Comparative analysis of activins A and B in the adult mouse epididymis and vas deferens

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Abstract

Activin A regulates testicular and epididymal development, but the role of activin B in the epididymis and vas deferens is unknown. Mouse models with reduced activin A (*Inhba*^{+/-} and *Inhba*^{BK/+}), or its complete absence (*Inhba*^{BK/BK}), were investigated to identify specific roles of activins in the male reproductive tract. In 8-week-old *Inhba*^{+/-} mice, serum activin A decreased by 70%, with a 50% reduction of gene expression and protein in the testis, epididymis and vas deferens. Activin B and the activin-binding protein, follistatin, were similar to wild-type. Testis weights were slightly reduced in *Inhba*^{+/-} mice, but the epididymis and vas deferens were normal, while the mice were fertile. Activin A was decreased by 70% in the serum, testis, epididymis and vas deferens of *Inhba*^{BK/+} mice and was undetectable in *Inhba*^{BK/BK} mice, but activin B and follistatin levels were similar to wild-type. In 6-week-old *Inhba*^{BK/BK} mice, testis weights were 60% lower and epididymal weights were 50% lower than in either *Inhba*^{BK/+} or wild-type mice. The cauda epididymal epithelium showed infoldings and less intra-luminal sperm, similar to 3.5-week-old wild-type mice, but at 8 weeks, no structural differences in the testis or epididymis were noted between *Inhba*^{BK/BK} and wild-type mice. Thus, *Inhbb* can compensate for *Inhba* in regulating epididymal morphology, although testis and epididymal maturation is delayed in mice lacking *Inhba*. Crucially, reduction or absence of activin A, at least in the presence of normal activin B levels, does not lead to major defects in the adult epididymis or vas deferens.

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Introduction

Activins belong to the transforming growth factor-beta (TGFB) superfamily of cytokines and regulate cell growth and differentiation in many tissues (Hedger & de Kretser 2013). Activin A is composed of two inhibin beta A subunits, encoded by the *Inhba* gene (Vale *et al.* 1986, Robertson *et al.* 1987), and activin B is a dimer of inhibin beta B subunits, encoded by *Inhbb* (Nakamura *et al.* 1992). Inhibins are composed of an inhibin beta subunit and an -alpha subunit, encoded by the *Inha* gene, and act as antagonists of the activins (Robertson *et al.* 1985). Activin bioactivity is more widely regulated by the activin-binding protein, follistatin, which is encoded by the *Fst* gene (Robertson *et al.* 1987).

Activins act via classical TGFB signalling mechanisms involving heterodimeric serine-threonine kinase receptors (Wijayarathna & de Kretser 2016). Initially, both activin A and activin B bind to an activin type 2 cell surface receptor (ACVR2A or ACVR2B). Activin A and B bind to type 2 receptors with equal affinity (Mathews & Vale 1991). The type 2 receptor-protein complex recruits an activin type I receptor that initiates

downstream signalling via the Smad2/3/4 signalling pathway. While activin A recruits the type 1 receptor ALK4 (ACVR1B), activin B can recruit either ALK4 or ALK7 (ACVR1C) (Tsuchida *et al.* 2004). As a result of the ability to recruit both ALK4 and ALK7, activin B may possess a wider range of biological actions than activin A (de Kretser *et al.* 2012).

While activin A and B have substantially similar actions in most organ systems, the potency of each activin in regulating biological processes shows considerable variation. For example, while both activin A and B regulate embryonic mesoderm induction, activin B is more potent (Sokol *et al.* 1991, Nakamura *et al.* 1992). Furthermore, activin B plays a more important role in embryonic development of the central nervous system (Dutta *et al.* 2014, Feijen *et al.* 1994). On the other hand, activin A appears to be a more effective regulator of testicular development compared to activin B, because *Inhba*^{BK/BK} mice, in which activin A has been replaced by an activin B-producing transgene, show a delay in testicular development (Brown *et al.* 2000) and altered postnatal germ cell maturation

(Mithraprabhu *et al.* 2010). Moreover, activin B is a weaker agonist than activin A in regulation of FSH secretion by the anterior pituitary and in inducing erythroleukaemic cell differentiation (Nakamura *et al.* 1992).

