Analysis of spatial and temporal expression patterns of bone morphogenetic protein family members in the rat uterus over the estrous cycle

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Abstract

Recent studies have demonstrated that bone morphogenetic proteins (BMPs) play fundamental roles in female fertility. This is particularly evident in terms of the ovary. One major question that is just beginning to be addressed is the role of BMPs in the non-pregnant uterus. To help fill this gap, we used *in situ* hybridization to investigate the expression of BMP family members in the rat uterus over the estrous cycle. We found that the endometrial/uterine cycle is accompanied by the expression of several components of the BMP pathway – including ligands, receptors and antagonists. The mRNAs encoding BMP receptors are expressed in the epithelial (BMP-RIA, -RIB and -RII), periluminal stroma (BMP-RIA and -RII) and smooth muscle cells (BMP-RIA and -RII). The expression of all three receptors showed clear cyclic variations. The mRNAs encoding BMP ligands were highly ex-

pressed in the periluminal stroma (BMP-2 and -7) and glandular epithelium (BMP-7). The expression of BMP-2, but not BMP-7, was cyclical. Notably, the periluminal stroma expressed noggin mRNA. In the blood vascular system, BMP-4, -6 and -RII mRNAs were expressed in myometrial endothelial cells. Interestingly, follistatin, noggin, and BMP-4, -6 and -7 mRNAs were expressed in eosinophilic leukocytes, suggesting unexpected roles for eosinophil-derived BMPs in uterine function. We conclude that BMP ligands, receptors and antagonists are expressed in spatially and temporally restricted patterns that are consistent with a physiological role for these regulatory molecules in promoting uterine cellular processes including cell proliferation, differentiation and apoptosis during the cycle.

Journal of Endocrinology (2004) **182**, 203–217

Introduction

Understanding the mechanisms regulating the endometrial cycle is an area of intense research because of its importance to normal fertility and reproductive function. During the menstrual and estrous cycles, the uterus undergoes cyclical changes that involve precise coordination of cell proliferation, differentiation and apoptosis in the endometrium and myometrium. The ovarian hormones, estrogen (Lubahn *et al.* 1993, Couse & Korach 1999) and progesterone (Lydon *et al.* 1995, Graham & Clarke 1997, Conneely *et al.* 2002, Rider 2002) play critical roles in coordinating uterine cellular processes during the cycle. In a broad sense, estrogen stimulates cell proliferation in the epithelial and stromal tissues of the endometrium during the proliferative phase while progesterone promotes glandular differentiation as well as inhibits both cell proliferation and myometrial contractile activity (Graham & Clarke 1997). The primary purpose of the tissue remodeling in the non-pregnant uterus is to create an environment suitable for blastocyst implantation.

The functional responses to these steroid hormones are mediated by specific progesterone and estrogen receptor (PR and ER respectively) signaling pathways in uterine target cells (Graham & Clarke 1997, Couse & Korach 1999). In normal cycling rodents (Katsuda *et al.* 1999, Pelletier *et al.* 2000, Wang *et al.* 2000, Andersson *et al.* 2001, Mendoza-Rodríguez *et al.* 2003) and humans (Mertens *et al.* 2001, Punyadeera *et al.* 2003), ER α is the dominant subtype expressed in the uterus, being localized to the epithelium, stroma, myometrium and some blood vessels. By comparison, ER β is more weakly expressed. Studies in knockout models indicate that ER α , but not ER β , is responsible for mediating estrogen action in the uterus (Couse & Korach 1999). PR-A and PR-B are co-expressed in target cells of the rodent (Ohta *et al.* 1993, Hodges *et al.* 2002) and humans (Mote *et al.* 1999, Punyadeera *et al.* 2003, Arnett-Mansfield *et al.* 2004) being expressed in the epithelium, stroma and myometrium. PR-A is the predominant isoform in the mammalian uterus. Knockout studies indicate that PR-A, but not PR-B, is essential for eliciting progesterone-dependent responses in the uterus (Conneely *et al.* 2002).

It has become increasingly clear that estrogen and progesterone do not operate in isolation, but rather function together with growth factors to control the uterine cycle. The most intensely studied and best understood growth factors are the insulin-like growth factors (Giudice *et al.* 1998, Wathes *et al.* 1998), transforming growth factor- β (TGF- β ; Godkin & Dore 1998, Ingman & Robertson 2002), TGF- α (Nelson *et al.* 1992), epidermal growth factor (Curtis *et al.* 1996, 1999), and the inhibin/activin/follistatin proteins (Jones *et al.* 2002). The conclusion emerging from these investigations implicates locally produced growth factors as critical mediators of steroid-hormone-dependent responses in the uterus (Pollard 1990, Cooke *et al.* 1997, Kurita *et al.* 2000).

