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Microvascular endothelial cells of the corpus luteum

John S Davis*[1], Bo R Rueda[2] and Katherina Spanel-Borowski[3]

Address: [1]Olson Center for Women's Health, Department of Obstetrics and Gynecology, University of Nebraska Medical Center, Omaha, Nebraska 68198 and VA Medical Center, Omaha, Nebraska 68105, USA, [2]Vincent Center for Reproductive Biology, Department of Obstetrics and Gynecology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA and [3]Department of Anatomy, University of Leipzig, Liebigstrasse 13, 04103 Leipzig, Germany

Email: John S Davis* - jsdavis@unmc.edu; Bo R Rueda - brueda@partners.org; Katherina Spanel-Borowski - spanelb@medizin.uni-leipzig.de

* Corresponding author

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Abstract

The cyclic nature of the capillary bed in the corpus luteum offers a unique experimental model to examine the life cycle of endothelial cells, involving discrete physiologically regulated steps of angiogenesis, blood vessel maturation and blood vessel regression. The granulosa cells and theca cells of the developing antral follicle and the steroidogenic cells of the corpus luteum produce and respond to angiogenic factors and vasoactive peptides. Following ovulation the neovascularization during the early stages of corpus luteum development has been compared to the rapid angiogenesis observed during tumor formation. On the other end of the spectrum, the microvascular endothelial cells are the first cells to undergo apoptosis at the onset of corpus luteum regression. Important insights on the morphology and function of luteal endothelial cells have been gained from a combination of *in vitro* and *in vivo* studies on endothelial cells. Endothelial cells communicate with cells comprising the functional unit of the corpus luteum, *i.e.*, other vascular cells, steroidogenic cells, and immune cells. This review is designed to provide an overview of the types of endothelial cells present in the corpus luteum and their involvement in corpus luteum development and regression. Available evidence indicates that microvascular endothelial cells of the corpus luteum are not alike, and may differ during the process of angiogenesis and angioregression. The contributions of vasoactive peptides generated by the luteal endothelin-1 and the renin-angiotensin systems are discussed in context with the function of endothelial cells during corpus luteum formation and regression. The ability of two cytokines, tumor necrosis factor alpha and interferon gamma, are evaluated as paracrine mediators of endothelial cell function during angioregression. Finally, chemokines are discussed as a vital endothelial cell secretory products that contribute to the recruitment of eosinophils and macrophages. The review highlights areas for future investigation of ovarian microvascular endothelial cells. The potential clinical applications of research directed on corpus luteum endothelial cells are intriguing considering reproductive processes in which vascular dysfunctions may play a role such as ovarian failure, polycystic ovary syndrome (PCOS), and ovarian hyperstimulation syndrome (OHSS).

Background

The vascular endothelium of the mature ovarian follicle maintains the capacity for rapid growth in response to angiogenic signals elaborated during the periovulatory process. New blood vessel growth is essential for the formation and function of the corpus luteum. Analogous to events occurring in the corpus luteum, the vascular endothelium of other tissues responds to extracellular signals during the physiologic processes of embryonic development and wound healing, and in the pathologic process of tumor angiogenesis. Although the corpus luteum is a transient tissue, it is one of the most highly vascularized tissues in the body [1] with endothelial cells representing greater than fifty percent of the total cells [2,3]. Endothelial cell proliferation and vascular changes have been examined throughout the luteal phase in rat [4,5], rabbit [6], pig [7], sheep [8,9], cow [10-13], horse [14], marmoset [15], macaque [16], and human [17] corpora lutea. The vascular elements in these studies were determined using markers of endothelial cells (von Willebrand factor VIII related antigen, FVIIIr antigen; angiotensin-converting enzyme, ACE; lectin binding, *e.g.*, Griffonia Simplicifolia agglutinin and Ulex Europaeus agglutinin; and platelet/endothelial cell adhesion molecule-1, PECAM-1). Evidence of proliferating endothelial cells was determined by the presence of Ki-67 antigen-positive cells, bromodeoxyuridine-positive cells, or [^{3}H] thymidine-positive cells. Collectively, these studies demonstrate that the rate of endothelial cell proliferation is highest during corpus luteum formation, then decreases and remains low during the mid-luteal phase and structural regression of the corpus luteum. The establishment of an extensive vascular network is an essential component of corpus luteum development, since the inhibition of angiogenesis during corpus luteum formation is associated with inadequate corpus luteum function [3,12,18-21].

The regulation of endothelial cell proliferation in the corpus luteum of pregnancy is less clear. Treatment with human chorionic gonadotropin (hCG) to simulate early pregnancy resulted in elevated progesterone secretion and sustained luteal weight but was not associated with an increase or maintenance of endothelial cell proliferation in monkeys or humans [16,17]. However, more recent morphometric evidence [22] suggests that hCG rescue of the human corpus luteum is associated with a second wave of angiogenesis and vascular stabilization.

The onset of the structural regression of the corpus luteum involves alterations in the vascular elements of the corpus luteum [12,23-28]. The endothelial cells lose tight junctions and the permeability barrier of the vascular wall is disrupted. As a result, capillary endothelial cells detach from the basement membrane and occlude small blood vessels. Capillaries disappear, whereas arterioles with a thickened wall appear, apparently as a result of increased numbers of smooth muscle cells [14,15,29]. It has been suggested that the arteriole thickening during corpus luteum regression represents a degenerative event [30]. The observed alterations in endothelial cell degeneration clearly demonstrate vascular changes concurrent with a reduction in the functional status of the corpus luteum.

Given the pivotal role that endothelial cells play in corpus luteum formation, maintenance and regression it is critical to understand the nature of the microvascular endothelial cells at various stages during the life span of the corpus luteum. The goal of this review is to summarize what is known about the morphological and functional characteristics of the microvascular endothelial cells of the corpus luteum. This review also summarizes evidence for the regulation of luteal endothelial cells by vasoactive peptides (angiotensin II and endothelin-1) and cytokines (tumor necrosis factor alpha and interferon gamma) during angiogenesis and/or angioregression. The review also discusses chemokines as endothelial cell secretory products that contribute to the recruitment of eosinophils and macrophages. A greater understanding of the specific endothelial cell types involved in the angiogenic response during corpus luteum formation and the vascular regression during structural regression will enable a greater appreciation for the endocrine, paracrine, and autocrine factors; intercellular cross-talk; and specific signaling mechanisms that control corpus luteum function.

Endothelial cells of the corpus luteum

Morphological characteristics

Over the past twenty years microvascular endothelial cells have been isolated from the corpora lutea of different species: the rabbit [31], pig [32], sheep [33], cow [34-38], rhesus monkey [39], and human [40]. These studies have provided important clues about the presence and role of specific endothelial cells types found in the corpus luteum despite species differences and various methods for endothelial cell isolation and culture. The ability to successfully freeze and recover endothelial cells from frozen stocks has allowed the opportunity to further explore endothelial cell function *in vitro*. Together these studies provide evidence for *in vitro* regulation of the proliferation, function and demise of luteal endothelial cells.

