Regulation of Prostate Branching Morphogenesis
by Activin A and Follistatin

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Ventral prostate development occurs by branching morphogenesis and is an androgen-dependent process modulated by growth factors. Many growth factors have been implicated in branching morphogenesis including activins (dimers of βA and βB subunits); activin A inhibited branching of lung and kidney in vitro. Our aim was to examine the role of activins on prostatic development in vitro and their localization in vivo. Organ culture of day 0 rat ventral prostates for 6 days with activin A (+/- testosterone) inhibited prostatic branching and growth without increasing apoptosis. The activin-binding protein follistatin increased branching in vitro in the absence (but not presence) of testosterone, suggesting endogenous activins may reduce prostatic branching morphogenesis. In vivo, inhibin α subunit was not expressed until puberty, therefore inhibins (dimers of α and β subunits) are not involved in prostatic development. Activin βA was immunolocalized to developing prostatic epithelium and mesenchymal aggregates at ductal tips. Activin βB immunoreactivity was weak during development, but was upregulated in prostatic epithelium during puberty. Activin receptors were expressed throughout the prostatic epithelium. Follistatin mRNA and protein were expressed throughout the prostatic epithelium. The in vitro evidence that activin and follistatin have opposing effects on ductal branching suggests a role for activin as a negative regulator of prostatic ductal branching morphogenesis.

Key Words: activin; follistatin; prostate; branching morphogenesis.

INTRODUCTION

Branching morphogenesis of the prostate is an androgen-sensitive process elicited via epithelial–mesenchymal interactions modulated by growth factors (Cunha et al., 1995). The rat prostate begins to develop late in gestation, when the urogenital sinus mesenchyme induces formation of epithelial buds from the urogenital sinus epithelium (Sugimura et al., 1986; Hayashi et al., 1991; Timms et al., 1994). These buds elongate and form solid epithelial cords, which grow toward the ventral mesenchymal pad (Timms et al., 1995; Thomson and Cunha, 1999). The solid prostatic buds elongate and branch under the influence of androgens and subdivide the ventral mesenchymal pad into caps of mesenchyme that surround individual epithelial bud tips. Concurrent with ductal branching, epithelial differentiation begins in the more proximal ductal regions near the urethra and then extends distally. This process involves differentiation of the solid epithelial cords into canalized ducts lined by columnar epithelial cells underlaid by a discontinuous layer of basal cells (Hayward et al., 1996a). The surrounding mesenchyme differentiates into smooth muscle or fibroblastic cells (Hayward et al., 1996b). Ductal branching morphogenesis and epithelial differentiation continue in the rat until about 40–50 days when branching ceases and canalization is completed in the ductal tips (Sugimura et al., 1986). Rising androgens at puberty initiate secretory function of the epithelium (Lopes et al., 1996).

The actions of androgens during prostatic development are elicited via reciprocal epithelial–mesenchymal interactions modulated by growth factors. For example, fibroblast growth factor (FGF)-7 and FGF-10 are implicated in androgen-induced ductal growth and branching morphogenesis of the prostate (Sugimura et al., 1996; Thomson and Cunha, 1999). These factors are also implicated in branching morphogenesis of the lung (Peters et al., 1994; Belluscio et al., 1997). Many other factors also regulate the complex
interactions during branching morphogenesis including members of the transforming growth factor (TGFβ) superfamily, such as bone morphogenetic proteins (BMPs) and activins. Activin A reduces branching morphogenesis in the developing pancreas, salivary gland, and kidney in vitro (Ritvos et al., 1995). In the prostate, the process of branching morphogenesis is similar to other organs, although the developmental process is androgen-dependent.

Activins are members of the TGFβ superfamily, and consist of disulphide-linked homo- and heterodimers of βα and ββ subunits (forming activins A, AB, and B). Inhibins consist of a βα or ββ subunit linked to an inhibin α subunit (forming inhibins A and B). Activins and inhibins were originally isolated as gonadal proteins that can regulate pituitary follicle-stimulating hormone secretion (Burger et al., 1988; De Jong, 1988). Inhibins are endocrine hormones produced by the gonads, which inhibit follicle-stimulating hormone secretion. Activins are growth and differentiation factors with diverse roles in development (Welt and Crowley, 1998). Activin A acts as a mesoderm inducer in Xenopus embryos (Thomsen et al., 1990; van den Eijnden-Van Raaij et al., 1990; Smith et al., 1990; Albano et al., 1990), and activins A and B can induce digit formation in chick embryos (Merino et al., 1999). Mammary-derived activin βα subunit is required for normal mammary gland development (Robinson and Hennighausen, 1997). Mammary tissue recombinants with βα subunit-deficient mice show that stromal activin βα subunit is essential for normal ductal elongation and alveolar morphogenesis (Robinson and Hennighausen, 1997).

The actions of activins cannot be considered in isolation due to the activity of the activin-binding and -neutralizing glycoproteins, follistatins (FS). Several isoforms occur, resulting from differential mRNA splicing to generate the proteins FS-288 and FS-315. FS-288 binds to heparin and is found on the cell surface and in extracellular matrix, whereas FS-315 is secreted from cells and does not bind to heparin (Sugino et al., 1993). Both forms of follistatin bind activins and block their biological activity (Sugino et al., 1993). Activins, inhibins, and follistatin are expressed in the human and rodent prostate (Risbridger et al., 1996; Thomas et al., 1998; van Schaik et al., 2000). Activin βα subunit is present in basal and secretory epithelial cells, and activin ββ subunit is predominantly in basal epithelial cells of the adult human prostate (Thomas et al., 1997, 1998; Ying et al., 1997); this expression is regulated by testosterone (Risbridger et al., 1997). The localization of activin subunits during prostatic development is not known.