With respect to the TGF- β superfamily, considerable attention has been paid to the bone morphogenetic protein (BMP) family in regulating mammalian reproduction (Shimasaki *et al.* 2004). This attention is particularly intense in the ovary where BMP ligands (Dong *et al.* 1996, Galloway *et al.* 2000, Yan *et al.* 2001), BMP receptors (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001, Yi *et al.* 2001) and BMP-binding proteins (Guo *et al.* 1998) have been shown to be essential for normal folliculogenesis and female fertility. BMPs are the largest family of growth factors in the TGF- β superfamily. The BMP ligands exert a wide range of physiological functions in the target cells (Hogan 1996, Wozney 1998, 2002, Botchkarev 2003, Canalis *et al.* 2003, Shimasaki *et al.* 2004) through binding to specific cell surface type I (BMP-RIA/ALK-3 and BMP-RIB/ALK-6) and type II (BMP-RII) serine/threonine kinase receptors (Balemans & Van Hul 2002, Shimasaki *et al.* 2004). Upon binding of the BMP ligand the type II receptor transphosphorylates the type I receptor, which in turn evokes BMP signaling through Smad-dependent pathways (Von Bubnoff & Cho 2001, Shimasaki *et al.* 2004). In vertebrates a number of extracellular antagonists of BMPs have been identified that include noggin, chordin, follistatin, follistatin-related gene, ventropin, twisted gastrulation and the differential screening-selected gene aberrative in neuroblastoma (DAN) family members such as DAN, cerberus and gremlin (Balemans & Van Hul 2002, Canalis *et al.* 2003). These BMP antagonists can prevent BMP signaling by binding BMPs, thus precluding their binding to specific cell surface receptors.

Although much is known about the expression of BMPs in the ovary (Erickson & Shimasaki 2003), the question of the role for BMPs in uterine physiology is just beginning to be addressed. To date, most of the experimental investigations of uterine BMPs have used pregnant rodents. The general principle to emerge from this work is that the early stages of implantation and decidualization are characterized by the spatiotemporal pattern of expression of the mRNAs encoding several members of the BMP family. These include BMP-2 in the decidualizing stromal cells (Ying & Zhao 2000,

Paria *et al.* 2001), BMP-4 and Smad1 in vascular endothelial cells (Ying & Zhao 2000), BMP-7 in the endothelium and subjacent stroma (Ozkaynak *et al.* 1997, Paria *et al.* 2001), BMP-8a in decidual cells (Zhao & Hogan 1996), BMP-RIB in the endometrium and endometrial glands (Yi *et al.* 2000), follistatin in decidual tissue (Kaiser *et al.* 1990, Mercado *et al.* 1993) and noggin and DAN in the stromal layer immediately underlying the luminal endothelium (Paria *et al.* 2001). This evidence has suggested that BMPs in the uterus have a potential role in pregnancy.

By comparison to the pregnant uterus, the expression and cellular consequences of the BMP family in the non-pregnant mammalian uterus remain largely unexplored. The purpose of this study is to fill this basic gap in our knowledge by analyzing the spatiotemporal pattern of expression of BMP ligands, receptors and binding proteins in the rat uterus over the estrous cycle.

Materials and Methods

Animals

Adult, female Sprague-Dawley rats (2–3 months of age) were housed under controlled lighting conditions (14 h light:10 h darkness; lights on from 0500 to 1900 h) at 23 °C; they were provided with formulated rat chow and water and were allowed to feed *ad libitum*. Vaginal smears were taken daily, and rats exhibiting two or more consecutive 4-day estrous cycles were used. The animals (two at each time of the cycle) were killed on proestrus (P1000 h and P2000 h: follicular phase), estrus (E0200 h and E1000 h: ovulatory phase), diestrus I (DI, 1100 h: luteal phase, luteinization) and diestrus II (DII, 1100 h: luteal phase, luteolysis). The rats were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the University of California, San Diego Animal Subjects Committee.

