Review

Species Comparison of the Role of p38 MAP Kinase in the Female Reproductive System

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Abstract: The p38 mitogen-activated protein kinases (MAPKs) are members of discrete signal transduction pathways that have significant regulatory roles in a variety of biological processes, depending on the cell, tissue and organ type. p38 MAPKs are involved in inflammation, cell growth and differentiation and cell cycle. In the female reproductive system, p38 MAPKs are known to regulate various aspects of the reproductive process such as mammalian estrous and menstrual cycles as well as early pregnancy and parturition. p38 MAPKs have also been implicated in alterations and pathologies observed in the female reproductive system. Therefore, pharmacologic modulation of p38 MAPKs, and inter-connected signaling pathways (e.g., estrogen receptor signaling, c-fos, c-jun), may influence reproductive physiology and function. This article provides a critical, comparative review of available data on the roles of p38 MAPKs in the mammalian female reproductive system and in reproductive pathophysiology in humans and preclinical species. We first introduce fundamental differences and similarities of the mammalian female reproductive system that should be considered by toxicologists and toxicologic pathologists when assessing the effects of new pharmacologic agents on the female reproductive system. We then explore in detail the known roles for p38 MAPKs and related molecules in female reproduction. This foundation is then extended to pathological conditions in which p38 MAPKs are thought to play an integral role. (J Toxicol Pathol 2009; 22: 109–124)

Key words: p38 MAPK, female, reproduction

Introduction

The p38 mitogen activated protein kinases (MAPKs) are members of discrete signaling transduction pathways that play significant regulatory roles in a variety of biological processes including inflammation, cell differentiation, and cell growth^{1–4}. By participating in phosphorylation cascades, p38 modulates the regulation and activity of several transcription factors (i.e., activating transcription factors-1 and -2 (ATF-1, ATF-2); the p53 tumor suppressor protein; and CCAAT/Enhancer Binding Proteins-beta (C/EBP β)^{5–8}. These transcription factors lead

to cytokine production, cell growth, apoptosis, and other cellular processes. Therefore, because of p38's involvement in inflammation, specific p38 inhibitors are under development as anti-inflammatory medicines^{3,9}.

p38 MAPKs are activated by dual phosphorylation at residues thr180 and tyr182. Some p38 inhibitors (i.e., SB203580) compete for the ATP binding pocket and inhibit direct enzymatic activation, while other inhibitors (i.e., BIRB796) stabilize a conformation that is unable to bind ATP¹⁰.

p38 MAPK, along with a variety of intracellular signaling pathways such as Estrogen Receptor α (ER α), c-jun and c-fos, orchestrate physiological events in the uterus during the menstrual cycle in both humans and in preclinical species such as nonhuman primates (NHPs). Emerging data suggests that uterine function effects may be attributable to the close interrelationships of these signaling pathways and their modulation¹¹. c-fos and c-jun interact with the transcription factor activator protein-1 (AP-1) which translocates to the nucleus and binds to the AP-1 enhancer element to initiate a cascade of gene induction events that lead to cell proliferation¹². MAPKs regulate AP-1 transcriptional activity and c-fos expression in the uterus and mediates mechanical stretch-induced c-fos expression in myometrial smooth muscle cells^{13–16}.

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Estrogen stimulates DNA synthesis, and cellular proliferation and differentiation in the uterus of mammals^{17,18}. Binding of estrogen to its receptor (ER) contributes to uterine cellular proliferation via increased expression of immediate early response genes^{13,18–21}. It has also been suggested that the initial steps in the mechanism of mitogenesis by estrogen involve activation of c-fos gene expression in the rat uterus, emphasizing the orchestrated effort of these molecules in directing uterine function¹⁹.

p38 MAPKs are thought to contribute to parturition^{15,22,23}. Marked increases in the p38 kinase activity in the human uterus was observed on day 19 of gestation and during labor, and declined to the control levels post-delivery²³. p38 MAPK has also been shown to be present in endometriotic cells from humans and activated by pro-inflammatory agents²⁴. Moreover, various aspects of the mammalian estrous and menstrual cycles, as well as early pregnancy, involve regulatory roles for p38 MAPKs^{5–8,25–28}, and p38 MAPK has been implicated in alterations and pathologies observed in the female reproductive system^{1,12,29}. Considerable “cross talk” between the MAPK signaling pathways (e.g., p38, JUNK) may also play an important role throughout all stages of the female reproductive process.

Collectively, these observations imply a close interrelationship between ER, c-fos, c-jun, and p38 in modulating uterine function during the estrous cycle and early embryonic processes. Therefore, pharmacologic modulation of one or more of these signaling molecules, coupled with interspecies differences in uterine cyclicity, may influence uterine function. This article provides a critical, comprehensive review of the known roles for p38 MAPKs and related pathways in the mammalian female reproductive system in of humans and in preclinical species, and signaling in reproductive pathophysiology. We first introduce fundamental differences and similarities in the mammalian reproductive system in humans and in preclinical species. We then explore in detail the known roles for p38 MAPKs pathways in female reproduction. With this foundation, we then provide a review of the pathological conditions in the female reproductive system under which p38 MAPKs may play a role.

Mammalian Estrous and Menstrual Cycles

Much of our knowledge of the female reproductive cycle is drawn from research conducted on a variety of species. The rat, dog, and monkey are common laboratory animals used for testing new drug candidates developed for use in human medicine. However, there are significant differences among these preclinical species in the female reproductive system. Such differences should be taken into consideration by toxicologists and toxicologic pathologists when assessing the effects of new pharmacologic agents on the female reproductive system.

We first introduce these species differences in uterine cyclicity. The mechanism of early pregnancy trophoblast

invasion is similar in rodents and the NHPs. The rat plays a prominent role in female reproduction research, making it a good model to detail the mammalian estrous cycle. There are significant interspecies differences in uterine cyclicity that may influence uterine functions and modulate the signaling pathways of MAPKs, c-fos, c-jun, and/or ER. For example, rats exhibit cyclic changes in the expression of c-fos and ER α ¹¹. The comparative aspects of the estrous cycle in the rat and dog and menstrual cycle in NHPs and human are summarized in Table 1 and Figs. 1 and 2.

