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# Vascular endothelial growth factor and angiopoietins during hen ovarian follicle development

Dongwon Kim [1], Jeeyoung Lee, A.L. Johnson *

*Center for Reproductive Biology and Health, Department of Animal Science, and Cell and Developmental Biology, Huck Institute of Life Science, The Pennsylvania State University, 227 Henning Building, University Park, PA 16802, USA*

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ABSTRACT

Growth and maturation of ovarian follicles in the hen (*Gallus gallus*) requires a network of blood vessels that increases in complexity during development. The present studies investigate expression of vascular endothelial growth factor A (*VEGF*), angiopoietin1 (*ANGPT1*) and *ANGPT2* mRNAs together with their associated receptors (*VEGFR* and *TIE2*, respectively) during maturation. Elevated expression of *VEGF* and its receptors is associated with healthy, compared to atretic, follicles. Levels of *VEGF* significantly increase, while antagonistic *ANGPT2* decrease, in granulosa cells (GC) at follicle selection. By comparison, levels of *VEGF*, *VEGFR1*, *VEGFR2*, *ANGPT1*, *ANGPT2* and *TIE2* within the theca layer do not change ($P > 0.05$) relative to developmental stages surrounding follicle selection (6–8 mm *versus* 9–12 mm follicles). Prior to selection, treatment with transforming growth factor β1 (TGFβ1) significantly increases levels of *VEGF* in undifferentiated GC from prehierarchal (6–8 mm) follicles and actively differentiating GC from selected (9–12 and F4) follicles. Moreover, subsequent to selection follicle stimulating hormone (FSH) increases *VEGF* expression in GC from 9 to 12 mm follicles, and eventually luteinizing hormone (LH) promotes VEGF expression in GC from more mature preovulatory follicles. It is concluded that prior to follicle selection VEGF expression is regulated by autocrine and paracrine actions of TGFβ1 (but not FSH), and that a comparatively limited extent of vasculature is sufficient to maintain prehierarchal follicles in a viable and undifferentiated state. At follicle selection, FSH- and subsequently LH-induced VEGF production within the GC layer enhance angiogenesis within the theca layer, which facilitates the rapid growth of preovulatory follicles via enhanced incorporation of yellow yolk.



## 1. Introduction

Angiogenesis represents the process of new blood vessel formation from pre-existing vasculature and is critical for various reproductive processes, including ovarian follicle maintenance and growth (Smith, 2001; Robinson et al., 2009). Studies of the mammalian ovary have demonstrated that angiogenic factors secreted by granulosa cells (GC) play critical roles in follicle growth and survival via new blood vessel formation within the theca layer (Chowdhury et al., 2009; Kuo et al., 2011). Any misregulation of blood flow within the ovary can contribute to disorders such as anovulation, infertility or polycystic ovary syndrome (PCOS) (Ferrara et al., 2003).

The network of ovarian blood vessels is established by angiogenic factors, including vascular endothelial growth factors (VEGF) and angiopoietins (ANGPT). In particular, VEGF isoforms represent potent mitogens for vascular endothelial cells and agonists of angiogenesis (Hoeben et al., 2004), and have been implicated in both follicle growth plus the formation of the mammalian corpus luteum (Robinson et al., 2009; Rico et al., 2014). The VEGF family is composed of six isoforms (VEGFA, -B, -C, -D, -E, and -F), of which VEGFA (referred to hereafter as VEGF) has been well studied in mammalian ovarian follicles (Achen and Stacker, 1998; Holmes and Zachary, 2005; Olsson et al., 2006). VEGF is expressed at comparatively lower levels during early follicle development, and levels significantly increase within the granulosa and theca layers during follicle maturation (Barboni et al., 2000; Fortune et al., 2001; Greenaway et al., 2005). This growth factor family exerts biological effects by binding to three structurally related VEGF receptor tyrosine kinases, VEGFR1, VEGFR2, and VEGFR3 (Olsson et al., 2006). In particular, VEGF signaling through VEGFR2 in mammals has been documented to promote proliferation, migration, and survival of endothelial cells (Hoeben et al., 2004).

