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Review Article

Insights into Ovarian Follicle Angiogenesis: Morphological and Chronological Vascular Remodeling from Primordial to Ovulating Follicles

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Abstract

Ovarian function is dependent on the establishment and continual remodeling of a complex vascular system. The present review focuses on the ovarian morphological angiogenic processes that involve swine ovarian folliculogenesis and their modulatory molecular mechanisms: from the primordial follicles recruitment to the antral periovulatory stage. The process of angiogenesis, in particular, during the final stage of follicle development (from antral to periovulatory follicular phase) is deepened with a kinetic approach adopted by using an experimental prepubertal model where antral follicle's selection, growth and ovulation are pharmacologically and timely controlled. Since the cyclicity of follicular angiogenesis is inducible under experimental conditions in several experimental and domestic animals using validated hormonal treatments, it may become a reproducible model to study in vivo molecular mechanisms and pathways involved in angiogenesis and blood vessel remodeling.

The present review has also focused the attention on the regulatory role of vascular endothelial growth factor A in controlling ovarian follicular development as orchestra conductor of the angiogenic process. The main animal model considered in the present review is the pig. The reproductive processes of this domestic animal is of a high translational value by considering the similarity with women of the long periovulatory window (40-44 hours from luteinizing hormone surge to ovulation). This animal model could become, for this reason, a valuable model in understanding the regulatory pathways involved in the final stage of follicular maturation.

Introduction

Angiogenesis is an active process that can be classified as the new blood vessels formation from pre-existing structures. It requires the cooperation of several growth factors and the activation in target cells, enzymes, adhesion and cell cycle molecules thus generating a balance between positive and negative regulatory mechanisms required for tissue homeostasis. This process plays an essential role for tissue homeostasis during fetal and adult life. The differentiation as well as the growth of organs during fetal development is, indeed accompanied by the tropic supply of a dynamic blood vessels network. In adult tissues angiogenesis is more limited, and blood vessels remain quiescent until angiogenic stimuli occur, such as hypoxia [1]. Besides the essential physiological influence of angiogenesis on supporting metabolic tissue requests (es. muscle aerobic exercise) or during wound healing, in adults, blood vessel remodeling play a crucial role also in several pathological situations like tumor growth that was first proposed in 1971 by Judah Folkman who described tumors as "hot and bloody" [2].

The process of angiogenesis involves a complex and dynamic interaction between Endothelial Cells (ECs) and the corresponding extracellular environment. In vivo, angiogenesis occurs either by the sprouting of vascular ECs from pre-existing capillary endothelia into the surrounding tissues, or by intussusception (non-sprouting angiogenesis), which involves the division of capillaries by tissue pillars into two or more daughter vessels [3].

The creation of new blood vessels in different tissue seems to follow either mechanical or chemical stimulation. The mechanical stimulation of angiogenesis is low characterized even if the migration of ECs, pericyte/smooth muscle cell migration and tube formation to newly formed endothelial sprouts are considered as mechanosensory-dependent and growth factor-independent mechanisms [4]. Authors show that the shear stress acting on capillaries causing angiogenesis [4,5], and current knowledge suggests that increased muscle contractions may increase angiogenesis [6]. This may be due to an increase in the production of nitric oxide during exercise that causes vasodilation of blood vessels [6,7].

The chemical stimulation of angiogenesis, instead, is better defined recognizing various angiogenic codified proteins, including several growth factors. On the basis of the current knowledge,

angiogenesis appeared to be a heavily regulated process involving a balance between pro- and anti-angiogenic factors [3].

The progress of vascular structures strongly argues the alternance of destabilization/stabilization mechanisms influencing the existing vasculature that have the autonomous fate control achieved by blood vessels. This improvement is primarily mediated by ECs which appear to have intrinsic mechanisms to sense environmental changes and accordingly to remodel blood vessels. New vessel formation is triggered by ECs activation coordinated with controlled detachment of the surrounding mural cells, and proteolytic remodeling of the basement membrane and the Extracellular Matrix (ECM) [8]. In fact, after angiogenic stimulus by up-regulating pro-angiogenic factors, such as Vascular Endothelial Growth Factor A (VEGF), the existing vessels start to destabilize through the disruption of endothelial and mural cellular contacts. At the same time, numerous proteases are activated and the extracellular matrix is degraded. Once connected and aligned, the ECs form a lumen and the newly formed vessel is then stabilized by the recruitment of pericytes [1].

The growing sprout moves along a VEGF gradient. VEGF gradient is recognized by specialized ECs that acquire a specific tip cell phenotype characterized by the formation of numerous filopodia that extends towards the direction in which ECs migrates. The ECs that trail tip cells called 'stalk cells' are less motile but support sprouting and vessel extension [9]. In the initial steps ECs at the leading edge extend filopodia and migrate towards avascular regions where is the angiogenic signals: where VEGF levels are highest, VEGF activates VEGF receptor 2 (VEGFR2) to stimulate cell migration [10]. VEGFR2 internalization and activation of ERK1/2 signaling are important for sprouting, likely because rapid receptor turnover and signaling is essential for ECs at the vascular front to respond strongly and quickly to angiogenic signals. Such dynamic responses necessitate rapid clearing of activated receptors to finely tune the speed and direction of vessel branching [10,11]. Vessel sprouting requires coordination between migrating ECs and proliferative stalk cells. This management is regulated by signal molecules, such as Notch [8]. ECs with activated VEGFR2 compete for the tip position by increasing expression of the Notch [10,12]. The ligand binds to Notch receptors on neighboring ECs and releases a specific Notch intracellular domain. This domain acts as a transcriptional regulator, decreasing VEGFR2 expression while increasing the levels of VEGFR1, which traps VEGF [9] and renders stalk cells less responsive to VEGF. Therefore, Notch blockade induces vessel hyper branching, while gain of function causes the opposite effect [8].

