

## Review

# New insights into the normal menstrual cycle-regulatory molecules

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**Summary.** Although the growth and regression of the endometrium is primarily a function of the ovarian hormones, recent studies indicate a potential autocrine/paracrine role for regulatory molecules. Thus, growth factors, angiogenesis stimulating factors and proliferating cell markers are high in the proliferative phase endometrium contributing to its regeneration. At the same time, other proteins promote endometrial cell survival by preventing extracellular matrix degradation and apoptosis. As glandular proliferation persists in the early secretory phase of the menstrual cycle, the activity of some proteins stimulating growth remains unchanged, but declines significantly thereafter, shifting the balance between proliferation and apoptosis in favour of apoptosis. During this period, several other regulatory substances are expressed at high levels, suggesting a role in endometrial maturation. If, however, implantation of a fertilized ovum fails to take place, menstruation occurs probably as the result of matrix metalloproteinases which antagonizes the anti-degradation factors (inhibitors of metalloproteinases). This review examines the changing endometrial patterns of a normal menstrual cycle in relation to these regulatory molecules.

**Key words:** Menstrual cycle, Autocrine/paracrine regulation, Growth factors, Angiogenic factors, Matrix metalloproteinases

### Introduction - The normal menstrual cycle

Unlike other tissues of the body, the female genital tract and the ovary undergo repetitive cyclical changes in response to oestrogen and progesterone stimulation. These changes, which occur throughout the reproductive years at approximately monthly intervals, are most striking in the endometrium; the prospective site for blastocyst implantation. Failure to accomplish this

objective is followed by menstruation. This hormone-dependent endometrial activity is associated with a series of continuously changing endometrial patterns which are an essential part of the menstrual cycle (Speroff et al., 1994).

A typical menstrual or endometrial cycle lasts for about 28 days, although its length varies from 21-35 days ( $28 \pm 7$ ) (Munster et al., 1992). The first day of the menstrual bleeding is designated day 1 of the cycle, but this is not universally accepted and in North America it is the day of ovulation that is usually taken as day 1 of the cycle. Ovulation occurs typically on day 14.

For convenience of description the menstrual cycle has been traditionally divided into three main phases that are correlated with the functional activities of the ovary. These are: the pre-ovulatory or proliferative phase; the post-ovulatory or secretory phase; and the menstrual phase (Buckley and Fox, 2002). Although these distinctions are not entirely arbitrary, it must be noted that the whole process is a continuous repetitive cycle of endometrial growth and regression (Speroff et al., 1994). Thus, in the proliferative phase of the menstrual cycle (early, mid and late), the endometrium regenerates and grows under the influence of oestrogens secreted by the developing follicle. In the secretory phase of the cycle (early, mid and late), the endometrium is fully matured and undergoes secretory transformation under the combined influence of oestrogen and progesterone produced by the corpus luteum. In the menstrual phase, which follows the abrupt fall in circulating oestrogen and progesterone levels as a result of degeneration of the corpus luteum, the functional layer of the endometrium is shed.

It is appreciated that these endometrial responses to ovarian hormones are accomplished through specific intranuclear binding proteins, the oestrogen and progesterone receptors, which are present in both epithelial and stromal cells (Oehler et al., 2000; Critchley et al., 2001a,b). Recent studies, however, indicated that the endometrial growth and regression is also dependent on plethora of regulatory molecules, including growth factors, angiogenesis stimulating factors and enzymes.

**Regulatory molecules**

These are mainly polypeptides having a regulatory function. They are produced in a variety of cells, epithelial, stromal and migratory, dispersed throughout the body. Most remarkable, such molecules are acting locally either in the cells in which they are produced – “autocrine function” or in adjacent cells – “paracrine function” (Osteen et al., 1999). Regulation factors of this type, often called local hormones, operate by binding to specific receptors on the cell surface of the target cells. The receptors usually contain an intracellular component with tyrosine activity which is stimulated by a binding-induced conformational change that induces phosphorylation of cell proteins (Speroff et al., 1994). Some factors, however, may act through other messenger systems, such as cyclic AMP (cAMP) or inositol 1,4,5-triphosphate (IP<sub>3</sub>) (Speroff et al., 1994). It is emphasized that autocrine/paracrine regulation factors are not operating in an autonomous fashion but interrelated closely with classical hormones produced in ductless glands and transported at the site of action via the circulation - classical “endocrine function”.