Based on previous studies showing that activin reduced ductal branching of pancreas, salivary gland, and kidney (Ritvos et al., 1995), we examined the effects of exogenous activin and follistatin on prostatic development in vitro and found that activin can regulate branching morphogenesis. We then examined the spatio-temporal expression of activin βα and ββ subunits and follistatin during prostatic development to determine whether the pattern of expression was consistent with a role for activin in prostatic development in vivo.

**METHODS**

**Animals**

Intact male Sprague-Dawley outbred rats from days 0 to 20 and day 90 postpartum were killed. Ventral prostate lobes were microdissected from newborn rats and processed for organ culture, reverse transcriptase-polymerase chain reaction (RT-PCR), or immunohistochemistry. All animals were obtained from Central Animal Services, Monash University. Procedures and animal care were administered according to the requirements, and with the approval of the Standing Committee of Ethics in Animal Experimentation, Monash University. Two sets of six ventral prostate lobes at each age examined (days 0, 5, 10, 15, 20, and 90) were used for RNA isolation as previously described (Cancilla et al., 2000). For immunohistochemistry, tissues were fixed in Bouins, processed to paraffin, and 3-μm serial sections were cut.

**Organ Culture**

Organ culture was carried out as previously described (Lipshutz et al., 1997; Jarred et al., 2000). Briefly, microdissected day-0 ventral prostates were cultured on Millicell CM filters (Millipore Corp., Bedford, MA) floating on 500 μl of nutrient media at 37°C in a humidified 5% CO2 incubator. A basal medium of DMEM/Ham’s F-12, 1:1 (vol/vol), supplemented with insulin (10 μg/ml) and transferrin (10 μg/ml) was utilized in all experimental groups, and media was replenished every 48 h. In order to reduce variability of growth between animals, one ventral prostate lobe from each gland was used for experimental treatment and the other for control cultures. Different doses of activin A (10–100 ng/ml) and follistatin (0.1–3.5 μg/ml) were tested; the optimal dose of activin A was 40 ng/ml, and follistatin was 3.5 μg/ml. Human recombinant activin A was a gift from Biotechnology Australia Pty. Ltd. Purified bovine follistatin, containing FS-288 and FS-315 isoforms, was kindly provided by Prof. D. de Kretser (Monash Institute of Reproduction and Development, Australia). Explants were incubated with activin A or follistatin in the presence or absence of 10 nM testosterone. The organs were harvested after 6 days of culture. Explants were photographed, fixed in Bouins for 2 h at room temperature, then processed to paraffin. At least four pairs of explants were subjected to each condition and dose tested.

**RT-PCR**

Reverse transcription (RT) and polymerase chain reaction (PCR) were performed sequentially in the same reaction tube by using a GeneAmp RNA PCR kit (Perkin Elmer, Foster City, CA) as previously described (Cancilla et al., 2000). PCR was performed for 35 cycles, with 30 s at 95°C for denaturation, 30 s at 55°C for annealing, and 1 min at 72°C for extension. Controls for RT-PCR omitted the reverse transcriptase (non-RT control) or RNA (H2O control), which were replaced with water. To confirm the presence of RNA in all samples, primers for cytoskeletal β actin were used (Tokunaga et al., 1986). The forward primer was 5′-GTTGCGCCCGTATTAGCCACCA and the reverse primer was 5′-CTCTTGTAGTGCAGCCGATTTTC and generated an RT-PCR product of 540 bp. To detect inhibin α, primers used were identical.
to those described previously for rat inhibin α subunit (Sonoyama et al., 2000) and generated an RT-PCR product of 656 bp.

**Antibodies**

Activin βa subunit was detected by using clone E4 (raised against amino acids 82–114 of human activin β subunit) at 2–4 μg/ml. Activin βb subunit was detected using biotinylated clone C5 (raised against amino acids 82–114 of human activin βb subunit) at 7 μg/ml; this antibody cross-reacts with βb subunit (Lillingworth et al., 1996). FS-315 was detected by using clone H10 (raised against a C terminus peptide of human FS-315; CDEDQDYSPISSILEW; Sato et al., 1991) at 5 μg/ml. This antibody has been used previously for immunohistochemistry (MPherson et al., 1999). E4, C5, and H10 were kind gifts from Prof. N. P. Groome (Oxford Brookes University). Antibodies against three activin receptors, anti-activin RIA, anti-activin RIIa, and anti-activin RIIb antibodies (R & D Systems, Minneapolis, MN), were used at 10 μg/ml. Smooth muscle cells were detected by using monoclonal anti-smooth muscle α-actin (Sigma, St. Louis, MO) at 7 μg/ml, or with FITC-labeled anti-smooth muscle α-actin (Sigma) at 19 μg/ml. Undifferentiated prostatic epithelial cells and differentiated basal cells were detected by using anti-high molecular weight cytokeratins (Dako, Carpinteria, CA) at 1 μg/ml. Proliferating cells were detected by using monoclonal anti-Ki-67 antibodies (NCL-Ki67-MM11; Novocastra Laboratories) at a dilution of 1:50.