The tip cells adhere to the ECM, mediated by integrins, and migrate toward guidance signal molecules (e.g., semaphorins and ephrins), while stalk cells trail behind the tip cell and proliferate to allow sprout elongation and lumen formation by specific signaling. So, as vascular migration/directionality (by tip cells) and elongation of the shaft (by proliferating stalk cells) are assured [13]. When two tip cells meet, they fuse making anastomose [10]. This mechanism is assisted by macrophages, which accumulate at sites of vascular anastomosis to act as bridge cells by interacting with the neighboring tip cells. Once contact between the tip cells has been established adhesion molecule, such as VE-cadherin, further strengthen the connection [8,9]. Perivascular macrophages further stimulate sprouting by producing angiogenic factors or proteolytically liberating them from the extracellular matrix. The stalk cells also deposit basal membrane and recruit pericytes, thus stabilizing the forming vessel. Pericyte precursors are attracted to vessels by EC-expressed PDGF. By TGF-β, these mesenchymal precursor cells were differentiated in pericytes to determine decrease ECs migration, proliferation, and vascular leakage, resulting in nascent vessel stabilization [14]. Studies indicate pivotal contribution made by vascular mural cells, especially pericytes, which can stabilize vessels in part through modulating the endothelial phenotype [15].

Once fusion has occurred, a connected lumen is formed to allow blood flow through the new vessel. This perfuses the hypoxic tissue, and the resultant oxygen and nutrient delivery leads to decreased levels of angiogenic signals, inactivation of ECs oxygen sensors, and increased proquiescent molecules that lead to ECs quiescence [8]. ECs resume a quiescent phalanx phenotype in a tightly apposed monolayer with a streamlined surface that conducts the blood flow and regulates tissue perfusion. Perfusion induces vascular maturation by re-establishment of cell-cell junctions, pericyte maturation, and basal membrane deposition [8,15].

In contrast with this initial phase, endothelial cells stop migration and proliferation in the maturation step and restore the barrier function of vessels. This vessel stabilization requires activation of distinct cellular signaling pathways different from those that initiate vascular cell mobilization [3].

Angiogenesis is essential to adult tissue remodeling and regeneration [3]. Amongst the adult tissues that physiologically require blood vessels adaption, a particular role is exerted by the female reproductive organs that modulate their quiescent and active phase by adjusting the supporting blood vessels [3,16,17]. The ovary, in particular after puberty, undergoes continual cyclical changes by alternating follicular and luteal phases in order to offer at each organism a reproductive chance [17]. The follicular phase start the recruitment of a so-called follicle cohort (medium antral size follicle/s) that in few days increases its volume and, within this, the selection of one/some mature follicle/s (preovulatory follicle/s) in depending of monocous or polytocous species, respectively, becoming an active steroids endocrine structure (estradiol is the steering hormone). Preovulatory follicle/s undergoing Luteinizing Hormone (LH) surge becoming periovulatory follicle/s. The follicular phase ends with ovulation of a periovulatory antral follicle/s with the release into the female genital tract of a mature metaphase II oocyte. Successively, the Corpus Luteum (CL) is differentiated on the vestigial of ovulated follicles thank to follicular theca cells that luteinize into small luteal cells (thecal-lutein cells) and follicular granulosa cells that luteinizing into large luteal cells (granulosa-lutein cells). In addition, starting the breakdown of basal lamina of the ovulatory follicle to begin angiogenesis required for CL formation. In fact, the CL is composed of a large number of vascular endothelial cells that account for up to 50% of the CL cells, while steroidogenic large and small luteal cells constitute about 30% [18,19].

This review focuses on morphological changes occurring during folliculogenesis, by addressing the analysis in a chronological way: from primordial follicles to the antral periovulatory ones.

The knowledge of follicular angiogenesis mechanism is of fundamental importance to understand the processes that ensure the reproductive success [16-19]. In fact, ovulation requires coordinated vascular development and adaptations in ovarian developed follicle

[16-21]. The link existing between these time-separated processes (follicle development and blood vessel remodeling) are not well characterized even if it allows the success of ovulation. The multitude of factors necessary to guide angiogenesis and the complexity of their temporal-spatial regulating activities, it suggests that more than one factor may be needed for the robustness of angiogenesis processes associated with ovulation. The complexity of the angiogenesis orchestration is also suggests by the ovulatory impairment induced by single factor inhibition/modification [19,20].

Moreover, the ovary is an excellent model in order to clarify the angiogenic mechanism controlling the homeostasis of other adult tissues as it represent an example of adult tissue that undergoes cyclically at intense vascular morphogenesis. The tissues in the ovary undergo constant remodeling either to induce the maturation of follicle or the formation and regression of the CL. This physiological cyclicity of follicular angiogenesis can be induced by using validated hormonal treatments better in prepubertal animals [19,22-25]. The reproducibility of these events under inductive pharmacological protocols make folliculogenesis an ideal experimental model to study angiogenesis under controlled in vivo parameters. This validated hormonal treatment has been developed in several domestic animal to control individual reproductive performances [23,26]. In particular follicular growth in pig is induced by the i.m. injection of a single dose of Equine Corionic Gonadotrophin (eCG), while ovulation is triggered by i.m Human Corionic Gonadotrophin (hCG) treatment. The ovulatory stimulation can be performed only when most of these follicles have reached a preovulatory diameter (from 60 to 72 h after eCG injection) and before the spontaneous LH surge will occur [22,25,27], These in vivo protocols than can be adopted to manage the reproduction of domestic animals represent a powerful experimental instrument to induce and control follicular angiogenesis offering the possibility to study the follicle-related events in a precise phase of their development (Figure 1). Besides, it is important to point out how the use of the ovarian follicles consents to overcome many typical problems correlated with the use of the transgenic models [28], often related to the creation of biological artefacts that inevitably require a physiological confirmation. The mammal animal model is used to study follicular dynamics in women to some similarities in reproductive events such as follicular waves, hormonal concentration changes, age-related decline in fertility, and pathological condition. Among the used models there are cow [29], ovine [30,31], mare [32,33], pig [22,24,25,27,34]. Swine reproductive processes have a high translational importance for the similarities with woman of the long window of periovulatory phase (40-44 hours from LH surge) thus becoming a valuable study model for understanding the regulatory pathways involved in the control of ovarian physiology and oocyte maturation [22,24,27]. Particularly, the Statistical Office of the Home Office 2016 (Statistic of Animal Science Procedures) puts the pig's use among the most commonly used species in applied human medicine studies.

Special emphasis will be put in the present review on morphological mechanism involve in swine follicular angiogenesis, and on the role of VEGF (VEGF A) in regulating them.