In situ hybridization

In situ hybridization was performed as described previously (Erickson & Shimasaki 2003). Sections (10 μ m) were hybridized with 35 S-labeled RNA probe. After hybridization the slides were dehydrated in graded ethanol concentrations, immersed in Kodak NTB-2 photographic emulsion, air dried and exposed at 4 °C for 4 weeks. The slides were developed, fixed and stained with hematoxylin (H) or hematoxylin and eosin (H&E). The probes were prepared by RT-PCR using total RNA from adult rat ovaries. The primers were derived from the cDNA clones at the following nucleotide numbers: 511–528 and 1066–1083 (accession number of the cDNA clone is M22489) for BMP-2 (Wozney *et al.* 1988); 878–897 and 1292–1311

Figure 1 Photomicrographs of sections of the non-pregnant adult rat uterus stained with H&E. (A) Longitudinal section showing myometrium (M), endometrium (E), and uterine cavity (UC) at E0200 h ($\times 4$). (B) Higher magnification of E in panel A; section through the endometrium showing the histology ($\times 10$). (C) Higher power magnification of M in panel A; section through the myometrium showing the histology ($\times 20$). (D) Section showing uterine eosinophils at E1000 h ($\times 40$).

(accession number D49494) for BMP-3b (Takao *et al.* 1996); 737–757 and 1181–1200 (accession number Z22607) for BMP-4 (Chen *et al.* 1993); 6–25 and 475–494 (accession number U66298) for BMP-6; 497–514 and 865–882 (accession number X56906) for BMP-7 (Ozkaynak *et al.* 1991); 441–460 and 876–895 (accession number D38082) for BMP-RIA (Takeda *et al.* 1994);

528–547 and 965–984 (accession number U89326) for BMP-RIB; 525–544 and 895–904 (accession number AF003942) for BMP-RII (Beppu *et al.* 1997); 94–113 and 404–423 (accession number U31203) for noggin (Valenzuela *et al.* 1995). These primers were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome

Table 1 The adult non-pregnant rat uterus expresses mRNAs encoding the BMP family (ligands, receptors and binding proteins)

	BMP ligands						BMP receptors			BMP antagonists	
	2	3b	4	6	7	15	RIA	RIB	RII	Follistatin	Noggin
Tissue											
Endometrium											
Luminal epithelium	—		—	—	—	—	+	+	+	—	—
Glandular epithelium	—	—	—	—	+	—	+	+	+	—	—
Periluminal stroma	+	—	—	—	+	—	+	—	+	—	+
Myometrium											
Circular muscle	—	—	—	—	—	—	+	—	+	—	—
Longitudinal muscle	—	—	—	—	—	—	+	—	+	—	—
Vascular system											
Endothelial cells	—	—	+	+	—	—	—	—	+	—	—
Smooth muscle cells	—	—	—	—	—	—	—	—	—	—	—
White blood cells	—	—	+	+	+	—	—	—	—	+	+

DNA contaminants. All PCR products were cloned into pBluescript SK+ and their DNA sequences confirmed. The probe for follistatin was made from the rat follistatin cDNA clone as described previously (Shimasaki *et al.* 1989).

Signal intensity

The *in situ* hybridization experiments were performed twice for each BMP ligand, receptor and binding protein. The intensity of the hybridization signals was determined as previously reported (Erickson & Shimasaki 2003). A subjective comparison of eight tissue sections per rat hybridized with the same concentrations of sense and antisense cRNAs and exposed for the same time (4 weeks) was made. The hybridization signal was estimated on a scale of 1+ to 3+ as originally described by Meunier *et al.* (1988): +, silver grains sparse, but positive hybridization; ++, silver grains are numerous but do not cover the cell type in question; +++, silver grains are very numerous and begin to merge in some places; +/-, there was heterogeneity in the hybridization signal, e.g. some of the histological units contained a signal, others did not; —, no detectable hybridization signal. In each experiment, tissues hybridized with the sense probe were used as the negative control. As a positive control, the specific hybridization of the antisense probes for BMP-2, -3b, -4, -6, -7, -15, -RIA, -RIB, -RII, and follistatin was confirmed in adjacent ovary tissue in the same uterine tissue sections as described previously (Erickson & Shimasaki 2003).