Although all endothelial cells have some common functional and/or morphological characteristics; the endothelial cells of large and small blood vessels from either the same or different organs vary in morphology, surface molecule expression, and function. A substantial amount of information has been accumulated on endothelial cells from the bovine corpus luteum [34-38]. Five distinct subtypes of bovine microvascular endothelial cells have been characterized by different morphology, surface molecule expression and function [34,41-47]. Each cell type exhibited contact-inhibited growth and maintained unique morphological characteristics in long-term culture. Figure 1 shows representative phase-contrast photomicrographs and growth curves for each of the five cell types.

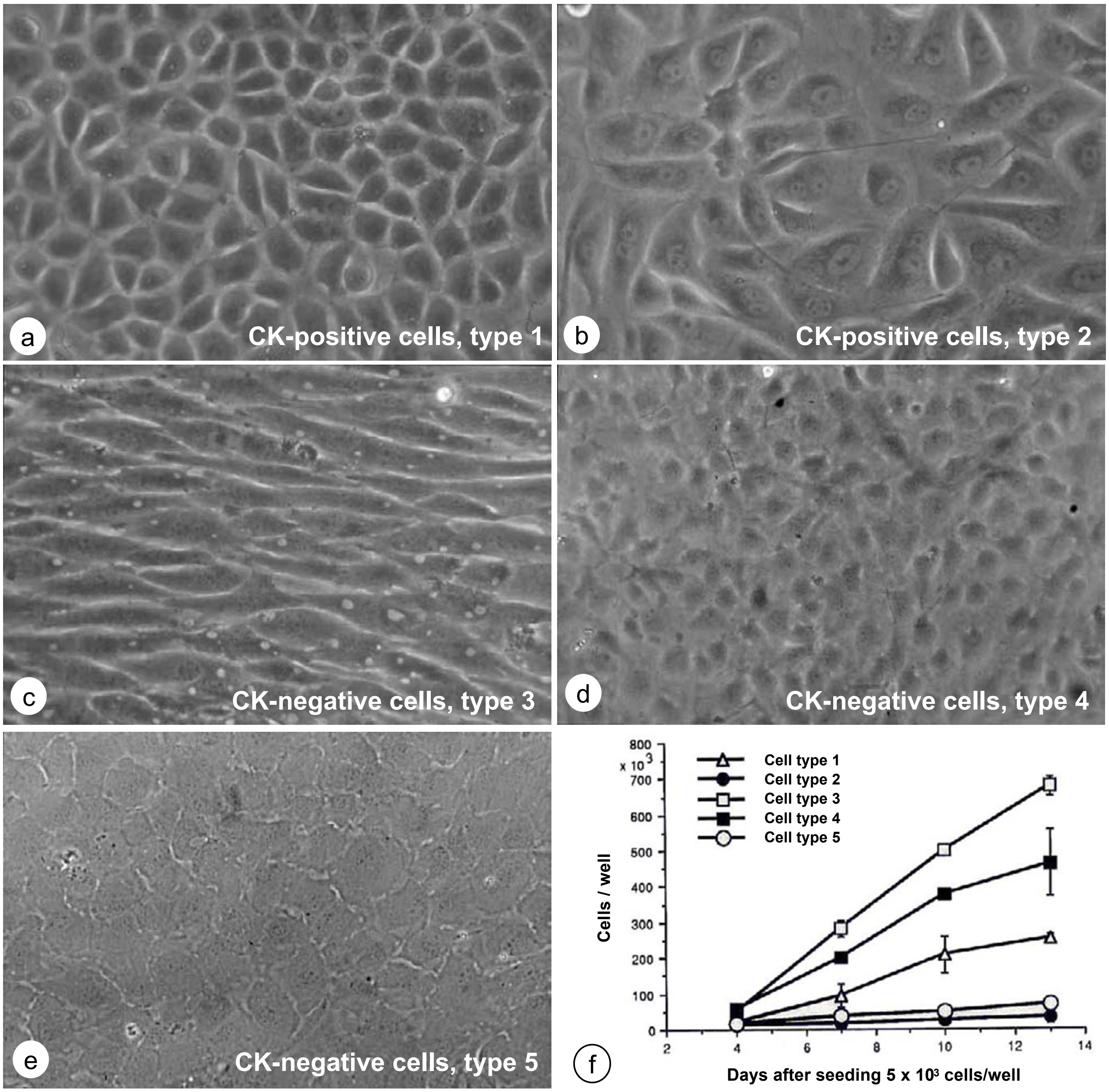

Figure 1
The five different endothelial cell types derived from bovine corpus luteum are shown by phase contrast microscopy as described by Spanel-Borowski and van der Bosch [41] and Fenyves *et al*., [44]. Panels a–f: In cytokeratin-positive cell types 1 and 2, the cobble-stone like pattern is distinct (panels a and b); in cytokeratin-negative cells, the monlayer consists of spindle-like cells with prominent "vacuoles" in cell type 3 (panel c); the monlayer of polygonal opaque cells appear in cell type 4 (panel d). Type 5 cells are judged as granulosa-like cells (panel e). The five cell types differ in growth rate (panel f).

