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Postnatal uterine development in Inverdale ewe lambs

Kanako Hayashi, Anne R O'Connell[1], Jennifer L Juengel[2], Ken P McNatty[3], George H Davis[1], Fuller W Bazer and Thomas E Spencer

Department of Animal Science, Center for Animal Biotechnology and Genomics, Texas A and M University, 442 Kleberg Center, 2471 TAMU, College Station, Texas 77843-2471, USA, [1]AgResearch, Invermay Agricultural Centre, Private Bag, Mosgiel, New Zealand, [2]AgResearch, Wallaceville Animal Research Centre, PO Box 40063, Upper Hutt, New Zealand and [3]School of Biological Sciences, Victoria University, PO Box 600, Wellington, New Zealand

Correspondence should be addressed to K Hayashi; Email: kanako@tamu.edu

K Hayashi and A R O'Connell contributed equally to this work

Abstract

Postnatal development of the uterus involves, particularly, development of uterine glands. Studies with ovariectomized ewe lambs demonstrated a role for ovaries in uterine growth and endometrial gland development between postnatal days (PNDs) 14 and 56. The uterotrophic ovarian factor(s) is presumably derived from the large numbers of growing follicles in the neonatal ovary present after PND 14. The Inverdale gene mutation (*FecXI*) results in an increased ovulation rate in heterozygous ewes; however, homozygous ewes (II) are infertile and have 'streak' ovaries that lack normal developing of preantral and antral follicles. Uteri were obtained on PND 56 to determine whether postnatal uterine development differs between wild-type (++) and II Inverdale ewes. When compared with wild-type ewes, uterine weight of II ewes was 52% lower, and uterine horn length tended to be shorter, resulting in a 68% reduction in uterine weight:length ratio in II ewes. Histomorphometrical analyses determined that endometria and myometria of II ewes were thinner and intercaruncular endometrium contained 38% fewer endometrial glands. Concentrations of estradiol in the neonatal ewes were low and not different between ++ and II ewes, but II ewes had lower concentrations of testosterone and inhibin-α between PNDs 14 and 56. Receptors for androgen and activin were detected in the neonatal uteri of both ++ and II ewes. These results support the concept that developing preantral and/or antral follicles of the ovary secrete uterotrophic factors, perhaps testosterone or inhibin-α, that acts in an endocrine manner to stimulate uterine growth and endometrial gland development in the neonatal ewes.

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Introduction

Uterine development is initiated in the fetus; however, it is not completed until after birth in domestic animals, laboratory rodents, and humans (Bartol *et al.* 1999, Spencer *et al.* 2005*a*). A major developmental event in the neonatal uterus is the differentiation and development of the endometrial glands or adenogenesis (Gray *et al.* 2001*b*, Spencer *et al.* 2005*b*). Alteration or ablation of the endometrial glands and/or their secretory products compromises blastocyst survival and implantation (embryo/fetus and associated extra-embryonic membranes) in the mouse, rat, and sheep (Stewart *et al.* 1992, Bartol *et al.* 1995, Zhu *et al.* 1998, Gray *et al.* 2001*c*, 2002). In humans, the secretory products of the endometrial glands appear to be a primary source of nutrition for conceptus growth during the first trimester of pregnancy (Burton *et al.* 2002).

In sheep, uterine adenogenesis after birth involves budding and differentiation of the endometrial glandular epithelium (GE) from the luminal epithelium (LE), specification and development of the intercaruncular endometrial stroma, development of endometrial folds, and, to a lesser extent, growth of endometrial caruncles and myometrium (Wiley *et al.* 1987, Taylor *et al.* 2000). Endometrial adenogenesis begins between postnatal days (PNDs) 1 and 7 when shallow epithelial invaginations appear along the LE in presumptive intercaruncular areas. Between PNDs 7 and 14, the nascent GE buds proliferate into the stroma and form tubules or ducts that begin to coil and branch by PND 21 as they proliferate through the stroma toward the inner circular layer of the myometrium. By PND 56, uterine morphogenesis is essentially complete, as the aglandular caruncular and glandular intercaruncular endometrial areas are histoarchitecturally similar to that of the adult uterus. The caruncular areas of endometrium, which are the sites of superficial implantation and placentation for the formation of placentomes, do not contain glands. Uterine adenogenesis is a critical developmental event in ewes, because inappropriate exposure to progestins or estrogens from birth to PND 56

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permanently ablates endometrial gland development, resulting in a uterine gland knockout (UGKO) phenotype in the adult (Bartol *et al*. 1999, Gray *et al*. 2001*a*, Carpenter *et al*. 2003*b*, Hayashi *et al*. 2004). The adult UGKO ewes are infertile and exhibit recurrent early pregnancy losses (Gray *et al*. 2001*b*, 2002). Therefore, it is important to understand the mechanisms regulating postnatal growth and differentiation of the uterus.

