Complex expression patterns support potential roles for maternally derived activins in the establishment of pregnancy in mouse

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Abstract

Maternal–fetal communications are critical for the establishment of pregnancy. Embryonic growth and differentiation factors produced by the oviduct and uterus play essential roles during the pre- and early post-implantation phases. Although several studies indicate roles for activin in embryonic development, gene-knockout studies have failed to identify a critical role in mammalian embryogenesis. We hypothesized that activin is produced by maternal tissues during the establishment of pregnancy, and thus maternally derived activin could compensate for the absence of embryonic activin in null homozygotes during critical developmental stages. We investigated the expression of inhibin α , activin β A, and β B subunits in the mouse oviduct and uterus during the estrous cycle and early pregnancy, and in the early conceptus. Inhibin α subunit was weakly expressed, while activin β A and β B subunits were strongly expressed in oviduct and uterus at estrous, and dramatically upregulated in the uterus on each day of pregnancy between days 3.5 and 8.5 post coitum. Prior to implantation, activin β A and β B subunits were immunolocalized to oviductal and uterine epithelial cells; following implantation they were expressed in the stroma, in a wave preceding decidualization. Later in pregnancy, activin β A and β B subunits were present in decidua basalis, trophoblast giant cells, and labyrinth zone of the developing placenta. Expression of activin β A subunit was also detected in blastocysts and early post-implantation embryos. These data are consistent with a role for maternally derived activins in the support of the pre-implantation embryo, and during gastrulation and embryogenesis.

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Introduction

Embryo development occurs in a highly specialized maternal environment within the oviduct and uterine lumen during the pre-implantation phases, and subsequently within the uterine implantation site. Cytokines and growth factors secreted by maternal cells support embryo development, as demonstrated by an improved developmental rate when blastocysts are co-cultured *in vitro* with endometrial and oviductal cells (Seta 2001). Although cytokines, such as granulocyte macrophage colony-stimulating factor and leukemia inhibitor factor, are reported to facilitate mouse embryo development (Robertson & Seamark 1992, Kauma & Matt 1995, Tsai *et al.* 2000), many maternally derived factors remain to be identified. This is critical in order to understand the regulation of early post-implantation

embryo development, and improve culture conditions to more closely mimic the maternal environment for embryo culture following *in vitro* fertilization (IVF).

Activins are potential candidate factors for maternal–embryo interactions, due to their roles in regulating cell proliferation, differentiation and apoptosis, and their abundant expression and actions in remodeling tissues. They are abundantly expressed by reproductive tissues, including the uterus, and have been attributed with roles in decidualization, embryogenesis, and organogenesis in a variety of species (Jones *et al.* 2002).

Activins are dimeric glycoproteins belonging to the transforming growth factor- β (TGF- β) superfamily. Activin A is a dimer of β A subunits, while activin B is formed from β B subunits. These two subunits can also heterodimerize to form activin AB (β A: β B), or alternatively can dimerize with

a distinct α subunit to form inhibin A (α : β A) or inhibin B (α : β B). Activins A and B are believed to have similar actions, with differences in specific functions ascribed to distinct spatial and temporal expressions. Inhibins are potent inhibitors of activins, acting as competitive antagonists through activin receptors (Harrison *et al.* 2005). Activins bind to cell-surface type II receptors, ActRIIA or ActRIIB, which then recruit the type I receptor, ActRIB/Alk-4. This initiates a signaling cascade through serine/threonine kinase activity, and the phosphorylation and nuclear translocation of intracellular Smads.

Studies in both mice and human provide evidence for roles for activins during the establishment of pregnancy. Mouse, bovine, and human embryos express activin receptors during the pre-implantation phase (Lu *et al.* 1993, Yoshioka *et al.* 1998, He *et al.* 1999). Receptor expression (ActRIB/Alk-4, ActRII, and ActRIIB) is maintained in the post-implantation embryo, and during organogenesis, receptors are expressed in a tissue-specific manner (Feijen *et al.* 1994, Manova *et al.* 1995, Verschueren *et al.* 1995, Roberts *et al.* 1996). Moreover, pre-implantation mouse and human embryos express activin β A and β B subunits; at the blastocyst stage, they become localized to the inner cell mass (Albano *et al.* 1993, Lu *et al.* 1993, He *et al.* 1999). The expression of activin β subunit in the early post-implantation phase is controversial (van den Eijnden-van Raaij *et al.* 1992, Manova *et al.* 1995), but strong expression is detected from E8.5 to 10.5 onwards (Feijen *et al.* 1994, Roberts & Barth 1994).