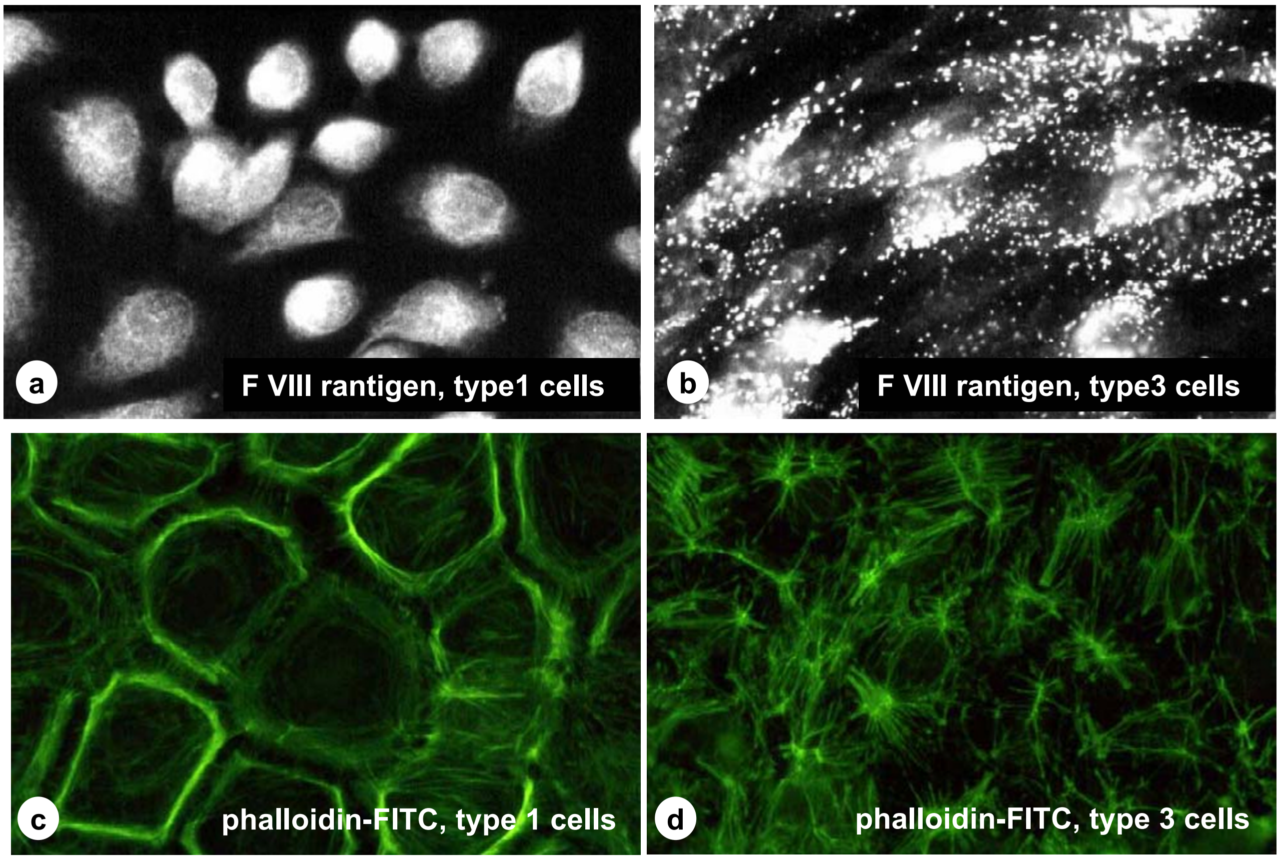

Figure 2
Distinctive morphological features of type 1 and type 3 endothelial cells. Panels a and b: Staining for von Willebrand factor VIII related antigen (FVIIIr antigen) shows a diffuse perinuclear pattern in type 1 cells (panel a) and a distinctive granular appearance in type 3 cells (panel b). Panels c-d: Immunofluorescence for actin filaments using phalloidin-FITC. The cytokeratin-positive (CK-positive) type 1 cells demonstrate a peripheral band of microfilaments (panel c); the cytokeratin-negative (CK-negative) cells of type 3 show a starburst-like pattern (panel d) [44].

Monolayers of type 1 and 2 cells appear as polygonal epithelioid cells with a prominent cobblestone pattern. Cells of type 3 form a polymorphic monolayer with spindle-shaped cells with and without intracytoplasmic vacuoles. Cells of type 4 form a monolayer of round cells with an opaque appearance, while type 5 cells form a monolayer of polygonal cells with a flat phase-dense appearance. With the notable exception of type 5 cells, the cells of types 1–4 form tubules. Immunocytochemical staining revealed that each of the five types of endothelial cells expresses FVIIIr antigen. The FVIIIr antigen staining varies among the cell types; type 1 cells exhibit a diffuse perinuclear pattern and type 3 cells exhibit a distinctive granular pattern (Figures 2a and 2b). All types of endothelial cells express ACE, and they internalize acetylated low-density lipoprotein (Ac-LDL).

Each endothelial cell type displays a unique pattern of microtubules; actin and vimentin filaments; and fibronectin matrix [45-49]. Figures 2c and 2d demonstrate the distinctive actin filament arrangement in type 1 and type 3 endothelial cells. The type 1 cells express E-cadherin as a molecule of adhesion plaques, while only the type 5 cells display N-cadherin molecules. Furthermore, the expression of neuronal cell adhesion molecule (NCAM-140) is found in cell types 1, 2 and 5, but not types 3 and 4 [48]. The immunoreactivity of NCAM-140 in cell type 1 is localized to the perinuclear region, whereas, in types 2 and 5 it is predominately localized at the lateral surface outlining the contact zones between adjacent cells. It seems likely that NCAM-140 may play a role in maintaining the extensive contact observed between microvascular endothelial cells and steroidogenic cells of the corpus luteum.

Of the original classifications of bovine microvascular endothelial cells, the type 5 endothelial cells are now thought to represent immature granulosa cells [50]. The type 5 cells and granulosa cells have similar morphological features *in vitro*, and the type 5 endothelial cells have characteristics different from other endothelial cell types. Type 5 cells exhibit weak staining for FVIIIr antigen and ACE [45], and display overlapping cell borders as determined by electron microscopy [49], and N-cadherin expression [45] that are not present in the contact-inhibited microvascular endothelial cells. When compared simultaneously, type 5 cells display features similar to bovine granulosa cells isolated from small antral follicles [50], *i.e.*, the endothelial cells have morphological characteristics of granulosa cells, produce small quantities of progesterone and are unresponsive to treatment with luteinizing hormone (LH). Spanel-Borowski *et al.* [50] suggested that these cells may represent stem cells for the renewal of luteal cells. More recently, Antczak and Van Blerkom [51] proposed that subpopulations of human and murine ovarian granulosa cells behave as specialized endothelial-like cells. They demonstrated that granulosa cells possess many markers used for the identification of endothelial cells. Furthermore, some subpopulations of granulosa cells engage in tubule-forming activity and produce a range of molecules in response to hypoxia [52]. The overlapping phenotypes presented in these studies using mouse, bovine and human ovarian cells suggest that under the appropriate conditions granulosa cells may perform functions normally ascribed to endothelial cells and vice versa. Studies are needed to determine whether a common stem cell present in the ovary contributes to the development of follicles and vascular cells.

Microvascular endothelial cells of the bovine corpus luteum have been also classified by the presence or absence of cytokeratin 8, 9, and 18 filaments [34,41-49]. Type 1 and 2 endothelial cells contain these cytokeratins and, *in vitro*, they display characteristics similar to cytokeratin-positive endothelial cells from the aorta [53]. Based on their limited appearance in the corpus luteum, the cytokeratin-positive endothelial cells have been classified as a rare luteal microvascular endothelial cell type. While both cytokeratin-positive and negative endothelial cells share characteristics typical of other endothelial cells (typical lectin binding patterns [46], positive for FVIIIr and internalization of Ac-LDL [54]), the cytokeratin-negative cells exhibit five-fold greater FVIIIr antigen binding and ten-fold greater Ac-LDL uptake [54] than the cytokeratin-positive cells. The cytokeratin-negative cells also have a greater proliferation rate than do the cytokeratin-positive cells [44]. The cytokeratin-positive cells produce tubules with helically arranged cells originating from a trunk-like structure *in vitro* [Figure 3a], whereas the cytokeratin-negative cells spontaneously form tubules that develop into a prominent tubule network as shown in Figures 3b and 3c[41,45].

