DOTTORATO DI RICERCA IN

"BIOTECNOLOGIE CELLULARI E MOLECOLARI"

XIX CICLO

Sede: Università Degli Studi Di Bologna

Coordinatore: Chiar.mo Prof. Lanfranco Masotti

SSD MIUR: VET/02

ANGIOGENESIS AND ANGIOREGRESSION GENE EXPRESSION ANALYSES IN SWINE CORPUS LUTEUM

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Anno Accademico 2005-2006

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ABSTRACT

The corpus luteum (CL) lifespan is characterized by a rapid growth, differentiation and controlled regression of the luteal tissue, accompanied by an intense angiogenesis and angioregression. Indeed, the CL is one of the most highly vascularised tissue in the body with a proliferation rate of the endothelial cells 4- to 20-fold more intense than in some of the most malignant human tumours. This angiogenic process should be rigorously controlled to allow the repeated opportunities of fertilization. After a first period of rapid growth, the tissue becomes stably organized and prepares itself to switch to the phenotype required for its next apoptotic regression. In pregnant swine, the lifespan of the CLs must be extended to support embryonic and foetal development and vascularisation is necessary for the maintenance of luteal function. Among the molecules involved in the angiogenesis, Vascular Endothelial Growth Factor (VEGF) is the main regulator, promoting endothelial cells proliferation, differentiation and survival as well as vascular permeability and vessel lumen formation. During vascular invasion and apoptosis process, the remodelling of the extracellular matrix is essential for the correct evolution of the CL, particularly by the action of specific class of proteolytic enzymes known as matrix metalloproteinases (MMPs). Another important factor that plays a role in the processes of angiogenesis and angioregression during the CL formation and luteolysis is

the isopeptide Endothelin-1 (ET-1), which is well-known to be a potent vasoconstrictor and mitogen for endothelial cells. The goal of the present thesis was to study the role and regulation of vascularisation in an adult vascular bed. For this purpose, using a precisely controlled *in vivo* model of swine CL development and regression, we determined the levels of expression of the members of VEGF system (VEGF total and specific isoforms; VEGF receptor-1, VEGFR-1; VEGF receptor-2, VEGFR-2) and ET-1 system (ET-1; endothelin converting enzyme-1, ECE-1; endothelin receptor type A, ET-A) as well as the activity of the Ca^{++}/Mg^{++}-dependent endonucleases and gelatinases (MMP-2 and MMP-9). Three experiments were conducted to reach such objectives in CLs isolated from ovaries of cyclic, pregnant or fasted gilts.

In the Experiment I, we evaluated the influence of acute fasting on VEGF production and VEGF, VEGFR-2, ET-1, ECE-1 and ET-A mRNA expressions in CLs collected on day 6 after ovulation (midluteal phase). The results indicated a down-regulation of VEGF, VEGFR-2, ET-1 and ECE-1 mRNA expression, although no change was observed for VEGF protein. Furthermore, we observed that fasting stimulated steroidogenesis by luteal cells. On the basis of the main effects of VEGF (stimulation of vessel growth and endothelial permeability) and ET-1 (stimulation of endothelial cell proliferation and vasoconstriction, as well as VEGF stimulation), we concluded that feed restriction possibly inhibited luteal vessel development.

This could be, at least in part, compensated by a decrease of vasal tone due to a diminution of ET-1, thus ensuring an adequate blood flow and the production of steroids by the luteal cells.

In the Experiment II, we investigated the relationship between VEGF, gelatinases and Ca^{++}/Mg^{++}-dependent endonucleases activities with the functional CL stage throughout the oestrous cycle and at pregnancy. The results demonstrated differential patterns of expression of those molecules in correspondence to the different phases of the oestrous cycle. Immediately after ovulation, VEGF mRNA/protein levels and MMP-9 activity are maximal. On days 5–14 after ovulation, VEGF expression and MMP-2 and -9 activities are at basal levels, while Ca^{++}/Mg^{++}-dependent endonuclease levels increased significantly in relation to day 1. Only at luteolysis (day 17), Ca^{++}/Mg^{++}-dependent endonuclease and MMP-2 spontaneous activity increased significantly. At pregnancy, high levels of MMP-9 and VEGF were observed. These results suggested that during the very early luteal phase, high MMPs activities coupled with high VEGF levels drive the tissue to an angiogenic phenotype, allowing CL growth under LH (Luteinising Hormone) stimulus, while during the late luteal phase, low VEGF and elevate MMPs levels may play a role in the apoptotic tissue and extracellular matrix remodelling during structural luteolysis.

In the Experiment III, we described the expression patterns of all distinct VEGF isoforms throughout the oestrous cycle. Furthermore, the mRNA

expression and protein levels of both VEGF receptors were also evaluated. Four novel VEGF isoforms (VEGF144, VEGF147, VEGF182, and VEGF164b) were found for the first time in swine and the seven identified isoforms presented four different patterns of expression. All isoforms showed their highest mRNA levels in newly formed CLs (day 1), followed by a decrease during mid-late luteal phase (days 10–17), except for VEGF182, VEGF188 and VEGF144 that showed a differential regulation during late luteal phase (day 14) or at luteolysis (day 17). VEGF protein levels paralleled the most expressed and secreted VEGF120 and VEGF164 isoforms. The VEGF receptors mRNAs showed a different pattern of expression in relation to their ligands, increasing between day 1 and 3 and gradually decreasing during the mid-late luteal phase. The differential regulation of some VEGF isoforms principally during the late luteal phase and luteolysis suggested a specific role of VEGF during tissue remodelling process that occurs either for CL maintenance in case of pregnancy or for noncapillary vessel development essential for tissue removal during structural luteolysis.

In summary, our findings allow us to determine relationships among factors involved in the angiogenesis and angioregression mechanisms that take place during the formation and regression of the CL. Thus, CL provides a very interesting model for studying such factors in different fields of the basic research.

INTRODUCTION

The ovarian cycle is characterized by repeated patterns of cellular proliferation and differentiation that accompany follicular development as well as the formation and regression of the corpus luteum (CL). Ovulation is the critical event that initiates the transformation of the fluid-filled preovulatory follicle into the solid CL. After the ovulation, profound and radical changes occur in the theca and granulosa layers, which enable CL formation and maturation. During the luteal phase, the CL undergoes definitive structural and functional changes until regression and corpus albicans formation or, when pregnancy occurs, its function is maintained until term. This rapid growth and regression of ovarian tissues are accompanied by equally rapid changes in their vascular beds (Kaczmarek et al., 2005). The formation of a dense capillary network (angiogenesis) in the ovary enables the hormone-producing cells to obtain oxygen, nutrients and also precursors necessary to synthesize and release different hormones essential for maintenance of the ovarian functions (Kaczmarek et al., 2005). In the vascularisation of the CL the angiogenesis is most intense. Indeed, the CL has the highest blood supply per unit mass of any tissue in the body, eight times that of the kidney. Over 85% of proliferating cells in the CL are endothelial cells and endothelial cells make up around 50% of all cells in the mature ovarian gland (Fraser & Duncan, 2005). In contrast, in non-reproductive tissues, endothelial cells,

once differentiated, normally remain functional for 2-3 years (Fraser & Duncan, 2005).

Within two weeks, however, in the absence of pregnancy, the CL regresses, becoming an avascular remnant (Fraser & Duncan, 2005). Consequently, in each ovarian cycle there is a highly regulated angiogenesis and vascular regression. In contrast, in a fertile cycle, the lifespan of the CL, with its attendant vasculature, is prolonged. Therefore, within a short time, the CL must accommodate a period of angiogenesis followed by either controlled regression or maturation of the vasculature in the non-fertile or fertile cycle, respectively.

Angiogenesis refers to the formation of new blood vessels and is essential for normal tissue growth and development (Folkman & Klagsbrun, 1987). The angiogenic process begins with capillary sprouting and culminates in formation of a new microcirculatory bed composed of arterioles, capillaries and venules. The initiation of angiogenesis consists of at least three processes: 1) breakdown of the basement membrane of the existing vessels, 2) migration of endothelial cells from the existing vessels towards an angiogenic stimulus, and 3) proliferation of endothelial cells (Klagsbrun & D'Amore, 1991). New blood vessel development is completed by formation of capillary basal lamina and differentiation of new capillaries into arterioles and venules.

Aside from luteal tissue, wound healing and some pathological processes, including neoplasia, the vascular system in the adult is generally quiescent. Since it is almost certain that some forms of ovarian dysfunction are associated with abnormalities of the angiogenic process, the ovary therefore represents an exceptional and highly relevant tissue in which to study the physiological and pathological control of blood vessel development (Fraser & Duncan, 2005). On the contrary to that observed during pathological tissue growth (e.g. tumor growth), the angiogenic process in female reproductive tissues is limited and, therefore, must be tightly regulated (Reynolds et al., 1992).

It is established that angiogenesis is regulated by a series of complex interactions among stimulatory, modulatory and inhibitory factors. Several potential regulators of angiogenesis have been identified including acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), angiopoietins (Angs), insulin-like growth factors (IGFs), transforming growth factors (TGFs), interleukin-8 (IL-8) (Ferrara, 2000). Several laboratories over the last several years have elucidated the pivotal role of Vascular Endothelial Growth Factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara, 2004). Because of the potential benefits in being able to manipulate angiogenesis in the clinic, inhibitors are being developed to prevent their action for treatment of solid tumours, rheumatoid arthritis, diabetic retinopathy, macular degeneration and

psoriasis. Pro-angiogenic strategies are being developed to stimulate the process where it is lacking, such as in ischemic heart disease. These molecules may also be employed to elucidate the processes involved in physiological angiogenesis in the female reproductive tract, and to explore possible new approaches to the regulation of fertility, the treatment of infertility and reproductive tract pathologies (Fraser & Duncan, 2005).

1. CORPUS LUTEUM

The oestrous cycle length varies among domestic animals; in swine is around 21 days. The corpus luteum (CL) is critical for successful maintenance of entire pregnancy period (114 days) because it is the primary source of progesterone production, necessary to the survival and development of the embryos and foetus.

The CL is a temporary endocrine structure with a short lifespan in the non pregnant state. It is formed from a fluid-filled preovulatory follicle composed by an avascular granulosa layer and two vascular theca interna and externa layers. After the ovulation induced by the luteinising hormone (LH) surge, the follicle undergoes remarkable changes and is converted into CL. The granulosa cells form large luteal cells (LLC) while the theca interna cells form small luteal cells (SLC). The granulosa-lutein cells begin progesterone (P4) secretion, and newly formed luteal tissue becomes highly vascularised. The very early CL is characterised by haemorrhage into the ovulatory cavity and individual sprouting vessels can be identified within 1 to 2 days after ovulation (Geva & Jaffe, 2000). The developing CL continues to growth for approximately one-third of the duration of the ovarian cycle until reaches its maximal size. As the CL forms, progesterone production increases and becomes maximal during the midluteal period. The mature CL is characterized by the typical lipid pink colour, reflecting its endocrine function. In conjunction with these cellular changes, there are significant changes in

the extracellular matrix (ECM) of the forming CL. For example, the type of collagen present in the basement membrane of the follicle (type IV) is replaced by a fibrillar (type I) collagen that comprises a major component of the CL (Luck & Zhao, 1993). This collagen component comprises up to one sixth of the luteal weight in the mature bovine CL (Luck & Zhao, 1993), reflecting the importance of the ECM in the overall structure of the CL.

By day 14-16 of the luteal phase, in case of pregnancy does not occur, the uterine Prostaglandin F2α (PGF2α) promotes the regression of the fully developed CL. The luteal regression includes two phases. The first phase is a functional luteolysis that is characterised by a rapid decline in progesterone production and secretion. The second phase is a slower, prolonged structural luteolysis of the CL. This structural luteolysis has been postulated to occur in part by an apoptotic mechanism (Paavola, 1979; Bacci et al., 1996; Forni et al., 2003) and in part by proteolysis and destruction of the luteal ECM by metalloproteinases (MMPs) (Kiya et al., 1999). This destruction of the luteal extracellular scaffolding may involve invading macrophages, which increase in number in the involuting CL and produce MMPs (Brannstrom & Friden, 1997).

The switch between growth and regression is mainly regulated by LH (luteotrophyn) and PGF2α (luteolysin) and their receptors balance as well as by cytokines, growth factors, apoptosis/oncogenes related factors and plasminogen activator/ MMP activators and inhibitors (Neuvians et al., 2004;

Diaz & Wiltbank, 2005). In fact, to allow repeated opportunities of fertilization, the duration of CL lifespan is rigorously programmed. Five to ten grams of luteal tissue grow and disappear at each ovarian cycle.

Figure 1 demonstrates the life history of a follicle destined to ovulate. The ovum of a mature follicle is surrounded by the cumulus oophorus and is situated within a fluid-filled cavity (antrum). Tissue degeneration within the follicular-ovarian surface contact leads to stigma formation and rupture. Upon ovulation the follicle is transformed into transient progesterone-producing CL.

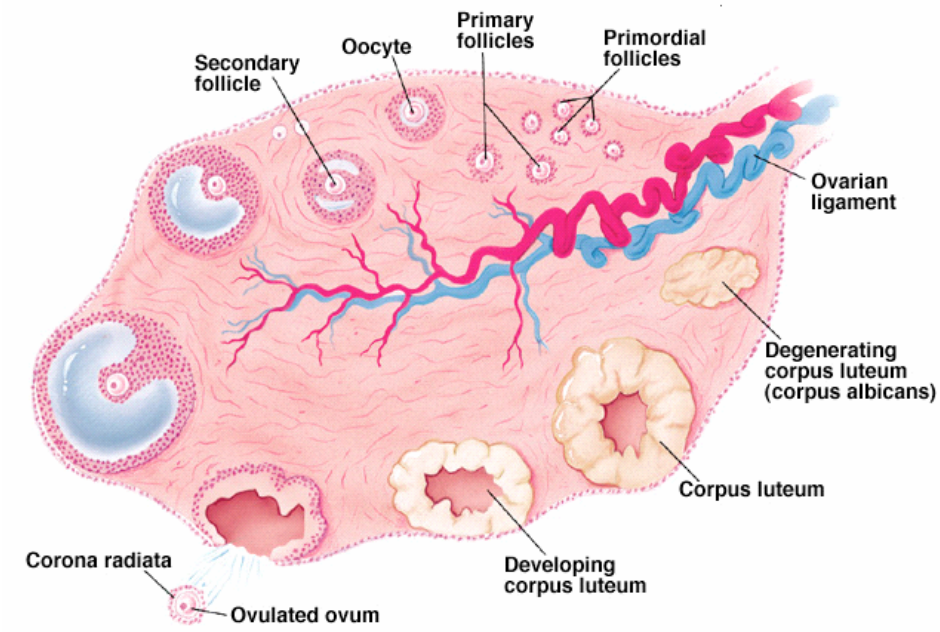

Fig. 1. Schematic ovary depicting the life history of a follicle destined to ovulate (counter-clockwise beginning from primordial follicles).

1.1 Role of the vascularisation during CL lifespan

Follicular development and atresia, ovulation, formation and regression of CL are processes characterised by dramatic tissue remodelling and angiogenesis.

The angiogenic process in the developing CL begins with dissolution of the basal membrane between granulosa and theca interna layers. Following this, the expansion of theca capillaries is initiated by sprouting into the avascular granulosa layer to form a dense network of capillaries surrounding the luteinising granulosa cells. The high density of capillaries is demonstrated by the fact that microvascular endothelial cells are the most abundant cell type in the CL, with each luteal cell in apparent contact with at least one neighbouring capillary (Gaytan et al., 1999). In fact, 85% of the dividing cells in the primate CL during the early luteal phase are endothelial cells and the rate of endothelial cells proliferation is 4- to 20-fold more intense than in some of the most malignant human tumours (Christenson & Stouffer, 1996). This intense blood vessel formation in the newly forming CL, often compared with angiogenesis in rapidly growing and aggressive tumours, enables mature CL to receive one of the greatest rates of blood flow of any tissue in the body (Kaczmarek et al., 2005).

Maturation of the newly formed vascular bed is characterised by recruitment of mural cells. Endothelial cells of arterioles and venules recruit smooth muscle cells to stabilise the vessels and control their vasotonia; whereas

endothelial cells in microvessels recruit pericytes to ensheath the capillaries and influence vessel function (Carmeliet, 2000). Some reports have indicated that up to 60% of microvessels in the mature CL contain pericytes and that, as well as for endothelial cells, pericytes migrate into the granulosa from the thecal capillaries and proliferate in the developing CL (Goede et al., 1998; Reynolds & Redmer, 1999). These data would suggest that microvessels maturation in the CL falls somewhere between that observed in most adult tissues (100% pericytes) and human tumours (15-40%). This is an important issue because the degree of maturation influences vessel integrity, function, and sensitivity to angiogenic or anti-angiogenic factors, and ultimately vessel degeneration.

The start of blood vessels regression (angioregression) during luteolysis varies among species, but, in several of them, this occurs at the beginning of the CL regression (Stouffer et al., 2001). This decline may be related to morphologic changes in endothelial cells including cell death (via apoptosis) or loss by shedding into the circulation. The degeneration of the microvasculature ensures loss of luteal structure-function and restores the ovaries to a state in which the next dominant follicle could develop. While portions of the luteal capillary beds degenerate during early luteolysis, some of the large microvessels are maintained (and arteriolisation may occur), perhaps to assist the resorption of luteal mass, and ultimately vascular shut-off of the luteal residue, i.e., corpus albicans (Stouffer et al., 2001).

1.2 Angiogenic and angiolytic factors throughout the CL lifespan

Maintenance of luteal function has been demonstrated to depend to a great extent on angiogenesis, while luteal regression is associated with its inhibition (Fraser et al., 2000).

Vascular Endothelial Growth Factor (VEGF) system

VEGF is the main angiogenic factor; promotes proliferation, migration and survival of endothelial cells as well as stimulates microvascular leakage which allows tissue infiltration of plasma proteins (hence the alternative name vascular permeability factor - VPF; Connolly et al., 1989; Keck et al., 1989).

The importance of VEGF throughout CL lifespan has been demonstrated in several species. In the newly forming CL, VEGF mRNA and protein expression are observed both in the granulosa- and theca-derived luteal cells. In several species, however, expression levels were higher in granulosa-derived than in theca-derived luteal cells (Kamat et al., 1995; Endo et al., 2001; Boonyaprakob et al., 2003). Highly expressed VEGF in granulosa-derived luteal cells may act as a chemoattractant for endothelial cells in order to initiate the invasion of avascular granulosa layer, establishing an extensive capillary network that nourishes the developing CL and maintains the luteal function throughout its' lifespan (Mattioli et al., 2001).

The duration of the intense angiogenic phase in CL varies among species, but appears to be completed by day 8 of the luteal phase in caprine (Kawate

et al., 2003) and in bovine (Acosta et al., 2003), and by day 6 in primates when capillaries surround most luteal cells and capillary dilation is evident (Lei et al., 1991; Christenson & Stouffer, 1996). In general, VEGF expression is higher during this period of intense angiogenesis (Redmer et al., 1996; Berisha et al., 2000; Ribeiro et al., 2007). However, in human (Otani et al., 1999; Endo et al., 2001) and equine CL (Al-zi'abi et al., 2003), high levels of VEGF mRNA and protein are found to be still maintained in the mid-luteal phase. Furthermore, in the macaque (Hazzard et al., 2000) and caprine CLs (Kawate et al., 2003), VEGF mRNA expression was even higher during the mid-luteal than early luteal phase. This continued expression of VEGF beyond the period of intense angiogenesis may serve as a survival factor for the newly-formed endothelial cell and as a permeability factor for increasing the uptake of cholesterol to luteal cells (Otani et al., 1999). In fact, administration of anti-VEGF antibody during the mid-luteal phase suppressed the production of progesterone in the marmoset CL, supporting the concepts that beside mitogenic activity, VEGF is also a modulator of the vascular permeability in the CL (Dickson et al., 2001). The decrease in permeability of capillaries can deprive the luteal cells of both the necessary precursors for P4 production and the efficient spreading of their products into the bloodstream (Dickson et al., 2001).

In regressing CL, VEGF expression decreases along with gradual dissolution of small blood vessels and decline of blood flow. However, VEGF expression

during luteolysis of the CL is regulated differentially depending on species-specific mechanisms involved in luteal regression. In domestic animals such as sheep, cow, pig and horse, the decline of VEGF expression in the regressing CL seems to be associated with PGF2α secretion (Al-zi'abi et al., 2003; Acosta & Miyamoto, 2004; Neuvians et al., 2004), suggesting that the cessation of VEGF support for the CL may play a role during structural luteolysis.

Many factors may regulate VEGF production and release, such as gonadotropins, cytokines and growth factors. Besides these factors, VEGF expression has also been shown to be influenced by nutrients, such as fructose (Feletou et al., 2003) and taurine (Boujendar et al., 2003). Some reports have been demonstrated the effect of feed restriction on reproductive hormone in ewes (Kiyma et al., 2004), on hormonal control of reproduction in pig (Prunier & Quesnel, 2000; Almeida et al., 2000), on follicular development and luteal function in cows (Burns et al., 1997), and on VEGF production by growing pig ovarian follicles (Galeati et al., 2003). We recently demonstrated that fasting significantly reduced the VEGF mRNA expression in newly formed pig CL after 3 days of food deprivation (Galeati et al., 2005).

Endothelin-1 (ET-1) system

Another factor, ET-1, has been demonstrated to stimulate VEGF and FGF-2 expression (Peifley & Winkles, 1998; Davis et al., 2003). ET-1, the only isopeptide synthesized and secreted by vascular endothelial cells (Levin, 1995) and initially defined as a potent vasoconstrictor, is synthesised as a prepropeptide, which is cleaved by endopeptidases to big ET-1 (proET-1). A zinc metallopeptidase, endothelin-converting enzyme (ECE-1), converts big ET-1 into an active ET-1, which acts through two receptors ET-A and ET-B. The ET-1 system has been shown to regulate oocyte maturation, ovulation and CL functions (Flores, 2000; Berisha et al., 2002) as well as to inhibit progesterone production in luteal cells after administration of PGF2α (Girsh et al., 1996; Miyamoto et al., 1997).

Angiopoietins (Ang) system

Co-ordination of blood vessel formation, maintenance, stabilisation and regression also involve other factors other than VEGF (Fraser & Duncan, 2005). These include the angiopoietins Ang1 and Ang2, which act via the tyrosine kinase receptor, Tie2. The angiopoietins are of particular interest because they influence the stabilisation of newly formed vasculature, as well as the destabilisation of existing vascular network (Thurston, 2003). Specifically, Ang1 activation of Tie2 enhances the maturation and stabilisation of newly formed blood vessels. Ang2 also binds to Tie2, but can

act as an endogenous antagonist, blocking Ang1 mediated receptor phosphorylation. In the presence of VEGF, increased autocrine expression of Ang2 by the vascular endothelium is associated with angiogenesis, while in the absence of VEGF or other pro-angiogenic factors, its expression is associated with degenerative changes in the vasculature (Maisonpierre et al., 1997). At luteolysis, VEGF mRNA decreased and the Ang2:Ang1 ratio increased. It was proposed that this change would destabilise vessels in the CL and lead to vascular regression via apoptosis (Maisonpierre et al., 1997).

Endocrine gland VEGF (EG-VEGF)

Another regulator of ovarian angiogenesis, EG-VEGF, was identified in human ovary and has been proposed as a steroidogenic endocrine gland specific angiogenic regulator (LeCouter et al., 2001). Although highest levels were found in the ovary, testis and adrenal, EG-VEGF is also present at lower levels in other tissues such as the small intestine, where its action appears to be in regulating contraction of gastrointestinal smooth muscle (Li et al., 2001). EG-VEGF mRNA has been reported by in situ hybridisation in the human CL (Ferrara et al., 2003a). However, the regulation of this factor throughout CL lifespan in domestic animals needs to be elucidated.