Although VEGF is a major initiator of angiogenesis, newly established capillaries must also undergo structural and functional

* Corresponding author at: 227 Henning, The Pennsylvania State University, University Park, PA 16802, USA.
*E-mail address:* alj14@psu.edu (A.L. Johnson).
[1] Post-Doctoral Research Fellow, Department of Dermatology, Johns Hopkins School of Medicine, 1550 Orleans Street, Baltimore, MD 21231.

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maturation. ANGPTs are known to function in modulating vessel remodeling and maturation by inducing the migration of mural cells such as pericytes and smooth muscle cells. While there are four structurally related isoforms (ANGPT1 to -4), ANGPT1 and ANGPT2 are the most commonly studied with respect to blood vessel formation, and they exert their functions via the TIE2 receptor and its associated tyrosine kinase activity (Iivanainen et al., 2003). ANGPT1 binding to the TIE2 receptor initiates downstream signaling pathways, including the phosphoinositide 3-kinase (PI3K) pathway (Brown et al., 2005). By comparison, ANGPT2 binds TIE2 with similar affinity but antagonizes ANGPT1-induced TIE2 signaling and disrupts angiogenesis (Maisonpierre et al., 1997; Yuan et al., 2009). In the mammalian ovary, ANGPT1, ANGPT2 and TIE2 are expressed in growing follicles and the corpus luteum (Shimizu et al., 2007; Scotti et al., 2013). A disrupted balance of ANGPT1 and ANGPT2 levels following a local injection of ANGPT2 into a preovulatory follicle inhibits follicular angiogenesis, ovulation, and the formation of corpus luteum in primates (Xu and Stouffer, 2005). This implies that some appropriate ratio of ANGPT1 to ANGPT2 is critical for the maintenance and stability of blood vessels.

Previous studies also provide unequivocal evidence that follicle viability is dependent upon VEGF production and an adequate blood supply (Mattioli et al., 2001; Pancarcı et al., 2011; Altermatt et al., 2012). Conversely, follicle atresia is closely associated with an inadequate supply of blood vessels surrounding and penetrating throughout the theca layer (Wulff et al., 2001; Acosta, 2007). Gonadotropins induce expression and secretion of angiogenic factors (including VEGF and ANGPTs) within mammalian GC (Chowdhury et al., 2009; Kuo et al., 2011). Moreover, several members of the transforming growth factor beta (TGFβ) superfamily, including TGFβ1 and Bone Morphogenetic Protein 4 (BMP4) have been demonstrated to induce VEGF expression in GC from the cow and human (Kuo et al., 2011; Shimizu et al., 2012).

In the domestic hen ovary, blood vessels are not uniformly distributed throughout the stages of follicle development. Prior to the entry of a slow growing follicle into the rapid growth stage (*e.g.*, before follicle selection) hen follicles are sparsely populated with small diameter blood vessels. Beginning at follicle selection the number and size of blood vessels associated with each preovulatory follicle are visibly increased, and this provides for rapid follicle growth and dramatically increased yolk incorporation during the final days before ovulation. While it has been estimated that the two largest preovulatory follicles each receive 12.4–17.4% of the total blood flow to the ovary, blood flow to all follicles less than 8 mm diameter combined (*e.g.*, all follicles other than those already selected into the preovulatory hierarchy) is estimated to total 12.6% (Scanes et al., 1982). Nevertheless, there is essentially no information currently available regarding the hormonal regulation of angiogenesis within hen ovarian follicles relative to stage of development, and in particular, at follicle selection. Accordingly, the objectives of the present study were to: (1) evaluate levels of *VEGF/VEGFR* and *ANGPT/TIE2* expression in hen follicles surrounding the time of follicle selection; and (2) identify factors (autocrine, paracrine and endocrine) that regulate *VEGF* and *ANGPT* expression both prior and immediately subsequent to follicle selection.