Of the various types of endothelial cells, the cytokeratin-positive cells are predominantly found in the early stages of bovine corpus luteum development, and to a much lesser extent in functional and regressing corpora lutea of the estrous cycle, and almost never in the corpus luteum of pregnancy [49]. The cytokeratin-positive endothelial cells are observed in the corpus luteum at the onset of angiogenesis and are often located at the branching point, or tips of sprouting capillaries. The expression of specific keratins may provide some advantage, since epithelial cells expressing keratins 8 and 18, are less susceptible to cytokine-mediated apoptosis [55]. The endothelial cell types also differ in their expression of adhesion molecules [54], recruitment of leukocytes [56], and ability to form intercellular junctions [57]. These *in vitro* and *in vivo* results, together with the selective presence of cytokeratin-positive cells in early corpus luteum development, provide evidence that cytokeratin-positive microvascular endothelial cells are morphologically and functionally different from the common cytokeratin-negative endothelial cell in the corpus luteum. Both cytokeratin-positive and -negative cell types are likely to be involved in blood vessel formation, maintenance and regression; yet exerting their influence in these events differently.

In the corpus luteum, microvascular endothelial cells are likely to mediate the cyclic accumulation of specific subsets of leukocytes [58-61] by the expression of cell adhesion factors [54-57] and secretion of chemokines [60-64]. Adhesion molecules expressed on endothelial cells are involved in cell migration, maintaining intercellular contacts and anchoring cells to the extracellular matrix. Resting cytokeratin-positive endothelial cells contain the cell adhesion factors CD-29, CD-31 (PECAM-1), CD-49A and CD-49E (integrin $\alpha 5$), but the level of expression is significantly lower than in cytokeratin-negative endothelial cells [54]. In contrast to the cytokeratin-negative cells, the cytokeratin-positive cells fail to express CD-49B (integrin $\alpha 2$), or MHC Class II [54]. Each of the endothelial cell types differs in their basal and interleukin-1 stimulated adhesion of granulocytes [56]. These results suggest that different types of endothelial cells have unique extracellular matrix-endothelial cell interactions and are differentially involved in the selective attachment and migration of leukocytes into the corpus luteum.

Differences in morphology, cell growth patterns, and lectin binding parameters have also been reported for microvascular endothelial cells isolated from corpora lutea of pregnant (second trimester) and non-pregnant (mid luteal phase) cows. Plendl *et al.* [36] used a flow cytometry approach to isolate endothelial cells from these physio-

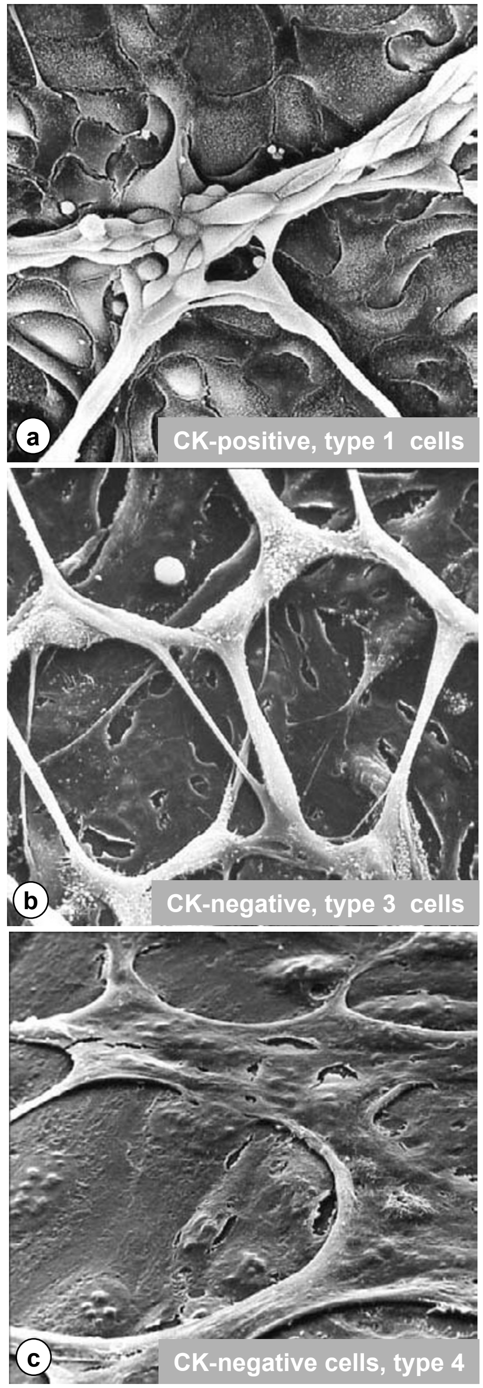

Figure 3
Endothelial cells of the bovine corpus luteum form tubule-like structures *in vitro* as demonstrated by scanning electron microscopy [41,42]. Panel a: In cytokeratin-positive, type 1 cell cultures, the tubule-like structures derive from a junction point and develop distinct borders. A longitudinal cut through a cytokeratin-positive tubule reveals the "inside out" model, i.e. a core of extracellular matrix. Panel b: In cytokeratin-negative, type 3 cells the tubule-like structures depict a reticular network without distincl cell borders. True tubules are formed as verified by the presence of an extracellular matrix core. Panel c: Cytokeratin-negative, type 4 cell cultures form a reticular arrangement of pseudo-tubules lacking the three-dimensional structure of true tubules (panel c).

logic distinct luteal tissues. Bovine corpora lutea from pregnant and non-pregnant animals each produced two populations of endothelial cells that were characterized as either having a cobblestone growth pattern or an arcuate growth pattern. Cultures of endothelial cells of the corpus luteum of pregnancy exhibited spontaneous angiogenic activities including cell migration and formation of ring-like structures. These intriguing observations lead Plendl *et al.* [36] to speculate that the endothelial cells isolated during pregnancy produce specific angiogenic factors not present or present in limited quantities in endothelial cells isolated from the corpus luteum of the estrous cycle. Heterogeneity between luteal endothelial cells of pregnant and non-pregnant animals was also observed in lectin binding studies which revealed that specific endothelial cell surface carbohydrates were correlated to the status of pregnancy [36].

Angiogenic factors and receptors

The maturation of preovulatory follicles as a prelude to ovulation and corpus luteum formation requires the development of a microvasculature sufficient for the delivery of adequate levels of hormones and lipoprotein-bound cholesterol. It has been appreciated that the granulosa cells and theca cells of the developing follicle produce angiogenic activities [65,66]. Following ovulation, the burst in blood vessel growth during the onset of corpus luteum development has been compared to the rapid angiogenesis observed during tumor formation. The angiogenic activities produced by granulosa, theca and steroidogenic luteal cells have been ascribed to basic fibroblastic growth factor (bFGF) and to vascular endothelial cell growth factor (VEGF). Conversely, microvascular endothelial cells also produce these growth factors and thus support their own mitosis as well as that of granulosa cells [67]. VEGF and probably bFGF serve as angiogenic factors as well as survival factors for microvascular endothelial cells [3,68].

