

INTRODUCTION

Ovulation is the central event of the ovarian cycle. The preovulatory surge of luteinizing hormone (LH) initiates several parallel biochemical pathways that in a highly synchronized and cooperative fashion lead to follicular rupture with release of the oocyte around 36 h later in humans (Andersen *et al.*, 1995). Animal studies have indicated that the collagenous layers in and around the theca layer and in the overlying tunica albuginea make up the tensile strength of the follicle and breakdown of these structural proteins seems to be a prerequisite for follicular rupture to occur. Several studies, mainly in experimental animals, have shown that there is an increased expression of a number of matrix metalloproteinases (MMPs) and their endogenous inhibitors (tissue inhibitor of metalloproteinases, TIMPs) during the ovulatory process (Curry and Osteen, 2003) and it is assumed that their major role is to degrade the collagen fibrils and networks of the follicular wall, which facilitates rupture of the follicle. The studies in this thesis examine the collagen composition of the human ovary and investigate the expression of the proposed ovulatory mediators, MMPs and their inhibitors TIMPs, in the three major cell compartments of the periovulatory follicle during four distinct ovulatory phases of the menstrual cycle. These studies aim to provide some insight into whether these pathways are important in the human ovulatory process.

Extracellular matrix

The extracellular matrix (ECM) is a dynamic and multifaceted meshwork that is vital to sustain the structural integrity of all tissues. Formerly believed to serve merely as a passive scaffold for cells it is now known that the ECM also has an effect on cell shape, cell adhesion, cell migration and differentiation as well as cell death (Hay, 1992). The ECM is composed of fibrous proteins set in a gel-like, polysaccharide foundation, the main components of which are heparin sulphate proteoglycans. Moreover, the ECM contains adhesion proteins including fibronectin and laminin as well as cell surface receptors such as the integrins. An exclusive ECM is formed in each type of tissue according to the variation and organization of diverse matrix components in different tissues. Thus, the ECM becomes calcified in bone and teeth, transparent in the cornea, stretchable in the lung and forms rope-like, strong fibres in ligaments and tendons. Collagens are the main structural proteins of the ECM and the single

most abundant protein type in mammalian tissues. The basal lamina (BL) is one such specific type of ECM. Since there are two BLs in the preovulatory follicle, which have a specific importance during the ovulatory phase, this explicit type of ECM will be discussed in brief below.

A basal lamina (BL) is a thin sheet of specialized ECM composed of a web-type network composed of collagen type IV and laminin (Timpl and Brown, 1996). BLs are present at the epithelial/mesenchymal border of most tissues and the different BL-components influence cell regulatory functions, tissue compartmentalization. BLs also serve as selective barriers and provide structural support (Yurchenco *et al.*, 2004). Heparin sulphate proteoglycan and other macro-molecules are associated with the collagen type IV-laminin network forming unique BL-compositions that vary from tissue to tissue, or within the same tissue at different periods of remodelling.

Collagens

Some 20 different collagens, formed from a combination of diverse collagen chains, have been reported (Song *et al.*, 2006). The collagens are characterized by a rope-like construction of three polypeptide chains that are twisted around each another forming a triple helix. Collagens may be divided into several classes (Table I) according to the polymeric structure they form or correlated structural features (Prockop and Kivirikko, 1995; Gelse *et al.*, 2003): Fibril-forming collagens (types I, II, III, V, and XI); network forming collagens (types IV, VIII and X), fibril-associated collagens with interrupted triple helices (FACITs) that are found on the surface of collagen fibrils (types IX, XII, XIV, XVI, and XIX); the collagen that forms beaded filaments (type VI); the collagen that forms anchoring fibrils for BL (type VII); transmembrane-domain collagens (types XIII and XVII); and newly discovered collagen types that are not yet fully characterized. The repetitions of the tripeptide Glycine X-Y (X and Y are often proline or hydroxyproline) are characteristic for the collagen family and essential for the formation of the triple helix (Hay, 1991). Each collagen subtype has different features that make it uniquely suited for performing specific tissue tasks and in most tissues there are combinations of different collagens.

In fibril-forming collagens the triple helical molecules form cross-links and hydrogen bonds between each other resulting in the formation of fibrils and finally collagen fibers to make up the basic structural components of connective tissues (Song *et al.*, 2006). The fibril-forming collagens are synthesized as soluble precursors (procollagens) which are enzymatically processed into insoluble collagens. Collagen type I is the most abundant and well studied type of fibril-forming collagen and provides tensile stiffness or tensile strength depending on the tissue-type. The triple helix of collagen type I is often formed as a heterotrimer composed of two identical $\alpha 1(I)$ -chains and one $\alpha 2(I)$ -chain. It is the major collagen of tendons, ligaments, skin, cornea, and numerous interstitial connective tissues, with the exception of hyaline cartilage, brain tissue, and the vitreous body of the eye. In addition, it constitutes 90% of the organic mass of bone (Gelse *et al.*, 2003). Collagen type I is predominantly integrated into complexes with either collagen type III as in skin and reticular fibres (Fleischmajer *et al.*, 1990), or collagen type V as in bone tissue, tendons and the cornea (Niyibizi and Eyre, 1989). Collagen type III is a homotrimer composed of three $\alpha 1(III)$ -chains. It is abundant in elastic tissues and therefore is a vital constituent of the reticular fibres in the interstitial tissue of the lungs, spleen, liver, dermis and vessels (Gelse *et al.*, 2003).

Table I Features of Collagen.

Collagen class	Collagen types	Tissue distribution
Fibril-forming	I	The majority of connective tissues
	II	Cartilage and vitreous humor
	III	Extensible connective tissues (e.g., skin, muscle, lung, blood vessels)
	V	Tissues containing collagen types I and III
	XI	Tissues containing collagen type II
Network-forming	IV	Basal laminae
Anchoring filaments	VII	Attachment of basal lamina to underlying connective tissue
Fibril-associated	IX	Tissues containing collagen type II
	XII	Tissues containing collagen type I
	XIV	Tissues containing collagen type I
	XVI	Many tissues

Remodelling of the ECM takes place in most physiological conditions such as tissue and organ development, wound healing, ovulation and menstruation as well as in pathological situations such as cancer and arthritis. During recent decades, activity of MMPs, and their regulation by TIMPs, have been postulated to play key roles in ECM remodelling especially in the degradation of collagens.

Matrix metalloproteinases

The tight regulation of ECM remodelling is a prerequisite for normal development and function of all organisms. Modulation of cell-matrix communication takes place through the action of proteolytic enzymes that are not only responsible for protein degradation but also control signals produced by other matrix molecules. It was first shown that diffusible enzymes produced by skin from the resorbing tail of the metamorphosing frog could degrade the triple helix of collagen (Gross and Lapiere, 1962). Since then the matrix metalloproteinase (MMP) family, including the collagenases described in the frog, has grown to comprise 23 members in the human (Nagase *et al.*, 2006). These MMPs (Table II) are all zinc-dependent, neutral endopeptidases that synergistically degrade the major components of the ECM, in particular collagens and proteoglycans (Birkedal-Hansen *et al.*, 1993). There are also two other large families with major roles in extracellular proteolysis, namely the ADAM family (proteins with a disintegrin-like and metalloprotease domain) and ADAMTS family (ADAM with thrombospondin type I repeats) (Sternlicht and Werb, 2001; Somerville *et al.*, 2003). The MMP family can be subdivided into collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs) and others based on their substrate specificity and molecular structure (Visse and Nagase, 2003). A typical MMP is composed of a signal peptide that is cut off during secretion into the extracellular space, a propeptide domain involved in activation of the precursor form (proMMP) and a catalytic domain linked to a hemopexin/vitronectin-like domain by a hinge region (Fig. 1). The catalytic domain contains a zinc-binding site which is essential for the stability and enzymatic activity of the MMP and the hemopexin/vitronectin-like domain is central for protein-protein interaction in the regulation of proteolytic activity (Woessner and Nagase, 2000). Additionally, gelatinases

contain three repeats of fibronectin type II modules inserted into the catalytic domain, which are necessary for the binding of gelatin (Allan *et al.*, 1995).

Table II Representatives of the matrix metalloproteinases.

A summary of the MMP family members modified and redrawn from Somerville *et al.*-03; Curry and Osteen-03; and Nagase *et al.*-06. Collagen substrates are listed. ECM substrates mentioned elsewhere in this thesis are listed.

MMP	Alternative name	Representatives of substrates
Collagenases		
MMP-1	Collagenase-1 Interstitial collagenase	Collagen types I, II, III, VII, VIII, X. Gelatin, nidogen, versican and perlecan and other ECM substrates.
MMP-8	Collagenase-2 Neutrophil collagenase	Collagen types I, II, III, VII, VIII, X. Gelatin, laminin, nidogen, fibronectin and other ECM substrates.
MMP-13	Collagenase-3	Collagen types I, II, III, IV, V, IX, X, XI. Gelatin, fibronectin, laminin, perlecan and other ECM substrates.
Gelatinases		
MMP-2	Gelatinase-A 72kDa gelatinase	Collagen types I, IV, V, VII, X, XI, XIV. Gelatin, elastin, fibronectin, laminin, nidogen, versican and other ECM substrates.
MMP-9	Gelatinase-B 92kDa gelatinase	Collagen types IV, V, VII, X, XIV. Gelatin, elastin, fibronectin, laminin, nidogen, versican and other ECM substrates.
Stromelysins		
MMP-3	Stromelysin-1	Collagen types II, III, IV, V, VII, IX-XI. Gelatin, elastin, decorin, fibronectin, laminin, nidogen, versican and other ECM substrates.
MMP-10	Stromelysin-2	Collagen types III, IV, V. Gelatin, fibronectin, elastin, laminin, nidogen,
MMP-11	Stromelysin-3	Laminin, fibronectin.
Matrilysin		
MMP-7	Matrilysin-1	Collagen types I, IV, and X. Gelatin, fibronectin, elastin, laminin.
MT-MMP		
MMP-14	MT1-MMP	Collagen I, II, III, IV. Gelatin, fibronectin, elastin, laminin.
MMP-15	MT2-MMP	Gelatin, laminin, fibronectin.
MMP-16	MT3-MMP	Collagen I, III. Fibronectin.
MMP-17	MT4-MMP	Gelatin, fibronectin
MMP-24	MT5-MMP	Gelatin, fibronectin
MMP-25	MT6-MMP Leukolysin	Collagen type IV and gelatin

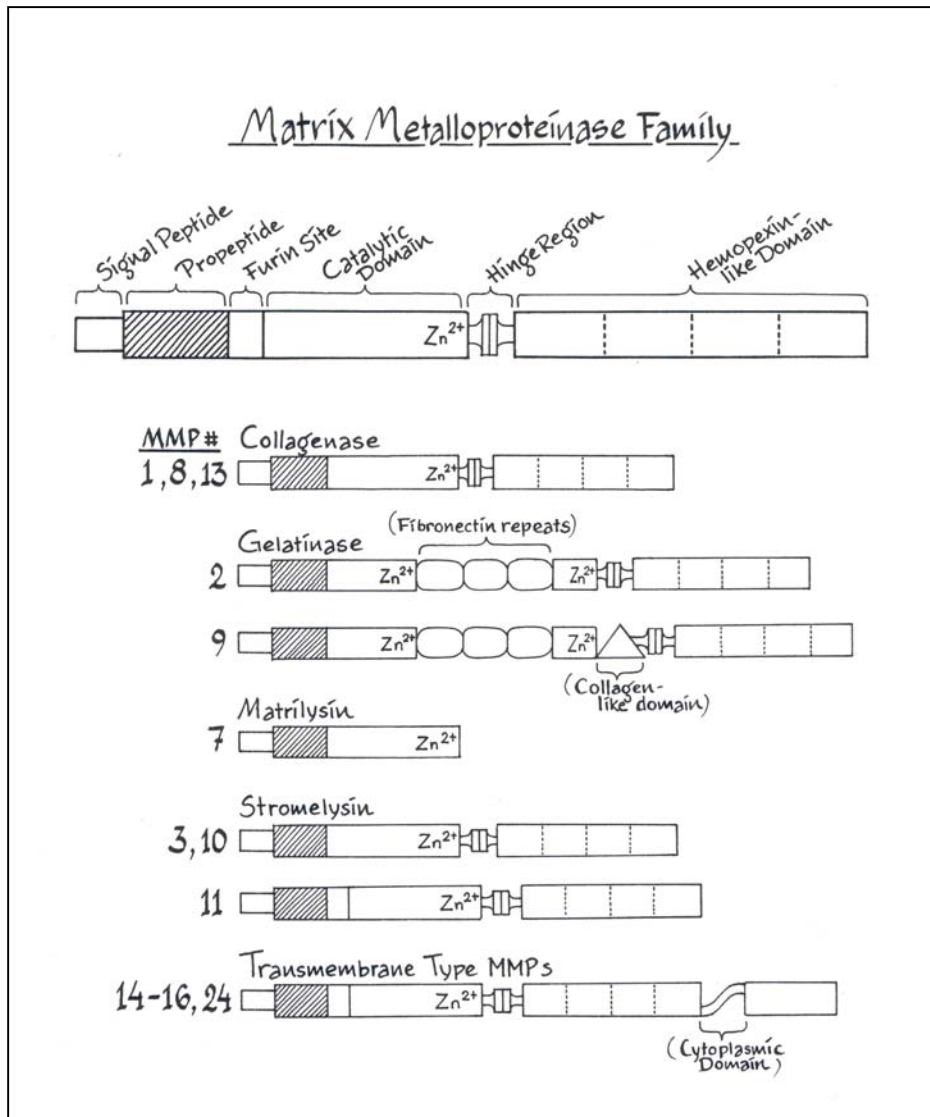


Figure 1 Domain structure of the MMP-family.

Functions of MMPs

Members of the MMP family are unique in that they can cleave highly structured fibrillar collagens under physiological conditions. This denaturation of the collagen molecule by collagenases results in gelatin that can be further degraded by gelatinases and stromelysins. Furthermore, gelatinases as well as stromelysins are able to degrade most of the components of BLs such as collagen type IV and laminin. Gelatinase A (MMP-2) has also been shown to cleave fibrillar collagen type I *in vitro* (Aimes and Quigley, 1995). In this study it was demonstrated that both human and chicken TIMP-free MMP-2, but not MMP-9, was able to cleave soluble as well as fibril-formed collagen type I.

The MMPs play important roles in a range of normal and pathological conditions that involve degradation and remodelling of the ECM. For instance, MMPs are highly expressed during the specific physiological processes taking place repetitively in the reproductive organs and several MMPs are expressed during wound healing. Similarly, MMPs participate in the tissue destruction that occurs during tumour invasion. Moreover, by cleaving large insoluble ECM components and ECM-associated molecules, MMPs modulate cellular behaviour and cell-cell communication (McCawley and Matrisian, 2001; Mott and Werb, 2004).

Regulation of MMPs

In order to carry out their physiological (and potential pathological) role in ECM remodelling, MMPs are strictly regulated on multiple levels, including the gene transcription level as well as post translational influences such as activation of proMMPs and regulation by extracellular inhibitors (Fig. 2). On transcriptional level, MMP expression is regulated by a number of cytokines and growth factors, reviewed in (Sternlicht and Werb, 2001), such as interleukins (ILs), interferons (IFs), vascular endothelial growth factor (VEGF), tumour necrosis factor- α (TNF- α) and extracellular matrix metalloproteinase inducer (EMMPRIN), also known as basigin. Most MMPs are secreted as proenzymes and are activated extracellularly in a stepwise fashion by proteinases, including other MMPs, serine-proteinases like the plasminogen-activator/plasmin system, as well as by non-proteolytic compounds, reviewed by (Nagase, 1997). This stepwise activation of proMMPs is partly explained by the “cysteine switch” mechanism, by which a covalent bond between the cysteine residue in the propeptide domain and the essential zinc atom in the catalytic domain is disrupted (Van Wart and Birkedal-Hansen, 1990). There is a special activation mechanism for MMP-2, which is activated at the cell surface through interaction with MT-MMPs (Atkinson *et al.*, 1995).

The proteolytic activity of MMPs is strictly controlled by endogenous inhibitors, including specific tissue inhibitors of metalloproteinases (TIMPs) as well as humoral inhibitors like α 2-macroglobulin, pregnancy zone protein, α 1-macroglobulin (in rodents, rabbits and guinea pigs), and the ovomacroglobulins (e.g. ovostatins, found in avian and reptilian egg white). The balance between the activity of MMPs and TIMPs is postulated to be essential for the maintenance of ECM homeostasis.

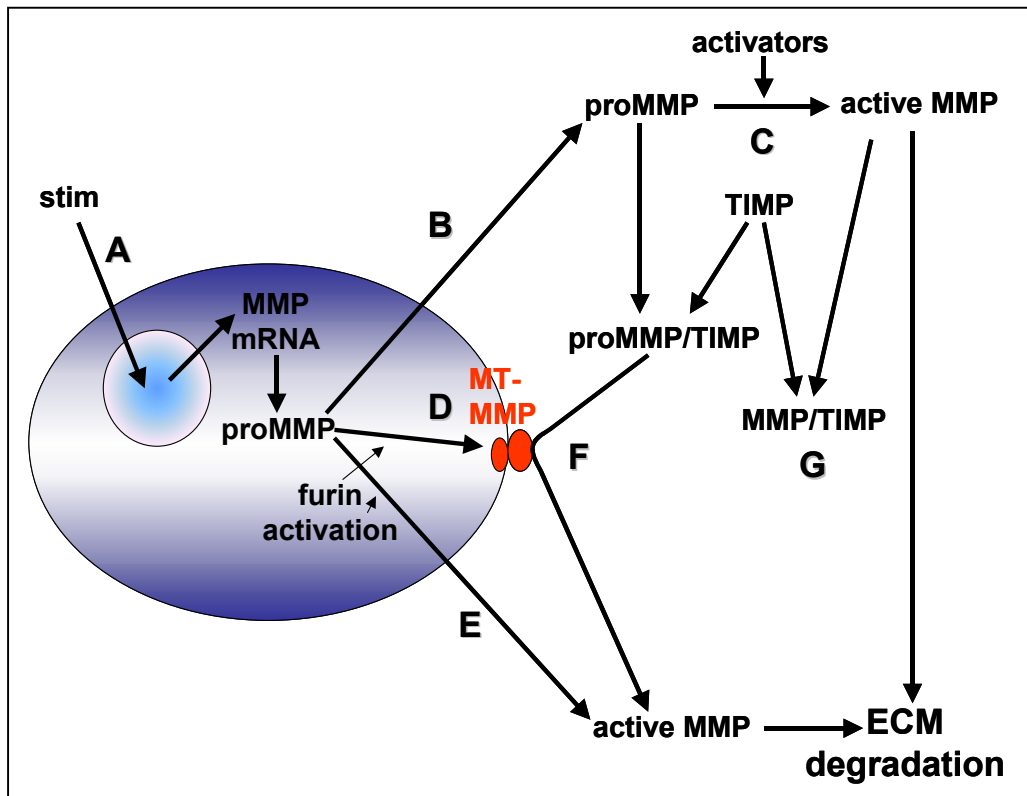


Figure 2 Regulation of MMPs. Modified and redrawn from (Sternlicht and Werb, 2001; Curry and Osteen, 2003). MMP expression is strictly regulated on the gene transcription level, **A**. Most MMPs are secreted as proenzymes, **B**, and are activated extracellularly in a stepwise fashion by proteinases, including other MMPs, serine-proteinases like the plasminogen-activator/plasmin system, as well as by non-proteolytic compounds, **C**. MT-MMPs and MMP-11 contain a furin recognition sequence between their propeptide and catalytic domains, and are activated intracellularly by furin before they reach the cell surface, **D**, or are secreted as active enzymes, **E**. MMP-2 is activated at the cell surface through interaction with MT-MMPs and TIMP-2, **F**. Active MMPs can be inhibited by TIMPs, **G**.

Tissue inhibitors of metalloproteinases

The TIMPs are significant regulators of ECM remodelling through their ability to control the activity of MMPs. Vertebrates have four types of TIMPs, TIMP-1, -2, -3 and -4, reviewed by (Gomez *et al.*, 1997; Brew *et al.*, 2000; Woessner and Nagase, 2000; Lambert *et al.*, 2004). The TIMPs were first identified in 1975 as inhibitors of collagenases. Later studies have revealed that they inhibit most active MMPs although there are extensive variations in the efficacy of the different TIMPs with respect to each MMP. In addition, some TIMPs bind to proMMPs to inactivate them on the zymogen level. Thus, TIMP-2, -3 and -4 bind to

proMMP-2, and TIMP-1 and -3 bind to proMMP-9. Moreover, a complex formation between proMMP-2, TIMP-2 and MMP-14 (membrane-type-1 MMP) is crucial for the activation of proMMP-2 at the cell surface (Strongin *et al.*, 1995). It has been suggested that the TIMP-2/MMP-14 complex acts as a receptor for proMMP-2 while active MMP-14, not in complex with TIMP-2, acts as an activator (Butler *et al.*, 1998). TIMPs are secreted by a diversity of cell types and are present in most tissues and body fluids. TIMP-1 and TIMP-2 are soluble but TIMP-3 is bound to the ECM. All four TIMPs inhibit MMP activity by forming non-covalent 1:1 stoichiometric complexes, one TIMP molecule inhibits one molecule of active MMP.