Rat Estrous Cycle

Rats are continuously polyestrous, with an average estrous cycle length of 4–5 days. The cycle is subdivided into four phases: proestrus (P), estrus (E), metestrus (M) (diestrus I), and diestrus II (D)^{30,31}. The P phase begins when progesterone (P₄) levels decline as a result of luteolysis and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion from the anterior pituitary. In the adult female rat, the concentrations of the preovulatory estradiol (E₂), which is produced by ovarian follicles, peak during the P phase (Fig. 1). As E₂ levels drop during the E phase, there is a corresponding lack of glandular and luminal epithelial growth and, in parallel, an increased apoptosis in these cells. Stromal cell proliferation is also seen¹⁸. The uterine lumen is dilated during both the P and E phases. As a result of the pre-ovulatory E₂ surge, ovulation and mating behavior occur at the beginning of the M phase. Notably, of the cell types comprising the uterus (stromal, epithelial, and myometrial), only the epithelial cells proliferate in response to E₂ in the adult rat and mouse. However, all cell types respond in the immature rodent²¹. P₄, produced by the newly formed ovarian corpora lutea, also begins to rise and reaches a maximum peak during the longest phase of the cycle: the D phase. During the D phase, epithelial cell division and maturation is inhibited, but due to lowered levels of P₄ in the rat, typical endometrial gland secretion is not observed.

The histologic appearance of the uterus varies with the stage of the reproductive cycle. The P phase is characterized by distention of the uterine lumen with clear fluid and the lumen is usually lined by large low columnar cells³¹. Smooth muscle hypertrophy, endometrial stromal cell proliferation, stromal edema, proliferation of luminal and glandular epithelium, and a few mitotic figures are present during proestrus (Fig. 3A). In the E phase, the uterine lumen is lined by very large, tall columnar epithelium. There is myometrial hypertrophy, and many of the luminal and glandular epithelial cells undergo vacuolar degeneration and necrosis (Fig. 3B)^{30,31}. In the M phase, the epithelial cells of the luminal lining are reduced in height, the stroma becomes denser, and mitotic activity decreases (Fig. 3C)^{30,31}. During the D phase, the uterus is quiescent and appears shrunken with dense endometrial stroma, atrophied myometrium, very small lumen, and cuboidal glandular and luminal epithelium.

Studies in several species have addressed the role of P₄ in the modulation of E₂ activity and maintenance of the

Table 1. Comparative Aspects of the Estrous Cycle vs. Menstrual Cycle in Human and Animal Species (Days¹)

	Species (Cycle length)	Humans (28 days)	Primates (28–30 days)	Dog (120 days)	Rat (96–120 hrs)
Cycle phases	Proliferative (follicular)	6 to 13	6 to 13	–9 to 9	–12 to 12 ²
	Secretory (luteal)	14 to 28	14 to 28	10 to 56	34 to 88 ²
	Menses	0 to 5	0 to 5	NA	NA
	Estrus	NA	NA	0 to 9	0 to 12 ²
	Metestrus	NA	NA	NA	12 to 33 ²
	Diestrus	NA	NA	10 to 56	34 to 88 ²
	Anestrus	NA	NA	57 to 198	NA
	Proestrus	NA	NA	199 to 208	89 to 100 ²
	Ovulation	14 to 15		2 to 3	6 to 12 ²
	Luteolysis factor	Ovarian PGF _{2α}	Ovarian PGF _{2α}	Uterine PGF _{2α}	Uterine PGF _{2α}
Hormones	Peak estrogen	12 to 13	12 to 13	A, E	P
	Peak progesterone	21 to 24	21 to 24	D	D
Early pregnancy	Receptivity	20 to 24	20 to 24	3 to 6	72 to 120 ²
	Pregnancy recognition	6 to 9	3 to 5	24 to 25	Post coitum several hrs post ovulation
	Pregnancy factor	HCG	HCG	None	PRL
	Conceptus attachment	23 to 26	23 to 26	16 to 18	120 to 144 ²
	Implantation	7 to 9	21 to 23	18 to 20 ⁴	120 to 144 ²
Gestation		9 ³	5.3 to 5.6 ³	2.1 to 2.3 ³	0.7 to 0.8 ³

Note the significant interspecies differences in uterine cyclicity. Such differences may influence uterine functions and pharmacologic modulation of the signaling pathways of MAPKs, c-fos and/or ER^{11,30,31,53,117,118,119}. Note: primates means Old-World monkey of the subfamily Cercopithecinae (macaques, baboons and their phylogenetic kin).

NA=Not applicable; E₂=Estrogen; E=Estrus; A=Anestrus; P=Proestrus; P₄=Progesterone; D=Diestrus; Mths=Months; hCG=Human chorionic gonadotropin; PRL=Prolactin-like hormones.

¹ All value ranges are provided in days, unless otherwise noted.

² Values expressed in hours.

³ Values expressed in months.

⁴ Implantation as such does not occur.

uterus in a state of quiescence or inactivity^{23–35}. P₄ is secreted for only a limited time by the rat, unless a leuteotropic signal from the pituitary is received³⁶. In rats, that signal is prolactin, which is released upon cervical stimulation (copulation)³⁷. With a new release of pre-implantation E₂ from the ovary, P₄ stimulates glandular secretion, endometrial stromal cell proliferation, and myometrial transformation. Furthermore, luminal epithelial cells undergo differentiation while preparing to receive the blastocyst.

Interestingly, in the absence of pre-implantation E₂ in pregnant rats at this time, P₄ maintains the uterus in a neutral phase and the blastocyst in dormancy. Co-activators and repressors of steroid receptors govern appropriate E₂/P₄ synergism³⁸. For example, estrogen receptor (ER) negative uteri are hypoplastic, while P₄ receptor negative uteri are hyperplastic. This stringent regulation is required for appropriate uterine receptivity and embryo implantation in many species^{39,40}.