It is well established that the development of a vascular bed comprises a coordinated collaboration between VEGF and its receptors, fms-like tyrosine kinase-1 receptor (Flt-1) and Flk-1 (fms-like kinase insert domain-containing receptor, a.k.a. KDR). As discussed in recent reviews [12,18-20], each of these components is present in the corpus luteum of rodents, farm animals and primates. Available evidence indicates that both mRNA and protein for VEGF and its receptors are highly expressed in the newly formed corpus luteum, maintained, albeit at lower levels, in the mid-luteal phase, and then decreased dramatically during corpus luteum regression. Based on *in situ* hybridization and immunohistochemistry studies VEGF is present mainly in steroidogenic luteal cells, while Flt-1 and Flk-1 appear to be present in both steroidogenic cells and endothelial cells in human corpora lutea [69-71], but only in the endothelial cells of bovine corpora lutea [68-72]. A recent report by Tscheudschilsuren *et al.* [73] on the expression of angiogenic factors and their receptors in cytokeratin-positive and -negative microvascular endothelial cells from the bovine corpus luteum shows that both types of microvascular endothelial cells produce VEGF mRNA. Thus, based on reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemical analysis, follicular granulosa and theca cells as well as steroidogenic luteal cells are not the sole source of VEGF in the bovine ovary. Both endothelial cell types also possess mRNA for the Flt-1 receptor. Further analysis revealed that cytokeratin-negative, but not cytokeratin-positive cells, contain Flk-1 mRNA. Based on RT-PCR analysis cytokeratin-negative cells possess Flk-1 mRNA levels 100 times greater than that of Flt-1 [73].

Additional angiogenic factors, such as the angiopoietins are also implicated in the regulation vascular development and regression [74]. Angiopoietin-1 and angiopoietin-2 are ligands for the Tie-2 receptor (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains). Angiopoietin-1 stimulates sprouting and maturation of blood vessels *in vitro*, whereas angiopoietin-2 inhibits this effect by acting as a competitive inhibitor by binding to the Tie-2 receptor without activating intracellular signaling pathways. In the bovine corpus luteum, similar levels of angiopoietin-1 and angiopoietin-2 mRNA are present in developing and fully functional corpora lutea of the estrous cycle [68]. However, the angiopoietin-2 to angiopoietin-1 ratio shifts markedly during blood vessel regression. It has been suggested that elevation of angiopoietin-2 results in regression of the corpus luteum [68,75]. Neither cytokeratin-positive or -negative endothelial cell types contain angiopoietin-1 mRNA, whereas only cytokeratin-negative endothelial cells contain mRNA for angiopoietin-2 [73]. These results further underscore the differences in these cell types. Also of interest in these studies are the observations that hypoxia, which may play a role in the initial process of luteal vascularization [76] had no effect on the levels of mRNA for hypoxia-induced transcription factors, HIF-1α and HIF-1β [73]. Hypoxia did not alter the levels of VEGF, Flk-1 or Tie-2 mRNA in cytokeratin-positive or negative endothelial cells. However, hypoxia did elevate Flt-1 and angiopoietin-2 mRNA levels in cytokeratin-negative cells. At present, it is unclear whether the mRNA for angiogenic factors and their receptors are efficiently translated into protein and have functional significance in the intact corpus luteum. If so, this would indicate that luteal endothelial cells are not only responsive to, but also actively contribute angiogenic signals in support of the angiogenic factors produced by the steroidogenic cells.

As a sign of *in vitro* angiogenesis, tubules form in four to six week-old confluent cultures of bovine endothelial cell types 1–4 [44]. The tubules represent a true three-dimensional "inside out" model with the apical cell side towards the culture medium and the basal side towards a core of extracellular matrix. Tubules formed by cytokeratin-positive endothelial cells have a braided-like aspect in the scanning electron microscope because of distinct cell boarders arranged helically around the tube axis [Figure 3a]. Since small arterioles of the human ureter show a similar endothelial cell helix in microvessel corrosion casts [77], the cytokeratin-positive tubules may represent arteriole-like tubules. Tubules of cytokeratin-negative type 3 endothelial cells have a smooth surface texture and are anchored to the monolayer by extremely long cell processes [Figure 3b]. Cytokeratin-negative type 4 cells form pseudotubules having a two-dimensional reticular appearance (Figure 3c) [41]. The differences in tubule formation further underscore the different endothelial cell types and point to possible differences in the origin of the microvascular bed and the involvement of different angiogenic factors (or the lack thereof) in tubule formation.

The signaling pathways that regulate angiogenesis of the corpus luteum are poorly understood. The receptors for angiogenic factors are analogous to other growth factor receptor tyrosine kinases. Studies employing endothelial cells from other tissues suggest that the phosphatidylinositol 3-kinase/Akt-signaling pathway is a key regulator of the angiogenic phenotype and survival of endothelial cells [reviewed in [78]]. More recently, glycogen synthase kinase-3β (GSK-3β) has been suggested to be an essential signaling mediator for controlling endothelial survival and migration *in vitro* and angiogenesis *in vivo* [79]. In these studies, GSK-3β was characterized as being under the control of Akt and mitogen-activated protein kinase signaling pathways. Thus, GSK-3β may serve as a point of conversion for multiple signaling pathways that coordinate endothelial responses to different angiogenic inputs. It will be important to discover the signaling pathways necessary to promote the rapid vascularization of the corpus luteum and the signaling pathways required for maintaining this elaborate vascular network.

Regulation by vasoactive peptides

Renin-Angiotensin System

Components of the renin-angiotensin system have been identified in the ovaries of a number of species [80]. A role for angiotensin II has been proposed in growth and atresia of follicles, oocyte maturation, ovulation, corpus luteum formation, steroidogenesis, and corpus luteum regression. Angiotensin I is produced from the precursor angiotensinogen by the enzymatic activity of renin, which is located in granulosa, theca and luteal cells [80,81]. Microvascular endothelial cells characteristically possess the angiotensin-converting enzyme (ACE) and are capable of converting angiotensin I into angiotensin II. In the bovine corpus luteum, tissue levels of angiotensin II remain constant throughout the estrous cycle, although levels of angiotensin II are higher in the ovarian vein than the jugular vein [82,83]. Early studies using pharmacologic approaches indicate that receptors for angiotensin II are present on steroidogenic cells; however, the presence of either angiotensin II type 1 (AT1R) or type 2 (AT2R) receptors varies among species [80]. Recent studies using a semi-quantitative RT-PCR approach indicate that bovine luteal cells and microvascular endothelial cells possess both AT1R and AT2R [81,82]. AT1R expression did not change over the bovine estrous cycle, while AT2R expression was lowest during the mid luteal phase and highest during the late luteal phase.

Angiogenesis

Available evidence suggests that angiotensin II may play a role in early corpus luteum development. Angiotensin II has been shown to stimulate the mRNA levels for bFGF in bovine luteal cells [84] and increase the proliferation of bovine aortic endothelial cells [85]. Furthermore, estradiol in combination with either VEGF or bFGF increases the production of angiotensin II in cytokeratin-negative type 3 bovine luteal endothelial cells [81]. In bovine corpora lutea collected during the early luteal stage (days 1–4), infusion of VEGF or bFGF using an *in vitro* microdialysis system stimulated the secretion of angiotensin II, progesterone and prostaglandin F2α [86]. Angiotensin II administration using either *in vitro* [86] or *in vivo* [83] microdialysis systems stimulated progesterone secretion from early luteal phase corpora lutea. Although a direct cause and effect relationship remains to be established, it appears that the renin-angiotensin system is active during the early luteal phase and endothelial cells are involved.

