

The role of smooth muscle in regulating prostatic induction

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Accepted 21 January 2002

SUMMARY

We have examined the role that smooth muscle plays during prostatic organogenesis and propose that differentiation of a smooth muscle layer regulates prostatic induction by controlling mesenchymal/epithelial interactions. During development of the rat reproductive tract, an area of condensed mesenchyme involved in prostatic organogenesis is formed. This mesenchyme (the ventral mesenchymal pad, VMP) is found in both males and females, yet only males develop a prostate. We demonstrate that a layer of smooth muscle differentiates between the VMP and the urethral epithelium, and that there is a sexually dimorphic difference in the development of this layer. Serial section reconstruction showed that the layer formed at approximately embryonic day 20.5 in females, but did not form in males. In cultures of female reproductive tracts, testosterone was able to regulate the

thickness of this layer resulting in a 2.4-fold reduction in thickness. We observed that prostatic buds were present in some female reproductive tracts, and determined that testosterone was able to stimulate prostatic organogenesis, depending upon the bud position relative to the smooth muscle layer. *In vitro* recombination experiments demonstrated that direct contact with the VMP led to the induction of very few epithelial buds, and that androgens dramatically increased bud development. Taken together, our data suggest that differentiation of a smooth muscle layer regulates signalling between mesenchyme and epithelium, and comprises part of the mechanism regulating prostatic induction.

Key words: Prostate, Organogenesis, Androgens, Smooth muscle, Urogenital development, Rat

INTRODUCTION

Sexually dimorphic development of the reproductive organs has been studied for many years, and androgens have been shown to play a central role in organogenesis of the male reproductive tract. What remain poorly understood are the mechanisms by which androgens elicit development of male specific organs such as the prostate and those derived from the Wolffian duct. The pioneering work of Jost established that testicular factors were required for the formation of the male reproductive organs (Jost, 1947; Jost, 1953). This work demonstrated that development of the reproductive tract was under hormonal control and was independent of the genetic mechanism of sex determination. Administration of androgens to embryos in utero led to the masculinisation of females and development of secondary sex accessory organs such as the prostate. The ability of androgens to masculinise the embryonic female reproductive tract declined with increasing age suggesting that there was a temporal limit on the mechanism by which androgens regulate reproductive organogenesis (Cunha, 1975). Androgens were able to induce prostatic budding in embryonic female reproductive tracts cultured *in vitro* (Takeda et al., 1986), which confirmed that androgens are a primary factor in prostatic organogenesis. Androgens act via the androgen receptor (AR), a member of

the nuclear receptor family of transcription factors, and mice carrying non-functional androgen receptor (*tfm*) do not develop a prostate.

Prostatic organogenesis requires interactions between mesenchyme and epithelium. In addition, androgen receptor expression in the mesenchyme is required for the development of the prostate (Cunha and Chung, 1981). During prostatic induction, AR is expressed in mesenchymal cells but is absent, initially, from epithelial cells (Takeda et al., 1985). Androgen signalling in the mesenchyme is both necessary and sufficient for prostatic organogenesis and epithelial androgen receptor is not required for development of the prostate (Cunha and Chung, 1981). These observations led to the hypothesis that androgens regulate the activity of paracrine-acting factors made by the mesenchyme, which regulate epithelial development. At present, mesenchymal paracrine regulators of prostatic growth have been identified (e.g. FGF7, FGF10 and IGF1) but how androgens may regulate their activity is unclear. It appears that androgens do not directly regulate the genes for FGF7 or FGF10 (Thomson and Cunha, 1999; Thomson et al., 1997), though other studies have suggested that these factors may be androgen regulated (Lu et al., 1999; Yan et al., 1992). As it was possible that expression of paracrine factors was not androgen regulated, alternative mechanisms by which androgens might control development of the prostate were examined.

The mesenchyme involved in prostatic induction includes the peri-urethral mesenchyme and ventral mesenchymal pad (VMP), a condensed pad of mesenchyme peripheral to the urethral epithelium that is found in both males and females. Tissue recombination studies have shown that the VMP of females is able to induce prostatic development of a heterologous epithelium in response to testosterone (Timms et al., 1995). As the VMP is present in both sexes, it does not appear that androgens are involved in the genesis of the VMP. Furthermore, there appears to be constitutive expression of fibroblast growth factor 10 (FGF10) in the VMP of both males and females (Thomson and Cunha, 1999). FGF10 has been shown to function as a regulator of lung branching morphogenesis and limb induction (Min et al., 1998; Sekine et al., 1999). FGF10 is a key mesenchymal regulator of prostate development and is required for prostatic organogenesis (A. Donjacour and G. R. C., unpublished). FGF10 expression is constitutive in embryonic males and females and the *Fgf10* gene does not appear to be directly regulated by testosterone in cells or organs grown in vitro (Thomson and Cunha, 1999). This raised the question of how androgens might regulate the prostatic inductive activity of the VMP and led to the study of the role of smooth muscle (SM) in regulating prostatic induction.