Matrix Metalloproteinases (MMP) system

As mentioned above, the CL formation, maintenance and regression are cyclic events that depend upon extensive luteal tissue and ECM remodelling. Components of the ECM modulate cellular processes (including gene expression, cellular proliferation, migration and differentiation; and apoptosis) via cellular surface receptors and serve as a reservoir for a number of biologically active factors. Consequently, the controlled degradation of ECM by the action of a specific class of proteolytic enzymes known as matrix metalloproteinases (MMPs) and their associated endogenous inhibitors (TIMPs: Tissue Inhibitors of Metalloproteinases) may be essential for preserving a microenvironment appropriate to luteal function (Smith et al., 2002).

The role of the MMPs and their inhibitors throughout the oestrous cycle and pregnant CLs has been described in several species, such as porcine (Pitzel et al., 2000; Ribeiro et al., 2006), bovine (Goldberg et al., 1996; Zhang et al., 2003), ovine (Ricke et al., 2002; Towle et al., 2002), mouse (Waterhouse et al., 1993; Hagglund et al., 1999; Liu et al., 2003), rat (Nothnick et al., 1995; Liu et al., 1999; Li et al., 2002), primates (Young et al., 2002; Chen et al., 2006), and human (Duncan et al., 1996; Duncan et al., 1998).

Although there are species differences in the luteal expression patterns of the various MMPs and TIMPs, it is readily apparent that the changes, which occur in the expression of the MMP system, parallel the luteal formation,

maintenance and regression. A general model for the MMP system in luteal formation is one in which MMPs and TIMPs are elevated during the period of extensive tissue and ECM remodelling that occurs as the postovulatory follicle is transformed into CL. After the CL is fully formed, steroidogenesis is maximal during the midluteal period, and MMPs/TIMPs expression and activity are at basal levels. With the onset of structural regression, the MMPs are again called into action for the ECM remodelling and removal of the regressing CL. These dynamic changes must be accompanied by a delicate balance between the activity of the MMPs and their inhibitors to allow precise remodelling of the ECM while at the same time limiting the type of matrix to be remodelled, the site-specific location, and the extent of proteolytic degradation (Curry & Osteen, 2003).

Currently, the MMP family encompasses at least 25 related proteolytic enzymes that can be subdivided into collagenases, gelatinases, stromelysins, membrane type MMPs (MT-MMPs) and others (Table 1; Smith et al., 2002; Curry & Osteen, 2003). These proteinases exhibit numerous structural and functional similarities (Fig. 2). Common features of the MMP family include: 1) the presence of zinc in the active site of the catalytic domain, 2) synthesis of the MMPs as preproenzymes that are secreted in an inactive form, 3) activation of the latent zymogen in the extracellular space, 4) recognition and cleavage of the ECM by the catalytic domain of the enzyme,

and 5) inhibition of enzymes action by both serum-borne inhibitors (macroglobulins) and TIMPs in the extracellular environment.

Table 1. Summary of the nomenclature and actions of MMPs.

Family	Enzyme	MMP Number	Matrix substrates
Collagenases	Interstitial collagenase, Neutrophil	MMP-1	Collagens I, II, III, VII, X, IGFBP-3
	Collagenase	MMP-8	Collagens I, II, III
	Collagenase-3	MMP-13	Collagens I, II, III
	Collagenase-4 (Xenopus)	MMP-18	Collagen I
Gelatinases	Gelatinase A	MMP-2	Gelatins, collagens IV, V, VII, X and XI, fibronectin, laminin, plasminogen
	Gelatinase B	MMP-9	Gelatins, collagens IV, V and XIV, fibronectin, plasminogen
Stromelysins	Stromelysin-1	MMP-3	Gelatins, fibronectin, laminin, collagens III, IV, IX and X, vitronectin, proteoglycan, ECAD, IGFBP-3, activates proMMP-1
	Stromelysin-2	MMP-10	Fibronectin, collagen IV
	Stromelysin-3	MMP-11	Fibronectin, laminin, collagen IV, gelatine
	Enamelysin	MMP-20	Amelogenin
Membrane-type MMPs	MT1-MMP	MMP-14	Collagens I, II and III, fibronectin, laminin, vitronectin: activates proMMP-2 and proMMP-13
	MT2-MMP	MMP-15	Fibronectin, gelatin, laminin: activates proMMP-2, collagen I and III, nidogen, tenascin, aggrecan, perclean
	MT3-MMP	MMP-16	Collagen III, fibronectin, gelatin, activates proMMP-2
	MT4-MMP	MMP-17	Gelatin, proMMP-2, proTACE-substrate
	MT5-MMP	MMP-24	ProMMP-2
	MT6-MMP (Leukolysin)	MMP-25	Collagen IV, gelatin, fibronectin, fibrin
Others	Matrilysin	MMP-7	Fibronectin, gelatin, laminin, collagen IV, plasminogen, proteoglycan
	Metalloelastase	MMP-12	Elastin, plasminogen
	RASI-1	MMP-19	Fibronectin, gelatin, collagen I, IV
	Xenopus MMP (xMMP)	MMP-21	Not known
	Chicken MMP (cMMP)	MMP-22	Casein
	Cysteine array MMP (ca-MMP)	MMP-23	Not known
	Matrilysin-2 (Endmetase)	MMP-26	Fibrinogen, fibronectin, vitronectin, gelatin, collagen I and IV, proMMP-9
	Human paralog of MMP-22	MMP-27	Not known
	Epilysin	MMP-28	Casein

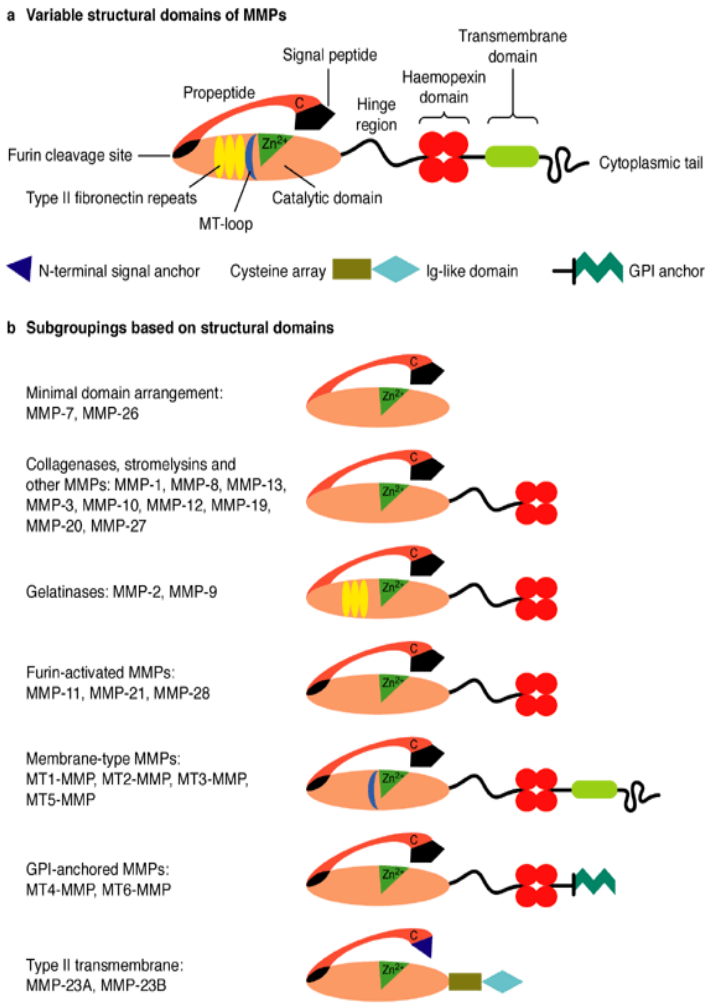

Fig. 2. Schematic representation of the MMP family (Derived from Lafleur et al., 2003).

The regulation of MMP synthesis can be both tissue- and MMP-specific (Fig 3). Subsequent to MMP synthesis, activation of the pro-MMPs in the extracellular space occurs via proteinases, including other MMPs, serine proteinases such as the plasminogen activator/plasmin system, cysteine proteinases, as well as by nonproteolytic agents such as reactive oxygen species (ROS), sulfhydryl reactive agents, and denaturants. However, MMPs

that are resistant to serine proteinase activation, such as MMP-2, are activated via an alternative mechanism involving the MT-MMPs and TIMP-2. This mechanism of MMP-2 activation may also occur for the activation of other MMPs, such as MMP-13, although other inhibitors may be involved (Knauper et al., 1996; Murphy et al., 1999). Although most MMPs are activated following secretion, stromelysin-3, MMP-23 and all MT-MMPs can be activated intracellularly by furin, a serine protease or by other unknown activators (Smith et al., 2002).

The inhibition of MMPs activity in the extracellular environment is principally controlled by TIMPS that are highly abundant in reproductive tissues, locally produced, hormonally regulated, and has been hypothesized to coordinate numerous ovarian and uterine processes including matrix turnover, cell growth, and steroidogenesis.

Currently, four different TIMPs have been identified. TIMP-1 has been shown to be a secreted glycoprotein (29 kDa) that binds to and inhibits the active form of MMPs on a 1:1 stoichiometric basis. Since the initial discovery of TIMP-1, other TIMPs have been identified including TIMP-2, TIMP-3 (which is glycosylated), and TIMP-4. TIMP-2 is differentially regulated from TIMP-1 and has been proposed to act selectively on different MMPs (Stetler-Stevenson et al., 1989). For example, TIMP-2 has a high affinity for MMP-2, whereas TIMP-1 preferentially binds to MMP-9. TIMP-3 also exhibits differential preference for the MMPs, having a high affinity for MMP-9 and

being able to inhibit MT1-MMP, unlike TIMP-1, which cannot act on MT1-MMP. However, unlike TIMP-1 or TIMP-2, TIMP-3 is secreted and then bound to the ECM. By residing in the ECM as opposed to being free in the extracellular fluid, TIMP-3 has been suggested to act as an additional regulatory stop point by acting at the site of MMP action (Leco et al., 1994). TIMP-4 has recently been cloned and shown to be present in reproductive tissues (Simpson et al., 2003). TIMP-4 has been shown to act on numerous MMPs, and has been postulated that this TIMP is a good inhibitor for all classes of MMPs without remarkable preference for special MMPs.

Several evidences indicate that TIMPs, in addition to their classical ability to regulate MMP action, may act as autocrine/paracrine factors in reproductive processes involving cellular proliferation, differentiation, and neovascularization (Fassina et al., 2000). Some reports have been demonstrated that TIMPs stimulate cellular proliferation of endothelial cells and fibroblasts (Hayakawa et al., 1992), inhibit angiogenesis (Moses & Langer, 1991; Johnson et al., 1994), promote embryo growth and development (Satoh et al., 1994) and stimulate steroidogenesis (Boujrad et al., 1995). Finally, there are correlative reports of TIMP-3 mRNA expression associated with healthy follicular development in the rat (Simpson et al., 2001).

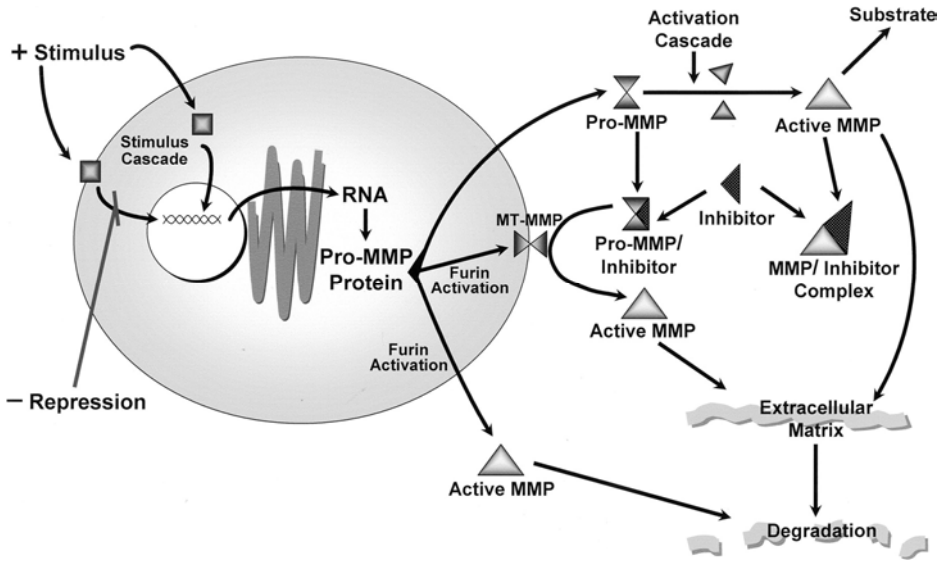

Fig 3. Schematic representation of the MMP system regulation. The MMP are generally translated into a latent or pro-form of the MMP protein. Certain MMPs, such as the MT-MMPs and MMP-11, are activated intracellularly via a furin proteolytic processing pathway and are secreted or inserted into the membrane in an active form. The majority of the pro-MMPs, however, are secreted in a latent form that requires activation in the extracellular space by other proteinases. This activation cascade can occur via other MMPs, such as the MT-MMPs, serine proteinases such as the plasmin-plasminogen activator pathway, or other proteinases. Once the MMP is active, it can cleave the ECM, resulting in focal degradation. Alternatively, the active MMP can be bound by MMP inhibitors, such as TIMPS, resulting in an inhibition of MMP action (Derived from Curry & Osteen, 2003).

1.3 Vascular bed regulation at pregnancy

When pregnancy is recognized, the lifespan of the CL must be extended to support embryonic and foetal development. In the CL of pregnancy, vascularisation seems to be necessary for the enhancement of luteal function (Fraser & Wulff, 2003). However, it is not clear whether this involves further

angiogenesis. No increase in CL angiogenesis was observed during early pregnancy in marmosets (Rowe et al., 2002), sheep (Jablonka-Shariff et al., 1993) and hCG-induced pregnancy in rhesus monkey and human (Christenson & Stouffer, 1996; Rodger et al., 1997), suggesting that the CL vascular bed required at pregnancy is already established during the luteal phase. In contrast, intensive proliferation of endothelial cells was observed in the rodent CL during early pregnancy (Tamura & Greenwald, 1987) and in rescued human CL (Wulff et al., 2001), indicating that a second wave of angiogenesis may take place in the CL of pregnancy. Certainly, the survival of the CL during pregnancy requires a stable vasculature with increased requirement of pericytes and prolonged endothelial cell survival in addition to prolongation of the lifespan of hormone-producing cells (Jablonka-Shariff et al., 1993; Rodger et al., 1997; Wulff et al., 2001).

The molecular mechanisms that regulate the angiogenic process in pregnant CLs are not fully elucidated, but clearly involve the expression of the VEGF and its receptors. The expression of VEGF mRNA in the CL of pregnancy was found to be higher than that during the miluteal phase in bovine (Berisha et al., 2000), swine (Ribeiro et al., 2006) and in women (Sugino et al., 2000). Moreover, VEGF mRNA and protein is up-regulated in the human CL during stimulated pregnancy (Wulff et al., 2000; Wulff et al., 2001). The increased expression of VEGF suggests a role in either angiogenesis or endothelial cell survival. On the contrary, no differences in mRNA expression of VEGF and

its receptors between CL of pregnancy and preregressing CL (late luteal phase) in marmosets were found, suggesting that the fully-formed, cyclic CL has already established a mature vascular system and the molecular capacity to synthesize VEGF and its receptors (Rowe et al., 2002).

Furthermore, Pauli et al., (2005) reported that the administration of anti-VEGFR-2 antibodies disrupted maternal ovarian functions in pregnant rats through elimination of pre-existing luteal blood vessels and caused abnormalities during the embryonic development probably due to the cessation of progesterone support. Therefore, during pregnancy, the activated VEGF/VEGFR-2 pathway is of critical importance for the survival and maintenance of luteal vasculature in the ovary.

2. ANGIOGENESIS AND ANGIOREGRESSION MECHANISMS

2.1 Angiogenesis

Blood vessels differentiate from endothelial precursors (angioblasts) by a process called vasculogenesis during embryo development. In adults, however, further vessel development from pre-existing vasculature occurs by intussusception or sprouting by a process called angiogenesis. This process is limited in adults, except for wound healing, several pathological conditions (including neoplasia), formation and growth of bone as well as in female reproductive cycle (Fraser & Duncan, 2005). The process and molecular basis of angiogenesis are detailed bellow and schematized in figure 4.

Vascular permeability, endothelial cell proliferation and migration

Angiogenesis initiates with vasodilation, a process involving nitric oxide (NO). Vascular permeability increases in response to VEGF, thereby allowing extravasation of plasma proteins that lay down a provisional scaffold for migrating endothelial cells. Ang1, a ligand of the endothelial Tie2 receptor, is a natural inhibitor of vascular permeability, tightening pre-existing vessels.

Endothelial cells need to loosen interendothelial cell contacts and relieve periendothelial cell support to migrate from their resident site; so, mature vessels need to become destabilized. Ang2, an inhibitor of Tie2 signalling, may be involved in detaching smooth muscle cells from the extracellular matrix (Maisonpierre et al., 1997; Gale & Yancopoulos, 1999). Proteinases of the plasminogen activator, MMP, chymase or heparanase families influence

angiogenesis by degrading basal membrane and extracellular matrix molecules as well as by activating or liberating growth factors (bFGF, VEGF and IGF-1) sequestered within the extracellular matrix (Coussens et al., 1999). MMP-3, MMP-7 and MMP-9 affect angiogenesis in neonatal bones (Vu et al., 1998) and tumours (Bajou et al., 1998), whereas TIMP-1, TIMP-3 or a naturally occurring fragment of MMP-2, by preventing binding of MMP-2 to $\alpha_v\beta_3$ integrin, may limit the extent of migration and invasion of endothelial cells during tumour angiogenesis (Brooks et al., 1998).

Once the path has been cleared, proliferating endothelial cells can migrate to distant sites. Ang1 phosphorylates tyrosine in Tie2 and is chemotactic for endothelial cells, induces sprouting and potentiates VEGF, but fails to induce endothelial proliferation (Suri et al., 1998). In contrast to VEGF, Ang1 itself does not initiate endothelial network organization, but stabilizes networks initiated by VEGF, presumably by stimulating the interaction between endothelial and periendothelial cells. This indicates that Ang1 may act at later stages than VEGF (Gale & Yancopoulos, 1999). Ang2, at least in the presence of VEGF, is also angiogenic. VEGF and its receptor VEGFR-2 affect physiological and pathological angiogenesis and are therapeutic targets, although much remains to be learned about the involvement of the distinct VEGF isoforms or of the heterodimers of VEGF family members.

Members of the fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) family are redundant during normal development; both affect

angiogenesis probably by recruiting mesenchymal or inflammatory cells (Zhou et al., 1998; Lindahl et al., 1998). TGF-β1 and tumour necrosis factor (TNF)-α can either stimulate or inhibit endothelial growth, and may be involved in tumour dormancy (Gohongi et al., 1999). Molecules involved in cell-cell or cell-matrix interactions, such as $\alpha_v\beta_3$ integrin, which localizes MMP-2 at the endothelial cell surface, mediate endothelial spreading, explaining why $\alpha_v\beta_3$ antagonists inhibit angiogenesis (Varner et al., 1995). Nitric oxide, a downstream effector of VEGF, TGFβ-1 and other angiogenic factors, is not essential for embryonic vascular development, but affects pathological angiogenesis and improves the re-endothelialisation of naked vessels (Murohara et al., 1998). A growing list of molecules is being discovered that are angiogenic after exogenous administration, but which molecules function as endogenous angiogenic factor remain undetermined (Carmeliet, 2000).

Angiogenic sprouting is controlled by a balance of activators and inhibitors. Angiogenesis inhibitors, suppressing the proliferation or migration of endothelial cells, include angiostatin (an internal fragment of plasminogen; O'Reilly et al., 1994), endostatin (a fragment of collagen XVIII; O'Reilly et al., 1997), antithrombin III, interferon-β (IFN-β), leukaemia inhibitory factor (LIF) and platelet factor 4 (PF4) (Carmeliet, 2000).

Lumen formation

Migrating endothelial cells often assemble as solid cords that subsequently acquire a lumen. Intercalation or thinning of endothelial cells and fusion of pre-existing vessels allow vessels to increase their diameter and length (Carmeliet, 2000). Specific VEGF isoforms play distinct roles in this moment, i.e. VEGF189 isoform decreases luminal diameter, whereas VEGF121, VEGF165 and their receptors increase lumen formation, in addition to increasing vessel length. Ang1 in combination with VEGF also increases luminal diameter (Suri et al., 1998). Other molecules affecting lumen formation are integrins ($\alpha_v\beta_3$ or α_5) and the myocyte enhancer binding factor 2C (MEF2C) transcription factor. Excessive proteolysis may lead to cystic assembly of endothelial cells and prevent tube formation. Thrombospondin (TSP)-1 is an endogenous inhibitor of lumen formation (Carmeliet, 2000).

Endothelial survival and differentiation

Once assembled in new vessels, endothelial cells become quiescent and survive for years (Carmeliet et al., 1999). Endothelial apoptosis is a natural mechanism of vessel regression in the retina and ovary after birth and a frequent (therapeutic) inhibitor of angiogenesis. Endothelial apoptosis is induced through deprivation of nutrients or survival signals when the lumen is obstructed by spasms, thrombi or the shedding of dead endothelial cells, or when a change in the angiogenic gene profile occurs (Jain et al., 1998; Gerber et al., 1999). The survival function of VEGF depends on an

interaction between VEGFR2, β-catenin and vascular endothelial (VE)-cadherin (Carmeliet et al., 1999). Ang1 also promotes, whereas Ang2 suppresses, endothelial survival, at least in the absence of angiogenic stimuli (Gale & Yancopoulos, 1999; Holash et al., 1999). Haemodynamic forces are essential for vascular maintenance, as physiological shear stress reduces endothelial turnover and abrogates TNF-α mediated endothelial apoptosis. Endothelial apoptosis can be also induced by NO, ROS, angiostatin, TSP-1, the metallospondin METH-1, IFN-γ, tissue factor pathway inhibitor (TFPI) and vascular endothelial growth inhibitor (VEGI) (Carmeliet, 2000).

To accommodate local physiological requirements, endothelial cells acquire specialized characteristics that are determined in part by the host tissue (Risau, 1998). For example, an interaction of astroglial cells expressing glial fibrillary acidic protein, pericytes and normal angiotensinogen levels is essential for development of the blood-brain barrier (Lindahl et al., 1998). In contrast, endothelial cells in endocrine glands, involved in the exchange of particles, become discontinuous and fenestrated; this is possibly mediated by interactions between VEGF and the extracellular matrix.

Remodelling, vessel maturation and stabilisation

The remodelling of the endothelial network involves the pruning of capillary-like vessels with uniform size, and irregular organization into a structured network of branching vessels. Intussusception, resulting in replacement of vessels by extracellular matrix, underlies pruning and branching. Gene

inactivation studies indicate a morphogenetic function for VEGF and VEGFR-3 (Carmeliet et al., 1996; Ferrara et al., 1996; Dumont et al., 1998), the endothelial receptor Tie1 (Patan, 1998), integrin, fibronectin and others (Carmeliet, 2000).

