Activin A Determines Steroid Levels and Composition in the Fetal Testis

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Activin A promotes fetal mouse testis development, including driving Sertoli cell proliferation and cord morphogenesis, but its mechanisms of action are undefined. We performed ribonucleic acid sequencing (RNA-seq) on testicular somatic cells from fetal activin A-deficient mice (Inhba KO) and wildtype littermates at embryonic day (E) E13.5 and E15.5. Analysis of whole gonads provided validation, and cultures with a pathway inhibitor discerned acute from chronic effects of altered activin A bioactivity. Activin A deficiency predominantly affects the Sertoli cell transcriptome. New candidate targets include Minar1, Sel1l3, Vnn1, Sfrp4, Masp1, Nell1, Tthy1 and Prss12. Importantly, the testosterone (T) biosynthetic enzymes present in fetal Sertoli cells, Hsd17b1 and Hsd17b3, were identified as activin-responsive. Activin-deficient testes contained elevated androstenedione (A4), displayed an Inhba gene dose-dependent A4/T ratio, and contained 11-keto androgens. The remarkable accumulation of lipid droplets in both Sertoli and germ cells at E15.5 indicated impaired lipid metabolism in the absence of activin A. This demonstrated for the first time that activin A acts on Sertoli cells to determine local steroid production during fetal testis development. These outcomes reveal how compounds that perturb fetal steroidogenesis can function through cell-specific mechanisms and can indicate how altered activin levels in utero may impact testis development. (Endocrinology 161: 1–16, 2020)

Key Words: spermatogenesis, steroidogenesis, Hsd17b1, Hsd17b3, 11-ketotestosterone

Perturbations during fetal life that affect development of either testicular somatic or germ cells are considered to underpin disorders of male reproduction. This includes hypospadias and cryptorchidism, which are evident at birth, and testicular cancer or reduced fertility, which become apparent in adulthood (1, 2). Knowledge of the mechanisms by which normal testis development occurs or is disrupted in humans is limited for ethical and practical reasons; however, rodent and primate studies are informative because many processes underlying male fertility are conserved.
When the mammalian testis is first forming immediately after sex determination, the plethora of genes selectively upregulated in male gonads involves several transforming growth factor-beta (TGF-β) superfamily ligands, including activin A (reviewed in (3, 4)). These signaling components mediate establishment of the somatic and germ cell populations in correct proportions, and they contribute to the transition of sexually indifferent primordial germ cells into unipotent gonocytes, the precursors of sperm.

The developmental interval from embryonic day (E) 11.5 to birth in mice establishes the foundations for sperm production in adult males (reviewed in (3)). Following Sry expression at E11.5, the bipotential gonads adopt the male differentiation pathway and initiate testis cord formation by E12.5 (reviewed in (5)). A somatic cell precursor population expressing Sf-1 (encoded by Nr5a1) differentiates to form Sertoli and Leydig cells, which interact with other interstitial cell subtypes to mediate testis cord formation (5, 6). Encapsulated by Sertoli cells within the cords, the male germ cells (termed gonocytes or prospermatogonia) proliferate until E13.5, then enter G1/G0 cell cycle arrest (7) for the remainder of fetal development. Ongoing Sertoli cell proliferation drives testis cord elongation and expansion (8). Further somatic cell differentiation within the developing testis creates the specialized organ that will support ongoing spermatogenesis through adult life. Fetal Sertoli cells jointly form a basement membrane of dynamic composition with the peritubular myoid cells that closely surround the cords (9, 10). The cords are embedded within an interstitium containing the fetal Leydig cells, immune cells, fibroblasts, and vasculature.

Androgens are central to the development and maintenance of male characteristics in fetal life. Steroid production in the mouse testis initiates with the conversion of cholesterol to androstenedione in fetal Leydig cells, catalyzed by CYP11A1, HSD3B1, and CYP17A1 (11). In adults, Leydig cells are the cellular site of testosterone (T) production; however, in the fetal testis, the conversion of androstenedione to T occurs within Sertoli cells mediated by the HSD17B1 and HSD17B3 enzymes (12). Androgen responsiveness in gonocytes contributes to their exit from the cell cycle after E13.5 (13), and it also determines peritubular cell functions (14). Thus, local production and actions of androgens involve multiple cell types and influence the earliest stages of testis formation.