Results

Histology of the adult rat uterus

Figure 1 shows the histology of the normal adult rat uterus at estrus after staining with H&E. It consists of an outer layer of smooth muscle cells, the myometrium, and an inner layer of glandular mucosa, the endometrium

(Fig. 1A). The endometrium consists of two epithelial cell types, the luminal epithelium and glandular epithelium (Fig. 1B). The luminal epithelium invaginates to form numerous tubular uterine glands that extend down into a relatively thick endometrial stroma (Fig. 1B). Juxtaposed to the luminal epithelium is a mass of densely packed cells termed the periluminal stroma or compact zone (Fig. 1B). It is rich in capillaries and undergoes growth and differentiation to form the decidual tissue of the maternal placenta (Abrahamsohn & Zorn 1993). The stroma at the myometrium–endometrium junction, termed the basal zone, is characterized by the presence of uterine blood vessels (Fig. 1B). The myometrium consists of outer longitudinal and inner circular smooth muscle with a layer of loose connective tissue and blood vessels between (Fig. 1C). As with the basal zone (Fig. 1B), large numbers of eosinophilic leukocytes with typical ring-shaped nuclei (Tchernitchin 1973, Ross & Klebanoff 1966) are commonly seen within the myometrium, particularly in the circular muscle layer (Fig. 1C and D).

Expression of BMP family in uterine cells

As seen in Table 1, the mRNAs encoding the BMP ligands (BMP-2, -4, -6 and -7), BMP receptors (BMP-RIA, -RIB and -RII) and BMP antagonists (follistatin and noggin) were expressed in the rat uterus in a tissue-specific manner. In general, BMP-2, -4, -6, -7, BMP-RIB and follistatin mRNAs were particularly abundant, whereas the messages for BMP-RIA, RII and noggin appeared in lower amounts. No hybridization signals above background were detected for BMP-3b and -15 in the uterine specimens studied.

BMP-2 mRNA expression

BMP-2 mRNA is strongly expressed in the non-pregnant uterus, and over the cycle has a periluminal stroma-

restricted expression pattern (Fig. 2A, B and C, and Table 1). The hybridization signal for BMP-2 showed a clear cyclic variation, being strong at diestrus II (DII1100 h, +++) and proestrus (P1000 h, +++; P2000 h, +++), decreased at estrus (E0200 h, ++; E1000 h, +), and undetectable at diestrus DI (DI1100 h, -).

BMP-4 mRNA expression

BMP-4 mRNA was expressed in blood vessels and eosinophilic leukocytes (Fig. 2D to H and Table 1). In blood vessels, BMP-4 message was localized to the endothelial cells of large veins in the myometrium (Fig. 2F). BMP-4-positive eosinophils were particularly abundant in the connective tissues near the endometrial-myometrial junction or basal zone (Fig. 2D, E, G and H). A positive BMP-4 hybridization signal is seen at all stages of the cycle, but appeared strongest at estrus (E0200 h, ++; E1000 h, ++).

BMP-6 mRNA expression

The message encoding BMP-6 was expressed in the blood vessels (Table 1). It appeared restricted to the endothelial cells of small arteries located between the circular and longitudinal muscle layers of the myometrium (Fig. 3A, B and C). The BMP-6 labeling intensity (++++) of the endothelial cells appeared unchanged over the estrous cycle. Eosinophilic leukocytes showed a positive hybridization signal for BMP-6 on estrus (E0200 h and E1000 h), but the signal appeared weak and variable (+/-).

BMP-7 mRNA expression

A strong hybridization signal for BMP-7 was observed in all uterine samples examined (Fig. 3D to H and Table 1). In the endometrium, BMP-7 mRNA was restricted to the periluminal stroma and glandular, epithelial cells (Fig. 3F, G and H). There was no detectable change in the levels of BMP-7 expression (+++) in the periluminal stroma and glandular epithelium over the estrous cycle. Eosinophilic leukocytes located within the basal zone appeared to display a weak signal (+) for BMP-7 at estrus.

BMP-RIA mRNA expression

BMP-RIA mRNA appeared to be broadly expressed in the uterus (Table 1). In the myometrium, the signal was localized to the circular and longitudinal smooth muscle cells (Fig. 4A to D). In the endometrium, BMP-RIA was expressed in both the luminal and glandular epithelial cells (Fig. 4C, D and E). A weak hybridization signal for BMP-RIA appeared in the periluminal stroma (Fig. 4E). The levels of BMP-RIA mRNA expression appeared to change during the cycle, being abundant on proestrus (P1000 h, ++; P2000 h, ++), low on estrus (E0200 h,

+/-; E1000 h, +/-) and diestrus I (+/-), and abundant on diestrus II (+++).

BMP-RIB mRNA expression

BMP-RIB message was strongly expressed, being restricted to the luminal and glandular epithelial cells (Fig. 4F, G and H, and Table 1). The level of expression of BMP-RIB showed a dynamic cyclic variation: it was highly expressed at diestrus I (+++) and diestrus II (+++), but appeared very low or undetectable at proestrus and estrus.