Despite the demonstration that activin A is obligatory for mesoderm induction in *Xenopus* (Smith *et al.* 1990), gene manipulation studies in the mouse have failed to demonstrate an absolute requirement for activins in mammalian embryogenesis (Vassalli *et al.* 1994, Matzuk *et al.* 1995). This discrepancy could be attributed either to compensatory actions of multiple activin β subunits or to maternal supply of activins during pregnancy. As activin β A null mice die neonatally, it has not been possible to establish whether embryo development is adversely affected by the absence of maternal activin. In support of the latter, *in vitro* studies with mouse and bovine embryos indicate that exogenous activin A promotes morula and blastocyst development (Orimo *et al.* 1996, Yoshioka *et al.* 1998, Mtango *et al.* 2003).

In humans, activin β A and β B subunits are abundantly expressed by uterine epithelial glands and decidual cells during the peri-implantation phase and in early pregnancy (Leung *et al.* 1998, Otani *et al.* 1998, Jones *et al.* 2000). Furthermore, activin A is secreted into the uterine fluid that supports pre-implantation development (Petraglia *et al.* 1998). Throughout the first trimester of pregnancy, endometrial glandular products, including activin A, are secreted into the intervillous space and thus have sustained roles in supporting embryonic and placental development (Hempstock *et al.* 2004). Throughout pregnancy, inhibin and activin subunits are

also abundantly produced by the placenta, and have been implicated in the regulation of placental development and hormone production (Petraglia *et al.* 1989, Caniggia *et al.* 1997).

Expression of activin β A subunits by the decidua in rat and mouse in the early post-implantation phase has been reported (Manova *et al.* 1992, Feijen *et al.* 1994, Gu & Gibori 1995). However, maternal production of inhibin/activin subunits during the pre-implantation phase, and in the implantation site in the mouse, has not been characterized, leaving major gaps in our understanding of potential maternal-embryo communication through the activin system. We hypothesize that maternally derived activins play critical roles in promoting pre- and early post-implantation embryo development, and subsequently in the differentiation events and tissue remodeling during uterine decidualization and placentation.

We report here the expression and production of inhibin α , β A, and β B in the uterus across the mouse estrous cycle and during the establishment of pregnancy. Spatial/temporal expression, and relative abundance of β A and β B subunits were examined to indicate whether there are overlapping or distinct functions. These data provide evidence consistent with a potential role for maternally derived activin during embryo development/implantation, and verify that the mouse would be a suitable model for functional studies to provide a greater understanding of activin involvement in human implantation.

Materials and Methods

Animals

Non-pregnant and pregnant Swiss White out-bred mice (*Mus musculus*) were used (Animal Services, Monash University). Mice were housed and handled according to the Monash University animal ethics guidelines on the care and use of laboratory animals. Experimental procedures were approved by the Monash University Animal Ethics Committee (Committee B, Monash Medical Centre, Melbourne, Australia) for animal experimentation and conducted in accordance with the Australian National Health and Medical Research Council (NHMRC) guidelines.

The stage of the estrous cycle of non-pregnant mice (8–10 weeks, weight 20–22 g) was determined by analysis of vaginal smears. Stage of cycle was determined by the characteristic presence or absence of inflammatory leukocytes, and the histological appearance of epithelial cells. Mice were killed by CO₂ asphyxiation and cervical dislocation during the pro-estrous, estrous, metestrous, and diestrous phases ($n=2/\text{stage}$), and oviducts and uteri dissected. Ovaries were collected from randomly cycling mice to use as positive controls.

Female mice (8–10 weeks, weight 20–22 g) were mated with male mice, and checked for vaginal plugs at 0900 h the following morning. Mice were killed on days 3.5, 4.5, 5.5, 6.5, 8.5, 10.5, and 12.5 post coitum (pc); where day 0 is morning of vaginal plug and uteri dissected ($n=2$ /day of pregnancy). On day 3.5 pc, ovaries were examined for corpora lutea to confirm ovulation. On day 4.5 pc, implantation sites were identified by tail vein injection of Chicago blue dye (1% in saline, 0.1 ml/mouse), 5 min before killing. Uterine tissues were dissected out immediately and processed for immunohistochemistry and RNA extraction. Implantation and inter-implantation sites were collected separately on days 4.5–6.5 of pregnancy; thereafter only implantation sites were collected. On day 10.5, the implantation site was dissected to separate the embryo from the implantation site.

Samples were divided into two portions: for immunohistochemistry, tissues were immersion fixed in formalin overnight at 4 °C, prior to thorough washing in Tris-buffered saline (TBS, pH 7.6) and paraffin embedding; the remaining samples were snap-frozen for subsequent RNA extraction.