Postnatal ovine uterine development is complex and involves the endocrine actions of pituitary-derived prolactin, as well as a number of autocrine and paracrine actions of the uterine-derived growth factor systems including the fibroblast growth factors, hepatocyte growth factor, insulin-like growth factors (IGFs), and WNTs (Taylor *et al*. 2000, 2001, Carpenter *et al*. 2003*b*, Hayashi *et al*. 2005, Hayashi & Spencer 2006). Experiments with ovariectomized ewe lambs established that the ovary also influences uterine growth and endometrial gland morphogenesis after PND 14 (Carpenter *et al*. 2003*a*). The neonatal ovine ovary contains both the preantral and antral ovarian follicles at birth, which decline on PND 14, increase to maximum numbers on PND 28, remain high from PNDs 42 to 56, and decline thereafter (Kennedy *et al*. 1974, Tassell *et al*. 1978). These changes in the ovarian follicular dynamics are temporally correlated with the ontogeny of endometrial gland development in the neonatal ewe (Wiley *et al*. 1987, Taylor *et al*. 2001). Estrogen is not likely to be the uterotrophic factor from the ovary, because the circulating levels of 17β-estradiol are essentially below the detectable limits after birth (Carpenter *et al*. 2003*b*). The uterotrophic ovarian factor presumably emanates from the large number of developing follicles in the ovary of the neonatal ewe. Indeed, in sheep, the activin–follistatin system has been implicated in ovarian regulation of neonatal uterine development and differentiation (Carpenter *et al*. 2003*a*, Hayashi *et al*. 2003).

The Inverdale ewe is a valuable model for investigating uterine development in the absence of factors that would normally be derived from developing ovarian follicles. The Inverdale high fecundity gene (*FecXI*), a major gene for prolificacy in sheep, was first identified in the descendants of a Romney ewe which consistently produced large litters that resulted from a dysfunctional mutation in bone morphogenetic protein 15 (*BMP15*) gene (Galloway *et al*. 2000). Ewes heterozygous for the *FecXI* gene (I+) have an average of one more ovulation during each estrous cycle than the wild-type ewes (++) and 0.6 more lambs per ewe lambing (Davis *et al*. 1991, Shackell *et al*. 1993). In the wild-type ewes, the first wave of ovarian follicle growth occurs in the fetus between days 100 and 120 of gestation (Juengel *et al*. 2002). However, homozygous (II) ewes are infertile and incapable of normal ovarian follicular development beyond the primary (type 2) stage (Braw-Tal *et al*. 1993*b*, Smith *et al*. 1997), resulting in a 'streak' ovary phenotype (Davis *et al*. 1992, Galloway *et al*. 2002). In the adult II ewes, the circulating levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are high and comparable with those for ovariectomized wild-type (++) ewes, because the II ewes have very low circulating levels of 17β-estradiol and inhibin (Shackell *et al*. 1993). The mean ovarian volume in adult II ewes is about 77% less than that for the I+ or ++ genotypes (Smith *et al*. 1997). Moreover, uteri of 1.5-year-old II ewes are much smaller than those for ++ ewes (Davis *et al*. 1992). However, the circulating levels of FSH and LH are not different between the ++ and I+ genotypes in either fetal or adult ewes. Furthermore, no differences in the ovarian secretion rates of inhibin, estradiol, and progesterone have been reported between the ++ and I+ genotypes (Shackell *et al*. 1993), suggesting that both ++ and I+ Inverdale ewes have similar levels of ovarian follicular activity (Smith *et al*. 1997). Thus, the effects of ovarian factors in I+ and ++ ewe lambs would be similar. Therefore, available evidence supports the working hypothesis that uterine growth and differentiation is compromised in homozygous Inverdale ewes due to the lack of uterotrophic ovarian factors.

The objective of this study was to test the hypothesis that the Inverdale gene mutation influences postnatal development of the uterus. The results from this study strongly support the hypothesis that a uterotrophic factor(s) from the developing ovarian follicles of the neonatal ewe acts in an endocrine manner to regulate postnatal growth and differentiation of the uterus.

Results

Morphology and morphometry of neonatal ovary and uterus

Ovary

The combined weight of the ovaries from II ewes was 87% lower ($P<0.002$) than for ++ ewes on PND 56 (Table 1). As expected, the ovaries from II ewes were devoid of the antral follicles (data not shown).

Uterus

Uteri from II ewes weighed 48% less ($P<0.03$) than those from ++ ewes (Table 1). Uterine length for II ewes tended ($P<0.07$) to be shorter, and the weight:length ratio of the uteri was 68% less ($P<0.04$) for II ewes.

Uterine histoarchitecture

Examples of differences in uterine histology are presented in Fig. 1, and the results of morphometric analyses are summarized in Table 1. When compared with the wild-type, the thickness of caruncular and intercaruncular areas of uterine endometrium was reduced by 16% ($P<0.03$) and 34% ($P<0.0001$) respectively in II ewes.