The effects of activin A on testis development are relatively well-characterised (Barakat *et al.* 2008, Archambeault & Yao 2010, Mithraprabhu *et al.* 2010, Mendis *et al.* 2011), but the roles of the activins in the remainder of the male tract have received much less attention. Both activin A and B proteins are produced in the epithelial lining of the epididymis and vas deferens in the adult mouse (Wijayarathna *et al.* 2017). The activins and follistatin are differentially expressed in the epididymis and vas deferens, with the highest level of activin A and B in the caput epididymis, and the highest level of follistatin in the vas deferens (Winnall *et al.* 2013, Wijayarathna *et al.* 2017). These observations suggest an interactive regulatory role for these proteins in the male reproductive tract, which is supported by the observation of structural and functional alterations in the epididymis and vas deferens of mice deficient in follistatin (Wijayarathna *et al.* 2017). Crucially, *Inhba* has been shown to be required for coiling of the anterior Wolffian duct during embryonic development of the mouse epididymis in mice lacking the *Inhba* gene (*Inhba*^{-/-}) (Tomaszewski *et al.* 2007). Studies on the adult reproductive tract in the *Inhba*^{-/-} mouse are limited by the fact that these mice die at birth. Moreover, the specific functional role of activin B in the epididymis and vas deferens has not been studied.

The effects of reduced *Inhba* expression and subsequent activin A deficiency in testicular development have been studied in *Inhba*^{+/-} and *Inhba*^{BK/BK} mice. The *Inhba*^{+/-} mouse bears only a single allele of the *Inhba* gene and hence produces reduced levels of activin A (Matzuk *et al.* 1995b). In the *Inhba*^{BK/BK} mouse, an *Inhbb* transgene has been inserted in the *Inhba* locus, with activin B completely replacing activin A (Brown *et al.* 2000). Decreased activin A levels in the *Inhba*^{+/-} mice resulted in a decrease in the number of Sertoli cells in the neonatal mouse testis (Mendis *et al.* 2011). Germ cell maturation and Sertoli cell proliferation in the juvenile *Inhba*^{BK/BK} mouse testis were affected due to the complete absence of activin A (Mithraprabhu *et al.* 2010), and the *Inhba*^{BK/BK} mouse showed a 9-day delay on the onset of fertility due to delayed testis maturation (Brown *et al.* 2000). However, the rest of the male reproductive tract of these models has not been studied in depth, and the actual levels of activin B in these mice have not been measured. This study utilised the *Inhba*^{+/-} and *Inhba*^{BK/BK} mouse models to investigate the comparative roles of activin A and activin B in the regulation of the structure and function of the epididymis and vas deferens. Crucially, these studies provide evidence for a hitherto unidentified role for activin B in male reproductive tract function.

Materials and methods

Animals and tissue collection

Inhba^{+/-} mice and *Inhba*^{BK/BK} mice were generated as described previously (Mithraprabhu *et al.* 2010, Mendis *et al.* 2011). All experiments were approved by the Animal Ethics Committee

Figure 1 Serum activins and follistatin in adult *Inhba*^{+/-} mice. (A) Serum activin A, (B) activin B and (C) follistatin levels in 8-week-old *Inhba*^{+/-} mice compared to wild-type (WT) littermates. ($n=8$ WT and 8 *Inhba*^{+/-}). Values are mean ± S.E.M. Unpaired *t*-test. * $P<0.05$; Absence of statistical notation indicates that there were no significant differences between groups.

Figure 2 Activin A, B and follistatin gene and protein expression in the adult wild-type (WT) and *Inhba*^{+/-} testis, epididymis and vas deferens. (A) *Inhba* expression, measured in the testis, caput, corpus and cauda epididymis, and the proximal (P. vas) and distal (D. vas) segments of the vas deferens. (B) *Inhbb* gene expression. (C) *Fst* gene expression. (D) Activin A protein, (E) activin B protein and (F) follistatin levels in the testis, caput and cauda epididymis and vas deferens ($n=4$ WT and 5 *Inhba*^{+/-}). Values are mean $\pm$ S.E.M. Unpaired *t*-test. Tissues with different letter notations differ significantly ($P<0.05$). Absence of statistical notation indicates that there were no significant differences between groups.