BMP-RII mRNA expression

As with BMP-RIA, the message encoding BMP-RII appeared widely expressed throughout the uterus (Table 1). In the endometrium, the luminal and glandular epithelial cells exhibited a moderate hybridization signal (Fig. 4I, J and K), with a weaker signal appearing in the periluminal stroma (Fig. 4K). In the myometrium, the circular and longitudinal smooth muscle cells showed a positive, albeit weak, hybridization signal (Fig. 4I and J), as did some vascular endothelial cells. The expression of BMP-RII mRNA appeared cyclical; the signal was moderate at proestrus (P1000 h, ++; P2000 h, ++) and very low or undetectable at other stages of the cycle.

Noggin mRNA expression

Noggin mRNA was detected in the periluminal stroma (Fig. 5A, B and C, and Table 1). Noggin mRNA in the periluminal stroma was detected only at diestrus II 1100 h. The eosinophils at estrus also appeared to show a positive, albeit weak, hybridization signal for noggin (Table 1).

Follistatin mRNA expression

In a previous study using solution hybridization-RNAase protein assay, Mercado *et al.* (1993) found high levels of follistatin mRNA in the non-pregnant rat uterus at estrus; however, the cellular site of expression was not identified. Here, we find that follistatin mRNA expression was restricted to the eosinophilic leukocytes, located in the endometrium and the interstitial tissue of the myometrium (Fig. 5D, E and F, and Table 1). Over the cycle a positive hybridization signal for follistatin was evident at estrus (E0200 h, +++; E1000 h, ++). Because follistatin mRNA was detected only in eosinophils, the abundance of follistatin message in the uterus appears tightly correlated with the number of eosinophils present in the uterine stroma during the cycle.

Discussion

This study shows that the endometrial cycle in the non-pregnant rat uterus involves a precise spatial and

Figure 2 *In situ* hybridization of BMP-2 and -4 mRNAs in the non-pregnant rat uterus. Dark-field (B, E and H) and bright-field (A, D and G) micrographs of the same sections of uterus. M, myometrium; E, endometrium. Magnifications are: panels A, B, D and E, $\times 4$; G and H, $\times 20$; C and F, $\times 40$. Panels A to E stained with hematoxylin; panels F, G and H stained with H&E. Using dark-field and bright-field optics, white and black dots respectively represent silver grains. (A, B and C) Note BMP-2 hybridization in the periluminal stroma of the endometrium; D11100 h. (D to H) Note BMP-4 hybridization signal in endothelial cells and eosinophilic leukocytes; E0200 h.

Figure 3 *In situ* hybridization of BMP-6 and -7 mRNAs in non-pregnant rat uterus. Dark-field (B, E and G) and bright-field (A, D and F) micrographs of the same sections of uterus. Magnifications: panels A, B, D and E, $\times 4$; panels F and G, $\times 20$; panels C and H $\times 40$. Panels A to G stained with H&E; panel H stained with hematoxylin. (A, B and C) Note BMP-6 (boxed in, panel B) is selectively expressed in endothelial cells of small arteries in the myometrium; E0200 h. (D to H) Note BMP-7 hybridization signal in periluminal stroma (PS) and glandular epithelial cells (GE); P2000 h.

Figure 4 *In situ* hybridization of BMP receptor mRNAs in the non-pregnant uterus. Dark-field (B, D, G and J) and bright-field (A, C, F and I) micrographs of same sections of uterus (M, myometrium; E, endometrium). Magnifications: panels A, B, E, G, I and J, $\times 4$; panels C and D, $\times 10$; panels E, H and K, $\times 40$. Panels A, B, C, D, E, I and J stained with H&E; panels F, G, H and K stained with hematoxylin. (A to E) Note the expression of BMP-RIA in the myometrium (circular and longitudinal muscle) and the endometrium (periluminal stroma and epithelial cells; P2000 h. (F, G and H) Note the selective expression of BMP-RIB in endometrial luminal and glandular epithelium; DII1100 h. (I, J and K) Note BMP-RII hybridization in endometrial luminal and glandular epithelium and weaker hybridization in myometrium and periluminal stroma; P2000 h.