Folliculogenesis Process

The mammalian ovary is comprised of two distinct portions: the cortex and the medulla (Figure 1). The cortex is an outermost part of connective tissue (stroma) with inside follicles and corpora luteal at several development stages. The medulla is the inner region that contains loose connective tissue highly vascularized and originating from ovarian arteries. The vessels of medulla form plessus that give rise to small branches destined to stroma and to follicles and the corpora luteum. The branches of smaller diameter which enter the cortex of the ovary tend to spiralizing. Small arteries, leading to the periphery of the follicle, subdivide forming a basket around the follicle one. Histologically the limits between these two regions are not well defined. In 1900 Clark [35] showed, for the first time, ovarian vascular organization that, plus recently, has been better described by combining SEM analysis to the technique of vascular corrosion cast [22,36].

This complex vascular network supports, in the ovarian parenchyma, follicles at various stages of development with different morphological architecture and functional asset.

The folliculogenesis process takes place within the cortex from the formation of the primordial follicle (immature follicles) developing to the periovulatory stage (follicular-luteal stage). The predominant and smallest follicle in the ovary is the primordial follicle, which is surrounded by squamous, flattened granulosa cells. Primordial follicles leaving the resting phase are called primary follicles. The transition from non-growing to growing follicles is a gradual process, which begins shortly after the formation of the primordial follicles and continues throughout reproductive life. This transition represents a commitment of primary follicles to subsequent stages of follicular development, and is also an irreversible process because a

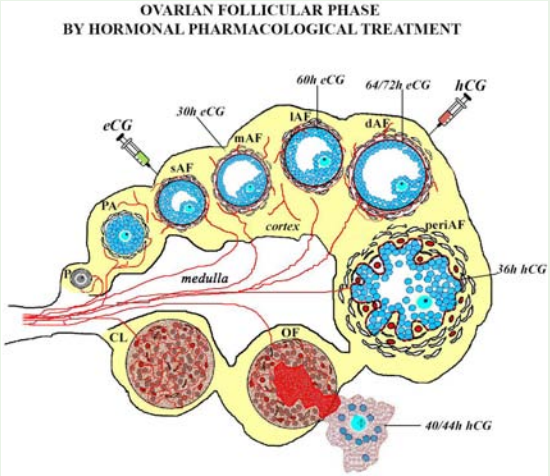

Figure 1: Representative scheme describing the ovarian follicular phase. Follicles are visualized by considering the specific morphological architecture (blue: granulosa cells; white/black: theca cells) and the relative blood vessel networks (red). Follicle-related events in a precise phase of their development can be obtained by validated hormonal treatments: the figure summarized an example of protocol used on prepubertal gilts to induce follicle selection/growth and ovulation. The follicular phase can be induced by injecting a single dose of equine chorionic gonadotropin (eCG, FSH-like activity), thus obtaining in 64-72 hours the development of the antral preovulatory follicles (dominant follicles). The subsequent injection of a single dose of human chorionic gonadotrophin (hCG, LH like activity) at 60-64 hours from eCG, injecting bring about ovulation (in pig 40-44 hours after hCG injecting). P: Primary/Primordial Follicle; PA: Preantral Follicle, sA: Small Antral Follicle; mA: Medium Antral Follicle; lA: Large Antral Follicle; dAF: Dominant Antral Follicle; periA: Periovulatory Follicle; OF: Ovulated Follicle; CL: Corpus Luteum.

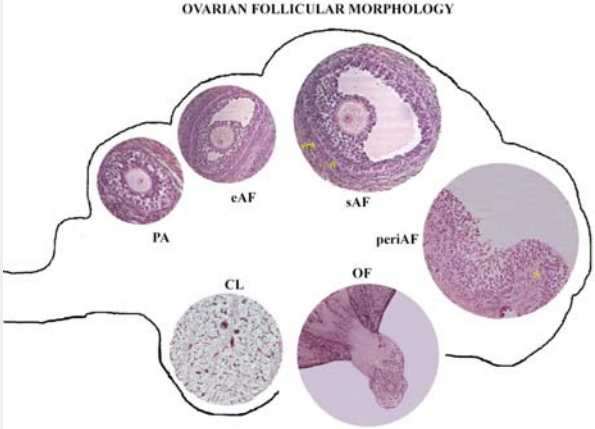

Figure 2: Representative morphology of folliculogenesis process (Hematoxylin and eosin stain; author data). PA: Preantral Follicles. eAF: Early Antral Follicle. sAF: Small Antral/Tertiary Follicle (asterisk: inner theca; double asterisk outer theca). periAF: Periovulatory Follicle (asterisk: inner theca infolding characteristic of this follicular stage). OF: Ovulated Follicles. 7: CL: Corpus Luteum.

follicle will continue to grow until its eventual demise, either through atresia or ovulation. Primary follicles are lined by a single layer of cuboidal granulosa cells that become proliferative and separated from the stroma of the ovary by a basal lamina. Granulosa cells continue to proliferate into several layers, forming multilayered secondary follicles (Figure 2, PA). Three events characterize the development of secondary follicles: the initiation of the zona pellucida assembly; fluid accumulation (liquor folliculi) between the granulosa cells, and the distinction of a cellular shell or theca that separates the ovarian stroma from the granulosa cell multilayer (Figure 2, AF). When the small intercellular spaces between granulosa cells contain follicular fluid and coalesce later to form a large space (Figure 2, sAF), the antrum, follicles became tertiary. The theca cell is organized into two distinct layers around each follicle: the theca interna and the theca externa (Figure 2, sAF asterisks).

The development of immature follicles is modulated by a variety of autocrine, paracrine and endocrine signals [18]. In fact, as a consequence of adequate stimuli, the primordial structures are activated and the follicles entering into the growing phase characterizing by morphological and functional modifications [37]. The initial activation and development of follicles (from primordial to the primary and secondary stages) is considered to occur independent of pituitary gonadotropin [18,37]. These follicles lack gonadotropin receptors and are far to ovarian blood supply, and are therefore not influenced by the fluctuation of hormone levels that occurs with each reproductive cycle. Follicle somatic cell proliferation (i.e., granulosa cell) and oocyte growth depend on complex and sequential communication by various oocyte-, granulosa cell, theca cell--derived factors between and within individual follicles. Platelet-Derived Growth Factor (PDGF), Bone Morphogenic Protein-6 (BMP6), Anti-Mullerian Hormone (AMH), BMP15, kit ligand, Basic Fibroblast Growth Factor (bFGF), BMP4/7, GDF-9, activin, and inhibin are produced by and act on the various early-stage follicle cells to stimulate growth and differentiation [18,37,38]. Oocyte development also dependent on growth factors, particularly those in the EGF-like family [39].