Activin A also contributes to the establishment of a fully functional testis during the week after sex determination in mice (8, 15). Its synthesis by Leydig cells drives Sertoli cells to proliferate and thereby enables cord expansion (8), while simultaneously establishing the numerical balance between Sertoli and germ cells that exists at the onset of spermatogenesis (15). Nodal, TGF-β and bone marrow morphogenetic protein 7 are additional TGF-β superfamily members that influence germ and Sertoli cell numbers and fate in the fetal mouse testis (16-19), and crosstalk within the family is expected to contribute to cell-specific responses. Many TGF-β ligands are highly conserved. Activin A is a dimer of INHBA subunits encoded by the Inhba gene that is 100% identical between mouse and human. Several TGF-β ligands are implicated in testis development and relevant to pathologies in a range of species from Drosophila to human (3, 20-22). However, the molecular mechanisms of action are not understood, a knowledge gap that the present study was designed to address.

Because the absence of activin A results in reduced Sertoli cell proliferation and compromised testis cord formation in the fetal testis, we hypothesized that activin A would mediate these effects by modulating target gene expression in somatic cells of the developing testis. Microarray and ribonucleic acid sequencing (RNA-seq) were used to identify messenger RNAs that are regulated by activin A in testicular somatic cells during the interval when the Inhba transcript level is rapidly rising and when the absence of activin A impairs Sertoli cell proliferation and testis cord expansion. Several differentially expressed transcripts were identified by comparing samples from mice lacking activin A with those from their wild-type counterparts. Examination of whole gonads validated these as activin A target genes and revealed a new and highly specific role for activin A in modulating fetal testis steroid production. These outcomes illustrate the specific cells and critical functions affected by dynamic activin A levels as the fetal testis develops.

**Materials and Methods**

**Animals**

Mice were housed at the Monash Animal Research Platform (MARP) or at the Monash Medical Centre Animal Facility in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, with a 12 hour light and 12 hour dark cycle and with food and water available ad libitum. This study was approved by the Monash University Animal Ethics Committee (MMCB/2017/41). All experimental work, except the RNA-seq, was performed on samples collected from the Inhba (Inhba\(^{tm1Zuk/J}\) MARP) mouse line lacking the Inhba exon 2 sequence (23), termed here Inhba KO. To enable exclusion of germ cells for RNA-seq analysis of somatic cells, the Inhba x Oct4-GFP line was
established by crossing Inhba heterozygous (HET) mice on a C57Bl6 background with Oct4 [Pou5f1]-eGFP (OG2) transgenic mice (24) on a pure 129T2svJ background (25), therefore the samples we analyzed were on a C57Bl6/129T2svJ mixed background. The Inhba line is congenic, and both lines were maintained through repeated heterozygote breeding.

**Tissue collection**

The presence of a vaginal plug after mating was used to identify E0.5 of pregnancy. Pregnant mice were killed by cervical dislocation, and the embryos quickly removed from the uterus for dissection under a binocular microscope. Embryonic age was confirmed using forelimb and hindlimb development. Gonads were dissected from the mesonephros, immediately snap frozen on dry ice, and stored at −80°C for RNA isolation, fixed for histological analyses, or placed in phosphate-buffered saline (PBS) prior to dissociation for fluorescence-activated cell sorting (FACS). Paired tests for hormone measurements were weighed using a Sartorius Supermicro S4 Ultra-Microbalance prior to snap freezing on dry ice and storage at −80°C.

**Sexing polymerase chain reaction and genotyping**

Embryo sex was ascertained from gonad morphology, based on the presence (male) or absence (female) of testis cords and/or by polymerase chain reaction (PCR) using primers to detect Sly on the Y chromosome and Xlr on the X chromosome (26). PCR conditions were 95°C for 3 minutes, 35 cycles of 95°C for 15 seconds, 50°C for 30 seconds, 72°C for 15 seconds, and a final elongation at 72°C for 5 minutes. Amplification of Sly yields a 280 bp product, amplification of Xlr yields 480 bp and 685 bp products.