Smooth muscle appears in the rat urogenital sinus at approximately embryonic day (E) 15 and is formed by the differentiation of mesenchymal cells, probably in response to epithelial signals (Hayward et al., 1998). The mesenchyme surrounding the urethral epithelium can be subdivided into three zones. The first zone of peri-urethral mesenchyme lies immediately adjacent to the basement membrane and remains mesenchymal during prenatal stages. This subepithelial zone is surrounded by a zone that undergoes SM differentiation starting at approximately E15. This layer is in turn surrounded (partially) by a third mesenchymal zone that contains the VMP. The SM layer surrounds the urethra and extends cranially as part of the detrusor muscle. In the bladder, the SM layer is thick and provides support and elasticity required for bladder function. In the urethra, SM forms a tube encasing the urethral epithelium and peri-urethral mesenchyme. The SM layers of the urethra and bladder meet below the base of the bladder in the region destined to form the prostate, which contains the VMP. The pattern of SM distribution in the prostate, and other organs, appears to be regulated by epithelial signals. This was demonstrated by tissue recombination studies using either human or rat urogenital epithelia. Human prostatic epithelium induced mesenchymal differentiation into thick layers of SM, while rodent prostatic epithelium induced thin layers of SM (Hayward et al., 1998). The nature of the epithelial-to-mesenchymal signalling involved in SM differentiation is not yet known, though members of the TGF β family stimulate expression of smooth muscle markers in cultures of stromal cells (Peehl and Sellers, 1998). Other molecules involved in the differentiation of SM in visceral organs throughout the body include Pod1 (Hidai et al., 1998; Lu et al., 1998) and sonic hedgehog (Ramalho-Santos et al., 2000), though it is not known if these regulate SM pattern in the urogenital tract. The differentiation of circular and longitudinal layers of SM at the periphery of the gut is regulated by sonic hedgehog (Sukegawa et al., 2000).

We have examined the role that smooth muscle might play

in regulating prostatic induction. Our hypothesis is that the differentiation of smooth muscle during prostatic development regulates signalling between mesenchyme and epithelium, and constitutes a mechanism involved in regulating prostatic induction. In particular, it appears that SM forms a layer separating prostatic inducing mesenchyme in the VMP from prostatic buds that have emerged from the urethra. We show that androgens are able to regulate the thickness of this SM layer, and that androgens have little effect upon SM mitogenesis. Prostatic buds were present in a significant proportion of female rat embryos, and those showing advanced buds developed prostate-like structures in response to testosterone only if the buds had penetrated the SM layer and could interact with the VMP. We propose that androgens control prostatic induction by regulating differentiation of the SM layer and consequently signalling between the VMP and prostatic buds.

MATERIALS AND METHODS

Histology and serial section reconstruction

Animals used in our studies were outbred rats of Wistar and Sprague Dawley strains. Embryos were obtained from mated animals where the observation of a copulatory plug was taken as E0.5 and the day of birth was designated P0. Tissues were fixed in Bouin's fluid or 4% paraformaldehyde, stored in 70% ethanol, and processed for histology followed by paraffin wax embedding and sectioning.

Images of whole reproductive tracts or organs grown in vitro were obtained using a Leica MZ6 dissection microscope, a Leica ICA camera and a Mac G3 computer with Adobe Photoshop and Scion Image software. Photomicrographs of histological sections were taken on an Olympus Provis microscope with a Kodak DCS330 camera and a Mac G3 computer with Adobe Photoshop software. Serial section reconstruction was carried out as previously described (Timms et al., 1994), by tracing the outline of anatomical regions using surface rendering software (SURFdriver, University of Hawaii) and creating 3D images of the developing UGT.

Organ culture and tissue recombination

Neonatal female UGTs were micro-dissected from P0 Wistar rats and grown in serum-free organ culture. Organs were grown and treated with testosterone as previously described (Thomson et al., 1997). For in vitro recombination studies, female UGTs (devoid of epithelial buds) were treated with 1% trypsin in 50:50 DMEM:Hams F12 for 1-1.5 hours at 4°C, followed by mechanical isolation of the epithelium using fine forceps. The trypsin was neutralised by removal of trypsin-containing medium and replacement with 50:50 medium containing 10% foetal calf serum.

Immunohistochemistry

Paraffin sections were dewaxed in xylene and rehydrated through graded ethanol dilutions. Endogenous peroxidase activity was inhibited by incubation of slides in 3% hydrogen peroxide/methanol solution at room temperature for 30 minutes, followed by rinsing with water and a 5 minute incubation in Tris-buffered saline pH 7.4 (TBS). Next, slides were incubated with 20% normal rabbit serum in 5% BSA diluted in TBS for 30 minutes at room temperature. Mouse monoclonal anti- α smooth muscle actin (Sigma, Poole, UK) was diluted 1:5000 in 20% normal rabbit serum/5% BSA/TBS and added to the sections followed by incubation overnight at 4°C. Slides were washed for 5 minutes in TBS three times, followed by incubation with rabbit anti-mouse biotinylated antibody (Dako, Denmark) for 30 minutes at room temperature. Slides were washed in TBS for 5 minutes three times. ABC-HRP complex (Dako, Denmark) was added

for 30 minutes at room temperature, followed by three washes of 5 minutes each with TBS. Antibody localisation was detected by addition of the DAB chromogen (Dako, Denmark) for 1-5 minutes until staining was visible, followed by washing with TBS/water. Slides were counterstained with Haematoxylin, dehydrated with graded ethanols and mounted in pterex.

AR and SM co-localisation studies were performed using antibodies to smooth muscle α actin (monoclonal, Sigma, Poole, UK) and AR (rabbit, Santa Cruz Biotech, Santa Cruz, CA), and antibodies were visualised with anti-mouse Cy5 (Amersham Pharmacia, Little Chalfont, UK) and biotinylated anti-rabbit (Vector labs, Burlingame, CA) with avidin FITC (Sigma, Poole, UK). Sections were incubated with Propidium Iodide (20 μ g/ml), washed in TBS, and observed on a Zeiss LSM confocal microscope. Mitogenic rates were measured by immunostaining of samples with anti-BrdU antibody (Sheep, Fitzgerald Industries International, Concord, MA) and co-localisation with smooth muscle α actin.