Control sections were incubated with concentration-matched mouse IgG or goat IgG (Dako) instead of primary antibodies. Specificity of E4, C5, and H10 was confirmed by preabsorption of antibodies with 10χ excess immunizing peptide for 3 h prior to incubation on tissue sections.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (Cancilla et al., 2000) with the following modifications. Sections (except those stained for activin receptors) were subjected to microwave antigen retrieval in 10 mM citrate buffer (pH 6.0). In addition, sections stained for high molecular weight cytokeratins were incubated with 0.01% trypsin, 0.2% CaCl2 for 10 min. All sections were then treated with 3% (vol/vol) H2O2 in PBS for 15 min, and blocked with CAS (Zymed) and incubated with double stain enhancer (Zymed) for 10 min. Sections were then incubated with biotinylated-C5 antibody overnight at 4°C. Riboprobes were detected with alkaline phosphatase-conjugated goat anti-digoxigenin (Boehringer Mannheim, Australia) for 30 min at 37°C, stopped with glycine, and treated with acetic anhydride. Sections were then prehybridized at 42°C for 30 min. Riboprobe (at 200 ng/ml) was hybridized onto sections overnight at 42°C. Riboprobes were detected with alkaline phosphatase-conjugated goat anti-digoxigenin (Boehringer Mannheim) and visualized with 5-bromo-chloro-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Boehringer Mannheim).

**Detection of Apoptosis**

Apoptosis was analyzed by a TdT Tag In Situ Apoptosis Detection Kit (Intergen, Purchase, N.Y.). Briefly, sections were dewaxed, incubated in Equilibration Buffer (Intergen), then treated with TdT enzyme in Reaction Buffer (Intergen) for 1 h at 37°C. Sections were washed in Stop/Wash Buffer (Intergen) for 30 min at 37°C, treated with 3% (vol/vol) H2O2 in PBS for 15 min, and blocked with CAS block (Zymed). Apoptotic cells were detected by anti-digoxigenin conjugate (Intergen) for 30 min at room temperature and color-reacted with DAB. The reactions were stopped in water, and sections were counterstained with Mayer’s haematoxylin, dehydrated, cleared, and mounted.

**In Situ Hybridization**

In situ hybridization was performed by using digoxigenin-labeled follistatin riboprobes as previously described (Thomas et al., 1997). This follistatin riboprobe detects both mRNA variants of follistatin. Briefly, sections were dewaxed, rehydrated, digested with 2–5 μg/ml proteinase K (Boehringer Mannheim, Castle Hill, NSW, Australia) for 30 min at 37°C, stopped with glycine, and treated with acetic anhydride. Sections were then prehybridized at 42°C for 30 min. Riboprobe at 200 ng/ml was hybridized onto sections overnight at 42°C. Riboprobes were detected with alkaline phosphatase-conjugated goat anti-digoxigenin (Boehringer Mannheim) and visualized with 5-bromo-chloro-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Boehringer Mannheim).

**RESULTS**

Prostatic Development in Vivo

The development of the rat ventral prostate was divided into three overlapping stages: branching morphogenesis, which occurred between days 0 and 6 postpartum; differentiation, which occurred in a proximal to distal manner and was most pronounced between days 6 and 10; and matura-

tion of secretory function, which began with the onset of puberty and was well established in the adult.
From days 0–4, the prostate consisted of solid epithelial buds (positive for high molecular weight cytokeratins) that grew out from the urogenital sinus toward the ventral mesenchymal pad. These buds branched into and subdivided the mesenchymal pad into caps of aggregated mesenchyme surrounding the tip of each epithelial bud (Figs. 1A–1D). This mesenchymal cap was maintained at all epithelial bud tips until epithelial branching ceased. The solid epithelial cords differentiated to form a polarized epithelium characterized by cuboidal or columnar epithelial cells and basal cells, concurrent with lumen formation (canalization) (Figs. 1E–1H). The developing stroma differentiated into thin smooth muscle sheaths surrounding the epithelial ducts and a fibroblastic stroma containing invading blood vessels (Figs. 1C and 1D). Between days 4 and 10, the solid epithelial cords of distal bud tips continued to branch. Progressive epithelial differentiation and ductal canalization occurred as the prostatic ducts continued to grow (Figs. 1E–1H). Between days 10 and 15, extensive maturation of the differentiated prostatic ducts occurred (Figs. 1G–1J) and secretory activity began.

Prostatic Development in Organ Culture

Development and differentiation in vitro were similar to that observed in vivo. The prostatic explants grew over the 6 days of organ culture in the presence of 10 nM testosterone from a small explant at day 0 with two main ducts per side already having two to three branch points (Fig. 2A) to a much larger organ rudiment with extensive branching of the prostatic epithelial ducts (Fig. 2B). In section, immunohistochemistry for high molecular weight cytokeratins and smooth muscle α-actin revealed a similar architecture to that observed in vivo, with canalized ducts surrounded by a smooth muscle sheath at the center of the explant, and solid epithelial cords (high molecular weight cytokeratin positive) at the periphery of the explant (Fig. 2C). A cap of condensed mesenchyme surrounded the tips of the branching solid epithelial cords (Fig. 2D) similar to that observed in vivo (see above). Cellular proliferation was also examined by using Ki-67, and many positive cells were observed in the ductal tips and mesenchyme (Fig. 2E). Activin A can induce apoptosis of adult prostatic epithelial cells (Wang et al., 1996; McPherson et al., 1999), and therefore we examined the level of apoptosis in control explants cultured with testosterone alone. We found that, although the explant was growing, some apoptotic cells were detected (Fig. 2F).