MAPK signaling pathways are activated during implantation in the rat⁴¹. Uterine receptivity, also known as the window of implantation, is identified by loss of progesterone (*Pgr*) gene expression from the epithelia⁴² and

by expression of a number of extracellular matrix molecules (e.g., secreted phosphoprotein 1 or osteopontin) and integrin heterodimers (e.g. αVβ3) that also initiate p38 MAPK signaling^{40,43}. If no embryo implantation follows, the uterine endometrium, via an oxytocin (OT)-mediated mechanism, produces excess prostaglandin (PG), particularly PGF_{2α}, which is received by the ovary. As a result, P₄ production is decreased by the ovary and luteolysis occurs, marking the end of the D phase.

Human and Nonhuman Primate Menstrual Cycle

There are several benefits to using the NHP, in particular Old-World monkeys, in female reproduction studies^{44,45}. In general, NHP reproductive uterine and ovarian cycles and circulating steroid binding proteins, resemble those in humans, although other aspects (i.e., cycle length, gestation length) are different (Table 1). Our discussion draws upon both human and NHP studies, with species differences noted. For purposes of this review, NHP means Old-World monkey of the subfamily Cercopithecinae (macaques, baboons and their phylogenetic kin).

While the estrous and menstrual cycles have numerous

Fig. 1.

Fig. 2.

similarities in humans and NHPs, a few primary differences or conventions should be noted (Table 1). In contrast to the estrous cycle, which begins and ends at E phase and/or ovulation, the menstrual cycle begins and ends at menses, with ovulation occurring mid-cycle. Also, while the estrous cycle uses follicular (P and E phases) and luteal (M and D phases) phases, the menstrual cycle is most commonly divided into proliferative (follicular) and secretory (luteal) phases, which describes endometrial thickness⁴⁶. Note that the proliferative phase occurs prior to ovulation in the menstrual cycle, although further proliferation does take place at the beginning of the secretory phase. Finally, humans and NHPs slough their endometrium when conception does not occur, whereas in other mammals it is reabsorbed. During the proliferative phase the endometrium begins to thicken and growth of all endometrial cells (endothelial, myometrial and stromal) takes place (Fig. 2). Mitoses are present in the endometrial glandular epithelium during the follicular ("proliferative") phase of the cycle, and in the stroma during the early luteal ("secretory") phase. No evidence of mucus secretion or vacuolation is present during the proliferative phase. In the secretory phase, the endometrium slows its growth, stromal edema is evident, basal sub-nuclear secretory vacuoles are present in the glandular epithelium, and there is secretory exhaustion. Stromal edema is usually present at two times in the menstrual cycle, once in the mid-follicular/proliferative phase and once in the mid-luteal/secretory phase¹¹.

However, the menstrual and estrous cycles have more similarities. During the follicular phase paralleling the P and E phases, follicle stimulating hormone (FSH) and luteinizing hormone (LH) are released from the anterior pituitary under hypothalamic control, leading to ovarian follicular recruitment for ovulation and increased E_2 production. The endometrium thickens and P_4 levels are minimal. The mature ovarian follicle eventually secretes sufficient E_2 to

promote a LH surge, which culminates in ovulation. Growth of all endometrial cells (endothelial, myometrial and stromal) takes place in response to this E_2 stimulation.

P_4 levels increase in the stroma and remain elevated throughout the secretory phase, while levels in the epithelium decrease. The ruptured ovarian follicle develops into the corpus luteum and secretes P_4 and E_2 during the secretory phase. P_4 suppresses E_2 proliferation and causes a shift in proliferative activity to the stromal cells, which triggers epithelial cell differentiation in preparation for implantation.

The changing pattern of E_2 and P_4 secretion during the NHP menstrual cycle is essential for the hormonal regulation of endometrial growth and differentiation, and P_4 action is essential for the proper maturation of the endometrium⁴⁷. The transition from a proliferative (E_2 -dominated) to secretory (P_4 -dominated) endometrium results in the appropriate differentiation that permits implantation⁴⁷. In NHP, P_4 primes the stromal cells to respond to pre-implantation (nidatory) E_2 for decidualization in early pregnancy. Stromal cell proliferation during the menstrual cycle is reported into the early to mid-secretory phase, when the endometrium reaches maximum thickness. Unlike the rat, P_4 levels in humans and NHPs produce glandular secretory activity. A pre-decidualization process, manifested by stromal edema, can be seen, as early as 10 days following the LH surge in humans. The reaction is characterized by eosinophilic and enlarged stromal cells that begin to surround prominent spiral arteries. Over the subsequent 3–4 days, this reaction spreads to the upper two thirds of the endometrium⁴⁸, preparing a suitable environment for embryo attachment, successful implantation, and protection from invasive trophoblasts of the mother. Notably, pre-decidualization is part of the normal human menstrual cycle and the late luteal phase of macaque endometrium show decidual changes^{48,49}.

Fig. 1. Rat estrous cycle and differences in p38, c-fos and ER expression during various stages of the cycle. The average estrous cycle length in rats is 4–5 days. The cycle is subdivided into four phases: proestrus, estrus, metestrus, and diestrus. The proestrus phase begins when progesterone (P_4) levels decline as a result of luteolysis and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion from the anterior pituitary. In the adult female rat, preovulatory estradiol (E_2), which is produced by ovarian follicles, concentration peaks during the proestrus phase. As a result of the pre-ovulatory E_2 surge, ovulation occurs during the estrus phase. In proestrus, endometrial stromal cell proliferation, stromal edema, and proliferation of luminal and glandular epithelium with subsequent increase in endometrial thickness take place. Myometrial hypertrophy also is seen during estrus. In the metestrus and diestrus phases, the luminal lining epithelial cells are reduced in height, the stroma becomes denser, and the uterus becomes quiescent with subsequent decreases in endometrial thickness. Overall, p38 strongly is upregulated during proestrus, estrus, and metestrus phases. c-fos is strongly upregulated in proestrus and metestrus and mildly upregulated in estrus. ER α expression is highest during proestrus and lowest in metestrus^{11,30,31,119}.