In vitro fertilization and embryo culture

Oocytes

Six-week-old females were superovulated by an i.p. injection of 10 IU equine chorionic gonadotropin (Folligon, Intervet International, Boxmeer, The Netherlands) followed by 10 IU hCG (Chorulon, Intervet International, The Netherlands) in 0.2 ml saline 50 h later. At 13–14 h after hCG injection, oocyte–cumulus complexes were collected from the oviducts by incubation for 5 min in HEPES-KSOMaa (Biggers *et al.* 2000; BDH Chemicals Pty., Ltd, Melbourne, Victoria, Australia) containing 60 IU/ml hyaluronidase (Sigma type IV-S; Sigma Chemicals Co.). Adhering cumulus cells were removed from oocytes by gentle pipetting and washed in HEPES-KSOMaa. Oocytes were then placed into 20 μ l drops of modified KSOMaa previously equilibrated under mineral oil in 5% CO₂ in air at 37 °C. Oocytes were incubated for 1 h at 37 °C in 5% CO₂ in air before they were used for IVF.

Sperm

Sperm were collected from 10- to 12-week-old males. Briefly, both the caudae epididymides were dissected from the mice and a small slit was made in each cauda before transfer to 2 ml modified Tyrode's medium (Fraser 1984; BDH) and equilibrated in 5% CO₂ in air at 37 °C in a 35 mm culture dish (NUNC, Roskilde, Denmark) under oil. The tissue was then removed from the dish 1 h later, and the sperm concentration was examined and adjusted to $3\text{--}5 \times 10^6$ sperm/ml. Sperm were capacitated for a total of 2 h before IVF.

In vitro fertilization (IVF)

For IVF, cumulus-free oocytes (30–50) were incubated for 5 h, under mineral oil, in 2 ml capacitated sperm solution ($3\text{--}5 \times 10^6$ sperm/ml). Oocytes were removed from the sperm solution after 5 h, washed in HEPES-KSOMaa, and cultured at 37 °C in modified KSOMaa under mineral oil in 5% CO₂ in air. Six hours later, oocytes were examined for signs of normal fertilization. Oocytes with two pronuclei and a second polar body were regarded as fertilized. These oocytes were separated from unfertilized oocytes and cultured in modified KSOMaa at 37 °C under 5% CO₂ in air up to 96 h for development to the blastocyst stage *in vitro*. Two and four cell embryos, morulae, and blastocysts (unhatched; $n=3$ /stage) were collected after 24, 48, 56–60, and 72 h culture respectively, placed directly into 100 μ l TRIZOL reagent (Invitrogen, Mt Waverly, Victoria, Australia). Samples were then snap-frozen prior to RNA extraction.

RNA extraction and purification

Total RNA was extracted from frozen uterine samples by acid guanidinium thiocyanate–phenol–chloroform extraction (GTC; Sigma) as previously described (Nie *et al.* 2000), followed by purification through RNeasy spin columns (Qiagen) according to the manufacturer's instructions. RNA was extracted from oviducts using RNeasy spin columns. RNA samples were treated with RNase-free DNase I (Ambion, Austin, Texas, USA) to remove genomic DNA contamination, and analyzed by spectrophotometry to determine RNA concentration, yield, and purity. RNA concentrations were also analyzed by Ribogreen fluorescence RNA assay (Invitrogen) as previously described (Jones *et al.* 2004).

RNA was isolated from embryos frozen in TRIZOL using a modified protocol. Samples were allowed to thaw and incubated at room temperature for 30 min, followed by vortexing. Thereafter, the manufacturer's instructions were followed, with the exception of the inclusion of a second chloroform – only extraction to prevent phenol carryover. RNA samples were resuspended in 12 μ l diethylpyrocabonate (DEPC) H₂O, and the entire volume was utilized for RT.

Real-time RT-PCR

Reverse transcription and validation

Total RNA (1 μ g) was reverse transcribed in triplicate reactions using random hexameric primers and AMV-RTase (Roche), at 46 °C for 90 min. Omission of reverse transcriptase served as a negative control. Triplicate RTs were analyzed for efficiency and reproducibility of the RT reaction by real-time PCR for ribosomal 18S subunit using a Light Cycler (Roche) as previously described (Jones *et al.* 2004). Primers used were: sense 5'-CGG CTA CCA CAT

CCA AGG AA-3' and antisense 5'-GCT GGA ATT ACC GCG GCT-3'. Amplified DNA quantitation was performed using SYBR Green I, by comparison with serially diluted cDNA standards, generated by PCR as previously described (Jones *et al.* 2004). Cycle conditions were as follows: 95 °C for 10 min, 35 cycles of 95 °C for 15 s, 60 °C for 5 s, and 72 °C for 10 s. 18S concentrations (pg/μl) were compared between triplicates and the intra-assay variability of the RT and PCR steps was evaluated. Within triplicates, samples outside 15% variability of the average 18S concentration were excluded as outliers. Otherwise, triplicates were pooled, creating cDNA reaction products that are close representations of the initial mRNA population. Negative controls were performed by omission of RT. All PCR products were DNA sequenced to confirm identity.