Table 1 Morphometric analyses of uteri from wild type (++) and homozygous Inverdale (II) ewe lambs.

Measurement	++	II	S.E.M.	*P* value
Ovarian weight (g)	0.89	0.12	0.07	0.002
Uterine weight (g)	5.88	3.09	0.64	0.03
Uterine length (cm)	7.17	5.75	0.40	0.07
Uterine weight:length ratio	0.80	0.54	0.06	0.04
Caruncular endometrial thickness (μm)	874	735	43	0.03
Intercaruncular endometrial thickness (μm)	363	242	11	<0.0001
Gland invaginations per section	50.4	50.8	2.5	0.9
Gland number per section	737.8	458.5	32.7	<0.0001
Gland density (stratum compactum)	2.45	2.88	0.13	0.02
Gland density (stratum spongiosum)	9.18	8.50	0.26	0.07
Myometrial thickness (μm)	398	303	16	<0.0001

Myometrial thickness was reduced ($P<0.0001$) by 24% in the uteri of II ewes. The intercaruncular endometria of ++ ewes contained numerous coiled and slightly branched glands extending radially from the LE through the stroma to the inner circular layer of myometrium (Fig. 1). The intercaruncular endometria of II ewes also contained glands, but they were fewer in number (Fig. 1). Although the number of gland invaginations from the uterine lumen was not different ($P>0.10$) between ++ and II ewes, the total number of endometrial glands in the uterus was reduced ($P<0.0001$) by 38% in II ewes. Gland density was 18% greater ($P<0.02$) in the upper stratum compactum endometrium of II ewes, whereas gland density in the lower stratum spongiosum tended ($P<0.07$) to be lower for the uteri of II ewes, suggesting a reduction in coiling and branching morphogenetic growth of the endometrial glands.

Circulating levels of estradiol, testosterone, and inhibin in the Inverdale ewe

The concentrations of 17β-estradiol, testosterone, and inhibin-α in plasma from ++ and II ewes between PNDs 0 and 56 are presented in Fig. 2.

17β-Estradiol

The circulating concentrations of 17β-estradiol were affected by day ($P<0.0001$), but not genotype or their interaction ($P>0.10$). Overall, the concentrations of 17β-estradiol were the highest on PND 0 (3.92 ± 0.26 pg/ml) and declined to low levels (0.6–0.7 pg/ml) thereafter (fifth-order effect of day, $P<0.02$, $r^2=0.61$).

Testosterone

The concentrations of testosterone in serum were affected by day and genotype (day×genotype, $P<0.002$). As illustrated in Fig. 2B, the concentrations of testosterone were low in ++ ewes on PND 0 (0.34 ± 0.08 ng/ml) and remained relatively unchanged on PND 56 (0.24 ng/ml; quadratic effect of day, $P<0.05$, $r^2=0.12$). By contrast, the concentrations of testosterone in serum were high in II ewes on PND 0 ($0.90+0.09$ ng/ml), declined on PND 14 (0.12 ng/ml), and remained low thereafter (cubic effect of day, $P<0.01$, $r^2=0.48$).

Inhibin-α

The circulating concentrations of inhibin-α were affected by day ($P<0.0001$) and genotype ($P<0.0001$), but not by their interaction. Overall, the concentrations of inhibin were about threefold higher ($P<0.0001$) in ++ (40.5 ± 2.2 ng/ml) compared with II (16.6 ± 2.2 ng/ml) ewes. The circulating levels of inhibin declined from

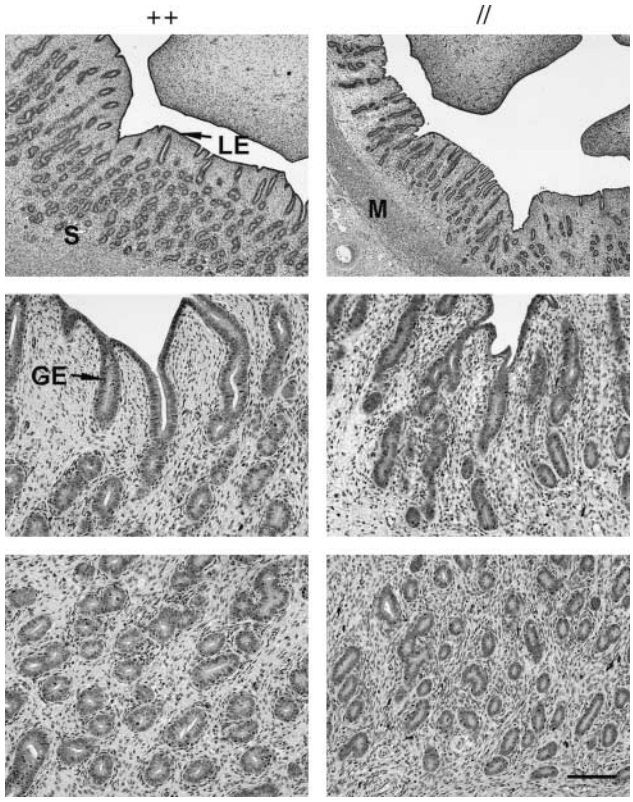

Figure 1 Representative photomicrographs illustrating the histoarchitecture of uteri from wild-type (++) and homozygous (II) for *FecX*^{I} Inverdale ewes on postnatal day 56. LE, luminal epithelium; GE, glandular epithelium; S, stroma; M, myometrium. Scale bar=100 μm.