Fig. 2. Human menstrual cycle and differences in p38, c-fos and ER expression during various stages of the cycle. The menstrual cycle begins and ends at menses, with ovulation occurring mid-cycle. The menstrual cycle is divided into proliferative (follicular) and secretory (luteal) phases, which describes endometrium thickness. During the follicular phase, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) release from the anterior pituitary under hypothalamic control, takes place and leads to ovarian follicular recruitment for ovulation and increased estradiol (E_2) production. The endometrium thickens and progesterone (P_4) levels are minimal. The mature ovarian follicle eventually secretes sufficient E_2 to promote a LH surge, which culminates in ovulation. Growth of all endometrial cells (endothelium, myometrium and stroma) takes place in response to this E_2 stimulation. During the proliferative phase, the endometrium begins to thicken and growth of all endometrial cells takes place. In the secretory phase, the endometrium slows its growth. Overall, p38, c-fos, and ER α are strongly upregulated during the proliferative phase and lack expression in the secretory phase^{11,53,117}.

Fig. 3. Histological appearance of the rat uterus during proestrus (A), estrus (B), and metestrus (C). Smooth muscle hypertrophy (short arrows), endometrial stromal cell proliferation, stromal edema, and few mitotic figures are present during **proestrus** (A). In **estrus** (B), the uterine lumen is lined by very large, tall columnar epithelium, there is myometrial hypertrophy, and many of the luminal and glandular epithelial cells undergo vacuolar degeneration and necrosis (long arrows). In **metestrus** (C), the luminal lining epithelial cells are reduced in height, there is cytoplasmic vacuolation of luminal epithelial cells (arrowheads), the stroma becomes denser, and mitotic activity decreases.

In primates, the ovarian cycle is uterine independent whereas it is uterine dependent in subprimate mammals. Luteolysis is driven by the withdrawal of the ovarian steroids, E_2 and P_4 , and release of $PGF_{2\alpha}$. $PGF_{2\alpha}$ is released by the uterus in rats and other mammals, and the ovaries in NHPs³⁷. Mediation of this luteolytic event by OT, although controversial, is reported in the NHP and also thought to be important in the rat^{37,50}, but appears unlikely to play a role in human menses^{48,49}. Cytokines, proteases, and PGs also play a role in menses^{51,52}.

Role of p38 MAPK Signaling Throughout the Normal Estrous and Menstrual Cycles and Early Pregnancy

As previously noted, p38 MAPKs are involved in cytokine and growth factor production, secretion and receptor signaling, cell growth and differentiation, and the cell cycle. All of these processes occur during normal uterine tissue cycling; therefore, it should not be surprising that p38 is expressed during both human menstrual and rat estrous cycles (Figs. 4A-F)¹¹.

$PGF_{2\alpha}$ is a luteolytic factor during the normal cycling in large animal species, pseudopregnant rodents, and in the human and NHP menstrual cycle^{30,53,54}. However, there are species differences in their luteolytic dependence on OT and site of $PGF_{2\alpha}$ production (Table 1)³⁷. Evidence for OT involvement in luteolysis at late D phase in ruminants is well established^{37,55}. By coupling to the G protein, OT promotes $PGF_{2\alpha}$ synthesis in a manner dependent on the ovarian steroids and their receptors. There is indirect and somewhat controversial evidence for the role of OT in $PGF_{2\alpha}$ production and luteolysis in the rat^{37,55}. However, OT receptor mRNA levels rise during the D phase in the rat uterus, reaching a maximum during the P phase, which emphasizes the importance of this receptor during the proliferative phase.

The mechanism of OT-induced PG synthesis has been investigated in rat OTR-transfected Chinese Hamster Ovary (CHO) cells⁵⁶. It was found that there are two signaling pathways triggered by the OT-OTR complex⁵⁶. Pathway (a) is G protein and cyclooxygenase (COX)-2-mediated and leads to PG synthesis through ERK2 MAPK, but not p38 MAPK activation. Similarly, $PGF_{2\alpha}$ production in sheep is shown to be ERK1/2-mediated. The parallel pathway (b), regulating free intracellular Ca^{2+} ion concentrations, involves coupling to the G_i protein and activation of p38 MAPK. Notably, a similar OT-OTR triggering event involving Ca^{2+} is reported to take place in the myometrium at the time of parturition in a variety of species. In contrast, regulatory events, especially those involving uterine sensitization to E_2 and $ER\alpha$, are thought to be different than those events seen during cycling⁵⁵. In the rat and human, this OT-mediated parturition event is modulated by both G_q - and G_i -coupling proteins. Thus, although the $PGF_{2\alpha}$ -mediated luteolysis does not involve p38, other events involving G_i -OTR coupling during the estrous cycle likely do involve p38.

Fig. 4. (A) p38 expression in human uterus during the secretory phase. No epithelial p38 expression. (B) p38 expression in nonhuman primate (*Cynomolgus macaque*) uterus during the proliferative phase. Strong glandular epithelial cytoplasmic expression. (C) p38 expression in nonhuman primate (*Cynomolgus macaque*) uterus during the secretory phase. No epithelial expression. (D) p38 expression in rat uterus during proestrus. Strong glandular and luminal epithelial cytoplasmic expression. (E) p38 expression in rat uterus during estrus. Strong glandular and luminal epithelial cytoplasmic expression. Note strong myometrial smooth muscle cells cytoplasmic staining. (F) p38 expression in rat uterus during metestrus. Strong glandular and luminal epithelial cytoplasmic expression. Immunohistochemical stain, original magnification $\times 10$. Radi ZA, Khan NK, *Toxicologic Pathology* (34(4)), pp. 327–335, copyright 2006. Reprinted by Permission of SAGE Publications.