Figure 5 *In situ* hybridization of *noggin* and *follistatin* mRNAs in the adult rat uterus. Dark-field (B and E) and bright-field (A and D) micrographs ($\times 4$) of the same uterus sections (M, myometrium; E, endometrium). All sections stained with H&E. Higher magnification bright-field micrographs: panel C, $\times 40$; F, $\times 100$. (A, B and C) Note *noggin* signal in the periluminal stroma; D11100 h. (D, E and F) Note *follistatin* hybridization to eosinophilic leukocytes; E0200 h.

temporal pattern of expression of the genes encoding BMP receptors, BMP ligands and BMP antagonists. Thus, the regulated expression of BMPs might add another mechanism by which cell-specific modulation can occur during the uterine/endometrial cycle. The data are as yet too preliminary to know what cellular effects the BMPs might regulate. However, inasmuch as BMPs have important functions in controlling proliferation, differentiation and apoptosis in other reproductive tissues (Shimasaki *et al.* 2004), it is not unreasonable to assume that uterine BMP actions could play a role in the mechanisms governing tissue remodeling during the endometrial cycle. It is important to acknowledge that the interpretation of our

studies are limited by the fact that thus far, there is no evidence that the BMP mRNAs are translated into functional proteins. In this context, it should be kept in mind that: (1) translation may take time and therefore drawing conclusions about the stages of the cycle at which BMPs act cannot be assumed from the mRNA levels alone; (2) the sites of action of ligands may differ from sites of synthesis.

Our results indicate that the genes encoding cell surface BMP receptors are expressed in the non-pregnant uterus. The mRNAs encoding BMP-RIA, -RIB and -RII are found in the epithelium and BMP-RIA and -RII are found in the periluminal stroma and myometrium. These

observations suggest that both the endometrium and myometrium are targets for BMP signaling. The further observation that the levels of BMP receptor expression appear to change during the cycle raises the possibility that the control of receptor gene expression may play a role in governing the level of BMP signaling in uterine target cells. In support of this possibility, deletion of *BMP-RII* in mice severely disrupts uterine structure such that the endometrial lining is thin, the epithelial cells remain undifferentiated and there are few if any uterine glands (Yi *et al.* 2001). This study provides clear support for a causal role of BMP-RII in uterine gland morphogenesis. Our studies demonstrate that BMP-RII mRNA is expressed exclusively in the luminal and glandular epithelial cells, and only during the secretory (luteal) phase of the cycle, i.e. at diestrus I and II. Collectively, these lines of evidence argue for a model in which the expression of BMP-RII in the epithelial cells at diestrus I and II plays a causative role in promoting the growth and development of uterine glands. It will be important to determine the mechanism by which BMP-RII promotes uterine gland development. In this light, it is notable that the luminal and glandular epithelial cells show maximal mitosis at diestrus (Bertalanffy & Lau 1963, Burroughs *et al.* 2000, Mendoza-Rodríguez *et al.* 2003). Thus, the concept that the regulated expression of BMP-RII might be involved in controlling epithelial cell proliferation seems to be a good working hypothesis. In this hypothesis, it will be interesting to consider whether the intense expression of PR in the epithelial cells during diestrus (Ohta *et al.* 1993) has a role in the regulation of epithelial BMP-RII gene expression.

During the cycle, we found that the messages encoding BMP-2 and -7 are highly expressed in the endometrium of the non-pregnant rat uterus. That is, BMP-2 and -7 are expressed in the periluminal stroma and BMP-7 in the glandular epithelium. Therefore, any model of BMP receptor signaling in the uterus must take into account the regulation of BMP-2 and -7 expression in the endometrium. Classic mesenchymal-epithelial BMP pathways have been shown to direct a variety of developmental phenomena (Hogan 1996, 1999). Our observation that the periluminal stroma expresses BMP-2 and -7 and the epithelium expresses BMP-RIA, -RII and -RII, fits with a classic mesenchymal-epithelial BMP pathway. In this BMP-2/BMP-7 model, Cooke *et al.* (1997) made the interesting finding that estrogen-stimulated mitosis in the uterine epithelium is a paracrine event mediated by ER α in the periluminal stroma. Further studies are required to test the intriguing hypothesis that stroma-derived BMP-2 and/or -7 may be paracrine factors that mediate estrogen-dependent epithelial mitosis in the mammalian uterus. It is known that secretions by the uterine glands are required for uterine receptivity and implantation in rodents (Gray *et al.* 2001). Hence, the question: Could endometrial-derived BMP-2 and -7 play a role in this important

process? Finally, it is notable that the periluminal stroma in the non-pregnant rat uterus expresses noggin message at DII. This finding confirms and extends studies in pregnant mice showing noggin expression in endometrial stroma cells during implantation (Paria *et al.* 2001). Noggin binds several BMPs (including BMP-2 and -7) with very high (picomolar) affinities, and by binding tightly to BMPs, noggin prevents BMPs from interacting with their receptors (Zimmerman *et al.* 1996). We suggest, therefore, that if noggin is secreted from the periluminal stroma at DII it could antagonize BMP-2 and -7 signaling.