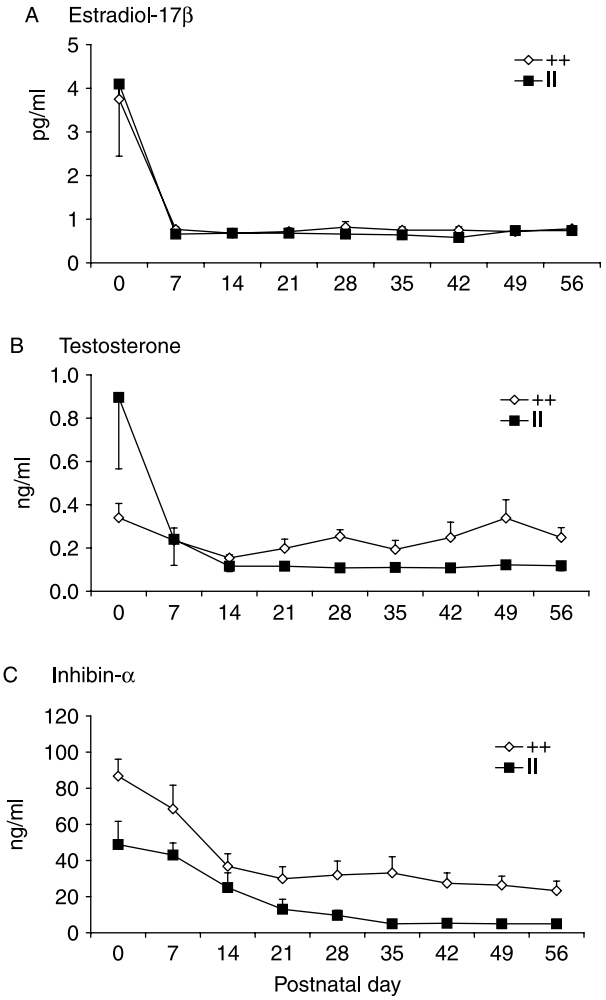

Figure 2 Concentrations of 17β-estradiol (A), testosterone (B), and inhibin-α (C) in the serum from blood samples collected between postnatal days 0 and 56 from wild-type (++) and homozygous Inverdale (II) ewes.

PNDs 0 to 56 in both ++ (cubic effect of day, $P<0.05$, $r^2=0.55$) and II ewes (quadratic effect of day, $P<0.001$, $r^2=0.63$).

Androgen receptor and activin receptors in the neonatal uteri of Inverdale ewe

Immunohistochemical analyses were performed to localize androgen receptor and activin receptor proteins in the uteri from ++ and II ewe lambs in Fig. 3. Overall, the abundance and pattern of androgen receptor, ActRIA, IB, and II proteins was not different in the uteri of ++ and II ewes.

Androgen receptor

Immunoreactive androgen receptor protein was detected in all uterine cell types and was most abundant in the endometrial LE and GE in both ++ and II ewes.

ActRIA and IB

ActRIA and IB proteins were detected in all cell types and were most abundant in endometrial LE and GE in both ++ and II ewes.

ActRII

In the uteri of both ++ and II ewes, ActRII protein was most abundant in endometrial LE, GE, and stroma. The antibody used for immunohistochemistry detects both ActRIIA and IIB (R&D systems, Minneapolis, MN, USA).