In a first phase (basal follicular growth), follicles grow slowly and follicular growth rate is tightly related to proliferation of granulosa cells. Basal follicular growth is mainly under the control of growth factors of paracrine origin. In these follicles, Follicle-Stimulating Hormone (FSH) may exert an indirect mitogenic effect on granulosa cells by enhancing expression of growth factors or growth factor receptors. In a second phase (terminal follicular growth), follicular growth is rapid and occurs by enlargement of the antrum. For this reason, the antrum formation expressing the maturing ovarian follicle also correspond to the highest degree of functional development assessed in terms of steroidogenetic activities. In addition, it is accompanied by important changes in differentiation of follicular cells. Terminal follicular development is strictly dependent on gonadotrophins [18,37].

During this developmental phase, the antral follicle, also known as tertiary graafian or preovulatory follicle, (Figure 2, sAP) the formation of the antrum soon segregates the granulosa cells with respect to the primary oocyte into two specific regions: the cumulus granulosa cell region (cumulus oophorous), in proximity to the primary oocyte, and the mural granulosa cell region lining the wall of the follicle Theca externa is a connective tissue capsule-like layer, continuous with the ovarian stroma. In contrast, the theca interna is a well-vascularized cell layer adjacent to the basal lamina of the developing follicle. It consists of elongated cells with small lipid droplets in the cytoplasm acquiring the characteristics of steroid-secreting cells. Cells of the theca interna secrete androstenedione, an androgen precursor that is transferred across the basal lamina to the granulosa cells for testosterone production. Testosterone is then converted to estradiol by aromatase. Granulosa cells lack of enzymes required for the direct production of estrogens [17,18,37]. As a result, granulosa cells cannot produce steroid precursors during folliculogenesis. The granulosa, avascular compartment, is crucial not only for the follicle endocrine function but also to define the fate of the germ cell contained in this layer. In fact, follicle is the oocyte incubator and, in particular the granulosa allow the germ cell maturation in term of endocrine and paracrine modification. FSH plays determinant roles in enhancing granulosa cell differentiation and survival. These actions are mediated or modulated in an important way by paracrine factors, particularly steroids and growth factors. The effect of FSH is potentiated by LH that stimulates steroidogenesis in theca cells and sustains terminal maturation of granulosa cells in follicles. After LH surge to ovulation follicle/s are defined as periovulatory (Figure 2, periAF). Luteinizing Hormone (LH) surge induces a progressive disorganization of follicle basal membrane caused by proteolityc enzymes activation. Moreover, it modifies the steroid enzymatic pathway transforming an estrogen secreting preovulatory follicle to a progesterone generating periovulatory follicle. Morphological changes occurring at this stage (see later) lead the follicle to ovulation where the expanded egg-cumulus complex leaving the follicle through the stigma (Figure 2, OF). The remaining cells in the follicle wall (both granulosa and thecal) fold into the empty follicle develop CL (Figure 2, CL).

The follicular growth can be divided into four distinct stages: 1) activation of primordial follicles and transition 2) from primary to preantral, 3) from preantral to tertiary/antral follicles , and 4) from dominant follicle/s to ovulation. During these latter developmental phases, ovarian follicles become an active endocrine structure and sustain the growing phase of the oocyte that acquires the ability to

undergo meiotic progression at the time of antral differentiation [18,37]. So, the mammalian ovarian folliculogenesis regulation is a complex process of cellular interactions able to create the local condition to sustain the development of a competent oocyte and the secretion of adequate steroids level in order to drive the reproductive cycle [40]. Even if the process of follicle recruitment occurs cyclically, few of the growing follicular structures reach the final stages by ovulating metaphase II oocytes (mature oocyte). Indeed, the majority of growing follicles undergo to regression, through a process known as atresia, which produces an irreversible and progressive loss of follicles with the relative germ cells [18,37]. Clearly, trophic needs change in relation of the stage reached by ovarian follicles as a consequence of different number of cells and the degree of steroid secretion involved. The blood vascular network remodeling is the physiological response of such dynamic follicles development. Therefore, follicle growth is accompanied by an increase and differentiation of blood vessels that become a limiting step in the selection of the dominant follicle and in ovulation [17,19,21,25]. Alternatively, a low development or regression of follicular blood vessel network represents an early signs of follicles atresia even if the cause effect relation is still unknown [21,39].

Understand Folliculogenesis through Angiogenesis

Primordial and primary follicles (Phase 1)

The primordial and primary follicles do not have a specific vascular network (are located in the avascular region of the ovarian cortex) and receive sufficient nutrients and oxygen by passive diffusion from blood vessels present in the surrounding stroma. However, a tiny vascularization [41] can be rarely observed around primary follicles (Figure 3) suggesting that the formation of an individual capillary network around each follicle may be required for follicles to grow. In fact, only when follicular growth is activated and a layer of thecal starts to get recruited, a follicle develops its own vascular network [42]. This is also supported by the evidence that primordial and primary follicles, despite being usually avascular, show VEGF, VEGFR1 and VEGFR2 immunopositivity in pregranulosa/granulosa cells. These data provide further support that VEGF signaling plays a role in the maintenance and/or activation of primordial follicles too [43].

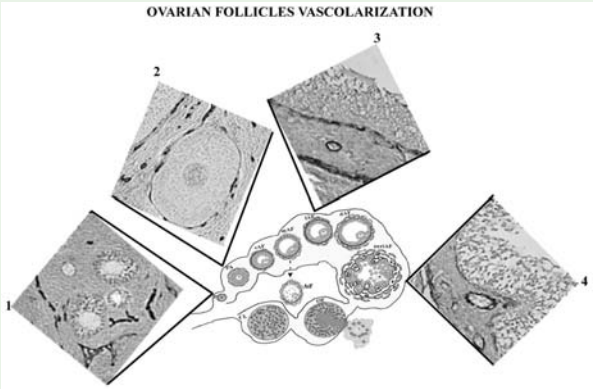

Figure 3: Representative vascular network morphology in different stage of ovarian follicles. Blood vessels were identified by von Willebrand Factor (black colour obtained by immunohistochemitry with avidina-peroxidasi - DAB nichel-method; author data). 1. Primary follicles: they do not presented an autonomous vascular supply. 2. Preantral follicle: a own vascular network is evident characterized by a ring of blood vessels near to basal membrane and spots of vessel at its periphery. 3.Antral/tertiary follicle: present two concentric blood vessel networks connected to each other by anastomotic vessels. 4. Periovulatory follicle: vascular network near to basal membrane lose their rounded appearance to the formation of infolding toward the granulosa compartment.