Real-time PCR for inhibin/activin subunits

Pooled RT samples (diluted in the ratio of 1:10) were amplified in a Light Cycler for inhibin/activin subunits, using primers: inhibin α : 5'-GCC TTG GCC TTG GTC TCC TGC G-3'/5'-ACG CGT AGG GAG GTC ATG CTC C-3'; activin β A: 5'-GGC TTG AGT GCG ACG GC-3'/5'-GCA GCC ACA CTC CTC-3'; activin β B: 5'-TCT TCA TCG ACT TTC GGC TCA T-3'/5'-TGT CAG GCG CAG CCA CAC TCC T-3' (Sigma; Albano *et al.* 1993). Cycle conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 55/62/60 °C for 5 s, and 72 °C for 15 s. PCRs were run in duplicate or triplicate. Mouse ovarian cDNA, diluted in the ratio of 1:5, was included in each run as a positive and quality control.

Immunohistochemistry for inhibin/activin subunits

Inhibin/activin α , β A, and β B subunits were immunolocalized using affinity purified rabbit polyclonal antibodies raised against the individual inhibin subunits (anti-porcine inhibin α ¹⁻²⁶, anti-porcine inhibin β A⁸¹⁻¹¹³, and anti-human inhibin β B⁸⁰⁻¹¹², kind gift from Prof. Wylie Vale, La Jolla, CA, USA; Vaughan *et al.* 1989), as previously described (Jones *et al.* 2000), but with modifications for optimal staining of mouse tissues. Mouse ovary was included as a positive control. In brief, microwave antigen retrieval was performed (5 min HIGH, 5 min LOW in a 1 kW microwave oven, followed by 20-min cooling), and endogenous peroxidase activity blocked with aqueous H₂O₂. Primary antibodies were applied at 2 μg/ml in a non-immune block containing 20% goat serum and 0.1% Tween-20 in TBS. Negative controls were performed by substitution of primary antibody with 2 μg/ml rabbit IgG (X0936; Dako, Glostrup, Denmark). Antibody binding was thereafter detected by sequential application of conjugate (Dako) for 1 h at room temperature, with chromogen diaminobenzidine (DAB; Dako), forming an insoluble brown precipitate. Tissue sections were counterstained with Harris's hematoxylin, dehydrated, and mounted with DPX mounting medium (Sigma).

Results

Expression of inhibin/activin α , β A, and β B subunits in uterus in non-pregnant cycling mice

A positive signal was obtained for all primer pairs for ovarian RT samples included as a positive control in real-time PCRs (data not shown). No amplification product was detected in negative controls. Inhibin α subunit mRNA was almost undetectable in cycling uterine samples (Fig. 1A). In contrast, activin β A mRNA was abundantly expressed in non-pregnant uterine samples, with fluctuations across the estrous cycle. mRNA levels were increased greater than threefold from the proestrous to estrous phases, thereafter levels decreased in the metestrous phase (Fig. 1A). A similar pattern was detected for β B subunit mRNA (Fig. 1A). Moreover, both activin β subunits were expressed by the oviduct, with an identical cyclical expression pattern, and maximal upregulation observed in estrous phase (data not shown).

Immunohistochemistry verified this expression pattern at the protein level and identified the cellular location. Intense immunostaining was detected for α , β A, and β B subunits in ovarian follicles and corpora lutea in agreement with known established patterns reviewed by Findlay (1993) (Fig. 1B and C). β A and β B subunits immunolocalized to epithelial cells lining the oviduct (Fig. 1D), and in the uterus, to the luminal and glandular epithelium (Fig. 1E–L). Consistent with mRNA expression patterns, endometrial immunostaining levels varied markedly during the estrous cycle. In the proestrous phase, β A subunit was present in the luminal epithelium (Fig. 1E). In the estrous phase, immunostaining levels were increased in luminal and glandular epithelial cells (Fig. 1F), and in addition, intensely stained single cells (possibly infiltrating immune cells) were identified in the sub-epithelial zone (Fig. 1G). Following estrous, β A immunostaining was minimal or absent in metestrous (Fig. 1H) and diestrous phases (data not shown). β B subunit protein expression was low in proestrous endometrium in both the luminal and the glandular epithelium (data not shown), but was elevated in the estrous phase in all epithelial cells (Fig. 1I). No intensely stained stromal cell was detected in the sub-epithelial zone (Fig. 1J). In metestrous (Fig. 1K) and diestrous (Fig. 1L) endometria, β B immunostaining was severely diminished in luminal epithelial cells, but retained in epithelial glands during both post-estrous phases. Low immunostaining was detected for α subunit in oviduct (Fig. 1B), whereas none was detectable in the uterus (Fig. 1M).