Discussion

The results of this study strongly support the hypothesis that a uterotrophic factor emanating from the developing ovarian follicles regulates postnatal uterine growth and endometrial differentiation in neonatal ewes. Ovariectomy of the neonatal ewe at birth did not affect the initial genesis and development of endometrial glands on PND 14 (Bartol *et al*. 1988). However, ovariectomy of ewe lambs on PND 7 resulted in a 50% reduction in uterine wet weight by PND 56 (Carpenter *et al*. 2003*a*). Furthermore, ovariectomy of ewes on PND 7 results in reduced growth of the endometrium and myometrium, as well as the total number of uterine endometrial glands on PND 56 (Carpenter *et al*. 2003*a*). In the present study, II ewes mirrored the effects of neonatal ovariectomy on PND 7, because II ewes, on PND 56, had uteri with reduced weight, growth of the endometrium and myometrium, and also fewer endometrial glands. In addition, II ewes, on PND 56, had reduced uterine growth including reduced endometrial and myometrial thickness. In the neonatal ewes, treatment with either estrogen or progestins from birth or during critical developmental periods ablates endometrial adenogenesis, reduces uterine growth, and decreases endometrial and myometrial thickness (Gray *et al*. 2000, Carpenter *et al*. 2003*b*, Hayashi *et al*. 2004). By contrast, treatment of neonatal ewes with prolactin from birth increases endometrial gland number and intercaruncular endometrial and myometrial thickness (Carpenter *et al*. 2003*c*). In fact, steroid disruption of uterine growth and endometrial adenogenesis in the neonatal ewe involves alterations in several growth factor systems including the IGFs, fibroblast growth factors, hepatocyte growth factor, WNTs, activin, and follistatin (Gray *et al*. 2000, Carpenter *et al*. 2003*b*, Hayashi *et al*. 2004, 2005, Hayashi & Spencer 2005, 2006). Thus, endometrial and myometrial growth in ewes with the II genotype may result from changes in epithelial–mesenchymal interactions due to alterations in intrinsic growth factor pathways that decrease the development of endometrial glands.

In the present study, the uteri of II ewes had fewer endometrial glands, but no differences in the number of

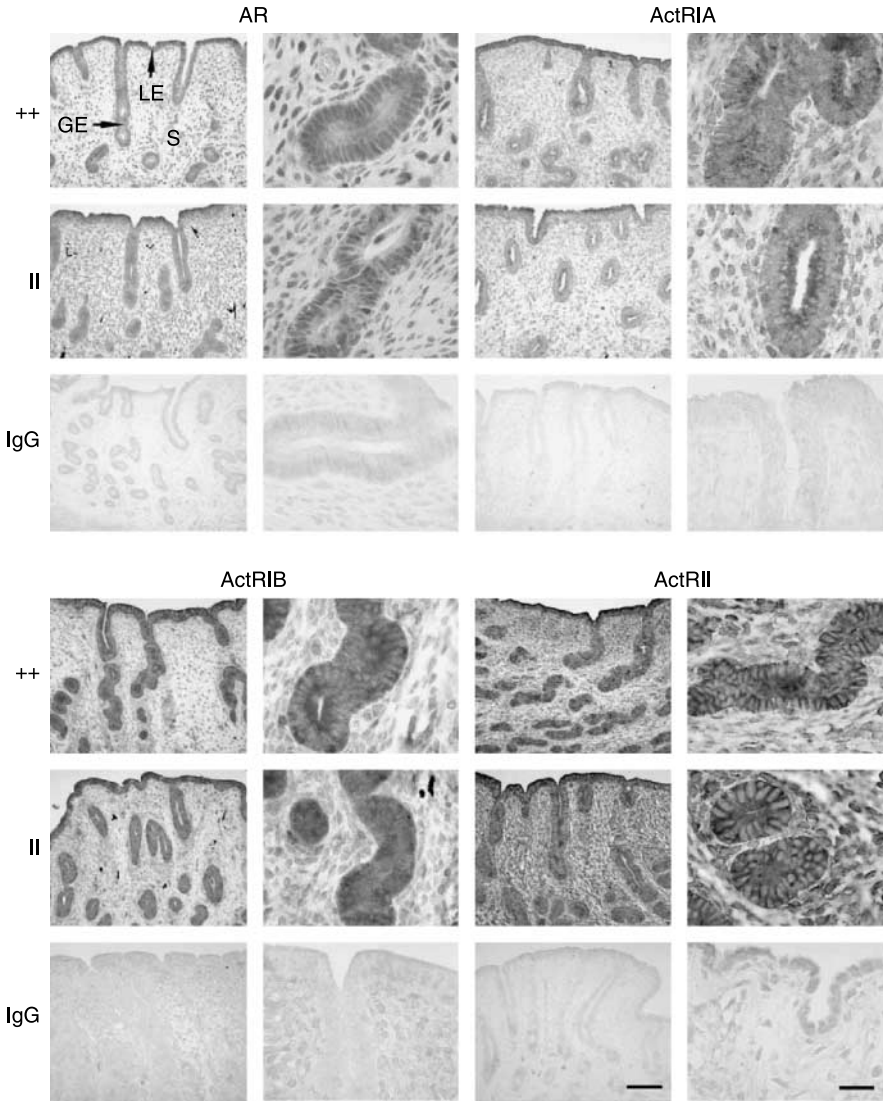

Figure 3 Immunohistochemical localization of androgen receptor (AR) and activin receptors (ActRIA, IB and II) in the uteri of ++ and II ewes. GE, glandular epithelium; LE, luminal epithelium; S, stroma. The scale bar indicates 100 μm at low magnification and 25 μm at high magnification.

endometrial gland invaginations from the LE were detected. Initial genesis and development of endometrial glands between birth and PND 14 is not dependent on the ovary (Bartol *et al.* 1988) and ovariectomy does not affect the numbers of endometrial gland invaginations from the uterine lumen (Carpenter *et al.* 2003*a*). Therefore, budding differentiation of the endometrial glands from the LE must be regulated by local factors within the uterus. However, the results of the present study indicated that total number and density of endometrial glands in the stratum spongiosum stroma are reduced in uteri from II ewes, and this was also reported for ewes ovariectomized on PND 7 (Carpenter *et al.* 2003*a*). Therefore, an ovarian-derived factor regulates, in part, coiling and branching morphogenesis of endometrial gland development that occurs between PNDs 14 and 56 in the neonatal ewe.