Figure 3 Adult *Inhba*^{+/-} testicular and epididymal morphology. (A) *Inhba*^{+/-} testicular weight and (B) epididymal weight compared with wild-type (WT) at 8 weeks of age. ($n=8$ WT and 12 *Inhba*^{+/-}). Values are mean $\pm$ S.E.M. Unpaired *t*-test. *** $P<0.001$. (C) PAS-stained sections of WT and *Inhba*^{+/-} testes. H&E-stained sections of WT and *Inhba*^{+/-}, (D) cauda epididymis (segment 9) and (E) proximal region of the vas deferens. E, epithelium; IT, interstitial tissue; L, lumen; M, smooth muscle layer. Scale bar = 50 μ m.

of the Monash University, and were performed in accordance with guidelines of the NHMRC Code of Practice for the Care and Use of Animals for Experimental Purposes. Tissues from *Inhba*^{+/-} mice and matching wild-type littermate controls were collected at eight weeks of age. Tissues from *Inhba*^{BK/BK} mice, heterozygous *Inhba*^{BK/+} mice and matching wild-type littermates were collected at six weeks and eight weeks of age. During the collection of epididymides for protein and mRNA analyses, the organ was cut into 3 parts, guided by the clearly defined connective tissue boundaries between the segments: the caput (epididymal segments 1–5), corpus (segments 6–7) and cauda (segments 8–10) (Turner *et al.* 2003). Note that the caput includes the initial segment (segment 1) in all data presented. All tissues were collected and processed as described in Wijayarathna *et al.* (2017).

Fertility assessments were conducted by analysing breeding records over a period of two years. *Inhba*^{BK/BK} and *Inhba*^{BK/+} mice have reduced health and fertility compared with wild-type mice. Notably, *Inhba*^{BK/BK} mice, in particular, can die unexpectedly after 6 weeks, apparently due to complications arising from the absence of activin A (Brown *et al.* 2000, 2003). These fertility and viability issues limited the number of the *Inhba*^{BK/BK} animals and corresponding tissues available for analysis, such as serum, epididymides and vasa deferentia for gene/protein analysis.

Histology

Bouin's fixed tissues were paraffin-embedded and longitudinally sectioned at 5 μ m thickness. Tissue sections were transferred to glass slides and stained using haematoxylin and eosin (H&E) or Periodic acid-Schiff (PAS) protocols. Histological assessment of stained sections was performed using an Olympus BX50 microscope (Olympus) and the Cellsens dimensions software 1.7.1 (Olympus).

Microarray and mRNA expression analysis

Total RNA from the entire caput epididymis of 4 wild-type mice and 4 *Inhba*^{+/-} mice was extracted using the RNeasy kit (Qiagen) for microarray analysis. The RNA samples had an RNA integrity number of greater than or equal to 8.0. Cyanine-3 (Cy3)-labelled cRNA was prepared from 0.2 μ g of total RNA using the

Figure 4 Microarray analysis of caput epididymal gene expression in wild-type (WT) and *Inhbba*^{+/-} mice ($n=4$ per genotype). (A) Boxplot showing normalised intensity for *Inhbba* expression. (B) Principal Component Analysis plot. (C) Heat map of top 500 genes showing highest fold change. Agilent SurePrint G3 Mouse gene expression array.

One-Color Low Input Quick Amp Labelling Kit (Agilent, v6.6 September 2012) according to the manufacturer's instructions, followed by Qiagen RNeasy column purification. 600 ng of Cy3-labelled cRNA (specific activity >6 pmol Cy3/ μ g cRNA) was fragmented and hybridised onto an Agilent SurePrint G3 Mouse gene expression 8 $\times$ 60K microarray slide (Design ID 028005) (Agilent Technologies). Slides were scanned on the Agilent DNA microarray scanner and the images were analysed with Feature Extraction Software 11.0.1.1 (Agilent). All array data were preprocessed using the R package limma (Smyth 2004), and Principal Component Analysis (PCA) was performed on the normalised data. Before proceeding with differential expression analysis, the normalised data for the 8 samples were filtered to remove invariant genes. Out of 55691 probe sets, 14672 probe sets having a variation coefficient greater than 0.03 across all samples were kept for further exploration. The normalised and filtered data were tested for significant differential expression between *Inhbba*^{+/-} and wild-type samples using the limma package (Smyth 2004). A linear model was fitted to the (log) normalised expression data and moderated t statistics were computed and adjusted for multiple testing using the Benjamini and Hochberg method. Data analysis was also performed using the Agilent Gene Spring 12.0 software package as described in Dairi *et al.* (2016).