The maturation of the neovascular bed is characterised by a recruitment of mural cells. Endothelial cells from large vessels recruit smooth muscle cells whereas endothelial cells in microvessels recruit pericytes. The mural cells stabilise nascent vessels by inhibiting endothelial proliferation and migration, and by stimulating production of a new basal membrane and extracellular matrix. They thereby provide haemostatic control and protect new endothelium-lined vessels against rupture or regression. Indeed, vessels regress more easily as long as they are not covered by smooth muscle cells (Benjamin et al., 1998). PDGF-B secreted by the endothelial cells recruits pericytes and smooth muscle cells (Lindahl et al., 1998). VEGF also promotes mural cell accumulation, presumably through the release of PDGF-B or binding to VEGF receptors (Benjamin et al., 1998). Ang1, Tie2 and Ephrin-B2 affect growth and maintenance of blood vessels by stabilizing the interaction of mural cells with the nascent endothelial channel, and by inducing branching and remodelling (Suri et al., 1996; Maisonpierre et al., 1997; Gale & Yancopoulos, 1999). TGF-β1, TGF-βR2 (Transforming Growth Factor-β Receptor II), endoglin (an endothelial TGF-β binding protein) and Smad5 (a downstream TGF-β signal) are involved in vessel maturation in a

pleiotropic manner: they inhibit endothelial cell proliferation and migration, induce smooth muscle differentiation and stimulate extracellular matrix production (Dickson et al., 1995; Li et al., 1999).

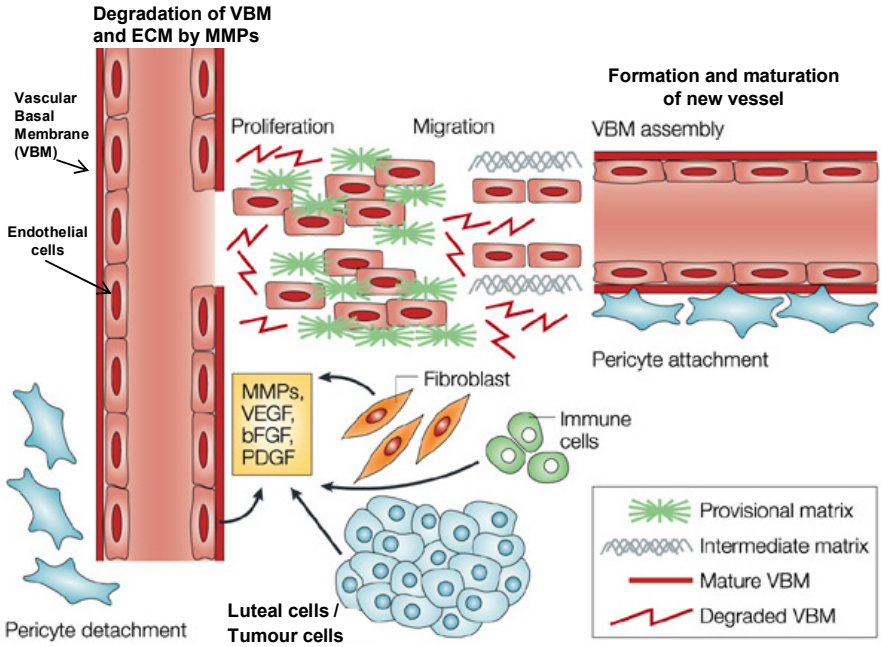

Fig. 4. Schematic angiogenic process showing the degradation of the ECM and vascular basal membrane (VBM) of existing blood vessel, proliferation and migration of endothelial cells towards an angiogenic stimuli, and formation and maturation of the new blood vessel sprout with the recruitment of pericytes.

2.2 Angioregression

The regression of blood vessels is an intrinsic feature of both physiological and pathological processes. The regression of vessels occurs under a variety of settings and is tightly correlated with apoptosis of the endothelial cells. In some tissue, the regression is a consequence of macrophage-induced apoptosis of the cells within the vessels (Ito & Yoshioka, 1999). However, in certain case, the regression precedes apoptosis; with the loss of cell-cell and cell-matrix contacts as the vessels begins precipitating apoptosis of the cells (Bayless & Davis, 2004; Saunders et al., 2005; Mavria et al., 2006). These observations suggest that regression is a regulated step in the angiogenic process (Im & Kazlauskas, 2006).

There is also evidence to indicate that extracellular factors can induce regression. For instance, Ang2, in absence of angiogenic stimuli and endothelial survival factors, destabilises the vessels, inducing vessel regression (Ito & Yoshioka, 1999; Tsigkos et al., 2003). However, Ang2 is also capable of promoting vessel formation in other vascular beds (Oshima et al., 1994; Lobov et al., 2002). The critical conditions that regulate the action of Ang2 seem to be the maturity of the vessels and the presence of other angiomodulators (such as VEGF) (Oshima et al., 2005).

Other specific factors that can be capable to drive regression include angiostatin and endostatin. These molecules induce vessel regression by disruption of the interaction between cell surface integrins and the

extracellular proteins (Sim, 1998; Chavakis & Dimmeler, 2002). The regulation of endothelial cells by vasoactive peptides (ET-1 and angiotensin II) and cytokines (TNFα and IFNγ) play also an important role during angioregression (Filippatos et al., 2001; Davis et al., 2003).

Figure 5 shows a tightly coordination between angiogenesis and angioregression. More specifically, stable, quiescent vessels must first be destabilized. This transition is associated with a loss of a functional interaction with pericytes (von Tell et al., 2006). In presence of angiogenic stimuli, the endothelial cells migrate out of the vessel and proliferate, resulting in angiogenesis. On the contrary, the destabilized vessels may regress in absence of these stimuli as well as of endothelial survival factors.

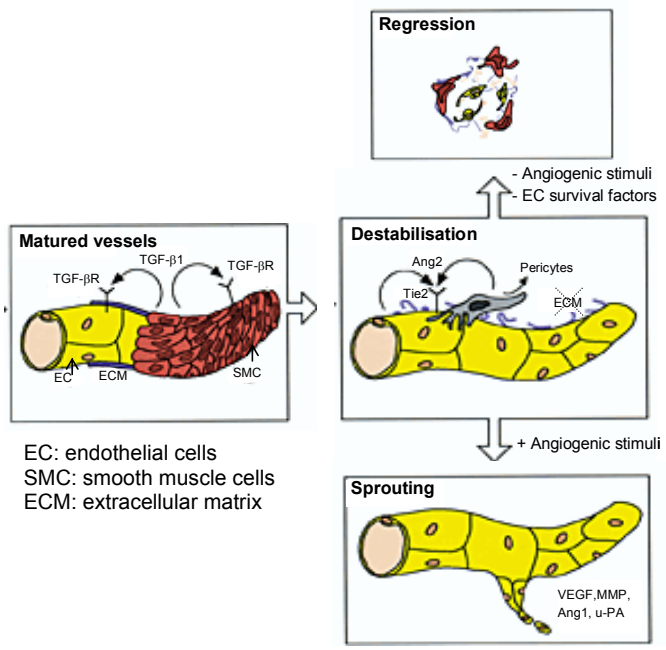

Fig. 5. Formation of new vessel or regression of existing vasculature. The first step of the angiogenic program is a process by which stable vessels become destabilised. This step is a prerequisite for subsequent responses such as sprouting or regression. The presence of growth factors and other angiomodulators contribute to the fate of the destabilized vessel.

3. VEGF FAMILY AND RECEPTORS

The VEGF family currently comprises several members, including the first identified molecule VEGF-A (also referred as VEGF), Placental Growth Factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E (a viral homolog of VEGF). PlGF is restricted to the placenta, while VEGF-B is particularly abundant in heart and skeletal muscle. VEGF-C and VEGF-D are involved in lymphangiogenesis and maintenance of the lymphatic vessels (Robinson & Stringer, 2001; Ferrara et al., 2003b).

The VEGF proteins exert their biological functions almost exclusively through a family of closely related receptor tyrosine kinase: fms-like tyrosine kinase (Flt-1 or VEGFR-1), fetal liver kinase-1/kinase insert domain-containing receptor (Flk-1/KDR or VEGFR-2), and VEGFR-3 (Flt-4). However, PlGF, VEGF-B, VEGF-E and certain isoforms of VEGF-A bind selectively to two neuropilins (NRP-1 and NRP-2), which are transmembrane non–tyrosine kinase receptors, identified firstly on neuronal growth cones as mediators of semaphoring/collapsing control of axonal guidance (Gluzman-Poltorak et al., 2000; Stouffer et al., 2001; Robinson & Stringer, 2001) (Fig 6).

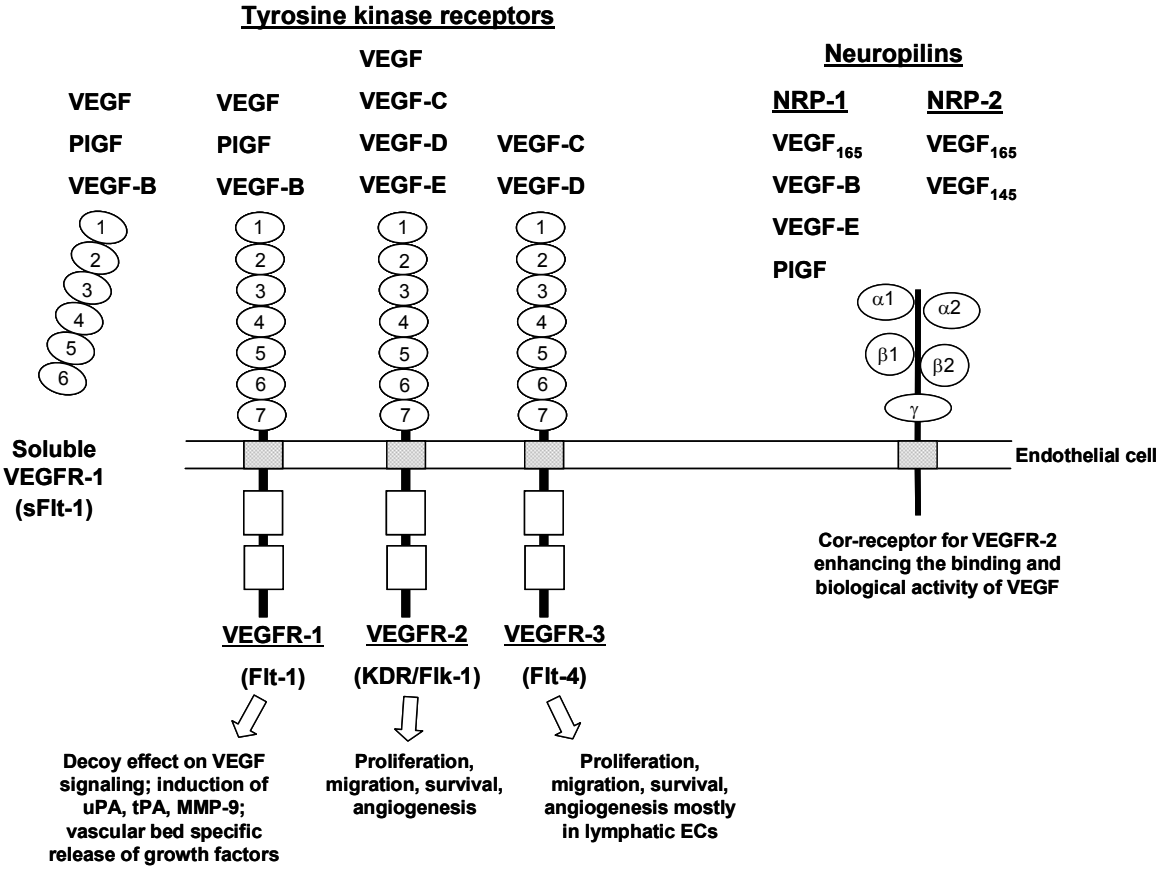

Fig 6. The endothelial cell surface receptor for members of VEGF family and their biological activities. VEGF tyrosine kinase receptors consist of seven extracellular Ig-like domains (numbered), a transmembrane region and an intracellular tyrosine kinase domain interrupted by a kinase-insert sequence. The soluble VEGFR-1 contains only the first six Ig-like domains. The neuropilins are isoforms-specific receptors for certain VEGF family members. The α1-α2 region has homology to components of the complement system; β1-β2 shares homology with coagulation factors V and VIII, whereas γ domain contain a MAM domain (Meprin/A5-neuropilin/Mu), a protein sequence also found in the metalloprotease meprin and receptor phosphatase μ (Robinson & Stringer, 2001).

2.1 VEGF gene and protein structure

The VEGF gene contains eight exons (Houck et al., 1991; Tischer et al., 1991). Alternative splicing of a single pre-mRNA generates several distinct isoforms identified by the exon composition and amino acid length of the final secreted protein (Fig 7).

All VEGF isoforms are secreted as covalently linked homodimers. The signal peptide (exon 1 and four residues of exon 2) is cleaved off during secretion (Leung et al., 1989). An N-glycosylation site exists at Asn74 that appears to have no effect in VEGF function but is required for efficient secretion (Peretz et al., 1992; Claffey et al., 1995).

Site-directed mutagenesis identified three acidic residues (Asp63, Glu64 and Glu67) in exon 3 and three basic residues (Arg82, Lys84 and His86) in exon 4 essential for binding to VEGFR-1 and VEGFR-2, respectively.

2.2 VEGF expression

Many cytokines and growth factors upregulate VEGF mRNA or induces VEGF release. These include PDGF, TNF-α, TGF-α, TGF-β, FGF-4, keratinocyte growth factor (KGF/FGF-7), epidermal growth factor (EGF), IL-1α, IL-1β, IL-6 and IGF-1. Several lack direct angiogenic effects but exert angiogenic activity through VEGF and bFGF (Brogi et al., 1994; Pertovaara et al., 1994).

Hypoxia induces a rapid and strong increase in VEGF mRNA levels, which is particularly noticeable around necrotic areas of tumours (Shweiki et al., 1992). Interestingly, the other VEGF family members and bFGF are not induced by hypoxia; therefore VEGF might be the main mediator of hypoxia-induced neovascularisation (Brogi et al., 1994; Enholm et al., 1997).

A hypoxia response element (HRE) acts upstream of the VEGF gene as an enhancer (Levy et al., 1995; Liu et al., 1995). This HRE contains a consensus binding site for hypoxia-inducible factor 1 (HIF-1), a heterodimer of the transcription factor HIF-1α and ARNT (aryl hydrocarbon receptor nuclear translocator) (Forsythe et al., 1996). Low oxygen tension increases HIF-1 levels at a post-transcriptional level and increases its DNA-binding ability (Jiang et al., 1996).

Hypoxia increases the half-life of VEGF mRNA, which is intrinsically labile owing to the presence of three synergistic sequence elements within the 5' and 3' untranslated regions (Dibbens et al., 1999). Binding of a hypoxia-induced stability factor (HuR) increases the half-life of this mRNA 3 to 8-fold (Levy et al., 1998). An alternative transcription-initiation site allows VEGF mRNA translation from a downstream ribosomal entry site. This might be advantageous under hypoxic stress, when cap-dependent translation can be inhibited (Stein et al., 1998).

2.3 VEGF splice variants

In human, at least eight VEGF isoforms (VEGF121, VEGF145, VEGF148, VEGF165, VEGF165b, VEGF183, VEGF189 and VEGF206) are generated by alternative splicing of a single VEGF mRNA (Fig 7). In non-primate animals, the VEGF isoforms present one amino acid shorter at the exon 2; so their nomenclatures are represented with a minus one amino acid.

VEGF isoforms are distinguished by the presence or the absence of the peptides encoded by exons 6a, 6b, 7a and 7b of the VEGF gene. VEGF121 lacks all these exons, VEGF189 lacks only the exon 6b, VEGF165 lacks exons 6a and 6b (Tischer et al., 1991), while VEGF145 lacks exons 6b, 7a and 7b (Poltorak et al., 1997). A conserved alternative splicing donor site within exon 6a originates the VEGF183 isoform; as a consequence, an 18-bp section from the C-terminal of the exon 6a is missing (Lei et al., 1998). VEGF148 lacks exons 6a, 6b and 7b, changing the reading frame and producing a premature stop codon into exon 8a (Whittle et al., 1999). VEGF206 is the full length form (Houck et al., 1991).

More recently, an inhibitor splice variant of VEGF165, named VEGF165b, has been described. This isoform, formed by distal splice site selection in the terminal exon of VEGF, predicts an open reading frame encoding an alternate C-terminal sequence, named exon 8b, that has the same number of amino acids in the mature protein. This predicted the translation of a protein of the same length as VEGF165, but with a different sequence. The C-

terminal six amino acids usually coded by exon 8 (CDKPRR) is replaced by six different amino acids (SLTRKD) coded by 18 bases of mRNA spliced 66 bases downstream of the usual acceptor splice site for exon 8 (Bates et al., 2002; Cui et al., 2004; Woolard et al., 2004).

Most VEGF-producing cells appear preferentially to express VEGF121, VEGF165 and VEGF189. VEGF183 also has a wide tissue distribution and may have avoided earlier detection through confusion with VEGF189 (Lei et al., 1998; Jingjing et al., 1999). In a model of systemic hypoxia, the VEGF183 and VEGF189 splice variants have been shown to be the most upregulated isoforms in response to a hypoxic challenge in rabbit meniscus (Hofstaetter et al., 2004). In contrast, VEGF145 and VEGF206 are comparatively rare, seemingly restricted to cells of placental origin (Anthony et al., 1994; Cheung et al., 1995). Interestingly human skin mast cells normally express VEGF121, VEGF165 and VEGF189 but can be induced to express VEGF206 as well by incubation with phorbol myristate acetate (PMA), which stimulates protein kinase C (PKC) activity (Grutzkau et al., 1998).

VEGF165 is secreted as ~46-KDa homodimers, which have a basic character and moderate affinity for heparin, owing to the presence of 15 basic amino acids within the 44 residues encoded by exon 7 (Ferrara & Henzel, 1989). In contrast, VEGF121, which lacks this region, is a weakly acidic protein and does not bind heparin. VEGF121 is freely released from producing cells, whereas 50-70% of VEGF165 remains cell and ECM

associated, probably owing to heparan sulphate proteoglycan (HSPG) interactions (Houck et al., 1992). VEGF189 and VEGF206 contain addition sequence encoded by exon 6 and bind heparin strongly. These isoforms are completely sequestered in the ECM and to a lesser extent at the cell surface (Houck et al., 1992; Park et al., 1993). VEGF183, although lacks 18 bp at the end of exon 6a, it still contains the heparin-binding site and its binding characteristics should be similar to that of VEGF189 (Lei et al., 1998).

The exon-6a-encoded sequence of VEGF145 confers an affinity for heparin similar to that of the exon-7-encoded sequence of VEGF165 (Poltorak et al., 1997). However, this sequence also mediates binding to components of the ECM that is independent of heparin or heparan sulphate. ECM-bound VEGF145 remains active as an endothelial cell mitogen (Poltorak et al., 1997). The 24-residue exon 6 peptide contains 12 basic amino acids, including the sequence ^{126}K-R-K-R-K-K^{131} identified as a cell-surface retention sequence (CRS). Since VEGF145 is freely released from producing cells, it is though that a combination of CRS binding protein-1 (CRSBP-1) and heparan sulphate interactions with the extended region derived from exons 6 and 7 is responsible for the cell-surface retention of VEGF189 and VEGF206 (Poltorak et al., 1997; Jonca et al., 1997). The sequence encoded by exon 6 has also been shown to release bioactive bFGF from the ECM and cell surface and thus confers to VEGF189 the ability to exert some of its biological effects through bFGF signalling pathways (Jonca et al., 1997).

VEGF147 splice variant is a truncated form of VEGF164, with mitogenic property but without the ability to bind heparin. VEGF147 is the only isoform presenting a truncated exon 8a and, as a consequence, it can possibly lack biological activity; nevertheless, it may affect the function of other VEGF isoforms, even though its physiological importance still remains to be elucidated (Whittle et al., 1999).

VEGF isoforms in the ECM constitute a reservoir of growth factor that can be slowly released by exposure to heparin, heparan sulphate (HS) and heparinases or more rapidly mobilised by specific proteolytic enzymes such as plasmin and urokinase-type plasminogen activator (uPA) (Houck et al., 1992; Plouet et al., 1997). These enzymes already contribute to angiogenesis through ECM depolymerisation, and might also regulate VEGF bioactivity by releasing sequestered VEGF from the cell surface and ECM. Recombinant VEGF189 and VEGF206 are unable to stimulate endothelial cell mitogenesis (Houck et al., 1991), because protein folding in these larger isoforms obscures regions responsible for receptor binding. VEGF189 binds VEGFR-1 but requires enzyme maturation by uPA or plasmin to bind VEGFR-2 and exert its mitogenic effects on endothelial cells (Plouet et al., 1997). uPA cleavage towards the C-terminal end of the exon-6-encoded region generates a truncated factor (uPA-VEGF189) that has an endothelial cell mitogenicity equivalent to that of VEGF165. Although not all VEGF isoforms contain a site for uPA cleavage, they can all be cleaved by plasmin.

The Pl-VEGF (VEGF110), by comparison with VEGF165 and uPA-VEGF189, elicits a 50-fold reduced mitogenic effect on endothelial cells, which is similar to that observed for VEGF121 (Keyt et al., 1996; Plouet et al., 1997). This demonstrates that the VEGF sequences encoded by exons 6 and 7 do more than just regulate the bioavailability of VEGF through HSPG binding: they actually enhance mitogenic signalling.

VEGF165b is an inhibitory isoform, described firstly to be down-regulated in human renal cell carcinoma (Bates et al., 2002). Further studies have been demonstrated that this isoform is an endogenous splice variant expressed in normal cell and tissues, and that could counteract the angiogenic and mitogenic activity of the VEGF165 isoform, by inhibiting the activation of VEGFR-2, and hence preventing phosphorylation and downstream signaling of this receptor (Cui et al., 2004; Woolard et al., 2004). Moreover, a potential role of VEGF165b in the control of human tumor growth was also demonstrated (Woolard et al., 2004).

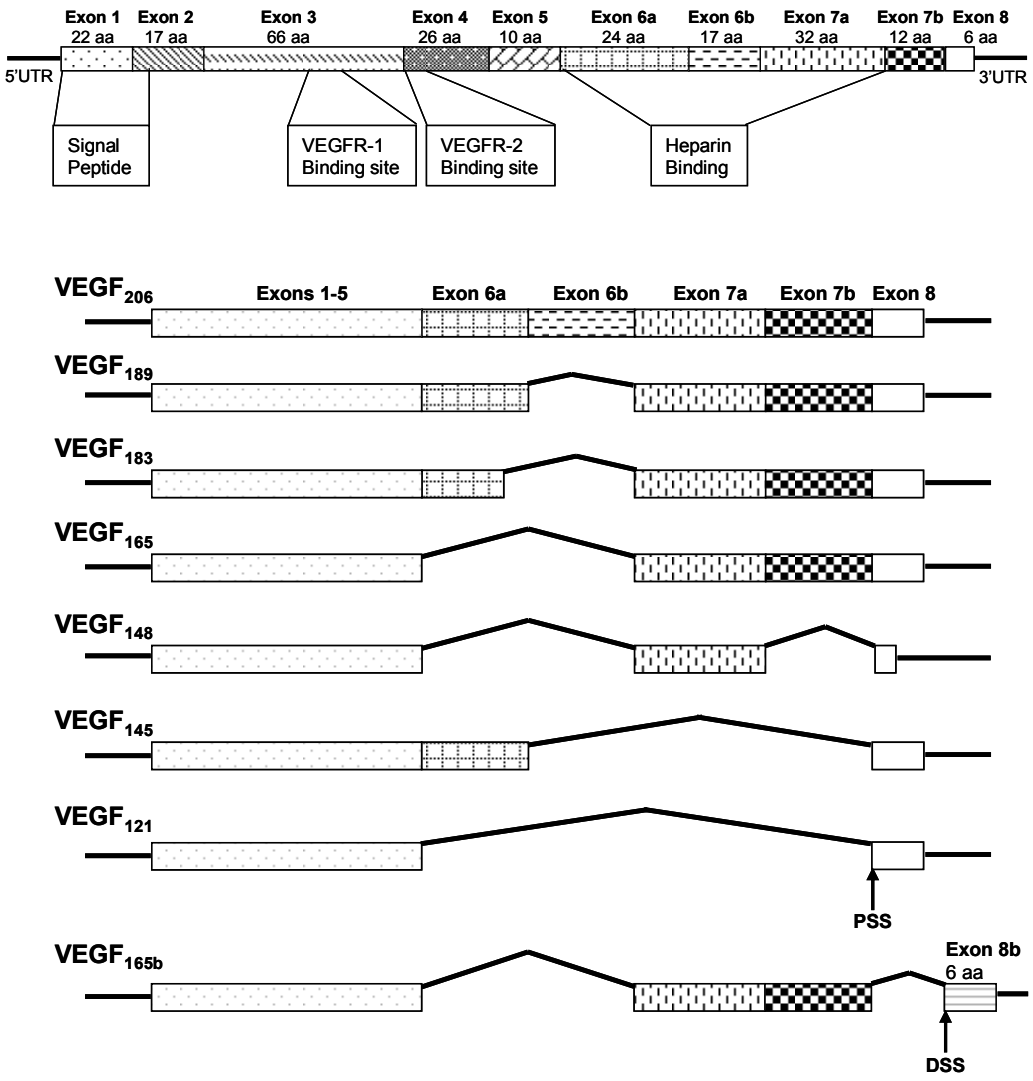

Fig. 7. The splice variants of human VEGF. The VEGF gene, through alternative mRNA splicing, produces currently seven isoforms plus an additional VEGF165b inhibitory isoforms. The functional domains and the exons from which they derived are represented at the bottom of the figure. Exon 1 contain 5'-untranslated region (UTR) and signal peptide; exon 2, NH2 terminus; exon 3 and 4 contain VEGFR-1 and VEGFR-2 binding sites, respectively; exon 6 and 7, heparin binding domains (PSS, Proximal Splice Site; DSS, Distal Splice Site) (Woolard et al., 2004).