Preantral follicles (Phase 2)

A follicular specific blood vessel network appears for the first time in theca layer of secondary stage follicles while the granulosa layer remaining avascular (Figure 3). Follicular angiogenesis begins as spots vessels that increasing become a thin, roughly structured surrounding the follicle. Only in the late stage of Preantral Follicle (PA) the vascular architecture begins to organize into a double concentric network: a ring of blood vessels near to basal membrane and spots of vessel at their periphery. This binary vascular network continues in early follicles and ultimate only when follicular antrum is completely formed thus the follicle is both morphologically and physiologically mature.

The PA give all those structures presenting multilayered mass of granulosa cells without antrum cavity. For this reason, these typologies of ovarian follicles are generically classified in early and late when the granulosa compartment was organized in less or more than four granulosa layers respectively [18,19,37,41]. Since the late PA are follicles from four to more than ten layers of granulosa cells, appear to be very heterogeneous population. For this reason, our research group has further classified late PA into also sub-classes [44] on the basis of mean follicular diameter and number of granulosa layers evidencing as during PA growth changing follicular and vascular morphological parameters (Figure 4).

The development classification is: class 1 and 2 (early PA, less than four granulosa layers), class 3 (five-seven granulosa layers, then class 4 and 5 (middle PA, eight-thirteen granulosa layers) and class 6 (later PA, more than 14 granulosa layers). This precise morphological classification is associated in simultaneous analysis of important parameters as somatic and endothelial proliferation index, vascular

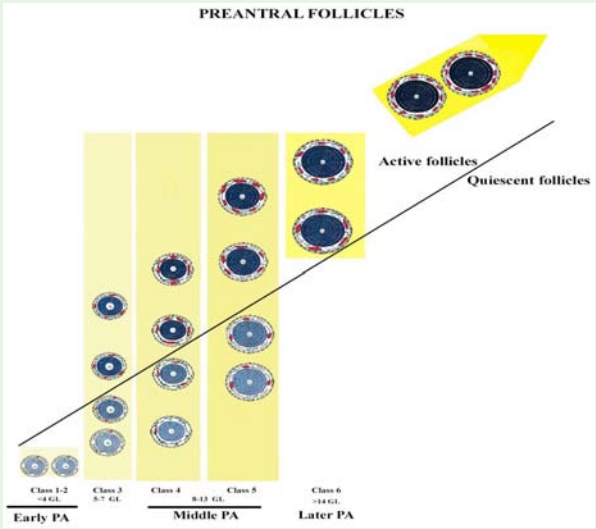

Figure 4: Schematic changes during preantral follicular development.

area and VEGF expression (VEGF mRNA and protein). Our research showing that pig early PA was also supported from blood vessels running into the surrounding stroma while an autonomous vascular supply starts to be evident in PA of class 3. This organization of an individual capillary network requires the local synthesis of VEGF able to stimulate and drive follicular vascular remodelling [16,17]. In fact, while VEGF expression was occasionally observed in the follicles of classes 1-2, on the contrary, it was always detected in PA during the middle/late stages of development. Furthermore, the analysis of VEGF expression revealed that the angiogenic stimulus progressively increases passing from class 3 to classes 4-5, reaching its highest levels during the final stage of development (class 6). Interestingly, the theca VEGF mRNA, that appeared low in preantral follicles from classes 3 to 5, becomes significantly higher in class 6, when in parallel an increasing in VA was recorded [44].

Stable and high levels of VEGF expression both in the theca and in granulosa compartments characterizes the final stage of PA development when the follicular walls are colonized by a widespread capillary network near the basal membrane and high levels of angiogenic factors may be required to maintain and stabilize these immature blood vessels [16]. Besides, high levels of VEGF appeared within the follicular structure just before the process of antrum formation (class 6). In this contest, major micro vessel permeability may be imposed by VEGF to stimulate plasma extravasations allowing the accumulation of fluids within the differentiating follicular cavity [45]. The VEGF within preantral follicles seems to be dependent also on the oocyte that is able to express and synthesize the angiogenic factor. Similar to other molecules, VEGF could be included amongst the signals involved in the bi-directional control of oocyte-follicle function [46,47], by guarantying that follicle development and synchronized with the growth of the metabolically active germinal cells.

Follicular angiogenesis in classes 3 and in middle PA resulted quite variable (es. proliferating endothelial cells ranged between 20 to 60%) suggesting that the follicular angiogenesis in these follicles could be switched-off or on position in response to specific and unknown stimuli. On the contrary, when preantral follicles reached the late stage of growth (class 6), probably under the stimulus of high and stable levels of VEGF, the process of angiogenesis remained active and the endothelial PI recorded resulted constantly high [44]. The correlation analysis amongst VEGF mRNA, VA and endothelial proliferation index *vs* the somatic proliferation index evidenced that both small and middle PA structures recognize two different sets of healthy follicles: one, with a widespread vascular network that have the pre-requisite to grow, and a second group angiogenetic inactive that may represent a set of quiescent follicles (Figure 4, active and quiescent follicles, respectively). During the preantral stage of folliculogenesis the process of angiogenesis appears crucial to sustain the increasing metabolic requirements in the growth process. In addition, endothelial cells and nascent vessels have been directly associated to the growth and differentiation of tissues/organs providing growth and/or morphogenetic signals probably VEGF mediated [45,48]. The strict correlation recorded between somatic and vascular parameters may represent an additional evidence that VEGF may be capable to stimulate directly the somatic components, over its indirect influence on follicular development through the control on vascular physiology [10,45].

The positive correlation existing between the morphological and vascular parameters disappeared in late PA, when somatic PI and the angiogenic functions (VEGF expression, VA and endothelial PI) remained on high and stable levels probably to the sensitivity for the FSH systemic stimulatory effect in this final stage of secondary follicle [44].

