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Activin over-expression in the testis of mice lacking the inhibin α-subunit gene is associated with androgen deficiency and regression of the male reproductive tract

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ABSTRACT

Regionalised interaction of the activins, follistatin and inhibin was investigated in the male reproductive tract of mice lacking the inhibin α-subunit (*Inha*$^{-/-}$). Serum and intratesticular activin B, although not activin A and follistatin, were increased in *Inha*$^{-/-}$ mice at 25 days of age, but all three proteins were elevated at 56 days. None of these proteins were altered within the epididymis and vas deferens at either age. At 25 days, histology of the epididymis and vas deferens was similar to wild-type. At 56 days, the testis contained extensive somatic cell tumours, leading to Leydig cell regression and testosterone deficiency. The epididymis and vas deferens showed epithelial regression and increased prominence of the interstitial stroma. Immunoregulatory and fibrotic gene expression in the epididymis and vas deferens were unchanged. Thus, absence of the inhibin α-subunit has marginal effects on activins in the epididymis and vas deferens, and regression of these tissues is associated with androgen deficiency.

1. Introduction

Activins, cytokines belonging to the transforming growth factor-β superfamily, are dimers of the inhibin β_A-subunit, encoded by the *Inhba* gene, or β_B-subunit, encoded by the *Inhbb* gene (Wijayarathna and de Kretser, 2016). Activins were initially isolated for their ability to stimulate the release of follicle stimulating hormone (FSH) from the anterior pituitary (Ling et al., 1987; Vale et al., 1986), but are now known to be important regulators of cell proliferation and differentiation in a wide range of tissues, including the reproductive and immune systems (Hedger et al., 2011). Inhibins are heterodimers of a β_A- or β_B-subunit and a homologous α-subunit (encoded by *Inha*), produced by the testis in the male (Ling et al., 1985; Robertson et al., 1985). The α-subunit binds competitively to the β-subunits to form inhibin, thereby reducing the formation of activin homodimers, but inhibin is also able to block binding of the activins to the activin receptors, thereby inhibiting activin signalling (Martens et al., 1997). In most species, including mice, the majority of inhibin produced by the testis is a heterodimer of the α- and β_B-subunits, while the β_A-subunits preferentially form the activin A homodimer (Hedger and Winnall, 2012).

Activins and inhibin are important regulators of testicular development (Barakat et al., 2008; de Kretser et al., 2002). Activin A plays a vital role in the developing testis by controlling Sertoli cell proliferation (Mendis et al., 2011) and germ cell differentiation (Mithraprabhu et al., 2010). The roles of the activins in the remainder of the adult male reproductive tract remain to be fully elucidated.

The highest level of *Inha* expression and inhibin production in any tissue occurs within the testis (Winnall et al., 2013). Within the male reproductive tract, *Inhba* expression and activin A production

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is highest in the caput epididymis (Wijayarathna et al., 2017; Winnall et al., 2013). While *Inhbb* expression is highest in the testis and caput epididymis (Wijayarathna et al., 2017; Winnall et al., 2013), relatively little is known about the distribution of activin B protein. In contrast to the activins, the endogenous activin-binding protein, follistatin (encoded by the *Fst* gene), is most highly expressed in the vas deferens (Winnall et al., 2013; Wu et al., 2012). Deficiency in follistatin leads to hypogonadism, reduction in epididymal duct diameter, and minor disruption of the anterior-posterior identity of the male reproductive tract (Wijayarathna et al., 2017). These data indicate that a balance of activins and their inhibitors is important for regionalised control of structure/function in the male reproductive tract. In order to test this hypothesis, we examined activin and follistatin production and the structural organisation of the male reproductive tract in the *Inha*-null mouse ($Inha^{-/-}$) (Matzuk et al., 1992). This mouse model lacks inhibin due to the deletion of exons 1 and 2 of the two-exon inhibin alpha gene, confirming that the mutant allele is a null allele. The mice develop testicular stromal tumours after 28 days of age, and therefore, were examined before (at 25 days of age) and after tumour development (56 and 77 days of age).

2. Materials and methods

2.1. Animals and tissue preparation

Male mice on a C57Bl/6 genetic background, having a homozygous deletion of the *Inha* gene, originally produced by Dr. Martin Matzuk (Baylor College of Medicine, Houston, Texas), were maintained by heterozygote breeding at the Animal Research Laboratories, Monash University, Clayton, Australia. Males and females with a heterozygous loss of function mutation in the *Inha* gene ($Inha^{+/-}$) were mated to generate $Inha^{-/-}$ mice. The $Inha^{+/+}$ (wild-type) and $Inha^{+/-}$ littermates were used as controls. Investigations were approved by the Monash University Standing Committee on Ethics in Animal Experimentation. All experiments were conducted according to the NHMRC Code of Practice for the Care and Use of Animals for Experimental Purposes.