In addition to their role as inhibitors of MMPs and as such key regulators of ECM homeostasis, recent studies have concluded that TIMPs are multifunctional proteins that exhibit growth factor-like activity and can inhibit angiogenesis (Lambert *et al.*, 2004). These biological activities seem to be, at least in part, independent of MMP-inhibitory activity.

The female reproductive cycle

The main function of the female gonad, the ovary, is the differentiation and release of a fully mature oocyte, which through ovulation is made available for fertilization and this process thus constitutes the foundation for the survival of the species. Furthermore, the ovary produces steroids that lead to the development of female secondary sex characteristics and also support pregnancy. The menstrual cycle of the human (usually 28-32 days in duration) may be divided into three parts; a phase of follicular development (the follicular phase), the ovulatory phase initiated by the LH-surge ending with follicular rupture, and the phase after ovulation dominated by the corpus luteum (the luteal phase). The follicular phase by definition starts at the first day of menstruation (usually defined as cycle-day 1) and ends 10-18 days later with the start of the gonadotropin-surge of LH and follicle stimulating hormone (FSH; Fig. 3). The dominant follicle, which reaches the preovulatory stage during the last part of the follicular phase is the major source of cyclic secretion of ovarian oestrogen, mainly oestradiol (E2). The length of the follicular phase shows substantial inter-individual variation, reflected by a disparity in total menstrual cycle length, since the duration of the luteal phase is comparatively constant. The ovulatory phase, triggered by the gonadotropin-surge, is approximately 36 hours in the human (Hanna *et al.*, 1994; Andersen *et al.*, 1995).

After ovulation, the luteal phase begins and the ruptured follicle transforms into the corpus luteum with secretion of both E2 and progesterone (P4). The luteal phase ends with luteal regression with a substantial drop in P4 secretion leading to shedding of the endometrium when a new cycle begins. Cyclicity is under the control of a large number of growth factors and hormones, which cooperate to regulate the hypothalamic-pituitary-ovarian axis.

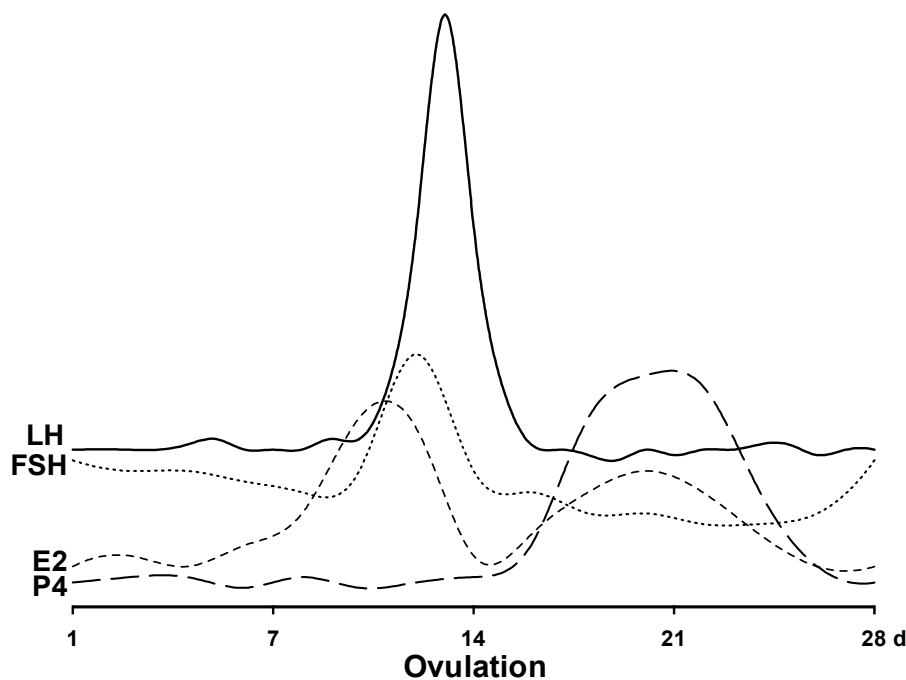


Figure 3. Hormonal changes during the human menstrual cycle.

Follicular development

Follicular development is a dynamic process, distinguished by a striking proliferation and differentiation of the different cells of the follicle in order to provide the optimal environment for the maturation of the oocyte, reviewed by (Gougeon, 1996; McGee and Hsueh, 2000; Richards, 2001; Richards, 2005). Oogonia, in the fetal gonadal ridge, proliferate by mitosis before some transform into primary oocytes and enter the first stage of meiosis at around 11-12 weeks of gestation. Others undergo atresia, a form of programmed cell death, also known

as apoptosis (Hsueh *et al.*, 1994; Vaskivuo *et al.*, 2001). Following meiotic arrest, the oocytes become surrounded by single layers of elongated pregranulosa cells to form primordial follicles. Around the time of birth, the oocytes have entered a resting state in the prophase of the first meiotic division, and make up the so called dormant primordial follicle pool.

The development from the smallest follicle, the primordial follicle, into a preovulatory (Graafian) follicle can be divided into four separate stages (Fig. 4): 1) initial (primary) recruitment, during which primordial follicles enter the growth phase; 2) cyclic (secondary) recruitment, when a pool of growing follicles begin to grow rapidly; 3) selection, a process whereby follicles are selected for further growth; and 4) dominance, during which the dominant follicle undergoes rapid development whilst the growth of subordinate follicles is suppressed (Goodman and Hodgen, 1983; McGee and Hsueh, 2000).

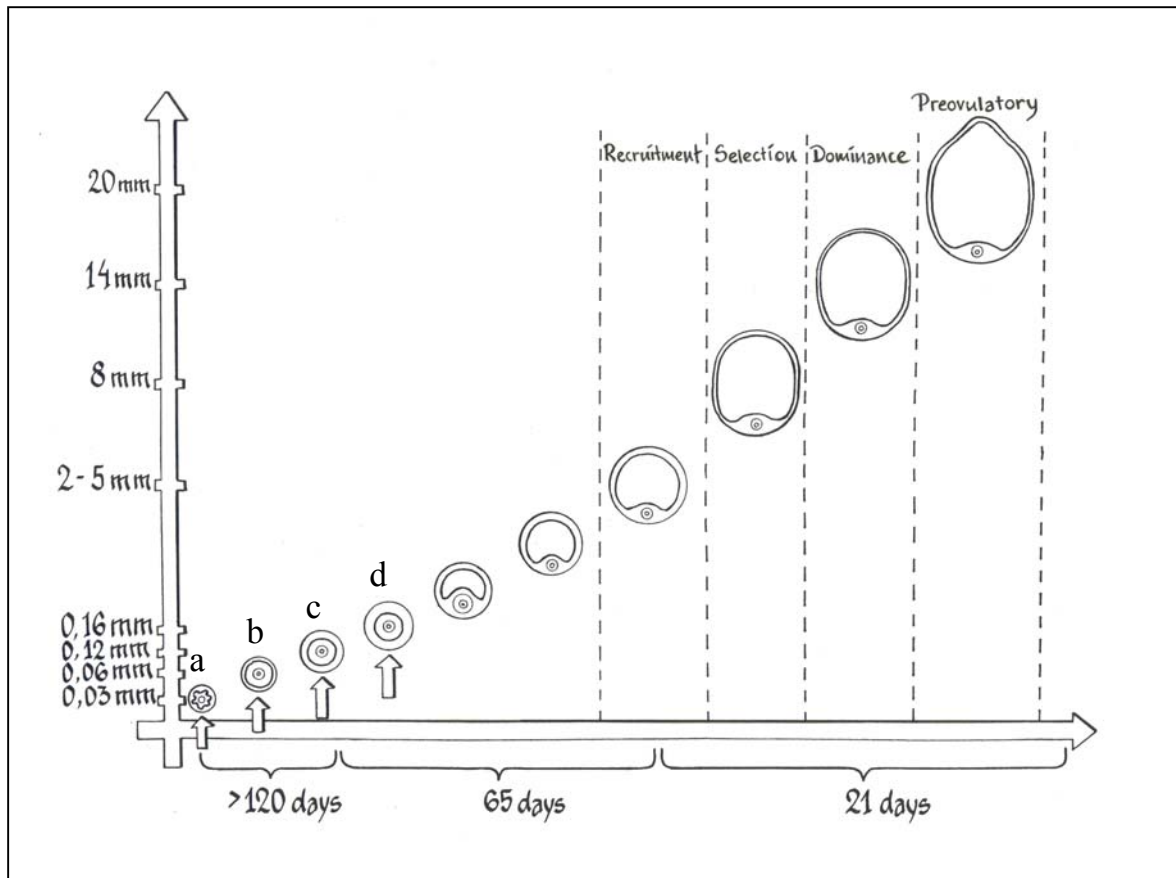


Figure 4. Follicular development in the human. Size of follicle (in mm) on Y-axis and time on X-axis. **A.** primordial follicle, **B.** primary follicle, **C.** secondary follicle, **D.** early antral follicle. Entry of primordial follicles from the pool of dormant follicles into the growth phase (initial recruitment) occurs at a steady rate throughout the reproductive life. It takes >120 days for the primordial follicle to grow into an early antral follicle. Approximately two menstrual cycles later the follicle is 2-5 mm and is now available for cyclic recruitment or undergoes atresia. If recruited, the follicle can be selected for further growth into the dominant follicle of 12-14 mm and prior to the LH-surge, a preovulatory follicle of 18-20 mm. (modified from Dahm-Kähler, 2006, with kind permission).

Entry of primordial follicles from the pool of dormant follicles into the growth phase (initial recruitment) occurs at a steady rate throughout the reproductive life span and is initially characterized by alteration of the flattened pregranulosa cells into a single layer of cuboidal granulosa cells surrounding the oocyte. The primordial follicle has thereby acquired the morphological signs of a primary follicle. Although, still under debate, this first stage of follicular development is generally considered to be gonadotropin-independent, since there is no evidence that the gonadotropins act as regulators at this early stage (Gougeon, 1996). It has been proposed that this developmental phase is initiated by local ovarian factors such as members of the transforming growth factor- β (TGF- β) superfamily, by paracrine or autocrine

mechanisms (Richards, 2001; Findlay *et al.*, 2002; Juengel and McNatty, 2005). Moreover, the oocyte by itself is an important regulator in this context (McNatty *et al.*, 2004), producing factors such as GDF9, reviewed by (Pangas and Matzuk, 2005). In the growing follicle, an ECM made up of mucopolysaccharides and specific glycoproteins (e.g ZP proteins) (Rankin *et al.*, 2001), called the zona pellucida (Chiquoine, 1960), is formed around the oocyte. The zona pellucida assists gap junction contact, which allows for oocyte-granulosa cell communication (reviewed by Kidder and Mhawi-02). During further granulosa cell differentiation, the cells start to express membrane receptors for FSH (Gougeon, 1996), cytoplasmic receptors for estrogens, (Drummond, 2006) and receptors for androgens (Hillier and Tetsuka, 1997). They also become coupled by gap junctions (Kidder and Mhawi, 2002). The basal lamina (BL), upon which the theca cells will become organized, is then formed with the theca layer developing from interstitial stroma cells. These changes occur at the end of the primary follicle stage, before it develops into a secondary follicle. The spindle-shaped theca cells that are in proximity to the BL become epitheloid and rounder in appearance, and are now referred to as theca interna (TI) cells. The more peripheral layer of theca cells keep their spindle-shaped form, merge with the stroma cells and are referred to as theca externa (TE) cells. Once entering the growing pool (initial recruitment), most growing follicles progress to the antral stage, at which point they unavoidably undergo atresia before puberty. As the follicle grows and an antrum is formed, granulosa cells separate into two subtypes. The cumulus granulosa cells surround the oocyte and are in intimate metabolic contact with the oocyte. The mural granulosa cells are in close contact with the BL, separating the granulosa cells from the thecal cell layer. The transition from pre-antral to antral stage is an important period during which the oocyte acquires the capacity to resume meiosis. The exact mechanism of the formation of the follicular antrum is not clear but the appearance of the fluid-filled follicular antrum during this phase, by definition, transforms the follicle into an antral follicle, and the oocyte acquires an eccentric position surrounded by the specialized cumulus granulosa cells. A cyclic recruitment of growing follicles only starts after pubertal onset, initiated by the increased FSH secretion, and about 10 follicles per ovary are usually selected each cycle (Hodgen, 1982).

During a single menstrual cycle, only one out of a pool of up to 20 selected follicles will acquire dominance and develop into the dominant follicle. The process of selection is not

completely understood but may involve an ability of specific follicles to respond to FSH, modulated by the inhibin-activin system (Campbell and Baird, 2001). One follicle, presumably at random, will produce more E2 than the other follicles during midfollicular phase, and become dominant. The production of large amounts of E2 by the dominant follicle will increase its sensitivity to FSH and it will then begin to grow and expand its follicular antrum with a further gain of LH receptors also in the granulosa cells. The dramatic increase of E2 produced by the dominant follicle results in a negative feedback regulation at the pituitary level and FSH secretion decreases, which may lead to the non-dominant follicles progress into growth arrest and atresia, (Tilly et al., 1991). The dominant follicle is surrounded by thecal cells that selectively bind more LH than the cells surrounding the non-dominant follicles (DiZerega *et al.*, 1980; Zeleznik *et al.*, 1981). Moreover, the vascularisation of the theca of the dominant follicle becomes more prominent than that of other follicles (Kanzaki *et al.*, 1981). The increased vascularisation of the dominant follicle may lead to an increased delivery of LH to the theca cells, and FSH to the granulosa cells, thereby supporting further follicular growth. This final phase of follicular development is considered to be highly dependent on FSH. However, it has been suggested that LH may be a significant regulator of granulosa cell differentiation at this time, perhaps even replacing FSH as the key controller of granulosa cell function just prior to ovulation (Filicori *et al.*, 2003).

Thus in summary, from a peak of approximately 7 million oocytes at mid-gestation, the number falls drastically so that less than 1 million remain in the ovaries at birth (Block, 1952; Forabosco *et al.*, 1991; Gougeon *et al.*, 1994). There is a continuous, non-gonadotropin dependent depletion of the pool of primordial follicles as a result of atresia, or by entry into the growth phase. During the reproductive years of a woman, as few as 300-400 follicles and thereby oocytes will eventually ovulate and when menopause is reached, there are just a few hundred left (Vaskivuo *et al.*, 2001). Consequently, only 0.1% of the total number of follicles will ovulate and the vast majority of follicles will undergo atresia. The general concept of reproductive biology has been that female mammals lose the capacity to regenerate germ-cells (oocytes) during fetal life and that a finite, non-renewable pool of primordial follicles is present at birth. However, this doctrine has been questioned by several research groups.

Bukovsky et al demonstrated in 1995 by *in vivo* methodology that the ovarian surface epithelium (OSE) may be a source of germ cells in the adult human ovary (Bukovsky *et al.*, 1995). Recently the same group made observations *in vitro*, suggesting that the OSE of adult human ovaries is a bipotent source of oocytes and granulosa cells, indicating follicular renewal (Bukovsky *et al.*, 2005). Moreover, new data from experiments in mice indicates the existence of proliferative germ cells which sustain the capacity to develop into an oocyte within a follicle (Johnson *et al.*, 2004). Furthermore, mice which have been sterilized by chemotherapy are claimed to be able to restore their oocyte production by bone marrow transplantation (Johnson *et al.*, 2005). These results imply that the bone marrow may be a potential source of germ cells, which may lead to oocyte production in adulthood. These data are quite spectacular since they challenge a dogma that has existed for a long time. Thus, further studies in other species are needed to confirm and implement the data.

ECM in follicular development

During the development from a primordial to a Graafian follicle, repeated remodelling of the follicular ECM and follicular wall occurs as the follicle grows. In particular, degradation of structural collagens within the thecal cell layer and overlying stroma must occur, to allow for the expansion of the follicle. The follicular BL must also enlarge with continuing growth.

The matrices of developing follicles have each been studied to varying extents. A large contribution in the field of matrix research, during folliculogenesis, has been done by Rodgers and co-workers, who used the cow as a model for a monoovular species. They have shown that the granulosa cell compartment of each follicle is enclosed by a BL separating it from the adjacent stroma in primordial follicles or from the theca in antral follicles (van Wezel and Rodgers, 1996). It is suggested that BLs influence epithelial cell migration, proliferation and differentiation, and selectively regulate the passage of molecules in and out of the interior of the follicle. The follicular BL in particular, is thought to influence granulosa cell proliferation and differentiation (Luck, 1994). A number of the components of the BL are thought to be produced by granulosa cells (Rodgers *et al.*, 1995; Zhao and Luck, 1995; Rodgers *et al.*, 1996). BLs are, as described earlier in this text, composed of a scaffold-like network of collagen type IV, and are stabilised by the binding of nidogen to this structure. Six different

α -chains of collagen type IV exist ($\alpha 1$ - $\alpha 6$)(Hay, 1991) and each molecule of collagen type IV consists of three α -chains. Consequently, several different combinations of α -chains are possible in one collagen type IV molecule. In the same way, laminins are multidomain heterotrimers composed of α - (5 different), β - (3 different) and γ -chains (3 different) (Aumailley *et al.*, 2005). Furthermore, at least twenty different isoforms of fibronectin have been described. BLs with many different properties may therefore arise simply due to the various combinations that make up these structures. In this way, follicular BL changes its composition during follicular development (Rodgers *et al.*, 1998; van Wezel *et al.*, 1998; Irving-Rodgers and Rodgers, 2006). Thus, $\alpha 1$ - $\alpha 6$ chains of collagen type IV are present around the primordial follicle, but as the follicle grows into an antral follicle, $\alpha 3$ - $\alpha 6$ chains cease to be expressed. Laminin chains $\alpha 1$, $\beta 2$ and $\gamma 1$ are present at all stages of folliculogenesis and the quantity increases with follicular size. As a consequence, the BLs change their composition to become less collagenous and more laminin-rich.

Follicular BL also contains the heparin sulphate proteoglycan, perlecan. Perlecan can bind a number of growth factors and can thus form a pool of growth factors or act as a barrier to their movement through the BL (McArthur *et al.*, 2000). Versican, another ECM component, is localised to the granulosa cell layer and theca layer of larger follicles (Mc Arthur *et al.*-00). Fibronectin can interact with other matrix components such as collagens and cell-surface integrins. Numerous fibronectin variants have been localized to the follicle ECM (De Candia and Rodgers, 1999) but the precise expression patterns of fibronectin isoforms during follicular development have, not yet been explored.

Structures known as Call-Exner bodies are found in the granulosa cell layer in all growing follicles (van Wezel *et al.*, 1999). A Call-Exner body was primarily described as “a ring of granulosa cells disposed radially around a central cavity filled with fluid” (Motta and Nesci, 1969). Electron microscopy has shown that Call-Exner bodies contain aggregates of convoluted BL or unassembled BL-like material which changes composition in a similar manner to the follicular BL during follicular growth (van Wezel *et al.*, 1999). However, the function of this structure is unknown. Recently, a new type of BL matrix made up of aggregates of BL material interspersed between granulosa cells was demonstrated in larger follicles after selection, and is similar in composition to the follicular BL. This new structure

has been named focal intra epithelial matrix, focimatrix, and it has been speculated to play a part in the luteinisation of granulosa cells (Irving-Rodgers *et al.*, 2004).

The theca cell layer (once it is formed) contains a number of BLs; sub-endothelial BL of small blood vessels and BL of the smooth muscle cells of arterioles. The $\alpha 1$ and $\alpha 2$ chains of collagen type IV together with different laminin chains have been observed in these BLs as well as throughout the theca interna, but not in association with any conventional BL (Rodgers *et al.*, 1998; van Wezel *et al.*, 1998). Electron microscopy-studies of this matrix have demonstrated fragments of BL-like material in the theca interna and the authors have named it “thecal matrix” (Rodgers *et al.*, 2000). It remains to be seen whether changes in focimatrix and thecal matrix occur during folliculogenesis. The role of these matrices in differentiation of the follicle is likely to be important and if other matrix structures may also likely to be found and characterized in the future.

Collagen distribution will be described in Discussion.

Ovulation

The ovulatory process begins at the moment the endogenous surge of luteinising hormone (LH) initiates local alteration in and around the preovulatory follicle, and ends with follicular rupture and the release of a fertilizable oocyte. Thus, the ovulatory process usually refers to simultaneous changes in and around the follicle that lead to follicular rupture, resumption and completion of meiosis, and luteinisation of the steroidogenic cells. In the human, this process takes approximately 36 hours (Hanna *et al.*, 1994; Andersen *et al.*, 1995). LH sets off numerous intraovarian regulatory systems that together, synchronously or sequentially, act to either degrade the ECM of the follicular apex or to induce vascular changes (Fig. 5).