Expression of activin subunits in mouse pre-implantation embryos

Real-time PCR was conducted for activins β A and β B on embryos at the two cell, four cell, morula, and blastocyst

Figure 1 Expression of inhibin, inhibin/activin subunit mRNA, and protein in non-pregnant cycling mouse reproductive tract. (A) mRNA expression of inhibin α , activin βA , and activin βB during the proestrous (PRO), estrous (EST), and metestrous (MET) of the estrous cycle of the mouse. Values are the average of two independent observations and error bars show the range. (B)–(M) Immunostaining for inhibin/activin subunits in non-pregnant female uterus and oviduct. (B) Inhibin α in ovary and oviduct, (C) activin βB in ovary, (D) activin βA in oviduct, (E)–(G) uterine expression of activin βA in (E) proestrous phase, (F) estrous, (G) high magnification of strongly stained subepithelial immune cells in estrous phase. (H)–(L) Uterine expression of activin βB in (H) proestrous, (I) estrous, (J) high magnification showing absence of staining in subepithelial immune cells in estrous phase, (K) metestrous, and (L) diestrous phases. Inset is negative control showing no staining. (M) Inhibin α staining was absent in all uteri examined. Scale bar=100 μm , except (B)=200 μm and (D, G and J)=50 μm .

(unhatched) stages ($n=3$ pooled embryo RNA/stage). A positive signal for both activin βA and βB subunits was detected in ovary included as a positive control (12.8 fg βA mRNA/pg 18S, 4.25 fg βB mRNA/pg 18S). βA mRNA was below the limit of detection in all embryos except for blastocysts (3.5 fg βA mRNA/pg 18S); βB expression was not detectable in embryos, including blastocysts (Fig. 2). 18S was used as a control for RNA integrity.

Expression of inhibin/activin α , βA , and βB subunits during establishment of pregnancy

Inhibin α mRNA was undetectable by real-time PCR in the uterus in the peri-implantation phase (days 3.5–4.5 pc;

Fig. 3A). Very low levels of expression were detected in the implantation site from days 5.5 to 8.5. In contrast, activin βA mRNA was markedly upregulated on day 3.5 pc compared with non-pregnant (NP) uteri (greater than tenfold; Fig. 3B, inset). On the day of embryo attachment (day 4.5 pc), activin βA was increased in one out of two implantation site samples, while no change in mRNA expression was seen in inter-implantation sites. From day 5.5, activin βA subunit expression rose further, with expression levels approximately tenfold higher in both implantation and inter-implantation sites compared with day 4.5 (Fig. 3B). Despite considerable variation, reflecting the heterogeneity of this dynamic tissue, a further increase (approximately threefold) in βA mRNA

Figure 2 Real-time PCR for 18S, activin β A, and β B in pre-implantation mouse embryos, at the two cell, four cell, morula, and blastocyst stages ($n=3$ embryos/stage). Ovary (Ov) was included as a positive control.

expression was detected on each of the days 6.5 and 8.5 pc in implantation sites, while levels remained static in inter-implantation sites. At day 10.5, expression levels were examined in the uterus and embryo separately. This showed a sharp decline in uterine activin β A mRNA expression and low embryonic expression of β A subunit.

A similar expression pattern was detected for β B mRNA; however, expression levels were consistently 50–100 times lower than β A mRNA levels (Fig. 3C). Furthermore, the increase in β B subunit expression with the onset of pregnancy was delayed compared with that of β A, with only a slight increase in activin β B mRNA from non-pregnant to day 4.5 (Fig. 3C, inset). However, on day 5.5, β B mRNA was upregulated more than 30-fold in implantation sites, and fivefold in inter-implantation sites, compared with day 4.5 (Fig. 3C). Expression increased a further threefold in implantation sites on day 6.5 and thereafter a slight decline was detected on days 8.5–10.5 in the uterine tissues. Activin β B mRNA expression was detected in the embryo, but at very low levels compared with activin β A mRNA expression.