Effect of Activin A on Prostatic Organ Cultures

Administration of exogenous activin A inhibited ductal branching morphogenesis (Fig. 2G). In the presence of testosterone, overall explant growth was reduced (Figs. 2G and 2H), with fewer branches than the pair-matched control (Figs. 2B and 2C). In activin-treated explants, the epithelial tips and surrounding mesenchymal cap were expanded (Fig. 2I) compared to testosterone-treated explants (Fig. 2D). Stromal differentiation was also inhibited in the activin-treated explants compared to controls. Smooth muscle differentiation, which extended to near the periphery of the explant in control cultures (Fig. 2C), was only observed in the center of the activin-treated explants (Fig. 2H). An expanded zone of undifferentiated mesenchyme was also
observed at the periphery of activin-treated explants (Figs. 2H and 2I) compared to controls (Figs. 2C and 2D). A reduction in proliferation was observed following activin treatment as determined by staining for Ki-67 (Fig. 2J). The alterations in the extent of branching and maturation of the ducts and stroma in the presence of activin A were not due to increased apoptosis in any segment or compartment of activin-treated explants (Fig. 2K) compared to controls (Fig. 2F). A similar effect of activin was observed in the absence of testosterone, where the limited branching was even further reduced by activin A, causing stunted branches and an expanded undifferentiated mesenchymal zone (data not shown).

**Effect of Follistatin on Prostatic Organ Cultures**

Explants treated with follistatin in the presence of testosterone were no different than control explants treated with testosterone alone (Figs. 3A and 3B). Prostatic explants cultured with 10 nM testosterone were growing under maximal growth conditions, thus a further increase in growth may not be observed. We therefore examined prostatic explants in the absence of testosterone. In control explants grown in the absence of testosterone, limited branching was observed (Fig. 3C). In section, these explants had a poorly differentiated stroma with few smooth muscle cells (Fig. 3D). The solid epithelial cords had differentiated in the absence of testosterone to form high molecular weight cytokeratin-positive basal cells and high molecular weight cytokeratin-negative epithelial cells but without canalization of the ducts (Figs. 3D and 3E). Limited proliferation and apoptosis were observed in these explants (Figs. 3F and 3G). Under these conditions, addition of exogenous follistatin increased growth and branching of the prostatic explant (Fig. 3H) beyond that observed in untreated controls (Fig. 3C). The increase in growth with follistatin alone (Fig. 3H) was less than that observed with androgens (Figs. 3A and 3B). The stroma of follistatin-treated explants was more differentiated than controls with smooth muscle surround-

![Figure 2](image_url)

**FIG. 2.** Prostatic development in vitro in the presence of androgens, and the effect of activin A treatment. (A) Day-0 rat ventral prostate with two main ducts that have branched into the ventral mesenchymal pad. (B) Extensive growth and branching is seen in the prostatic explant following 6 days of culture with 10 nM testosterone. (C) Section through the explant, with high molecular weight cytokeratin-positive solid epithelial buds (brown staining) at the periphery and canalized ducts with a smooth muscle sheath (purple) at the center. (D) A solid epithelial bud (high molecular weight cytokeratin-positive) with surrounding mesenchymal cap (arrowheads) is shown at higher magnification. (E) Many proliferating cells (Ki-67 positive, brown staining) are observed in the epithelium and mesenchyme. (F) Some apoptosis was detected in mesenchyme and epithelium (arrows) following organ culture with 10 nM testosterone alone. (G) Pair-matched prostatic explant from the same animal as in (B) following 6 days of culture with 10 nM testosterone and 40 ng/ml activin A. Activin A inhibited branching morphogenesis compared to controls. (H) Section through the explant in (G) showing stunted epithelial buds (high molecular weight cytokeratin-positive), with an expanded cap of mesenchyme (arrowheads), shown at higher magnification in (I). (J) Ki-67 staining revealed a marked decrease in proliferation following activin A treatment compared to control explants (E). (K) No increase in apoptosis was detected (arrows) following organ culture with activin A compared to control (F). Bar in (A), 500 \( \mu \)m in (A–C), (G), and (H). Bar in (D), 50 \( \mu \)m in (D–F) and (I–K).
ing epithelial ducts further toward the periphery of the explant (Figs. 3I and 3J). The epithelial ducts in follistatin-treated explants were similar to untreated controls, with high molecular weight cytokeratin-positive basal cells and high molecular weight cytokeratin-negative epithelial cells (Fig. 3J). Even though the epithelium in the proximal portion of the explant consisted of columnar epithelial cells with basal cells, these ducts did not have a lumen. Cellular proliferation was increased in FS-treated explants (Fig. 3K) compared to controls (Fig. 3F) reflecting the increased growth and branching in these explants. Again, no changes in the level of apoptosis were observed in cultures with the addition of follistatin compared to controls (Figs. 3G and 3L).