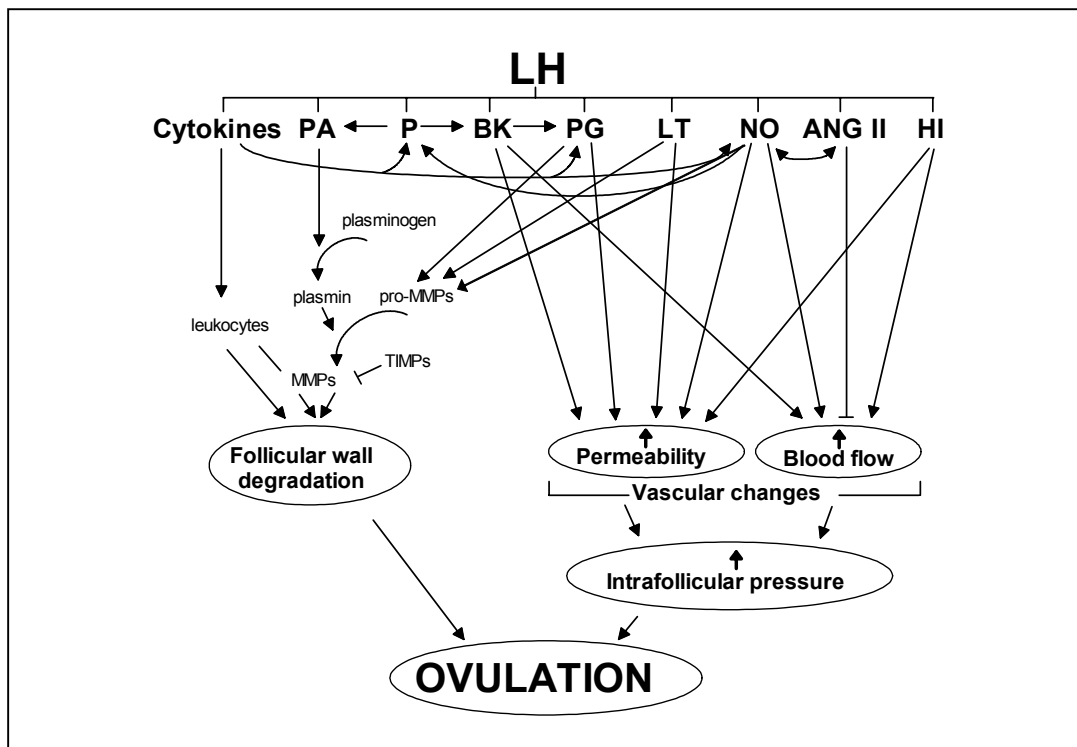


Figure 5. An overview of biochemical mediators in ovulation and their proposed effects in the ovulatory process. Plasminogen activator (PA), progesterone (P), bradykinin (BK), prostaglandin (PG), leukotriene (LT), nitric oxide (NO), angiotensin II (ANG II), histamine (HI), matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs).

The Graafian follicle and structural changes during the ovulatory phase

The preovulatory, dominant follicle is known as the Graafian follicle and is characterized by a central, fluid-filled antrum. The Graafian follicle protrudes markedly from the ovarian surface. The follicle wall, at the apex, is composed of five distinct cell layers: surface epithelium, tunica albuginea (TA), theca externa (TE), theca interna (TI) and the granulosa cell layer (Fig. 6). A BL separates the surface epithelium from the underlying TA and another BL separates the TI from the granulosa cell layer.

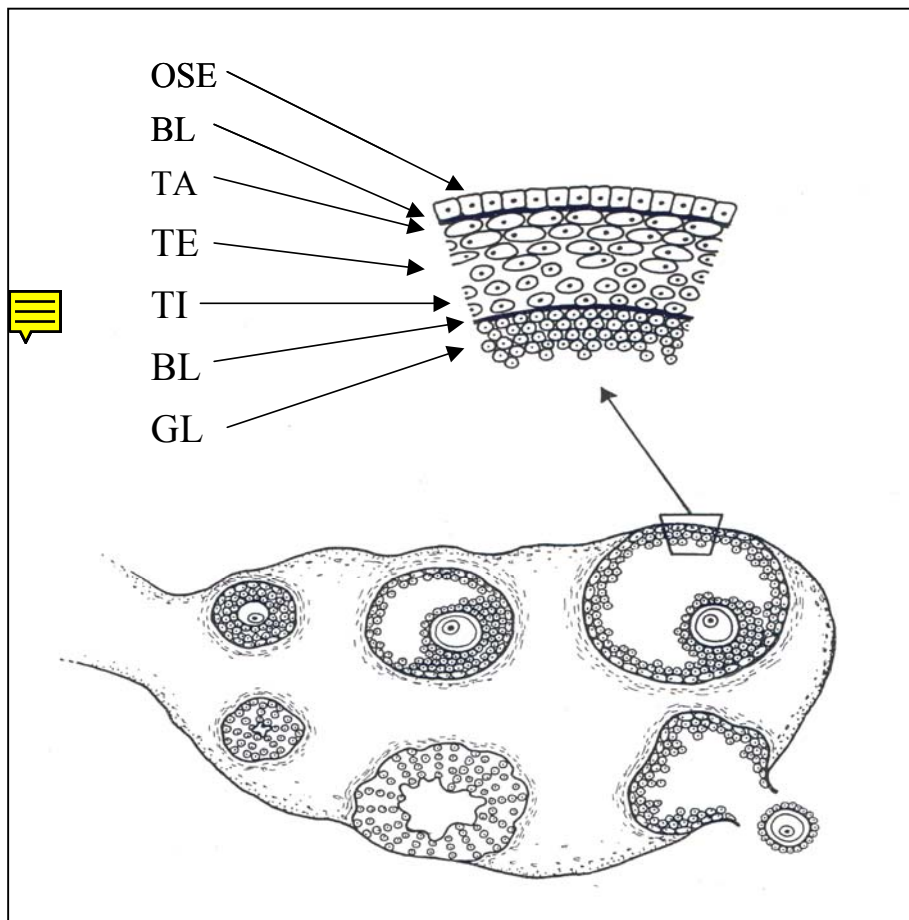


Figure 6. Schematic representation of the human ovary containing follicles and corpora lutea of different stages. Magnification shows the follicle wall, at the apex, composed of five distinct cell layers: ovarian surface epithelium (OSE), tunica albuginea (TA), theca externa (TE), theca interna (TI) and the granulosa cell layer (GC). A basal lamina (BL) separates the OSE from the underlying TA and another BL separates TI from GC.

The single layered ovarian surface epithelium (OSE) is the part of the pelvic peritoneum that covers the ovary and it is loosely attached to the underlying BL (Kruk *et al.*, 1994). It is held together by tight junctions containing claudin proteins (Zhu *et al.*, 2004). The tunica albuginea is collagen-rich and about twice as thick as the OSE (personal unpublished observation). Under the tunica albuginea is the thecal cell layer. The thecal layer consists of two major sub-layers, TI and TE and is supplied with blood and lymph vessels.

It contains both adrenergic and cholinergic nerves (Roby and Terranova, 1998a; Roby and Terranova, 1998b). The theca interna, which is approximately 3-5 cell layers thick, is highly vascularised and comprises steroidogenic cells adjoining the BL separating the granulosa cell layer and the theca cell layer. The TE, on the other hand, is mainly a collagenous connective tissue consisting of non-steroidogenic cells, overlying the TI (Magoffin, 2005). Studies in rats (Amsterdam *et al.*, 1977; Walles *et al.*, 1978) and humans (Walles *et al.*, 1990) have indicated the presence of muscle cells in the TE suggesting a role in contraction of the follicle and extrusion of the oocyte. In a recent mouse study, it was shown that endothelin-2 (EDN2) induced smooth muscle contraction in the thecal externa layer of the periovulatory follicle (Ko *et al.*, 2006). It was demonstrated, by immunohistochemistry, that individual follicles were surrounded by a smooth muscle layer in the theca externa except at the apex region of the periovulatory follicle. Administration of an endothelin receptor antagonist into the ovarian bursa or directly into the ovarian medulla, resulted in a dose-dependent impairment of ovulation (Ko *et al.*, 2006).

Several hours prior to follicular rupture, striking morphological changes occur in the follicle. Thus, the cells of the OSE increase in size, accumulate lysosomes and eventually parts of the OSE disappear from areas of the apex (Bjersing and Cajander, 1974a; Bjersing and Cajander, 1974b). An oedema appears in the TI and the inner region of the TE, later spreading to all layers of the follicle wall (Bjersing and Cajander, 1974a). These changes are primarily localized to the apex. The extensive granulosa cell proliferation, which took place in the Graafian follicle then arrests. In addition, the LH surge induces an increase in cell size and stimulates morphological signs of luteinization in the granulosa cell layer. The mural granulosa cells penetrate the lamina propria (Bjersing *et al.*, 1981) and some cells become detached and migrate into the follicular antrum (Parr, 1974; Parr, 1975). Both the mural and cumulus granulosa cells dissociate, due to production of mucopolysaccharides, primarily hyaluron sulphate (Rankin *et al.*, 2001), in a process referred to as cumulus expansion (Hillensjö *et al.*, 1982) among the granulosa cells.

The most prominent structural change is the degradation of the connective tissue, especially in the TA and the TE, which leads to follicular rupture. Preceding rupture in the rabbit, dissociation of collagen fibres in the theca was observed (Espey, 1967b), the tensile strength of collagenous tissue in the follicle wall decreased (Espey, 1967a) and it has been suggested that specific proteolytic enzymes are involved in this process of degradation (Espey and Lipner, 1994). The thinning and disintegration of the follicular wall is most prominent in the apical area and a thin translucent stigma cone forms at the apex as a final sign of imminent rupture (Blandau, 1955; Löfman *et al.*, 2002; Dahm-Kähler *et al.*, 2006a).

As early as 1916, Schochet suggested that proteases may play a role in ovulation by digesting the theca folliculi, thereby weakening the follicle wall (Schochet, 1916). It was later shown that injection of proteolytic enzymes into the rabbit follicular antrum led to ovulation (Espey and Lipner, 1965). During the following decades, studies on follicular rupture have focused on two proteolytic enzyme systems, the MMPs and the plasmin/plasminogen activator (PA) system. The plasmin/PA system will be mentioned briefly in the section “*Ovulation-associated mediators*” and the role of MMPs in ovulation will be explored in the Discussion-section of this thesis.

Vascular changes

The ovary is an exceptionally vascularized organ in which also dramatic cycle-dependent changes in the localization and extent of vascularization occur (Kerban *et al.*, 1999). Throughout follicular growth, the capillaries in the highly vascularized TI, which encircles the avascular antrum/granulosa cell compartment, proliferate and anastomose to form a basket-like structure (Murakami *et al.*, 1988). Soon after the preovulatory LH-surge there is a distinct dilatation of the vasculature surrounding the ovulating follicles (Kranzfelder *et al.*, 1992), which contributes to an increase in ovarian blood flow as has been shown in the rabbit using a

radioactive microsphere technique (Janson, 1975), and in cows (Acosta and Miyamoto, 2004) and humans (Brännstrom *et al.*, 1998) using color-Doppler ultrasonography. Moreover, an increased permeability in the follicular microvasculature has been described (Okuda *et al.*, 1983) as well as extravasation of blood components into the pericapillary space (Brännstrom *et al.*, 1993) and development of oedema in the follicular wall (Bjersing and Cajander, 1974a). This alteration in permeability, in combination with an increase in ovarian blood flow taking place during the same period, may cause a major fluid transudation from the vascular wreath in the TI to other parts of the periovulatory follicle. It has been suggested that this up-regulation of the fluid supply to the follicle is required for the increase of intra follicular pressure that is observed prior to ovulation (Matousek *et al.*, 2001). Furthermore, in a study in which the ovarian blood flow was reduced by ligation of the major arteries supplying the ovary, the number of ovulations was diminished (Zackrisson *et al.*, 2000). This further indicates the importance of ovarian blood flow in the ovulatory process.

Ovulation-associated mediators

A multifaceted network of autocrine and paracrine interactions results in follicular rupture in the normal menstrual cycle. Numerous biochemical mediators are mobilized in the ovary after the preovulatory LH-surge. These ovulation-associated mediators cooperatively facilitate the changes in the follicle necessary for follicular rupture and some of them will be described briefly below.

The plasmin/plasminogen activator system has been suggested to be one of the main actors in the degradation of the follicular connective system prior to follicular rupture. The key components of the plasmin/PA system are the proteolytic activators, tissue-type PA (tPA) and urokinase-type PA (uPA), the proenzyme plasminogen and its enzymatically active degradation product, plasmin, together with the central inhibitors of this system, PA inhibitor-1 and -2 (PAI-1, PAI-2), reviewed by (Ny *et al.*, 2002; Liu, 2004). This system is activated by the release of tPA or uPA from specific cells, initiated by external signals such as cytokines, growth factors and hormones, reviewed by (Myohanen and Vaheri, 2004). Plasmin, the end product of the PA cascade, is able to cleave ECM proteins, regulate growth factor activity and convert some proMMPs into active MMPs.

The expression and secretion of PAs and PAIs preceding ovulation is induced by gonadotropins in a cell-specific and time-coordinated manner in several species such as the rat (Peng *et al.*, 1993), the mouse (Hägglund *et al.*, 1996), the pig (Politis *et al.*, 1990), and the rhesus monkey (Liu *et al.*, 2004). Thus, in the rat, tPA expression in granulosa and thecal interstitial cells, was induced by gonadotropins, and PAI-1 was up-regulated in the theca interstitial cells and surrounding stroma 6 hours before follicular rupture (Peng *et al.*, 1993). By comparison, uPA, expressed by granulosa cells, appears to be the most abundant and noticeably up-regulated PA during the ovulatory process in the mouse (Hägglund *et al.*, 1996). There was also a synchronized up-regulation of tPA in thecal interstitial tissue, also in the mouse (Hägglund *et al.*, 1996). Moreover, in the rhesus monkey prior to ovulation, accompanying the highest granulosa cell-produced tPA expression, the theca-derived PAI-1 declined to a minimal level which may assist in the breakdown of the follicular wall (Liu *et al.*, 2004). However, the PA-system has not been extensively investigated during ovulation in the human. A limited number of studies have been performed on granulosa lutein cells from IVF-cycles showing relative abundance of PAI, yet little or no PA (Jones *et al.*, 1988; Jones *et al.*, 1989). The differences between animals and humans are somewhat surprising but could, at least in part, be species specific.

Even though a considerable body of indirect evidence obtained from different species has indicated that the PA-system plays a role in ovulation, studies on knockout (KO) mice with single deficiencies for either of the components of the PA system, as well as on tPA/uPA double deficient mice, have shown that these mice are fertile although the ovulation rate is reduced to some degree in the double KO mouse (Leonardsson *et al.*, 1995; Ny *et al.*, 1999). This indicates that the PA-system may be of less importance for follicular rupture and that the activation of MMPs may be involved in this process. However, it is likely that a great redundancy has been built into this very important reproductive process, so that other pathways may be able to compensate for the deficiency of one pathway.

Nitric oxide (NO), a free radical gas with a half-life of <5 s, is one of the smallest known bioactive products of mammalian cells (Nathan, 1992). Nitric oxide is formed from the essential amino acid L-arginine through oxidation by nitric oxide synthase (NOS) and participates in a variety of physiological and pathophysiological conditions such as regulation

of local blood flow, inflammatory response and tissue remodelling processes, reviewed by (Thippeswamy *et al.*, 2006). Accordingly, the importance of NO has also been demonstrated during the ovulatory process. Thus, ovulation is stalled by the administration of NOS inhibitors in the rat (Shukovski and Tsafiriri, 1994). This was confirmed by studies on perfused rat ovaries *in vitro* in our own laboratory (Mitsube *et al.*, 1999) and by others (Bonello *et al.*, 1996). Moreover, KO mice for NOS have a reduced number of ovulations (Olson *et al.*, 1996; Jablonka-Shariff and Olson, 1998; Klein *et al.*, 1998; Drazen *et al.*, 1999; Jablonka-Shariff *et al.*, 1999). The importance of NO in the ovulatory process has primarily been thought to be caused by the vascular functions of NO (Mitsube *et al.*, 2002) and by its role as a regulator of ovarian steroidogenesis (Bonello *et al.*, 1996; Jablonka-Shariff *et al.*, 1999; Mitsube *et al.*, 1999). In addition, NO has been suggested to modulate proteolytic activity by participating in the activation of neutrophil collagenase (MMP-8) in human neutrophils (Okamoto *et al.*, 1997), induction of MMP-9 activity in the mouse (Yoshimura *et al.*, 2006) as well as modulation of MMP-2 activity in the rat (Robinson *et al.*, 2006).

Progesterone (P₄), produced by the luteinizing granulosa cells, is the most central, intraovarian regulator of the ovulatory process, reviewed by (Chaffin and Stouffer, 2002). Soon after the LH surge, there is an overall stimulation of steroid production due to induction of P450 side chain cleavage enzyme (Goldring *et al.*, 1987). The concentration of P₄ remains elevated throughout the periovulatory phase, whereas E₂ production declines towards the expected time of ovulation due to decreased aromatase expression (Hoff *et al.*, 1983; Chaffin *et al.*, 1999a). The physiological effects of P₄ are mediated by the interaction of the hormone with specific intracellular P₄ receptors (PRs) and it has been shown that LH increases PR mRNA expression within 4-6 hours in granulosa cells in the rat (Natraj and Richards, 1993) and within 12 hours in the rhesus monkey (Chaffin *et al.*, 1999b). Furthermore, LH/hCG was shown to have a stimulatory effect on P₄ production in human theca cells (Bergh *et al.*, 1993). In studies on isolated perfused rabbit ovaries, LH-induced P₄ production was enzymatically blocked without changing the ovulation rate (Holmes *et al.*, 1985; Yoshimura *et al.*, 1987). However, it was found in the rat that P₄ has an important mediatory role in LH-induced ovulations (Brännstrom and Janson, 1989). Administration of a selective PR antagonist to the perfused rat ovary reduced ovulation rate only when administrated in the early period after

LH stimulation, suggesting that P₄ dependency is restricted to the initial phases of the ovulatory process (Pall et al., 2000). This result, from our laboratory, was in line with results from an earlier study in the rhesus monkey where PR expression increased within 12 hours after the ovulatory stimulus (Chaffin *et al.*, 1999b). This corresponds to an early phase of the ovulatory cycle of the rhesus monkey, which extends over about 36 h.

Progesterone controls proteolytic enzyme activity in the sheep (Murdoch *et al.*, 1986) and in the rat (Iwamasa *et al.*, 1992) suggesting a possible role in regulating degradation of the follicular wall, which is necessary for ovulation. Moreover, P₄ regulates the expression of MMP-1 and TIMP-1, in the rhesus monkey (Chaffin and Stouffer, 1999).

Taken together, there is a body of evidence indicating that P₄ is a key coordinator of ovulation. This has been further highlighted by studies on PR KO mice, exhibiting pleiotropic reproductive abnormalities, such as inability to ovulate, uterine hyperplasia, limited mammary development and abnormal sexual behaviour (Lydon et al., 1995). More recently, the PR KO mice were found to exhibit reduced expression of two PR-induced proteases, ADAMTS -1 and cathepsin L (Robker et al., 2000) supporting their role in follicular remodelling.

Prostaglandins (PGs), members of the eicosanoid family, are important ovulatory mediators as shown by studies in rats (Armstrong, 1981) as well as humans (Killick and Elstein, 1987). Free arachidonic acid is metabolized into PG by cyclooxygenase (COX) enzymes. There are two isoforms of COX and both have been detected in the ovary. Thus, COX-1 is constitutively expressed in theca cells (Wong and Richards, 1991) and COX-2 is induced by LH in granulosa cells (Wong and Richards, 1991; Morris and Richards, 1995). The importance of PGs in the ovulatory process has further been demonstrated in studies showing that inhibition of the PG synthesis by non-steroid-anti-inflammatory drugs (NSAIDs) decreases the ovulation rate in both the cow (Orczyk and Behrman, 1972) and the human (Killick and Elstein, 1987; Pall *et al.*, 2001). In a study on perfused rabbit ovaries, *in vitro* administration of a nonselective COX inhibitor blocked and addition of PGF₂ α restored ovulation in all ovaries (Holmes *et al.*, 1983). Also the administration of selective COX-2 inhibition has been shown to reduce the LH/hCG-stimulated production of prostanoids and the number of ovulations both *in vivo* and *in vitro* (Mikuni *et al.*, 1998) in the rat. The importance of COX-2 activity in the ovulatory process has also been shown in humans in a

study where a selective COX-2 inhibitor inhibited ovulation (Pall *et al.*, 2001). COX-2 is suggested to be the main catalyser of PG-production in the ovary, since female KO mice lacking this enzyme are infertile (Dinchuk *et al.*, 1995; Lim *et al.*, 1997). To further underscore the importance of PGs in the ovulatory process it has been reported that female mice lacking COX-2 or the PGE(2) receptor EP2 are infertile and show decreased ovulation rate with abnormal cumulus expansion (Ochsner *et al.*, 2003).

In cultured granulosa cells from the rhesus monkey, PGE2 decreased LH-stimulation of MMP-1 mRNA and PGF2 α reduced LH-stimulated TIMP-1 mRNA levels proposing a regulatory function of PGs also in the proteolytic cascade (Duffy and Stouffer, 2003).