Morphometric measurements

Smooth muscle thickness in immunostained sections was measured using Image Pro Plus software (Media Cybernetics, Maryland, USA). Measurements were made from sections cut in both the longitudinal plane of section as well as transverse plane of section, to minimise possible artifacts introduced by sectioning.

RESULTS

The anatomy of P0 male and female reproductive tracts is shown in Fig. 1 (A,B are male; C,D are female). The VMP in females corresponds to an area of condensed mesenchyme surrounding the ventral and lateral urethral epithelium (Fig. 1C, labelled VMP and outlined in grey) and is in a position analogous to that of the ventral prostate in the male (Fig. 1A, labelled VP and outlined). The rat VMP is visible from approximately E17 until P12-15 in females. Tissue recombination studies (in vivo) have shown that the prostatic inductive activity of the rat VMP is retained until approximately P12-15 (P. Young and G. R. C., unpublished). Studies in the mouse (in vivo) have shown that the ability of the female UGT to respond to testosterone by inducing prostate is gradually lost between P1 and P5 (Cunha, 1975). We propose that, for nomenclature purposes, VMP corresponds to the structure of condensed mesenchyme before E20-E21, which is devoid of epithelia (urothelium), and that after invasion by epithelial buds the structure is termed the VP.

We speculated that there might be a mechanism to regulate the inductive activity of the VMP and that it was possible that a layer of SM might form between the VMP and urethral epithelium. This SM layer might affect contact or signalling (or both) between the urethral epithelium and the VMP. To examine the anatomy of the SM, VMP and urethra in detail, serial sections of male and female UGTs (three samples per timepoint and sex) were stained with an antibody against smooth muscle α actin. The stained serial sections were then used for 3D reconstruction to show the spatial distribution of SM, urethral epithelium, VMP and prostatic buds emerging from the urethra. Samples are illustrated with shading and surface rendering to simulate the 3D nature of the structures, and the VMP is drawn as semi-transparent in order to show the underlying urethra and SM layer. Comparison of VMP and SM anatomy in male and female embryos between E17-E20 is shown in Fig. 2. At E17-E19, both male and female exhibit a

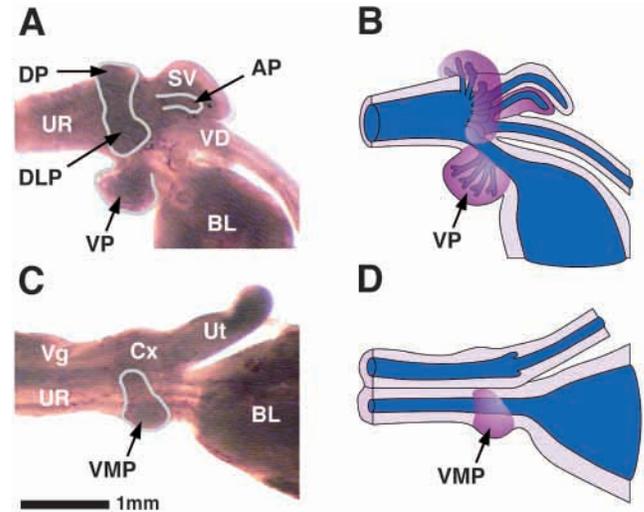


Fig. 1. Anatomy of perinatal rat urethra and associated reproductive organs. Orientation is as follows: top of figure is dorsal, bottom is ventral, left is caudal and right is cranial. (A) A P0 male rat urogenital tract: DP, dorsal prostate; DLP, dorsolateral prostate; AP, anterior prostate (also termed coagulating gland); VP, ventral prostate; UR, urethra; VD, vas deferens; BL, bladder. (C) A P0 female urogenital tract: VMP, ventral mesenchymal pad; UR, urethra; BL, bladder; Vg, vagina; Cx, cervix; Ut, uterus. (B,D) Diagrams of A,C showing the relative positions of the VP and VMP. Scale bar: 1 mm.

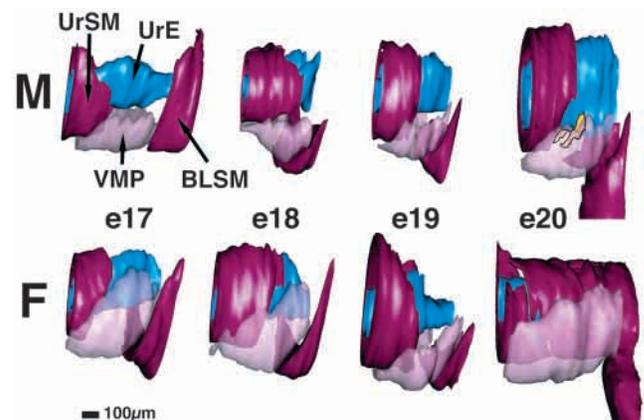


Fig. 2. Serial section reconstruction of embryonic (E17-E20) male and female urethral UGT. The urethral epithelium (UrE) is shown in blue, SM originating from the urethra (UrSM) and bladder (BLSM) is shown in magenta, and the VMP is shown in pink. At E20, in the male, a prostatic bud emerging from the urethra is shown in yellow. Comparison of male with female shows a discontinuity between the urethral SM and the bladder SM. The VMP aligns with the SM discontinuity. In both males and females the SM is discontinuous from E17 to E19. At E20, in females the SM layer has become continuous, while in males it remains discontinuous.

gap in the SM at the junction of urethral SM and bladder SM immediately below the bladder where the prostate develops. This gap was coincident with the position of the VMP, and there was no SM between the VMP and urethral epithelium. At E20 there appeared to be a difference between male and female, as the SM layer had become confluent in the female

but remained discontinuous in the male. A ventral prostatic bud (yellow) is visible emerging from the urethra in the male at E20. This, and other ventral buds, will grow towards the VMP through the gap between peri-urethral and bladder SM, make contact with the VMP, and undergo branching morphogenesis.