Collectively, the organ culture data showed that activin A and follistatin modulated prostatic ductal branching, development, and growth in vitro. We therefore determined whether other members of the activin and inhibin family were involved during prostatic development by examining their expression and localization during prostatic development and differentiation.

Expression of Inhibin Subunit during Prostatic Development

RT-PCR with β-actin primers showed that mRNA was present in all prostate samples from day 0 to adult (Fig. 4A).

![Fig. 3](image-url) Prostatic development in vitro, and the effect of follistatin treatment. (A) Prostatic explant following 6 days of culture with 10 nM testosterone (T), showing extensive growth and branching. (B) Pair-matched prostatic explant following treatment with 10 nM testosterone and 3.5 μg/ml follistatin (+T + FS) grew and branched similarly to the control testosterone-treated explant. Follistatin had no effect under maximal growth conditions (10 nM T). (C) Prostatic explant following 6 days of organ culture in the absence of testosterone (−T); limited ductal branching is seen. (D) Section through the explant showing few branches with some smooth muscle differentiation (purple staining). (E) At higher magnification, the solid epithelial cords have differentiated into high molecular weight cytokeratin-negative epithelial cells and high molecular weight cytokeratin-positive basal cells (brown staining), but these ducts have not canalized in the absence of testosterone. Few proliferating cells can be seen in these explants (F), and apoptosis in these organs is limited (G). (H) Pair-matched prostatic explant from the same animal as in (C) following 6 days of culture with 3.5 μg/ml follistatin in the absence of testosterone (−T + FS). Follistatin increased growth and branching compared to control (C) but not as much as testosterone-treated explants (A, B). (I) Section through the explant in (H) showing epithelial ducts and differentiating smooth muscle stroma. (J) At higher magnification, follistatin treatment in the absence of testosterone resulted in ducts with epithelia that have differentiated into basal cells with overlying cuboidal/columnar epithelial cells. Again, these ducts have not undergone canalization. In addition, the mesenchymal cap is reduced and the differentiating stroma reaches closer to the tip of the differentiating duct than that of controls or testosterone-treated explants. (K) An increase in proliferating cells (Ki-67) is seen in follistatin-treated explants compared to controls. (L) No difference is observed in the levels of apoptosis with follistatin treatment compared to controls. Bar in (A), 500 μm in (A–D), (H), and (I). Bar in (E), 50 μm in (E–G) and (J–L).
RT-PCR showed that inhibin α subunit mRNA was not expressed in the developing prostate (day 0 and 5) but some expression was detected once the prostate had begun to mature at day 10 (Fig. 4B). Expression was then maintained in the pubertal prostate at day 15, day 20, and in the adult prostate at day 90 (Fig. 4B). This indicates that inhibin synthesis does not occur until the prostate begins maturing into a secretory organ. No RT-PCR products were observed in any of the non-RT or H2O controls.

Inhibin α subunit mRNA was not expressed in the developing rat prostate, thus inhibin proteins are not involved in prostatic development. We therefore examined the localization of activin βA and βB subunit proteins and their binding protein, follistatin, during rat prostatic development.

**Immunolocalization of Activin βA Subunit**

Immunolocalization of βA subunit was observed in the rat prostate throughout development during branching morphogenesis, differentiation, and maturation of secretory function (Fig. 5). Distinct immunoreactivity for βA subunit was localized to the cap of undifferentiated mesenchyme surrounding the epithelial bud tips (Figs. 5A and 5B). Immunoreactivity for βA was maintained in the mesenchymal cap at all epithelial bud tips (Figs. 5B–5E) until branching morphogenesis ceased. No βA subunit protein was observed in the undifferentiated mesenchyme at the periphery of the developing organ (Fig. 5A) or in the differentiated stroma that surrounded the ductal epithelium (Figs. 5B–5D). Weak immunostaining for βA subunit was present in the solid epithelial buds at day 0, and was maintained in the epithelial cords as they elongated and branched (Figs. 5A–5E). As the prostate ceased developing and began matura-

![FIG 4. Expression of inhibin α subunit in the developing rat prostate. (A) RT-PCR products for the positive control cytoskeletal β actin (540 bp) were detected in all samples at all ages. (B) RT-PCR products of 656 bp for inhibin α subunit mRNA were not detected until day 10 postpartum. Inhibin α subunit mRNA expression was then maintained in the maturing and adult prostate. For each sample, “+” and “−” indicate the presence or absence of RT and the water control is labeled H2O.](image)

![FIG 5. Localization of activin βA subunit in the developing rat prostate. Immunoreactivity for βA subunit was localized to the mesenchymal cap (arrowheads) surrounding the epithelial bud tips at days 2 (A), 4 (B–D), and 8 (E). Weak immunostaining for βA subunit was seen in the epithelium from days 2–10 (A–E) and was upregulated in the epithelium at day 15 (G–F). βA subunit was not localized to the differentiating smooth muscle stroma (asterisks) in the developing and maturing prostate. This was shown by double staining the sections for βA with α smooth muscle actin (B–D and G–I). Preabsorption of the antibody with the immunizing peptide showed no immunostaining (J, K). Bar, 50 μm for (A) and (E–K), and 100 μm for (B–D).](image)
tion during puberty, immunostaining for \( \beta_1 \) subunit was observed in epithelial cells of canalized ducts at day 10 (Fig. 5F), and was present throughout the epithelium at day 15 (Fig. 5G). No staining was observed in the smooth muscle surrounding the differentiating ducts (Figs. 5H and 5I). This pattern was maintained in the adult rat (data not shown), consistent with activin A as a secretory product of the prostatic epithelium (Risbridger et al., 1996). No staining was observed when the antibodies were preabsorbed with the corresponding activin \( \beta_1 \) subunit immunizing peptide (Figs. 5J and 5K).