Inhba and Oct4-GFP genotypes were determined from tail samples, either by commercial vendor using real-time PCR with gene-specific probes (Transnetyx, TN, USA), or in-house (Inhba only) (15).

**Microarray**

Microarray analysis was performed on E15.5 testes from Inhba WT (wild type, +/+ ) and Inhba KO (knockout, −/−) mice, with 2 independent RNA samples per genotype, each corresponding to testes pooled from 5 to 10 different animals from multiple litters. RNA was prepared using the RNeasy Mini Kit (Qiagen). Biotinylated complementary deoxyribonucleic acid (DNA) was generated from 10 µg total RNA, fragmented and hybridized to Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA), and stained in accordance with the manufacturer’s protocol using the Fluidics Station 450 (Affymetrix). GeneChips were scanned using a laser confocal slide scanner (GeneChip Scanner 3000, Affymetrix) and quality was checked and quantified using GeneChip Operating Software (GCOS) v1.2 (Affymetrix). All reactions and microarray hybridization procedures were performed in the Laboratory for Biotecnology and Bioanalysis I at Washington State University.

Mass normalization (27, 28) was performed and the presence/absence calls for each probe set were used to determine which transcripts were detected at E15.5, and the transcripts that showed a ≥ 2-fold difference between Inhba WT and KO testes were considered (All supplementary material are located in a digital research materials repository: Supplementary Table 1(29)). From this data, the top 30 up- and downregulated differentially expressed genes (DEGs) were identified (Supplementary Table 2 (29)). Comparison with cellular expression patterns delineated in purified embryonic gonadal cells at E13.5 (GEO Profiles dataset GDS3995 (30)) was used to deduce the most likely expressed cell type. Genes were identified as being predominantly expressed in one cell type relative to another if they showed a difference of one log order of magnitude.

**FACS sorting and RNA isolation for RNA-seq**

Green fluorescent protein negative (GFP−) somatic cells were collected using FACS, as previously described (31), from paired testes of E13.5 and E15.5 Inhba x Oct4-GFP littermates. Single cell suspensions were obtained by dissociation in 0.25% trypsin (Gibco) for 8 to 10 minutes at 37°C. Dissociation was halted using 10% fetal bovine serum/Dulbecco’s modified Eagle medium (Bovigen/Gibco), then the cell suspension was filtered (35 µm), centrifuged gently (1020 RCF), and the pellet resuspended in 300 µL 0.4% bovine serum albumin (BSA)/PBS containing 2 µg/mL propidium iodide to identify nonviable cells. FACS was performed by Monash Flowcore Platform staff using the Influx or ARIA Fusion (Becton Dickinson). Gating was set to sort viable (propidium iodide negative) GFP− and GFP+ cells, resulting in the collection of 27 000 to 90 000 GFP− somatic cells from E13.5 and E15.5 paired testes from individual animals. RNA was isolated and treated with deoxyribonuclease using the NucleoSpin RNA XS kit (Macherey-Nagel) according to the manufacturer’s instructions.

**RNA-seq, bioinformatics, and statistics**

RNA-seq (ACRF Centre for Cancer Genomic Medicine, MHTP Medical Genomics Facility; Melbourne, Australia) was performed on RNA from FACS-purified E13.5 and E15.5 Inhba x Oct4-GFP somatic cells collected using FACS, as previously described (31), from paired testes of E13.5 and E15.5 Inhba x Oct4-GFP littermates. Single cell suspensions were obtained by dissociation in 0.25% trypsin (Gibco) for 8 to 10 minutes at 37°C. Dissociation was halted using 10% fetal bovine serum/Dulbecco’s modified Eagle medium (Bovigen/Gibco), then the cell suspension was filtered (35 µm), centrifuged gently (1020 RCF), and the pellet resuspended in 300 µL 0.4% bovine serum albumin (BSA)/PBS containing 2 µg/mL propidium iodide to identify nonviable cells. FACS was performed by Monash Flowcore Platform staff using the Influx or ARIA Fusion (Becton Dickinson). Gating was set to sort viable (propidium iodide negative) GFP− and GFP+ cells, resulting in the collection of 27 000 to 90 000 GFP− somatic cells from E13.5 and E15.5 paired testes from individual animals. RNA was isolated and treated with deoxyribonuclease using the NucleoSpin RNA XS kit (Macherey-Nagel) according to the manufacturer’s instructions.

