasculogenic rather that angiogenic origin. Obviously, to validate this experiment, it would be necessary to achieve a complete filling of the gonadal circulatory system.

R.H. Christofferson: What angiogenic mediator(s) is (are) responsible for the angiogenesis in the chick gonads? I think your study would gain by, e.g., *in situ* hybridization for detection of VEGF and bFGF mRNA.
Authors: The authors ignore the angiogenic mediator(s) responsible for the angiogenic processes in the chick gonads. However, recent papers (Flamme *et al.*, 1995a,b)

Table 1. Mean number and mean diameter with their standard deviations of the angiogenic holes of the subepithelial capillary network of the left ovary in the stages 35 H-H and 40 H-H. The number of angiogenic holes was measured on micrographs covering a constant area of the capillary network of approximately 180 mm^2. The number of samples thus measured was: [a]n = 9 embryos, [b]n = 11 embryos; and [c]n = 52 angiogenic holes.

	Stage 35 H-H	Stage 40 H-H	Comparison between means*
Number	22.88 (± 6.71)[a]	7.18 (± 6.36)[b]	$p < 0.001$
Diameter	7.02 (± 6.33)[c]	12.58 (± 7.27)[c]	$p < 0.001$

*The difference between means is considered to be statistically significant when $p < 0.05$ (Student's t-test).

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suggest a major role for VEGF in vasculogenesis and angiogenesis during embryogenesis in different species. The idea of the reviewer is very interesting and will probably have to be developed in the future.

R.H. Christofferson: Why does the right avian ovary regress? I find this intriguing: is it by apoptosis or by inhibition of angiogenesis? Have you kept your specimens for a review with this question at issue?
Authors: This work is part of a study of vascular morphogenesis of the gonads in chick embryos that includes several developmental stages: stage 18 of Hamburger and Hamilton (H-H) (3 days of incubation), stage 30 H-H (6.5-7 days of incubation), stage 35 H-H (8.5-9 days of incubation), stage 40 H-H (14 days of incubation), stage 46 H-H (21 days of incubation, newly hatched chick) and 8 week-old chicks. Unpublished data shows that cell death in the right ovary takes place by means of apoptotic mechanisms. Surprisingly, apoptosis can be found both in parenchymatous and endothelial cells. Whether regression in the right ovary is produced by death of the parenchymatous cells, degeneration of the endothelium that causes vascular malfunction and trophic problems, or a combination of both remains unclear.

A. Lametschwandtner: You describe "open angiogenic holes" and interpret them as a stage in angiogenesis, i.e., a stage of vessel growth. What evidence do you have that these "open angiogenic holes" (1) do not represent closed meshes which simply could not be cast because blood or plasma was trapped when the mesh was filled from both sides, (2) do not represent a stage of vessel regression (involution), where the area of the open site represents the obliterated part of the mesh?
Authors: We have used CLSM (see discussion with **R.H. Christofferson**) to observe the vascular wall and Mercox® filling the lumen of the vessel (Fig. 20). By this means, no incompletely filled vessels or vessels with trapped erythrocytes could be seen. As commented before, in our conditions, chick embryonic erythrocytes were fluorescent (unpublished data) and were, therefore, easy to detect. About the second question, we have observed tissue at the open sites of the open angiogenic holes using CLSM. Unfortunately, it is impossible to discern with the methodology used if this obliteration of the mesh is due to an angiogenic or involutive process.

A. Lametschwandtner: Endothelial fenestrations of capillaries are reported to have a diameter from 50-80 nm. The "fenestrations" you mark in Figure 16 are much larger. Are fenestrations in the capillaries of the chick embryo of this size or are these "fenestrations" pores or even sinusoids?
Authors: Two kinds of fenestrations can be observed in Figure 16: (1) large fenestrations (240-1950 nm) similar to those found in the sinusoidal endothelium of the mammalian bone marrow or liver (Castenholz, 1983). Some of them exhibit smaller fenestrations inside. These large fenestrations could be observed only in some areas of the capillary network; (2) small fenestrations (approximately 90 nm) that are compatible with the definition of fenestrae or pores of visceral capillaries.

A. Lametschwandtner: In Figures 7-12, the surfaces of cast capillaries impose as rather smooth. From Figure 16, one could expect that the large "fenestrations," which are seen to open deeply into the surrounding tissue, give way to excessive leakage. The casts you show, however, lack any extravasations. Please, could you comment upon this obvious discrepancy?
Authors: The authors think that the large fenestrations shown in Figure 16 may be a possible site of leakage. However, it is true that no excessive leakage can be found in our casts. For us, the only explanation is that the small fenestrations seen inside the large fenestrations are fenestrae or pores closed by a diaphragm of cell membrane that prevents from leakage. Fenestrae or pores of visceral capillaries usually exhibit this type of diaphragm (Fawcett, 1987). Correlative TEM analyses will be necessary in the future to clarify this point.

Reviewer V: Have you ever observed apoptosis near to "angiogenic holes"?
Authors: In TEM sections, we have observed morphological signs of apoptosis in the endothelial cells of the subepithelial capillary network of the right ovary, the one that regresses (unpublished data). Unfortunately, we are not able to assure whether this endothelial cells correspond to those forming the tissue pillars that produce the angiogenic holes in the casts.

Reviewer V: Embryonic material is delicate, and therefore, fixation prior to casting would protect them from being ruptured. Why was this protocol not applied to your studies?
Authors: Theoretically, this is true. However, we previously standardized a procedure to perform injections of the chick embryos (Carretero *et al.*, 1993) and determined that when rinsing of the vascular system with heparinized Tyrode solution (37°C) and perfusion-fixation glutaraldehyde (0.5% in Tyrode solution) were executed before the injection of the casting resin, replication of the vascular system was not adequate. Therefore, these steps were omitted.

Reviewer V: You state that "enlarged capillaries are the result of endothelial proliferation." Can you prove this?
Authors: No, we cannot directly prove this. However, enlarged capillaries have been related to endothelial proliferation before in many instances (Grunt *et al.*, 1986; Spanel-Borowsky *et al.*, 1987; Bugajski *et al.*, 1989; Zama *et al.*, 1991; Forsman and McCormack, 1992). Aharinejad and Böck (1992, 1994) related dilatations of the capillaries of the rat exocrine pancreas to areas without pericytes that were easily distended during casting medium perfusion. They showed that these dilatations were defined by furrows corresponding to pericyte processes. In the gonadal subepithelial capillary network, not all of the enlarged capillaries were defined by furrows. We, therefore, related these enlargements to areas of endothelial proliferation.

Reviewer V: It is not clear to me how the furrows shown near to some holes are caused. Would not the processes of pericytes, cells which are thought to be involved in the regulation of endothelial growth, be candidates? (Aharinejad and Böck, 1994).
Authors: Observations of tertiary angiogenic holes in the CLSM showed thickenings of the capillary wall related to the circular narrowings of the angiogenic holes. The authors hypothesize that these thickenings may correspond to pericytes and their processes surrounding the capillary. Further studies like exhaustive serial sectioning have to be done to elucidate this hypothesis.

Reviewer V: Did you examine testicles or ovaries of older or adult animals with regard to the angiogenic holes?
Authors: Yes, this and other questions raised by the reviewers led us to measure the number of angiogenic holes and their mean diameter in stages 35 H-H and 40 H-H (see Table 1).

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