## 2. Materials and methods

### 2.1. Animals and reagents

Single-comb White Leghorn hens (Hy-Line W-36 variety; Elizabethtown, PA, USA) 34–55 weeks of age and laying sequences of 5 or more eggs were used in the studies described. Animals were housed individually in laying batteries within a windowless, environmentally controlled facility and were provided free access to feed and water, under a controlled photoperiod of 15 h light, 9 h dark. Hens were euthanized by cervical dislocation 3–7 h following a mid-sequence ovulation and the ovary was immediately removed and transferred into ice-cold sterile 1% NaCl w/v solution until GC were isolated. All procedures described herein were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committees, and were performed in accordance with The Guiding Principles for the Care and Use of Laboratory Animals.

Recombinant human (rh) TGFβ1 was purchased from PeproTech (Rocky Hill, NJ, USA) while rhFSH and ovine LH (oLH) were provided by the National Hormone and Pituitary Program (Torrance, CA, USA). Type II collagenase (335 U/mg) was from Worthington Biochemical Corporation (Lakewood, NJ, USA).

### 2.2. Tissue collection, incubations and cell culture

Ovarian follicles from individual hens were collected according to stage of development, and GC and theca layers from 3–5 mm and 6–8 mm follicles, the most recently selected (9–12 mm) follicle, the fourth (F4) and third (F3) largest preovulatory follicles were isolated (Tilly et al., 1991a; Woods and Johnson, 2005). While the GC layer represents a single cell type, the theca layer contains a variety of functional cell types, including steroidogenic and endothelial cells. To evaluate mRNA expression levels during follicle development, GC and theca layers were collected separately from 3 to 5 mm follicles, the largest 6–8 mm follicle, the single 9–12 mm follicle, and the F4 follicle. Theca layers were vigorously rinsed with DMEM to remove any remaining yolk and GC (Kowalski et al., 1991) prior to the processing for the analysis of mRNA expression. Atretic follicles are not common in the ovary from a hen laying regular sequences of eggs, and in such healthy ovaries are almost always limited to follicles $\leqslant$8 mm (see Tilly et al., 1991b). The combined granulosa and theca layers from individual 3–5 mm viable or atretic follicles were collected for RNA, and levels of *VEGF* and *VEGFR* were compared. B-cell lymphoma-extra large (*BCL-XL*) gene, an anti-apoptotic cell survival factor, was used as a definitive marker to differentiate normal from atretic follicles (Johnson et al., 1996b).

For short-term (4 h) incubations, GC layers were collected and immediately dispersed into small groups of cells (without collagenase) by gentle, repeated pipetting with a 1 ml pipette, then aliquoted into 12 × 75 mm polypropylene tubes containing 1 ml of Dulbecco's Modified Eagle Medium with high glucose (DMEM; HyClone, Thermo Scientific, USA) containing 2.5% FBS v/v (PAA Laboratories, Piscataway, NJ, USA), 1% antibiotic-antimycotic solution v/v (Gibco, Grand Island, NY, USA) and non-essential amino acids (Gibco) (Ocón-Grove et al., 2012). Each tube contained the equivalent of one GC layer from 3 to 5 mm follicle, one-half GC layer from 6 to 8 mm follicles, one-third layer from the single 9–12 mm follicle or one-quarter layer from the third largest (F3) preovulatory follicle (approximately $5 \times 10^5$ cells per tube). The tubes were incubated in a shaking water bath at 40 °C in the absence (control) or presence of 10 ng TGFβ1/ml, based upon the previously established minimal effective dose (Johnson et al., 2004) or gonadotropins (10 ng rhFSH/ml; Kim et al., 2013, or 10 ng oLH/ml; Tilly and Johnson, 1989) for 4 h.

For cell cultures, granulosa layers were dispersed using 0.3% collagenase II w/v to achieve a single cell suspension. Approximately $5 \times 10^5$ cells per well were cultured in 12-well polystyrene culture plates (Beckton Dickinson, Franklin Lakes, NJ, USA) containing 1 ml of DMEM plus 2.5% FBS, 0.1 mM non-essential amino acids (NEAA), and 1% antibiotic-antimycotic v/v mixture (Invitrogen Life Technologies, Carlsbad, CA, USA) at 40 °C in an atmosphere of 95%