RNA-seq (ACRF Centre for Cancer Genomic Medicine, MHTP Medical Genomics Facility; Melbourne, Australia) was performed on RNA from FACS-purified E13.5 and E15.5 GFP− somatic cells collected from WT and Inhba KO mice testes (n = 4 independent RNA samples per genotype). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies), and approximately 20 ng of high-quality RNA (RNA integrity number 8.4-9.6) were used to prepare libraries using the Nugen Trio Library preparation kit. Libraries were sequenced on an Illumina HiSeq3000 using 80 bp reads to generate 30 to 40 million reads per sample, and 96% of the reads had a quality score > 30.

Bioinformatic analysis was performed by the Monash Bioinformatics Platform. Reads were aligned against GRCh38 (ensembl release 84) with STAR (v2.5.2b; RRID:SCR_015899) (32, 33) and read counts (nonstranded) generated with featureCounts (subread v1.5.2; RRID:SCR_012919) (34, 35) using RNAseq analysis pipeline (v1.4.8). File manipulations
were performed with SAMtools (v1.4.1; RRID:SCR_002105) (36, 37). RNA-seq data are available via accession number: GSE143847. Differential expression was evaluated with limma after voom normalization (v3.34.1; RRID:SCR_010943) (38-40) via theDegust interface (v3.1.0; RRID:SCR_001878) (41). This identified differences between transcript levels (counts per million, cpm) in WT and Inhba KO at E13.5 and 15.5 and was reported using a false discovery rate (FDR); differences were considered to be significant when FDR values were < 0.05 (Supplementary Table 3 (29)). A heat map was generated using ClustVis (RRID:SCR_017133) (42, 43). The list of DEGs was submitted to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Functional Clustering Annotation Tool (RRID:SCR_001881) (44) to identify significantly associated functions.

**Hanging drop testis cultures**

Testes collected at birth (0 days postpartum [dpp]) from C37BL/6J MARP male mice were dissected free from epidermides into media and cultured in 30 µL of hanging drops (45) on inverted Petri dishes in media (α-minimum essential medium; Gibco), 1% BSA, 1% penicillin-streptomycin, 1% insulin-transferrin-selenium (both Gibco) at 37°C in 5% CO₂ for 24 hours. Media was supplemented with 10 µM SB 431542 (Sigma Aldrich) or dimethyl sulfoxide (DMSO; vehicle control). From a single animal, each testis was cut into 2 halves that were cultured together in a single drop, with either SB or vehicle alone. Postincubation, both testis fragments were snap frozen together and stored at −80°C. RNA was prepared using TRIZOL (Invitrogen) according to manufacturer guidelines.

**RNA preparation for Fluidigm analysis**

For the Inhba fetal age series, RNA was prepared from paired testes (E12.5-E15.5) using the RNeasy Mini Kit (Qiagen). For cultured 0 dpp testis fragments, RNA was prepared using TRIZOL (Invitrogen). Genomic DNA was eliminated using DNAfree (Applied Biosystems) according to the manufacturer’s guidelines. RNA quantity was determined using Nanodrop (Thermo Scientific). RNA quality in a subset of samples was assessed by Agilent 2100 Bioanalyzer. From a single animal, each testis was cut into 2 halves that were cultured together in a single drop, with either SB or vehicle alone. Postincubation, both testis fragments were snap frozen together and stored at −80°C. RNA was prepared using TRIZOL (Invitrogen) according to manufacturer guidelines.