The renin-angiotensin system (RAS) is recognized mainly as a vasopressor system that maintains blood pressure via its vasoconstrictor as well as its fluid- and electrolyte-conserving actions. However, investigations during the last two decades have indicated that RAS has much broader functions and that the ovary is one of many tissues having its own local RAS (Speth and Husain, 1988). This ovarian RAS comprises: renin, the proteolytic enzyme that covert angiotensinogen into angiotensin I (Ang I); angiotensinogen, the major substrate for renin and the crucial precursor of angiotensin II (Ang II); angiotensin converting enzyme (ACE) the enzyme that converts Ang I into Ang II; and AngII receptors initiating the cellular effects of the active hormone Ang II, reviewed by (Yoshimura, 1997). The importance of RAS in the ovulatory process was suggested by the finding that the concentration of Ang II in follicular fluid increases after the hCG/LH-surge in the human (Lightman *et al.*, 1987) and rabbit (Yoshimura *et al.*, 1994). Additionally, it has been shown that Ang II induces ovulation in the rabbit (Yoshimura *et al.*, 1996) and that a nonselective Ang antagonist reduces the ovulation rate in rats (Pellicer *et al.*, 1988). A recent study on perfused rat ovaries showed that intrabursal injection of Ang II reduced the ovarian blood flow in vitro (Mitsube *et al.*, 2003), indicating that the ovarian RAS is involved in the regulation of ovarian blood flow.

Cytokines are a group of peptides with numerous functions including autocrine and paracrine regulation of immune cells, which are essential for several immunological, inflammatory and infectious diseases. More than 100 cytokines have been reported, including the interleukins (ILs), tumour necrosis factors (TNFs), interferons (INFs), colony stimulating factors (CSFs)

and the group of chemotactic cytokines called chemokines. These biologically potent mediators are produced by a wide variety of cell types, such as leukocytes, endothelial cells and most tumour cells. Furthermore, cytokines act on many different cells, although many share similar functions and are in that context characterized by a significant redundancy. This complexity is further compounded by the fact that a cell is rarely exposed to only one cytokine, and exposure of several cytokines to one cell can have different effects depending on the combination of the specific cytokines and the tissue context. In view of the fact that cytokines regulate many inflammatory reactions they have also gained interest in the perspective of the ovulatory process, which has been recognized as an inflammatory-like reaction, and the emerging concept is that the cytokines are also instrumental in the regulation of follicular development, ovulation and corpus luteum function. Numerous cytokines have been implicated in some of these cyclic ovarian events (Wang and Norman, 1992; Brännstrom *et al.*, 1995; Runesson *et al.*, 2000). IL-1 stands out as a cytokine, which has key effects on functional and structural alterations within the ovary at all stages of the ovarian cycle, and its role during ovulation will be briefly summarized.

The IL-1 system includes two bioactive ligands, IL-1 α and IL-1 β , and one natural receptor antagonist (IL-1ra). These molecules bind to type 1 (IL-1R1) and type 2 receptors (IL-1R2).

The IL-1s are primarily synthesized as 31kDa precursors (pro-IL-1), which are further cleaved to produce a 17 kDa mature IL-1. However, the precursor can be as biologically active as the mature form intracellularly as shown for IL-1 α (Roux-Lombard, 1998).

During the ovulatory process there is a massive influx of neutrophils and macrophages into thecal layer of the periovulatory follicle (Brännstrom *et al.*, 1993; Brännstrom *et al.*, 1994). These leukocytes have a great capacity for IL-1 secretion and activation. In an initial study on the potential influence of the IL-system in ovulation, a positive correlation was found between the IL-1 levels in human follicular fluid and plasma from women analysed during IVF-cycles (Wang and Norman, 1992). The IL-1 levels in follicular fluid were about half of the plasma levels but the follicular IL-1 concentration was still physiologically relevant. Furthermore, IL-1 β induction has been detected in preovulatory follicular aspirates from IVF-cycles, and IL-1ra and IL-1R expression has been shown in different compartments of the human ovary (Hurwitz *et al.*, 1992). An apparent role for IL-1 in ovulation was first demonstrated in perfused rat ovaries where IL-1 β independently induced ovulations as well as potentiated the

LH-induced ovulatory effect by increasing the number of ovulated oocytes (Brännstrom *et al.*, 1993). A more recent study in mares, confirmed these observations by showing that an intrafollicular injection of IL-1 β at the preovulatory stage could induce ovulation, and that IL-1ra administered the same way reduced the ovulation rate or delayed the time of ovulation (Martoriati *et al.*, 2003).

A number of studies have been carried out to elucidate whether IL-1 affects any of the other LH-induced mediatory systems involved in the ovulatory process. Thus, IL-1 is proposed to intervene in PG production mainly by regulating COX-2 synthesis (Ando *et al.*, 1998) and IL-1 β is able to restore ovulation in COX-2 null mice (Davis *et al.*, 1999). In a study on rat ovaries, it was shown that IL-1 β induced secretion of gelatinase-B (MMP-9) in a dose-dependent manner in cultures of whole ovarian dispersates (Hurwitz *et al.*, 1993). Furthermore, IL-1 β inhibits PA activity in cultured granulosa cells in the rat (Hurwitz *et al.*, 1995). This suggests that IL-1 interacts with both the MMP and PA system and thereby operates as a regulator in the ECM remodelling process necessary for follicular rupture.

Chemokines are cytokines with chemotactic activity. A number of chemokines are present at increased levels in the ovary during the ovulatory process (Runesson *et al.*, 2000; Wong *et al.*, 2002; Zhou *et al.*, 2005). As the chemokines have the capacity to control the movements of leukocytes (Luster, 1998) they have been suggested to play an important role in the migration and activation of leukocytes in the follicle prior to rupture. Monocyte chemotactic protein-1 (MCP-1) has chemotactic activity on monocytes, T-lymphocytes and basophils. The main effect of MCP-1 is the ability to recruit circulating monocytes into different tissues (Luster, 1998). In a recent study from our own laboratory it was shown that MCP-1 is highly expressed in the human periovulatory follicle and that MCP-1 is induced by IL-1 in the theca layer (Dahm-Kähler *et al.*, 2006b).

Corpus luteum

The midcycle LH-surge initiates major structural and functional changes that transform the large preovulatory follicle into a corpus luteum (CL). Luteal formation, known as luteinization, is the transformation of TI cells and granulosa cells into mainly P₄ producing lutein tissue. This process begins prior to, and appears to be required for, follicular rupture

(Kamat *et al.*, 1995). This is most likely explained by activation of PR and secondary effects as discussed above. In response to the LH-surge, blood vessels in the thecal area invade the previously avascular granulosa layer.

The BL dividing the theca and granulosa layers disintegrate to allow for further angiogenesis and cell migration towards the centre (Bjersing and Cajander, 1974c; Bjersing and Cajander, 1974d). Following follicular rupture, the wall of the follicle collapses, the theca lutein layer folds into the granulosa lutein layer, and a rosette-like structure is formed. There may be some associated bleeding from the capillaries of the theca interna, resulting in the formation of a central blood clot. Fibroblasts grow into the gland from the periphery and an extensive neovascularisation permits immune cells to migrate from the blood stream into the theca lutein and granulosa lutein areas (Brännstrom and Fridén, 1997; Stouffer, 2004). This swift and extreme angiogenesis results in the construction of an extraordinarily dense capillary plexus present throughout the mature CL (Murakami *et al.*, 1988) and luteal blood flow is among the highest tissue blood flows in the body (Janson *et al.*, 1981).

Within this newly formed CL, two sub-populations of steroidogenic cells are evident. During luteinization there is a marked hypertrophy of the steroidogenic cells with an increase of granules and lipid droplet content, indicating the accumulation of the steroid substrate cholesterol within the cells to enable increased capacity of P₄ synthesis. The two cell populations are referred to as large luteal cells most likely of granulosa cell origin, and small luteal cells presumable of thecal cell origin, based on the noticeable morphological differences (O'Shea *et al.*, 1989). The smaller theca lutein cells are found peripheral to the more numerous, larger granulosa lutein cells, as shown by immunohistochemistry (Maybin and Duncan, 2004).

Connective tissue elements also migrate into the developing CL from its periphery, forming a network around the lutein cells and gradually converting the resolving blood clot in the central cavity into a fibrous core. In a recent study on the human CL two different types of ECM were identified. Subendothelial BLs composed of collagen type IV $\alpha 1$ and laminin were found, which were localized to an interstitial matrix between non-vascular cells at irregular intervals (Irving-Rodgers *et al.*, 2006). Versican was localized to the connective tissue margins of the CL (Irving-Rodgers *et al.*, 2006).

In non-fertile cycles, the CL undergoes gradual regression, luteolysis, starting after the mid-luteal P₄ peak (Morales *et al.*, 2000). This demise occurs in two phases. Firstly, functional luteolysis occurs when the CL loses its ability to produce P₄ and thereby allows for the development of new follicles. Secondly, the lutein cells degenerate, which is referred to as structural luteolysis. This phase commences after the decrease in P₄ secretion, although complete degeneration of the CL takes numerous cycles (Stouffer, 2004). Until quite recently, it was generally assumed that the final fate of the CL is the formation of a white scar, the corpus albicans (CA), through fibrosis and hyalinization of luteal tissue. However, Morales *et al.* demonstrated in a morphologic study of over 600 ovaries from cycling women that CAs were absent in about 28% of the women, indicating that alternative luteolytic patterns may exist (Morales *et al.*, 2000). It was suggested that only CLs presenting a large blood-filled cavity give rise to typical large CAs.

MATERIALS AND METHODS

Ethical considerations

This work was solely performed on human tissues. The study was approved by the Ethics Committee of the Sahlgrenska Academy at Göteborg University. Each woman was given both written and verbal information about the study. Informed written consent was obtained from all women before they were included. One woman had a prophylactic oophorectomy because of germline BRCA mutation. All other women, who participated in the study, underwent planned surgery for non-ovarian diseases at the Division of Gynecology and Reproductive Medicine at Sahlgrenska University Hospital.

Human subjects

Paper I

Sections from whole ovaries were obtained from 5 regularly menstruating women (age 44 - 51 years). These women did not have any chronic systemic diseases and were not at the time of the study on any hormonal medication. The women underwent bilateral oophorectomy as a part of the surgical treatment for cervical cancer (n=4) or prophylactic oophorectomy due to familial breast-ovarian cancer (n=1). In addition, precisely timed ovarian samples were obtained during the periovulatory interval from patients undergoing laparoscopic sterilization (see below).

Paper I-IV

Thirty-two women (age 30-39 years, mean 35.4), with previously proven fertility (para >1, mean 2.9), and who menstruated regularly (cycle length 26-32 days, mean 29 days), with no chronic systemic diseases and who were planned for laparoscopic bilateral tubal ligation (sterilization) were included in the study. The patients had not been on any hormonal contraceptive for a period of at least 3 months prior to surgery. These patients were monitored extensively before and during the cycle of tissue harvesting, as described below (Table III).

Table III Characteristics of the study population. All data is given as means \pm SEM, n=7 in each group (paper II), n=5 in each group (paper I, III-IV).

	Preovulatory phase	Early ovulatory phase	Late ovulatory phase	Postovulatory phase
Follicle size mm	15.8 \pm 0.6 range 14-17.5	16.3 \pm 0.3 range 15-17	16.9 \pm 0.6 range 15-20	16.7 \pm 0.4 range 15-18
Hours after rhCG		14.0 \pm 0.9 range 12-18	21.9 \pm 0.8 range 18.5-24.0	49.1 \pm 0.6 range 44-70
Progesterone nmol/L	0.4 \pm 0.5 range 0.3-0.6	1.0 \pm 0.13 range 0.5-1.4	2.0 \pm 0.4 range 1.4-3.0	3.1 \pm 1.2 range 0.5-9.0
Estradiol nmol/L	0.4 \pm 0.1 range 0.13-1.0	0.6 \pm 0.1 range 0.4-0.9	0.7 \pm 0.1 range 0.6-0.9	0.3 \pm 0.0 range 0.21-0.56
LH IU/L	4.9 \pm 0.4 range 2.9-6.0	9.7 \pm 4.3 range 3.0-34.9	34.2 \pm 9.0 range 2.1-64.2	16.6 \pm 3.8 range 0.21-31
Cycle day of surgery	14 \pm 0.3 range 13-15	12.6 \pm 0.5 range 11-14	13 \pm 0.6 range 11-15	13.1 \pm 0.6 range 11-15
Cycle length (days)	30.1 \pm 0.9 range 28-35	28.1 \pm 0.8 range 26-32	29.8 \pm 0.8 range 28-32	29.4 \pm 0.9 range 27-34
Age (years)	35.2 \pm 1.1 range 30.8-37.7	34.6 \pm 1.0 range 30.1-37.8	36.7 \pm 0.4 range 34.9-38.2	35.4 \pm 0.7 range 33.2-37.0

Follicular development monitoring and experimental groups (paper I-IV)

To ascertain that normal development of a dominant follicle occurred during a menstrual cycle prior to the study cycle, all patients were repeatedly examined by transvaginal ultrasound (TVU; Aloka SSD-900/2000, Aloka Co Ltd, Tokyo, Japan), during a minimum of one menstrual cycle (mean 2 cycles) before surgery. During the first half of the menstrual cycle, during which the laparoscopic sterilization took place (the cycle when the tissue was collected), TVU was performed every 1-2 days to enable the surgical procedure to be planned during the periovulatory interval, as described in detail below.

The study was designed to monitor the whole periovulatory period and to surgically resect the entire preovulatory/ovulatory follicle. Prior to the study, it was discussed whether the endogenous gonadotropin surge or exogenous hormones should be used to trigger ovulation. The best would of course be to use the physiological gonadotropin surge as the ovulatory trigger in these experiments. However, previous experience with self-measurements of the LH-surge (by the use of urinary LH sticks) in a research study in our laboratory showed that it was often difficult for the patient to determine the transition from a negative to a weakly positive test and also that some patients misinterpreted a negative test as positive (Brännstrom *et al.*, 1998). In that specific study, some patients were called in for TVU-Doppler examination during what was thought to be the ovulatory phase, according to the patient's test, but subsequent analysis of blood samples did not indicate elevated LH-levels. Thus, for these reasons and since it would be impossible to plan the surgery to specific days, when relying on the endogenous gonadotropin surge, this alternative was ruled out.

The alternatives that were discussed for exogenous hormonal stimulation were recombinant human LH, recombinant hCG, and urinary hCG. The use of recombinant human LH would be the preferred choice, since this is the natural hormone to induce ovulation. Contacts with the pharmaceutical industry revealed that even if recombinant human LH was available on the market it was not produced to act as an ovulatory trigger. Thus, the doses and concentrations which are used for stimulation of folliculogenesis as a complement to recombinant FSH, were too low to be used by subcutaneous (sc) administration to induce ovulation. About 5 ml had to be administered to give the patient the quantity of LH that would trigger ovulation, and these volumes could only be administered intravenously. Because the ideal time of administration

would often fall outside of office hours and it would be preferable if the patient herself could give the injection, we sought a s.c. route of administration.

Several studies have shown that urinary preparations of hCG induces ovulation in menstrual cycles (Hanna *et al.*, 1994) and in clomiphene citrate stimulated cycles (Andersen *et al.*, 1995). Human CG binds to the LH-receptors, and should then set in motion the same intracellular events as LH. However, the biological half-life of hCG is considerably longer than the half-life of LH and this may of course influence the results. It was decided to use recombinant hCG instead of a urinary hCG preparation since this would avoid effects caused by possible contaminants of the urinary hCG and also that a higher batch-to-batch consistency would be expected.

Several studies in the literature indicate that a dominant follicle of a size larger than 12 mm is fully LH/hCG-responsive and has the cellular and inter cellular machinery necessary to go through the entire ovulatory process (Gougeon, 1996; van Dessel *et al.*, 1996; Macklon and Fauser, 1998). In the present study a minimum follicular diameter of 14 mm was used as a cut off for a definition of a true preovulatory follicle. This limit of 14 mm allows for errors of measurement and also gives some margin for the expected biological variation.

In order to determine the approximate time of follicular rupture after rhCG administration, a pilot study was performed. Five healthy volunteers (aged 25-38 years) with no current hormonal contraception participated. When the dominant follicle had reached a diameter of >14 and up to 17 mm on TVU, a s.c. injection of 250µg rhCG (Ovitrelle®; Serono International S.A., Genève, Switzerland) was given. Frequent TVU examinations determined that rupture occurred between 30 and 36h after hCG administration. Luteinization, as a part of ovulation, was confirmed by elevated serum progesterone levels. A previous larger study using 6000 IU of urinary hCG detected follicular rupture around 38 h after injection (Andersen *et al.*, 1995), a result which is in line with the results of the pilot study. Moreover, the dose of 250 µg of recombinant hCG, used in the present study, is equally effective as urinary preparations (Driscoll *et al.*, 2000; Al-Inany *et al.*, 2005). Thus, the results from these studies and from our pilot study convinced us that rhCG at a dose of 250 µg is a suitable substitute for the LH-surge.

The study population was divided into four groups (preovulatory phase, early ovulatory phase, late ovulatory phase and post ovulatory phase) to distinguish between the different stages of the ovulatory process. The work of the present thesis is part of a bigger project to examine the genome-wide transcriptional consequences of the LH surge. Part of the cellular material from granulosa cells, theca cells and perifollicular stroma were analyzed by gene microarray to detect genes that are significantly up- or downregulated by LH. Some animal studies, mostly in rodents, have used various techniques to detect LH-induced changes in ovarian expression. These studies have all used hCG as the ovulatory trigger and they have had many (typically more than 5) time-points throughout the ovulatory interval to examine the expression levels. It would, for practical reasons, not be possible to recruit enough patients to obtain material from several time points during the human ovulatory interval and we therefore decided to examine the preovulatory stage, as a possible baseline and to compare this stage to two phases during the ovulatory process and to one interval after follicular rupture. The first phase during ovulation (early ovulatory phase) was predicted to capture the rapidly expressed genes and the second phase during ovulation (late ovulatory phase) was predicted to capture the more slowly expressed genes.

The preovulatory phase was defined as the stage when the dominant follicle was ≥ 14 mm and ≤ 17.5 mm. For the patients in this group, surgery was performed at this preovulatory stage without giving rhCG. Measurements of serum levels of estradiol and progesterone did not suggest initiated ovulation/luteinization. The majority of women received a s.c. injection of 250 μ g rhCG (Ovitrelle®) when the dominant follicle was of a diameter of ≥ 14 mm and ≤ 20 mm. These patients had surgery during either of three different time intervals after rhCG-injection: Early ovulatory phase, defined as 12 h to ≤ 18 h after rhCG, late ovulatory phase >18 h to ≤ 24 h after rhCG or post ovulatory phase 44 h to 77 h after rhCG (Fig. 7). Samples for measurement of serum levels of progesterone and oestradiol were drawn immediately before surgery to confirm their ovulatory phase category. The characteristics of the patient material are shown in Table I. When the study was planned the goal was to narrow the early ovulatory phase to 12-14 h after hCG, but it was later realised that it was extremely difficult to plan the surgery at such exact times, since we had to fit the operations into a the daily operating plan and the exact durations of the other operations at a specific operating theatre

could not be determined beforehand. The goal was to get samples from the late ovulatory phase at a time around 30 h post injection to be able to capture the genes that were expressed within a few hours before anticipated follicular rupture. However, during a small number of operations performed at this time of the ovulatory phase, the follicle would rupture during surgical manipulation, even if care was taken to avoid any trauma to the apical part of the follicle. When the follicle ruptured during surgery a large amount of the granulosa cells would be lost as spillage into the abdominal cavity. Thus, it was decided to obtain the late ovulatory phase samples at a somewhat earlier time point, which was less well separated in time from the early ovulatory phase. This is of course a disadvantage when we try to distinguish between rapidly and slowly induced genes in the human ovulatory process.

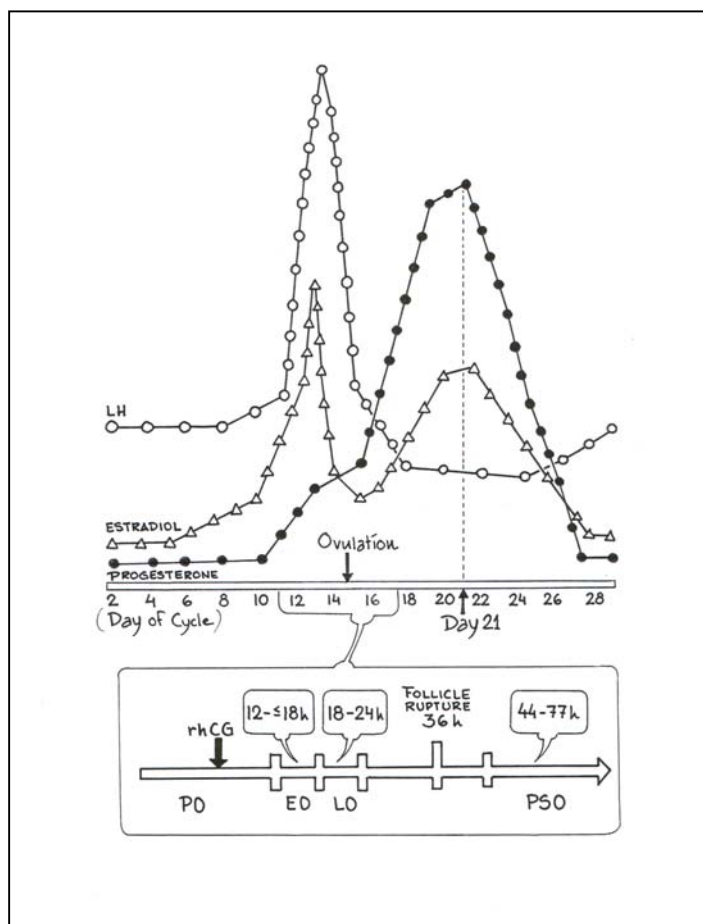


Figure 7. Hormonal changes during the menstrual cycle. Magnification shows the ovulatory phases when the human material of paper I-IV were collected. Preovulatory phase (PO), early ovulatory phase (EO), late ovulatory phase (LO) and postovulatory phase (PSO). Recombinant hCG (rhCG).