Interestingly, the level of BMP-2 mRNA expression undergoes dynamic changes over the estrous cycle. That is, BMP-2 mRNA is expressed at relatively high levels throughout the cycle, except at E1000 h and DI when the message appears to be very low or undetectable. This result could be interpreted to mean that uterine BMP-2 gene expression (like that of BMP-RII) is tightly regulated and thus of physiological importance. An important question concerns the nature of the regulation of BMP-2 expression in the endometrium and its physiological function. In the case of regulation, it is noteworthy that the time at which BMP-2 mRNA is expressed at low levels coincides with luteinization (Butcher *et al.* 1974). Thus, it is possible that progesterone secreted by the corpus luteum of the cycle could act to inhibit BMP-2 expression in the periluminal stroma. Consistent with this idea is the expression of PR in the periluminal stroma (Ohta *et al.* 1993). In the case of function, it is notable that DII is characterized by the highest rates of apoptosis in the endometrial epithelial cells (Spornitz *et al.* 1994, Burroughs *et al.* 2000, Mendoza-Rodríguez *et al.* 2003). This, coupled with the ability of BMP-2 to cause apoptosis in target cells (Macias *et al.* 1997, Song *et al.* 1998, Kimura *et al.* 2000, Hay *et al.* 2001, Kawamura *et al.* 2002), raises the possibility that the temporal pattern of BMP-2 expression could have a role in the regulation of epithelial apoptosis/proliferation during the cycle.

In contrast to BMP-2, we found no evidence that the levels of endometrial BMP-7 mRNA change over the cycle. Thus, the concept that the periluminal stroma and glandular epithelial cells constitutively express high levels of BMP-7 during the endometrial cycle is supported by our results. In a previous paper, Ozkaynak *et al.* (1997) reported that administration of exogenous estradiol markedly inhibits uterine BMP-7 transcripts *in vivo*. Similarly, studies on the chick oviduct have demonstrated an inhibitory effect of estrogen on BMP-7 gene expression (Monroe *et al.* 2000). Therefore, our finding that BMP-7 expression was not suppressed by increases in circulating estradiol levels during the estrous cycle was unexpected. The reason for this apparent discrepancy is not known; however, it may involve the pituitary. In Ozkaynak's study, normal non-pregnant adult mice (with pituitary) were injected for 4 days with increasing doses of estradiol. In the oviduct experiments intact chickens were implanted with

Figure 6 Schematic model showing the existence of a BMP system in the non-pregnant rat uterus, complete with ligands, receptors and antagonists. In the endometrium (E), endogenously produced BMP-2 and -7 could act as ligands for the BMP receptors (BMP-RIA, -RIB and -RII) expressed in the periluminal stroma and epithelial cells. Such BMP signaling pathways may directly influence physiological responses during the endometrial cycle by autocrine and paracrine mechanisms (arrows). In the myometrium, BMPs produced by vascular endothelial cells and eosinophils may participate directly in the control of smooth muscle and blood vessel activities by receptor-mediated signaling. Presumably, eosinophil-derived noggin and follistatin could be modulators of BMP-receptor-dependent responses.

diethylstilbestrol (DES) pellets for at least 2 weeks. While it is clear the exogenous estrogen will directly stimulate cellular responses in the uterus and oviducts, it will also initiate responses in other target tissues, including the pituitary. Consistent with a role of the pituitary, we have observed that hypophysectomy results in the loss of BMP-7 message in the rat uterus (Erickson, unpublished observations). This evidence suggests that the estrogen-induced down-regulation of uterine BMP-7 message may be a secondary response to estrogen-induced changes in pituitary hormones, such as elevated prolactin secretion. That prolactin receptors are expressed in the endometrium (Bole-Feysot *et al.* 1998) could be regarded as general support for this pituitary/prolactin hypothesis.