Immunohistochemistry for inhibin/activin subunits

Consistent with mRNA expression patterns, β A protein was upregulated on day 3.5 of pregnancy, in luminal and glandular epithelium (Fig. 4A) and also in the stromal zone underlying the luminal epithelium, particularly in the anti-mesometrial region. With blastocyst attachment on day 4.5, stromal immunostaining became more intense and polarized at the anti-mesometrial zone, where the primary decidual response occurs (Fig. 4B and C). Immunostaining for β A was downregulated in the luminal epithelium in implantation site compared with NP. A distinct staining pattern was observed throughout inter-implantation sites, with intense immunostaining in

Figure 3 Quantitation of (A) inhibin α , (B) activin β A, (C) activin β B subunit mRNA expressions in mouse uterus during the establishment of pregnancy. Day post coitum is shown on the x-axis. NP, non-pregnant; imp, implantation site; inter, inter-implantation site; emb, embryo; OV, ovary. For (B) and (C), NP-day 4.5 is magnified in inset, due to the differences in scale. Data are the mean expression ($\pm$ S.D.) from triplicate PCR runs, for two individual mice at each time point.

epithelial glands, while luminal epithelium and stroma possessed minimal immunoreactivity (Fig. 4D). By day 5.5 pc, activin β A expression exhibited a 'butterfly-like' pattern, with intense immunoreactivity in the anti-mesometrial and mesometrial zones distant to the implanting embryo, and with stroma immediately surrounding the embryo devoid of immunostaining (Fig. 4E and F). Basal epithelial glands maintained strong activin β A expression (Fig. 4E). Between days 6.5 and 8.5 pc, uterine immunoreactivity for activin β A diminished and became limited to the basal zone (bz) adjacent to the myometrium (Fig. 4G and J). From day 5.5 onwards, intense immunostaining for activin β A was observed in and surrounding the developing embryonic and extra-embryonic structures (Fig. 4F, H and I).

An almost identical pattern of activin β B was observed in the implantation site (Fig. 5). Intense staining was detected in epithelial glands throughout early pregnancy

Figure 4 Immunohistochemical localization of activin β A in mouse uterus during early pregnancy. Photomicrographs are orientated such that the mesometrium is at the lower right-hand side in each case. (A) Day 3.5 post coitum (pc), (B) and (C) day 4.5 showing blastocyst (collapsed) attachment to the anti-mesometrial pole and activin β A staining in the primary decidual zone (pdz), (D) day 5.5 pc inter-implantation, and (E) day 5.5 pc implantation site, showing staining in the embryo (em) and epithelial glands (gl), (F) high magnification of embryo at day 5.5 pc, (G) day 6.5 pc implantation site, illustrating the restriction of activin β A staining to the embryo and basal zone (bz) of the decidua, (H) high magnification of embryo at day 6.5 pc, (I) activin β A immunostaining in the embryo and developing placenta, (J) anti-mesometrial zone showing staining restricted to the basal zone, and faint staining in giant cells (gc). Scale bars = 100 μ m, except (B, E, G) = 200 μ m and (F, H) = 50 μ m.

Figure 5 Immunohistochemical localization of activin β B in mouse uterus during early pregnancy. Photomicrographs are orientated such that the mesometrium is at the lower right-hand side in each case. (A) Activin β B staining on day 3.5 post coitum (pc), primarily in epithelial glands (gl), (B) uterus on day 4.5 showing staining in the primary decidual zone (pdz), (C and D) low and high magnification images of β B in day 5.5 pc implantation site, showing staining in the embryo (em) and in maternal cells surrounding the embryo, (E) day 6.5 pc, and (F) day 8.5 pc implantation sites, illustrating faint staining for β B subunit in the mesometrial decida, but with no staining generally absent in the embryo, (G) intense immunostaining in epithelial glands in the basal zone on day 8.5 pc, (H) β B immunostaining becomes focused to the decida basalis on day 9.5 pc. Scale bars = 100 μ m, except (C and E) = 200 μ m, and (D and G) = 50 μ m.

(e.g., Fig. 5A and G). Activin β B immunostaining was faint or absent in the embryo in the early post-implantation phases (Fig. 5C–F), but it intensely immunolocalized to individual maternal cells surrounding the implanting embryo on day 5.5 (Fig. 5D), and was generally present in the mesometrial zone surrounding the developing extra-embryonic structures on days 6.6–8.5 (Fig. 5E and F).