2.4 VEGF Receptors

The three signalling tyrosine kinase receptors (VEGFR-1, VEGFR-2 and VEGFR-3) contain an extracellular region with seven immunoglobulin (Ig)-like loops, a single transmembrane region and an intracellular tyrosine kinase domain interrupted by a kinase-insert sequence (Robinson & Stringer, 2001; Cross et al., 2003).

The ~180-KDa glycoprotein VEGFR1 has the highest affinity for VEGF (Kd 10-30 pM; de Vries et al., 1992; Quinn et al., 1993). VEGFR1 is also shared by the related growth factors PlGF and VEGF-B. Its expression in mice is localised to the endothelium in adult and embryonic tissues, as well as to the neovasculature of healing skin wounds (Peters et al., 1993). The presence of VEGFR-1 mRNA in quiescent as well as proliferating endothelial cells suggests a continued role in endothelial maintenance. Tyrosine phosphorylation of VEGFR-1 in response to VEGF stimulation is hard to detect, and, in endothelial cells, no direct proliferative, migratory or cytoskeletal effects mediated by this receptor are apparent (Park et al., 1994; Seetharam et al., 1995). However, VEGFR-1 has been implicated in upregulated endothelial expression of tissue factor, uPA and plasminogen activator inhibitor 1 (PAI-1) (Clauss et al., 1996; Olofsson et al., 1998). In other cell types VEGFR-1 has different roles, such as tissue factor induction and chemotaxis in monocytes, and enhancing matrix metalloproteinase

expression by vascular smooth muscle cells (Barleon et al., 1996; Wang & Keiser, 1998).

VEGFR-2 is a 200-230-KDa high-affinity receptor for VEGF (Kd 75-760 pM; Terman et al., 1992; Quinn et al., 1993), as well as for VEGF-C, VEGF-D and VEGF-E. VEGFR-2 is expressed normally in endothelial cells, nascent haematopoietic stem cells and umbilical cord stroma. However, in quiescent adult vasculature, VEGFR-2 mRNA appears to be downregulated (Millauer et al., 1993; Quinn et al., 1993). Although VEGFR-1 has the greater affinity for VEGF, VEGFR-2 is tyrosine phosphorylated much more efficiently upon ligand binding and in endothelial cells leads to mitogenesis, chemotaxis and changes in cell morphology (Quinn et al., 1993; Waltenberger et al., 1994).

Both VEGF receptors are glycosylated; this is not essential for VEGFR-1 ligand binding (Barleon et al., 1997a), but only the mature glycosylated form of VEGFR-2 can efficiently autophosphorylate (Takahashi & Shibuya, 1997).

The interaction of VEGF with VEGFR-1 and VEGFR-2 occurs in two separate domains located at one end of the VEGF monomer. In the mature VEGF dimer, the monomers are linked in a rough “head-to-tail” fashion (with a large overlap) by disulfide bridges so that the main VEGFR-2 binding domains are at opposite ends of the molecule, as are the main VEGFR-1 binding domains (Fig. 8). The positioning of these receptor-binding interfaces at each pole of VEGF seems to facilitate receptor dimerisation, which is essential for transphosphorylation and signalling (Siemeister et al., 1998).

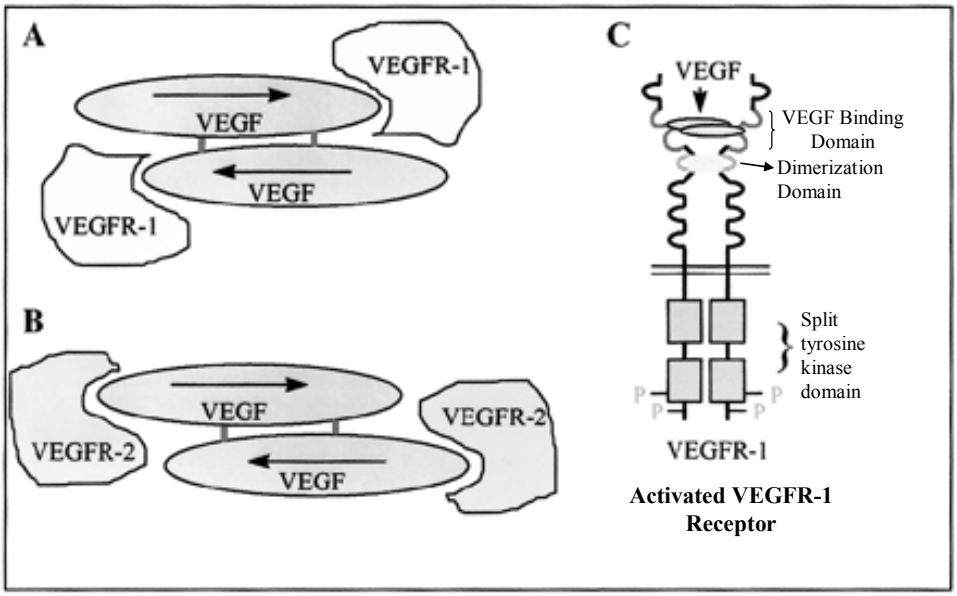

Fig 8. Interaction of VEGF with the binding sites of VEGFR-1 (A) and VEGFR-2 (B). The two VEGF monomers are shown in a head-to-tail orientation, indicated by arrows and held together by disulfide bonds. The main VEGF binding domains of the VEGFR-1 and VEGFR-2 receptors is located in Ig-like loop 2, but loop 3 also participates in the binding. The two VEGFR-1 receptors form a dimer that undergoes autophosphorylation on tyrosine residues located in the cytoplasmic part of the VEGFR-1 receptors (P), leading to the initiation of signal transduction. The dimer is held together by the interaction of each VEGFR-1 with a common VEGF dimer and is further stabilised by interactions between amino acids located at the loop 4 dimerisation domain (C) (Derived from Neufeld et al., 1999).

A soluble truncated form of VEGFR-1 (sFlt-1) that contains only the first six Ig-like domains has been cloned from a HUVEC cDNA library (Kendall et al., 1996). sFlt-1 binds to VEGF as strongly as does full-length VEGFR-1 and inhibits VEGF activity by sequestering it from signalling receptors and by forming non-signalling heterodimers with VEGFR-2. VEGF-B and PlGF also bind to sFlt-1. Particularly high levels of sFlt-1 occur in the placenta, where it

might control VEGF activity at particular stages of pregnancy (Clark et al., 1998; He et al., 1999). A truncated form of VEGFR-2, lacking the C-terminal half of the kinase domain, is expressed in normal rat retina (Wen et al., 1998). This truncated form is expressed at lower levels than full-length VEGFR-2 in these cells, but seems to be activated by VEGF at least as efficiently.

VEGFR-3 (Flt-4) expression is restricted mainly to the lymphatic endothelium of adult tissues (Pajusola et al., 1992; Kaipainen et al., 1995). VEGFR-3 binds VEGF-C and VEGF-D, but not VEGF, and is though to control lymphangiogenesis.

As regarding neuropilins (NRP-1 and NRP-2), some reports have been indicated that certain tumour and endothelial cells express cell-surface VEGF binding sites distinct from VEGFR-1 and VEGFR-2, and that the exon 7-encoded basic sequences are required for binding to this putative receptor (Soker et al., 1996; Soker et al., 1998). Subsequently, it was demonstrated that NRP-1 binds specifically to VEGF165 (Soker et al., 1998), while NRP-2 binds both VEGF165 and VEGF145 (Gluzman-Poltorak et al., 2000). When coexpressed in cells with VEGFR-2, NRP-1 enhanced the binding of VEGF165 to VEGFR-2 and VEGF-mediated chemotaxis (Soker et al., 1998). It has been proposed that NRP-1 presents VEGF165 to VEGFR-2 in a manner that enhances the effectiveness of VEGFR-2 mediated signal transduction. Binding to NRP-1 explains, in part, the greater mitogenic

potency of VEGF165 relative to VEGF121 (Soker et al., 1998). There is no evidence that NRP-1 or NRP-2 signal after VEGF binding (Neufeld et al., 2002). In contrast, NRP-1 is required for the development of the vascular system in mice embryos (Kawasaki et al., 1999) and zebrafish (Lee et al., 2002). Furthermore, both NRP-1 and NRP-2 are expressed in rat uterus (Pavelock et al., 2001).

2.5 Receptors expression

Upstream control elements confer endothelial-cell-specific transcription on both VEGFR-1 and VEGFR-2 (Morishita et al., 1995; Patterson et al., 1995). Not surprisingly, hypoxia, as well as inducing VEGF release, is also a potent stimulator of VEGFR-1 and VEGR-2 expression in vivo (Tuder et al., 1995; Li et al., 1996). In common with VEGF, VEGFR-1 has a HIF-1 consensus in its promoter region (Gerber et al., 1997). VEGFR-2 has no similar sequence and transcription might be slightly downregulated by hypoxia (Thieme et al., 1995; Gerber et al., 1997). However, VEGFR-2 is though to be upregulated at the post-transcriptional level by an unidentified paracrine factor released from ischemic tissue (Brogi et al., 1996; Waltenberger et al., 1996).

Several growth factors, including VEGF (Wilting et al., 1996; Barleon et al., 1997b), affect VEGF receptors expression. VEGF stimulation of VEGFR-2 both upregulates expression of VEGFR-2 gene and increases cellular VEGFR-2 levels (Shen et al., 1998). bFGF is known to synergise with VEGF in inducing angiogenesis and one mechanism for this is through upregulation

of VEGFR-2 in endothelial cells (Pepper et al., 1998). Interestingly, cell-cell and cell-matrix contact might also affect VEGF receptors expression: VEGFR-2 levels vary according to cell density and the geometry of the culture conditions (Pepper & Mandriota, 1998), and expression of both VEGFRs is influenced by the levels of platelet endothelial cell adhesion molecule 1 (PECAM-1: Sheibani & Frazier, 1998).

2.6 Heparan sulphate proteoglycans and VEGF isoforms

Heparin and heparan sulphate (HS) are found on the cell surface and in the ECM of almost all mammalian cells, and are component of the heparan sulphate proteoglycans (HSPGs). Heparin/HS affinity can affect VEGF function through several mechanisms. HSPGs affect VEGF bioavailability through sequestration at the cell surface/ECM. This VEGF reservoir may be protected from enzymatic degradation (Houck et al., 1992), although ECM-bound VEGF189 does not appear to be protected from uPA or plasmin maturation (Plouet et al., 1997). VEGF can displace other HSPG-bound growth factors from the ECM, most notably bFGF, which can then synergise with VEGF in stimulating angiogenesis (Jonca et al., 1997). Heparin/HS also protects the heparin-binding isoforms from natural inhibitory and degradatory processes by preventing their interaction with proteins such as α2-macroglobulin and platelet factor 4 (Soker et al., 1993; Gengrinovitch et al., 1995). Furthermore, HSPGs may regulate the interaction of several heparin

binding growth factors with their respective receptors and, consequently, their biological activity (Schlessinger et al., 1995).

A novel function of heparin may be in the restoration of function to damaged VEGF. Oxidising agents and free radicals, both common around areas of inflammation and wound healing, can inactivate VEGF. Under such conditions, heparin binding by VEGF165 could prolong its biological activity compared with VEGF121 by partially restoring lost function (Gitay-Goren et al., 1996). In fact, glypican-1, an HSPG known to be present on endothelial cells, has been shown to play a chaperone-like function by partially restoring VEGF165 activity after oxidative damage and promoting VEGFR-2 binding (Gengrinovitch et al., 1999).

AIMS

The experiments realized during my PhD originated three original papers published on international journals.

The first study (Experiment I) was aimed at verifying whether feed restriction influences the VEGF production as well as the mRNA expression of VEGF and its receptor VEGFR-2 and the ET-1 system members (ET-1, ECE-1 and ET-A) in swine CL.

Galeati et al., 2005. Domestic Animal Endocrinology, 28:272-284.

The second study (Experiment II) was aimed at characterizing the temporal expression pattern of VEGF, gelatinases and endonuclease throughout the lifespan of swine CL and pregnancy and investigating whether the expression levels of those molecules are related to CL functional stage.

Ribeiro LA et al., 2006. BMC Developmental Biology, 6(1):58.

The third study (Experiment III) was aimed at determining the pattern of different VEGF isoform and receptor mRNAs expression and protein levels in swine CL during estrous cycle.

Ribeiro LA et al., 2007. Molecular Reproduction and Development, 74:163-171.

EXPERIMENT I

Fasting influences steroidogenesis, vascular endothelial growth factor (VEGF) levels and mRNAs expression for VEGF, VEGF receptor type 2 (VEGFR-2), Endothelin 1 (ET-1), Endothelin Receptor Type A (ET-A) and Endothelin Converting Enzyme -1 (ECE-1) in newly formed pig corpora lutea.

Domestic Animal Endocrinology, 2005, 28:272-284.

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Domestic Animal Endocrinology 28 (2005) 272–284
DOMESTIC ANIMAL ENDOCRINOLOGY
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Fasting influences steroidogenesis, vascular endothelial growth factor (VEGF) levels and mRNAs expression for VEGF, VEGF receptor type 2 (VEGFR-2), endothelin-1 (ET-1), endothelin receptor type A (ET-A) and endothelin converting enzyme-1 (ECE-1) in newly formed pig corpora lutea

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Received 20 August 2004

Abstract

This study was designed to verify whether fasting influences vascular endothelial growth factor (VEGF) production and VEGF, VEGF receptor-2 (VEGFR-2) as well as endothelin (ET) system members (endothelin converting enzyme-1, ECE-1; ET-1; endothelin receptor type A, ET-A) mRNA expression in pig corpora lutea; furthermore, we wanted to assess whether fasting affects steroidogenesis in luteal cells. Eight prepubertal gilts were induced to ovulate and were randomly assigned to two groups: (A) $n = 4$, normally fed; and (B) $n = 4$, fasted for 72 h starting 3 days after ovulation. At the end of fasting, ovaries were removed from all the animals and corpora lutea (CLs) were collected. VEGF and steroid levels in luteal tissue were determined by ELISA and RIA, respectively; VEGF, VEGFR-2, ET-1, ET-A and ECE-1 mRNAs expression was measured by real-time PCR. VEGF protein levels were similar in the two groups, while all steroid (progesterone, testosterone, estradiol 17β) concentrations were significantly ($P < 0.001$) higher in CLs collected from fasted animals compared

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0739-7240/$ – see front matter
doi:10.1016/j.domaniend.2004.11.002

with those from normally fed gilts. VEGF, VEGFR-2, ET-1 and ECE-1 (but not ET-A) mRNA expression was significantly lower ($P < 0.05$) in fasted versus normally fed animals. The overall conclusion is that all the parameters studied are affected by feed restriction, but the mechanisms activated at luteal level are possibly not fully adequate to compensate for nutrient shortage.

Keywords: Angiogenesis; Gilt; Progesterone; Testosterone; Estradiol 17β

1. Introduction

The corpus luteum (CL) undergoes rapid growth, differentiation and controlled regression during every ovarian cycle [1–3]. Maintenance of luteal function has been demonstrated to depend to a great extent on angiogenesis, the growth and development of new blood vessels from pre-existing ones, while luteal regression is associated with its inhibition [4]. The main angiogenic factor is the vascular endothelial growth factor (VEGF), whose importance in CL development and function has been demonstrated in several species [4–7] for a review on the angiogenic process at luteal level see [8]. In addition, luteal tissue has been found to be a source of VEGF (cattle [9], caprine [10], equine [11], humans [12], rat [13], sheep [14]). In the pig CL, Boonyaprakob et al. [15] reported the expression of VEGF and its receptors (VEGFR-1 and VEGFR-2) mRNA throughout the luteal phase. In situ hybridization and immunohistochemistry studies provided evidence for the presence of VEGF mRNA mainly in steroidogenic cells, while VEGF receptors appear to be present in both steroidogenic and endothelial cells in human corpora lutea [12,16]. In the bovine species, receptors for VEGF are only expressed in endothelial cells [9] and VEGFR-2 (but not VEGFR-1) mRNA expression is clearly regulated throughout the estrous cycle [9].

Endothelin-1 (ET-1), the only isopeptide synthesized and secreted by vascular endothelial cells [17], has been demonstrated to regulate oocyte maturation, ovulation and CL functions [18,19]. It is derived from an inactive intermediate peptide, big ET-1, by endothelin converting enzyme-1 (ECE-1). Endothelin-1, initially defined as a potent vasoconstrictor, has recently gained increasing attention due to its diverse biological functions, such as cardiovascular homeostasis, embryo development and angiogenesis [20–22] and the endothelin system appears to play an important role in ovarian physiology [23,24]. ET-1 has been demonstrated to stimulate both VEGF and basic fibroblast growth factor (bFGF) production [25–27] and it may also inhibit progesterone production in luteal cells [28] this inhibitory effect is potentiated by PGF2α [29]. Thus, ET-1 may play a role during both physiological and induced luteolysis [30], as it appears to be responsible for the decrease of blood flow in the mid-cycle CL after induction of luteal regression [31].

Besides being up-regulated by luteinizing hormone (LH), insulin-like growth factor-I (IGF-I) and other factors [32], VEGF production has also been shown to be influenced by nutrients such as fructose [33] and taurine [34]. Thus, a reduction in the availability of nutrients could modify luteal VEGF production which, in turn, may alter blood vessel growth during CL function. Recently, we have demonstrated that short-term fasting in gilts

significantly increases VEGF levels in follicular fluid from large ovarian follicles as well as the permeability of follicular blood vessels. We also observed an increase in VEGF mRNA levels in the theca (but not in granulosa) layer [35].

Both VEGF and ET-1 systems are, therefore, involved in modulating luteal angiogenesis and impaired metabolic conditions occurring during the early CL development could likely affect their expression and function.

On the basis of these observations, this study was designed to verify whether fasting influences VEGF production and VEGF and VEGFR-2 mRNA expression as well as expression of members of the ET system (ECE-1, ET-1, ET-A) in pig corpora lutea and whether this is related to changes in luteal steroidogenesis. The experimental model we chosen (72 h fasting) has been recently demonstrated to be effective in significantly reducing glucose while increasing both leptin and non-esterified fatty acids (NEFA) plasma levels [36]; this approach may be, therefore, useful to determine the effects of impaired metabolic conditions on the factors involved in the angiogenic process.

2. Materials and methods

2.1. Animals and hormonal stimulation protocols

Eight prepubertal Large White gilts, with an average weight of 98 ± 1.67 kg (mean ± S.E.M.), were treated with 1250 IU equine chorionic gonadotropin (eCG; Folligon, Intervet, Holland) and 750 IU hCG (Corulon, Intervet) 60 h later (day 0); this treatment induces ovulation about 44 h after hCG administration. Five days after hCG treatment, animals were randomly divided into two groups: group A, normal feeding with a commercial diet ($n = 4$); and group B, 72 h of fasting with water available at all times ($n = 4$).

On day 8, ovaries were recovered by laparotomy; animals were pre-anesthetized by an injection of azaperone (240 mg/gilt; Stresnil, Janssen, Belgium) and atropine sodium salt (2 mg/gilt; Industria Galenica Senese, Italy), and maintained under thiopental sodium (1.5 g/gilt; Pentothal Sodium; Gellini, Latina, Italy) anaesthesia. Immediately after removal, ovaries were transported to the laboratory where single CLs were isolated. The CLs obtained from each ovary were counted and cut in two halves with a razor blade. One half was weighed and homogenised in PBS (0.1 g/ml) on ice bath by an Ultra Turrax. The homogenate obtained was processed as follows: 500 μl were centrifuged at $2000 \times g$ for 10 min at 4 °C and supernatant was stored at −20 °C until VEGF determination; the remainder of the homogenate was kept frozen until steroid (progesterone, P4; testosterone, T; and estradiol 17β, E2) measurement. All CLs were processed for steroid and VEGF determination. Total RNA was isolated from the second half of the CLs (10 CLs/gilt) with the Tri-Pure isolation reagent, according to the manufacturer's instructions (Roche Diagnostic GmBH, Mannheim, Germany), and stored at −80 °C until VEGF and its receptor mRNA as well as ET-1, ET-A and ECE-1 mRNA levels were determined as described below. All the samples were frozen by 2 h from the surgical removal of the ovaries.

All animals were housed and used according to EEC animal care guidelines. The experimental procedures had previously been submitted to and approved by the Ethical Committee of Bologna University.

2.2. VEGF assay

VEGF concentrations were measured in 100 μl samples of homogenate supernatants by a specific enzyme linked immune-adsorbent assay (ELISA, Quantikine, R&D Systems, Minneapolis, MN, USA) previously validated for the measurement of porcine VEGF [35,37,38]. This highly specific sandwich assay recognises VEGF 165 as well as VEGF 121, while it exhibits negligible cross-reactivity with all the cytokines/growth factors tested (the complete list is reported in the manufacturer's booklet). A 96-well plate reader (Biomek 1000, Beckman Instruments, Fullerton, CA, USA) set to read at 450 nm emission was used to quantify the results. The sensitivity of the assay was 5 pg/ml, and the intra- and inter-assay coefficients of variation were less than 6 and 10%, respectively. All data are expressed as pg/mg protein; protein concentration was determined according to Lowry method [39] using a protein assay kit (Sigma Diagnostics, St. Louis, MO, USA).

2.3. Steroid assays

Steroid hormone concentrations in CL homogenates were measured by validated RIAs as previously described (progesterone and estradiol 17β [40], testosterone [41]).

2.4. Estradiol 17β (E2) and testosterone (T)

In brief, aliquots (500 μl) from each homogenate CL were extracted with 5 ml diethyl ether. After centrifugation, ether was collected and dried under a N_2 stream. Dried ether extracts were resuspended in 500 μl of phosphate buffer and sets of two 100 μl or 20 μl aliquots were then assayed for E2 and T content, respectively.

The sensitivities were 1.6 pg/tube for T assay and 1.1 pg/tube for E2 assay. The intra- and interassay coefficients of variation were 3.4 and 10.9%, respectively, for T and 5.8 and 11.2% for E2. The results are expressed in pg/mg tissue.

2.5. Progesterone (P4)

Aliquots of 20 μl from each homogenate CL were extracted with 5 ml petroleum ether. After centrifugation, ether was collected and dried under a N_2 stream. Dried ether extracts were resuspended in 1 ml phosphate buffer, diluted 1:50 and aliquots of 50 μl were then assayed.