In order to validate the microarray data, relative mRNA expression of *Inhbba*, *Inhbb*, *Fst*, *Ido1*, *Cox2*, *Hox10* and *Il10* in the epididymal caput was measured by qRT-PCR using the same primer sequences and conditions described in Wijayarathna *et al.* (2017). Furthermore, these genes were measured by qRT-PCR in the corpus and cauda epididymis and vas deferens, as well.

Immunoassays for protein measurements

Activin A was measured in serum and tissue lysates using a specific activin A ELISA employing antibodies supplied by Oxford Brookes University, as described previously

(Knight *et al.* 1996, O'Connor *et al.* 1999). The limit of detection was 33.2 pg/mL. The mean inter-assay coefficient of variation (CV) was 8.6%, while the mean intra-assay CV was 8.7%.

Activin B was measured in serum and tissue lysates were assayed using a specific activin B ELISA, as reported by Ludlow and coworkers, using antibodies from Oxford Brookes University (Ludlow *et al.* 2009). The assay was validated for measurement of activin B in mouse tissues. The limit of detection was 28.4 pg/mL. The mean inter-assay CV was 9% and the mean intra-assay CV was 9.6%.

Total follistatin in serum and tissue lysates was measured using a discontinuous radioimmunoassay, as previously described (O'Connor *et al.* 1999, Winnall *et al.* 2013). This assay measures both forms of follistatin, produced by alternative splicing. The assay detection limit was 0.6 ng/mL. The mean intra-assay CV was 10.3%.

Statistical analysis

Statistical tests were conducted using Graphpad Prism 6 software (Graphpad Software). Student's t -test (two-tailed distribution) was used for comparisons between two groups. Multiple groups were compared using either one-way or two-way analysis of variance depending on the number of variables, followed by Tukey's multiple comparison test. Statistical significance was achieved when $P < 0.05$. All data are presented as mean $\pm$ standard error of mean (S.E.M.).

Results

Activin B and follistatin levels in serum were maintained in *Inhbba*^{+/-} mice, despite a reduction in activin A

The *Inhbba*^{+/-} mice showed a 70% reduction in serum activin A levels relative to wild-type littermates at 8 weeks of age (Fig. 1A). Although serum activin B levels

Figure 5 Serum activins and follistatin in adult *Inhba*^{BK/+} mice. (A) Serum activin A, (B) activin B and (C) follistatin levels in 8-week-old *Inhba*^{BK/+} mice compared with wild-type (WT) littermates ($n=4$ WT and 4 *Inhba*^{BK/+}). Values are mean $\pm$ S.E.M. Unpaired *t*-test. * $P<0.05$. Absence of statistical notation indicates that there were no significant differences between groups.

in *Inhba*^{+/-} mice showed an apparent small reduction compared to wild-type mice, this difference was not statistically significant (Fig. 1B). Serum follistatin levels were also similar to the wild-type (Fig. 1C).

Activin B and follistatin gene and protein levels were maintained in the male reproductive tract of *Inhba*^{+/-} mice

Inhba expression, measured in the testis, caput, corpus and cauda epididymis, and the proximal and distal segments of the vas deferens, showed approximately a 50% reduction relative to wild-type littermates

at 8 weeks of age (Fig. 2A). In contrast, *Inhbb* gene expression (Fig. 2B) was similar to that of the wild-type throughout the male reproductive tract. Both *Inhba* and *Inhbb* showed their highest expression in the caput region. In both wild-type and *Inhba*^{+/-} mice, the maximum level of *Fst* gene expression was found in the vas deferens (Fig. 2C).

Consistent with the reduced expression of *Inhba*, activin A protein levels showed a 50% reduction throughout the testis, caput and cauda epididymis and vas deferens of *Inhba*^{+/-} mice (Fig. 2D). Activin B protein also followed the *Inhbb* expression, and was the highest in the caput region, similar to the wild-type (Fig. 2E). Activin B showed a much more restricted distribution pattern than that of activin A downstream of the caput epididymis. Follistatin protein levels in the testis, epididymis and vas deferens were similar to the wild-type, and largely followed the *Fst* gene expression pattern, despite the difference observed in the gene expression within the distal vas deferens.