Extensive remodeling of the endometrium during the menstrual phase involves repeated tissue breakdown and regeneration⁷. During this time, leukocytes and endometrial cells secrete cytokines, chemokines, and proteases and also promote proliferation of endometrial cells, reepithelialization, and angiogenesis during reconstruction. These activities are modulated in the endometrium, in part,

by the protease-activated receptor 2 (PAR2) via stimulation of IL-8 secretion in stromal and epithelial cells, which induce stromal cell proliferation and activate metalloprotease 7 in epithelial cells⁷. p38 and other MAPKs have been shown to mediate PAR2 activation and are therefore critical to the regulation of endometrial remodeling⁷. Furthermore, in the late secretory phase of the

human menstrual cycle, as ovarian steroid levels decrease, superoxide dismutase (SOD) catalysis of the conversion of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) decreases, which increases reactive oxygen species (ROS)⁵¹. Increased ROS production activates the nuclear factor kappa B (NF- κ B), an inflammatory response transcription factor, which is thought to regulate COX-2 and subsequent synthesis of $PGF_{2\alpha}$. In rat skeletal muscle, it was shown that p38 inhibition impairs contraction-mediated I κ B kinase phosphorylation, the precursor step to NF- κ B activation⁵⁶. Translationally, it is plausible that p38 plays a role in NF- κ B regulation of $PGF_{2\alpha}$ during luteolysis.

E₂-Induced Endometrial Cell Proliferation and Possible Roles for p38 MAP Modulation: The E₂-ER α complex

Infertility and lack of uterotrophic response has been reported in ER α knockout mice⁵⁷. As previously noted, endometrial cell proliferation in the rat estrous cycle and NHP menstrual cycle is triggered by the release of E₂ from developing ovarian follicles. The ER is a zinc finger-containing transcription factor and member of the nuclear receptor superfamily with two isoforms, α and β , which are differentially expressed in a tissue-dependent manner in the uterus and ovary, respectively⁵⁸. In the uterus, E₂ binds to ER α . ER α is then targeted to the nucleus where it stimulates the growth of normal and transformed endometrial cells of the female reproductive system (Fig. 5). To facilitate this activity, ER α is localized in nuclei of uterine cells in a variety of species^{11,59,60}.

Although ER α predominates over its ER β analog, ER β mRNA and ER β protein are expressed in the nuclei of glandular epithelium in rats, macaques, and humans, suggesting a possible role for this isoform in modulating E₂ action^{61,62}. Furthermore, nuclear receptors are not the only the key mediators in the female reproductive system. Non-genomic membrane ERs, upon E₂ binding, can also induce G-protein activation and MAPK pathways important to cellular processes (Fig. 5)⁶³⁻⁶⁵.

During the proliferative phase of the menstrual cycle, ER α mRNA and protein are expressed in all major uterine cells, including glandular epithelial, stromal, and uterine wall smooth muscle cells (Fig. 6A). Protein concentrations of both receptors decline during the secretory phase⁵⁹. Similarly in the adult rat, ER α expression is lowest during the P and E phases, and consistent with cell proliferation patterns, ER α is only expressed in the glandular and luminal epithelia (Fig. 6B). ER α concentration rises significantly in these cells during the M phase in response to E₂ release^{18,62}.

In the luminal epithelial cells, proliferation continues to rise and although ER α levels drop, ER β in uterine glandular epithelium of the rat is debatable^{18,62}. In NHP uterine sections, strong endometrial stromal cell nuclear ER α staining, moderate to strong glandular epithelial nuclear staining, and mild luminal epithelial and myometrial smooth muscle cell nuclear staining were seen during the

proliferative phase (Fig. 6C)¹¹.

During the secretory phase, mild glandular epithelial nuclear staining, negative myometrial smooth muscle cell and luminal epithelial nuclear staining, and strong endometrial stromal cell staining are observed. The changing pattern of E₂ and P₄ secretion during the NHP menstrual cycle is essential for the hormonal regulation of endometrial growth and differentiation and P action is essential for the proper maturation of the endometrium⁴⁷. The transition from a proliferative (E₂-dominated) to secretory (P₄-dominated) endometrium results in the appropriate differentiation that permits implantation⁴⁷. The numbers of ER in non-human primate endometrium are low when serum P₄ levels are elevated during the secretory phase of the menstrual cycle, but rise two to three fold when P₄ levels decline during the proliferative phase⁶⁶. In humans, extracellular signal-regulated kinase 1 (ERK1) is weakly expressed in glandular cells, but nearly undetectable in stromal cells of endometrial sections. ERK2 exhibited distinct glandular expression in both the proliferative and secretory phases, and a weak stromal cell expression⁶⁷.

The mechanism of E₂ cell growth stimulation in the uterus is mediated through the expression of a series of genes (Fig. 5). Unlike other ligand-dependent receptors (e.g., glucocorticoid receptor), translocation back into the cytosol is not observed upon E₂ binding⁶², although it is interesting to note that other non-classical ligands (e.g., the ER α antagonist, ICI 182,780) can translocate ER α to the cytoplasm via a p38-mediated mechanism⁶⁸.

The ligand binding region of ER α has two transactivating domains, which are thought to act cooperatively. These are E₂-independent activation function (AF1) and E₂-dependent AF2 domain, located in the N- and C-terminal regions, respectively⁶⁹. When E₂ binds to the AF2 region, a conformational change occurs that permits receptor binding to co-activating proteins (and co-repressors) that are necessary for transcription. Some of these co-activators include estrogen receptor-associated protein 160 (ERAP160), a splice variant of the progesterone receptor (PR) co-activator steroid receptor coactivator-1 (SRC-1) and member of the p160/SRC-type steroid receptor co-activators, and CBP/p300, a known co-activator of the nuclear receptor transcription factors cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and activating protein-1 (AP-1)⁷⁰.

The E₂-independent AF1 domain is thought to be responsible for tissue and target specificity of the receptor molecule. AF1 activation, also leading to co-activator recruitment, is accomplished by MAPK phosphorylation. Importantly, direct phosphorylation and activation of ER α by p38 has been shown in human uterine endometrial cancer cells, suggesting that a similar mechanism is expected to play a role in normal uterine cell proliferation⁶⁹. MAPKs have also been shown to phosphorylate ER α ^{71,72} and other nuclear hormone receptors containing AF1 and AF2 domains (e.g., peroxisomal proliferators activated receptor [PPAR- γ])⁷³, thereby modulating their activity.