The concept of tissue-specific expression of BMPs in the uterine vasculature is supported by our findings that BMP-4, -6 and -RII mRNAs appear to be constitutively expressed in the vascular endothelial cells. This concept is further supported by the expression of BMP-4 and Smad1 in vascular endothelial cells of the pregnant mouse uterus (Ying & Zhao 2000). Two of the most striking features of the vascular BMPs in the non-pregnant uterus are that:

(1) they appear restricted to blood vessels in the myometrium; (2) BMP-4 and -6 show very different patterns of expression, being located in endothelial cells of veins and arteries respectively. Consequently, to understand the potential for cellular functions of the BMPs in the uterine vasculature, one must consider this spatial pattern of BMP-4 and -6 expression within the myometrial endothelial cells. In this concept, it should be pointed out that estrogen plays a major role in remodeling the vasculature of the rodent uterus (Clark *et al.* 1977, Couse & Korach 1999, Celia & Osol 2002, Page *et al.* 2002), perhaps by activating ER α expressed in the endothelial cells (Andersson *et al.* 2001). The degree to which the endothelial-derived BMPs might contribute to the estrogen-dependent structural remodeling of the uterine vasculature remains to be determined. It is noteworthy that BMP-6 knockout mice have normal sized litters (Solloway *et al.* 1998). This result could imply that BMP-6 expressed by endothelial cells of small myometrial arteries is not essential for uterine vascular remodeling in the rat.

Regarding blood cells, our study indicates that uterine eosinophils express BMP antagonists (follistatin and noggin

mRNAs) and BMP ligands (BMP- 4, -6 and -7 mRNAs) at estrus. An interesting question is whether the expression of BMPs in eosinophils is applicable to uterine homeostasis. It has been known for many years that the uterine cycle involves the recruitment and infiltration of large numbers of circulating eosinophils into the perivascular uterine stroma at estrus (Rytomaa 1960). During estrus, the uterine eosinophils undergo lysis and release their contents into the interstitial spaces (Ross & Klebanoff 1966, Kelenyi & Nemeth 1972). This process is associated with marked uterine edema. The importance of estrogen in provoking eosinophil chemotaxis into the uterus is clear (Tchernitchin *et al.* 1974); however, the cellular mechanisms and the functions of this uterine phenomenon remain poorly understood. Two molecules required for estrogen-dependent chemotaxis of eosinophils into the uterine stroma are interleukin-5 (IL-5; Perez *et al.* 1996) and eotaxin (Gouon-Evans & Pollard 2001). In IL-5- and eotaxin-deficient females, the virtual absence of eosinophils has little or no effect on structure-function relationships in the uterus. Indeed, in eotaxin-deficient females, the estrous cycles and reproductive functions are normal (Gouon-Evans & Pollard 2001). Thus, the increase in eosinophils at estrus does not appear to play a critical role in establishing uterine homeostasis. Nonetheless BMP-2 (Kim *et al.* 1999, Willette *et al.* 1999), BMP-4 (Cunningham *et al.* 1992, Miyazaki *et al.* 2003) and BMP-7 (Gould *et al.* 2002, Lee *et al.* 2003) have been linked to chemotaxis in a variety of cell types (including blood cells), leaving open the question of whether BMPs (in addition to IL-5 and eotaxin), might also play a role in eosinophil infiltration into the uterus. By *in situ* hybridization, we detected BMP-RIA and -RII in the myometrium. It will be interesting to learn whether eosinophil-derived BMPs might affect the circular and smooth muscle cells, perhaps contributing to the known activation of apoptosis in these cells at estrus (Burroughs *et al.* 2000).

In conclusion, the general principle to emerge from this work (Fig. 6) is that the normal endometrial/uterine cycle is accompanied by the spatiotemporal pattern of expression of at least nine BMP genes, which fit into three functional classes: ligands (BMP-2, -4, -6 and -7), receptors (BMP-RIA, -IB and -II) and antagonists (follistatin and noggin). Given the fact that these BMPs can control mitosis, differentiation and apoptosis, the findings presented here may have new implications for understanding uterine growth, glandular morphogenesis, vascular remodeling and cell fate specification. Further studies should build on our finding by exploring both the regulation of tissue-specific BMP gene expression in the uterus and the functional consequences of their expression. It should be apparent that understanding BMP responses in the uterus could have potential relevance in the area of infertility and pathology, including endometriosis, leiomyomas and endometrial cancer.

Acknowledgements

We thank Andi Hartgrove for preparing the manuscript and for her work on the Figures.

Funding

This work was supported by NIH U54 HD12303 as part of the Specialized Cooperative Centers Program in Reproduction Research.

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Received 30 April 2004

Accepted 11 May 2004