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Sex specific retinoic acid signaling is required for the initiation of urogenital sinus bud development

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ABSTRACT

The mammalian urogenital sinus (UGS) develops in a sex specific manner, giving rise to the prostate in the male and the sinus vagina in the embryonic female. Androgens, produced by the embryonic testis, have been shown to be crucial to this process. In this study we show that retinoic acid signaling is required for the initial stages of bud development from the male UGS. Enzymes involved in retinoic acid synthesis are expressed in the UGS mesenchyme in a sex specific manner and addition of ligand to female tissue is able to induce prostate-like bud formation in the absence of androgens, albeit at reduced potency. Functional studies in mouse organ cultures that faithfully reproduce the initiation of prostate development indicate that one of the roles of retinoic acid signaling in the male is to inhibit the expression of *Inhba*, which encodes the βA subunit of Activin, in the UGS mesenchyme. Through in vivo genetic analysis and culture studies we show that inhibition of Activin signaling in the female UGS leads to a similar phenotype to that of retinoic acid treatment, namely bud formation in the absence of androgens. Our data also reveals that both androgens and retinoic acid have extra independent roles to that of repressing Activin signaling in the development of the prostate during fetal stages. This study identifies a novel role for retinoic acid as a mesenchymal factor that acts together with androgens to determine the position and initiation of bud development in the male UGS epithelia.

Introduction

The prostate develops from the urogenital sinus (UGS), an endodermal structure derived from the cloaca. The UGS has a bipotential fate depending on the sex of the embryo and will differentiate into the prostate and bulbourethral gland in males or contribute to the vagina during development but not in the adult in the female (Kurita, 2010; Marker et al., 2003). This sexual differentiation is driven by the presence or absence of androgens. In males, the production of hormones by the fetal testis ensures the development of secondary male specific structures, including the prostate. Two types of tissue are present in the UGS; the epithelium (UGE) and the surrounding mesenchyme (UGM), and prostate development depends on their interaction (Cunha, 2008). The UGM has been shown to specify prostate identity on epithelium from different sources, including bladder (Cunha et al., 1983). Androgens signal through the androgen receptor (AR) and analyses of tissue recombinants with tissue from AR deficient mice have shown that mesenchymal AR is required for prostate formation from the UGS (Cunha and Lung, 1978).

Prostate development is initiated in response to androgens with the UGE budding out and growing into the surrounding mesenchyme. In the mouse this occurs in the fetal period at around E16.5–17.5 (embryonic day 16.5–17.5). The buds then elongate, branch, canalize and cytodifferentiate to become secretory at 12–20 days after birth. The initial steps of bud development are faithfully reproduced in ex vivo organ cultures and these have been used to determine the factors involved in prostate differentiation (Doles et al., 2005; Lopes et al., 1996). However, the molecular pathways involved in prostate development initiation are poorly understood. One factor that has been identified to be important is FGF10, which is produced by the UGM in both sexes (Thomson and Cunha, 1999). Mice that are deficient for FGF10 show rudimentary prostate bud formation that does not progress, even in the presence of androgens (Donjacour et al., 2003).

The vitamin A derivative, retinoic acid (RA), has been implicated in many processes during embryogenesis. In the prostate, RA has been shown to increase prostate budding at embryonic stages but has also shown to inhibit prostate ductal growth and branching at

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postnatal stages (Aboseif et al., 1997; Seo et al., 1997; Vezina et al., 2008). The levels of the highly biologically active RA are tightly controlled in the embryo in space and time. The dietary form of vitamin A, retinol, is converted to RA in two steps catalyzed by two types of enzymes, the alcohol dehydrogenases, ADH, which are widely expressed, and the aldehyde dehydrogenases, ALDH, with restricted expression patterns. The presence of RA is further restricted by inactivation through metabolizing enzymes such as those belonging to the CYP26 family. RA acts as a ligand for the retinoic acid receptors (RARs) that bind DNA together with the retinoic X receptors (RXRs) at specific retinoic acid response elements (RARE) and contribute to transcriptional control.

In this study we show that RA, synthesized in the male UGM in a sex specific manner, is required for the initial step of prostate formation from the UGS. Addition of RA is able to induce prostate bud formation in the absence of androgens, albeit at reduced potency. Expression and functional analysis revealed that one of the roles of RA is to repress the production of Activin by the UGM that inhibits prostate bud formation. Our study suggests that the combined action of androgen and RA determines early prostate development in the UGE.

Methods and materials

Mouse strains

All mouse work was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Female MF1 outbred mice were purchased from Harlan UK Ltd. at 6–8 weeks old and mated with male MF1 outbred mice to obtain wild type embryos. For embryo staging, observation of a vaginal plug was designated as embryonic day 0.5 (E0.5). RARE-LacZ mice were generated by Janet Rossant and were kindly provided by Peter McCaffery, University of Aberdeen (Rossant et al., 1991). *Inhba*$^{-/-}$ (*Inhba* mutant) animals lacking the *Inhba* coding sequence were maintained through heterozygote breeding because *Inhba* mutants are neonatal lethal, as previously described (Matzuk et al., 1995).