The identification of a sexually dimorphic difference in SM distribution led us to investigate if testosterone might play a role in the development of this difference in SM patterning. Many studies have examined the development of a prostate in response to testosterone in embryonic female reproductive tracts (both in vivo and in vitro), and thus we decided to use postnatal female reproductive tracts as our model system. Initially, we studied the effect of testosterone on the distribution and thickness of SM in cultures of P0 female urethra and VMP grown for 6 days in vitro under serum free conditions. In these P0 UGT specimens, the SM layer was present at the start of the culture period. Culture of P0 female UGT with or without testosterone lead to minor changes in the gross morphology of the VMP and urethra (shown in Fig. 4B), but an immunohistochemical study of SM α actin in UGT cultures showed an effect of testosterone on the SM layer (Fig. 3). Fig. 3A,B, show the SM thickness in UGT cultured in the absence of testosterone, while Fig. 3C,D show SM thickness after culture in the presence of testosterone. A significant reduction in SM thickness occurred in samples grown in the presence of testosterone, when compared with those grown without testosterone. The pattern of SM thickness in samples grown without testosterone was almost identical to that in vivo observed in P0-6 female UGT (P3 female UGT shown in Fig. 3E), suggesting that the culture system was a good model of the in vivo situation. To quantitate the effect of testosterone on SM thickness, we made morphometric measurements of the SM layer and the results are shown in Fig. 3F. Treatment with testosterone lead to a 2.4-fold reduction in SM thickness in the P0 female UGT grown for 6 days in vitro (628 measurements; 36 specimens; five experiments). Quantitation of SM α actin by western blotting in cultures of VMPs has demonstrated a two- to fourfold reduction of α actin after treatment with testosterone (data not shown).

We next examined the effect of testosterone on the mitogenic rate of cells in the SM layer in BrdU incorporation studies. VMPs were grown in the presence or absence of testosterone for 6 days, and treated with BrdU for 2 hours before fixation. Samples were sectioned and stained for BrdU and SM α actin (not shown), and the percentage of SM cells positive for BrdU determined. In the presence of testosterone 2.43% of cells in the SM layer were positive for BrdU (92/3779 cells), while in the absence of testosterone, 3.11% of cells were positive for BrdU (268/8608; $n=4$ experiments). We were able to count more cells in the samples grown without testosterone because the SM layer was thicker in these samples. Statistical comparison of the mitogenic rates by paired t -test showed that there was no difference ($P=0.05$) in the growth rate in the presence or absence of testosterone. Additionally, we examined if there might be a change in cell size in response to testosterone. Morphometric measurement of the SM area followed by division by the cell number allowed us to estimate the average cell volume, and there was little or no difference between samples grown in the presence or absence of testosterone.

During the course of our studies we observed that a small

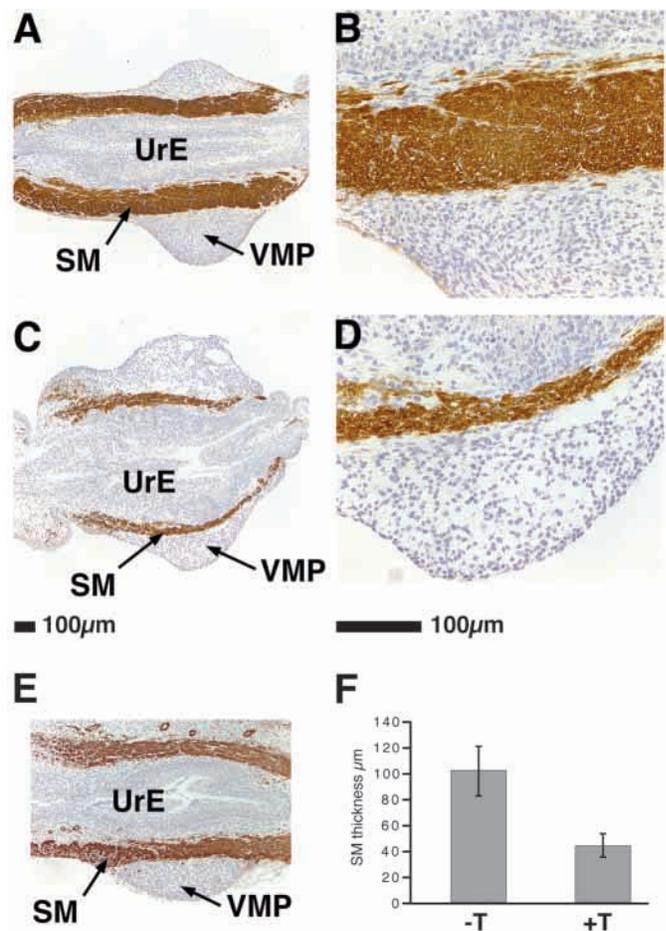


Fig. 3. The effect of testosterone on SM in P0 female UGTs grown in vitro. (A,B) SM distribution in P0 female UGT grown in vitro for six days in the absence of testosterone; (C,D) a similar culture to which 1×10^{-8} M testosterone has been added. Scale bars shown represent 100 μm . (E) UGT from a P3 female. (F) A graph of SM thickness in response to testosterone (\pm s.d., five experiments, 628 measurements). Testosterone treatment led to a 2.4-fold reduction in SM thickness.

percentage of P0 female UGTs had epithelial prostate-like buds that had emerged from the urethra (Fig. 4A). The epithelial buds observed in females varied in size and position, as well as in the frequency with which they were observed. The appearance of female UGTs with prostate-like buds was highly variable, and in many litters no female UGTs with buds were observed. In some litters, up to 50% of the females UGTs showed epithelial buds. We were not able to identify what might cause this variability in the appearance of epithelial buds in females, though it has been suggested that intra-uterine position of embryos may be a factor (Timms et al., 1999). A series of female UGTs showing different sizes and positions of epithelial buds is shown in Fig. 4A. On the left-hand side is a female UGT without buds, while specimens to the right show increasing development of buds (bud position indicated by arrowheads). The two rightmost specimens show buds that have emerged from the urethra and have extended into the VMP.