**Immunolocalization of Activin \( \beta_1 \) Subunit**

Immunostaining for \( \beta_1 \) subunit during prostatic development was different from that of activin \( \beta_2 \). In contrast to the focal localization of \( \beta_2 \) protein in the mesenchymal cap, \( \beta_1 \) staining was only observed in some cells of the mesenchyme and fibroblastic stroma (Figs. 6A–6D). Weak immunostaining was observed in the solid epithelial cords and canalized ducts (Figs. 6A–6C) during development. In the maturing prostate, strong immunostaining was observed in columnar epithelial cells of the more mature canalized ducts at day 15 (Fig. 6D). No \( \beta_2 \) subunit immunostaining was present in the differentiated smooth muscle stroma (Fig. 6D). Activin \( \beta_2 \) subunit was present throughout the epithelium at day 20 (data not shown) and this pattern of localization was maintained in the epithelium of the adult rat (data not shown).

**Expression and Immunolocalization of Follistatin**

In situ hybridization was performed to detect follistatin mRNA by using a riboprobe that detects both splice variants of follistatin mRNA (FS-288 and FS-315). Follistatin mRNA was expressed throughout the prostatic epithelium during development and differentiation (Figs. 6E–6G), and was maintained in the epithelium of pubertal (Fig. 6H) and adult animals. In differentiating prostates (days 10–15), follistatin mRNA was reduced in the maturing epithelium toward the center of the gland (Fig. 6H). No staining was observed in sense controls at any age (Figs. 6I–6L). Using a rat-reactive antibody to FS-315, this follistatin protein was localized to prostatic epithelium during development and differentiation (Figs. 6M–6O). Immunolocalization was maintained in the epithelium of pubertal (Fig. 6P) and adult animals consistent with the mRNA expression of follistatin in prostatic epithelium.

**Immunolocalization of Activin Receptors**

Activin type I and type II receptors were expressed during prostatic development (Fig. 7). ActRIIA was localized throughout the prostatic epithelium and to the developing stromal compartment (Fig. 7A) with staining in the stroma and developing blood vessels. ActRIIA was strongly localized throughout the prostatic epithelium (Fig. 7B). ActRIIB (Fig. 7C) was also localized predominantly to the developing prostatic epithelium. No immunostaining was observed in the goat IgG negative control (Fig. 7D).

**DISCUSSION**

Activins are growth and differentiation factors with diverse roles in development. This study provides evidence for a functional role of activin A and follistatin in prostatic development. The specific pattern of prostatic expression of activin \( \beta_1 \) and \( \beta_2 \) subunits is consistent with a role for activin A rather than other activin or inhibin ligands in prostatic development. In other systems, the bioactivity of activins may be controlled by follistatin, or regulated by the interplay between activins and inhibins (Hsueh et al., 1987; McLachlan et al., 1987; Risbridger and Cancilla, 2000). Although we detected the inhibin \( \alpha \) subunit in the tubular prostate, inhibin \( \alpha \) subunit was not expressed in the developing prostate. Instead, we detected follistatin mRNA and protein in vivo, and showed that follistatin had an opposing action to activin A in vitro. The predominantly epithelial localization of activin receptors in the developing prostate and co-localization of epithelial activin with follistatin suggests that the precise localization of endogenous activin A within the tubal mesenchyme is required for paracrine control of branching morphogenesis by activin A. These data show that the interplay between activin A and follistatin is important in regulating prostatic branching morphogenesis (summarized in Fig. 8).

Our study and that of Ritvos et al. (1995) provide functional evidence of a role for activin A in branching morphogenesis. The inhibition of branch formation and epithelial elongation observed in the prostate gland was similar to that observed in the kidney, pancreas, and salivary gland (Ritvos et al., 1995). However, the dose of activin A required to achieve inhibition of branching varied between the different organs examined. A lower dose of activin A (40 ng/ml) was required in the prostate (present study) and pancreas (Ritvos et al., 1995), but higher doses of activin A were required for disruption of kidney and salivary gland development. This differential response could be due to the disparate distributions of endogenous activin and follistatin in these structurally distinct branching organs. In the kidney, follistatin expression was localized to the peripheral mesenchyme (Ritvos et al., 1995) in contrast to the epithelial expression we observed in the prostate.