Previous studies led to the hypothesis that the uterotrophic ovarian factor(s) was from the large number of developing follicles in the neonatal ovary (Carpenter *et al.* 2003*a*, Hayashi *et al.* 2003). The numbers of ovarian preantral and antral follicles in ewe lambs at birth (455 and 935 per ovary respectively) increase on PND 28 (683 and 1100 per ovary) and then decline on PND 84 (100 and 287 per ovary; Kennedy *et al.* 1974). These changes in the ovarian follicular dynamics are temporally correlated with rapid growth and development of the uterus and specifically the uterine glands in the neonatal ewe (Taylor *et al.* 2000). In II ewes, follicular development does not continue beyond the primary stage, which results in 'streak' ovaries without preantral (i.e., types 3 and 4 follicles) and antral follicles (Braw-Tal *et al.* 1993*b*, Smith *et al.* 1997). Thus, available results strongly support the hypothesis that the uterotrophic ovarian factor(s) is from the preantral and/or antral follicles in the neonatal ovine ovary.

The nature of the uterotrophic ovarian factor(s) is not known, but available evidence indicates that the factor is not estrogen. Although numerous follicles are present in the neonatal ovine ovary, they do not synthesize or secrete appreciable amounts of estrogens between birth and PND 56 (Kennedy *et al*. 1974, Carpenter *et al*. 2003*b*). The developing preantral and antral follicles of the neonatal ovine ovary lack aromatase (K Hayashi, unpublished results), and ovariectomy on PND 7 did not affect circulating concentrations of estrogen between PNDs 7 and 56 (Carpenter *et al*. 2003*a*). In the present study, the circulating levels of 17β-estradiol were not different between ++ and II ewes. High concentrations of 17β-estradiol on PND 0 most likely reflect secretion by the maternal placenta during the periparturient period. Furthermore, administration of a non-steroidal aromatase inhibitor, CGS 20 267, from birth to PND 56 did not affect uterine growth or endometrial adenogenesis in the neonatal ewes (Carpenter *et al*. 2003*b*). Thus, there is no evidence to support a role for estrogens as regulators of uterine development in ewe lambs between PNDs 14 and 56.

A potential uterotrophic factor from ovarian follicles identified in the present study is testosterone. In the adult ovary, testosterone is synthesized by thecal cells of ovarian follicles (Shemesh & Hansel 1976). Testosterone levels were higher in II ewes at birth and then lower than those for ++ ewes after PND 14, suggesting that testosterone, presumably from ovarian follicles, is affecting uterine growth and development. Indeed, the lack of conversion of testosterone to estrogen due to the absence of aromatase enzyme activity in the developing ovarian follicles may result in higher levels of circulating testosterone in the neonatal ewes. Androgen receptors are present in the uteri of a number of species including pigs, mice, and primates (Slayden *et al*. 2001, Pope & Cardenas 2006). Similarly, the present study found that androgen receptor protein was present in all cell types in the neonatal ovine uterus, although no differences in abundance were detected between uteri of ++ and II ewes. Treatment of ovariectomized rats with testosterone restored uterine weight and increased IGF-I mRNA (Sahlin *et al*. 1994). Indeed, the intrinsic IGF-I system has been implicated in epithelial–stromal regulation of uterine growth and endometrial adenogenesis in the neonatal ewes (Taylor *et al*. 2001, Hayashi *et al*. 2004, 2005). Thus, further consideration of testosterone as an ovarian uterotrophic hormone in the neonatal ewe seems warranted.