**Lipid droplet detection**

Neutral lipid droplets in E15.5 Inhba WT and KO testes were detected using oil red O (ORO) in the Monash Histology Platform. Briefly, testes were fixed in 4% paraformaldehyde for 90 minutes at room temperature, placed in 30% sucrose at 4°C for < 5 days, embedded in Tissue-Tek OCT (optimal cutting temperature) compound (Sakura Finetek, Netherlands), snap frozen on dry ice, and stored at −80°C. Cryosections (6–8 µm) were stained with ORO (0.5% in isopropyl alcohol) for 10 minutes. Images were taken using brightfield microscopy (Olympus BX50 microscope with DP70 camera). ImageJ software (47) was used to quantify lipid droplet number, size, and percentage occupancy of the area (cord vs interstitium) within 3 to 8 testis cords/sample with clearly defined boundaries, and in 3 interstitial areas (average area = 1900 µm²/sample in a single cross section from E15.5 Inhba WT (n=5) and E15.5 Inhba KO (n=6) individual animals.

**Intratesticular steroid measurements by liquid chromatography–mass spectrometry**

Frozen E17.5 paired testes from individual Inhba WT (+/+), HET (+/-) and KO (-/-) mice were homogenized in 150 µL ice cold homogenization buffer (0.5% BSA, w/v), 5 mM ethylenediaminetetraacetic acid (EDTA) in PBS, pH 7.4) in a 5 mL glass tube for 20 seconds using an IKA T10 basic disperser on the highest setting; the probe was rinsed between samples in homogenization buffer. Samples were centrifuged (3000 rpm, 10 minutes, 4°C), and supernatants were transferred to new 1.5-mL plastic tubes for storage at −80°C until analysis.

Steroid hormones were measured for all samples in a single batch using liquid chromatography–tandem mass spectrometry assay as previously described (48). The profile of steroids comprised androstenedione (A4), T, 11-keto A4 (11-KA4), 11-keto T (11-KT), 3α-androstanediol, dihydroidosterone (DHT), and 11-keto DHT (11-KDHT).

**Statistical analysis**

All graphs were created using Prism 8 (GraphPad, version 8.2.0) and show mean with error bars indicating standard deviation.
Results

Activin A deficiency affects somatic cell gene expression in the embryonic mouse testis

Two approaches were used to investigate the overall transcriptional differences in embryonic somatic cells arising from activin A deficiency. An initial assessment of gene expression using microarrays compared pooled E15.5 whole testes from Inhba KO and WT littermates (n = 2 for each genotype). A total of 255 genes differed by more than 2-fold (Supplementary Table 1 (29)). Of the top-30 upregulated and downregulated transcripts (60 in total), 30 (50%) were found to be expressed at E13.5, primarily in somatic cells or at equivalent levels in all early fetal testis cell types (30). In addition, 22 (37%) had an unknown cell-type expression, and only 4 (7%) were expressed in germ cells (Supplementary Table 2 (29)).

We then used RNA-sequencing to define the impact of activin A deficiency on fetal somatic cells purified by FACS from E13.5 and E15.5 WT and Inhba KO littermates (Fig. 1A). This identified 22 334 unique transcripts, comprising mainly protein coding sequences, but also long noncoding RNAs, and pseudo-genes (GSE143847). Using an FDR < 0.05 as the cutoff for statistically significant differential expression between WT and Inhba KO samples, only Inhba was identified at both E13.5 and E15.5 (downregulated, as expected). More transcripts were significantly affected at E15.5 (n = 222; Figs. 1B, 1C and Supplementary Table 3 (29)) than at E13.5 (n = 1), identifying this as the interval when somatic cell responsiveness to activin emerges in the fetal mouse testis. This analysis identified several genes previously described to be activin responsive (Figs. 1B, 1C, Supplementary Table 3 (29)) including Ccl17 (49), Hsd17b1, Hsd17b3 and Serpina5 (50). Other activin-dependent transcripts were identified (Fig. 1B), and DAVID analysis revealed that activin-responsive transcripts in E15.5 somatic cells were significantly associated with UniProt keywords (biological terms) glycoprotein, secreted protein, extracellular matrix, membrane, transmembrane, collagen, cell junction, lipid biosynthesis, steroid biosynthesis, and lipid metabolism (Fig. 1D).