Fig. 5. E₂-ERα complex mediate cell proliferation via p38-mediated mechanism. ERα nuclear receptors are only one of the key actors in the female reproductive system; non-genomic membrane ERs, upon E₂ binding, can also induce G-protein activation and MAPK pathways important to cell processes such as cellular proliferation.

By binding to estrogen response elements (ERE), the nuclear E₂-ERα transcription complex enhances the expression of genes (i.e., PR, OT, OTR) and protooncogenes (i.e., c-fos, jun [c-jun, jun-B and jun-D] and c-myc)^{18,19}, regulates other non-ERE factors (i.e., epidermal growth factor (EGF), insulin-like growth factor (IGF-1) and their respective receptors, and cyclin D1, a first acting cyclin in cell cycle regulation and intra-cellular sensor of extracellular signals)⁷⁴.

Expression and localization of E₂-ERα, c-fos, c-jun, and c-myc in Rat and Human Uteri

Depending on the tissue type, the nuclear protooncogenes are key players in cell proliferation, differentiation, and tumorigenesis, due in part to the direct effect of E₂ on these genes and their direct regulation by p38 MAPK⁷⁵. E₂-ERα transcription products, c-Fos and c-Jun, are members of the AP-1 transcription factor complex. Because increased expression occurs early in E₂ signaling, these genes are called “immediate early genes”. Notably, and as it will be discussed, there are differences in protooncogene expression between primates and other species during the menstrual cycle.

Following E₂ treatment in mice, c-fos expression is observed only in the glandular and luminal epithelia, signifying a cell specific proliferative role⁷⁶. Mendoza *et al.* further detailed c-fos expression in the rat, reporting a rise and peak in c-fos mRNA in both the glandular and luminal epithelia during the M phase, with a drop in concentration in both cells during the D phase. While c-fos protein concentrations rise during the M phase, concentrations increase only slightly during the D phase¹⁸. The rate of c-fos gene expression parallels that of formation of the active nuclear E₂-ERα complex¹⁹. Studies in p300/CBP-associated factor (PCAF)^{-/-}/PCAF-B^{+/-} knockout mice have pinpointed

Fig. 6. A. ERα expression in human uterus during the proliferative phase. Strong glandular (long arrow) epithelial nuclear expression. Note strong interstitial and moderate smooth muscle cell nuclear ER expression (arrowheads). B. ERα expression in rat uterus during estrus. Moderate glandular and luminal epithelial nuclear expression. C. ERα expression in non-human primate (*Cynomolgus macaque*) uterus during the proliferative phase. Strong endometrial stromal cell nuclear expression and moderate glandular epithelial nuclear staining. Immunohistochemical stain, original magnification $\times 10$. Radi ZA, Khan NK, Toxicologic Pathology (34(4)), pp. 327–335, copyright 2006. Reprinted by Permission of SAGE Publications.

the involvement of E_2 -ER α co-activators p300/CBP and PCAF in mediating c-fos expression in normal uterine cell growth⁷⁶.

P_4 appears to have more effect on c-fos mRNA than on the c-fos protein, a finding also reflected in the mouse⁷⁵. Therefore, it is suggested that c-fos may have a role in the implantation period.

Similarly c-myc mRNA has been shown to rapidly increase in response to E_2 in the rat¹⁹, with more specific distribution details described in the mouse⁷⁷. Following E_2 injection in ovariectomized mice, c-myc protein was detected in uterine luminal and glandular epithelial nuclei during the P phase. P_4 also increased the number of c-myc positive stromal cells. Moreover, c-myc was detected in the nuclei of luminal and glandular epithelial cells during proestrus and on days 1 and 2 of pregnancy. These results suggest c-myc is a potent stimulator of cell proliferation¹⁹.

In further contrast to c-fos and c-myc, c-jun is repressed by the ER in the rat epithelium^{20,21}. c-jun expression was decreased in the epithelium and became evident in the stromal and myometrial cells early after E_2 stimulation in ovariectomized mice⁷⁵. This work suggests that other Jun proteins (i.e., Jun-B, Jun-D) and c-fos may be important in early epithelial cell proliferation in the rodent.

As might be expected from animal model studies, E_2 stimulates c-fos mRNA expression in the human endometrial epithelium and stroma during the proliferative phase of the menstrual cycle and occasionally during the mid-secretory phase^{78,79}. Additionally, ER α -dependent c-fos expression in decidual tissue during pregnancy is very low, paralleling the decline of ER α in these cells and providing further evidence on the relationship between c-fos and ER α .

c-myc is also expressed in the human endometrium during the menstrual cycle. Unlike in the rat, c-jun expression is strongly detected in both the proliferative and secretory phases of the menstrual cycle in humans⁷⁹. Bircan *et al.* showed, using immunohistochemistry, that c-jun expression occurs primarily in the proliferative phase of the menstrual cycle⁸⁰, which was further supported by Hong *et al.* showing a correlation between growth in cultured endometrial stromal cells and activated c-jun expression⁸¹.

AP-1 subunits are dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-jun, Jun-B and Jun-D), Fos (c-fos, Fos-B, Fra-1 and Fra-2), ATF (ATFa, ATF-2 and ATF-3), Maf, and Jun dimerization partner (JDP) subfamilies and recognize either phorbol-myristate-acetate (TPA)-response elements (TREs) or cAMP response elements (CRE) of DNA. Both Fos and Jun can act independently as transcription factors, activating transcription of growth-promoting genes or inhibiting growth-repressing gene transcription⁸².

AP-1 regulation of cell processes (i.e., proliferation, survival, differentiation) is dependent on dimer composition, cell stimulus, cell type, and cell environment. MAPKs, such as p38, contribute to AP-1 regulation by stabilizing the participating proteins through phosphorylation and

regulating their induction^{82,83}. For the latter, p38 has been shown to phosphorylate and activate *cis*-element binding proteins important for c-fos induction, including CREB or ATF (2), which occupy the CRE consensus sequence and ELK-1, a monomeric ternary complex factor (TCF). This TCF is recruited by the serum-response factor (SRF), the protein that recognizes the serum response element (SRE) in response to ultraviolet (UV) irradiation and interleukin-1 (IL-1) stimuli. It is therefore hypothesized that p38 may play a similar role in AP-1 regulation in uterine cell proliferation.