Furthermore, the VA increased for the first time when follicles passed from class 3 to middle class and, then during the late stage of growth to sustain the increased requirements in nutrients and gases in growing structures. The second increase may guarantee the major delivery of endocrine and paracrine molecules that metabolically change and drive the function of late preantral follicles [18,44]. Another interesting data is the association between VEGF and Notch in rat granulosa compartment during the transition from PA to antral follicles [45,48] to allows to sustain that the granulocyte compartment is incisive in the management of the various angiogenetic factors necessary for vascular growth that supports follicular development.

Observing these results, it is enticing to speculate that PA recruitable is based on vascular supply: the different vascular organization can also be defined as the ways in which the different degree of PA activation allows you to keep that essential cyclicity in the reproductive process [14,17,32].

Preovulatory follicles growth (Phase 3)

The antral follicles present two concentric blood vessel networks connected to each other by anastomotic vessels: i) an inner network made of relatively small diameter vessels, directly laying on the basal membrane, and ii) the outer one characterized by blood vessels of larger diameter (Figure 3). Depending on the diameter the antral follicles are divided into small (in pig < 3mm), medium and large (in pig >5 mm). Our experimental tests in vivo [49] showed that the gonadotropins not only determine the follicle growth, but also leads to an increase of the vascular network. In fact, the gonadotropin stimulation (FSH) able to induce follicle growth simultaneously activated ovarian VEGF production, according to follicles diameter and status. So, angiogenesis and steroidogenesis are in parallel maturating the follicle morphologically and physiologically. Paradoxically, an increase in VEGF expression does not affect all follicular sectors, but specifically the avascular granulosa layer only. Because vessels cannot cross the barrier represented by the basal membrane, it has led to the hypothesis that the granulosa create a angiogenic gradient that maintains the presence of blood vessels close to the basal membrane essentially to the sustenance of the egg cell. Follicular environment during this preovulatory phase must be supported by high oxygen tension, a particular difficult condition to obtain in large antral follicles where gases must diffuse from the vessels present in theca layers through basal membrane to reach the granulosa layer and the oocyte [48]. The persistence of this inner capillary network, whose importance has been highlighted by Moor and Seamark [50], appears to depend directly on VEGF accumulation in follicular fluid and when such a store is no longer available, as it occurs in early atretic follicles, it undergoes a marked reduction. By contrast, atresia does not affect the outer vessel network were basal thecal VEGF production was present while early atresia involves primarily the granulosa layer [49]. So, not only the vascular follicular wall network resulted incompatible with the health of follicle but started to exert its negative influence primarily on the granulosa layer started the process of apoptosis.

The medium antral follicles (in pig 4-5 mm in diameter) can classified as healthy under a stereomicroscope on the bases of the compactness and translucency of the wall. They did not revealed any morphological differences even if, on the contrary, a bimodal classification can be obtained by evaluating their intrafollicular levels of VEGF. Indeed, the biochemical analysis aimed to determine the intra-follicular VEGF content revealed that the healthy classified medium antral follicles recognized, at least, two different sub-population of high and low VEGF producers [49]. This different angiogenic response seems to suggest that follicle selection in pig is already operative within the medium antral category of follicles. The VEGF low producers pool may represent a silent sub-population that coexist with the high VEGF producers that may be able to increas the levels of secreting angiogenic factor after gonadotrophin stimulation. In fact, in pig the antral follicles with a diameter of 4-5 mm seems to be the border stage of development before activating angiogenesis leading the final phase of follicular growth (Figure 5). Likewise, negative influence on VEGF production may represent a mechanism to divert angiogenesis from resting or degenerative follicles (early atretic follicles) [18,37,49]. As far as, the physiological meaning of this modification in intrafollicular storage of VEGF is concerned indicate that only the follicles that accumulate high levels of VEGF within follicular fluid are able to increase their blood vessels network and activate in parallel steroidogenesis: the significantly wider vessel network in the wall of highly producers is certainly advantageous to follicles selection growing. Follicles producing high levels of VEGF improve their vascularization (vascular area) creating the trophic condition necessary to metabolic and oxygen contribution. In addition, blood vessels network could drive an increase in the vascular permeability thus supporting the delivery of large antral follicle such as lipid and the lipoprotein, the precursors of steroids [49].

The biological effects of VEGF are regulated by two membrane receptors, VEGFR1 and VEGFR2, and two soluble receptors, sVEGFR1 and sVEGFR2, which play an antagonistic role. Study showed as bovine non-dominant follicles maintain a greater concentration of the mRNA expression of both membrane and sVEGFR [51] to evidence as follicular dominance is related to a reduction in the mRNA expression of sVEGFR1 and sVEGFR2 , which may favour VEGF binding with VEGFR2 and, hence, improve the follicular development.

Several evidences seem to indicate the angiogenic-related mechanisms as fundamental in addressing the follicle fate through the reorganization of follicular blood vessel architecture [18,19,21,27,45,48]. Angiogenic pathways swicth-on and swicth off appears to be a key element to move follicles from a resting status towards growth and functional activation (Figure 5) or, alterntively, towards atresia (Figure 5, A) [49,51].

Preovulatory- periovulatory follicle transition (Phase 4)

The growth that takes place in the pre-ovulatory window is not well definite because after the LH surge, in very few hours, the follicle starts to ovulation. In this window, also called peri-ovulatory phase, the selected follicle/s (Figure 6, dominant follicle) modifies its hormonal asset becoming a structure able to synthesize high levels of progesterone and presenting an intense follicular reorganization finalizing to the CL differentiation, structure necessary to guarantee the pregnancy progression. In pig, follicle rupture occurs around 40-44h after an ovulatory stimulus, similar to humans with a range 34-46h [25,27]. In particular, follicular vascular remodelling is intense after the LH surge when the follicle, a limited blood supplied structure (blood vessel network was present only in theca layer), get transformed in a well vascularized organ, the CL (Figure 2, CL).