Male sex determination is normal

Candidate transcripts were selected for expanded analysis during the 4-day interval directly after sex determination (E12.5-E15.5) by Fluidigm in whole gonads from WT and Inhba KO male littermates. Targets were selected based on the magnitude of fold changes measured in the microarray, significance by FDR and low P values in the RNA-seq dataset, and biological relevance to sex determination.

In WT testes, Inhba levels progressively increased from E12.5 through E15.5, but this transcript was undetectable in Inhba KO samples (Supplementary Fig. 1A...
Figure 1. RNA-seq experimental approach and results summarizing E15.5 somatic cell population analysis. (A) Summary of the experimental workflow. Testes from E13.5 and E15.5 Inhba−/− Oct4-GFP embryos were collected and GFP− somatic cells purified by FACS for RNA sequencing. (B–E) RNA-seq analysis of testicular somatic cells from E15.5 Inhba KO and WT controls. (B) Heatmap showing 222 DEGs with an FDR < 0.05 and absolute fold change ≥ 1.5 identified between the Inhba KO and Inhba WT samples. Rows clustered using Euclidean distance and average linkage.
Selective effects of activin A deficiency on Sertoli cell transcription and identification of new target genes

We previously reported that Sertoli cell numbers at E13.5 and E15.5 were identical in Inhba KO mice and their WT littermates (15). This was supported by the new observation that Sertoli cell-specific transcripts including Ambr2, Fgf9, Jaknip2, Wt1, and Pvr were unaffected in Inhba KO testes between E12.5 and E15.5, although Sox8 and Sox9 were each transiently elevated at E12.5 and E14.5, respectively (Fluidigm analysis; Supplementary Fig. 2 (29)). To identify transcriptional differences that underpin the reduced Sertoli cell proliferation and cord expansion and continued germ cell proliferation observed at birth in activin A null animals (15), we focused on identifying activin-responsive genes transcribed in E15.5 Sertoli cells that could mediate these phenotypic changes.

Fluidigm analyses (E12.5-E15.5 testes) confirmed the activin A-dependent expression of several well-known activin target genes (Ccnd2, Cldn11, Serpina5, Ccl17) that are highly expressed in fetal Sertoli cells (30). Ccnd2 encodes a cyclin that promotes cell proliferation and is expressed in all fetal testis cell types at E15.5; this transcript was significantly reduced in Inhba KO testes at E14.5 and E15.5 (Fig. 2A), as previously reported (15, 51). Claudin 11 (encoded by Cldn11) is a membrane protein and component of tight junctions that is negatively regulated by activin A in postnatal Sertoli cells (52). In the absence of activin A, Cldn11 was significantly higher at all ages (Fig. 2A), indicating it is also negatively regulated by activin A in the fetal testis. Serpina5 is also activin dependent in postnatal Sertoli cells (50). This transcript was increased 55-fold between E12.5 and E15.5 in WT gonads but did not rise in Inhba KO testes (Fig. 2A). Ccl17 is a chemokine predominantly expressed in Sertoli cells in E13.5 gonads (30), and it is an activin A target gene in immune cells (53, 54). While Ccl17 transcription increased substantially between E14.5 and E15.5 in WT testes, it was not upregulated in Inhba KO testes (Fig. 2A), demonstrating its dependence on activin A in this organ.

In addition to known activin A target genes, a range of putative new activin A target genes that were identified by their differential regulation in Inhba KO compared with WT testicular somatic cells using RNA-seq (Fig. 1, Supplementary Table 3 (29)) were validated by Fluidigm analysis (Fig. 2B). These included Masp1, Kazal1, Slc38a5, Sel113, Serpina3a, Erbb3, and Col9a3, which are all selectively enriched in Sertoli cells, and Pdgfa, which is expressed in all fetal testis cell types (Fig. 2B). Other transcripts predominantly found in Sertoli cells showed no changes in Inhba KO testes (eg, Ambr2, Fgf9 and Wt1; Supplementary Fig. 2 (29)), demonstrating that a specific cohort of Sertoli cell transcripts is affected by activin A deficiency.