The sensitivity of the assay was 3.7 pg/tube. The intra- and interassay coefficients of variation were 6.3 and 9.6%, respectively. The results are expressed in ng/mg tissue.

2.6. Total RNA extraction and reverse transcription

Total RNA from CLs (10 CLs/gilt) was resuspended in RNAse-free water and was spectrophotometrically quantified (A_{260} nm). One microgram of total RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-RAD Laboratories Inc., CA, USA) in a final volume of 20 μl, according to the manufacturer's instruction. Transcrip-

tion reactions without reverse transcriptase were performed to determine a possible DNA contamination.

2.7. Real-time quantitative PCR

Swine primers were designed for ET-1, ET-A, ECE-1, VEGF, VEGFR-2 and β-actin, using the Beacon Designer 2.07 Software (Premier Biosoft International, Palo Alto, CA, USA). Their sequences, expected PCR product length and accession number are shown in Table 1. Real-time quantitative PCR was performed in the iCycler Thermal Cycler (Bio-RAD Laboratories Inc., Hercules, CA, USA) using SYBR green I detection. A master-mix of the following reaction components was prepared to the indicated end-concentrations: 1.5 μl forward primer (0.6 μM), 1.5 μl reverse primer (0.6 μM), 6.5 μl water and 12.5 μl IQ SYBR Green BioRad Supermix (Bio-RAD Laboratories Inc.). Three μl of cDNA were added to 22 μl of the master-mix. All samples were performed in duplicate for all genes. The two step real-time PCR protocol employed was: initial denaturation for 3 min at 95 °C, 40 cycles at 95 °C for 15 s and 60 °C for 30 s, followed by a melting step with a slow heating from 55 to 95 °C with a rate of 0.5 °C/s. The relative mRNA level was determined as the PCR cycle number that crosses an arbitrarily placed signal threshold (C_T). The C_T value correlates inversely with the amount of target mRNA in the sample. The housekeeping gene β-actin was used to normalize the amount of RNA. The expression of each gene was calculated as ΔC_T (target gene C_T − β-actin C_T) for all individual samples. To determine the relative amount of the target genes in the fasting group relative to control one, we calculated the $\Delta\Delta C_T$ (fasting group average ΔC_T − control group average ΔC_T). As the PCR amplification is an exponential process, a difference of two C_T signifies approximately a regulation by a factor of $2^{-\Delta\Delta C_T}$.

Real-time efficiencies were acquired by amplification of a standardised dilution series and corresponding slopes and PCR efficiencies were calculated using iCycler iQ Real Time PCR Detection System (Bio-Rad Laboratories Inc.).

Table 1
Forward and reverse primers sequences, RT-PCR product length and accession number (Acc. No.)

Primer	Sequence (5′–3′)	Product length (bp)	Acc. No.
ET-1	For.: CCTGTCTGAAGCCATCTC Rev.: AGTAAGGAACGGTCTGAAC	109	X07383
ET-A	For.: TCACCGTCCTCAATCTCTG Rev.: GGCTGTGACCAATGGAATC	98	S80652
ECE-1	For.: CCATCATCAAGCACCTCCTC Rev.: GCTCCTCAATCCTGGTTTCG	108	D89494
VEGF	For.: CCTTGCCTTGCTGCTCTACC Rev.: CGTCCATGAACTTCACCACTTC	101	AF318502
VEGFR-2	For.: AACGAGTGGAGGTGACAGATTG Rev.: CGGGTAGAAGCACTTGTAGGC	104	AJ245446
β-Actin	For.: ATGGTGGGTATGGGTCAGAAAG Rev.: TGGTGATGATGCCGTGCTC	103	AF054837

3. Statistical analysis

Statistical significance was determined using the independent samples T-test (SPSS Version 8.0 Inc., Chicago, IL, USA), after a preliminary statistical comparison between treatments based on evaluation of S.E.M. at 95% probability (significance level). Differences were considered significant at $P < 0.05$. Data on VEGF and steroid concentrations and mRNA expression of the target genes in luteal tissue are presented as mean ± S.E.M.

4. Results

4.1. VEGF and steroid concentrations in homogenate CLs

The total number of CLs isolated from the ovaries of gilts fed with normal plane of nutrition was 76 (19 ± 1.35 CLs/gilt, mean ± S.E.M.); fasting did not modify CLs number ($n = 81$; 20.25 ± 1.62 CLs/gilt). Differences in weight were not observed, neither per CL, nor per ovary (data not shown).

VEGF concentrations in CL extracts from A and B groups were not different (125.97 ± 4.57 and 115.97 ± 3.43 pg/mg protein, respectively).

The levels of steroids in CLs (Fig. 1) were markedly affected by feed regimens. Under control conditions, P4 concentrations were 23.49 ± 0.55 ng/mg protein, while E2 and T concentrations were 0.39 ± 0.02 and 14.58 ± 0.62 pg/mg, respectively. Fasting significantly ($P < 0.001$) increased P4 (30.44 ± 0.65 ng/mg), E2 (0.57 ± 0.02 pg/mg) and T (23.16 ± 1.09 pg/mg) levels.

4.2. ET-1, ET-A, ECE-1, VEGF and VEGFR-2 mRNA Expression

Table 2 and Fig. 2 summarize the ΔC_T of ET-1, ET-A, ECE-1, VEGF and VEGFR-2 mRNA expression in fasted and normally fed animals. The relative amount of ET-1 mRNA in CLs from fasted animals was significantly lower ($P = 0.002$) than that observed in normally fed animals, with a relative decrease of 34%. Similar results were observed for ECE-1, whose mRNA expression was reduced by 20% ($P = 0.032$) in fasted gilts, while no significant differences were observed between fasted and normally fed animals for ET-A mRNA expression. VEGF mRNA and VEGFR-2 mRNA expressions were significantly lower in fasted than in normoalimented animals ($P = 0.001$ and 0.015, respectively).

5. Discussion

The production of VEGF by cells from both cyclic and pregnant CLs as well as mRNA expression for VEGF and its receptors (VEGFR-1 and -2) has been reported in many species. Available data demonstrate that both protein and mRNA change throughout the estrous cycle in bovine [9] and equine [1] CL, with maximum levels during the early and low levels during the late luteal phase and particularly during luteal regression. High VEGF expression during the early luteal phase has also been shown in ovine

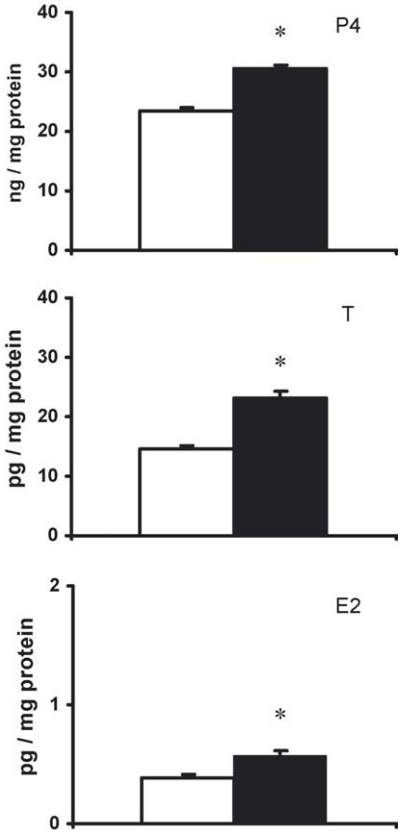

Fig. 1. Progesterone (P4), testosterone (T) and estradiol 17β (E2) concentrations in luteal tissue from normally fed (open bars) and fasted (closed bars) gilts. Asterisk indicates a significant difference ($P < 0.001$).

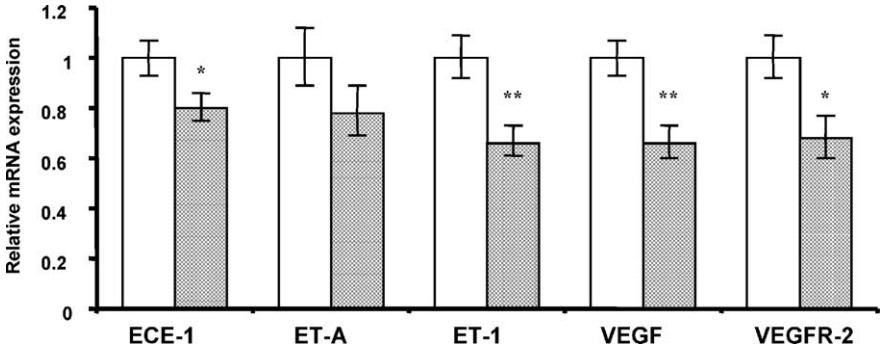

Fig. 2. Relative luteal tissue mRNA expression for ECE-1, ET-A, ET-1, VEGF and VEGFR-2 in normoalimented (open bars) and fasted (closed bars) gilts. Error bars represent the range of relative expression. Asterisks indicate significant differences ($^{*}P < 0.05$; $^{**}P < 0.005$).

Table 2
ET-1, ET-A, ECE, VEGF and VEGFR-2 mRNA expression in fasted and normally fed animals; the data are presented as the mean ± S.E.M.

Gene	Group	ΔC_T [a]	$\Delta\Delta C_T$ [b]	Relative expression to control [c]
ET-1	Control	4.94 ± 0.12	0 ± 0.12	1.0 (0.92–1.09)
	Fasting	5.53 ± 0.13**	0.59 ± 0.13	0.66 (0.61–0.73)
ET-A	Control	3.34 ± 0.16	0 ± 0.16	1.0 (0.89–1.12)
	Fasting	3.69 ± 0.18 n.s.	0.35 ± 0.18	0.78 (0.69–0.89)
ECE-1	Control	0.27 ± 0.10	0 ± 0.10	1.0 (0.93–1.07)
	Fasting	0.59 ± 0.10*	0.32 ± 0.10	0.80 (0.75–0.86)
VEGF	Control	0.71 ± 0.10	0 ± 0.10	1.0 (0.93–1.07)
	Fasting	1.3 ± 0.14**	0.59 ± 0.14	0.66 (0.60–0.73)
VEGFR-2	Control	0.87 ± 0.12	0 ± 0.12	1.0 (0.92–1.09)
	Fasting	1.44 ± 0.18*	0.56 ± 0.18	0.68 (0.60–0.77)

n.s.: not significant.

[a] ΔC_T = target gene C_T − β-actin C_T; β-actin level was similar in control and fasting group ($P = 0.24$).

[b] $\Delta\Delta C_T$ = fasting group − control group average ΔC_T. This is a subtraction of an arbitrary constant, so that the S.E.M. of $\Delta\Delta C_T$ is the same as the S.E.M. of the ΔC_T value.

[c] The relative expression is determined by $2^{-\Delta\Delta C_T}$. The range (numbers in parentheses) were calculated by $2^{-(-\Delta\Delta C_T - \text{S.E.M.})}$ and $2^{-(-\Delta\Delta C_T + \text{S.E.M.})}$, where S.E.M. = the standard error mean of the $\Delta\Delta C_T$ value.

* $P < 0.05$.

** $P < 0.005$.

CL [14]. These findings represent convincing evidence that luteal development is associated with VEGF expression in those species. In contrast, high levels of mRNAs encoding the VEGF 165 isoform and VEGFR-1 have been found during the mid and late luteal phase in caprine CL [10] allowing these Authors to suggest a non-angiogenic role of the VEGF system. In the pig, VEGF mRNA expression has been reported to be constant throughout the lifespan of CL and to decrease only at its end, while the expression of both VEGF receptors mRNA changes [15]. Although our experimental model does not allow us to clarify whether steroidogenic or endothelial cells (or both) are involved, results from this study confirm that porcine luteal tissue expresses mRNAs for both VEGF and VEGFR-2 as well as VEGF protein. The highly specific sandwich assay we used for VEGF determination recognizes VEGF 165 as well as VEGF 121 [37,38], while our method for VEGF mRNA quantification (real-time PCR) measures all VEGF isoforms even though the contribution of each to the total amount of VEGF mRNA cannot be determined.

A possible modulatory role for CL formation has also been suggested for members of the endothelin family [19]. In the bovine CL, ET-1 mRNA expression is high after ovulation, decreases during the mid and late luteal phases and increases again during luteal regression, while ET-A and ECE-1 mRNA levels are constant throughout all luteal stages. As both ECE-1 and ET-1 levels have been shown to be up-regulated in the bovine CL after PGF2α-induced luteolysis [42], the ET-1 system is thought to act as a vasoconstrictor and an inducer of cell apoptosis, likely through a positive-feedback mechanism with prostaglandins [28]. ET-1 seems also to be related with VEGF, as in other systems endothelin has been found to stimulate VEGF production [25]. Since both VEGF and endothelin-1 are expressed in the

newly ruptured follicle [43], we may hypothesise that endothelin-1 potentiates the VEGF-mediated angiogenesis in the newly developing corpus luteum.

Dietary restrictions have been shown to negatively affect reproductive performance in many species (ovine [44], swine [45–47], bovine [48,49]). In particular, a post-ovulatory food deprivation in sows has been reported to reduce cleavage rate of embryos [45] and to delay oocytes transport [50,51]. Several mechanisms are likely involved, among them an impairment of luteal development and function (for a review on nutritional influences on reproductive activity, see [52]). Our results demonstrate that both endothelin and VEGF systems are negatively affected by acute fasting, although VEGF concentrations in luteal tissue do not seem to be modified. Angiogenesis has been shown to be stimulated by nutrients shortage [53,54], which has been demonstrated to be also effective in increasing VEGF levels in follicular fluid from pig large follicles [35]. In our previous study we found that the overall content of VEGF mRNA in the follicle wall was unaffected by fasting, even though the distribution of VEGF gene expression shifted from the granulosa to the theca compartment; thus, the two follicle compartments differently contribute to the total amount of VEGF in follicular fluid. Both theca and granulosa cells differentiate into luteal cells; therefore, it is not surprising that VEGF concentrations do not differ between CLs from normoalimented and fasted animals in that theca- and granulosa-derived luteal cells may possibly compensate each other as for VEGF production. This does not seem to be the case for VEGF and its receptor mRNAs, which are significantly lower in fasted than in normoalimented gilts; similar data were obtained for all the components studied (except for ET-A) of the ET-1 system. ET-1 reduction may be, at least in part, responsible for the decrease in VEGF mRNA, as a stimulatory effect of ET-1 on VEGF has been demonstrated [25]. Nutrients shortage seems, therefore, to be effective in reducing the ET-1 system expression. In accordance with this, a fasting-induced decrease of plasma endothelin levels has been reported in rats [55], while high glucose levels are effective in increasing ECE-1 [56] and ET-1 has recently been shown to be higher in obese than in lean control patients [57]. In support of our observations, maternal malnutrition has been demonstrated to dramatically reduce VEGF and VEGFR-1 expression in fetus pancreatic cells [34], while taurine supplementation to the maternal diet prevents such under-expression. In addition, both VEGF and endothelin-1 have recently been found to be higher in diabetic patients than in healthy controls [58]. These observations may account for the concomitant reduction of the VEGF and ET-1 system gene expression we found in feed restricted gilts. The fasting-induced VEGF and ET-1 inhibition may be mediated through a reduction of gonadotropins and/or insulin-like growth factor-I (IGF-I) [44,48,59,60], which are effective in stimulating both VEGF [37,61–63] and ET-1 [64] production. On the basis of the main effects of VEGF (stimulation of vessel growth and endothelial permeability) and ET-1 (stimulation of proliferation of endothelial cells and vasoconstriction, as well as VEGF increase), we may conclude that feed restriction possibly inhibits luteal vessel development. This could be, at least in part, compensated by a decrease of vasal tone due to a suppression of endothelin-1, thus ensuring an adequate blood flow. Our data on steroid concentrations in luteal tissue indicate that fasting does not negatively affect steroidogenesis which, in fact, is stimulated. We have previously reported [35] that fasting modifies follicular steroidogenesis, by increasing progesterone secretion while decreasing both E2 and testosterone output. The increase in progesterone synthesis as induced by fasting seems, therefore, also to be confirmed in luteal tissue and may be due, at

least in part, to a reduction of the inhibitory effects of ET-1, which has been demonstrated to inhibit steroidogenesis in bovine [24,65] and porcine [66] luteal cells. In according to our luteal data, higher plasma progesterone concentrations were seen in feed-restricted than in normally fed gilts [67] and sows [45]. As suggested [57], the high P4 levels in plasma from fasted gilts may be due to a reduction of the metabolic clearance of the steroid, but our luteal data suggest that there may also be a higher secretion; similar conclusions have been drawn also in both sheep [44] and cows [68]. As for the increase observed in E2 and its precursor (T), we do not have any convincing explanation for this effect; however, since VEGF has been demonstrated to inhibit estradiol output by granulosa cells from pig large follicles [69], a fasting-induced inhibition of the VEGF system may account for the indirect stimulation of E2 which, in turn, may exert a powerful stimulatory effect on P4 secretion [70,71].

Taken together, the results from the present study indicate that acute fasting inhibits mRNA expression of ET-1 system, VEGF and VEGFR-2 in newly formed pig corpora lutea, even though it does not seem to affect VEGF levels in luteal tissue. Furthermore, feed restriction exerts a stimulatory effect on luteal steroidogenesis. According to our experimental model, we evaluated only one time point after fasting; therefore, we may hypothesize that the discrepancy between VEGF luteal levels and VEGF mRNA expression could depend on a temporal shift between the activation of a mechanism of adjustment of luteal cells at the level of gene and protein expression. The overall effects of fasting on luteal function may be clarified in the future by studying other luteal phases (i.e., luteal regression, when ET-1 system should be maximally expressed).

Acknowledgement

This work was supported by a MIUR-COFIN grant.

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EXPERIMENT II

Gelatinases, endonuclease and Vascular Endothelial Growth Factor during development and regression of swine luteal tissue.

BMC Developmental Biology, 2006, 6(1):58.

BMC Developmental Biology

Research article

Gelatinases, endonuclease and Vascular Endothelial Growth Factor during development and regression of swine luteal tissue

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Published: 30 November 2006

BMC Developmental Biology 2006, **6**:58 doi:10.1186/1471-213X-6-58

Received: 28 July 2006
Accepted: 30 November 2006

This article is available from: http://www.biomedcentral.com/1471-213X/6/58

Abstract

Background: The development and regression of corpus luteum (CL) is characterized by an intense angiogenesis and angioregression accompanied by luteal tissue and extracellular matrix (ECM) remodelling. Vascular Endothelial Growth Factor (VEGF) is the main regulator of angiogenesis, promoting endothelial cell mitosis and differentiation. After the formation of neovascular tubes, the remodelling of ECM is essential for the correct development of CL, particularly by the action of specific class of proteolytic enzymes known as matrix metalloproteinases (MMPs). During luteal regression, characterized by an apoptotic process and successively by an intense ECM and luteal degradation, the activation of Ca^{++}/Mg^{++}-dependent endonucleases and MMPs activity are required. The levels of expression and activity of VEGF, MMP-2 and -9, and Ca^{++}/Mg^{++}-dependent endonucleases throughout the oestrous cycle and at pregnancy were analyzed.

Results: Different patterns of VEGF, MMPs and Ca^{++}/Mg^{++}-dependent endonuclease were observed in swine CL during different luteal phases and at pregnancy. Immediately after ovulation, the highest levels of VEGF mRNA/protein and MMP-9 activity were detected. On days 5–14 after ovulation, VEGF expression and MMP-2 and -9 activities are at basal levels, while Ca^{++}/Mg^{++}-dependent endonuclease levels increased significantly in relation to day 1. Only at luteolysis (day 17), Ca^{++}/Mg^{++}-dependent endonuclease and MMP-2 spontaneous activity increased significantly. At pregnancy, high levels of MMP-9 and VEGF were observed.

Conclusion: Our findings, obtained from a precisely controlled in vivo model of CL development and regression, allow us to determine relationships among VEGF, MMPs and endonucleases during angiogenesis and angioregression. Thus, CL provides a very interesting model for studying factors involved in vascular remodelling.

Background

The growth rate of corpora lutea (CL) immediately after ovulation is 4- to 20-fold more intense than that of some of the most malignant human tumours and is sustained by the formation of new blood vessels from capillaries of the theca interna compartment [1]. Actually, 85% of the dividing cells in the primate corpus luteum during the early luteal phase are endothelial cells [2]. The duration of this intense angiogenic phase in the corpus luteum varies among species, but appears to be completed by day 6 of the luteal phase in primates when capillaries surround almost all luteal cells and capillary dilation is evident [3]. Vascular Endothelial Growth Factor (VEGF) effect on endothelial cells mitosis and differentiation is considered fundamental in vascular bed development. We have previously determined the ability of swine granulosa and theca cells of growing follicles as well as luteal cells to produce VEGF [4,5].

After the stimulation of endothelial cells mitosis for neovascular tubes development, the remodelling of extracellular matrix is necessary and different proteolytic enzymes are involved [6]. The extracellular matrix (ECM) has become recognized as a key regulatory component in cellular physiology, providing an environment for cell migration, division, differentiation, anchorage, and in some cases, an ultimate fate between cell survival or cell death [7]. The highly regulated control of ECM turnover and homeostasis occurs, in part, by the action of a specific class of proteolytic enzymes known as the matrix metalloproteinases (MMPs). The MMPs and their associated endogenous inhibitors act in concert to control aspects of reproductive function. In the ovary and uterus, the MMP system has been postulated to regulate all the dynamic structural changes that occur throughout the oestrous cycle [8].

To allow repeated opportunities of fertilization, the duration of CL life is rigorously programmed, in fact, after a first period of rapid growth the tissue becomes stably organized and prepares to switch to the phenotype required for its next apoptotic regression [9,10]. Five to ten grams of luteal tissue growth and disappear at each ovarian cycle (21 days in sow).

The switch between growth and regression is mainly regulated by LH (luteinising hormone – luteotrophyn) and PGF2α (prostaglandin F2α – luteolysin) and their receptors balance as well as by cytokines, growth factors, apoptosis/oncogenes related factors and plasminogen activator/matrix metalloproteinase activators and inhibitors [10-12]. If fertilization has not occurred, or implantation was unsuccessful, or the pregnancy ends, luteolysis is initiated whereby the CL rapidly loses its progesterone-producing ability followed by degradation of luteal tissue [13]. Luteal regression is thought to occur through apoptotic [9,14] and proteolytic [15] mechanisms; however, the molecular mechanisms underlining this event are not well characterized [8,16]. Apoptosis requires a wide extracellular matrix remodelling [17] as well as the action of a Ca^{++}/Mg^{++}-dependent endonuclease, that is developmentally regulated in rat luteal cell nuclei [18], leading to the degradation of genomic DNA into discrete oligonucleosome fragments. Therefore angiogenesis and apoptosis and their regulation in the CL play a fundamental role in the maintenance of reproductive performances. Furthermore, the ovary is the unique organ where strictly regulated tissue hyperplasia and regression take place in a cyclic manner under physiological conditions.

This study was aimed at characterizing the temporal expression pattern of VEGF, MMP-2, MMP-9 and Ca^{++}/Mg^{++}-dependent endonuclease throughout the lifespan of swine CL and investigating whether the expression levels of those molecules are related to CL functional stage.

Results

CL functional stage assessment

Both luteal progesterone (P4) concentration and prostaglandin F2α receptor (FPr) mRNA expression followed typical swine luteal phase patterns. P4 concentrations increased gradually during the formation of CL, reaching the highest level during the mid-late phase (days 10 and 14), and then declined significantly on day 17 (Fig 1A). Similarly, FPr mRNA levels increased gradually to a greater extent on day 14 (3.4-fold in relation to day 1, Fig 1B). At pregnancy, high levels of P4 and FPr mRNA (7.7-fold increase in relation to day 1) were observed.