Tissue harvesting

The side of the dominant follicle had been identified and documented by TVU. Only cycles where TVU examination showed the typical appearance of a follicle and with the predicted increase in size of around 2 mm per day (Zegers-Hochschild *et al.*, 1984) were scheduled for surgery. The first procedure after inserting the trocar was to closely study the ovary of the dominant follicle and ensure that the dominant follicle was accessible for surgery and not covered by adhesions. The dominant follicle, together with the immediately adjacent stroma, was then excised with scissors from the ovary. During this procedure a dissection plane was found immediately basal to the follicle and the ovarian capsule was held with forceps on each side of the follicle. Diathermy was not used, since this would most likely cause thermal damage to the tissue and the follicle with predicted damage also to mRNA and proteins. The entire intact follicle was taken out from the abdomen inside a laparoscopic sac through the trocar incision above the pubic bone. The surgical procedure continued with bipolar diathermy of the proximal parts of the oviducts and division by scissors to carry out the sterilization procedure.

The follicle was, immediately placed on ice, taken to the laboratory (2 minutes away), washed with ice cold RNAsfree phosphate buffered saline (PBS) and submerged into this solution.

The majority of the follicles were used to acquire stromal tissue, theca cells and granulosa cells (Fig. 8). This was accomplished by opening each follicle (kept on ice) with scissors to release and collect the loosely attached granulosa cells. The mural granulosa cells were then gently scraped off the interior follicle wall by use of the blade of small tissue forceps. The follicular fluid and cell suspension were combined and was centrifuged at 500 g to pellet the granulosa cells. Previous studies in our laboratory, using an identical technique to obtain granulosa cells, did not demonstrate any detectable contamination of white blood cells in the granulosa cell preparations, as compared to 11-13% contamination of white blood cells in preparations of granulosa-lutein cells from IVF-cycles (Runesson *et al.*, 2000). Since there is a considerable vascularisation of the thecal layer, these results indicate that the granulosa cell preparations of the present study are fairly pure. The exception is of course the oocyte, which most likely follows the granulosa cells in the preparations of the different cell types.

The theca cells were harvested mechanically from the remnants of the follicle by separating the thin layer of the theca interna from the underlying theca externa cell layer using watchmakers' forceps. The theca cells, as they are called in paper IV, is probably a mixture of theca interna cells, cells from the vasculature of that layer, immune cells and fibroblasts, since all these cells types are major components of this layer. However, since all these cells can be active in the ovulatory changes, by themselves or in collaboration with each other, we considered it appropriate to study them together. The remaining tissue was defined as perifollicular stroma, although no specific investigations were made to determine whether this cell mixture contained the theca externa cells or not. These samples also contain vascular cells, leukocytes, fibroblasts, the so called interstitial cells and follicular cells of small follicles. All cell preparations were snap frozen in liquid nitrogen and kept at -70°C until further analysis by quantitative western blot (Paper II) and real time PCR (Paper II-IV). Stroma and supplementary whole follicles were used for immunohistochemistry, and these tissues were fixed in 4% formaldehyde overnight and then embedded in paraffin.

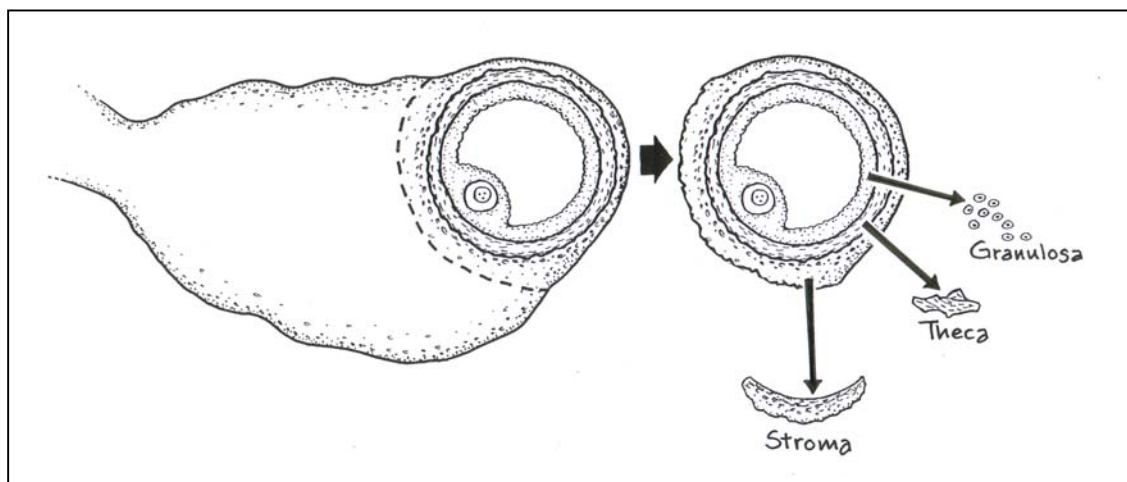


Figure 8. Schematic drawing of the tissue separation of the human follicle after surgery in paper I-IV. (with kind permission from Dahm-Kähler, 2006).

Sections from whole ovaries were obtained from women undergoing surgery including bilateral oophorectomy. When the ovary had been removed, it was bisected in the midline of its longitudinal axis, by the use of a scalpel. A second cut was made in one of the halves around 2 mm from the cut surface to get an ovarian slice with a thickness of about 2 mm. The ovarian section was then immediately fixed in 4% formaldehyde.

Primary antibodies

The following primary antibodies were used for immunohistochemistry (all monoclonal antibodies produced in mouse) (paper I): collagen type I (ab6308, dilution 1:600, Abcam, Cambridge, UK), collagen type III (ab6310, dilution 1:600, Abcam), collagen type IV (ab6311, dilution 1:400, Abcam), vimentin (555480, dilution 1:100, BD-PharMingen, San Diego, CA, USA) and CD 45 (v6630, dilution 1:400, Sigma-Aldrich, Saint Louise, MI, USA).

The following primary antibodies were used for both Western blot (paper II) and immunohistochemistry (paper II-IV): MMP-2 (cat.no.IM33L, mouse monoclonal anti human MMP-2, Calbiochem, EMD Biosciences, Inc/Merck KGaA, Darmstadt, Germany); MMP-9 (ab16306, rabbit polyclonal anti mouse MMP-9, Abcam, Cambridge, UK); TIMP-1 (cat.no.IM41L, mouse monoclonal anti human TIMP-1, Oncogene Research Products/Calbiochem); TIMP-2 (cat.no.IM11L, mouse monoclonal anti human TIMP-2, Oncogene Research Products).

The following antibodies were only used for Western Blot (paper II): α -actin (product no. A2066, rabbit polyclonal anti human α -actin, Sigma-Aldrich, Saint Louise, MI, USA) and α -tubulin (product no. T 5168, mouse monoclonal anti human α -tubulin, Sigma-Aldrich).

Chemicals

The different substances used are described in detail in the material and method sections of the individual papers.

Western Blotting (paper II)

Western blotting is used to detect the quantity of specific proteins in a tissue. Tissue protein extracts are prepared and equal amounts of denatured total protein are loaded into each well for 1D sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

A molecular weight marker is also loaded to be able to determine what size the resulting bands are of. The proteins are separated according to their molecular weight and are further transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane is blocked with a blocking agent to minimize the background. A primary antibody directed to the target of interest is added to the membrane for incubation. After washing, a secondary antibody, which is conjugated to an enzyme directed towards the primary antibody, is added.

Chemiluminescent detection uses an enzyme to catalyse a reaction that results in the production of visible light. Detection is achieved by exposing the membrane to x-ray film.

Semi-quantitative measurements of the optical density (OD) of each band are made by densitometry (fluor-STM Multimager, BioRad, Hercules, CA, USA), and the relative value is used for further statistical analysis.

Immunohistochemistry (IHC; paper I-IV)

Immunohistochemistry is used to study the localization of a protein. Tissues are fixed in formalin and embedded in paraffin. Tissue sections of approximately 4 µm are cut and subjected to antibody binding. This technique is based on the binding of a primary antibody to the antigen (protein) of interest. A secondary antibody, directed towards the primary antibody, is added and the secondary antibody is coupled to a specific enzyme complex. In the last step, the tissue antigen is localized by incubation with a substrate for the enzyme.

In these studies a system from Vector was used, based on a complex between avidin and a biotinylated enzyme. Following development of the complex, a substrate, NovaRED (Vector Laboratories, Burlingame, CA, USA) was used. A red reaction product at the antibody/antigen binding site can then be visualized by bright-field microscope.

Real time PCR

Real-time polymerase chain reaction (PCR) is used to detect changes in gene expression in a tissue, reviewed by (Bustin, 2002; Bustin and Nolan, 2004). This method is highly sensitive

and permits quantification of small variations in gene expression. The first step is to isolate mRNA from the particular cell/tissue sample. The quality of the extracted RNA is analyzed to confirm its purity before producing a deoxyribonucleic acid (DNA) copy of complementary DNA (cDNA) of each mRNA molecule using the enzyme reverse transcriptase; the process is known as reverse transcription (RT). This is required since RNA removed from its cellular environment is highly unstable, it is particularly sensitive to heat. The gene, the expression level of which is to be determined (the target gene) is then further amplified from the cDNA mixture together with a “housekeeping” gene. Housekeeping genes are expressed at relatively constant levels in all nucleated cell types because they are necessary for basic cell survival. In general, amplification of a given cDNA over time follows a curve, with an initial flat-phase, followed by an exponential phase. Lastly, as the experiment reagents are used up, DNA synthesis slows down and the exponential curve evens out into a plateau. The amount of cDNA is measured during each amplification cycle of the real time PCR. The threshold cycle (C_T) is the fractional cycle number at which the fluorescence detected passes a fixed threshold. After demonstrating that the efficiencies of the target and housekeeping gene (control) amplifications were approximately equal (Fig. 9) the comparative $2^{-\Delta\Delta C_T}$ method was used (Livak and Schmittgen, 2001). The quantification of changes in gene expression was then based on the relative expression of the target gene versus a reference gene (Fig. 10).

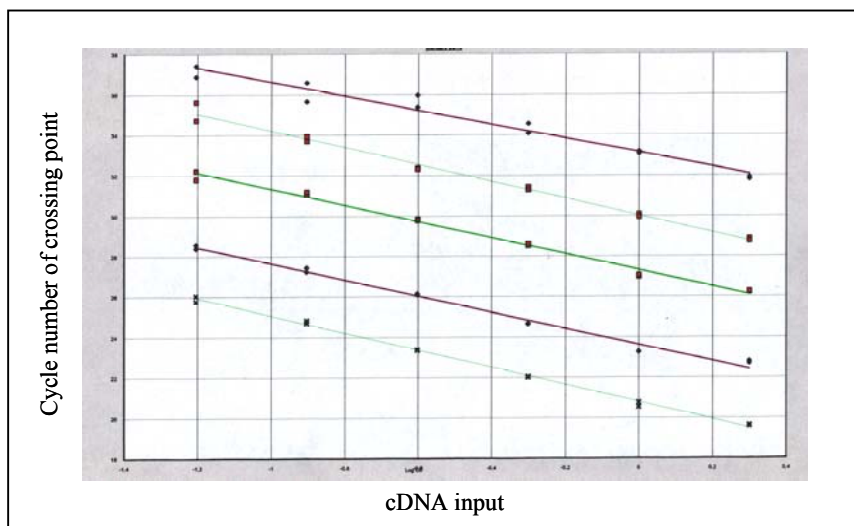


Figure 9. Determination of real time PCR efficiencies of the target genes and housekeeping gene. The corresponding real time PCR efficiencies were calculated according to the equation: $E = 10^{(-1/\text{slope})}$.

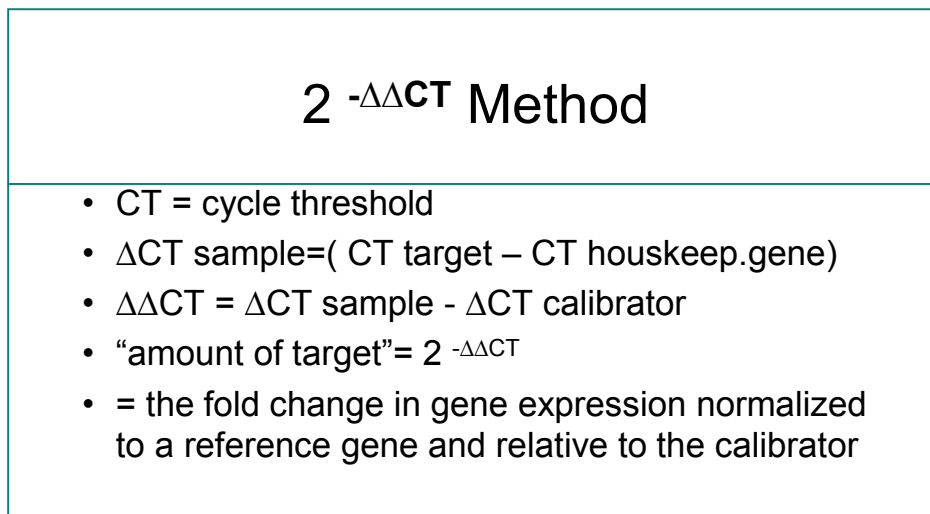


Figure 10. Schematic explanation of the comparative $2^{-\Delta\Delta CT}$ method.

Statistics

Statistical calculations were performed using SPSS Version 13.0 for Windows (SPSS, Chicago, IL, USA). The non-parametric analysis of variance by rank, the Kruskal-Wallis test, was used to detect significant differences in all papers at four time points. Thus, this test was used in the overall analysis of the results of immunoblotting (paper II) and real-time quantitative PCR (paper II-IV). Individual groups were then compared post hoc with the non-parametric Mann-Whitney U-test (paper II-IV). A p-value of less than 0.05 was considered to be significant.

RESULTS AND COMMENTS

Collagens in the human ovary and their changes in the perifollicular stroma during ovulation (paper I)

Ovulation is a highly regulated process, where remodelling of the ECM in and around the follicle is a major event. Previous studies in a restricted number of animal species had shown that there collagen fibres exterior to the preovulatory follicle and it was first assumed (Espey, 1967b) and later shown that the collagens of the ovarian ECM make up the tensile strength of the follicle wall (Espey, 1967a). The general concept has been that there are collagen fibres which run parallel to the surface of the ovary immediately underneath the surface epithelium. This collagen rich area is generally referred to as the tunica albuginea and could be considered as the more compact outer shell of the ovary. A detailed study (Bjersing and Cajander, 1974c) has revealed that the layer contains mostly collagen fibres but also scattered fibroblast, presumed to be the producers of these collagens. The border between the tunica albuginea and the layer deeper to that is not distinct. This tissue is generally referred to as only ovarian stroma. The area contains blood vessels, fibroblast and collagen fibres that are not arranged in such a parallel order as in the tunica albuginea. In the innermost region of this layer the pool of primordial follicles are situated. In the work of the present study this area is referred to as the capsular stroma. The capsular stroma layer is comparatively thick in the human, but will of course get thinner and more compressed when a dominant follicle is growing beneath and is pushing the tissue upwards. The outermost layer of the follicle is the theca externa, which also contains collagen.

There exists robust evidence from experiments in several animal studies that breakdown of collagens are critical for ovulation to occur and most of this breakdown is probably within the capsular stroma and the theca externa. Several studies in experimental animals have shown that there is an increased expression of a number of matrix metalloproteinases (MMPs) during the ovulatory process (Curry and Osteen, 2003) and it is assumed that their major role is to degrade these collagens.

The knowledge of the intraovarian events of the human ovulatory process regarding ovarian collagen distribution and degradation is very limited. The present study focused on the distribution of the interstitial fibril-forming collagens of type I and type III together with the network-forming collagen type IV in ovarian tissue as well as fibroblasts and leukocytes in

the ovary, particularly at ovulation. Since a major task for this thesis and for future work was to describe the possible LH regulated expression of ECM degrading proteins it was important to in more detail describe the collagen distribution in the human ovary to get some information on where these enzymes may be active.

Results

Whole ovarian slices from five women were examined by immunohistochemistry (Fig. 11). Collagens I and III were distributed in concentric layers in the capsular stroma with bundles of collagens connecting these layers to form a mesh. In the theca, collagen I was present in the externa and collagen III in the entire layer. Fibroblasts, most likely the main producing cells of collagens, visualized with positive immunostaining against vimentin was seen with similar distributions as collagen type I and III. Thus, these cells were seen at relative density at the sites of the concentric collagen layers. Immunostaining for leukocytes (antibody against CD 45), which are presumed to be the cells responsible for the breakdown of collagens by secretion of MMPs was also seen around the places where collagens were present but in general this immunostaining was more uniformly distributed in the ovarian capsular stroma. The staining intensity of collagens I and III in the perifollicular stroma was evaluated in a semi-quantitative way. It was found that the staining, and thereby presumably the collagen content, decreased from preovulatory stage. Collagen IV was present in the BL separating granulosa and theca cells but was not seen in the BL underlying the surface epithelium.

Comments

It has been suggested by an electron microscopic study that collagen bundles in tunica albuginea and theca externa in human follicles more or less disappear before ovulation (Okamura *et al.*, 1980) and by applying biochemical methods to follow collagen synthesis it was indicated that collagen in the apical region of the follicle undergoes substantial remodelling during the hours prior to ovulation (Dennefors *et al.*, 1982). These studies did not examine what collagens were present in the human follicle wall and where they were located in relation to each other and in relation to the cell compartments of the ovary.

This study shows for the first time that collagen I and III are abundant in and around the ovulating human follicle. The distribution was more clearly shown in whole ovarian sections,

which did not contain preovulatory follicles but follicles of smaller sizes. The function of the layers of bundles of fibres containing collagen type I and III are most likely to be the structural support for the ovary. Collagen IV was found to be present in the BL that separates the granulosa from the theca cells. This finding is in line with the general concept that this type of collagen is a main component of the BL.

The human ovarian tissue material from the perifollicular stroma and the excised follicles used in the present study, were well characterized regarding the timing in the ovulatory phase. The finding of decreased staining for collagens after exogenous gonadotropin stimulation suggests that there is a decrease in the collagen contents around the follicle at that time. The decrease may of course be due both to a shut down in the production of collagen molecules but also to an increased degradation. This latter aspect was examined more in detail in Paper II. Taking into account the abundance of collagens in the human follicular wall, as shown in this study, major site-directed degradation of collagens seems to be necessary for follicular rupture to occur.

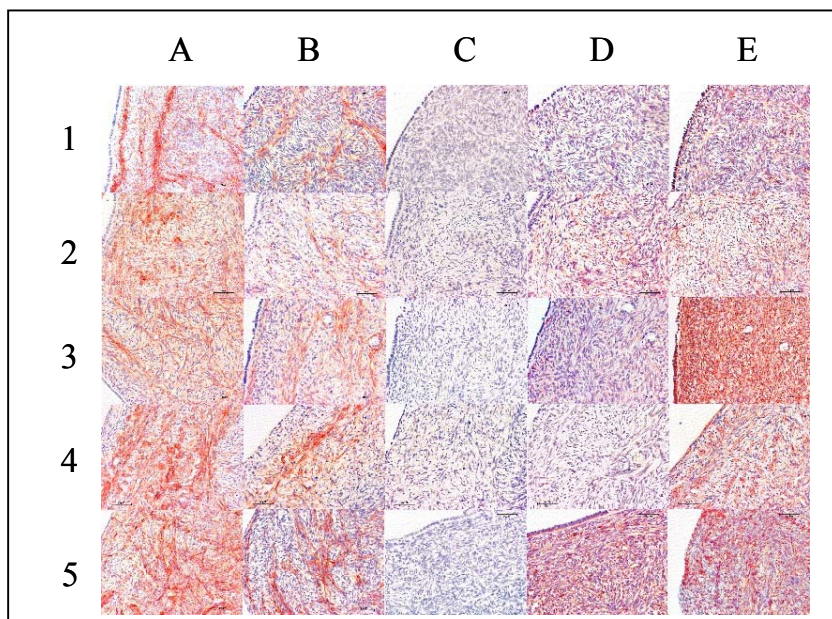


Figure 11. Immunostaining for collagen type I (A), collagen type III (B), collagen type IV (C), vimentin (D) and CD 45 (E) in the ovarian capsular stroma from the ovarian surface epithelium (OSE to the left in the photos) up to and including the stromal part with the primordial follicles. Photos are shown from altogether 5 patients. Scale bar represent 50 µm.