Activin B is involved in branching morphogenesis of other organs. The activin \( \beta_2 \) subunit is essential for normal ductal elongation and alveolar morphogenesis of the estrogen-dependent mammary gland (Robinson and Hennighausen, 1997). Specific roles for activins A and B have not been fully explored due to a lack of purified activin B ligands, but recent in vivo studies show that some of the functions of activin A can be replaced by activin B (Brown et al., 2000). Activin \( \beta_2 \) knockout mice were rescued from some of the major defects caused by the absence of endog-
enous $\beta_a$ subunit by replacing the missing activin $\beta_a$ gene with activin $\beta_b$. However, activin $\beta_b$ could not replace activin $\beta_a$ in developing testes or in ovarian function (Brown et al., 2000).

Previous studies have shown that activin A induces apoptosis of adult prostatic epithelial cells in vitro and in prostatic tumor cell lines (Wang et al., 1996; McPherson et al., 1999). In our study, we found that exogenous activin did not enhance cell death in the developing prostate at any dose examined; no increase in apoptosis was observed in the epithelium or mesenchymal stroma of prostates exposed to exogenous activin A in vitro. Similarly, apoptosis was not observed in epithelia of developing kidney, pancreas, and salivary gland exposed to activin A in vitro (Ritvos et al., 1995). Excess circulating activins in the inhibin $\alpha$ knockout mouse do not induce apoptosis in the developing gastric

**FIG. 6.** Localization of activin $\beta_b$ subunit, and follistatin expression and localization in the developing rat prostate. Weak immunoreactivity for $\beta_b$ subunit was observed in epithelium during development (A–C). At day 15, strong immunostaining was observed in columnar epithelial cells of the more mature canalized ducts (D). Immunostaining for activin $\beta_b$ subunit was also observed in some cells of the mesenchyme and fibroblastic stroma throughout development (A–D). In situ hybridization with follistatin antisense [FS (as)] riboprobes showed that follistatin mRNA was expressed throughout the prostatic epithelium during development, differentiation and maturation (E–H). At day 15, follistatin mRNA was reduced in the maturing epithelium toward the center of the gland (H). No staining was observed with follistatin sense [FS (s)] riboprobes (I–L). Follistatin protein (FS-315) was localized to prostatic epithelium during development, differentiation and maturation (M–P), identical to the pattern of mRNA expression. Bar = 50 $\mu$m for (A–D) and (M–P), and 500 $\mu$m for (E–L).
epithelium but instead the excess activins block differentiation of specific cell types in the gastric mucosa (Li et al., 1998). The lack of apoptosis in response to exogenous activin A may be due to neutralization by endogenous follistatin (McPherson et al., 1999). Alternatively, the developing prostate may not be competent to undergo apoptosis upon exposure to exogenous activin A. In contrast to an apoptotic role for activin A, we did observe a decrease in proliferation in response to activin A treatment, and a corresponding increase in proliferation in response to follistatin treatment. A similar response to activin A was observed in other cell types where activin A inhibited DNA synthesis (McCarthy and Bicknell, 1993; Yasuda et al., 1993; Carcamo et al., 1994).

Although activins are TGFβ family members, the reported actions of TGFβ on branching morphogenesis are not the same as those we have described for activin A. Exogenous TGFβ was shown to inhibit ductal branch formation, but did not inhibit elongation of existing ducts, suggesting that these two processes have distinct controlling mechanisms. This effect of TGFβ was observed in intact organs such as prostate, kidney, and lung (Cunha et al., 1995; Ritvos et al., 1995; Chinoy et al., 1998) as well as in cultures of kidney epithelial cells grown in a mesenchyme-free matrix (Santos and Nigam, 1993; Sakurai and Nigam, 1997). Conversely, exogenous activin A inhibited branch formation and elongation in the developing prostate such that the ductal tips did not bifurcate and elongate but continued to expand as a solid cluster of cells (Fig. 8). The predominantly epithelial localization of activin receptors in the developing prostate suggests that exogenous activin A can act in a paracrine manner on the developing epithelium. Autocrine activity of activin A in prostatic epithelium may be inhibited by the presence of endogenous follistatin.

In addition to epithelial localization, activin βs subunit was localized to the remnants of the ventral mesenchymal pad at the epithelial bud tips but not the surrounding mesenchyme or developing stroma. This pattern of activin βs expression is different from that of TGFβ, which was localized throughout the mesenchyme of the developing mouse and human prostate (Timme et al., 1995; Raghow et al., 1999). The localization of activin βs in the developing prostate was also different from that of another member of the TGFβ family, BMP-4 (Lamm et al., 2001), which was localized to the prostatic mesenchyme between epithelial branch points. Culture with exogenous BMP-4 reduced bud formation from the fetal urogenital sinus (Lamm et al., 2001), which was different from the effects of exogenous activin A on the newborn prostate (present study).

Interestingly, localization of activin βs subunit in the developing rat prostate is more similar to that described for BMP-7 in the developing kidney. BMP-7 was localized to the condensed nephrogenic mesenchyme at the ureteric bud tips, and was also localized to the ureteric epithelium (Godin et al., 1998). Treatment of kidney explants with exogenous BMP-7 in vitro inhibited branching morphogenesis of the ureteric bud (Piscione et al., 1997; Godin et al., 1998) and resulted in an expanded zone of mesenchyme (Dudley et al., 1999). This is similar to the effects of exogenous activin A on prostatic explants observed in the present study. Activin A may play a role in maintaining the tip mesenchyme in the prostate, as exogenous activin A increased the size of the mesenchyme surrounding the prostatic bud tips (Fig. 8).