From days 8.5 to 12.5 pc, strong staining for activin β A (Fig. 6A) and β B (Figs 5H and 6C) subunits was

detected in the mesometrial decida basalis, localized specifically to decidual cells, while uNK cells were unstained (Fig. 6B). During this time, immunoreactivity for activin subunits was also detected in the developing placenta. Inhibin α immunostaining was faint or absent in the developing placenta (Fig. 6D). Immunostaining for both activin subunits was present in enlarged giant cells (Fig. 6E) and in the trophoblast cells of the labyrinth zone (Fig. 6A and F). Activin β A was absent from the spongiotrophoblast layer (Fig. 6A and G),

while β B immunostaining was present, but at reduced levels compared with decidua and labyrinth (Fig. 6C and H). Of interest was the intense staining for β B in the cells lining the large maternal vessels of the spongio-trophoblast layer (Fig. 6H).

Discussion

Activin β A and β B subunits were found to be strongly expressed in the mouse female reproductive tract during the estrous cycle and establishment of pregnancy. In non-pregnant cycling mice, activin β subunit mRNA, and

Figure 6 Immunohistochemical localization of inhibin/activin subunits in mouse placenta on day 12.5 post coitum (pc). (A) Activin β A immunoreactivity is intense in labyrinth (lab) and decidua basalis (dec), but faint or absent in spongio-trophoblast (sp), (B) high magnification images of β A decidual cells, (C) activin β B positively immunostained labyrinth, spongio-trophoblast, and decidua, while (D) inhibin α immunostaining was absent from all placental layers, (E) activin β A staining in trophoblast giant cells, and (F) activin β B staining in placental labyrinth, (G) activin β A is absent from spongio-trophoblast, while (H) activin β B is present around blood vessels (v) in the spongio-trophoblast. Scale bars on (A, C and D)=200 μ m, on (H)=100 μ m, and on (B, E–G)=50 μ m.

protein expression levels fluctuated in a cycle-dependent manner in the uterus. During early pregnancy, both activin β subunits were strikingly upregulated in uterine tissues, and embryonic activin β A mRNA expression was detectable from the blastocyst stage. Immunohistochemical studies localized activin β A and β B subunits to the uterine epithelial and decidual cells, as well as to the developing embryo and placenta.

The predominant source of activin β subunits during the estrous cycle and pre-implantation phase were the epithelial cells lining the oviduct and endometrium. The strong expression of activin subunits by the oviductal epithelial cells is consistent with findings by Lu *et al.* (1993) and with a role for maternally derived activins, secreted into oviductal fluid, in facilitating pre-implantation embryo development. Both β A and β B subunits mRNA and protein were elevated at estrus, suggesting positive regulation by estrogen, and a 'priming' of the oviduct and uterus in anticipation of pregnancy. A further increase in activin β A expression occurred in the uterus on day 3.5, corresponding to the entry of morulae into the uterine lumen and the first embryonic differentiation steps of morula compaction and blastocyst development, hatching and implantation.

The elevation in maternally derived activin correlating these critical steps is consistent with an involvement for activin in facilitating embryonic development. Furthermore, embryonic expression of activin β A mRNA becomes detectable with blastocyst development. ActRs are expressed by the embryo during this period, and in the human, expression levels of both type I and II receptors increase with blastocyst formation (He *et al.* 1999). Indeed, treatment of mouse and bovine embryos with activin A *in vitro* accelerates morula and blastocyst development, blastocyst hatching, and increases blastocyst cell number (Orimo *et al.* 1996, Yoshioka *et al.* 1998, Mtango *et al.* 2003); while neutralization of endogenous activin with follistatin treatment *in vitro* significantly retards embryonic development (Yoshioka *et al.* 1998). Although activin action is not obligatory for pre-implantation development, as embryos lacking ActRs survive beyond the blastocyst stage (Gu *et al.* 1998, Song *et al.* 1999), it is likely that the deficit of maternal growth factors is responsible for the retarded development observed during embryo culture (Seta 2001). Therefore, the corroboration between the previous *in vitro* data and the specific temporal expression patterns for activin subunits in oviductal and uterine epithelia, support the hypothesis that activins contribute to the maternal support of pre-implantation embryo development.