Angioregression

In contrast to a supportive role in early corpus luteum development, angiotensin II may facilitate functional and structural regression of the corpus luteum. During the mid luteal phase treatment with prostaglandin F2α *in vivo* had no effect on levels of mRNA for either AT1R or AT2R (87). It did, however, enhance luteal ACE mRNA levels, increase levels of immunoreactive angiotensin II in endothelial cells and large luteal cells, and increase the secretion of angiotensin II [87]. In contrast, prostaglandin F2α had no direct effect on angiotensin II production in isolated microvascular endothelial cells *in vitro* [82].

Angiotensin II inhibits LH-stimulated progesterone secretion from primary cultures of bovine corpora lutea cells [84] and from mid luteal phase bovine corpora lutea in an *in vitro* microdialysis system [87,88]. Furthermore, angiotensin II, together with prostaglandin F2α, has a marked suppressive effect on progesterone secretion *in vivo* and hastens luteal regression [89]. Although a direct cytotoxic effect of angiotensin II on the luteal endothelial or steroidogenic cells has not been reported, in other tissues angiotensin II has been shown to directly induce apoptosis of microvascular endothelial cells and parenchymal cells [reviewed in Filippatos *et al.*, [90]]. A complex interaction is emerging among various principles that regulate angiotensin II secretion and its action in the corpus luteum. The contribution of vascular endothelial cells cannot be understated in this context. Experiments designed to determine the actions of angiotensin II on endothelial cells are clearly required for a deeper understanding of the events leading to angioregression.

Endothelin-1

Endothelin-1 is a potent vasoconstrictor and mitogen for endothelial cells, vascular smooth-muscle cells and various tumor cells [91]. Endothelin-1 expression correlates with the vascularization of tumors [92] and the malignancy in ovarian cancer [93]. Endothelin-1 also acts as a cell survival factor for rat fibroblasts, vascular smooth muscle cells, and endothelial cells [90]. The endothelins exert their physiological effects via two receptors, endothelin type A (ET_A) and type B (ET_B) receptors. The ET receptors are G-protein-coupled transmembrane receptors found in both vascular and nonvascular tissues. Endothelin-1 suppresses or induces apoptosis depending on cell type and cell-specific expression of ET_A and ET_B receptor subtypes [90]. The anti-apoptotic effects of the ET_A receptor are presumptively mediated through activation of the mitogen-activated protein kinase signaling system. In contrast, endothelin-1 may promote apoptosis by acting through ET_B receptors [90]. Despite the close relationship between endothelin-1 and vascular function, little is understood about the role of endothelin-1 in the regulation of blood vessel formation and maintenance in the corpus luteum.

Angiogenesis

The corpus luteum has a complete endothelin system that is influenced by endocrine and paracrine factors. Endothelin-1 (ET-1) is synthesized by proteolytic cleavage of a large precursor molecule, prepro endothelin-1, which is facilitated by a metalloproteinase, endothelin converting enzyme (ECE). In the bovine corpus luteum, prepro endothelin-1 mRNA appears to be expressed only in endothelial cells whereas, both the steroidogenic cells and luteal endothelial cells express ECE [87,94-96]. Thus, the steroidogenic cells and endothelial cells may differentially cooperate in the biosynthesis of mature endothelin-1. The ECE is expressed in the corpus luteum throughout the normal estrous or menstrual cycle [94-96]. However, ECE mRNA and protein levels peak at mid luteal phase and are reduced during corpus luteum regression suggesting that endothelin-1 plays a role in corpus luteum development. A recent report [94] describes two ECE isoforms, ECE-1a and ECE-1b, in steroidogenic cells and cytokeratin-negative endothelial cells of the bovine corpus luteum. Based on their cellular localization, the isoforms are capable of cleaving the intra- or extra-cellular endothelin-1 precursor molecule, respectively. Endothelial cells posses mRNA for both ECE-1 isoforms, whereas the steroidogenic cells posses only mRNA for the extracellular ECE-1b isoform. Treatment with LH and endothelin-1 for 24 hours down regulated ECE-1b mRNA levels, providing a preliminary indication that hormonal and feedback regulatory mechanisms may control the level of ECE-1 activity and ultimately endothelin-1 levels in the corpus luteum [94]. Other evidence suggests that in cultures of luteinized bovine and human granulosa cells treatment for periods of 4–5 days with either insulin-like growth factor-I (IGF-I) [94] or hCG [97] up regulates ECE levels. Thus, a complex pattern of ECE regulation may occur depending on the specific stage of follicle or corpus luteum development. In other systems endothelin-1 has been shown to stimulate endothelial cell growth through stimulation of VEGF production, and VEGF has been shown to stimulate endothelin-1 secretion [91]. Since VEGF and endothelin-1 protein are expressed in the newly ruptured follicle [68,94], it seems possible that endothelin-1 potentiates VEGF mediated angiogenesis in the developing corpus luteum.

Angioregression

Endothelin-1 has been implicated as a mediator of functional corpus luteum regression (reduced steroid synthesis, yet intact structure) in rat [98], ovine [99], bovine, [100] and human [101] luteal tissues. Although treatment with endothelin-1 has no effect on angiotensin II, oxytocin, progesterone or prostaglandin F2α secretion during the early luteal phase [83,86], endothelin-1 appears to play an essential role during prostaglandin F2α-induced regression of corpora lutea during the mid luteal phase. Following the administration of a sub-luteolytic dose of a prostaglandin F2α analogue during the mid luteal phase in cows, an intraluteal injection of endothelin-1 results in a rapid decrease in plasma progesterone [99]. Furthermore, intraluteal injection of an endothelin-1 receptor antagonist before administration of a luteolytic dose of prostaglandin F2α reduces the response to prostaglandin F2α [102].

Receptors for endothelin-1 have been documented in corpora lutea of many species [87,94,97,103,104]. The ET_A and ET_B receptors are present in the bovine ovary throughout the estrous cycle and pregnancy, but levels of ET_B are elevated around the time of spontaneous corpus luteum regression [87,96]. Although it is clear that the steroidogenic cells and endothelial cells of the corpus luteum have receptors for and respond to endothelin-1, detailed studies on the specific receptors present in endothelial cells are sparse. In autoradiographic studies of the human ovary [104], ET_A and ET_B were localized to the blood vessels, with ET_B predominantly localized to endothelial cells and ET_A localized to the smooth muscle of the vascular wall. *In situ* hybridization studies [103] of monkey and human ovaries revealed that ET_A receptor mRNA was predominantly located in smooth muscle cells of blood vessels adjacent to follicles and corpora lutea. The ET_B receptor mRNA was localized to the endothelial cells lining the blood vessels of corpora lutea. As determined by RT-PCR analysis of purified populations of bovine corpora lutea cells, cytokeratin-negative endothelial cells contain levels of ET_A mRNA greater than those found in either the large or small luteal cells [105]. Much work is required to clarify the cellular distribution of endothelin receptors among species and luteal cell types.