Regulatory molecules that either stimulate or inhibit cell proliferation and differentiation, angiogenesis, matrix synthesis and degradation have been located in the human endometrium. They include growth factors and growth factor receptors, i.e., transforming growth factors-beta (TGF-β) and their receptors (TGF-βR), angiogenesis stimulating factors, i.e., erythropoietin and vascular endothelial growth factor (VEGF), cytokines, i.e., interleukins (IL) of various forms, and enzymes, i.e., matrix metalloproteinases (MMPs), telomerase and polo-like kinase (PLK). Arachidonic acid metabolites, such as prostaglandins (PG), are also classed as regulatory molecules. All these biological active substances can operate in a cooperative, competitive or synergistic fashion with oestrogen and progesterone hormones for transforming the endometrium continuously during the menstrual cycle.

**Regulatory molecules at menstruation**

For many years, the initiation of normal menstruation was thought to be directed by the local haemodynamic effects of prostaglandins (PG) on the uterine tissues (Jensen et al., 1987). This view is considered speculative nowadays, as more and more data support a role for matrix metalloproteinases (MMPs) and leucocytes in endometrial breakdown (Salamonsen and Woolley, 1999; Critchley et al., 2001b; Henriot et al., 2002; Zhang and Salamonsen, 2002). The total complex factors implicated in the phenomenon of menstruation still remains to be defined (Critchley et al., 2001b).

The “prostaglandin theory”. Prostaglandins are synthesized by the endometrium, but are stimulated by the corpus luteum (Poyser, 1995); their action is cyclical. Secretion of PGF<sub>2</sub> alpha causes constriction of the spiral arterioles and contraction of the myometrium, resulting

in endometrial hypoxia and irregular lines of endometrial necrosis. PGE<sub>2</sub> causes dilatation of the spiral arterioles, while PGI<sub>2</sub> induces dilatation of the spiral arterioles and relaxation of the myometrial muscle (Jensen et al., 1987). It was postulated that the sequential action of PGF<sub>2</sub>, PGE<sub>2</sub>/ PGI<sub>2</sub> was responsible for the normal endometrial shedding.

The “metalloproteinase theory”. Recent studies, however, have suggested that menstruation results from the action of the lytic enzymes MMPs, with the contribution of activated endometrial stromal granulocytes, macrophages and mast cells (Salamonsen and Woolley, 1999; Critchley et al., 2001b; Henriot et al., 2002; Zhang and Salamonsen, 2002). In fact, leucocytes release MMPs, and the interactions between leucocytes and endometrial cells, both epithelial and stromal, induce and activate MMPs (Salamonsen et al., 2000, Salamonsen and Lathbury, 2000). As tissue inhibitors of metalloproteinases (TIMPs) remain constant throughout the menstrual cycle, it is the production and activation of MMPs that alters the MMPs/TIMPs ratio, resulting in tissue breakdown (Zhang and Salamonsen, 1997; Salamonsen et al., 2000). Besides, tissue plasminogen activators are also on the increase, facilitating fibrinolysis (dissolution of clots) and menstrual loss (Gleeson et al., 1993; Gleeson, 1994).

At the latter part of the menstrual phase, but well before menstrual bleeding has ceased, mitotic activity resumes and epithelial regeneration becomes evident, particularly in the denuded basalis (More, 1995). The new surface epithelium is created by horizontal growth from the stem cells of the glands remaining in the basalis, the layer which is not shed with menstruation and provides the regeneration of the endometrium. To this end, neoangiogenesis is important. New blood capillaries are formed by the stimulating effect of vascular endothelial growth factor (VEGF) (Zhang et al., 1998; Moller et al., 2001; Gargett and Rogers, 2001) and thymidine phosphorylase (TP) (Zhang et al., 1997; Sivridis et al., 2000) secreted by both epithelial and stromal cells. At the same time, continuous proliferation of stem cells is ensured by a high telomerase activity (Hiyama et al., 1995; Bonatz et al., 1998).

**Regulatory molecules at proliferative phase endometrium**

Endometrial remodeling, however, is the primary scope of the proliferative phase endometrium. At this phase, endometrial cell proliferation and growth is ensured by the rising levels of oestrogens and the secretion of specific regulatory molecules. Epidermal growth factor (EGF) and epidermal growth factor receptors (EGFR), in particular, emerge as the main mediators of cell proliferation affecting both epithelial and stromal cells (Pfeiffer et al., 1998; Sugino et al., 2002). Nonetheless, the expression is stronger in the glandular epithelium resulting in tortuosity of the glands. This is not counterbalanced by the basic fibroblast

growth factor receptor-1 (bFGF-R1) which induces growth of the endometrial stroma by stimulating fibroblasts (Sangha et al., 1997). Other important regulators of cell proliferation include the transforming growth factor-beta isoforms (TGF-beta 1, 2, and 3) and their receptors type I and II, which also have a similar preferential action on the endometrial glands (Tamura et al., 1999), the insulin-like growth factors (IGF-I and IGF-II) and the IGF-I receptor (IGF-IR) (Roy et al., 1999), and the transforming growth factor-alpha (TGF-alpha) (Pfeiffer et al., 1998; Zhang et al., 1999). The expression of most of these growth factors and growth factor receptors are particularly prominent at the peak of proliferative activity, i.e., during the early and late proliferative phase endometrium (Roy et al., 1999; Zhang et al., 1999). In all cases, the endometrial glands of the functional layer are produced by vertical growth from those in the basal layer.