Connective tissue remodeling enzymes in human ovulation: increased expression of tissue inhibitor of metalloproteinase-1 (TIMP-1; Paper II)

Mammalian ovulation is a finely tuned process which extends over about 36 h. It is generally assumed that tissue breakdown and vascular changes are the major mechanisms that eventually cause follicular rupture and oocyte release. Remodeling of the ECM of the stroma in the follicular wall around the preovulatory follicle by MMPs and TIMPs has been suggested to be crucial in this process. There are many MMPs and four different TIMPs that have been implied to be active in ovulation. Animal studies and studies on human granulosa lutein cells have shown that the gelatinases are expressed in the ovary. The availability of human tissue material to be used in the present study was restricted and it was decided to specifically study the expression patterns of the gelatinases and their tissue inhibitors. Gelatinase A has also been called the 72 kDa gelatinase but is currently most often referred to as MMP-2. Gelatinase B (92 kDa gelatinase) is generally named MMP-9. The objective of the present study was to investigate the protein expression of MMP-2 and MMP-9 together with their inhibitors TIMP-2 and TIMP-1 in the perifollicular ovarian stroma from women just before and during induced ovulation in the natural cycle during four specific ovulatory phases. It was decided to use western blotting as the primary method to investigate the levels of the mediators since this method measures the protein levels. The real time PCR method was then used to verify any interesting findings concerning protein levels. However, measurements of the protein levels should be considered to be more closely related to the predicted activity of the enzyme. This activity is of course related to both the content of the enzyme, in its active form, but also to the local concentrations of inhibitors. Thus, the activity of the protein does not have to be fully related to the protein levels. There exist zymographic methods to study the net gelatinase activity in a tissue but these methods were not used in the present study.

Results

All four proteins were expressed in the perifollicular stroma, but there were no detectable changes in the protein expression of MMP-2, MMP-9 and TIMP-2. Scattered immunostaining for MMP-9 and TIMP-2 was seen in the perifollicular stroma of all stages. Immunostaining

for MMP-2 was demonstrated in the perifollicular stroma with the immunoreactivity concentrated to a concentric layer. A clear increase of TIMP-1 protein was seen during the three ovulatory phases as compared to the preovulatory phase. Real time PCR verified the ovulatory increase of TIMP-1 expression also on the mRNA level. A strong and patchy immunostaining for TIMP-1 was seen in the perifollicular stroma. In biopsies containing the entire follicle wall, a weaker but general staining for TIMP-1 was also seen in the theca- and granulosa cell layers.

Comments

The present study focused on enzymes that are primarily involved in the regulation of collagen type IV-breakdown but these gelatinases are also important in the secondary degradation of interstitial collagens. These interstitial collagens are initially degraded by other MMPs into gelatin. Although the exact composition of the ECM of the human ovary is not clearly established, it is assumed that collagen type IV is an important ECM component of the follicular wall and the surrounding stroma (Irving-Rodgers and Rodgers, 2005). In paper I we showed that there is an abundance of collagen type IV in the BL that separates the theca and granulosa cells. The results of the present study indicate that there is no variation in the gelatinases and TIMP-2 in the perifollicular stroma during the ovulatory phases. However, this result could not rule out that there are fluctuations in the local concentrations. Thus, it would be interesting to study the apical perifollicular stroma in comparison to the basal part. In the present study, all the perifollicular stroma around the follicle was collected in the same sample. It may well be that there are greater signs of net activity of the gelatinases in the apex, which is the part that mainly is going to be degraded. There were some variations, although not statistically significant, of the protein levels for the gelatinases and it is also possible that these variations would reach a statistical significant level if the number of samples was greater.

The increased expression of TIMP-1 may reflect a specific temporal inhibition of collagenolysis and thereby a time dependent regulation of ECM breakdown in areas surrounding the apex of the follicle. This would apply mostly to the non-apical regions around the follicle. It should also be noted that parallel to the degradation of the top of the follicle the

machinery for reorganization of the follicle into a corpus luteum is operative. Tissue inhibitors of metalloproteinases, such as TIMP-1, may be important in these processes.

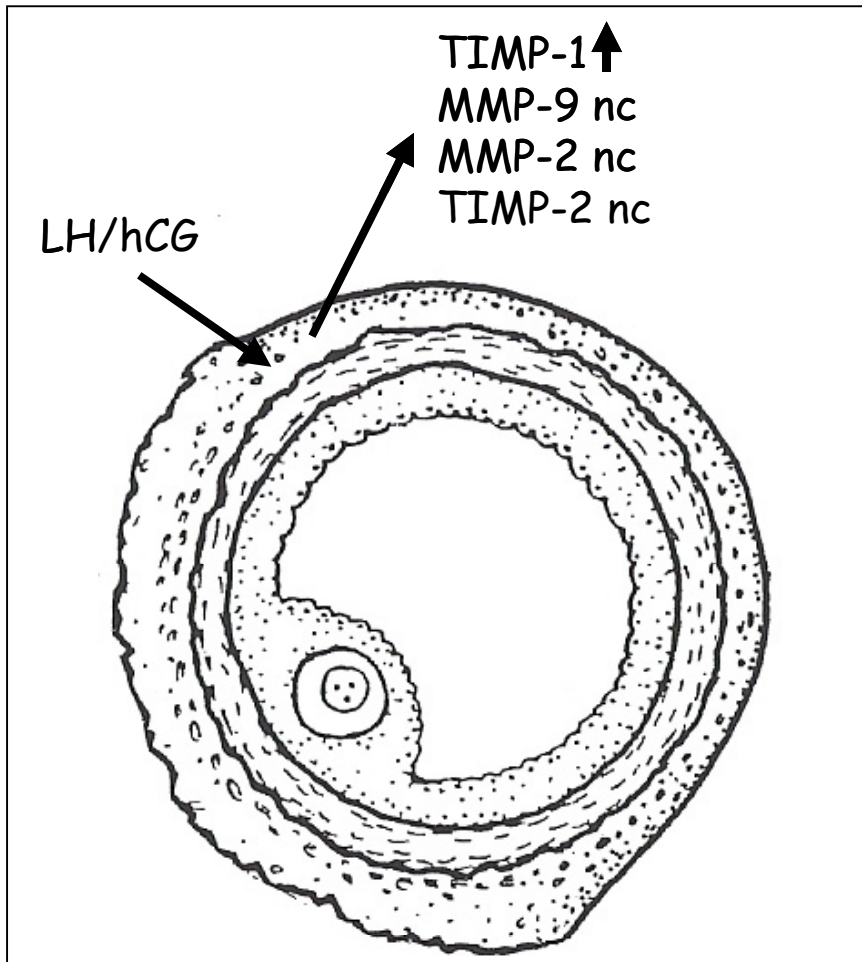


Figure 12. Major findings of paper II. An increase in expression is indicated as ↑, no change in expression is illustrated as "nc".

Temporal differences in expression of matrix metalloproteinases–2 and -9 (MMP-2, MMP-9) and of tissue inhibitors of metalloproteinase – 1 and -2 (TIMP-1, TIMP-2) in granulosa cells during human ovulation (Paper III)

The site-specific degradation on the apex of the ovulatory follicle wall results in dissolution of interstitial collagens of the tunica albuginea, capsular stroma and the theca externa as well as degradation of collagen type IV of the basal membranes (Bjersing and Cajander, 1974c). It has been suggested that MMPs are the key substances that carry out the degradation of the follicular ECM during ovulation (Curry and Osteen, 2003) Concerning human ovulation, there is a lack of studies that have looked at expression and function of the proposed ovulatory mediators in the different cell compartments during the ovulatory process of the menstrual cycle. In the present study we have evaluated the expression and localization of the gelatinases and their inhibitors in granulosa cells during human ovulation to increase the knowledge about the intraovarian regulation of ovulation. Granulosa cells are situated away from the main layers of interstitial collagens in the theca externa, capsular stroma and tunica albuginea. However, since the results of paper I showed that the main site for collagen type IV is the BL that borders the mural granulosa cells, we hypothesized that these cells would be main producers of the gelatinases and their inhibitors. Moreover, ovulatory mediators such as prostaglandins and plasminogen activators are mainly produced within the granulosa cells but with their proposed ovulatory effects mainly in the theca layer. The granulosa cell layer is avascular but diffusion of substances through the porous basal lamina would allow for effects in the theca interna, theca externa and also possibly in the stroma around the follicle.

Results

The mRNAs for MMP-2 and TIMP-2 mRNA were expressed at low constant levels during the preovulatory, early ovulatory and late ovulatory phases. There was an approximate 3-fold increase of TIMP-2 expression during the postovulatory phase, as compared to the earlier phases. MMP-2 was expressed at constant levels during the entire periovulatory interval. The expression of MMP-9 mRNA showed an early rise with a more than 25-fold increase at the early ovulatory stage as compared to the preovulatory stage. The expression levels then declined but stayed elevated throughout the periovulatory interval. The greatest increase was

seen for TIMP-1 which showed a 50-fold increase at the early ovulatory stage and a more than 150-fold increase at the late and postovulatory stages. The localization of the MMP proteins and TIMP proteins were investigated by immunohistochemistry. All enzymes were found within the granulosa layer but with different staining intensities. The staining intensity seemed to correspond to the fluctuations of the mRNA levels at the various phases.

Comments

This study demonstrates that the preovulatory LH-surge induces a rapid, major expression of TIMP-1 and MMP-9 in human granulosa cells during ovulation. This is the first study which has demonstrated the LH/hCG induced expression of these enzymes in the human. The increase in expression levels was very high in comparison to the increases previously found in animal species. A reason for this could be that the present study was on pure granulosa cells in comparison to many of the animal studies which have been on whole ovulatory. It may also be that the mono-ovulatory human ovary expresses relatively higher levels in the follicle that will ovulate. One can speculate that the enzyme levels have to be higher because only one follicle has to be responsible for the breakdown of the capsular stroma. Since the human capsular stroma is relatively thick, and thereby most likely contain more collagens than in other species, the amounts of MMPs and also their regulators may need to be higher. In the present study, there was a greater increase in TIMP-1 than in MMP-9. This does not necessarily mean that all the newly expressed MMP-9 will be effectively inhibited by the abundant TIMP-1 but rather that both of these enzymes are of importance in the remodelling of the follicle at ovulation. There are most likely important fluctuations of these enzymes which are not captured by the fairly broad ovulatory phases studied in the present study. There may also be differences in their expression levels among the cumulus granulosa cells, the mural granulosa cells which are closest to the antrum, the mural granulosa cells which are not bordering either the antrum or the BL, and the mural granulosa cells which are in contact with the BL.

There was a modest and late increase in TIMP-2. This may indicate that this enzyme is of importance in the luteinisation events that involves both controlled angiogenesis and structural reorganisation of the gland.

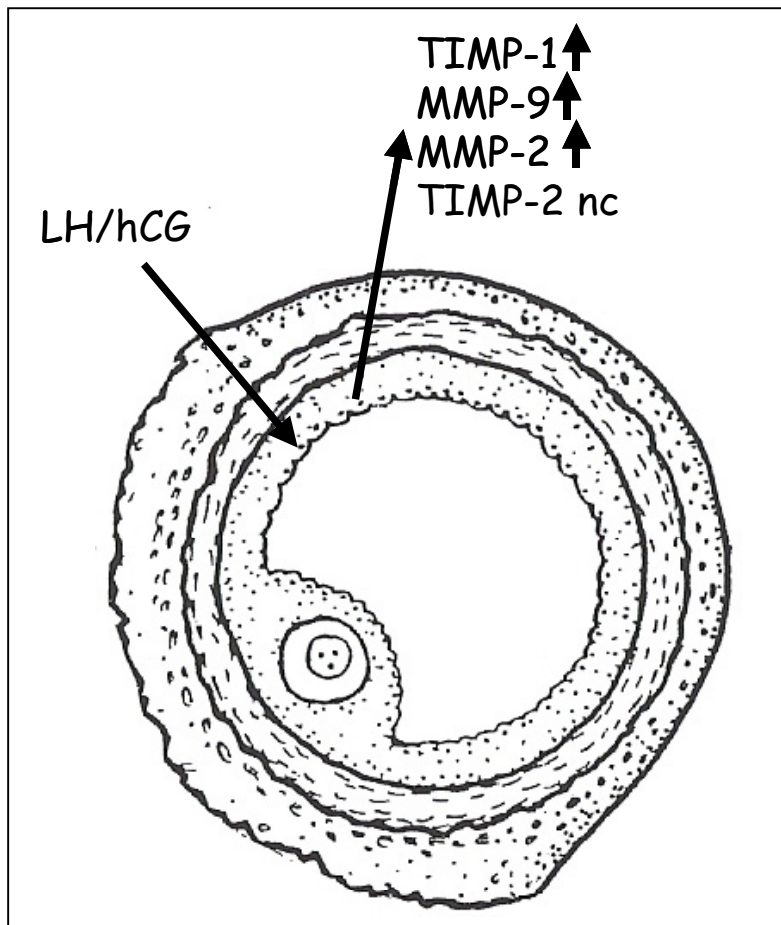


Figure 13. Major findings of paper III An increase in expression is indicated as ↑, no change in expression is illustrated as "nc".

Increased expression of MMP-9 and its endogenous inhibitor TIMP-1 in human theca cells during ovulation and luteinisation (Paper IV)

The ovulatory process is triggered by the LH-surge that initiates several biochemical processes in the ovary such as vascular changes (Brännström *et al.*, 1999) and remodelling of the ECM, particularly at the apex (Espey, 1967b; Okamura *et al.*, 1980). In the rabbit, after hCG to mimic the LH-surge, there first appeared a prominent oedema in the theca interna region of the follicular wall. This accumulation of extracellular fluid later spread to the theca externa to include the whole follicle wall. Parallel to this, there was fragmentation and disruption in the connective tissue just prior to follicular rupture (Bjersing and Cajander, 1974a). We have in the present study in particular studied the theca cell layer, since the first changes after the gonadotropin surge seems to appear in this layer. The same components of the gelatinase system, as studied in papers II and III, were examined in this study. The thecal compartments that were studied were from the same patients that are included in papers II and III.

Results

Both MMP-9 and TIMP-1 mRNA was expressed at low levels in PO. At LO a significant increase was found compared to PO, 10-fold for MMP-9 and 25-fold for TIMP-1.

MMP-2 and TIMP-2 mRNA was expressed at relatively high levels throughout the four different ovulatory phases and no significant changes were noted although a non-significant decrease in the expression levels were noted just before (LO) and after (PSO) ovulation compared to PO and EO. Immunohistochemistry showed staining for all four proteins in the theca layer while this immunostaining was more pronounced in the theca interna at LO and PSO.

Comments

Albeit the well-established role of MMPs and TIMPs during the ovulatory process most of these data are based on animal studies, for review see (Smith *et al.*, 1999; Curry and Osteen, 2003). There exist but a few studies in the human concerning the involvement of these enzymes at ovulation. One of the first changes after the gonadotropin surge seems to appear in

the theca layer and it has been proposed that there is a special “thecal matrix” (Rodgers *et al.*, 2000) composed of laminin and collagen type IV that has to be degraded. Gelatinases are known to cleave collagen type IV, a major component of the BLs, and further hydrolyze the denatured fibrils of collagens type I and III, following their initial cleavage by collagenases. We have demonstrated, for the first time in the human, an increased expression of mRNA for both MMP-9 and TIMP-1, and an unvarying, high expression of MMP-2 and TIMP-2 mRNA, in theca cells just before and after follicular rupture, indicating their significance in ovulation. The presence of all the four components already during the preovulatory phase suggest that there is changes in the connective tissue of the theca layer already at this phase. The increase in MMP-9 and TIMP-1 showed the same time course as in the granulosa cells but was of lower magnitude. It may well be that the granulosa cells have to produce larger quantities in order to have effect on the collagens, which are present in other cellular compartments. The theca interna cells are very centrally located to produce the enzymes that are necessary for the degradation of the BL towards the interior and the theca externa and capsular stroma towards the exterior.

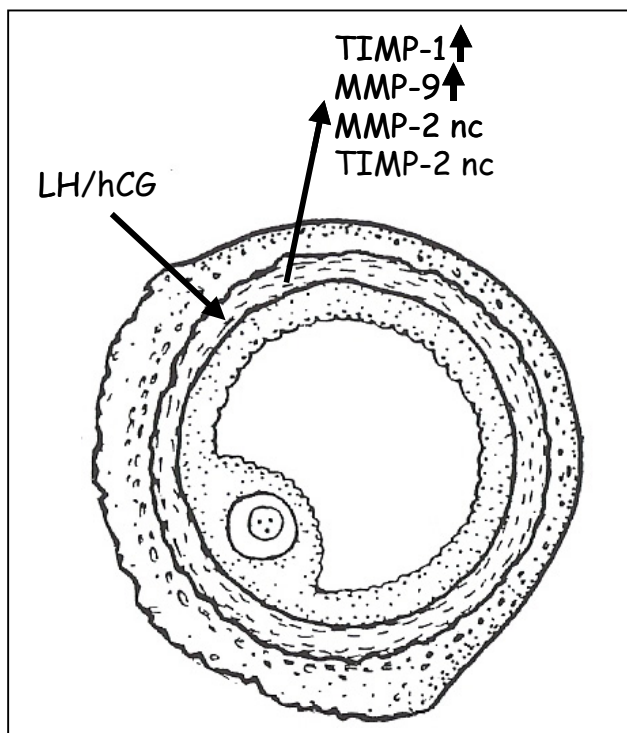


Figure 14. Major findings of paper IV. An increase in expression is indicated as ↑, no change in expression is illustrated as “nc”.

DISCUSSION

The ovulatory process is continuing over several hours and is regulated by countless of cooperatively and parallel-acting mediators (Brännström and Janson, 1991; Richards *et al.*, 2002), ultimately leading to the release of a fertilizable oocyte from the interior of the follicle to the exterior surface of the ovary. The most prominent aspects of this process involve dynamic physiological events, such as alterations of ovarian blood flow (Janson, 1975; Campbell *et al.*, 1993; Zackrisson *et al.*, 2000), and degradation of the follicular wall (Espey, 1967b). Many experimental models in several different animal species have been developed to enable studies of the ovulatory process *in vivo* since these dynamic events then can be studied undisturbed

In vivo models

Spontaneously ovulating, polyestrous rodents, such as the laboratory mice and rat, are excellent models for studying ovulation as they have frequent, rapidly occurring cycles unless mating takes place.

Mouse models to study ovulation can be done during the natural murine estrous cycle, occurring every 4-5 days, including diestrus (before the gonadotropin-surge), proestrus (the day of the gonadotropin surge), estrus (after ovulation), and metestrus (luteal phase) (Inderdeo *et al.*, 1996). The ovulatory process can also be studied during gonadotropin-induced ovulation in the mouse (Hägglund *et al.*, 1999). Briefly, immature female mice are injected with pregnant mare's gonadotropin (PMSG) to stimulate follicle growth and 48 h later with hCG to induce ovulation. Follicular rupture takes place 12-14 h after hCG in the mouse (Ny *et al.*, 1999).

Rat models follow the same principals as in mouse models, during natural cycles (Simpson *et al.*, 2001) as well as in gonadotropin stimulated cycles (Jo *et al.*, 2004). The gonadotropin surge initiates ovulation in the gonadotropin stimulated rat model 12-16 hours later (Curry *et al.*, 1992; Jo *et al.*, 2004).

Domesticated farm animals, like the cow, sheep, and pig, have been extensively used for studies of the ovulatory process.

Bovine models can be used during natural cycles, where usually ovaries from random stages of the estrous cycle are obtained from an abattoir (McCaffery *et al.*, 2000) or models where follicular development and timing of the preovulatory gonadotropin surge are synchronized in nonlactating cows (Pursley *et al.*, 1995; Bakke *et al.*, 2002). In short, gonadotropin-releasing hormone (GnRH) is injected to start a new wave of follicular growth and thus a new dominant follicle. To regress the corpus luteum, PGF_{2α} is given a week later. Then, a second GnRH injection is given 36 h after PGF_{2α} administration to induce a gonadotropin surge and ovulation of the new dominant follicle on average 29 h afterwards.

Ovine models have similarities as compared to bovine models. In brief, ewes are observed for oestrous behaviour in the presence of vasectomised rams. Two weeks later luteal regression is synchronized by injection of PGF_{2α}. Subsequently, GnRH is given after 36 h and the dominant follicles will ovulate 24 h later (Gottsch *et al.*, 2002). An alternate way to synchronize estrous cycles in the sheep is by progestin implants (Russell *et al.*, 1995). The latter model is followed by PMSG to induce follicular growth when the progestin implant is removed after 14 days. This is followed by hCG 24 h later and finally ovulation 24-36h later (Russell *et al.*, 1995).

Porcine models have been used to some extent by authors referred to in this text. Progestin treatment to synchronize the animals can be used (Driancourt *et al.*, 1998) and hCG to mimic the gonadotropin surge (Driancourt *et al.*, 1998; Shores and Hunter, 2000). Natural cycles can be studied as well (Smith *et al.*, 1994).

Nonhuman primates on a controlled ovarian stimulation protocol have been used to study periovulatory events (Chaffin and Stouffer, 1999; Duffy and Stouffer, 2003). Briefly, adult female monkeys are stimulated with recombinant human gonadotropins (rhFSH for 8 days and rhLH day 7 and 8) starting on day 1-3 of the 28 days long menstrual cycle to promote the development of multiple preovulatory follicles. Additionally, daily injections of a GnRH agonist are given to prevent the endogenous LH-surge. An injection of rhCG is given to induce periovulatory events. Ovulation occurs 36 h later in the rhesus monkey (Chaffin and Stouffer, 1999).