Our research group is the first that studied in vivo single follicle at known endocrine status, combining immunohistochemistry and Scanning Electron Microscopy (SEM) of vascular corrosion cast technique. To investigate the ultra structural modification occurring to vascular architecture, the corrosion cast technique seems to be an elective method [52]. Corrosion casting is an investigative method that perfused the vascular system with a low viscosity resin that polymerizes even into the microvasculature. The intraluminal space of blood vessels results full of polymer and, for this reason, resistant to the digestive treatment that degrade the nearby tissues [22,52]. An ultra structural morphological analysis of blood vessels can be then performed using a SEM thus observing at high magnification the blood vessel network structures surrounding the follicles in their three-dimensional native organization. Using SEM, the corrosion casts can provide useful data about vascular density, the presence of intussusceptive pillars and the orientation of vascular lining cells. High-quality casts can reveal endothelial nuclear imprints. Because

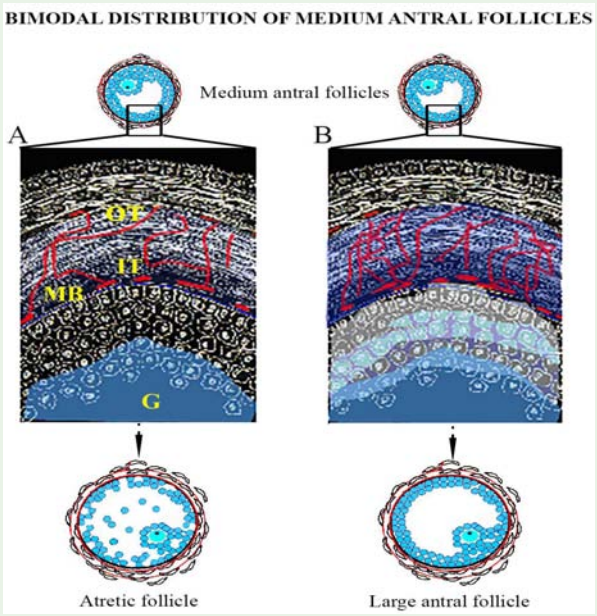

Figure 5: Representation of the bimodal distribution of medium antral follicles. Left image (A) representing the part of the antral population with a poorly developed vascular structure (low VEGF protein in antrum and in theca compartment, and low vascular area). Right image (B) representing the part of the antral population with a very developed vascular structure (high VEGF protein in antrum and in theca compartment, and high vascular area). G: Granulosa Compartment. BM: Basal Membrane. IT: Inner Theca. OT: Outer Theca. Blue colour: VEGF levels in antrum and in theca compartment. Red colour: blood vessels network. Light/dark colour: low/high presence of protein in the different follicular compartment, respectively. Angiogenesis mechanism continuously drives the reorganization of follicular blood vessel architecture that represents the preliminary condition to maintain the follicles in a resting status to drive them through growth and functional development (right follicle, B; large antral follicle) as well as to induce the definitive process of regression (left follicle, A; atretic follicle).

endothelial nuclei align with flow, these corrosion casts can provide a measure of cell density and the direction of blood flow [36,52]. In fact, with the aid of this technique different structural conformations, but also subtle ultra structural details can be described [22,36,52,53]. Furthermore, the great depth of SEM focus and the subsequent morphometry provide detailed quantitative data on blood vessels, such as diameters and branching degrees [52]. This combination results in a powerful method allow investigate the extent, the morphological and morphometrical appearance of the follicular vasculature in a stage of great changes and remarkable reproductive importance.

The obtained results allow us to hypothesize that, in terms of blood vessels remodeling, the pig periovulatory phase can be divided in two moments (Figure 6): an early and a late stage. In the early stage (from the LH surge to half of peri-ovulatory window, in pig 18h after hCG), a turn-off in follicular angiogenesis is observed. In these early peri-ovulatory follicles there is an essential vascular asset characterize by the inner vascular layer with low outer vascular network. The persistence of active inner capillary network evidenced the importance of the a correct trophic supply of oxygen and metabolites both to the avascular granulosa compartment and to the germinal cells [25,27,47,50], that in this phase is ending the first meiotic division and starting the second one. Early periovulatory follicles presenting few proliferating endothelial cells and rare angiogenic figures. This quiescence represents the starting point for the subsequent metamorphosing process, necessary to transform the periovulatory follicle into the next physiologic structure. In fact, in the late stage (the second half of the peri-ovulatory window, in pig 36h after hCG), close to ovulation, the metamorphosed follicles rapidly and strongly turns-on its angiogenic activity to sustain a successful CL formation [22,25,27]. The late periovulatory follicles showed tissues and vascular reorganization accompanied by high level of angiogenesis. The follicular morphology loses the typical roundish aspect acquiring an undulated one (Figure 2, periAF asterisk) [25,27]. This seems mainly consequence of the inner vascular plexus in folding toward the antrum, made by arterioles- or venules-like from the middle network near to basal membrane. The turning-on of angiogenesis in late periovulatory follicles is also demonstrated by a newly endothelial cell proliferation (similarly to periovulatory follicles), presence of Perivascular cells, and an increased endothelial area. Particularly, the improved vascular area is represented by sprouting and non-sprouting angiogenesis. Particularly, it is interesting to underline that the non-sprouting angiogenesis is present only in this stage and its characteristic of structures in rapid neovascularization. In contrast to sprouting, intussusceptive angiogenesis a) occurs in the virtual absence of endothelial cell proliferation, b) is achieved at low vascular permeability levels, and c) requires only 4-5 h for completion [53]. All of them are fundamental conditions, considering how quickly (about 4-8 hours) the follicle will ovulate to become a CL [22,53]. In addition, the different morphological non-sprouting angiogenesis features called pillars recorded in late periovulatory follicles suggest different outcomes: the continuous pillar formation and growth within the capillary bed leads to intussusceptive micro vascular growth represents a general and ubiquitous mechanism of capillary growth where the vascular bed can undergo to a rapid expansion without compromising vascular physiology or function [53]. Indeed, rows of round pillars that changed shape to acquire a slit-like configuration and then merged each other to form small vessels leads to intussusceptive arborisation; pillar formation occurring within small vessels can guide to a vascular branches remodeling leads to intussusceptive branching remodeling [22,53].