Angiogenesis is an integral part of endometrial remodeling, and important angiogenesis stimulating factors, such as VEGF and TP, are consistently expressed in the normal proliferative endometrium. VEGF is typically expressed in the glandular epithelium and, to a lesser extent, in the stromal cells; its activity is considerably higher at the beginning of the cycle (Zhang et al., 1998; Hyder and Stancel, 2000; Gargett and Rogers, 2001). By contrast, TP is initially expressed in the endometrial stroma but, as proliferative activity advances, TP expression shifts from the endometrial stroma to the endometrial glands (Zhang et al., 1997; Sivridis et al., 2000). Other growth factors, such as the fibroblast growth factor-2 (FGF-2) and its receptors FGF-R1 and FGF-R2, are evenly expressed in both glandular epithelial and stromal cells during the menstrual cycle (Sangha et al., 1997; Moller et al., 2001).

Mitoses, the defining feature of the proliferative phase endometrium, are indicated by the exuberant presence of proliferation markers, i.e., the proliferating cell nuclear antigen (PCNA) and Ki-67, in both glands and stroma. PCNA indices are progressively increased from early through late proliferative to early secretory endometrium (Fujishita et al., 1999), and this parallels an increase in telomerase activity, particularly in stem cells (Kyo et al., 1997; Shroyer et al., 1997; Saito et al., 1997; Lehner et al., 2002). At this phase, human telomerase reverse transcriptase (hTERT) mRNA is also expressed (Kyo et al., 1999; Lehner et al., 2002). Nucleolar organiser regions (NORs), the number of which was thought to reflect protein synthesis, show their highest activity in the proliferative phase (Wilkinson et al., 1990).

The regeneration process is further accentuated by the complete paucity of the apoptosis-related epitope cytokeratin 18 (CK18), and the accompanied increase in the anti-apoptosis protein bcl-2 (Morsi et al., 2000; Vaskivuo et al., 2000; Mertens et al., 2002). It is inferred that bcl-2 promotes cell survival by preventing apoptosis (Mertens et al., 2002). Interestingly, bcl-2 expression is

more predominant in the epithelial cells, particularly at the end of the proliferative phase, contributing, together with EGF and EGFR, to the glandular tortuosity (Gompel et al., 1994). Similar cyclic variations in bcl-2 expression display the surface lining epithelium, the endometrial stroma and the arterial capillary network, indicating a hormone-dependent bcl-2 regulation (Gompel et al., 1994). In addition, the surviving gene, another recently described anti-apoptotic gene, shows its highest expression in the late proliferative phase (Tarkowski et al., 2000).

Given that the cell cycle is regulated by the enzyme polo-like kinase (PLK), it is not surprising that the PLK expression by endometrial glandular cells correlates with the expression of Ki-67 and PCNA during the course of the menstrual cycle (Takai et al., 2000). This suggests that PLK is associated with a hormone-dependent cell proliferation function (Takai et al., 2000). The fact that many glandular endometrial cells that are positive for certain regulatory molecules, i.e., TGF-alpha, are also positive for oestrogen receptors (ER) (Niikura et al., 1996), this suggests a mutual interdependence between growth factor receptors and ovarian hormone receptors. Finally, the maintenance of endometrial integrity during the proliferative phase of the cycle is very much dependent on the continuous presence of tissue inhibitors of metalloproteinases (TIMPs) (Zhang and Salamonsen, 1997).

### Regulatory molecules at secretory phase endometrium

As glandular proliferation persists in the early secretory phase of the menstrual cycle, the activity of some, but not all, growth factors and growth factor receptors remain high. These include the epidermal growth factor (EGF), the transforming growth factor-beta isoforms (TGF-beta 1,2,3), the insulin-like growth factors I and II (IGF-I and IGF-II), and their corresponding receptors (Roy et al., 1999; Tamura et al., 1999). Thereafter, the expression of growth stimulating proteins declines, following a similar decrease in the enzymatic activity of the polo-like kinase (PLK) (Takai et al., 2000), the telomerase (Shroyer et al., 1997; Kyo et al., 1997), and the telomerase reverse transcriptase (TERT) mRNA which has virtually vanished (Kyo et al., 1999). Furthermore, the proliferative index PCNA (Ki-67) and the expression of bcl-2 is significantly reduced (Morsi et al., 2000; Vaskivuo et al., 2000; Mertens et al., 2002), shifting the balance between anti-apoptosis (bcl-2), proliferation (Ki-67) and apoptosis (M30) in favour of apoptosis (Gompel et al., 1994; Morsi et al., 2000).