DNase activity assay

A regulated Ca^{++}/Mg^{++}-dependent endonuclease expression and activity were detected in swine CL. Fig 2A illustrates the Ca^{++}/Mg^{++}-dependent endonuclease activity found in luteal nuclei obtained at days 1, 14 and 17. A marked activity was obtained with both Ca^{++} and Mg^{++} whereas the addition of Zn^{++} inhibited almost completely the enzyme activity.

The amount of activable DNase gradually increased during the oestrous cycle, maintaining high at pregnancy. Moreover, a high level of spontaneous apoptosis in the control group (No Salt) at day 17 was also observed (Fig 2B).

VEGF content

Both VEGF mRNA and protein presented similar patterns of expression throughout the oestrous cycle. The highest values of VEGF were observed immediately after ovulation, decreasing significantly at day 3, and remaining unchanged during the mid-luteal phase. At day 17, a sec-

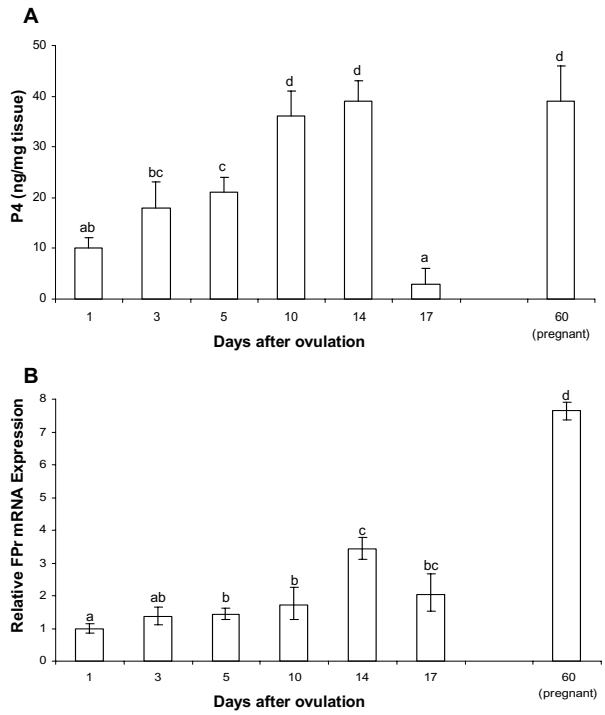

Figure 1
P4 and FPr mRNA levels in swine CL during different luteal phases and pregnancy. A: Changes in P4 levels (mean ± SEM). B: Changes in FPr mRNA expression in relation to day 1; error bars represent the range of relative expression. The statistical analysis were based upon the mean of 5 CLs per animal (n = 4/time point). Different letters represent significant differences ($p < 0.05$).

ond significant drop was observed. At pregnancy VEGF mRNA and protein levels were intermediate between those of day 1 and 3 (Fig 3).

MMPs activity assay

Three distinct bands of gelatinase activity corresponding to latent MMP-9 (proMMP9), latent MMP-2 (proMMP2) and active MMP-2 (actMMP2) were evidenced in the swine CL (Fig 4A). Gelatinolytic activities for both latent and active forms of MMP-2 were considered together for the analysis. The activities of MMP-2 and MMP-9 changed over the luteal phase. Constant levels of MMP-2 activity were observed during CL formation, increasing significantly in late (day 17) luteal phase (Fig 4B). In contrast, MMP-9 activity peaked in the early (days 1–3) and late (day 17) luteal phase, showing the lowest values in the midluteal phase (days 10–14 – Fig 4C). At pregnancy, basal levels of MMP-2 and high levels of MMP-9 were observed.

Discussion

The CL is a transient endocrine gland, which is formed from the remnants of an ovulated follicle [13]. During this process, a capillary network invades from the theca tissues into the granulosa layers through a dynamic angiogenesis process such that after its formation, the CL is one of the most vascularised organs in the body [16]. During VEGF driven angiogenesis, microvascular endothelial cells produce gelatinases (MMP-2 and -9), which breach the perivascular basement membrane and allow endothelial cells to migrate outward through the extracellular space [19-21]. MMPs are also involved, together with Ca^{++}/Mg^{++}-dependent endonuclease, in apoptotic tissue remodelling at luteolysis.

In this study, proteases with gelatinolytic activity that is consistent with MMP-9 and MMP-2 family members [7] were identified in CL collected during the oestrous cycle and at pregnancy. The gradual increase of FPr mRNA expression, peaking on day 14, together with the accumulation of Ca^{++}/Mg^{++}-dependent endonuclease, confirms the competence acquired by the luteal tissue around this moment to rapidly react to PGF2α and to initiate the regression. These characteristics are maintained in the pregnant CL which is always sensible to PGF2α. Moreover, elevated concentrations of P4 at day 14 corroborate the CL ability to sustain an eventual pregnancy.

Members of MMP-system may be involved in several of the proteolytic events that take place in the ovary during the reproductive cycle [16]. MMP-2 and MMP-9 have previously been detected by zymography in homogenates of rat ovaries [22,23], and in CL of bovine [24], human [15], primates [25], mouse [26], ovine [27] and swine [17]. The main role proposed for MMPs concerns the tissue remodelling associated to luteolysis.

Our findings demonstrated the presence of MMP-2 and MMP-9 in the corpus luteum during different ovarian phases. MMP-2 activity was basal during early-mid luteal phase and at pregnancy while was maximal during luteolysis. MMP-2 plays a role in various tissue remodelling processes, including trophoblast invasion [28] and tumour cell motility [20]. However, a persistence of MMP-2 activity throughout the CL formation supports suggestions that tissue remodelling continues throughout CL development and maintenance.

Concerning MMP-9 activity, high levels were observed during early luteal phase, luteolysis and pregnancy. MMP-9 is probably involved in the extensive tissue remodelling that occurs during CL formation, when extracellular material, composed primarily of follicular elements that represent a basement membrane-type ECM, is removed [15,29]. This clearance may create a more hospitable or

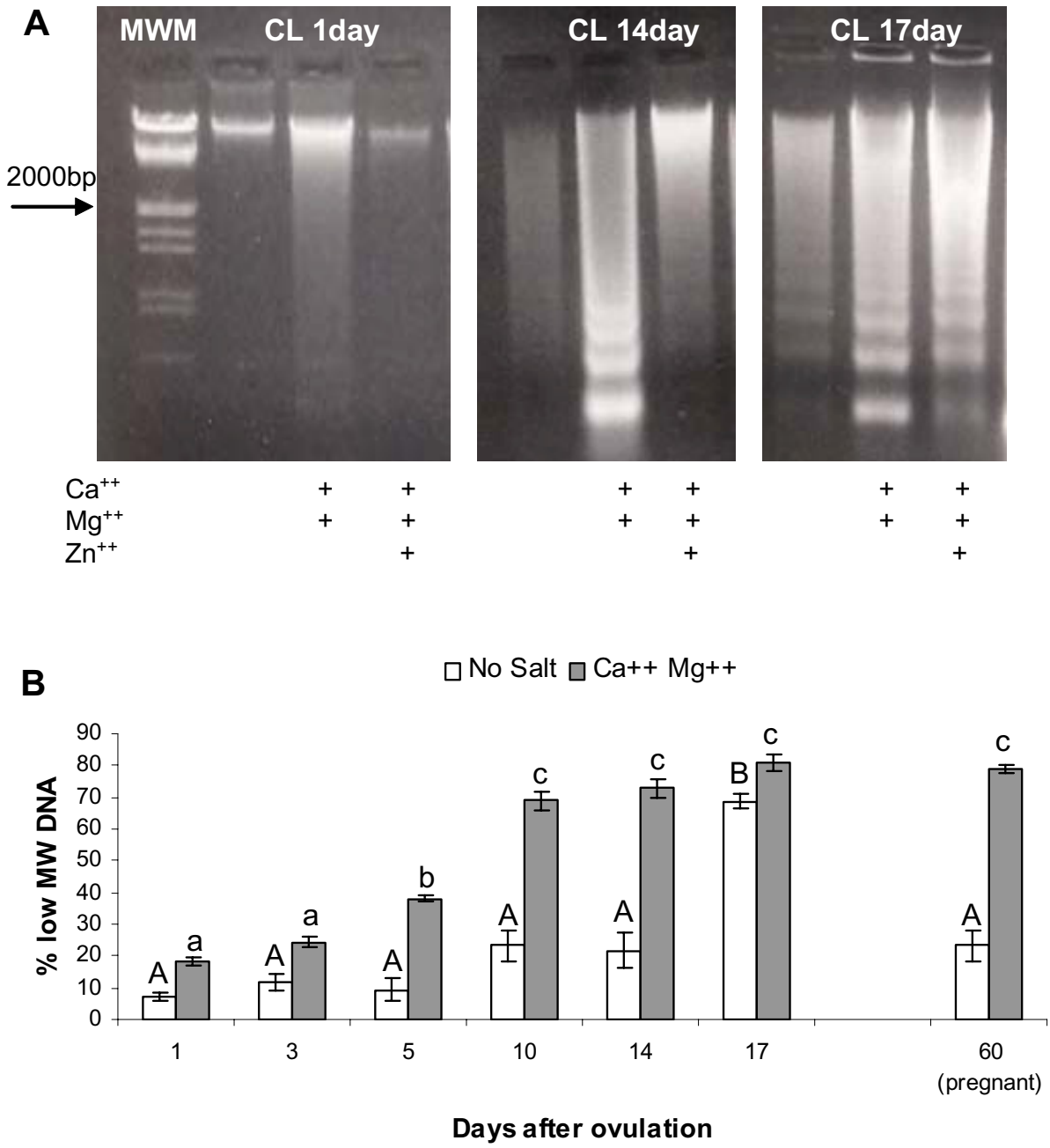

Figure 2
A: Representative electrophoresis profile of low molecular weight DNA from CL at day 1, 14 and 17. Each lane contains 10ug of DNA extracted from luteal nuclei after treatment with different cations. B: Nuclease activity in swine CL nuclei during different luteal phases and pregnancy. For each time point only No Salt (open bars) and $Ca^{++}+Mg^{++}$ (closed bars) samples were presented. Data represent means ± SEM of percentage of low molecular weight DNA (≤ 2000 bp). The statistical analysis were based upon the mean of 5 CLs per animal (n = 4/time point). Different capital and small letters represent significant differences ($p < 0.05$) for No Salt and $Ca^{++}+Mg^{++}$ groups, respectively.

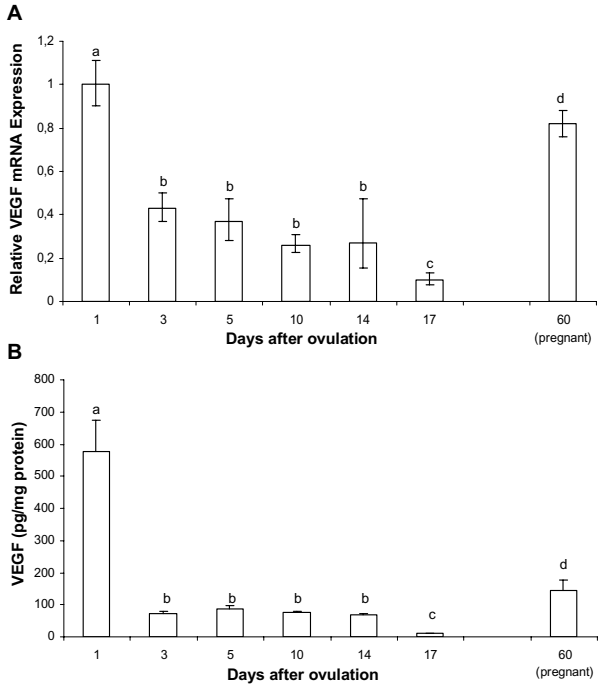

Figure 3
VEGF mRNA and protein levels in swine CL during different luteal phases and pregnancy. A: changes in VEGF mRNA expression in relation to day 1; error bars represent the range of relative expression. B: changes in VEGF content (mean ± SEM). The statistical analysis were based upon the mean of 5 CLs per animal (n = 4/time point). Different letters represent significant differences ($p < 0.05$).

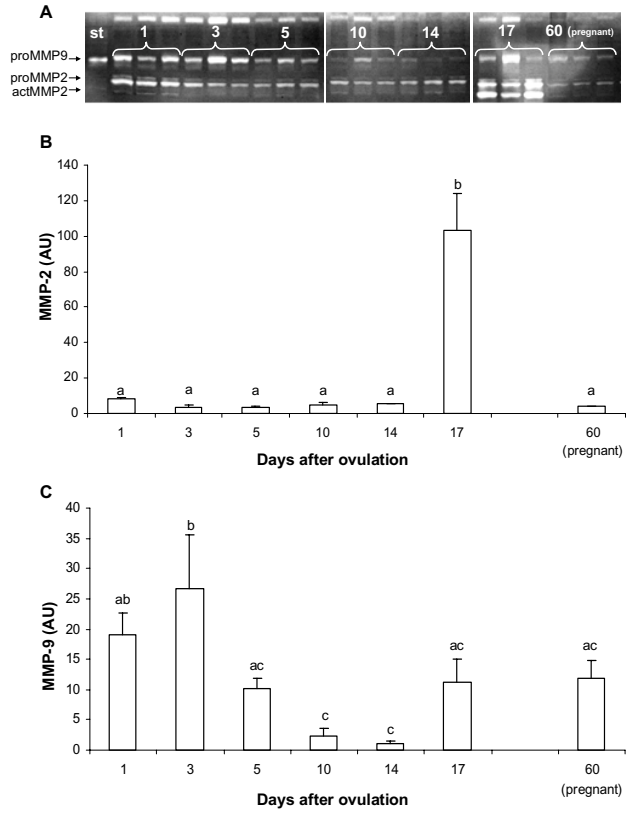

Figure 4
A: Representative gelatin substrate zymography gel showing gelatinase activity in swine CL during different luteal phases and pregnancy. B: Relative abundance of MMP-2 as determined by densitometric analysis expressed as means ± SEM. C: Relative abundance of MMP-9 as determined by densitometric analysis expressed as means ± SEM. The statistical analysis were based upon the mean of 5 CLs per animal (n = 4/time point). Different letters represent significant differences ($p < 0.05$).

spacious environment for the subsequent rapid proliferation and intermingling of luteal cells [21,30] and development of luteal ECM [31]. This hypothesis is supported by the findings that it is the primary metalloproteinase detected in follicles explants [32]. In addition, MMP-9 is the major MMP secreted into the culture medium of luteinised bovine [33] and human granulosa cells [34].

A different activity profiles for both MMP-2 and MMP-9 were also observed in human [15] and bovine [21] corpus luteum. In the porcine CL, mRNA expression for MMP-2 and MMP-9 was low during early luteal period (days 6–8 of the oestrous cycle), increasing significantly during mid-luteal period (days 9–11), and reaching the highest values during the late luteal period (days 13–15) [17]. These results agree well with ours, considering that the authors did not studied the earliest period of oestrous cycle (days 1–5 after ovulation), period in which we verified major alterations of MMP-9 activity.

In the early luteal phase, we also detected the highest levels of VEGF. As expected, VEGF mRNA and protein were detected during all days of the oestrous cycle and showed similar pattern of expression. High levels were verified immediately after ovulation, concomitantly with the intense luteal vascular growth and CL formation. Thus supporting the role of VEGF in the angiogenesis of the newly formed CL. Changes of VEGF levels in CL during the oestrous cycle have been reported also in other species [35-39]. Those reports, in accord with our results, demonstrate that the highest VEGF mRNA expression and VEGF protein concentration are detected during the early luteal phase, and are followed by a significant decrease of expression during the mid and late luteal phases.

Taken together, our results provide that in addition to intense angiogenesis (characterized by maximal VEGF concentration), elevated luteal gelatinases may contribute to the extensive luteal ECM and tissue remodelling that occurs as the postovulatory follicle is transformed into the CL [15,21]. Many studies relative to neoplastic growth well describe the interplay between VEGF and MMPs [40-43]. Bergers et al. [44] demonstrated that MMP9 is able to mobilize matrix attached VEGF isoforms and this action results essential for the switch between vascular quiescence to angiogenesis during carcinogenesis. Another study showed that VEGF regulated ovarian cancer invasion through secretion and activation of MMPs [45].

After the CL is fully formed, steroidogenesis is maximal during the midluteal period, and MMP activity and VEGF concentration are at basal levels. Interestingly, we previously reported an increased expression of the less abundant and matrix-attached VEGF splice variants (VEGF188 and VEGF182) as well as of the two VEGF receptors in this moment [5]. In this phase, luteal tissue prepares itself to the next step cumulating high levels of FPr and endonuclease making the tissue very sensitive to luteolytic stimulus. With the onset of structural regression, the MMPs are again called into action for the remodelling and removal of the CL [7].

The elevated expression of VEGF associated to the presence of MMP-9 at pregnancy suggests that initial angiogenic process during the early luteal phase may be renewed in swine pregnant CL. Increased angiogenesis at pregnancy in luteal tissue is controversial. Wulff et al. [46] demonstrated that luteal rescue is associated with a second wave of angiogenesis in human CL, while Rowe et al. [47] stated that no pregnancy-induced angiogenesis take place in marmoset CLs. Certainly, a stable and efficient vascular bed are required for the endocrine function of pregnant CL. Pauli et al. [48] reported that the administration of anti-VEGFR-2 antibody during the pre- and post-implantation periods in rodents, disrupted maternal ovarian function eliminating pre-existing luteal blood vessels. Thus, VEGF/VEGFR-2 pathway is critical to maintain the functionality of luteal blood vessels during pregnancy and might also be involved in regulating vascular permeability and P4 release into the bloodstream [49]. Therefore, the increase in VEGF at pregnancy is not unexpected but the increase in MMP-9 needs further clarifications. The different profile of VEGF/MMP-9 expression observed between fully formed (midluteal phase) and pregnant CLs should be taken into account when physiological effects will be monitored.

Conclusion

We have demonstrated that CL development and regression is a very useful model for studying VEGF/MMPs relationships. During the very early luteal phase, high MMPs activities coupled with high VEGF levels drive the tissue to an "angiogenic phenotype", allowing CL growth under LH stimulus. In the late luteal phase, low VEGF and elevated MMPs levels may play role in the apoptotic tissue and ECM remodelling during structural luteolysis.

Methods

Animal model

Synchronized folliculogenesis was obtained in 28 prepubertal Large White gilts (96 ± 2.13 Kg, mean ± SEM) by administration of 1250 IU equine chorionic gonadotropin (eCG; Folligon, Intervet, Holland) and 750 IU hCG (Corulon, Intervet) 60 h later. In this model, ovulation occurs around 42–44 h after hCG administration (day 0). Four animals were artificially inseminated 40 h after hCG administration and pregnancies were determined by ultrasonography 35 days after. Ovaries were recovered by surgical laparotomy on days 1, 3, 5, 10, 14 and 17 after ovulation (n = 4 animals/time point) and at day 60 of pregnancy. Animals were pre-anesthetized by using azaperone (240 mg/gilt; Stresnil, Janssen, Belgium) and atropine sodium salt (2 mg/gilt; Industria Galenica Senese, Italy), and maintained under thiopental sodium (1.5 g/gilt; Pentothal Sodium; Gellini, Latina, Italy) anaesthesia. Five CLs from each gilt, chosen totally random from both ovaries, were isolated, cut in three parts with a razor blade and employed to perform all the analysis.

One third was weighed and homogenized in PBS (0.1 g/ml) on ice bath by an Ultra Turrax. The homogenate obtained was processed as follows: 500 μl were centrifuged at 2000 × g for 10 min at 4°C and supernatant was stored at -20°C until VEGF determination and the remainder of the homogenate was kept frozen until progesterone (P4) measurement and MMPs activity evaluation.

Total RNA was isolated from the second third of the CLs with the Tri-Pure reagent (Roche Diagnostic GmBH, Mannheim, Germany) and stored at -80°C until VEGF and Prostaglandin F2α receptor (FPr) mRNA levels quantification. The last part of samples was immediately processed for nuclei extraction and Ca^{++}/Mg^{++}-dependent endonuclease activity evaluation. All the sampling procedures were executed within 2 hours from the surgical removal of the ovaries.

All animals were housed and used according to EEC animal care guidelines. The experimental procedures had been previously approved by the Ethical Committee of Bologna University.

P4 assay

Aliquots of 20 μl from each homogenate CL were extracted with 5 ml petroleum ether. After centrifugation, ether was collected and dried under a N_2 stream. Dried ether extracts were resuspended in 1 ml phosphate buffer, diluted 1:50 and aliquots of 50 μl were then assayed by a validated RIA as previously described [9].

The sensitivity of the assay was 3.7 pg/tube. The intra- and interassay coefficients of variation were 6.3 and 19.6%, respectively. The results are expressed in ng/mg tissue.

RNA extraction and Real-time quantitative RT-PCR

Total RNA from CLs, homogenized in Tri-Pure reagent (50 mg/ml), was extracted according to manufacturer's instructions (Roche Diagnostic GmBH, Mannheim, Germany). Purified RNA was resuspended in RNase-free water and quantified (A_{260} nm). One microgram of total RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-RAD Laboratories Inc., CA, USA) in a final volume of 20 μl, according to the manufacturer's instruction. Transcription reactions without reverse transcriptase were performed to control for an eventual DNA contamination.

Swine primers were designed for VEGF, FPr and HPRT (Hypoxanthine Guanine Phosphorybosyl Transferase), using the Beacon Designer 3.0 Software (Premier Biosoft International, Palo Alto, Ca, USA). Their sequences, expected PCR product length and accession number are shown in Table 1. Real-time quantitative PCR was performed in the iCycler Thermal Cycler (Bio-RAD Laboratories Inc., Hercules, CA, USA) using SYBR green I detection. The following reaction components was prepared to the indicated end-concentrations: 0.6 μM of each primer, 1X IQ SYBR Green BioRad Supermix (Bio-RAD Laboratories Inc.), 150 ng of cDNA and H_2O nuclease free to a final volume of 25 μl. All samples were performed in duplicate for all genes. The two step real-time PCR protocol employed was: initial denaturation for 3 min at 95°C, 40 cycles at 95°C for 15 sec and 60°C for 30 sec, followed by a melting step with a slow heating from 55 to 95°C with a rate of 0.05°C/s. The specificity of the amplified PCR products was verified by analysis of the melting curve, which is product-specific. The relative mRNA level was determined as the PCR cycle number that crosses an arbitrarily placed signal threshold (Ct). The Ct value correlates inversely with the amount of target mRNA in the sample. The housekeeping gene HPRT was used to normalize the amount of RNA. The relative changes in VEGF and FPr expressions were examined using the ΔΔCt method described previously [50], with $\Delta Ct = Ct_{target} - Ct_{HPRT}$ and $\Delta\Delta Ct = \Delta Ct_{(days\ 3,5,10,14,17,60)} - \Delta Ct_{(day\ 1)}$. As PCR amplification is an exponential process, a ΔΔCt difference denotes a shift in regulation by a factor of two ($2^{-\Delta\Delta Ct}$).

Real-time efficiencies were acquired by amplification of a standardised dilution series and corresponding slopes and PCR efficiencies were calculated using iCycler iQ Real Time PCR Detection System (Bio-Rad Laboratories Inc.).

Ca^{++}/Mg^{++}-dependent endonuclease activity assay

Tissues were immediately treated to isolate nuclei and to determine endonuclease activity in the samples [51].