Adult testicular weight was decreased but no histological abnormalities were observed in the testis, epididymis or vas deferens of *Inhba*^{+/-} mice

At 8 weeks of age, testicular weight showed a small reduction (20%) in the *Inhba*^{+/-} mice compared to wild-type (Fig. 3A). Nevertheless, no differences were observed in sections of the testes of *Inhba*^{+/-} mice compared to wild-type controls at the histological level (Fig. 3C); the mice produced sperm and were fertile. The seminal vesicle weight of *Inhba*^{+/-} mice was similar to that of wild-type littermates (data not shown), indicating that peripheral testosterone levels were normal.

The epididymal weight of *Inhba*^{+/-} mice was comparable to the wild-type at the age of 8 weeks (Fig. 3B). Histologically, the epididymis of *Inhba*^{+/-} mice showed no evidence of defects in coiling of the duct, reduction in epididymal weight or any other type of morphological abnormality (Fig. 3D). Similarly, the vas deferens of *Inhba*^{+/-} mice was histologically identical to the wild-type (Fig. 3E).

Total gene expression in caput epididymis of *Inhba*^{+/-} mice did not differ significantly from the wild-type

Microarray analysis of ~60,000 transcripts of the caput epididymis showed that despite a 50% reduction in *Inhba* expression in this tissue (Fig. 4A), no statistically significant differences (adjusted *P*-value <0.05) were found in gene expression between *Inhba*^{+/-} and wild-type. Furthermore, PCA analysis did not identify grouping of the *Inhba*^{+/-} and wild-type samples (Fig. 4B). Similarly, a heat map of the top 500 genes based on the log fold change value did not show clustering of samples based on the genotypes (Fig. 4C). Crucial highly expressed epididymal functional genes, including

Figure 6 Activin A, B and follistatin protein expression in the adult wild-type (WT), *Inhba*^{BK/+} and *Inhba*^{BK/BK} testis, epididymis and vas deferens. (A) Activin A, (B) activin B and (C) follistatin protein in *Inhba*^{BK/BK} testicular lysates compared with those of *Inhba*^{BK/+} and WT mice at the age of 8 weeks ($n=5$ WT, 6 *Inhba*^{BK/+} and 4 *Inhba*^{BK/BK}). (D) Activin A, (E) activin B and (F) follistatin protein in *Inhba*^{BK/+} testis, epididymis and vas deferens compared to WT mice ($n=5$ WT, 6 *Inhba*^{BK/+}). Values are mean $\pm$ S.E.M. Unpaired *t*-test. Tissues with different letter notations differ significantly ($P<0.05$). **** $P<0.0001$, *** $P<0.001$, ** $P<0.01$. Absence of statistical notation indicates that there were no significant differences between groups.

aquaporins (*Aqp1-Aqp9*, *Aqp11*, *Aqp12*), tight junction proteins (*Tjp1-Tjp3*) and caput-specific beta-defensins *Defb41* and *Defb42* (Jalkanen *et al.* 2005), were not differentially expressed. Moreover, preliminary qRT-PCR expression analysis on known and putative activin-responsive genes such as *Ido1*, *Cox2*, *Hox10* and *Il10* (Nüsing *et al.* 1995, Ciarmela *et al.* 2008, Wijayaratna *et al.* 2017) also did not show a difference between the genotypes (data not shown).

Altogether, the morphological, histological and gene expression data indicated that a 50% reduction in activin A expression in the *Inhba*^{+/-} mouse had no substantial effects on the structure and function of the

mature epididymis or vas deferens. Since these mice had essentially normal levels of activin B, the relative contributions of activin A and activin B were examined further in mice in which the *Inhba* gene had been partially (*Inhba*^{BK/+}) or completely (*Inhba*^{BK/BK}) replaced by *Inhbb*.