By contrast, other regulatory molecules, such as the neuregulin-1 alpha and beta, the betacellulin and the TGFβ3, are expressed at significantly higher levels at the luteal phase of the menstrual cycle, suggesting a role for these proteins in endometrial maturation (Srinivasan et al., 1999; Reis et al., 2002). It is unclear whether lectins, like *Canavalia ensiformis* (Concanavalin A), which are

increased as a consequence of progesterone stimulation, may have a similar function in the endometrium (Sivridis et al., 2000a,b).

Not only the epithelium but also the endometrial stromal cells secrete important proteins, including relaxin, prolactin, insulin-like growth factor-binding protein (IGFBP-1), and pregnancy associated endometrial  $\alpha$ 1-globulin ( $\alpha$ 1-PEG) (Waites et al., 1988; Bryant-Greenwood et al., 1993). The latter two proteins (IGFBP-1 and  $\alpha$ 1-PEG), which are responsible for the decidualization of the endometrial stroma, are secreted preferentially around the spiral arteries and the subepithelial regions (Waites et al., 1988; Bryant-Greenwood et al., 1993). A similar role may be played by the glucocorticoids, as glucocorticoid receptors are specifically expressed at this late secretory phase (Bamberger et al., 2001).

The intensity of gonadotrophin-releasing hormone (GnRH-II), as detected by immunohistochemical techniques, is stronger in the early and mid secretory phase of the menstrual cycle and may be responsible for blastocyst implantation (Cheon et al., 2001). Other proteins, such as haptoglobin, are almost exclusively expressed at this phase and may protect the fetus from a maternal allograft-like immune response (Berkova et al., 2001). Similarly, osteopontin, a progesterone induced glycoprotein which binds to integrin at the cell surface (Apparao et al., 2001) may be important in establishing pregnancy. The epithelial mucin episialin/MUC1 which is a transmembrane glycoprotein with anti-adhesion properties is expressed in abundance during the secretory phase of the menstrual cycle and may interfere with blastocyst implantation (Sivridis et al., 2002). Angiogenin and erythropoietin (Epo) potent stimulators of angiogenesis expressed in both epithelial and stromal/decidual cells of the normal human endometrium (Koga et al., 2001; Yasuda et al., 2000). At the time of implantation of the conceptus, the endometrial stroma peaks of matrix degrading enzyme deposition (Giudice, 1999; Salamonsen et al., 2001). At the same time, the endometrial immune system is flourishing, as the immunoglobulins IgA and IgM, along with J chain and secretory component, are increasing from early proliferative to late secretory phase of the menstrual cycle (Bjercke and Brandraey, 1993). They form part of the secretions of endometrial glands. A similar increase shows many interleukins (IL), including IL-6, IL-11 and IL-15 (Dimitriadis et al., 2000; Okada et al., 2000; von Wolff et al., 2002).

If implantation of a fertilized ovum fails to take place endometrial shedding occurs as a result of the combined action of prostaglandins and matrix metalloproteinases (Soini et al., 1997; Henriot et al., 2002; Zhang and Salamonsen, 2002). Metalloproteinases, as enzymes degrading basement membranes and components of the extracellular matrix, are expressed in abundance in the decidualized endometrium (Soini et al., 1997). In high numbers are also detected premenstrually the endometrial stromal

granulocytes, often referred to as endometrial granulated lymphocytes. These are mainly CD3+, CD4+, CD8+ T lymphocytes, and CD56+ NK-cells (Yeaman et al., 1998; Tian et al., 2000; King, 2000) demonstrating a high telomerase activity (Igarashi et al., 2001). Mast cells also show some increase premenstrually, particularly the degranulated forms (Sivridis et al., 2001), and may be a source of tumour necrosis factor (TNF) (Fajardo and Allison, 1997). TNF-alpha induces RANTES gene expression (Regulated upon Activation Normal T-cell Expressed and Secreted), a cytokine that stimulates recruitment of CD68+ macrophages in endometrial tissues (Hornung et al., 2001). Endothelin-1 and interleukin-1 receptor type II (IL-1RII), by increasing premenstrually, may play some role in menstruation (Ohbuchi et al., 1995; Boucher et al., 2001). It is, however, the expression of the bcl-2 gene that heralds apoptosis and menstrual shedding (Gompel et al., 1994).

From the above discussion it seems more probable that the endometrium is in itself an autocrine/paracrine diffuse endocrine organ, rather than a simple target tissue for sex-steroid hormones.

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