In brief, minced tissue was homogenized (1/10, w/v) with a Dounce homogenizer in a homogenization solution containing 10 mM Tris-Cl (pH 7.4), 3 mM $MgCl_2$, 3 mM EGTA, and 250 mM sucrose. The homogenate was filtered and then centrifuged at 800 g for 15 min at 4°C. The resulting pellet was resuspended in the homogenization solution supplemented with 0.5% (v/v) nonidet P40, incubated for 15 min at 4°C and then centrifuged at 800 g for 15 min at 4°C. The resulting pellet was resuspended in a solution containing 10 mM Tris-Cl (pH 7.4), 25 mM NaCl, and 340 mM sucrose. The reaction mixture for the DNA fragmentation assay was performed with 30 mg of luteal tissue and 1 mM Ca^{++} and 5 mM Mg^{++}, with or without the addition of 2 mM Zn^{++}. A control reaction, without salts, was also carried out. The reactions were performed at 37°C for 10 min, after which low and high molecular weight DNA were extracted; residual RNA was removed by addition of RNase A. DNA content was evaluated by densitometric scanning under a UV transilluminator after 2% agarose gel electrophoresis run. Low

Table 1: Sense and antisense primers sequences used for real time RT-PCR.

Primer	Sequence (5'-3')	Product size (bp)
HPRT sense	GGACAGGACTGAACGGCTTG	
HPRT antisense	GTAATCCAGCAGGTCAGCAAAG	115
VEGF sense*	CCTTGCCTTGCTGCTCTACC	
VEGF antisense*	CGTCCATGAACTTCACCACTTC	101
FPr sense	TCAGCAGCACAGACAAGG	
FPr antisense	TTCACAGGCATCCAGATAATC	151

*VEGF primers were located on a common region for all VEGF isoforms.

molecular weight DNA (≤ 2000 bp) was expressed as percent of total DNA in the sample.

VEGF assay

Luteal VEGF concentrations were measured in 100 μl samples of homogenate supernatants by a specific enzyme linked immune-adsorbent assay (ELISA, Quantikine, R&D Systems, Minneapolis, MN, USA) previously validated for the measurement of porcine VEGF [4]. This highly specific sandwich assay recognizes VEGF164 as well as VEGF120, while it exhibits negligible cross-reactivity with all cytokines/growth factors tested. A 96-well plate reader (Biomek 1000, Beckman Instruments, Fullerton, CA, USA) set to read at 450 nm emission was used to quantify the results. The sensitivity of the assay was 5 pg/ml, and the intra- and interassay coefficients of variation were less than 6 and 10%, respectively. All data are expressed as pg/mg protein; protein concentration was determined according to Lowry method [52], using a protein assay kit (Sigma Diagnostics, St Louis, MO, USA).

MMPs activity assay

MMP-2 and -9 activities were analyzed by use of gelatin zymography on 10% Tris-Glycine poliacrylamide pre-cast gels with 0.1% gelatin (10% Novex Zymogram Gels, Invitrogen U.K.). Aliquots containing 30 μg of total proteins, mixed with an equal volume of sample buffer (Novex Tris-Glycine SDS sample Buffer 2X, Invitrogen U.K.) were loaded into the gel. Electrophoresis was performed under non-reducing conditions at a constant voltage (125 V for 120 minutes). Following electrophoresis, gels were washed for 30 minutes in Novex Zymogram Buffer (Invitrogen U.K.), equilibrated at room temperature for 30 minutes in developing buffer (Novexα Zymogram Developing Buffer, Invitrogen U.K.) and then incubated at 37°C for 22–24 hours in fresh developing buffer. Band of gelatinolytic activity were developed after staining gels for 6–8 hours with Simply Blue Safe stain (Invitrogen U.K.) by comparison with a MMP-2 and -9 human standard (Chemicon International, CA, USA).

Gel images were captured with a computerized system (Geldoc 1000, Bio-Rad), and gelatinolytic bands were measured with densitometric analysis software (Quantity One, Bio-Rad). The resulting data are expressed as arbitrary units (AU).

Statistical analysis

The statistical analysis were based upon the mean of 5 CLs per animal (n = 4/time point), since no significant differences among CLs within the same animal were observed. Differences in relative mRNA expression of VEGF and FPr (using the ΔCt values), VEGF protein levels, progesterone contents and MMPs and Ca++/Mg++-dependent endonuclease activities were determined using one-way ANOVA (SPSS Version 13.0, Inc, Chicago, IL, USA), followed by the Duncan's post-hoc test. Data are presented as mean ± SEM. A value of $p < 0.05$ was considered significant.

Authors' contributions

All authors participated in experimental design and collected biological material. LAR carried out RNA extraction and real-time RT-PCR. MET performed MMPs activity and VEGF assays. AZ carried out endonuclease activity and P4 assays. MLB was responsible for animal care and surgical procedures. MF conceived and supervised the study. LAR and MF wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by grants from MIUR COFIN and CITO (Consorzio Interuniversitario Trapianti d'Organo).

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EXPERIMENT III

Characterization and differential expression of Vascular Endothelial Growth Factor isoforms and receptors in swine corpus luteum throughout estrous cycle.

Molecular Reproduction and Development, 2007, 74:163-171

Characterization and Differential Expression of Vascular Endothelial Growth Factor Isoforms and Receptors in Swine Corpus Luteum Throughout Estrous Cycle

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ABSTRACT Corpus luteum (CL) undergoes growth and regression during each estrous cycle; these processes are accompanied by growth and regression of the luteal vascular bed. Vascular endothelial growth factor (VEGF) is the main regulator of angiogenesis, inducing endothelial cell proliferation, migration, vascular permeability, and vessel lumen formation. VEGF presents several isoforms that are produced by alternative splicing of the same mRNA transcript. We determined by real time RT-PCR the expression patterns of VEGF isoform and receptor mRNAs, as well as the VEGF protein levels in pig CL throughout a whole estrous cycle. Four novel VEGF isoforms (VEGF144, VEGF147, VEGF182, and VEGF164b) were found for the first time in swine and the seven identified isoforms can be grouped in four different patterns of expression. The most expressed splice variants were VEGF120 and VEGF164. All isoforms showed their highest mRNA levels in newly formed CLs (day 1), followed by a decrease during mid-late luteal phase (days 10–17), except for VEGF182, VEGF188 and VEGF144 that showed a differential regulation during late luteal phase (day 14) or at luteolysis (day 17). VEGF protein levels paralleled the most expressed and secreted VEGF120 and VEGF164 isoforms. The VEGF receptors mRNAs showed a different pattern of expression in relation to their ligands, increasing between day 1 and 3 and gradually decreasing during the mid-late luteal phase. The differential regulation of VEGF isoforms may suggest specific physiological roles for some of them, particularly in angioregression occurring during the apoptotic structural luteolysis. *Mol. Reprod. Dev.*

Key Words: VEGF isoforms; VEGFR1; VEGFR2; corpus luteum; pig; Real Time

INTRODUCTION

Vascular endothelial growth factor (VEGF) is the fundamental regulator of angiogenesis both in physiological and pathological conditions including tumorigenesis (Neufeld et al., 1999; Grunstein et al., 2000). VEGF exerts its effects by stimulating endothelial cell migration, proliferation, and vessel lumen formation; it is also a potent vasodilator and a mediator of microvascular permeability (Robinson and Stringer, 2001; Bates et al., 2002b).

In adult tissues, angiogenesis is a highly controlled phenomenon and the female reproductive cycle is one of the few examples in which angiogenesis occurs at a rapid rate leading to describe the corpus luteum (CL) as a "transitory tumor" (Zhang et al., 2005).

The ovarian cycle is characterized by repeated patterns of cellular proliferation and differentiation that accompany follicular development and atresia as well as CLs formation and regression. Associated with CL lifespan are strong alterations in luteal vascular bed, being VEGF the most important regulator factor (Ferrara and Davis-Smyth, 1997).

In human, at least eight VEGF isoforms (VEGF121, VEGF145, VEGF148, VEGF165, VEGF165b, VEGF183, VEGF189, and VEGF206) are generated by alternative splicing of a single *VEGF* mRNA. The domain encoded by exons 1–5, conserved in all VEGF isoforms, contains the VEGF receptor [VEGFR-1/Flt-1 (fms-like-tyrosine kinase) and VEGFR-2/Flk-1 (fetal liver kinase-1)] binding sites. VEGF isoforms are distinguished by the presence or the absence of the peptides encoded by exons 6a, 6b, 7a and 7b of the *VEGF* gene (Fig. 1). VEGF121 lacks all these exons, VEGF189 lacks only the exon 6b, VEGF165 lacks exons 6a and 6b (Tischer et al., 1991), while VEGF145 lacks exons 6b, 7a and 7b (Poltorak et al., 1997). A conserved alternative splicing donor site within exon 6a originates the VEGF183 isoform; as a consequence, an 18-bp section from the C-terminal of the exon 6a is missing (Lei et al., 1998). VEGF148 lacks

Grant sponsor: MIUR PRIN; Grant sponsor: Consorzio Interuniversitario Trapianti d'Organo.

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Received 26 April 2006; Accepted 8 June 2006
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20589

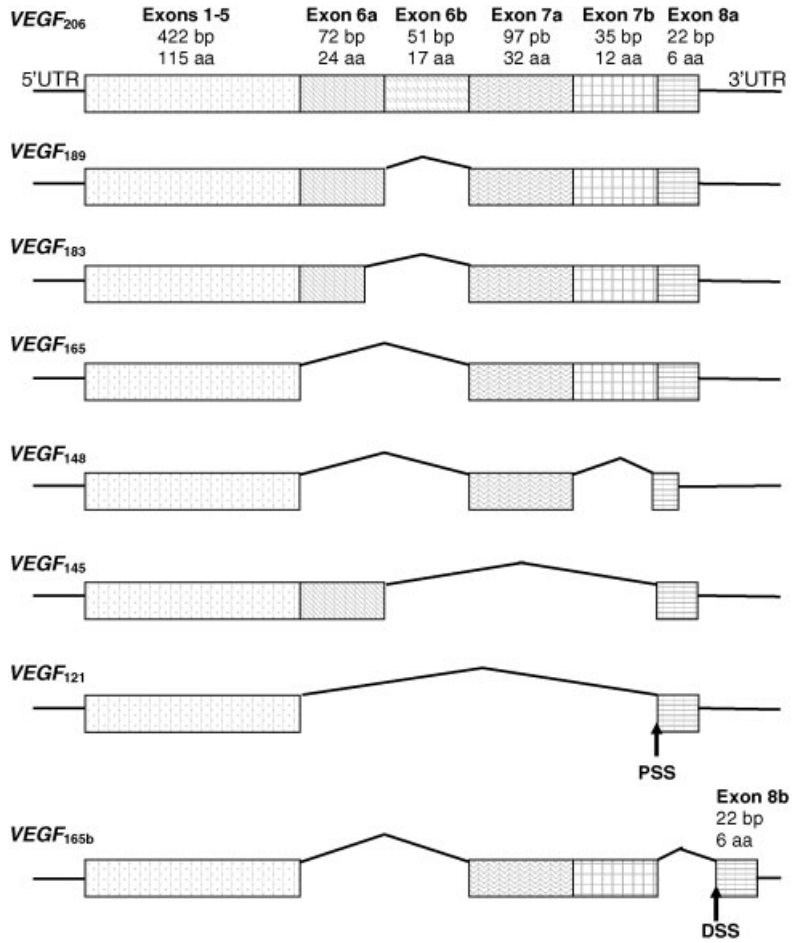

Fig. 1. Reported model for human VEGF pre-mRNA that generates the alternative splicing variants (Woolard et al., 2004). Exon 3 and 4 contain VEGFR-1 and VEGFR-2 binding sites, respectively; exon 6 and 7, heparin binding domains (UTR, untranslated region; PSS, proximal splice site; DSS, distal splice site).

exons 6a, 6b, and 7b, changing the reading frame and producing a premature stop codon into exon 8a (Whittle et al., 1999). VEGF206 is the full-length form (Houck et al., 1991).

More recently, an inhibitor splice variant of VEGF165, named VEGF165b, has been described. This isoform is generated by a distal splice site, 66 bases downstream of the usual acceptor splice site for exon 8a, which predicts an open reading frame encoding an alternate C-terminal sequence, named exon 8b (Bates et al., 2002a; Cui et al., 2004; Woolard et al., 2004).

The VEGF isoforms exhibit different secretion patterns (despite all members having an identical signal sequence), which suggests different physiological roles. VEGF121 is a weakly acidic protein; it does not bind heparin and is freely diffusible. In contrast, VEGF189 and VEGF206 bind to heparin with high affinity and are almost completely sequestered in the extracellular matrix (ECM) and, to a lesser extent, on the cell surface. VEGF165 and VEGF145 present intermediate properties; they are predominantly secreted, but a significant fraction remains bound to the cell surface and ECM. VEGF isoforms in ECM constitute a reservoir of growth factors that can be slowly released by exposure to heparin, heparin sulfate and heparinases, or more rapidly mobilized by specific proteolytic enzymes such as plasmin and urokinase-type plasminogen activator (uPA) (Robinson and Stringer, 2001).

Most cell types produce several VEGF variants simultaneously, VEGF121 and VEGF165 being the most highly expressed isoforms (Ferrara and Davis-Smyth, 1997). VEGF145 is one of the main VEGF isoforms expressed by several cell lines derived from carcinomas of the female reproductive system, reaching levels comparable with VEGF165 (Poltorak et al., 1997). VEGF206 is a very rare isoform that has been detected in a human fetal liver cDNA library (Houck et al., 1991) and in human mast cells stimulated with calcium ionophore and phorbol ester (Grutzkau et al., 1998).

In female swine, previous studies have demonstrated the presence of three VEGF isoforms (one amino acid shorter than the human ones, Sharma et al., 1995), VEGF120, VEGF164, and VEGF188 in follicles (Barboni et al., 2000; Shimizu et al., 2002), CL (Boonyaprakob et al., 2003), oviduct and endometrium

(Welter et al., 2003); however, these reports focused on the identification of single VEGF splice variants.

This study was aimed at determining the pattern of different VEGF isoform and receptor mRNAs expression and protein levels in CL collected at different stages of an induced estrous cycle.

MATERIALS AND METHODS

Animals

Twenty-four prepubertal large white gilts, with an average weight of 100 ± 2.18 kg (mean $\pm$ SEM), were treated with 1250 IU equine chorionic gonadotropin (eCG; Folligon, Intervet, Holland) and, 60 hr later, with 750 IU hCG (Corulon, Intervet); this treatment induces ovulation 44 hr after hCG administration (Barboni et al., 2000). Animals were randomly assigned to different groups ($n = 4$) and on day 1, 3, 5, 10, 14, and 17 after ovulation, the ovaries were recovered by surgical laparotomy. The animals were preanesthetized by an injection of azaperone (240 mg/gilt; Stresnil, Janssen, Belgium) and atropine sodium salt (2 mg/gilt; Industria Galenica Senese, Italy), and maintained under thiopental sodium (1.5 g/gilt; Pentothal Sodium; Gellini, Latina, Italy) anesthesia. Immediately after removal, ovaries were transported to the laboratory where single CLs (22 ± 5 CLs/gilt), chosen totally random from both the ovaries, were isolated and cut in two halves with a razor blade. One-half was homogenized in PBS (100 mg/ml) on ice bath by an Ultra Turrax. The homogenate obtained was processed as follows: 500 to 0.5 ml were centrifuged at 2,000*g* for 10 min at 4°C and supernatant was stored at −20°C until VEGF protein determination; the remainder of the homogenate was kept frozen until progesterone measurement or Western blotting analysis. The second half of the CLs was homogenized in Tri-Pure reagent (Roche Diagnostics GmbH, Gerrmany) and stored at −80°C until RNA extraction. Five CLs from each gilt were randomly selected to perform the analysis. These CLs were quite homogeneous as for morphology and weight.

All animals were housed and used according to EEC animal care guidelines. The experimental procedures had previously been submitted to and approved by the Ethical Committee of Bologna University.

Measurement of Luteal VEGF Content

VEGF concentrations were measured in 100 μl samples of homogenate supernatants by a specific enzyme linked immune-adsorbent assay (ELISA, Quantikine, R&D Systems, Minneapolis, MN) previously validated for the measurement of porcine VEGF (Barboni et al., 2000; Galeati et al., 2003). This highly specific sandwich assay recognizes VEGF164 as well as VEGF120, while it exhibits negligible cross-reactivity with all cytokines/growth factors tested. A 96-well plate reader (Biomek 1000, Beckman Instruments, Fullerton, CA) set to read at 450 nm emission was used to quantify the results. The sensitivity of the assay was 5 pg/ml, and the intra- and interassay coefficients of variation were 5.6% and 9.8%, respectively. All data are expressed as pg/mg protein; protein concentration was determined according to Lowry method (Lowry et al., 1951) using a Protein Assay Kit (Sigma Diagnostics, St. Louis, MO).

Western Blot

Protein homogenates (30 μg) were separated on NuPage 4%–12% Bis-Tris Gel (Invitrogen, Paisley, UK) for 60 min at 200 V. Proteins were then electrophoretically transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, UK). Blots were washed in PBS and protein transfer was checked by staining the nitrocellulose membranes with 0.2% Ponceau Red and the gels with Comassie Blue. Non-specific protein binding on nitrocellulose membranes was blocked with 5% milk powder in PBS-T20 (phosphate buffer saline, 0.1% Tween-20) for 1 hr at RT. The membranes were then incubated with rabbit polyclonal antibodies against human VEGFR-1 (1/100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse VEGFR-2 (1/100, Santa Cruz Biotechnology, Inc.) and human HPRT (1/250, Santa Cruz Biotechnology, Inc.) in Tris Buffered Saline-T20 (TBS-T20, 20 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.1% T20) overnight at 4°C. After several washings with PBS-T20 the membranes were incubated for 1 hr with a goat anti-rabbit IgG biotin conjugate antibody (1/80,000, Stressgen Biotechnologies, Inc., San Diego, CA) and then with a 1/1,000 dilution of an anti-biotin horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology, Inc., Danvers, MA). The Western blots were developed using a chemiluminescent substrate (Bio-Rad Laboratories, Inc., Hercules, CA), according to the manufacturer's instructions. The relative protein content was determined by the density of the resultant bands and expressed in arbitrary units (AU) relative to the HPRT content, using the Quantity One Software (Bio-Rad Laboratories, Inc.).

Progesterone (P4)

Aliquots of 20 μl from each homogenate CL were extracted with 5 ml petroleum ether. After centrifugation, ether was collected and dried under a N2 stream. Dried ether extracts were resuspended in 1 ml phosphate buffer; diluted 1:50 and aliquots of 50 μl were then assayed by validated RIA as previously described (Galeati et al., 2005).

The sensitivity of the assay was 3.7 pg/tube. The intra- and interassay coefficients of variation were 6.1% and 10.3%, respectively. The results are expressed as ng/mg tissue.

Total RNA Extraction and Reverse Transcription

Total RNA from CLs (5 CLs/gilt), homogenized in Tri-Pure reagent (50 mg/ml), was extracted according to manufacturer's instructions. Purified RNA was resuspended in 25 μl of RNAse-free water and was spectrophotometrically quantified (A260 nm). One microgram of total RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-RAD

Laboratories, Inc.), in a final volume of 20 μl, according to the manufacturer's instruction. Transcription reactions without reverse transcriptase were performed to control for an eventual DNA contamination.

Real-Time PCR Quantification

Based on the human and swine VEGF sequences present in GenBank, primers were designed for the specific VEGF isoforms and VEGF receptors, VEGFR-1 and VEGFR-2, using the Beacon Designer 2.07 Software (Premier Biosoft International, Palo Alto, CA). For VEGF205, VEGF188, VEGF182, VEGF147, VEGF144, and VEGF120 splice variants, a common forward primer located on the exon 4 was designed. For VEGF164 and VEGF164b isoforms, a forward primer spanning the exons 5 and 7a boundary was employed. The specific amplification of each splice variant was performed with specific reverse primers spanning the variant specific exon boundaries (Table 1).

Real-time quantitative PCR was performed in the iCycler Thermal Cycler (Bio-RAD Laboratories, Inc.) using SYBR green I detection. A master-mix of the following reaction components was prepared to the indicated end-concentrations: 1.5 μl forward primer (0.6 μM), 1.5 μl reverse primer (0.6 μM), 6.5 μl water and 12.5 μl IQ SYBR Green BioRad Supermix 2X (Bio-Rad Laboratories, Inc.). Three μl of cDNA were added to 22 μl of the master mix. All samples were performed in duplicate for all genes. The real-time PCR protocol employed was: initial denaturation for 3 min at 95°C, 40 cycles at 95°C for 15 sec and 60°C for 30 sec, followed by a melting step with a slow heating from 55°C to 95°C with a rate of 0.05°C/sec. Real-time efficiencies were acquired by amplification of a standardized dilution series and corresponding slopes and PCR efficiencies were calculated using iCycler iQ real time PCR detection system (Bio-Rad Laboratories, Inc.). The specificity of the amplified PCR products was verified by analysis of the melting curve, which is product-specific. The relative mRNA level was determined as the PCR cycle number that crosses an arbitrarily placed cycle threshold (CT). The CT value correlates inversely with the amount of target mRNA in the sample. The housekeeping HPRT (hypoxanthine-guanine phosphorybosyl transferase) gene was used to normalize the amount of RNA. The expression of each gene was calculated as ΔC_t (HPRT C_t—target gene C_t) for all individual CL.

Detection of VEGF205 Isoform by DIG-Labeled RT-PCR

To detect the rare VEGF205 isoform, a direct labeling of the RT-PCR product with digoxigenin-11-dUTP (DIG-dUTP) was performed, using primers flanking exon 6b. The PCR reaction was conducted in a final volume of 50 μl, containing the following components (end-concentrations indicated): 1X buffer iTaq (BioRad Laboratories, Inc.), 200 μM each dNTP (PCR DIG Labeling Mix, Roche Diagnostics GmbH, Mannheim, Germany), 0.6 μM forward and reverse primers, 3 mM $MgCl_2$ (Bio-Rad Laboratories, Inc.), 2.5 U iTaq DNA Polymerase (Bio Rad Laboratories, Inc.) and 300 ng cDNA. The primers used were located on exon 6a (forward, 5′-TCGAGGAAAGGGAAAGGG-3′) and exon 7a (reverse, 5′- CGTCTGCGGATCTTGTAC –3′). The real-time PCR protocol employed was the same described above. After amplification, the PCR products were electrophoretically separated in a 4% low melting agarose gel for 4 hr and subsequently transferred to a Nylon membrane (Hybond, Amersham Biosciences) to proceed to the chemiluminescent detection of the DIG-labeled RT-PCR, according to DIG Luminescent Detection Kit (Roche Diagnostics GmbH) instructions. The luminescent light signal was detected in a FluorS MultiImager instrument (BioRad Laboratories, Inc.).

Statistical Analysis

The statistical analysis was performed considering the mean of all 5 CLs per animal, since no significant differences among CLs within the same animal were observed.

Differences in mRNA expression of the target genes (using the ΔC_t values), VEGF and VEGF receptor protein levels and progesterone contents were determined using ANOVA, followed by the Duncan's post-hoc

TABLE 1. Forward and Reverse Primers Sequences Used for Real Time RT-PCR

Primer	Sequence (5′-3′)	Product size (bp)
HPRT forward	GGACAGGACTGAACGGCTTG	
HPRT reverse	GTAATCCAGCAGGTCAGCAAAG	115
VEGFR-2 forward	AACGAGTGGAGGTGACAGATTG	
VEGFR-2 reverse	CGGGTAGAAGCACTTGTAGGC	104
VEGFR-1 forward	TTGGACTGTTGGCACAAAGAC	
VEGFR-1 reverse	GCTGTTGCTCGTCAGAATGG	141
VEGF common forward	ATGCGGATCAAACCTCACCAAG	
VEGF205 reverse	ACAGCAGCGGGCACCAAC	197
VEGF188 reverse	CCACAGGGAACGCTCCAG	185
VEGF182 reverse	CCACAGGGACGGGATTTCTT	167
VEGF147 reverse	GTCACATCTTGCAACGCGAG	208
VEGF144 reverse	TCGGCTTGTCACATACGCTC	190
VEGF120 reverse	CGGCTTGTCACATTTTTCTTGCC	117
VEGF164/164b common forward	GAGGCAAGAAAATCCCTGTGG	
VEGF164 reverse	GTCACATCTGCAAGTACGTTCG	151
VEGF164b reverse	TCCTGGTGAGAGATCTGCAAG	156

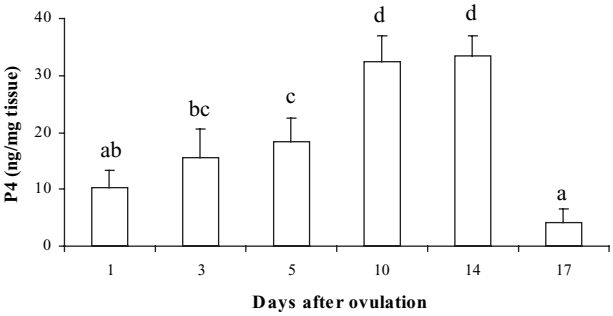

Fig. 2. Progesterone (P4) concentrations in swine CLs during different luteal phases. Results are presented as mean ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

test (SPSS Version 8.0, Inc., Chicago, IL). The data are shown as the mean ± SEM.