Next, we examined the effect of testosterone on P0 female UGTs in which buds were present or absent (Fig. 4B). The

UGTs on the left hand side did not have any buds present before culture and testosterone did not induce the formation of buds in these specimens when the specimens were examined at the end of the culture period. The effect of testosterone on female UGTs where buds were present before culture is shown on the right-hand side of Fig. 4B. Culture of UGTs with buds in the presence of testosterone led to prostatic organogenesis, while in the absence of testosterone the pre-existing buds were no longer visible. The size and position of the prostatic buds was a key determinant of their response to testosterone in culture. Samples showing small buds not extending to the VMP did not undergo bud development or branching morphogenesis in response to testosterone. By contrast, buds underwent branching morphogenesis in response to testosterone only if they were significantly advanced and closely juxtaposed to (or embedded in) the VMP ($n=6$ experiments, 20 organs). This led us to investigate if contact with the VMP was a key requirement for subsequent bud growth and branching morphogenesis.

To determine if contact with the VMP was a key determinant of bud development, we performed tissue recombination studies in vitro using female UGT and urethral epithelium. Our hypothesis was that direct contact between the VMP and urethral epithelium (applied by recombination) might lead to budding, as there would be no smooth muscle layer separating VMP and epithelium. Urethral epithelium (without buds) was isolated from P0 female UGT and recombined on top of the VMP of another UGT, followed by culture in vitro for 6 days in the presence or absence of testosterone ($n=7$ experiments, 62 organs). Fig. 5A shows a schematic diagram describing the recombination experiment and Fig. 5B shows the results of recombinants grown in vitro with or without testosterone. Recombined epithelium is indicated by arrowheads. In the absence of testosterone, there were few or no buds emerging from the recombined epithelia. In the presence of testosterone, there were numerous buds and perhaps some branching morphogenesis (Fig 5B).

Contact with the VMP did not appear to be sufficient to induce extensive epithelial budding, and testosterone stimulated budding in epithelium recombined with VMP and grown in vitro. Next, we examined the pattern of smooth muscle differentiation in recombinants of VMP and epithelium to determine if testosterone was regulating the pattern of SM differentiation in the recombination system. Fig. 6 shows the pattern of SM differentiation in recombinants of VMP and urethral epithelium grown with (Fig. 6C,D) or without (Fig. 6A,B) testosterone. In the absence of testosterone, a layer of smooth muscle differentiated directly adjacent to the recombined epithelium (marked by an arrow and arrowhead, respectively), and few or no epithelial buds were observed extending through the SM into the VMP. In the presence of testosterone, a layer of smooth muscle differentiated adjacent to the recombined epithelium on the surface of the VMP. The SM layer was traversed by epithelial buds undergoing branching morphogenesis within the VMP. SM was also observed in close association with the developing epithelial buds. Taken together, it appeared that testosterone did not affect the pattern of SM differentiation in the recombination model though there may have been effects on the amount or rate of SM differentiation. Testosterone altered SM thickness

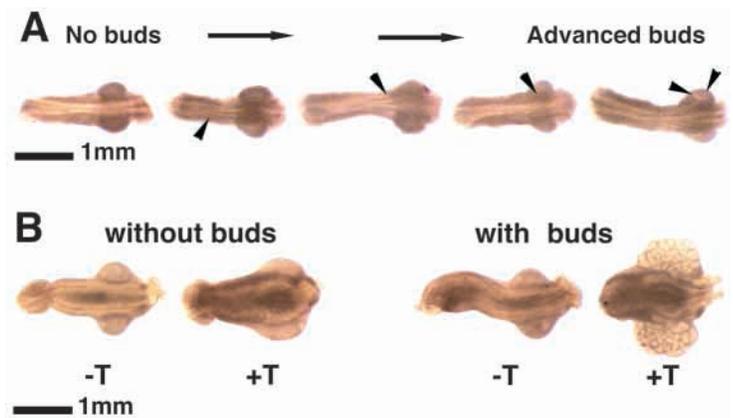


Fig. 4. Emergence of prostatic buds in the female UGT, and the effect of testosterone on female UGT with or without prostatic buds. (A) Five female UGTs with the VMP at the cranial end (right side). The leftmost sample shows no prostatic buds, while the next three samples show increasing bud size and proximity to the VMP, and the rightmost sample shows a bifurcated prostatic bud within the VMP. Buds are marked by an arrowhead. (B) Left side shows the effect of testosterone (T) on female UGTs without prostatic buds; right side shows female UGTs with prostatic buds grown in the presence or absence of testosterone.