Endothelin-1 (ET-1) peptide and ET_B mRNA levels are elevated during spontaneous corpus luteum regression [96]. Furthermore, administration of prostaglandin F2α at mid-cycle elevates mRNA levels for ECE, endothelin-1, ET_A and ET_B, as well as increasing endothelin-1 peptide expression in the bovine corpus luteum [87,94-96,98,99]. As determined by immunocytochemistry the increases in endothelin-1 occur in the large luteal cells and the endothelial cells [87]. These results suggest a functional role for endothelin-1 in the blood vessel changes observed in regressing corpus luteum. Perhaps endothelin-1 serves to coordinate the regression of capillaries, and the degenerative process in arterioles (arteriolarization) observed in the regressing corpus luteum [30]. This idea would be consistent with the observed increases in the proliferation of vascular smooth muscle cells and possibly pericytes during corpus luteum regression [14,15,29]. Currently, there is no evidence that endothelin-1 plays a role in the structural regression of the corpus luteum by directly affecting the viability of steroidogenic or endothelial cells, such as assumed for the renin-angiotensin system [90]. Studies to examine the actions of endothelin-1 in combination with other vasoactive peptides and cytokines may be required to reveal cytotoxic effects of endothelin-1.

Regulation by Cytokines

Role of Tumor Necrosis Factor Alpha (TNFα) in Angioregression

The cytokine TNFα exerts many actions on the corpus luteum [61,106,107]. Although it is generally assumed that immune cells contribute to intraovarian TNFα, endothelial cells and macrophages of the porcine corpora lutea secrete TNFα [108]. The steroidogenic cells and endothelial cells of the corpus luteum possess receptors for TNFα (TNFR) [109-111]. Levels of TNFR1 mRNA were elevated in physiologic or prostaglandin F2α-induced regression of the corpus luteum [111]. Microvascular cytokeratin-negative endothelial cells isolated from the bovine corpus luteum possess TNFR1. Furthermore, TNFα receptors are functionally coupled in microvascular endothelial cells to an increase in the production of both endothelin-1 and prostaglandin E2 [110]. As stated in the previous section, endothelin-1 has the ability to reduce progesterone secretion from the mid cycle bovine corpus luteum *in vitro* and *in vivo*. It seems likely, therefore, that TNFα-induced endothelin-1 secretion from endothelial cells may act in a paracrine manner to influence the steroidogenic capacity of luteal cells.

Two recent reports employing primary cultures of bovine luteal endothelial cells demonstrate that TNFα is cytotoxic for endothelial cells derived from the corpus luteum [38,111]. Apoptosis of luteal endothelial cells in response to TNFα was associated with both morphological and biochemical features of apoptosis, including shrunken nuclei, DNA fragmentation, and caspase-3 activation. In contrast, neither Fas ligand nor prostaglandin F2α altered the viability of these cells [38]. TNFα-induced cell death was associated with a rapid activation of members of the mitogen-activated protein kinase family (ERK, p38 and JNK), as well as, the activation of sphingomyelinase with a resultant accumulation of the second messenger ceramide [38]. Treatment with ceramide elevated the activity of JNK and induced apoptosis in endothelial cells. Of considerable interest were observations that pretreatment of endothelial cells with glutathione, an intracellular reducing agent and known inhibitor of reactive oxygen species, significantly attenuated TNFα-induced apoptosis [38]. These data suggest that TNFα or a member of this cytokine superfamily, may mediate the actions of prostaglandin F2α on regression of the microvascular capillary beds during structural regression of the corpus luteum *in vivo*.

Role of Interferon gamma (IFNγ) in Angioregression

The cytokine IFNγ exerts anti-proliferative effects on a number of primary cells and cell lines. This cytokine prominently figures in the regulation of corpus luteum function [61,106,107] and has a variety of effects on bovine luteal endothelial cells types 1–5. Under confluent monolayer culture conditions, treatment with IFNγ for three days resulted in a flattening and enlargement of cell types 1–4, with less evident changes in the morphology in cell type 5 [44,45]. In cytokeratin-negative cells of type 3, large intracytoplasmic vacuoles were present following treatment with IFNγ. These morphological changes were considered to be signs of senescence. Treatment with IFNγ also caused dramatic alterations in the actin cytoskeleton of endothelial cells types 1, 3, 4 and 5 [45] and generally reduced the content of fibronectin in all endothelial cell cultures [45]. IFNγ treatment selectively elevated the levels and localization of E-cadherin at cell borders in the cytokeratin-positive type 1 cells [45]. This was associated with increased levels of desmosomes and both tight and gap junctions and a reduction in permeability [57].

In addition to the notable effects on the cytoskeleton, treatment with IFNγ under low density and growth promoting conditions exerted a profound anti-proliferative, but mainly cystostatic, effect on cell types 1–4 [38,44]. However, type 5 cells (granulosa-like cells) were not growth inhibited by IFNγ. In consideration of the anti-proliferative actions on types 1–4 endothelial cells, together with the features of cell senescence, it appears that endothelial cells are generally negatively affected by IFNγ. This cytokine is produced by local immune cells [58-61] and may support the cytotoxic effect of TNFα on luteal endothelial cells [38,111] during corpus luteum regression.

Class I and II major histocompatibility (MHC) molecules are expressed in the corpus luteum and are thought to initiate a transient immune response during corpus luteum regression [61]. The class I and II MHC molecules are present on the endothelial and steroidogenic cells in the bovine corpus luteum. In cytokeratin-positive type 1 and 2 endothelial cells, basal expression of class I major MHC molecules is low, and increases 7–13 fold following treatment with IFNγ [43]. The class II MHC molecules are not detectable in un-stimulated cytokeratin-positive endothelial cells, expression of Class II MHC molecules is elevated in response to IFNγ. Endothelial cell types 3–5 have a high basal level of expression of Class I MHC antigens, which is moderately elevated only in type 5 cells. Interferon γ also induces expression (80 fold increase) of Class II MHC antigens in type 5 cells [43]. Others have observed that IFNγ-treated endothelial cells derived from veins express considerably more Class II molecules than arteries [112]. Given the differences in expression levels for Class II MHC molecules, this finding could point to a different origin for types 2 and 5 cells. Additionally, the differential expression of Class I and Class II molecules translates to different immunological responses which may help explain the temporal nature of endothelial cell loss during corpus luteum regression

Secretion of Chemokines

Regulated on activation, normal T cell expressed and secreted (RANTES)