Another potential ovarian-derived uterotrophic factor identified in the present study is inhibin-α. Inhibins and activins are the members of the transforming growth factor-β (TGF-β) superfamily, which regulate growth and differentiation of many branched epitheliomesenchymal organs, including mammary and prostate glands via autocrine, paracrine, and perhaps, endocrine mechanisms (Ritvos *et al*. 1995, Liu *et al*. 1996, Cancilla *et al*. 2001, Jones *et al*. 2006). Inhibins and activins are dimeric proteins (for review, see Welt *et al*. 2002, Jones *et al*. 2006). Activin consists of two β-subunits, βA and βB, that homodimerize or heterodimerize to form activin A (βA:βA), activin B (βB:βB), or activin AB. In Inverdale ewes, inhibin-α-subunit, activins, and follistatin are identified in small preantral follicles (type 3) and larger follicles in the wild-type ewes, but not in the follicles of II ewes that are only at the primordial (type 1) and primary (type 2) stages of follicular development (Juengel *et al*. 2000). In the neonatal ewe, activin subunits are present in the ovarian follicles (Carpenter *et al*. 2003*a*), endometrial LE and GE, and myometrium of the uterus (Hayashi *et al*. 2003). Inhibin consists of an α-subunit that heterodimerizes with a β-subunit to form either inhibin A (βA) or inhibin B (βB); thus, inhibin-α can inhibit activin activity and vice versa. Inhibin-α-subunit, activins, and follistatin are synthesized and secreted by the ovarian follicles of the neonate and the adult (Braw-Tal *et al*. 1993*a*, 1997, Braw-Tal 1994, McFarlane *et al*. 2002). In the neonatal ewe, inhibin-α-subunit is present in the granulosa cells of growing and antral follicles, but not in the uterus (Carpenter *et al*. 2003*a*, Hayashi *et al*. 2003). The biological activity of activins is mediated by receptor complexes consisting of activin receptor (ActR) type IA or ActRIB and ActRII. In the neonatal ewe, activin receptors are found in all uterine cell types, but are most abundant in endometrial LE and GE, and myometrium (Hayashi *et al*. 2003). One of the key features that distinguish the effects of activins from those of TGF-β is that binding of activins to their receptors can be blocked if activin binds to follistatin or if the inhibin-α-subunit binds to activin receptors (Ball & Risbridger 2001). Follistatin binds to activins with high affinity and neutralizes their activity (Phillips & de Kretser 1998). In the ewe, the circulating concentrations of follistatin increase in the fetus during parturition and remain relatively high until at least PND 7 (McFarlane *et al*. 2002). Follistatin is transcribed by the granulosa cells of growing and antral ovarian follicles in the neonatal ewes (Carpenter *et al*. 2003*a*) and also by the stromal and myometrial cells of the developing neonatal uterus between PNDs 21 and 56 (Hayashi *et al*. 2003). Available results support the hypothesis that inhibin, follistatin, activins, and activin receptors act as endocrine, autocrine, and/or paracrine regulators of uterine development and endometrial adenogenesis in the neonatal ewes (Carpenter *et al*. 2003*a*, Hayashi *et al*. 2003).

In the present study, the circulating levels of inhibin-α were significantly lower in the serum of II ewes from birth to PND 56 due to the lack of developing follicles in the streak ovary. Although follistatin was not measured in the present study, it is likely low in the II ewes, because follistatin is also synthesized and secreted by developing ovarian follicles (Braw-Tal *et al*. 1997, Carpenter *et al*. 2003*a*). As proposed for the effects of ovariectomy on

uterine development in the neonatal ewes (Carpenter *et al*. 2003*a*), a reduction in the circulating levels of inhibin-α-subunit and follistatin would increase the bioavailability of circulating activins and uterine-derived activins, which could retard uterine growth and endometrial adenogenesis. Studies of several epitheliomesenchymal organs, including salivary gland, pancreas, kidney, prostate gland, and mammary gland, indicate that exogenous activin inhibits gland development, whereas follistatin counteracts the inhibitory effects of activin by binding to individual βA- and βB-subunits to prevent activin receptor activation (Ritvos *et al*. 1995, Liu *et al*. 1996, Ball & Risbridger 2001, Cancilla *et al*. 2001).

Collectively, available evidence supports the hypothesis that a factor(s) from the ovary, such as testosterone, inhibin-α, or follistatin, acts in an endocrine manner to regulate uterine growth and development in the neonatal ewes. The Inverdale ewe, lacking functional ovaries, is an excellent natural model for studies to define ovarian uterotrophic factors. Discovery of such factors could be useful in efforts to enhance uterine capacity and to take advantage of prolificacy genes and associated biotechnologies designed to increase reproductive performance of ewes. Furthermore, it will be important to examine the role of ovarian factors in the regulation of endometrial growth and development in the human uterus which, unlike uteri of domestic animals, must regenerate after menstruation during each menstrual cycle. Indeed, the activin–follistatin system is implicated in human endometrial growth and function (Jones *et al*. 2002, 2006).

Materials and Methods

Animals and experimental design

All experiments were performed with the approval of the Animal Ethics Committee at Invermay Agricultural Centre in accordance with the 1999 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand.