***Inhba*^{BK/+} mice had reduced activin A, but normal serum activin B and follistatin levels, with normal activin B and follistatin levels in the testes of both *Inhba*^{BK/+} and *Inhba*^{BK/BK} mice**

At 8 weeks of age, *Inhba*^{BK/+} mice showed a 60% decrease in serum activin A (Fig. 5A) and 50% reduction in testicular activin A (Fig. 6A). As observed in *Inhba*^{+/-} mice, serum activin B (Fig. 5B) and follistatin (Fig. 5C) were similar to the wild-type (Fig. 5C). Insufficient blood samples were available for analysis from *Inhba*^{BK/BK} mice. Testicular activin B and follistatin were not different from the wild-type in the *Inhba*^{BK/+} and *Inhba*^{BK/BK} mice at 8 weeks of age (Fig. 6A, B and C) or at 6 weeks of age (data not shown).

***Inhba*^{BK/+} mice showed reduced activin A, but normal activin B and follistatin levels in the epididymis and vas deferens**

In the *Inhba*^{BK/+} mice at 8 weeks of age, the levels of activin A showed a 50% reduction in the testis, caput and cauda epididymis, and vas deferens relative to the wild-type (Fig. 6D). Similar to the observation in the *Inhba*^{+/-} mice and their wild-type littermates, activin B protein levels were similar to the wild-type, and highest within the caput region of the *Inhba*^{BK/+} mice (Fig. 6E). Follistatin protein levels were similar to the wild-type throughout the reproductive tract of the *Inhba*^{BK/+} mice (Fig. 6F). Insufficient samples from the *Inhba*^{BK/BK} mice were available to measure protein levels in the epididymis and vas deferens.

Figure 7 Testicular and epididymal weight of wild-type (WT), *Inhba*^{BK/+} and *Inhba*^{BK/BK} mice at 6 weeks and 8 weeks compared with controls. Testicular weights of *Inhba*^{BK/BK} mice compared with *Inhba*^{BK/+} and WT littermates at (A) 6 weeks and (B) 8 weeks. (C) Epididymal weight at 6 weeks and (D) 8 weeks ($n=5-6$ WT, 6 *Inhba*^{BK/+} and 2-3 *Inhba*^{BK/BK}). Values are mean $\pm$ S.E.M. Unpaired *t*-test. *** $P<0.001$. Absence of statistical notation indicates that there were no significant differences between groups.

Figure 8 Histological assessment of the wild-type (WT) and *Inhba*^{BK/BK} epididymis and vas deferens. WT cauda epididymis (segment 9) at the age of (A) 6 weeks and (B) 8 weeks, compared to the *Inhba*^{BK/BK} cauda at (C) 6 weeks and (D) 8 weeks. Proximal region of the vas deferens in WT mice at (F) 6 weeks and (G) 8 weeks, and *Inhba*^{BK/BK} mice at (H) 6 weeks and (I) 8 weeks. H&E-stained sections. Scale bar = 50 μm.

The epididymis and vas deferens of the *Inhba*^{BK/BK} mice were not mature at 6 weeks of age, consistent with a developmental delay in the maturation of the testis

At 8 weeks of age, the testis and epididymal weights of the *Inhba*^{BK/BK} mice were comparable to the *Inhba*^{BK/+} and wild-type mice (Fig. 7B and D). However, at 6 weeks of age, when wild-type mice are sexually mature, the testis and epididymal weights of the *Inhba*^{BK/BK} mice were reduced by ~60% and 50%, respectively, relative to *Inhba*^{BK/+} and wild-type littermates (Fig. 7A and C). This observation is consistent with the developmental delay in the *Inhba*^{BK/BK} testis, reported previously (Brown *et al.* 2000). Seminal vesicle weight, however, did not differ between the genotypes at either age examined (data not shown), indicating that the mice had normal androgen levels.

The *Inhba*^{BK/BK} epididymis at 8 weeks of age was histologically normal, with no evidence of reduced formation and coiling of the duct. At 6 weeks of age, however, the cauda epididymal epithelium in the *Inhba*^{BK/BK} mice was taller and had extensive folding (Fig. 8C), compared with the wild-type epithelium at this age (Fig. 8A). The amount of sperm present in the cauda epididymis of the *Inhba*^{BK/BK} mice at 6 weeks of age was also considerably reduced compared with the wild-type. The histological appearance of the epididymis of the *Inhba*^{BK/BK} mice at 6 weeks of age was similar to a wild-type epididymal epithelium at the age of 3.5 weeks (data not shown). However, at the age of 8 weeks, the *Inhba*^{BK/BK} cauda epididymis was comparable to the wild-type both in terms of epithelial morphology and sperm content (Fig. 8B and D).