UGS organ culture

Fresh UGS tissue was dissected from embryos in PBS by removing the bladder, urethra and ductal tissue using a 5 mm dissection knife, as previously described (Staack et al., 2003). Female UGS were used for all experiments, as they have not been exposed to fetal androgens. Similar organ culture results were also observed when using male UGS, although the degree of prostatic inhibition was variable. This variability was probably due to the presence of older embryos where prostate budding had already initiated at the time of dissection. UGS samples from E15.5 female embryos were chosen because of consistency of bud growth in culture. Similar results were obtained when E14.5 embryos were analysed. To grow tissue caudal to the prostate, the bladder and UGS were identified and the surrounding tissue carefully dissected with forceps until the tissue that will form the bulbourethral gland was located. A dissection knife was then used to remove the prostate and the bulbourethral gland and the intermediate tissue was used for culture. Dissected tissue was grown on 0.4 μm Biopore filters (Millipore, UK) in 2.5 ml of serum-free culture medium (DMEM/Hams F12 1:1) containing 1 x ITS (insulin, transferrin and sodium selenite) (Sigma, UK), 0.025 mg/ml gentamicin (Sigma, UK), 0.06 mg/ml benzylpenicillin sodium, 0.1 mg/ml streptomycin sulphate and 0.05 mg/ml ampicillin. Dihydrotestosterone (DHT) (Sigma, UK) was solubilised in 100% ethanol and added to the media at a concentration of 10^{-8} M. *All trans* retinoic acid (RA) (10^{-6} M), the ALDH inhibitor 4-diethylaminobenzaldehyde (DEAB) (50 μM), the pan RAR inverse agonist BMS493 (20 μM) and the activin inhibitor SB431542 (50 μM) were prepared in DMSO and added to the media (Sigma, UK). Control UGS were treated with the equivalent volume of vehicle. The dishes were placed in a humidified incubator at 37 °C in 5% CO_2 and media was changed at least every 48 h.

Bud number quantification

Bud number counting was performed on whole mount in situ stained UGS samples or from sections of *Inhba* mutants and controls. Positive buds were defined as those that stained for *Nkx*3.1 (Figs. 2A–C, 4C–E, and 5), were clear bud-like structures on a light microscope (Fig. 2D) or by histological features with haematoxylin and eosin staining (Fig. 4A). Statistical significance when two treatments were compared was calculated using the Student's two-tailed *T*-test.

Real-time PCR (RT-PCR)

RNA was extracted from tissue using an RNeasy Micro kit (Qiagen). cDNA was synthesized using OligodT primer and SuperscriptII Reverse Transcriptase (Invitrogen). mRNA accumulation of *Aldh*1*a*1, *Aldh*1*a*2, *Aldh*1*a*3, *Fgf*10, *Inhba* and *Hprt*1 mRNA was determined using the TaqMan system (Applied Biosystems, UK). Triplicates of each sample were analysed. Relative gene expression was calculated using the ΔΔCt method and the housekeeping gene *Hprt*1 as the normaliser. Statistical significance was calculated using the Student's two-tailed *T*-test.

Whole mount in situ hybridization analysis

In situ hybridization was carried out on an in situ processor (Intavis In Situ Pro) using a standard protocol as described previously (Val et al., 2006). Digoxigenin-labelled antisense RNA probes for *Aldh*1*a*1, *Aldh*1*a*2, *Aldh*1*a*3 and *Inhβa* were generated from PCR fragments containing T7 RNA polymerase recognition sites using the following primers.

*Aldh*1*a*1:5′AGCTCAAGACAGTCGCAATG3′ and
5′GTAATACGACTCACTATAGGGAGTCCTCCTCACCAAATGAG3′
*Aldh*1*a*2:5′TGCCAAGACTGCCACGTTTC3′ and
5′GTAATACGACTCACTATAGGGAAGGACTCAAAGCCACTGTC3′
*Aldh*1*a*3:5′GGCTAACAAGTAACACCTGG3′ and
5′GTAATACGACTCACTATAGGGCCTCCGTGTACTTACAGCTA3′
Inhβa: 5′ATT TGCTGAAGAGGAGAAGG3′ and
5′GTAATACGACTCACTATA GGGCGGCAAAGGTGATGATCTCC3′

Probes for *Nkx*3.1 and *Sox*9 have been described previously (Bhatia-Gaur et al., 1999; Thomsen et al., 2008). Stained whole-mount in situ hybridization samples were fixed in 4% paraformaldehyde, taken through a sucrose gradient before being frozen in OCT (R.A. Lamb) at −80 °C and then sectioned.

β-Galactosidase stain

Transgenic embryos and organ cultures expressing RARE-LacZ were fixed in 4% paraformaldehyde for 1 h at 4 °C, and then washed in PBS. LacZ stain solution (1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and 0.02% NP40) was applied to the tissue and incubated in the dark at 37 °C until stain was visible. Embryos from the same litter were used for each experiment to control for transgene heterogeneity.

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