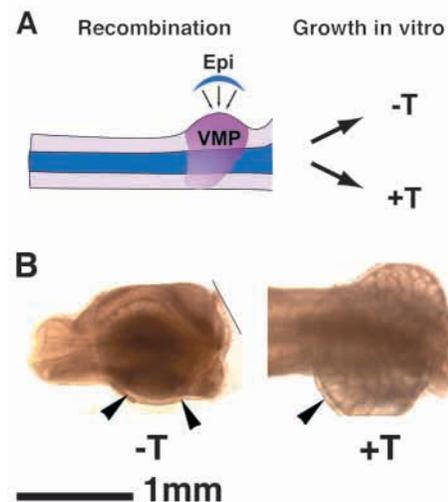


Fig. 5. Recombination of epithelium with female UGT/VMP. (A) A schematic diagram of the recombination, where isolated urethral epithelium was placed on top of the VMP of a female UGT, followed by 6 days growth in vitro with or without testosterone. (B) Left side shows a recombination grown in the absence of testosterone, and the recombined epithelium is indicated by arrowheads. Right side shows a recombination grown in the presence of testosterone; arrowhead shows recombined epithelium on the surface of the VMP; numerous epithelial buds can be seen extending into the VMP. Scale bar: 1 mm.

in the urethral SM in cultures of UGT (Fig. 3) but did not appear to alter SM thickness in the recombination model. It is possible that there are differences between endogenous urethral SM and SM induced by epithelium in the recombination model. In support of this, it is interesting to note that the urethral and prostatic SM exhibit differences in AR staining pattern (Fig. 7).

Because androgens were able to regulate SM thickness in

cultures of female UGT, we investigated expression of AR in the SM of P0 male and female reproductive tracts, as well as female UGT grown in vitro (\pm testosterone). We performed co-localisation studies by immunohistochemistry in which we compared expression of AR and SM alpha actin (Fig. 7). We used P0 male (Fig. 7A-D) and female (Fig. 7E-H) reproductive

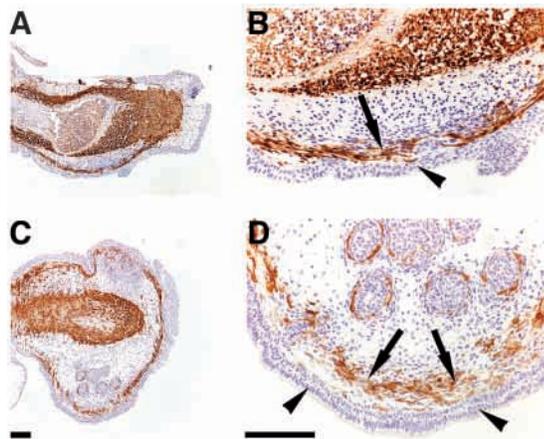


Fig. 6. Immunohistochemistry for SM α actin in female VMP/epithelium recombinants. (A,B) SM α actin distribution in recombinants grown in the absence of testosterone. (C,D) Recombinants grown in the presence of testosterone. Arrowheads mark the recombined epithelium; arrows mark SM induced by the recombined epithelium. Scale bars: 100 μ m.

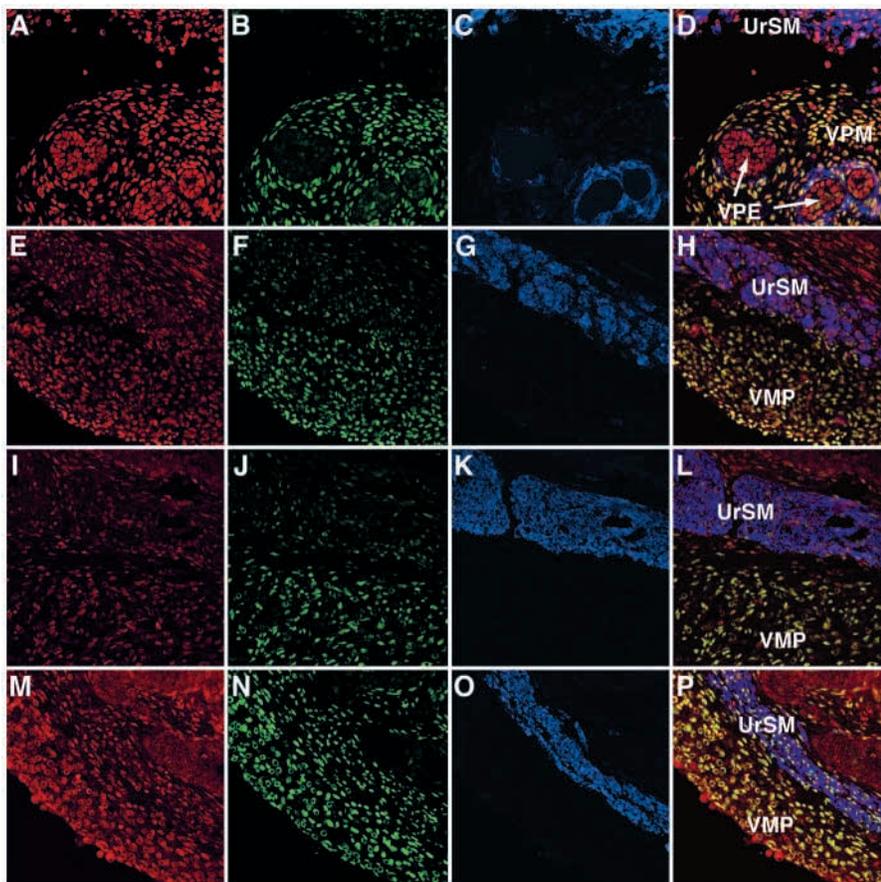


Fig. 7. Co-localisation studies of AR and SM. Propidium Iodide (PI) staining of nuclei is shown in red, AR is shown in green, SM is shown in blue and the merged images are shown on the right-hand side. PI/AR co-expression results in a yellow colour. (A-D) P0 male UGT, showing urethral SM (UrSM), epithelial buds of the ventral prostate (VPE) and prostatic mesenchyme (VPM). (D) UrSM contains little or no AR, whereas that SM associated with the VPE is AR positive, as is VPM. (E-H) P0 female UGT, where UrSM and VPM are visible. AR levels in the P0 female UrSM appear lower than those observed in the VPM. (I-L) A female UGT grown in vitro in the absence of testosterone; the SM layer shows lower levels of AR than the VPM. (M-P) A P0 female UGT grown in the presence of testosterone, where levels of AR in the SM layer are similar to those in the VPM. Comparison of L and P suggests that AR expression has been upregulated in the UrSM.