RESULTS

Progesterone Levels

Progesterone concentration in luteal tissue increased gradually during the formation of CL, reached the highest level during the mid-late phase (day 10 and 14) and decreased drastically on day 17 (Fig. 2).

VEGF mRNA Isoforms Expression

All splice variants were amplified, except for VEGF205 isoform that was not detected either with specific primers or with primers localized externally to the exon 6b. With the specific reverse primer, designed according to human sequence and located on the exon 6b, no band was detected. With primers positioned externally to the exon 6b, two products equivalent to the VEGF182 (102 bp) and VEGF188 (120 bp) isoforms were observed, whereas no band equivalent to the VEGF205 isoform (171 bp in human) was detected, even with the highly sensitive chemiluminescent detection.

The two most abundantly expressed splice variants were VEGF120 and VEGF164 followed by VEGF147, VEGF188, VEGF182, VEGF144, and VEGF164b in that order. Four patterns of expression were detected. VEGF164, VEGF147, and VEGF164b mRNA expression immediately dropped after day 1 (2.6-fold decrease), showing a second significant decrease between day 14 and 17 (3.3-fold decrease) (Fig. 3a). VEGF120 showed a three-step fall of mRNA expression, between day 1 and 3 (two-fold decrease), day 5 and 10 (1.7-fold decrease), and day 14 and 17 (1.6-fold decrease) (Fig. 3b). VEGF188 and VEGF182 mRNA levels progressively decreased until day 10 (four-fold decrease between day 1 and 10), then transiently increased on day 14 (2.3-fold increase), returning to value of day 10 on day 17 (Fig. 3c). VEGF144 showed a decline of mRNA expression until day 10 (five-fold decrease between day 1 and 10), followed by a progressive increase until day 17 (2.5-fold increase between day 10 and 17) (Fig. 3d).

VEGF Protein Levels

The pattern of VEGF luteal levels during the estrous cycle was similar to that of VEGF164 isoform, even if

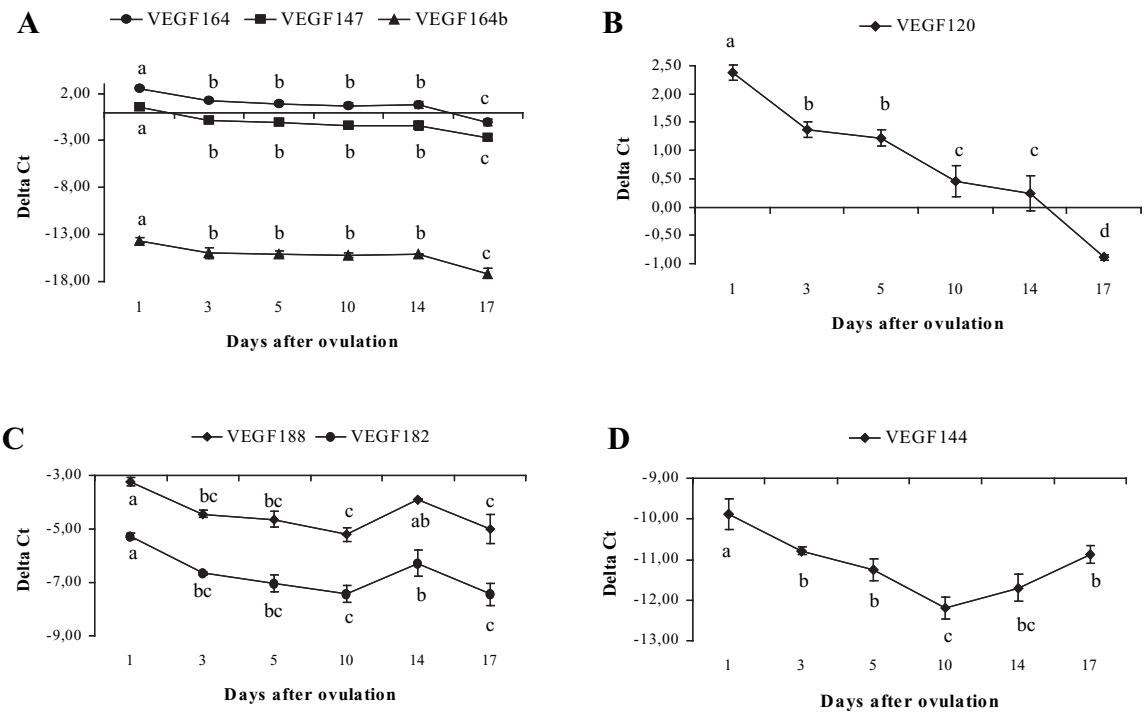

Fig. 3. Expression of VEGF mRNA isoforms in swine CLs during different luteal phases. **A**: VEGF164, VEGF147 and VEGF164b. **B**: VEGF120. **C**: VEGF188 and VEGF182. **D**: VEGF144. Results are presented as Delta C_t (HPRT C_t—target gene C_t) ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

with a different magnitude. After day 1, at which the highest values were observed, the levels of tissue VEGF drastically decreased, remaining unchanged during most of the luteal phase (day 3–14). A second significant drop occurred on day 17 (Fig. 4).

VEGFR-1 and VEGFR-2 mRNAs Expression

The VEGF receptor most abundantly expressed was the VEGFR-2. Both receptors showed a rapid increase in mRNA expression during the early luteal phase (a 1.7-fold increase between day 1 and 3 for VEGFR-1, and a 2.7-fold increase between day 1 and 3–5 for VEGFR-2), followed by a decrease during mid and late luteal phase, reaching levels similar to those reported for day 1 on day 17 (Fig. 5).

VEGFR-1 and VEGFR-2 Protein Levels

Both VEGFR-1 and VEGFR-2 proteins were detected during CL lifespan. A clear single band (180 kDa) was detected for VEGFR-1, while two faint bands (200 and 230 kDa) were observed for VEGFR-2. The relative expression of VEGFR-1 increased at days 10–14 and slightly decreased on late luteal phase (day 17). On the contrary, the highest levels of VEGFR-2 expression was observed on day 5, gradually decreasing until day 14, while no bands was detected on day 17 (Fig. 6).

DISCUSSION

Progesterone measurement in luteal tissue confirmed that our experimental protocol had been effective in stimulating an ovarian activity. Steroid levels gradually increased from day 1, reached the maximum values on day 10–14 and dropped on day 17, consistently with the establishment of luteal regression.

Angiogenesis represents a key aspect of the normal cyclical ovarian function and VEGF is its most important regulatory factor. Early studies have shown that VEGF and its receptors are temporally and spatially related to the proliferation of blood vessels in several reproductive tissues, including follicles (Barboni et al., 2000; Mattioli et al., 2001; Shimizu et al., 2002), endometrium (Charnock-Jones et al., 1993; Huang et al., 1998; Welter et al., 2003), placenta (Houck et al.,

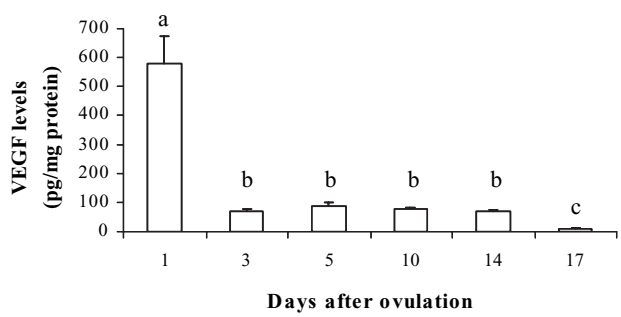

Fig. 4. Tissue VEGF concentrations in swine CLs during different luteal phases. Results are presented as mean ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

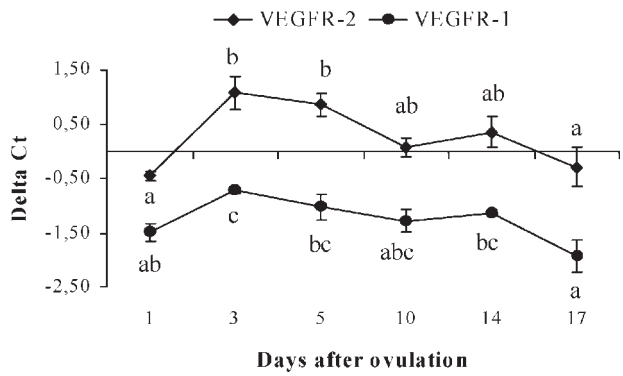

Fig. 5. Expression of VEGFR-2 and VEGFR-1 mRNAs in swine CLs during different luteal phases. Results are presented as Delta Ct (HPRT C_t—target gene C_t) ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

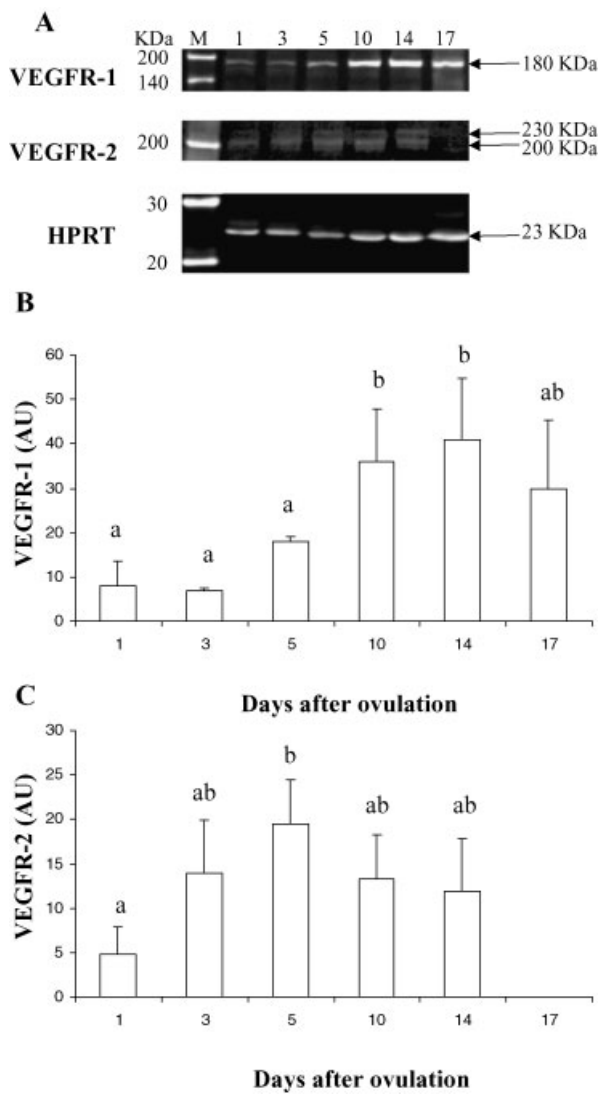

Fig. 6. Expression of VEGFR-1 and VEGFR-2 proteins in swine CLs during different luteal phases. Representative Western blotting of VEGFR-1, VEGFR-2 and HPRT (**A**) and relative VEGFR-1 (**B**) and VEGFR-2 (**C**) contents (AU, arbitrary units). Data represent the mean ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

1991; Cheung et al., 1995; Vonnahme and Ford, 2004b), uterus (Ancelin et al., 2002; Vonnahme and Ford, 2004a), fallopian tube and ovary (Gordon et al., 1996; Ferrara et al., 2003a), and CL (Berisha et al., 2000; Redmer et al., 2001; Boonyaprakob et al., 2003; Gabler et al., 2004).

Only three VEGF isoforms have been described in swine and only the VEGF164 isoform has been detected in CL (Boonyaprakob et al., 2003). This last report showed a constant level of expression on days 4, 7, 10, 13, and 15 after onset of estrus; these results agree well with our observation for the period examined as well as for the specific VEGF164 isoform.

Changes of VEGF levels in CL during the estrous cycle have been reported also in other species (Redmer et al., 1996; Berisha et al., 2000; Al-zi'abi et al., 2003). Those reports demonstrate that the highest VEGF mRNA expression and VEGF protein concentration are detected during the early luteal phase, and are followed by a significant decrease of expression during the mid and late luteal phase. Our findings on the two most expressed isoforms, VEGF120 and VEGF164, agree well with those observations.

Recently, Tesone et al. (2005), in an attempt to identify the different VEGF splice variants in monkey CL, detected only the two most expressed isoforms, VEGF121 and VEGF165. To our knowledge, our study is the first one reporting the multiple alternatively spliced VEGF mRNA isoforms in swine CL throughout the whole luteal phase.

Due to the VEGF splice variant complexity, we chose to apply an isoform specific panel of RT-PCR primers effective in differentiating the VEGF isoforms and its receptors; to provide quantitative data, a Real Time RT-PCR approach was used.

In our study, the predominant VEGF isoforms are VEGF120 and VEGF164, followed by VEGF147, VEGF188, and VEGF182, in this order. VEGF144 and VEGF164b are also expressed, although at very low level. In contrast, VEGF205 isoform has not been detected in any day of the estrous cycle, thus confirming that this isoform is possibly restricted to embryonic tissues (Ferrara and Davis-Smyth, 1997).

All isoform mRNAs are maximally expressed immediately after ovulation (day 1), then their expression decreases during mid-luteal phase. VEGF120 and VEGF164, that are involved essentially in the endothelial cell proliferation (Hofstaetter et al., 2004), reach the lowest mRNA expression levels during the CL functional regression (day 17). The same pattern of expression has been observed for the VEGF protein levels.

VEGF188 and VEGF182 isoforms showed a transient increase of expression on day 14. This might indicate a potential role of these isoforms in the mechanism of the maternal recognition of pregnancy that takes place, in pregnant sows, around this moment. In this period, in fact, CLs either start the process of regression or, if a pregnancy is in progress, have to survive, and VEGF may have a role in matrix maturation, necessary for CL maintenance (Bacci et al., 1996; Duncan, 2000). This hypothesis is supported by the fact that Uchida et al. (2003) suggested a possible role of VEGF188 in the healing process after a drill-hole injury in rat bones, as the responsible for matrix maturation, even if this isoform is inactive as a mitogen due to its inability to bind efficiently to VEGFR-2 (Plouet et al., 1997). In addition, progesterone has been shown to stimulate VEGF189 expression in human decidual cells (Ancelin et al., 2002), suggesting that the high progesterone levels observed at day 14 in our model may have up-regulated the VEGF188 mRNA expression. The involvement of VEGF188, and possibly VEGF182, in CL survival and remodeling may also be supported by the simultaneous decrease of both these isoforms and progesterone levels at day 17.

The increase of VEGF144 during the functional luteolysis (day 17) leads to hypothesize that the conditions of metabolic stress and hypoxia (observed during this phase) are responsible for the up-regulation of this isoform, as previously demonstrated in murine ovarian cancer cells under glucose starvation (Zhang et al., 2002). A possible explanation for the presence of this isoform during early luteolysis, despite the reduction and loss of capillaries (Bacci et al., 1996; Lei et al., 1998; Gaytan et al., 1999), should be the conspicuous development of noncapillary vessels in order to assist the absorption of degenerated luteal mass (Reynolds and Redmer, 1998; Bauer et al., 2003).

VEGF144/145 expression, in contrast to that of VEGF120/121, VEGF164/165, and VEGF188/189, which have been detected in most tissues and cells (Neufeld et al., 1999), seems to be restricted to reproductive tissue. This specific isoform has been demonstrated in human endometrium (Charnock-Jones et al., 1993), blastocysts (Krussel et al., 2001), breast and ovarian cancer (Stimpfl et al., 2002), and in several tumorigenic cell types originated from the female reproductive system (Poltorak et al., 1997), as well as in ovine placenta and fetal membranes (Cheung et al., 1995).

Hence, the present study identified in swine CL four novel isoforms, VEGF144, VEGF147, VEGF182, and VEGF164b, not yet described. Surprisingly, VEGF147 splice variant demonstrated high levels of expression during estrous cycle. This isoform is a truncated form of VEGF164, with mitogenic property but without the ability to bind heparin. VEGF147 is the only isoform presenting a truncated exon 8a and, as a consequence, it can possibly lack biological activity; nevertheless, it may affect the function of other VEGF isoforms, even though its physiological importance still remains to be elucidated (Whittle et al., 1999).

The pattern of VEGF182 mRNA expression is similar to that of VEGF188. Although this isoform lacks 18 bp at the end of exon 6a, it still contains the heparin-binding site, so its binding characteristics should be similar to that of VEGF188. In a model of systemic hypoxia, the VEGF182 and VEGF188 splice variants have been shown to be the most upregulated isoforms in response to a hypoxic challenge in rabbit meniscus (Hofstaetter et al., 2004).

VEGF165b is an inhibitory isoform, described firstly in human renal cell carcinoma (Bates et al., 2002a); its mRNA expression is very low throughout the whole luteal phase. The pattern of expression is similar to that of VEGF164, thus suggesting that the inhibitory isoform could counteract an excessive angiogenic and mitogenic activity of the abundant VEGF164 isoform (Cui et al., 2004). An anti-angiogenic effects of VEGF165b in vivo as well as its potential role in the control of human tumor growth have been already demonstrated (Woolard et al., 2004).

As for VEGF receptors, VEGFR-2 (which is known as the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF) is the most expressed in swine CLs throughout the estrous cycle.

Both VEGF receptors showed a differential regulation throughout estrous cycle. The patterns of mRNA and protein exhibited some differences, probably due to a different sensitivity of the employed techniques. All together, the results obtained showed that both VEGF receptors increased during CL development and decreased at luteal regression. VEGFR-2 reached maximal levels for both protein and mRNA at day 5 (presenting high mRNA levels already at day 3), gradually decreasing afterward. Similar patterns have been observed for VEGFR-2 mRNA levels in both bovine (Berisha et al., 2000) and swine (Boonyaprakob et al., 2003) CLs. This transitory increase observed during early luteal phase is inversely related to the contemporaneous decrease of all VEGF isoforms, thus suggesting that it may be involved in a negative feedback mechanism, responsible for the control of the VEGF-mediated luteal vascular growth.

On the contrary, conflicting results have been reported for VEGFR-1 expression. Boonyaprakob et al. (2003) demonstrated a gradual increase of mRNA expression between day 4 and 15 from onset of estrus in swine while no differences have been observed throughout the bovine estrous cycle (Berisha et al., 2000). Our findings on VEGFR-1 protein levels are consistent with those obtained by Boonyaprakob et al. (2003). The precise function of VEGFR-1 is still unclear; it is expressed in both proliferating and quiescent endothelial cells and is thought to be critical for VEGF-induced formation of vascular capillary tubes as well as for promoting a vascular bed-specific release of growth factors. Other data indicate that VEGFR-1 may also play a role in the regulation of VEGF activity by preventing its binding to VEGFR-2 [for review see (Ferrara et al., 2003b; Tamanini and De Ambrogi, 2004)]. This later role of VEGFR-1 could explain the high levels of this receptor observed in our study during mid-late luteal phase as well as during luteolysis.

In conclusion, luteal VEGF protein and transcript concentrations are high immediately after ovulation, when luteal vascular growth is rapid and tumultuous, thus supporting the role of VEGF in the angiogenesis of the newly formed CL. Subsequently, the increases of receptors expression as well as the coincident decrease of VEGF possibly assure a regulatory mechanism of angiogenesis in the mid-stage CL. Finally, the high mRNA expression of some VEGF isoforms during late luteal phase and luteolysis suggest a role of VEGF in the tissue remodeling necessary either for CL maintenance in case of pregnancy or for noncapillary vessel development essential for tissue removal during structural luteolysis.

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CONCLUSIONS

The results presented in this thesis, summarised in four main points, demonstrated:

1) the regulation of feed deprivation on luteal vessel development and hormone production by luteal cells;
2) the strict relationship between VEGF, MMPs and endonucleases and the functional CL stage throughout the oestrous cycle and at pregnancy;
3) the detection of novel VEGF isoforms for the first time in swine CL;
4) the differential expression of some VEGF isoforms principally during the late phase of the oestrous cycle; period in which the CL lifespan should be extended in case of pregnancy occurs or should undergoes structural and functional regression.

Taken together, these findings demonstrate that our model of CL development and regression is a very useful tool for studying the factors involved in the angiogenesis and angioregression mechanisms as well as their molecular interactions and regulation.

LIST OF ABBREVIATIONS

aFGF or FGF-1: acidic Fibroblast Growth Factor
bFGF or FGF-2: basic Fibroblast Growth Factor
Ang: Angiopoietin
Ang-1: Angiopoietin-1
Ang-2: Angiopoietin-2
ARNT: Aryl Hydrocarbon receptor Nuclear Translocator
CL: Corpus Luteum
CRS: Cell-surface Retention Sequence
CRSBP-1: Cell surface Retention Sequence Binding Protein-1
ECE-1: Endothelin Converting Enzyme
ECM: Extracellular Matrix
EGF: Epidermal Growth Factor
EG-VEGF: Endocrine Gland - Vascular Endothelial Growth Factor
ET-1: Endothelin-1
ET-A: Endothelin receptor Type A
ET-B: Endothelin receptor Type B
HIF-1: Hypoxia-Inducible Factor 1
HRE: Hypoxia Response Element
HS: Heparan Sulphate
HSPG: Heparan Sulphate Proteoglycan
HUVEC: Human Umbilical Vein Endothelial Cells
IFN-β: Interferon-β
Ig: Immunoglobulin
IGF: Insulin-like Growth Factors
IL-8: Interleukin-8
KGF/FGF-7: Keratinocyte Growth Factor
LH: luteinising hormone
LIF: Leukaemia Inhibitory Factor

LLC: Large Luteal Cells
MAM: Meprin/A5-neuropilin/Mu
MEF2C: Myocyte Enhancer binding Factor 2C
MMP: Metalloproteinases
MT-MMP: Membrane Type-Metalloproteinases
NO: Nitric Oxide
NRP-1: Neuropilin 1
NRP-2: Neuropilin 2
P4: Progesterone
PDGF: Platelet-Derived Growth Factor
PECAM-1: Platelet Endothelial Cell Adhesion Molecule 1
PF4: Platelet factor 4
PGF2α: Prostaglandin F2α
PKC: Protein Kinase C
PlGF: Placental Growth Factor
PMA: Phorbol Myristate Acetate
ROS: Reactive Oxygen Species
SLC: Small Luteal Cells
TFPI: Tissue Factor Pathway Inhibitor
TGF-βR2: Transforming Growth Factor-β Receptor type II
TGF: Transforming Growth Factor
TIMPs: Tissue Inhibitors of Metalloproteinases
TNF-α: Tumour Necrosis Factor-α
TSP-1: Thrombospondin-1
uPA: urokinase-type Plasminogen Activator
UTR: Untranslated Region
VEGF: Vascular Endothelial Growth Factor
VEGFR-1: Vascular Endothelial Growth Factor Receptor-1
VEGFR-2: Vascular Endothelial Growth Factor Receptor-2
VEGI: Vascular Endothelial Growth Inhibitor
VPF: Vascular Permeability Factor

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