In the kidney, BMP-7 was found to interact with FGF-2 to promote survival and maintain competence of metanephric mesenchyme (Dudley et al., 1999). These authors suggested that signaling between the two mesenchymal populations of the kidney (nephrogenic mesenchyme and surrounding mesenchyme) maintained the mesenchymal cap at branch tips during kidney development. An interaction between activin and FGF has been shown in the brain, where the neuroprotective effects of FGF-2 during stroke were due to the induction of activin A (Tretter et al., 2000). A similar mechanism may be acting in the prostate between FGFs and activin A, where the promotion of branching morphogenesis by FGFs in the prostate (Sugimura et al., 1996; Thomson and Cunha, 1999) may be modulated by activin A. Thus, a balance between maintenance and growth of ductal tip mesenchyme and rate of ductal branching morphogenesis is essential for proper organogenesis (Fig. 8).

We found follistatin expression and localization in prostatic epithelium throughout development. In addition to neutralizing epithelial activin during development, our in vitro data suggest that follistatin may be modulating the rate of branching morphogenesis of the prostate. A similar
A Epithelial bud growth

activin

follistatin

B Branch formation and expansion of mesenchyme

activin

follistatin

C Cleft stabilization

activin

follistatin

D Ductal elongation

activin

follistatin

mesenchymal cap (endogenous activin)

epithelium (endogenous activin, activin receptors and follistatin)

maturing stroma

FIG. 8. Schematic diagram of the roles of activin A and follistatin in prostatic branching morphogenesis. Branching can be divided into several stages: (A) epithelial bud stage, with a surrounding cap of condensed mesenchyme at the tip and differentiating stroma surrounding the developing epithelium; (B) cleft formation, where the epithelium begins to grow outward from the tip and a cleft is formed, and the mesenchyme expands to cover this extended structure; (C) cleft stabilization, where the newly formed cleft is stabilized by extracellular matrix production, and the expanded mesenchymal zone is now split between the newly formed epithelial branches; and (D) branch extension, where the newly formed branches grow and extend out from the cleft. Stromal development and maturation are concurrent with branch formation and epithelial extension. As the epithelial buds branch and grow, the stroma differentiates and surrounds the maturing epithelium. A balance between endogenous activin and follistatin is required to maintain this process. An excess of activin causes destabilization of the cleft, thus inhibiting branching of the epithelium, and a continual expansion of the mesenchymal cap, thus inhibiting stromal maturation. As proliferation is not inhibited, the epithelial buds continue to expand. An excess of follistatin has the opposite effect, with a reduction in the size of the mesenchymal cap, an increase in cleft stabilization, and more branching. Excess follistatin also causes an increase in maturing stroma surrounding the differentiating epithelial ducts.

effect was observed in the pancreas (Miralles et al., 1998), where the exocrine tissue of pancreatic rudiments does not develop in the absence of mesenchyme in vitro but could be induced by exogenous follistatin. In our study, follistatin was able to increase prostatic development and branching morphogenesis but was unable to mimic the full effects of testosterone on prostatic development in vitro. Our data suggest that a balance between activin and follistatin is important for prostatic branching morphogenesis, but that other factors or hormones are needed for proper ductal canalization and maturation. In addition to the regulation of activin activity, follistatin can also bind to members of the BMP family, such as BMP-4 and BMP-7 (Fainsod et al., 1997; Iemura et al., 1998), although with much lower affinity than for activin. The localization of BMP-7 in the prostate is not known. BMP-4 localization in the developing prostate (Lamm et al., 2001) is different from that of activin β1, but since both BMP-4 and activin A reduced prostatic growth, it is possible that the increase in growth observed with exogenous follistatin may include inhibition of endogenous BMP ligands in prostatic explants.

Activin β1 and follistatin knockout mice die just before birth, therefore the prostatic effects of an absence of activin A or follistatin cannot be determined in these mice (Matzuk et al., 1995a,c). Activin β1 (Vassalli et al., 1994) and activin receptor type II (actRII) knockout mice (Matzuk et al., 1995b) survive after birth and have some defects. Although these mice are fertile, their prostates have not been examined. Transgenic mice expressing a truncated actRII, which acts as a dominant negative activin receptor have been generated. This truncated receptor blocks signaling by activin and other TGF family members (Maeshima et al., 2000). These mice survived after birth and were apparently normal; however, analysis of their kidneys showed that
these mice have more glomeruli than wild-type littermate controls. Glomeruli (and their associated nephron) develop at the tips of the branched ureteric bud. Therefore, increased branching morphogenesis presumably occurred when activin signaling was reduced in the kidney (Mae-shima et al., 2000). We saw a similar effect when follistatin was added to prostatic organ cultures; activin signaling was attenuated, and an increased number of branches was observed.

In conclusion, the present results indicate that activin β, subunit and follistatin, but not activin β subunit or inhibin α, are important during prostatic development. The in vitro data on activin A and follistatin, together with the discrete localization of activin β, to the mesenchymal cap of ductal tips and localization of activin receptors to prostatic epithelium, suggest important roles for activin during branching morphogenesis of the prostate. Our data indicate that activin A acts as a negative regulator of branching morphogenesis, either directly through activin receptors or indirectly through other growth factors and/or inhibitors.

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