The vas deferens of *Inhba*^{BK/BK} mice at 6 weeks of age also showed reduced sperm content and a large number of round cells, which were most likely prematurely released immature germ cells (Fig. 8H). At 8 weeks, the *Inhba*^{BK/BK} vas deferens was indistinguishable from the wild-type (Fig. 8G and I). The *Inhba*^{BK/+} epididymis and vas deferens were indistinguishable from the wild-type at both time points studied (data not shown). Overall, comparison of the morphology of the testis and epididymis of the *Inhba*^{BK/BK} mouse at 6 weeks and 8 weeks of age was consistent with delayed maturation,

relative to the wild-type and *Inhba*^{BK/+} mice, which were already mature by 6 weeks of age.

Discussion

This study is the first to highlight the functional significance of activin B in the structural development of the murine epididymis. Using a specific activin B immunoassay, our data established that the caput epididymis contains the highest activin B protein levels, confirming our earlier report based on immunohistochemistry (Wijayarathna *et al.* 2017). Consistent with previous data, the highest *Inhba* gene expression in the male reproductive tract was also found in the caput epididymis of the adult mouse, but activin A protein levels were more broadly distributed downstream (Winnall *et al.* 2013, Wijayarathna *et al.* 2017). As activin B showed a more restricted distribution pattern downstream of the caput epididymis, this suggests that the clearance of activin B in the epididymis may be more efficient compared with activin A.

The high levels and regionalised expression of both activins A and B within the epididymis suggest critical roles for these proteins in epididymal development and function. *Inhba* is known to be an important regional paracrine regulator in the morphogenesis of the straight anterior Wolffian duct into the highly coiled epididymis. Tomaszewski and colleagues reported in 2007 that the Wolffian duct in *Inhba*^{-/-} mice, which completely lacked activin A, did not elongate and coil at E19.5 during development. *Inhba*^{-/-} mice die at birth (Matzuk *et al.* 1995a), so their postnatal epididymal phenotype could not be studied. We show here that *Inhba*^{BK/BK} mice, in which the *Inhba* locus is replaced by *Inhbb*, displayed normal formation and coiling of the mature epididymis. This indicates that *Inhbb*, in the absence of *Inhba*, plays a compensatory role in anterior Wolffian duct coiling and epididymal development. At 6 weeks of age, a lag in *Inhba*^{BK/BK} epididymal development was observed in the form of epithelial immaturity and reduced organ weight, compared to wild-type mice. This is consistent with the previous observation of a maturational delay of the testis in the *Inhba*^{BK/BK} mice (Brown *et al.* 2000). Nevertheless,

the *Inhba*^{BK/BK} epididymis was structurally comparable to the wild-type at the age of 8 weeks, providing further evidence that the lack of *Inhba* delays but does not completely prevent normal epididymal morphogenesis and development. This indicates that activin B can ultimately compensate for the complete lack of activin A in maintaining normal adult epididymal structure.

The mild hypogonadism seen in the *Inhba*^{+/-} mouse model does not cause overall fertility defects, as previously reported (Matzuk *et al.* 1995a). The reduction in adult testicular weight could be related to the decrease in Sertoli cell numbers observed in neonatal *Inhba*^{+/-} mice (Mendis *et al.* 2011). The absence of morphological abnormalities in the epididymis of *Inhba*^{+/-} mice is also reflected in the lack of transcriptomic differences between *Inhba*^{+/-} and wild-type caput epididymis. It was noted that substantial variation existed among individual samples used for the microarray analysis even among wild-type individuals, as evident from the heatmap. Hence, it is possible that a larger sample size could perhaps detect significant gene expression differences. The lack of effects of reduced activin A on adult epididymal and vas deferens morphology or gene expression in this study contrasts with the pronounced effects of increased activin A on epididymal and vas deferens morphology and gene expression in the follistatin-288 deficient mouse model (Wijayarathna *et al.* 2017). However, it is important to note that more subtle effects of reduced activin A on epididymal and vas deferens function may yet be found by a more detailed functional analysis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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