tracts, as well as P0 female VMPs cultured in the absence (Fig. 7I-L) or presence (Fig. 7M-P) of testosterone. Nuclei were stained with Propidium Iodide (red; Fig. 7A,E,I,M), AR was observed with FITC (green; Fig. 7B,F,J,N), SM was observed with Cy5 (blue; Fig. 7C,G,K,O) and merged images are shown in Fig. 7D,H,L,P. Nuclear AR staining is seen as yellow in merged images. In the P0 male UGT, AR was abundant in the ventral prostatic mesenchyme (VPM), as well as SM surrounding prostatic buds (VPE), but was low or absent in urethral SM (UrSM). In the P0 female UGT, AR was observed in the VMP, and at low levels in the urethral SM, in a similar pattern to that observed in males. In cultures of female VMP, it appeared that AR levels were low in SM of samples grown without testosterone but were increased in the urethral SM in the presence of testosterone.

DISCUSSION

Many studies have examined the effect of testosterone on the embryonic female UGT, and have shown that administration of testosterone leads to prostatic induction in vivo or in vitro (Cunha and Chung, 1981; Jost, 1947; Jost, 1953; Takeda et al., 1986). Androgen signalling via AR in mesenchymal cells is required for prostatic development, but other details of how prostatic induction occurs are not known. Comparison of P0 male and female rat reproductive tracts showed a similarity in the anatomy of the male VP and female VMP. The female VMP induces prostatic development in tissue recombination studies in vivo in response to testosterone (Timms et al., 1995). It is clear that mesenchyme in the VMP has prostatic inductive activity, and that androgens are involved in prostatic induction and growth. What is less clear is how androgens regulate inductive activity of the VMP mesenchyme.

We report that the position of the VMP was coincident with a gap in the SM at the

junction of the urethra and bladder. The discontinuity in peri-urethral SM was similar in both males and females from E17 until E19, after which a sexually dimorphic difference was observed. In E20 females, a layer of SM formed between the urethral epithelium and the VMP, whereas in males this SM layer did not become continuous. Ventral prostatic buds that emerged from the male urethra were anatomically positioned in the middle of the SM discontinuity and directly adjacent to the VMP. A model describing the chronology and anatomy of prostatic bud induction is shown in Fig. 8. On the left side is a UGT between E17 and E18.5 showing an 'open' SM layer (i.e. a gap between urethral and bladder SM). We propose that the gap in SM allows interaction between the VMP, urethral epithelium and prostatic buds that have emerged from the urethra. The gap in the SM layer may allow inductive signals from the VMP to reach the urethral epithelium and induce budding, or epithelial buds (perhaps made constitutively in males and females) to come into contact with the VMP. Buds of the VP are first observed at E18.5 in the rat (Timms et al., 1994). On the right of Fig. 8 are male and female UGTs at E21.5. Development of the UGT in females (without androgens) leads to thickening of the SM layer and inhibition of prostatic induction, though some budding may have occurred. We propose that separation or isolation of the VMP from the urethral epithelium by SM prevents prostate development in females. In males (with androgens), formation of the SM layer is inhibited or delayed, and prostatic buds emerging from the urethra can penetrate the VMP, which elicits further epithelial growth, mesenchymal/epithelial interactions and branching morphogenesis. This study demonstrates that testosterone can affect the thickness of the peri-urethral SM layer and suggests that inhibition of SM differentiation by androgens enables prostatic induction in males.

One of the questions raised by our observations is 'how do androgens regulate SM thickness in the peri-urethral SM?' We did not observe any difference in the rate of SM mitogenesis in the presence or absence of testosterone, in cultures of female UGT grown *in vitro*. Our experiments measuring SM growth rate determined the mitogenic rate after 6 days of culture (\pm testosterone) and it is possible that there may be an effect of

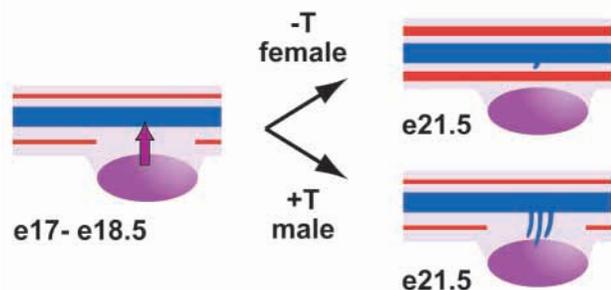


Fig. 8. A model describing ventral prostate induction. On the left-hand side is a UGT during the initial stages of prostatic induction. The SM layer is discontinuous and signalling between the VMP and urethra occurs. At E18.5, prostatic buds become visible, in males. On the upper right side of the figure is a female at E21.5, showing the SM layer has formed and that interaction between the VMP and urethra is prevented. Residual buds may be present, but these do not enter the VMP and eventually regress. On the lower right-hand side is a male at E21.5 showing a discontinuous SM layer, prostatic buds have emerged from the urethra and entered the VMP, where subsequent growth and branching morphogenesis takes place.

testosterone at earlier timepoints. After 6 days of culture, the SM layer became thinner in response to testosterone, which could have involved a decrease in mitogenic rate before the thinning was apparent – we are presently addressing this issue. Changes in SM thickness did not appear to be due to differences in cell volume, as this was not affected in samples grown with or without testosterone. We observed that AR was present in SM, though at levels below those observed in prostatic SM or in the VMP. The effect of androgens on SM may be direct as our experiments have shown that AR is expressed in SM in the UGT. However, it is possible that androgens may affect the differentiation of mesenchyme into SM, and thus function via an indirect pathway to result in changes in SM thickness or pattern. The molecular mechanism by which androgens and the AR regulate SM thickness is not known but represents an interesting avenue for future investigation.