Collection of tissue samples

Ewe lambs either homozygous for the Inverdale mutation in BMP15 (II; $n=5$) or their wild-type contemporaries (++; $n=6$) were identified by genotyping (Genomnz Laboratory, Mosgiel, New Zealand). Blood samples (1–3 ml) were obtained by jugular venipuncture on PNDs 0 (within 12 h of birth), 7, 14, 21, 28, 35, 42, 49, and 56 to obtain serum for the analyses. After pentobarbital overdose on PND 56, the ovaries and uteri were removed from the ewe lambs, photographed, measured, and weighed. Cross-sections of the uterine horns were fixed in 4% paraformaldehyde in PBS for 24 h and then transferred to 70% ethanol until being embedded in paraffin.

Determination of hormone concentrations

Laboratory chemicals were obtained from BDH Chemicals New Zealand Ltd (Palmerston North, New Zealand); Roche Diagnostics N.Z. Ltd; ICPbio Ltd, Auckland, New Zealand (BSA); and GE Healthcare, Auckland, New Zealand (17β-estradiol and testosterone tracer) respectively. The concentrations of 17β-estradiol, testosterone, and inhibin-α in serum from all blood samples were determined by RIA using the methods described previously (McNatty *et al*. 1981, 1992, Lun *et al*. 1998). The intra-assay coefficients of variation and assay sensitivities respectively were as follows: 6.6% and 0.05 ng/ml for testosterone, 6.6% and 0.05 pg/ml for 17β-estradiol, and 11.0% and 5 IU/ml for inhibin-α.

Histology and morphometry

Uteri were sectioned (5 μm) and stained with hematoxylin and eosin as described previously (Gray *et al*. 2001*c*, Hayashi *et al*. 2004). Uterine sections ($n=6$) from each ewe were photomicrographed, and the images analyzed using Scion Image software (Scion Corporation, Frederick, MD, USA) as described previously (Carpenter *et al*. 2003*b*, 2003*c*, Hayashi *et al*. 2004). Measurements were standardized using the image of a stage micrometer at the same magnification. The number of superficial ductal invaginations of GE from LE into the stroma was determined. The criterion for ductal gland invagination was invagination of the GE into the underlying stroma with a length of more than 10 μm, which could be visibly tracked to a cross-section of a gland. Endometrial gland number was determined by counting the total number of uterine glands in a complete cross-section of the uterine horn. A gland cross-section with a visible open lumen was counted as a single uterine gland. Endometrial gland density was determined by counting the number of glands in a 0.04 mm^2 area of the stratum compactum and stratum spongiosum areas of the intercaruncular endometrium. The number of ductal gland invaginations and endometrial gland number estimates were generated for at least three areas within five non-sequential sections from each uterine horn. Intra- and intersection estimates of repeatability for the determination of ductal gland invagination number and endometrial gland number by a single observer were 0.85 and 0.8 respectively. The thicknesses or widths of the intercaruncular and caruncular endometrium, as well as myometrium (inner circular and outer longitudinal layers), were measured using the Scion Image software from multiple points ($n=4$) of at least ten non-sequential uterine sections.

Immunohistochemistry

Immunolocalization of androgen receptor and activin receptors (types IA, IB, and II) proteins was performed in the cross-sections (5 μm) of paraffin-embedded uterine sections ($n=6$ ewes per genotype) using specific antibodies and a Vectastain ABC Rabbit or Mouse IgG Kit (PK-6101 or PK-6102; Vector Laboratories, Burlingame, CA, USA). Rabbit polyclonal antibody to androgen receptor (sc-816, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and mouse monoclonal

antibodies to ActRIA (MAB637; R&D Systems Inc.), ActRIB (MAB222; R&D Systems), and ActRII (MAB3391; R&D Systems) were used for immunohistochemistry (Carpenter *et al*. 2003*a*, Hayashi *et al*. 2003, Juengel *et al*. 2006). All antibodies used in the experiments have been validated for ovine tissues in our laboratory. The working antibody concentrations for immunohistochemistry were 1.0 μg/ml for androgen receptor, ActRIA, IB, and II. Negative controls were performed, in which the primary antibody was substituted with the same concentration of normal rabbit or mouse IgG (Sigma–Aldrich). Antigen retrieval using a boiling citrate buffer was performed as described previously (Taylor *et al*. 2000). Multiple tissue sections from each ewe were processed as sets within an experiment. Sections were not counterstained before affixing the coverslip.

Photomicroscopy

Representative photomicrographs of the uterine tissues were taken using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX, USA) fitted with a Nikon DXM1200 digital camera. Digital images were captured and assembled using Adobe Photoshop (Adobe Systems).

Statistical analyses

All quantitative data were subjected to least-squares analyses of variance (LS-ANOVA) using the general linear models procedures of the Statistical Analysis System (Cary, NC, USA). Histomorphometrical data were analyzed using an overall model that included main effects of genotype, section, and area, as well as their interactions. RIA data were analyzed using a model to determine the effects of day, genotype, and their interaction. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Data were then analyzed by least-squares regression analyses to determine the effects of day within genotype. $P \leq 0.05$ was accepted as indicating significance. Data are presented as least-square means of untransformed values with overall S.E.M.

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