Cell cycle regulation is carried out by cyclins that bind to cyclin-dependent kinases (cdks) or cdk inhibitors to regulate phosphorylation of the retinoblastoma protein (pRB) and cell cycle progression. Studies in the murine system have suggested that a central point of regulation involves E_2 -induced uterine epithelial cell proliferation by nuclear accumulation of Cyclin D1 and pRB activation via a PI3 kinase/AKT/GSK3 β -mediated pathway, which is inhibited by P_4 ⁸⁴. However, these studies also note the need for a parallel pathway to initially trigger DNA synthesis. Correspondingly, the c-jun protein product of E_2 -ER α transcription is known to promote DNA synthesis, or the S phase of the cell cycle, in cultured normal human endometrial glandular cells via binding to the AP-1 sequence, with subsequent activation of Cyclin D1⁸⁵. Many other factors have been shown to play a role in Cyclin D1 regulation in other systems in response to the E_2 -ER α interaction, including CREB, ATF-2, c-fos and pS1 in breast cancer cells. These studies reveal a host of complex signalling pathways that are most likely triggered upon E_2 and P_4 stimulation. Several pathways can involve p38 MAPK activation.

Role of p38 MAPK Signaling in Stromal Cell Differentiation and Proliferation During Normal Menstrual Cycling and Early Pregnancy

The purpose of cell proliferation upon E_2 exposure is to prepare the uterus for embryo implantation where P_4 and its progesterone receptor (PR) are critical. During the primate menstrual cycle, PR levels increase in the stroma during the proliferative phase and remain high during the secretory phase, while levels in the epithelium decrease with increasing P_4 and during the secretory phase. Thus, P_4 suppresses E_2 -modulated proliferation via ER α and causes epithelial cells to differentiate in preparation for implantation. Persistence or overexpression of ER α is associated with implantation failure and other disorders³⁹.

Fazleabas' group has detailed early conception in the non-human primate, describing three phases of uterine receptivity⁸⁶. Phase I of early pregnancy, regulated by E_2 and P_4 , is characterized histologically by the presence of columnar epithelium with microvilli and an increase in stromal cell proliferation. There is a loss of ER α and PR in the luminal and glandular epithelium, among other significant endometrial changes. Loss of receptors for estrogen and progesterone in uterine epithelia is a

prerequisite for implantation and maintenance of pregnancy⁸⁷. Phase II is induced by blastocyst signaling and is characterized by endometrial changes not observed in the absence of the blastocyst. In Phase III, blastocyst attachment and implantation occurs. Glandular hypertrophy, increased permeability of subepithelial capillaries, and stromal cell decidualization are initiated by the attachment reaction and are accomplished, in part, by expressed extracellular matrix proteins.

Pinipode formation characterizes the receptive endometrium or "implantation window"; and implantation occurs during the luteal phase (approximately day 21) of the menstrual cycle (Table 1)⁸⁸. In NHPs, in response to embryo signaling, P_4 primes the stromal cells to respond to preimplantation (nidatory) E_2 for decidualization in early pregnancy. It has been proposed that NHPs, especially macaques, may be a valuable experimental animal model to test the functional role of progesterone-regulated genes on endometrial receptivity⁸⁹. A study on isolated normal and endometriotic stromal cells showed normal expression of p38, ERK, and JNK MAPKs, and an increase in p38 MAPK phosphorylation rate, when stimulated with IL-1 β ⁹². It has been demonstrated in an endometriosis murine model that a p38 inhibitor, FR 167653, suppresses the development of endometriosis⁹⁰. This finding suggests an important regulatory role for p38 in endometrial cell growth. Indeed, a modulatory role for p38 in human endometrial stromal cell differentiation and proliferation in the uterus has been reported⁸¹. Growth factors from uterine stromal cells regulate the uterine epithelia, and many of them (e.g., fibroblast growth factors-7 and -10, hepatocyte growth factor) signal via p38 MAPK⁸⁷.

Another potential role for p38 may be in the regulation of the p53 protein, a known substrate for p38 evidenced to be involved in decidualization of the endometrial stroma critical for embryo implantation². The p53 protein is massively upregulated and sustained during cAMP-induced decidualization of cultured human endometrial stromal cells, and expressed *in vivo* in the stroma during the late secretory phase of the cycle⁶.

Successful implantation requires complete stromal cell decidualization. As described in human tissue, fibroblast-like mesenchymal cells differentiate into polygonal decidual cells that express new proteins such as the insulin-like growth factor binding protein-1 (IGFBP-1) and prolactin⁹¹. Species differ in embryonic signaling for the onset of decidualization (Table 1).

Type I and/or type II interferons (IFNs) are important in establishing uterine receptivity to implantation in mammals⁸⁷. In ruminants, the pregnancy recognition signal, interferon tau (IFNtau), a type I interferon, prevents luteolysis by inhibiting the expression of ER α and subsequently OTR⁹². It has been shown in bovine endometrial cells that IFNtau induces the activation of p38, implicating a role for this MAPK in establishing and maintaining pregnancy⁹³.

In the NHP, the release of chorionic gonadotropin (CG)

rescues the corpus luteum and begins preparing the uterus for implantation. CG binds to its receptor in the primate endometrial epithelial cells and has been shown to induce phosphorylation of the ERK1/2 MAPKs, leading to expression of COX-2 mRNA and PGE₂ production⁸⁶. CG along with the appropriate P_4/E_2 ratio, only initiates the process; decidualization is completed through an inflammatory-like response with the release of numerous cytokines.

An important and well-studied cytokine, expressed throughout the menstrual cycle, is Interleukin-1 (IL-1 α or IL-1 β). The expression of its receptor, IL-1 receptor type I, is low during the proliferative phase, moderate during ovulation and implantation, and peaks at the end of the cycle⁹⁴. Along with macrophages and uterine epithelial cells, trophoblast release IL-1, continuing, in the case of humans, or beginning, in the case of NHP, decidualization of stromal cells in early pregnancy⁹¹.