In the corpus luteum, microvascular endothelial cells are likely to mediate the cyclic accumulation of specific subsets of leukocytes. Eosinophils rapidly accumulate during the early phase of corpus luteum development, whereas, the number of macrophages is dramatically increased during the late luteal phase [58-61]. The combined action of adhesion molecules (as described in a previous section) and chemokines are required to recruit eosinophils and macrophages into the corpus luteum at precisely defined stages of the estrous cycle. A chemokine known to stimulate eosinophil recruitment, RANTES (regulated on activation, normal T cell expressed and secreted), is expressed in the bovine [54,62] and human ovary [63,64]. *In vitro* studies indicate that the common cytokeratin-negative bovine endothelial cells have significantly higher basal RANTES mRNA levels than do cytokeratin-positive cells [54]. Furthermore, TNFα up-regulates RANTES mRNA in both cell types. However, *in situ* hybridization studies indicate that RANTES mRNA positive macrophages are present in the former thecal layer of the corpus luteum, not in the corpus luteum itself, pointing to mRNA levels below the detection level [62]. Both RANTES mRNA and protein are present and up regulated by TNFα in cultures of luteinized granulosa cells from women undergoing *in vitro* fertilization [63]. It is possible that endothelial-like granulosa cell subpopulations as described by Antczak and Van Blerkom [51] may be involved in the recruitment of eosinophils. Additionally, the mRNA for granulocyte-macrophage colony-stimulating factor, a macrophage recruiting factor, was found in control and TNFα-stimulated cytokeratin-negative endothelial cells but not cytokeratin-positive cells [54]. This indicates that the common cytokeratin-negative endothelial cells may play a role in recruitment of macrophages during corpus luteum regression [64].

Monocyte chemoattractant protein-1 (MCP-1)

The chemokine, monocyte chemoattractant protein-1 (MCP-1) is produced in the regressing corpus luteum of rats, pigs, sheep, cows, and women [reviewed by Penny in [113]]. This chemokine is of particular interest because once expressed within blood vessels it facilitates the attachment and migration of immune cells, specifically monocytes, macrophages, and T-lymphocytes, from the bloodstream into sites of inflammation. In this regard, macrophage and lymphocytes have been demonstrated to accumulate in the regressing corpora lutea of many species and have been implicated in phagocytosis of luteal cells, degradation of extracellular matrix, and secretion of other pro-inflammatory mediators that may influence luteal steroidogenesis or apoptosis. Depending on the species studied, steroidogenic and/or endothelial cells appear to be the source of MCP-1 [60,113]. The endothelial cells of the bovine corpus luteum are clearly a source of MCP-1 and respond directly to cytokines, such as TNFα and IFNγ, with an elevation of MCP-1 mRNA and MCP-1 secretion [114]. However, in contrast to previous *in vivo* studies showing that prostaglandin F2α elevates MCP-1 mRNA during induced regression of the corpus luteum [113], treatment of primary cultures of luteal endothelial cells with prostaglandin F2α had no effect on MCP-1 mRNA expression or secretion. Further analysis revealed that these endothelial cells lacked prostaglandin F2α receptor (FP) mRNA and did not respond to prostaglandin F2α with elevations in well-characterized signal transduction pathways [114]. Since Girsch *et al*. [115] provided evidence that some luteal endothelial cells are responsive to prostaglandin F2α, different laboratory conditions or endothelial cell types (cytokeratin-positive versus cytokeratin-negative cells) may explain the divergent response to prostaglandin F2α. Thus, whether the stimulatory effect of prostaglandin F2α on MCP-1 secretion *in vivo* involves the activation of endothelial cells directly, or indirectly mediated by cytokine action on steroidogenic cells, endothelial cells or macrophages remain to be determined. However, previous studies employing *in situ* hybridization have not identified luteal endothelial cells as a source of prostaglandin F2α receptor mRNA [116].

Granulocyte-macrophage colony-stimulating factor (GM-CSF)
Granulocyte-macrophage colony-stimulating factor, another chemokine with macrophage recruiting properties, has been identified in endothelial cells. The mRNA for GM-CSF was found in control and TNFα-stimulated cytokeratin-negative endothelial cells but not cytokeratin-positive cells [54]. These studies indicate that the common cytokeratin-negative endothelial cells may play a role in recruitment of macrophages during corpus luteum regression. [64].

Relationship to tumor microvascular endothelial cells
As mentioned in the introduction, the angiogenesis associated with the developing corpus luteum is often compared to tumor-associated angiogenesis [3,11,12,20,117,118]. The growth and development of both are critically dependent an adequate supply of blood vessels. The analogy continues with the dependency on a number of shared angiogenic factors and vasoactive molecules, and their receptors. Despite these similarities, the index of proliferating capillary cells of the newly developing bovine corpus luteum (40.6 ± 11 %) was consistently higher than that observed in six different types of malignant tumors in humans (2–5 % for prostate, lung and mammary carcinomas; and as high as 8–10 % for colon, carcinomas, renal carcinomas and glioblastomas) [118]. Additional differences were noted in the extent of pericyte coverage (12–67 %) of the vasculature in these tumors compared to the developing (60 %) and mature (64 %) corpus luteum [118]. If coverage of the vasculature with pericytes is linked to the maturity of the vascular bed, then these data suggest that both tumor and corpus luteum vasculature beds are relatively immature. This functional immaturity allows the vascular elements to be targeted for remodeling. Thus, the very active angiogenesis observed during corpus luteum development is greater than observed in some tumors. In contrast to tumor angiogenesis, the vascular development in the corpus luteum is tightly regulated by endocrine and paracrine, and autocrine factors that regulate ovarian function. The plasticity of the vasculature of the mature corpus luteum presumptively allows for targeted destruction of the capillaries during corpus luteum regression. This also requires a tightly coordinated process in which angiogenic factors are reduced and angiostatic factors, vasoactive compounds, cytokines and chemokines are elevated.

Future directions
Much remains to be discovered about the processes that control vascular development and regression in the cyclic corpus luteum. Similarly, little is known about the factors responsible for maintaining the vasculature of the corpus luteum of pregnancy. Studies with specific microvascular endothelial cells will enhance efforts to understand which factors influence this complex physiologic process. New research is necessary to learn more about the role of the extracellular matrix and cell-to-cell interactions among endothelial cells, capillary pericytes, immune cells and steroidogenic cells. The involvement of steroid receptors and nitric oxide alone or in combination with vasoactive peptides and lipids, cytokines and/or chemokines are fertile fields of discovery using *in vivo* and *in vitro* approaches.

Conclusion
The study of the endothelial cells of the corpus luteum has provided new insights into the mechanisms required for normal corpus luteum development, function, and regression. Much remains to be discovered about the function of vascular specific growth factors, as well as the pleiotropic angiogenic cytokines present during angiogenesis and angioregression of the corpus luteum. Furthermore, the specific intracellular signaling pathways that promote and maintain the vasculature, induce regression of the vasculature and ultimately the regression of the corpus luteum remain to be worked out. The field of vascular-bed heterogeneity is well recognized, yet it remains to be specified for the vasculature of the corpus luteum of many animals. The qualitative and quantitative differences in ovarian angiogenesis in various animal models remains to be worked out. Research on all of these issues will help scientists to better understand the complexity of a fascinating biologic process, how blood vessels grow and regress during the life span of the corpus luteum. Furthermore, the potential clinical applications of this research are tremendous considering crucial reproductive processes in which vascular dysfunctions play a role.

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