It has been shown that patterning of SM surrounding prostatic epithelia is regulated by epithelial signals. In these studies, SM differentiated in intimate association with prostatic epithelial ducts and was patterned by the epithelial signals (Hayward et al., 1998). The urethral SM is not directly juxtaposed to the epithelium, and thus there may be differences in the function, type or differentiation mechanisms of SM present around the urethra and prostatic ducts. Our studies on AR expression suggest that there is a difference between urethral and prostatic SM, and this may affect the differentiation or function of these SM compartments.

The induction of prostatic organogenesis by testosterone is well established, yet our studies showed that we could not induce prostatic buds visible by whole-mount imaging in cultures of P0 female UGT by treatment with testosterone (Figs 3,4B). This was most probably due to the use of older (postnatal) tissue rather than embryonic tissue. We propose that thickening of the urethral SM layer contributed to the loss of bud induction in response to testosterone, though other temporal factors may be involved. *In vivo*, the ability to develop a prostate in response to testosterone is progressively lost with increasing age (Cunha, 1975) and this may be due to SM thickening or an age-dependent loss of molecules required for prostatic induction.

At present, it is not clear how prostatic buds arise or whether they occur ubiquitously in males and females. Perhaps formation of prostatic buds is a constitutive process and subsequent development of these buds within the VMP is regulated by androgens. Alternatively, perhaps signals from the VMP induce prostatic buds in the urethral epithelium. Our results favour the latter mechanism, but do not rule out the former. We observed prostatic buds in some female embryos and examined the effect of testosterone on these buds. When female UGTs with buds were grown with testosterone, bud position along the caudal/cranial axis of the urethra was a key determinant of the response to testosterone (Fig. 4), and a prostate-like structure formed only if buds were closely juxtaposed to (or embedded in) the VMP. In recombination experiments (Fig. 5), it appeared that direct contact between the VMP and epithelium induced very few buds and that testosterone stimulated or augmented bud induction. We propose that the role of androgens in regulating prostatic organogenesis is to inactivate an inhibitory mechanism that prevents interactions or signalling between urethral epithelia and the VMP. During later stages of prostatic ductal growth and branching morphogenesis, other regulatory mechanisms may

become active, and androgens may control some or all of these mechanisms.

Nkx3.1 is a transcription factor expressed in prostatic epithelia and in the urethral epithelium prior to bud induction (Bhatia-Gaur et al., 1999). The expression pattern of Nkx3.1 has led to the suggestion that there is a 'pre-pattern' in the urethral epithelium that defines the position at which epithelial buds will form. However, it is possible that Nkx3.1 expression is a response to inductive signals from the VMP, or that Nkx3.1 is a marker of a constitutive budding mechanism, that results in budding in both males and females. The observation that we could induce buds in urethral epithelia in recombination experiments supports the idea that Nkx3.1 may be induced in response to signals from the VMP.

Testosterone did not appear to affect the SM differentiation induced by epithelium in the recombination experiments (Fig. 6). This is in contrast to the effect of testosterone on peri-urethral SM thickness (Fig. 3). We propose that peri-urethral SM may be different from SM induced by recombined epithelium, or prostatic SM; and we observed differences in levels of AR expression in peri-urethral SM and prostatic SM (Fig. 7). In the recombination system, it is possible that addition of epithelium caused a rapid differentiation of SM, which led to changes in local growth factor signalling. In the presence of testosterone, the SM differentiation was delayed sufficiently to allow growth factor signalling and induction of buds. It is well established that epithelial signals pattern SM differentiation in the prostate (Hayward et al., 1998) and it will be important to address the kinetics of SM differentiation in response to testosterone in urethral SM and prostatic SM.

The VMP of males and females contains transcripts for FGF10, a factor required for prostatic development. Furthermore, *Fgf10* does not appear to be regulated by testosterone in vivo or in organs grown in vitro (Thomson and Cunha, 1999). It has been proposed that androgens regulate prostatic growth by controlling expression of paracrine acting factors (Lu et al., 1999; Yan et al., 1992). This may be true but, as yet, no mesenchymal paracrine factors have been shown (unequivocally) to be regulated by androgens in the prostate. There are many other possible mechanisms by which androgens might regulate the function of mesenchymal paracrine signalling. It is possible that regulatory molecules are expressed constitutively in mesenchyme of males and females but that androgens regulate the protein synthesis, distribution or activation of factors. Additionally, androgens may regulate expression of co-factors such as heparan sulphate glycoproteins or control signal transduction pathways. Our data suggest that androgens regulate differentiation of SM and thus control signalling involved in prostatic induction. This may be by limiting access to, or diffusion of, inductive factors. It is important to remember that prostatic organogenesis may involve several mechanisms and molecules; thus, it is possible that several mechanisms are active at different stages of prostatic development.

We thank Ted Pinner for help with figure preparation, and Denis Doogan and Jim MacDonald for technical assistance. We also thank Mike Millar and Sheila McPherson for advice on immunostaining and patient instruction in confocal imaging. Supported by the Medical Research Council and grants from the CDMRP Prostate Cancer Research Programme DAMD17-00-1-0034 (to A. A. T.); by the South Dakota Health Research Foundation (to B. G. T.); and by grants

DK47517, DK52708, CA64872, CA59831, CA84294 and CA89520 (to G. R. C.).

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