IL-1 β is a major secretory product of the conceptus and establishment of pregnancy in pigs⁹⁵. Following IL-1 β stimulation, a COX-2 pathway leads to PGE₂ synthesis, and is mediated by the p38 MAPK pathway. The IL-1-p38 mechanism noted above may be similar in other species, since both PGE₂ and PGF_{2 α} are known to induce decidualization in other hormonally-primed species, including the mouse and rat^{96,97}.

PGE₂ is known to play a role in endometrial vascular permeability, one of the first responses to blastocyst implantation. In the rat, vascular permeability, in response to PGE₂, occurs prior to stromal cell decidualization⁹⁸. Cyclooxygenases have a pathophysiologic role in various systems in the body⁹⁹. In the murine model, COX-2 was shown to be important during ovulation, fertilization, implantation, and decidualization¹⁰⁰. p38 MAPK has been shown to be crucial to COX-2 expression and the nuclear hormone receptor PPAR δ ²⁵. Expression of COX-2 in the human endometrium by Prokineticin 1 (PROK1), a recently described protein that can modulate the inflammatory process, is dependent on activation of the Gq-phospholipase C-beta-cSrc-epidermal growth factor receptor-MAPK/ERK kinase pathway¹⁰¹. Blocking the COX-2 pathway by inhibiting p38 MAPK blunts expression of PPAR δ and decreases the decidualization reaction. Interestingly, downstream from p38 activation are the ATF, CREB, and C/EBP factors; known co-activators of the ER α and cis elements of c-fos, and may be a link between E_2 and COX-2 activity. It has been shown that E_2 is involved in the induction of COX-2 activity during the D phase, as well as the P and E phases of the estrous cycle in rats¹⁰².

In the rat, PGs, including PGI₂ (prostacyclin), PGF_{2 α} , and PGE₂, play a significant role in the decidualization response^{96,103}. PGI₂ is thought to be a key player, but PGE₂ has been shown to act on four different G protein receptors. Both COX-1 and COX-2 are found in the epithelial cells of the endometrium and smooth muscle cells in the circular layer of the myometrium; expression of COX-2 in the latter cells increases with IL-1 β treatment¹⁰⁴. Further, COX-1 and

COX-2 proteins are increased from the non-pregnant D phase stage to day 18 of gestation, supporting the role of COX-2 in decidualization during early pregnancy. As noted in mice, p38 MAPK most likely mediates the decidualization process.

IGFBP-1 is a major secretory product in NHP decidualized endometrium. It plays a role in trophoblast invasion as it can stimulate (in the presence of cAMP) or inhibit (in the absence of cAMP) decidualization^{48,105}. In the rat, it has also been shown to be associated with implantation, modulating the proliferation of uterine cells and their production of PGI₂ during the peri-implantation period¹⁰⁶. In the baboon, it is known that both p38 and NF- κ B are activated during decidualization, followed by COX-2 and MMP-3 gene expression, which leads to extracellular matrix degradation, disruption of the cytoskeleton, and ultimately, IGFBP-1 production¹⁰⁷. A similar MAPK mechanism may be acting in rats.

Roles of p38 MAPKs Modulation During Pregnancy and Parturition

P₄ maintains the myometrium of the uterus in a state of quiescence during pregnancy by: 1) inhibiting the expression of contraction associated proteins (CAPs), which include connexin 43 and OTR¹⁰⁸ and 2) controlling PGF_{2 α} , in part, via modulation of PG dehydrogenase and COX activity¹⁰⁹. These possible roles for p38 have been previously discussed.

IL- β contributes to parturition, by stimulating the production of PGF_{2 α} via a COX-2-mediated mechanism. As in the normal cycle, PGF_{2 α} induces contraction and luteolysis. Takanami-Ohnishi *et al.* have shown, using human decidual stromal cells, that this process is mediated by p38 kinase²². Both COX-1 and COX-2 are differentially expressed in the rat uterus, specifically in the epithelial and myometrial cells, during the estrous cycle, increasing dramatically during parturition and pregnancy¹⁰⁴. Markedly up-regulated p38 MAPK activity has also been demonstrated in the uterus of term-pregnant non-laboring and spontaneously laboring women.

Role of p38 MAPKs in Female Reproductive Pathology

A variety of intracellular signals, including those involving ER α , c-fos, and p38 α , orchestrate physiological events in the uterus during the secretory and proliferative phases of the estrous cycle in humans and NHPs. It is suggested that p38 has a modulatory role on human endometrial stromal cell proliferation and differentiation⁸¹. It is known that MAPKs play a role in regulating cellular hypertrophy and hyperplasia via mechanical stretch of the uterus¹⁵. Therefore, p38 inhibitors could play a role in various uterine and cervical proliferative conditions and cancers, including the pathophysiology of endometriosis^{24,110}, endometrial, mammary, and ovarian cancers^{111–113}, leiomyoma, and uterine fibroids^{114,115}.

MAPKs are known to regulate COX-2 expression and therefore, cervical cancers¹¹⁶.

Conclusions

Toxicologists and toxicologic pathologists need to consider species differences when evaluating the effects of new pharmacologic agents on the female reproductive system. Experiments conducted on different preclinical species are not strictly comparable to that of humans because of significant interspecies differences in the physiology of uterine cyclicity. p38 MAPK signal transduction pathways are interconnected with ER, c-fos, c-jun and jun kinase, and regulate various aspects of the mammalian estrous and menstrual cycles, early pregnancy, and parturition. p38 is involved in: 1) the OT-OTR-mediated events and luteolysis, 2) regulation of uterine tissue breakdown and regeneration, 3) E₂-induced uterine cell proliferation and growth, 4) implantation and uterine receptivity, and 5) maintenance of uterine quiescence during pregnancy and onset of parturition. Finally, p38 MAPK signaling plays a role in various pathological conditions in the female reproductive tract.

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