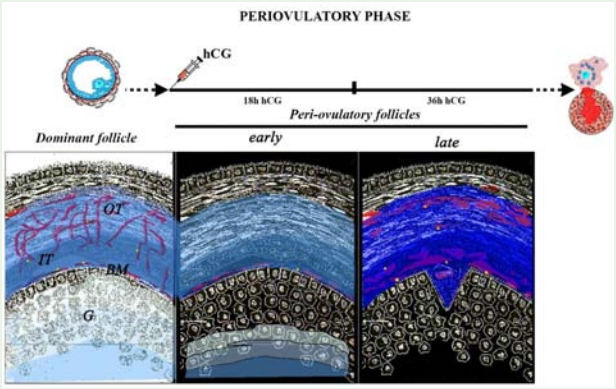

Figure 6: Representation the periovulatory phase: Dominant (pre-ovulatory) undergo LH surge becoming periovulatory follicle destined to ovulation. This follicular phase can be induced by validated hormonal treatments to injection of a single dose of human chorionic gonadotrophin (hCG, LH like activity) at 60-64 hours from eCG, injecting. Follicle were collected in a precise phase of their development: early (18h hCG) and a late periovulatory follicles (36h hCG) from hCG.

In pig [22], as well as woman [36] angiogenic figures are region-dependant distribution. Studies, in fact, revealed [52] marked regional difference in follicular blood flow with a sustained increased in the basal and lateral follicular wall and a concomitant decrease in the flow of their apical region. These change are probably required for the follicle rupture because minor blood supply at the stigma is necessary for the subsequent ovulation [21,36].

Besides, also blood vessels vascular diameter of peri-ovulatory follicles evidenced a great variability. The analysis of vascular geometric relations (in order to quantify vessel diameters and bifurcation angle) in periovulatory phase, allowed hypothesizing that the periovulatory vascular remodeling happens with the "maximum output with minimal outflow of energy" [22].

These morphological modification that occur in few hours, from LH surge to ovulation, evidence how, in term of angiogenesis, the periovulatory follicles is a highly dynamic structure. Also in this phase of follicular development VEGF is a crucial factor. It up regulated in dominant follicle/s selection process leading to ovulation [49], controls the crucial follicles transition from preovulatory to periovulatory stage to precedes ovulation [22,25]. To emphasize how the periovulatory phase vascular remodeling is closely linked a follicular compartment. In fact, our study, evidenced as the variations of the VEGF in the early periovulatory follicles were present only in granulosa layer. This layer showed a dramatic reduction in VEGF synthesis, while VEGF mRNA content remain unchanged, suggesting a post-transcriptional regulation of VEGF expression at the level of splicing. Instead, next to ovulation, theca and granulosa compartment re-started to synthesize new VEGF and to accumulate high levels of the protein within the extracellular matrix, while VEGF mRNA expression significantly increased only within the theca compartment. On the contrary, in follicular fluid the pattern of VEGF secretion progressively disappeared. This observation may

suggest a different solubility of the protein secreted by production of VEGF isoform with high molecular weight. Also, there could be a different VEGF bioavailability because it binds molecules bypassing in follicular fluid for increasing permeability characteristic of this periovulatory phase [25,27].

The turn-off follicular angiogenesis (early-periovulatory phase) can be attributed to LH surge [22,25], while the turn-on angiogenesis (late-periovulatory phase) coincides with the increase in progesterone production by the follicle. Study *in vivo* [27] have demonstrated that administration of P4 antagonist molecule, RU486, determine a different periovulatory follicular evolution: the follicles maintain the characteristic preovulatory morphology with circular aspect not in folding and immature vascular network determining an altered functionality of CL and the subsequent altered implantation and maintenance of pregnancy. Specifically, the inner vascular network of these follicles not modified maintaining the presence of blood vessels near to basal membrane, probably regularly controlled by the unchanged VEGF follicular fluid and granulosa content. On the contrary, outer network significantly decreased by absence of large blood vessels presenting in inner theca folds and the blood vessels resulting structurally immature prejudicing the physiological transition from periovulatory follicle to CL structure. Indeed, the progesterone role blocking by RU486, significantly decreased VEGF synthesis in vivo only in theca layer while it did not affect VEGF granulosa and fluid follicular content [27] evidencing how functional mechanisms are activated to maintain an adequate trophic supply of the germinal structure [22,23,41,47].

Conclusion

The knowledge of follicular angiogenesis is of fundamental importance not only to understand the mechanisms that ensure the reproductive success, but also in order to better clarify the angiogenic process in adult tissues. In this context, folliculogenesis becomes a very important experimental model for the cyclic nature of angiogenesis and because of its reproducibility by validated hormonal pharmacological treatments [19, 22-25, 27].

The prentral (PA) follicular population were classified in six different classes on the basis of number of Granulosa Layers (GL). Early PA presented spots vessels (red colour) in the theca compartment and were also supported from blood vessels running into the surrounding stroma while an autonomous vascular supply starts to be evident in preantral follicles of class 3.In class 3 and middle PA are present two sets of follicles: one pool with low-value (light blue colour) vascular parameters as endothelial proliferation index, vascular area and VEGF mRNA expression (yellow colour) and another pool with high-value (dark blue colour) parameters.

In the classes 4 and 5 low-values pool corresponds at high-value poll of classes in the previous stage. Later PA presenting only high-value of vascular parameters.

PA with low-value vascular parameters representing quiescent follicles , while PA with one high-value representing growing follicles. When the PA follicles reach the class 6 (late PA) continue their growth (PA in large dark yellow arrow) by entering the stage of antral follicles. In the classes columns the intensity of yellow indicates the increase in vascular parameter values. The different vascular organization can also be defined as the ways in which the different degree of PA activation allows you to keep that essential cyclicity in the reproductive process.

Large antral follicle (dominant/preovulatory follicle) was characterized by granulose layer (G) separated by Basal Membrane (BM) to theca compartment (inner and outer theca; IT, OT respectively). In early periovulatory follicles there is an essential vascular asset (red colour), represented by the maintenance of the inner vascular layer (red color near to BM). In late periovulatory follicles high level of angiogenesis are accompanied to tissues and vascular reorganization. In later phase the follicular morphology loses the typical roundish aspect acquiring an undulated one mainly consequence of the inner vascular plexus in folding toward the antrum. The active angiogenesis in late periovulatory follicles is also demonstrated by a newly endothelial cell proliferation (similarly to periovulatory follicles; yellow color). VEGF levels decreased in follicular fluid and granulose compartment (blue color) of early periovularory follicles (blue colour).On the contrary, in theca layer significantly increased in late periovulatory follicles (dark blue color).

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