Animal models allow us to control the environment and manipulate the interventions in a manner that can not be carried out to the same extent in humans. In this way, the understanding of the physiology of ovulation can be addressed. Several options to study this process and different ways to administer certain substances exist. Thus, a substance could be administered locally in the ovary, such as directly into the follicle. In an ovine study, neutralizing MMP-2 antibody or normal IgG, were given into preovulatory follicles (Gottsch et al., 2002) and a difference was seen in number of ovulations with fewer ovulations from follicles with MMP-2 antibody. Moreover, a substance could be given intrabursally to an animal having an ovarian bursa, such as the rat, the mice and the hamster. Studies with intrabursal injection have been performed and showed differences in ovulatory response, indicating that the substance can act from the outside of the ovary by influencing the surface epithelium or by diffusion through this cell layer to affect the TCs or GCs (Abisogun *et al.*, 1988; Van der Hoek *et al.*, 2000; Matousek *et al.*, 2001b).

In vitro studies

To investigate a multifaceted process such as ovulation *in vitro* using cell cultures are very limited. Even though cell culture studies can provide useful information about the immediate responses of certain cell types to different stimuli, the ovulatory process engages synergistic interactions between a number of cell types and the basic tissue architecture has to be preserved to interpret the complete sequence of events. The model of the *in vitro* perfused ovary was developed to permit advanced investigations of local ovarian biochemical variations involved in the ovulatory process under a more physiological state. Attempts to perfuse the ovary *in vitro* for studies of morphological alterations were first reported in the 1930s (Carrel and Lindbergh, 1935). The methodology for specific studies on ovulation in the *in vitro* perfused ovary was originally developed for the human (Stähler *et al.*, 1974) and the rabbit (Ahren *et al.*, 1972; Ahren *et al.*, 1975; Lambertsen *et al.*, 1976) ovary in the 1970s. The method was further developed or modified for the sheep (Seamark *et al.*, 1977), the rabbit (Janson *et al.*, 1982) and the rat (Koos *et al.*, 1984; Shaykh *et al.*, 1985; Brännstrom *et al.*, 1987) ovary, which enabled investigations of local ovarian biochemical events. A methodological study on ovulation in the perfused mouse ovary has also been put forward (Brännström and Flaherty, 1995). Substances, which are tested in an *in vitro* perfusion

system, would have direct effects on the ovary. It is also possible to measure the true ovarian output of certain substances, such as steroids and other ovarian mediators that are produced and released by the ovary. A new intravital microscopy method was recently developed for continuous long-term observation of ovulation *in vivo* in the rabbit (Dahm-Kähler *et al.*, 2006a). This method offers a new clear advantage since the ovary is possible to observe and can be manipulated, and that measurement for instance of blood pressure and IFP can be done *in vivo*.

Difficulties in imitating the specific physiological ovulatory process is the disadvantage with the *in vitro* models, as mentioned above. There is lack of several endogenous ovulatory factors that have been reported crucial for the ovulatory process such as nerve supply (Walles *et al.*, 1982; Walles *et al.*, 1986), a number of blood components such as angiotensin II (Mitsube *et al.*, 2003) and monocytes (Wu *et al.*, 2004). Consequently, observations of the ovulatory events *in vitro* may not fully mimic the physiological *in vivo* situation.

Collagens in the ovarian capsule and periovulatory follicle

The most prominent feature of the ovulatory process is the rupture of the follicle wall with the bleeding and expulsion of granulosa cells as recently documented in an intravital-microscopy study in the rabbit (Dahm-Kähler *et al.*, 2006a). Experiments in several animal studies have show that breakdown of collagens are critical for this process to occur. Thus, fragmentation and dissociation of collagens with decreased density of collagen fibers were demonstrated in the ovulatory rabbit follicle wall (Espey, 1967b; Bjersing and Cajander, 1974c; Bjersing and Cajander, 1974d). In the sheep ovary more pronounced changes in collagen architecture were noted in the apical region as compared to the basal region (Murdoch and McCormick, 1992). In intravital microscopic studies a rugged appearance and increased transparency was noted on the apex before rupture in the rat (Löfman *et al.*, 2002) and in the rabbit (Dahm-Kähler *et al.*, 2006a). Taken together, these studies indicate a site-directed ECM breakdown occurring in the apical wall of the follicle. This wall is composed of both the follicle and the overlying capsular stroma.

The knowledge of the intraovarian events of the human ovulatory process regarding ovarian collagen distribution and degradation is very limited. Expression of mRNAs and proteins for type I, III and IV collagens were recently demonstrated in biopsies from ovaries of fertile

women (Oksjoki *et al.*, 2004). However, it is not described from what area of the ovary the biopsies were taken and exactly what menstrual cycle the patients were in. Moreover, there was no attempt to describe the ovarian localisation.

In paper I we demonstrated the localisation of the interstitial fibril-forming collagens of type I and type III together with the network-forming collagen type IV in whole ovarian sections from regularly menstruating women. Collagen types I and III were distributed in concentric layers in the capsular stroma with bundles of collagens connecting these layers to form a mesh. Collagen type I was present in larger quantity in the outer layers and collagen type III showed the inverse distribution. In whole periovulatory follicles collagen type I was present in the theca externa and collagen III in the entire theca layer. It is evident from this distribution of the fibril-forming collagen types I and III that they make up a scaffold for the ovarian structure. It is not surprising that there is a robust scaffold of collagen fibres in the ovary so that the shape and architecture of the ovary can be preserved in spite of the dramatic physiological changes during folliculogenesis, corpus luteum formation and luteolysis.

Interestingly, a new form of ECM was recently demonstrated in the theca interna of the bovine follicle. By elaborate studies using electron microscopy, this matrix was demonstrated as fragments of BL-like material and the authors has named it “thecal matrix” (Rodgers *et al.*, 2000). We were not able to show immunostaining for collagen type IV, a BL component, in the theca interna. However, collagen type III was distributed in between the cells in theca interna and it is possible that collagen type III also is a constituent in this thecal matrix, at least in the human.

In paper I collagen type IV was present in the BL separating the granulosa and the theca cells as well as in the BL of small vessel walls in the follicle. This collagen type IV was also seen in blood vessels of the ovarian stroma visualised in the whole ovarian sections. Yet, collagen type IV is considered to be one of the main components of BLs (Timpl and Brown, 1996), it was surprising that we could not demonstrate immunostaining for collagen type IV in the BL separating the OSE and TA. This could be due to different collagen type IV α -chain compositions in the two main BL of the follicle. Thus, the antibody used in paper I to detect collagen type IV may not react with the collagen type IV chains beneath the OSE.

It has been suggested, by an electron microscopic study, that collagen bundles in tunica albuginea and theca externa in human follicles more or less disappear before ovulation

(Okamura *et al.*, 1980). Furthermore, by applying biochemical methods to follow collagen synthesis it was indicated that collagen in the apical region of the follicle undergoes substantial remodelling during the hours prior to ovulation (Dennefors *et al.*, 1982). In the latter study this was indicated by measuring collagen synthesis in tunica albuginea (TA) as the incorporation rate of radioactive proline into collagen. Dennefors and co-workers demonstrated that PGE₂ inhibits collagen synthesis in the apical part of the preovulatory follicle wall (Dennefors *et al.*, 1982). These findings are consistent with that the TA of the follicular apex demonstrated a lower collagen content than the areas of the ovarian capsule with no large follicles beneath (Postawski *et al.*, 1996). These latter studies did not specifically examine the collagen composition in the human ovary. In paper I we showed that the staining intensity of collagen types I and III in the perifollicular stroma decreased from preovulatory stage. The human ovarian tissue material from the perifollicular stroma and the excised follicles was well characterized by several methods. Importantly all women had demonstrated their fertility and they were not on any hormonal medication neither during the menstrual cycle of biopsy nor during the time when they were closely monitored. The administration of exogenous hCG to mimic the LH-surge also ensured that the biopsies were taken at the right time during the ovulatory phase. These finding of a decrease in the content of collagen types I and II further indicate that there is weakening of the follicle wall during the ovulatory process and that breakdown of collagens is an important factor to cause this.

Table IV. Summary of the general distributions of collagen types I, III and IV in the ovary, and particularly in the periovulatory follicle.

Collagen type	OSE	Stroma	TE	TI	BL	GC	Species	References
I				yes		no	cow	(Zhao and Luck, 1995)
		yes	yes				rat	(Palotie <i>et al.</i> , 1984)
		yes	yes	yes			cow, sheep	(Luck <i>et al.</i> , 1995)
						yes	sheep	(Huet <i>et al.</i> , 1997)
	yes						human	(Auersperg <i>et al.</i> , 1994)
III		yes	yes				rat	(Palotie <i>et al.</i> , 1984)
	yes						human	(Auersperg <i>et al.</i> , 1994)
		yes			weak		human	(Christiane <i>et al.</i> , 1988)
IV					yes		cow	(Zhao and Luck, 1995)
				yes		weak	human	(Yamada <i>et al.</i> , 1999)
					yes		rat	(Palotie <i>et al.</i> , 1984)
				weak	yes		cow, sheep	(Luck <i>et al.</i> , 1995)
			yes	yes	yes	yes	sheep	(Huet <i>et al.</i> , 1997)
			yes	yes	yes		rat	(Bagavandoss <i>et al.</i> , 1983)
	yes						human	(Auersperg <i>et al.</i> , 1994)
				yes	yes		cow	(Rodgers <i>et al.</i> , 1998)
				yes		rat, cow, pig	(Bortolussi <i>et al.</i> , 1989)	

In the ovary, collagen type IV, has been most consistently demonstrated. Thus, collagen type IV is localised to the BL, separating the granulosa- and theca cell layers, in several animal species such as the rat (Bagavandoss *et al.*, 1983; Palotie *et al.*, 1984), the sheep (Luck *et al.*, 1995; Huet *et al.*, 1997), the cow (Bortolussi *et al.*, 1989; Zhao and Luck, 1995; Rodgers *et al.*, 1998), and pig (Bortolussi *et al.*, 1989). Very few studies on the localisation of collagen type IV in the ovary exist on human material and, especially concerning the periovulatory follicle. By extensive search in the literature I have found four studies. There are two similar studies on human OSE (Auersperg *et al.*, 1994; Kruk *et al.*, 1994), where they showed that cultured OSE cells produce collagen type IV, as well as collagen types I and III. In a study on recently archived human ovarian tissue specimens from prophylactic oophorectomies, collagen type IV was localized to the BL separating the OSE and tunica albuginea (Roland *et al.*, 2003). The latter study concerned the OSE and preneoplastic morphologic changes and only focused on the OSE. By indirect immunohistochemistry on pre-ovulatory human

follicles collagen type IV was present in the theca interna and weakly in granulosa cells (Yamada *et al.*, 1999). They could show that granulosa cells from IVF cycles contained higher concentrations of collagen type IV than granulosa cells from follicles not exposed to hCG. In paper I we demonstrated that collagen type IV is localised to the BL separating the granulosa- and theca cell layers which is in line with the above mentioned animal studies and is now also demonstrated in humans as well. However, no immunostaining for collagen type IV was seen in the BL underlying the OSE in paper I. In the study by Roland *et al.*, a clear immunostaining was seen in this BL. There can be several explanations for this difference. The antibody we used recognises an epitope located on the $\alpha 1$ and/or $\alpha 2$ chains of human collagen type IV and it is recommended for immunohistochemistry by the supplier (Abcam, Cambridge, UK). Roland *et al.* used a similar antibody but from a different supplier (Dako, Glostrup, Denmark). There can be differences in dilution and it could be possible that with a higher concentration of our antibody there would be staining in this BL as well but on the other hand more unspecific staining could then be seen. In paper I collagen type IV was not localised to the granulosa cells or theca cells.

In summary, collagen types I and III forms a scaffold like network of concentric and basketlike structures in the ovarian capsule of the human ovary.

LH as the ovulatory trigger

The mid-cycle surge of LH is regarded as the primary physiological trigger for ovulation while the simultaneous release of FSH is considered to be essential for the recruitment of follicles for the next menstrual cycle (Hoak and Schwartz, 1980). Ovulation can be inhibited by antisera against LH, but not FSH, *in vivo* in the rat (Schwartz *et al.*, 1975) which further supports the function of LH as the ovulatory hormone. The LH-surge itself is triggered by the sustained high circulating levels of estradiol produced by the preovulatory follicle.

By binding to specific receptors, LH and FSH acts to induce post-receptor signalling systems mainly by adenyl cyclase, cyclic AMP production, and activation of protein kinase a (PKA) (Richards *et al.*, 1998). The lutropin/choriogonadotropin receptor (LHR) is located in both thecal and granulosa cells of mature, antral follicles (Camp *et al.*, 1991). The FSH-receptor (FSHR) is present in the granulosa cells, persisting throughout preovulatory follicular development (Camp *et al.*, 1991).

MMPs and TIMPs in ovulation

The ovulatory process is characterised by degradation of the connective tissue matrix in the follicle wall. Several morphological studies showed that the striking dissolution of this connective tissue matrix and of collagen fibres in the follicle wall is most pronounced in the apical portion of the preovulatory follicle, as demonstrated in the rabbit (Espey, 1967b), in the rat (Blandau, 1955) and in the human (Okamura *et al.*, 1980). The mature preovulatory follicle markedly protrudes from the ovarian surface but is also surrounded by the connective tissue matrix of the ovary. The major structural element, forming the framework of the follicle wall and adjacent ovary tissue, are collagens, as shown in Paper I. Even not specifically studied in the present study, the collagens and other ECM components of the ovarian capsular stroma overlying the pole of the follicle that are most likely compressed during the growth of the dominant follicle up to and including the preovulatory stage. During this stage of the follicular phase the follicle grows rapidly, mostly because of granulosa cell proliferation and especially the antrum increases in size. As shown in papers II-IV, the levels of the MMPs are low, in comparison to the ovulatory phase, and it is thus unlikely that degradation of the collagens already occur at this stage. This is also indicated by the observation in the rabbit that the tensile strength of the collagenous tissue in the follicle wall decreases after the LH surge but prior to follicular rupture (Espey, 1967a). In paper I, it was demonstrated that collagen types I and III form a scaffold of concentric layers in the ovarian capsule. Collagen type I was additionally localised to TE and perifollicular stroma of the periovulatory of the human, and that collagen type III is present in the entire theca layer as well as in the perifollicular stroma. The typical architecture of the collagen types I and III layers in the ovarian capsular stroma (Paper I) implies that the collagen fibres of the capsular stroma is produced in a highly organized fashion. The main producers of the collagens are considered to be the fibroblasts. Findings of Paper I indicated that these cells were localized to the same layers. The main role for the collagen layers of the capsular stroma is of course not to prevent ovulation but to be a structure barrier that protects the valuable pool of primordial follicles from damage. Collagen type IV is localised to the BL separating the granulosa and theca cells compartments and in the BL of blood vessels in the theca layer and in the ovarian capsule. Also the BL has to be degraded in a controlled manner to enable the oocyte to be released.

However, the BL is needed beforehand to maintain the structural and functional integrity of the granulosa cells, follicular fluid and oocyte.

There is today, robust evidence that both MMPs and their endogenous tissue inhibitors, TIMPs are critical in the remodelling of follicular ECM, a process which seems necessary for the occurrence of follicular rupture with the release of a mature ovum (Ny *et al.*, 2002; Smith *et al.*, 2002; Curry and Osteen, 2003; Goldman and Shalev, 2003; Curry and Smith, 2006). A vast majority of the data on MMPs and TIMPs in ovulation is based on animal studies. When the studies of this thesis were planned, we were aware of the extensive work on MMPs/TIMPs in ovulation in animals but the lack of studies in the human. Since the goal of a major part of the biomedical research is to increase the knowledge about human physiology/pathophysiology, it is of importance to find out whether the same pathways are operative in the human. Concerning the MMP/TIMP system, there exist some studies in nonhuman primate species, but as pointed out below, the results in non-human primates do not necessarily fully correspond to the human situation. Another important remark is that results of lack of phenotypic changes in the KO mice, with deletions of genes presumed to be expressed in the ovary during ovulation, does not rule out a role of these mediators in ovulation. Normal ovulation has been shown in KO mice with deletions in the gene encoding for TIMP-1 (Nothnick *et al.*, 1997) and MMP-9 (Vu, *et al.*, 1998). As for many ovulatory mediators it seems that two or more mediator pathways have to be inhibited to suppress ovulation. Since ovulation and many other processes are important for the propagation cycle of the species it is not surprising that such safety mechanisms are built into the systems.

Previous findings in the human are restricted to observations of increased collagenolytic activity towards ovulation within human follicular fluid (Puistola *et al.*, 1986) and a higher collagenase activity in ovarian tissue of menstruating women compared to after menopause (Postawski *et al.*, 1999). With reference to IVF-cycles, human granulosa lutein cells contained mRNA for TIMPs (Curry *et al.*, 1990). Expression of MMP-2 plus MMP-9 was lower in follicular fluid from IVF patients compared to normally ovulating women with the inverse relationship for TIMP-1 (D'Ascenzo *et al.*, 2004).

In papers II-IV we exclusively examined well defined human material from specific times throughout the periovulatory period and great effort was made to obtain human follicles only from normal menstrual cycles. By studying three different tissue compartments (perifollicular stroma, theca cell layer and granulosa cell layer) of the periovulatory human follicle the study would also elucidate whether there were any differences between these follicular compartments. As pointed out earlier the investigation of the present study was solely on the gelatinases, MMP-2 and MMP-9, together with their inhibitors, TIMP-2 and TIMP-1. It is believed that the gelatinases may play a key role in degradation of the BL separating the granulosa and theca layers as well as to further hydrolyze denatured fibrillar collagen following their initial cleavage by the collagenases. TIMP-1 and TIMP-2 are capable of inhibiting the activities of most MMPs and as such play a key roles in maintaining the balance between ECM deposition and degradation (Gomez *et al.*, 1997). The activity of the MMPs are not only regulated by the TIMPs but regulation also occurs by serum-borne inhibitors, such as α 2-macroglobulin, and by control of cleavage in the extracellular space of inactive pro-MMP into active MMP, which is carried out by several classes of proteinases including the MMPs themselves, serine proteinases such as plasmin, and cysteine proteinases (Woessner and Nagase, 2000).

Most of the animal research on expression and activity of various MMPs and TIMPs have been performed in rodent models. In these models, eCG has been used to prime follicular maturity and hCG has been used as the ovulatory trigger. Ovulation in rodents span over 12-15 h and several time points have been examined during the ovulatory phase to more exactly pinpoint the expression profiles during ovulation. The human model which was used in paper II-IV is in some ways similar to the rodent models since ovulation was triggered by hCG and since several periovulatory time points were examined. This enabled us to compare time courses for MMP and TIMP expression in the human with the previously published data from animal studies. However, the duration of our sampling periods were much wider.

In a stimulated mouse model, TIMP-1 mRNA from whole mouse ovaries increased 5-10 times by hCG and peaked at 12 hours (Hägglund *et al.*, 1999). This 12 h time-point would be comparable to a time just prior to follicular rupture and this would be about 30-36 h in the human. Exogenous hCG induced the mRNA expression for TIMP-1 in all three compartments

investigated in paper II-IV. The increase was highest in the granulosa cells (paper III), second highest in the theca cells (paper IV) and lowest in the perifollicular stroma (paper II). The increases found in Paper II-IV peaked in the late ovulatory and postovulatory phases, which is comparable with the findings in the mouse model. This pattern of TIMP-1 expression is similar to results of several other earlier studies in different species such as the rat (Mann *et al.*, 1993; Curry *et al.*, 2000; Komar *et al.*, 2001), the mouse (Inderdeo *et al.*, 1996; Hägglund *et al.*, 1999), the cow (Smith *et al.*, 1996; Li *et al.*, 2006), and the sheep (Smith *et al.*, 1994).

In paper III and IV, it was shown that MMP-9 was the gelatinase which was preferentially upregulated during ovulation. In contrast MMP-2 mRNA levels were stable throughout the ovulatory interval but increased in granulosa cells after ovulation (paper III). The result of a unique upregulation of MMP-9 is in line with results previously obtained in the mouse (Robker *et al.*, 2000). In that study gel zymography on whole ovarian extracts showed stable levels of MMP-2 and a transient increase of MMP-9 at 4 h after hCG, with a decline already at 8 h. The relative modest 2-fold induction, in comparison to that of the study in paper III and IV, may be explained by that greater increase in the preovulatory follicle is masked by a decrease in MMP-9 expression in other follicular compartments. In another study of MMP expression in the mouse, during the periovulatory interval, there was also a slight increase of the gelatinolytic activity of MMP-9 detected (Hägglund *et al.*, 1999) at 4 h post hCG but no changes in mRNA levels of any gelatinase could be seen. The time course of an early increase in MMP-2 (Robker *et al.*, 2000) in the mouse is in line with the results of paper III and IV, since a time interval of 4 h into the ovulatory period in the mouse would correspond to around 12 h in the human. These time intervals are about 1/3 into the ovulatory period between LH/hCG and follicular rupture. It should be noted that in this aspect the similarities between the mouse and the human seems to be greater than that between the rat and human, since the rat shows a periovulatory increase of MMP-2 (Curry *et al.*, 2000).