At the age of 25 days, 56 days and 77 days, $Inha^{-/-}$ mice and their heterozygous and homozygous littermates were euthanized by carbon dioxide asphyxiation for tissue-collection. Due to difficulties with breeding and health issues, the number of mice and tissues available for analyses was limited, and the number of mice of each genotype and age included in each experiment is recorded in the results section. Blood was collected by cardiac puncture, and body and organ weights were recorded. One testis, epididymis and vas deferens from each animal were fixed in Bouin's solution, processed and embedded in paraffin. The other testis, epididymis and vas deferens were snap-frozen and stored at −80 °C until further analysis. Prior to being snap-frozen, the epididymis was sectioned into caput (epididymal segments 1–5), corpus (segments 6 and 7) and cauda (segments 8–10) regions, as previously described (Wijayarathna et al., 2017). The vas deferens was sectioned into equal length proximal and distal parts in the 56 day old mice, but not in the 25 day old mice, due to limitations in the amount of tissue available.

2.2. Histology, immunohistochemistry and morphometry

Paraffin-embedded tissues were sectioned at 5 μm thickness, and stained using Masson's trichrome staining or Periodic Acid-Schiff reagent (PAS) techniques. Masson's trichrome stained sections of the epididymis were used for morphometric analysis of the epithelial height, tubule diameter and luminal diameter of the epididymis, as described (Wijayarathna et al., 2017).

Immunohistochemistry was performed as described previously (Michel et al., 2016; Sarraj et al., 2010). Briefly, heat-mediated antigen retrieval was performed, and 5% normal serum was used to block non-specific binding. Sections were incubated overnight with the appropriate primary antibody (Table 1). Negative controls used an isotype-matched immunoglobulin. The α-smooth muscle actin and cytokeratin staining was visualised following secondary antibody addition (goat anti rabbit IgG, Vector Laboratories Inc., USA), using a Vectastain ABC kit (Vector laboratories) and Diaminobenzidine (DAB) (Dako North America Inc., USA). A fluorescent conjugated secondary antibody (AlexaFluor 488 anti-rabbit IgG, Life Technologies) were used for detection of collagen I and collagen IV.

2.3. Quantitative RT-PCR

Expression of mRNA for the inhibin subunit and follistatin genes, *Inha*, *Inhba*, *Inhbb* and *Fst*, immunoregulatory genes *Ido1*, *Il6* and *Il10*, and the fibrotic genes, *Col1a1*, *Col1a2*, *Tgfb1* and *Acta2* were measured as described previously, using the same primers and conditions (Wijayarathna et al., 2017). The relative expression of each gene was quantified using the ΔΔCt method using the reference gene *Rplp0*, which was found to be stably expressed in all tissues and genotypes.

2.4. Immunoassays

Specific ELISA assays for activin A and B, and radioimmunoassays for follistatin and testosterone were performed, as described (Wijayarathna et al., 2017). Serum LH concentrations were measured using a discontinuous radioimmunoassay, as previously described (Itman et al., 2015).

2.5. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). One-way analysis of variance or two-way analysis of variance followed by Tukey's multiple comparison test was performed to compare more than 2 groups. For comparisons between 2 groups the Student's t-test was applied. Significant differences were accepted when $P < 0.05$. The Graphpad prism 6 software (Graphpad software, Inc., USA) was used for all analyses.

3. Results

3.1. Activin A, activin B and follistatin were increased in the serum of $inha^{-/-}$ mice

At 25 days of age (i.e. before testicular tumour development), serum activin B showed a significant elevation (~2-fold) in $Inhba^{-/-}$ mice, compared with their wild-type ($Inha^{+/+}$) or heterozygous ($Inha^{+/-}$) littermates, but activin A and follistatin levels were

Table 1
Antibodies used in the study.

Antigen	Primary antibody details
α-smooth muscle actin	Rabbit polyclonal. (1:200 dilution) Abcam, catalogue number: ab5694
Cytokeratin-5	Rabbit monoclonal. (1:200 dilution) Abcam (Catalogue number: ab52635)
Collagen I	Rabbit polyclonal. (1:200 dilution) Abcam (Catalogue number: ab21286)
Collagen IV	Rabbit polyclonal. (1:200 dilution) Abcam (Catalogue number ab6586)

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