The upregulation of activin β A and β B subunit expressions in the implantation site in early pregnancy is primarily due to expression by the developing decidua, and later, by the placental cells. Activin β A expression has been well characterized during decidualization in the rat (Gu *et al.* 1995), and here we

demonstrate a similar expression pattern for both β A and β B in the mouse decidua. Importantly, the upregulation of activin subunits in stromal cells precedes decidualization, with a coordinated wave of up- and downregulation spreading through the uterus in advance of the onset and spread of decidualization. This indicates a preparative role for activin prior to decidual differentiation. In human endometrial stromal cells, activin upregulates matrix metalloproteinases (MMPs; Jones *et al.* 2006), which have critical actions during decidualization, at least in the rat (Alexander *et al.* 1996, Rechtman *et al.* 1999). Interestingly, in the rodent uterus, MMP-2 and -3 have almost identical expression patterns to that of activin during early pregnancy (Alexander *et al.* 1996, Bany *et al.* 2000), suggesting an interaction between activins and MMPs during decidualization in the mouse. Later in pregnancy, activin promotes decidual apoptotic regression in the rat, allowing expansion of the implantation site for placentation and embryonic growth (Gu *et al.* 1995, Tessier *et al.* 2003). Interestingly, the primary decidual cells adjacent to the embryo on day 7.5 that stain intensely for activin subunits correspond to the site of maximum apoptosis (Alexander *et al.* 1996).

In the developing placenta, activin β subunit expression was evident in the invasive trophoblast giant cells at the maternal-fetal interface, possibly related with a role in promoting trophoblast invasion, as has been demonstrated in the human (Caniggia *et al.* 1997). In the placenta at day 12.5, activin β A and β B subunits were clearly localized predominantly to the syncytiotrophoblast of the labyrinth. This is identical with the spatial expression of activin/inhibin subunits in the villous syncytiotrophoblast of the human placenta (Lockwood *et al.* 1997); their expression is apparent immediately with the onset of syncytialization both *in vivo* and *in vitro* (Debieve *et al.* 2000, Jones *et al.* 2006).

The significance of multiple activin β subunits is not known. β A and β B subunits frequently exhibit distinct expression patterns, suggesting specific functions. For example, in developing embryos, widespread expression of β A in mesenchymally derived tissues, including gonads and brain, has been reported (Manova *et al.* 1992); while β B expression is primarily limited to reproductive tissues. However, in the uterus, β A and β B have almost identical expression patterns, but β B mRNA is consistently far less abundant than β A. This may explain the failure of previous studies to detect β B subunit in uterine and embryonic tissues using less sensitive methods (Manova *et al.* 1992). Female mice lacking activin β B subunit undergo normal implantation and early pregnancy, but experience perinatal loss of progeny, suggesting maternal activin B fulfils critical functions late in pregnancy (Vassalli *et al.* 1994). Fertility cannot be assessed in the β A knockout, due to neonatal lethality (Matzuk *et al.* 1995). However, when the β B subunit was 'knocked-in' into the β A gene locus of the β A-null mutant, the female mice reproduced, but

exhibited a severely decreased pregnancy rate (Brown *et al.* 2000). The uterine activin β A subunit deficiency contribution to reduced fertility has not been established. However, these gene manipulation models suggest distinct functions for β A and β B in the female reproductive system.

The present study provides evidence for the uterine epithelial (glandular) and decidual cells as a significant source of activin in the mouse at the time of implantation and early embryonic development, the uterine epithelial (glandular) and decidual cells are a significant source of activin in the mouse. Therefore, during gastrulation, when activin action is obligatory in *Xenopus*, lack of embryonic β A and β B could be compensated for by the abundant maternal contribution, giving a plausible explanation for the absence of gastrulation-related defects in the activin β A null ($-/-$) embryos (in activin β A subunit heterozygote ($+/-$) mothers). This is supported by the absolute requirement for activin signaling for mesoderm induction in mice, with arrested development prior to gastrulation observed in activin receptor knockout mice (Alk-4, and double ActRII/ActRIIB nulls; Gu *et al.* 1998, Song *et al.* 1999). The results of the present study strongly support the hypothesis that maternally derived activin fulfils a critical role in early embryonic development in the mouse.

In summary, we have characterized in detail the spatial and temporal expression patterns of inhibin/activin α , β A, and β B subunits during the establishment of pregnancy in the mouse. We demonstrate that maternally derived activin subunits, predominantly β A, are specifically upregulated in the uterine luminal epithelium during pre-implantation embryo development, and subsequently in the decidua of the implantation site, during the critical development events of gastrulation and organogenesis. These data suggest that activins secreted by the oviduct and uterus could facilitate the development of the embryo prior to implantation. Furthermore, our findings support the hypothesis that maternally derived activins play important roles in early post-implantation embryo development. The observed expression patterns in the mouse uterus bear marked resemblance to those in the human uterus during the preparation for implantation and in early pregnancy, thus suggesting it to be a suitable model to delineate the functional roles for activins in maternal–fetal interactions during the establishment of pregnancy.

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