Even if the results of the two studies in the mouse (Hägglund *et al.*, 1999; Robker *et al.*, 2000) and the present study suggest a role for MMP-9 in the remodelling events during ovulation, MMP-9 by itself does not seem to carry an essential role in ovulation, at least in the mouse, since mice lacking MMP-9 are fertile (Vu *et al.*, 1998). As discussed above, the lack of

effects could be explained by that other compensatory mechanisms may be used in this situation when MMP-9 is lacking in the ovary.

The rat is the species which has been most extensively studied in relation to expression, localization and function of the gelatinases. Concerning, MMP-2 and MMP-9, it was found that both these enzymes were increased during various stages of folliculogenesis in the immature eCG-primed model (Cooke *et al.*, 1999). With reference to expression during the ovulatory interval, experiments on whole ovary extracts revealed that the mRNA levels for MMP-2 increased about 3-fold, with a significant increase at the 8 and 12 h time point (Curry *et al.*, 2000). In the latter study, the mRNA levels for MMP-9 were unchanged during the periovulatory period.

The animal models which are considered to be closest to the human are any of the non-human primate models. In research on reproductive function, especially the baboon (Cseh *et al.*, 2002), the rhesus monkey(macaque) (Chaffin *et al.*, 1999a), and the marmoset (Cui and Matthews, 1994) have been used. The expression patterns of gelatinases were examined in macaque periovulatory granulosa cells (Chaffin and Stouffer, 1999). In this study, granulosa cells were obtained from rhesus monkeys undergoing controlled ovarian stimulation before (0h), 12, 24 or 36 h after administration of an ovulatory hCG bolus. The pattern of gelatinase expression was not analogous to that of the human granulosa cells evaluated in paper IV, even though the durations of the menstrual cycles and ovulatory phases are almost identical. In the macaque, the mRNA levels of MMP-9 were only significantly increased just prior to follicular rupture and an increase of MMP-2 was seen already at 12h (Chaffin and Stouffer, 1999). These observed differences point out that data from animal models, including nonhuman primates, can not at all times be extrapolated to the human situation.

The existence of a TIMP system in the human ovary was first established by demonstrating metalloproteinase activity in human follicular fluid of gonadotropin-stimulated IVF and gamete intrafallopian transfer (GIFT) cycles (Curry *et al.*, 1988). A positive correlation with progesterone and oestradiol concentrations of the follicular fluids was seen, suggesting a

hormonal regulated expression within the follicle of a tissue inhibitor which was not fully identified at this time.

The results of this thesis show a fairly parallel time course for the expression of MMP-2 and its inhibitor TIMP-2 (paper II-IV). It was only during the PO phase in granulosa cells that an increase in MMP-2 was seen. It is proposed that MMP-2 is active in events that form the CL.

The unbalanced increase of TIMP-1 message during the later stages of the ovulatory period, as found in paper II-IV, suggests that inhibition of MMP activity may be central to avoid excessive degradation of the follicle during ovulation and during luteinisation. In the present study, the highest TIMP-1 mRNA levels were found after follicular rupture. The role of TIMP-1 in the follicular luteal transition as suggested by the temporal expression pattern may also be related to TIMP-1 as a factor that regulates angiogenesis or other processes that are of importance in luteinization. In line with a role for TIMP-1 in establishment of CL function, is the finding that hCG stimulates the accumulation of TIMP-1 protein in luteinised human granulosa cells (Phan *et al.*, 2006).

The role for TIMP-1 that is produced by the granulosa cells may not only be to regulate MMP activity. Several studies point towards that TIMP-1 regulates steroidogenesis within the granulosa cells. Thus, a TIMP-1 like protein was reported to stimulate progesterone production in rat granulosa cells (Boujrad *et al.*, 1995). Moreover, recombinant human TIMP-1 stimulates oestradiol production in cultured mouse granulosa cells (Nothnick *et al.*, 1997) and mice deficient of TIMP-1 exhibit reduced blood levels of oestradiol (Nothnick, 2003) and progesterone (Nothnick, 2003). In human luteinised granulosa cells the MMP-2/TIMP ratio is inversely related to progesterone levels (Ben-Shlomo *et al.*, 2003). Thus, it is quite possible that TIMP-1 in the follicle may in some way facilitate the expression of the steroidogenic enzymes that are necessary for the continuous P₄ increase and the temporary E₂ increase that is seen after LH/hCG stimulation *in vivo* in the human.

Even though TIMP-1 synthesis is highly induced in the ovary by the gonadotropin surge, as shown in paper II-IV, and in other species such as the macaque monkey (Chaffin and Stouffer, 1999), sheep (Smith *et al.*, 1994), pig (Shores and Hunter, 2000), cow (Smith *et al.*,

1996), mouse (Hägglund *et al.*, 1999) and rat (Simpson *et al.*, 2001) the increase may not be obligatory for ovulation to occur. Thus, studies in mice lacking a functional TIMP-1 gene revealed that these mice were normal in regards to ovulatory frequency and fertility (Nothnick *et al.*, 1997). Thus, it may well be that the function of TIMP-1 is covered by other TIMPs in a redundant fashion.

However, it has to be emphasized that the results of the present thesis show a very large induction of TIMP-1 and MMP-9 in the granulosa cells, theca cells and partly in the stromal tissue during ovulation. The increase seen in the human is much larger than what has previously been described in animal species and suggests a critical, functional role for these ECM remodelling enzymes during ovulation and luteinization.

MMPs and TIMPs in other processes occurring in the female reproductive system

The main results of the present thesis are that both the MMP- and the TIMP system are highly involved in ovulation and luteinization. However, the MMPs and the TIMPs are considered to play essential roles in the remodelling of the ECM during other cyclic physiological processes of the female reproductive organs as well. Moreover, their participation in several pathological conditions affecting the female reproductive system is also acknowledged. In the section below I will briefly summarize what is known about the roles of MMPs and TIMPs in other human, female reproductive processes, and to some extent discuss the findings in paper I-IV in relation to that.

The cycling endometrium

During every normal menstrual cycle the endometrium undergoes extensive remodelling. Several MMPs and TIMPs are expressed in the various cell types and diverse compartments of the endometrium during the menstrual phases. The overall control of these cyclic, endometrial changes is carried out by the major ovarian steroids, progesterone and oestradiol, as thoroughly reviewed by (Salamonsen and Woolley, 1996; Salamonsen *et al.*, 2002; Curry and Osteen, 2003). The endometrium contains large quantities of collagen type I and III as well as collagen type IV (Iwahashi *et al.*, 1996). In paper I, we demonstrated a similar abundancy of collagen types I, III and IV in the ovarian stromal capsule, and around as well

as in the periovulatory follicle. In the present thesis the distribution of other collagens in the ovary was not specifically studied but this should be done in future studies. The fact that the collagens of type I, III and IV seems to be the most common in the endometrial stroma mediates that there will not be a large quantity of other collagens present in the ovary. The collagens in the endometrium are degraded upon the loss of progesterone support at menstruation and, as expected, the highest levels of both MMPs (MMP-1, -2,-3, and -9) and TIMPs (TIMP-1 and -2) are demonstrated during this time (Salamonsen and Woolley, 1996; Freitas *et al.*, 1999; Vassilev *et al.*, 2005). These enzymes have also been demonstrated in the human ovary at ovulation when an extensive remodelling of the follicular ECM is necessary (paper II-IV). The signal to MMP and TIMP production in the endometrium is the abruptly decreasing, circulating levels of P₄. In contrast, during the ovulatory process, increasing concentrations of P₄, leading to PR activation and expression, may be the overall signal. Expression of PR is induced by LH (Robker *et al.*, 2000) and it has been shown in PR-KO mice. However, in PR-KO mouse MMP-2 and MMP-9 do not seem to be targets of PR during ovulation. In the same study Robker and co-workers showed that ADAMTS-1 is induced by LH in granulosa cells of preovulatory follicles and depends on PR (Robker *et al.*, 2000). Furthermore, endometrial MMP-3 and -9 are increased in women with breakthrough bleedings using progestin-only contraceptives (Vincent *et al.*, 1999; Marbaix *et al.*, 2000).

Corpus luteum formation and luteolysis

After ovulation the ruptured follicle is transformed into the highly vascularised corpus luteum (CL) by extensive reorganization of the ECM, cellular migration and proliferation as well as angiogenesis. The role of MMPs and TIMPs in this transition, reviewed by (Smith *et al.*, 2002; Curry and Osteen, 2003; Murdoch and Gottsch, 2003), has mainly been studied in animal models, and in cultured human luteinised granulosa cells from IVF-cycles. However, few studies exist on human CL of the menstrual cycle or during pregnancy. In vitro studies in human cell systems have not shown any change in the mRNA expression of MMP-1, TIMP-1 and TIMP-2 during the luteal phase or after luteal rescue with hCG to mimic early pregnancy (Duncan *et al.*, 1996; Duncan *et al.*, 1998). In these studies, the patients were monitored by hormone profiles and ultrasound to get an accurate dating of the CL in relation to the LH-surge. It was also important to find the right time to administer hCG to rescue the CL. In fact,

we used the studies by Duncan and co-workers as model when we planned the human ovulation genomic expression analysis, which this thesis is part of. After reading their papers we were convinced that we would also be able to monitor patients before their planned surgery and exogenous hCG could be given for experimental purposes. A step forward in the methodology of the present study (paper I-IV) is that we were able to harvest the follicle/CL by a laparoscopic approach. Previous experience from our laboratory had demonstrated that the solid CL structure could be harvested by laparoscopic approach (Fridén *et al.*, 2000) but during the work of the present thesis, methodology to harvest the entire follicle was developed. In the studies on the CL (Duncan *et al.*, 1996; Duncan *et al.*, 1998) there was an increased expression of MMP-9 mRNA during early luteal phase (day 1-5 after the LH-peak) and of MMP-2 and MMP-9 mRNA in the late luteal phase (day 11-14 after the LH-peak). The finding of MMP-9 being highly expressed during the formation of the CL fits with the findings of paper III-IV where a high MMP-9 expression was seen during postovulatory phase. Since the postovulatory phase of this thesis was around 2-3 days after hCG, this would be during the interval of day 1-5 after LH (Duncan *et al.*, 1996). Luteal rescue inhibited the expression of MMP-2 (Duncan *et al.*, 1998; Manase *et al.*, 2002). This implies that MMP-2 is the major degradation enzyme in CL demise, which is in contrast to that of the follicle wall with signs of MMP-9 as being the important (paper III, IV). The more recent study by Manase and co-workers, however, showed an increased expression of mRNA and protein for TIMP-1 and TIMP-2 during the midluteal phase (4-11 days after ovulation) and a further increase of TIMP-2 during the late luteal phase (11-14 days after ovulation;). The present study (paper II-IV) showed an increased TIMP-1 expression during luteinization (PSO phase) and together the results suggest that there is a biphasic expression pattern of TIMP-1 with an early and midluteal rise. An increased expression of MMP-2 and its activator MMP-14 (MT1-MMP) was also reported, particularly during the late luteal phase (Manase *et al.*, 2002). Even though there are some differences between the studies from these two research groups, both MMPs and TIMPs are induced at the beginning of structural luteolysis, when major remodelling is needed. However, during the preceding ovulation, MMP-2 and TIMP-2 are constantly expressed (paper II-IV) indicating their different roles during stages of follicle/CL remodelling. An increased expression of TIMP-1 and TIMP-2 in the midluteal phase could imply that these two proteins are actually involved in influencing steroidogenesis, which is

maximal during this period. Additionally, during maternal recognition of pregnancy, hCG seems to avert the normal increase in MMP-2 in the late luteal phase to prevent luteolysis (Duncan *et al.*, 1998).

Pregnancy-placentation, cervical ripening and rupture of the foetal membranes

The balance between the secretion of MMPs from the trophoblasts and their inhibition by TIMPs appears to be necessary for successful implantation and placentation (Niu *et al.*, 2000). Degradation of collagen type IV, one of the main components of BL, is crucial to enable invasion of the trophoblast cells through the decidua and into the maternal vasculature. Degradation of collagen type IV-rich (paper I) BL separating the granulosa and theca cells compartments is in a similar way necessary for the release of the oocyte at ovulation. The gelatinases (MMP-2 and MMP-9) are regarded as key enzymes in the implantation process, reviewed by (Staun-Ram and Shalev, 2005; Cohen *et al.*, 2006). MMP-2 is also overexpressed in the hydatidiform mole (Petignat *et al.*, 2006) suggesting involvement in the uncontrolled trophoblastic proliferation occurring in this condition.

In paper III-IV it was demonstrated that there is a marked increase in the expression of the gelatinase MMP-9 but not MMP-2 in both granulosa and theca cells during ovulation. One can speculate that ECM homeostasis during ovulation also depends on this type of coordinated balance between deposition and removal of ECM components but that the degradation of collagen type IV is not as massive in ovulation as in implantation.

At the end of gestation, diminishing steroid levels and an inflammatory-like reaction initiate preparation for delivery and at partus, a prominent reorganization of the ECM in the cervix occurs. At the time of final cervix ripening, turnover and degradation of collagens are increased (Uldbjerg *et al.*, 1983) and in particular the collagenase MMP -8 (Sennstrom *et al.*, 2003) has been suggested to play a major role in this process. Moreover, at term, the mRNA levels of MMP-2 and MMP-9 were increased in the cervix compared with the nonpregnant state (Stygar *et al.*, 2002) indicating their participation in the final remodelling of connective tissue in the cervix preceding parturition. The main source of MMP-2 was found to be cervical stromal fibroblasts and smooth muscle cells, while the MMP-9 protein was identified in invading leukocytes (Stygar *et al.*, 2002). This last finding is of interest in comparison to the results of paper IV. It is known that there is a massive influx of leukocytes into the theca

layer at ovulation (Brännström *et al.*, 1994) and it may well be that the increased expression seen in the theca at ovulation may be due to expression in invaded leukocytes.

Degradation and breakdown of the foetal membranes (chorion and amnion) and the adjacent decidua are important for the progression of labour. Collagen types I, IV and V are components of the ECM in gestational membranes (Hampson *et al.*, 1997) and both MMP-2 and MMP-9 expression increase in the foetal membranes before (McLaren *et al.*, 2000) and during active labour (Bryant-Greenwood and Yamamoto, 1995; Vadillo-Ortega *et al.*, 1995). Furthermore, prostaglandin F_{2α} (PGF_{2α}) increases decidual gelatinolytic activity and the inhibition of PG by indomethacin reduces total gelatinolytic activity in foetal membranes which could possibly explain the labour-arresting effect of indomethacin (Ulug *et al.*, 2001). MMP-9 has also been suggested to be involved in placental separation (Tsatas *et al.*, 1999). All these mediators have been suggested to be essential also in human ovulation, as shown for the MMPs (paper II-IV) and PGs (Killick and Elstein, 1987; Pall *et al.*, 2001).

Endometriosis

The aetiology of endometriosis appears to be complex but the theory of retrograde menstruation with implantation of shed endometrial tissue on the peritoneal surface is accepted to be a part of the explanation. MMPs have been postulated to play a major role in the invasive establishment of the disease, reviewed by (Osteen *et al.*, 2003). Ectopic endometrium and peritoneal fluid from women with endometriosis express higher levels of MMP-2 and MMP-9 mRNA than eutopic endometrium and peritoneal fluid from women without endometriosis (Chung *et al.*, 2001; Ueda *et al.*, 2002). Moreover the MMP-9/TIMP-3 ratio in both eutopic and ectopic endometrium is higher in endometriosis patients (Chung *et al.*, 2001), implicating an increased proteolytic activity that may be one of the reasons for the invasive properties of the ectopic endometrium resulting in the development of endometriosis. A very recent study has reported an altered expression pattern of basigin/EMMPRIN, a regulator of MMP expression/activation, in ovarian endometriosis and in normal ovarian tissue indicating a possible role for dysregulated proteolytic MMP activity in endometriosis (Smedts *et al.*, 2006).

Polycystic ovarian syndrome

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in women of reproductive age. In a proportion of the patients, anovulation is a feature. The anovulatory aspect of PCOS and the thickened ovarian capsule as well as ovarian stroma make it likely that ECM of the ovary is changed in this condition both concerning its composition and breakdown. Only few studies exist concerning the involvement of MMPs/TIMPS in PCOS. Thus, in luteinised IVF granulosa cells from PCOS patients, a shift in the MMP/TIMP balance towards MMP-2 and MMP-9 was shown (Shalev *et al.*, 2001). Additionally, also in an IVF-setting, reduced levels of TIMP-1 in follicular fluid from women with PCOS were demonstrated as compared with women without ovulatory dysfunction (Lahav-Baratz *et al.*, 2003). This is of interest in relation to the findings of paper III, with very high expression of TIMP-1 in the granulosa cells bordering the follicular fluid, of normal ovulation. Reduced TIMP-3 mRNA expression has been observed in ovarian biopsies from PCO patients (Oksjoki *et al.*, 2004). Furthermore, in an in vitro study of luteinised granulosa cells from IVF cycles from patients with normal ovulatory function versus patients with PCOS, it was shown that progesterone production was inversely related to the ratio of MMP-9/TIMP-1 and that the highest MMP-9/TIMP ratios were found in the PCOS patients (Ben-Shlomo *et al.*, 2003). These findings may imply that the shift in the MMP/TIMP balance towards MMP, with increased proteolytic activity as a result, is a compensatory mechanism to degrade the thick ovarian capsule in PCOS, as suggested by Lahav-Baratz and co-workers (Lahav-Baratz *et al.*, 2003).

Unexplained infertility

The MMP/TIMP-system has also been explored to some extent in women with unexplained infertility and in couples with infertility due to a male factor. In all IVF programmes, some women are unsuccessful in achieving an ongoing pregnancy, even after several embryo transfer (ET) procedures. This could, in part, be due to an unfavourable endometrial environment. In natural cycles of IVF patients, with previous recurrent implantation failures, it has been shown that both MMP-9 and IL-1 β levels in the uterine cavity are higher during “the implantation window” compared with natural cycles of previously fertile women (Inagaki *et al.*, 2003). This is suggestive of an altered pattern of proteolytic activity and intra-

uterine cytokine concentration that may predispose for a less optimal environment for embryonic implantation in these patients. Both MMP-9 and IL-1 β are important in the normal implantation process as well as in ovulation (paper III-IV) (Brännstrom *et al.*, 1993b). However, it may be that if these mediators are expressed at too high levels, these finely controlled physiological processes may be disturbed as pointed out above and for ovulation as in ovarian hyperstimulation syndrome (Enskog *et al.*, 2001). Another reason for failed pregnancy in IVF programs could be aberrations in the oocyte-cumulus micro-environment in the follicle. In the present study MMP-9 levels in follicular fluid collected during oocyte retrieval were higher in women achieving pregnancy than in the group which did not become pregnant (Lee *et al.*, 2005). Moreover, the concentration of serum TIMP-1 was shown to be higher after ET in a pregnant group than in a non pregnant group (Shibahara *et al.*, 2005). Thus, following IVF-ET, MMP-9 expression in follicular fluid and serum concentrations of TIMP-1 at the time of oocyte retrieval were recommended as pre-diagnostic markers of successful implantation and pregnancy respectively (Lee *et al.*, 2005; Shibahara *et al.*, 2005).

Gynecological cancer

Invasion and metastasis of cancer cells involves degradation of the surrounding ECM, especially the BL, which facilitates detachment of cells, their crossing of tissue boundaries, and invasion into the adjacent tissue compartment. Both MMPs and TIMPs are associated with invasion and metastasis of several malignant tumours of the female genital tract. The gelatinases (MMP-2 and MMP-9) together with their inhibitors, TIMP-2 and TIMP-1, increase with tumour progression in cervical cancer, detected as an increased immunostaining in the tumour cells invading the underlying stroma (Asha Nair *et al.*, 2003). In paper II-IV we also focused on enzymes that are primarily involved in the regulation of collagen type IV-breakdown, since collagen type IV is the main component of BLs. Ovulation is an extensive tissue remodelling process where degradation of the two main BLs in the follicle wall is necessary. This is however, a controlled ECM degrading process as compared to cancer invasion.

Moreover, MMP-7 has been suggested to serve as a prognostic marker in high grade uterine endometrial carcinoma as it is mainly produced by the cancer cell itself, and increased MMP-7 expression is associated with a shorter disease-free interval after treatment (Misugi *et al.*,

2005). The gelatinases increase with an increase in malignant potential of ovarian tumours as shown by western blot, immunohistochemistry and gelatin zymography (Schmalfeldt *et al.*, 2001). The use of various protease inhibitors has been suggested as a new therapeutic approach in gynaecological cancer. However, more recent studies have indicated that MMPs can exhibit pro-metastatic as well as anti-metastatic roles (Deryugina and Quigley, 2006). It may not be as simple as inhibiting one pathway to get an effective block of tumour growth. In most biological processes, such as ovulation, there are safety mechanisms built in as exemplified by that inhibiting MMPs with phenanthroline during in vitro perfusion of rat ovaries (Brännstrom *et al.*, 1988) would not totally block ovulation. It seems as if generally two or more systems need to be non-functioning to get a total inhibition of ovulation. The MMP-TIMP system (paper II-IV) may be one such pathway.

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