To determine if the changes in selected Sertoli cell transcripts to activin A were likely to be direct (acute) or whether they were an indirect consequence of the chronic activin A deficiency in Inhba KO mice, we cultured newborn (0 dpp) mouse testes for 24 hours with the type 1 receptor (ALK 4/5/7) inhibitor, SB 431542, which inhibits activin, Nodal and TGF-β signaling (55) (Fig. 3). Treatment outcomes were concordant with the in vivo transcriptome data Hsd17b1, Slc38a5, Serpina5, Ccl17 and Hsd17b3 and were each reduced while Col9a3 was increased, indicating these are genes that respond directly to altered activin A levels. The Leydig cell transcript, Cyp11a1, was unchanged, in alignment with results from both RNA-seq measurements of isolated somatic cells (Fig. 1) and Fluidigm validation using whole testis (Fig. 2).

Activin A levels determine fetal testis androgen biosynthesis

The enzymes encoded by Cyp11a1, Hsd3b1 and Cyp17a1 convert cholesterol to the androgen precursor A4 and are synthesized in fetal Leydig cells (12, 30). The levels of these Leydig-expressed androgen biosynthesis
Figure 2. Activin A target genes predominantly expressed in Sertoli cells are regulated after E13.5 in the developing testis. Fluidigm analysis in whole testis samples from Inhba WT (black bars) and Inhba KO (white bars) showing: (A) Relative expression of known activin target genes Ccnd2, Cldn11, Serpina5, and Ccl17; (B) Genes identified by RNA-seq (by FDR cut off < 0.05) as novel activin A target genes. Graphs show relative expression normalized to Canx and Mapk1 measured in independent whole gonads aged E12.5–E15.5 (N = 3; age, genotype). Values represent mean ± SD. Significance determined using 1-way analysis of variance with the Sidak multiple comparisons test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. E, embryonic day; FDR, false discovery rate; KO, knockout; RNA-seq, ribonucleic acid sequencing; SD, standard deviation; WT, wild type.
genes were unaffected by activin A deficiency (Fig. 4A). In contrast, Hsd17b1 and Hsd17b3, which are required for conversion of A4 to T and expressed only in fetal Sertoli cells (12, 30) were reduced in Inhba KO testes compared with WT controls (Fig. 1B, 4B) and were acutely responsive to activin A inhibition in culture (Fig. 3). This is highly significant as it provides the first evidence that T production in the fetal testis is governed by activin A through its regulation of androgen biosynthesis in Sertoli cells.

Based on this finding, we hypothesized that a sustained decrease in Hsd17b1 and Hsd17b3 transcription in Inhba KO testes would reduce conversion of A4 to T within the fetal testis. Steroid levels were measured in whole testis homogenates from Inhba WT, HET and KO mice at the age (E17.5) when the T level peaks prior to birth (56). A4 levels were significantly increased (P < 0.001) while T levels tended to decrease (P = 0.08) in Inhba KO compared with WT control testes (Fig. 4C, left panel). Moreover, the T to A4 ratio revealed a highly significant dose-dependent decrease in androgen production that correlated with reducing Inhba gene dosage (Fig. 4C, center panel, P < 0.0001), directly illustrating the dependence of local steroid production on activin A actions in the Sertoli cells of the fetal testes. Importantly, increased levels of the 11-oxygenated androgens, 11-KA4 (11-keto androstenedione, also named adrenosterone) and 11-KT (11-ketotestosterone); Fig. 4C, right panel) were recorded in Inhba KO testes. This collectively demonstrates that an increased accumulation of A4 occurs due to its reduced conversion to T and ultimately changes the steroid hormone profile in this organ. Other cholesterol metabolites, including 3-adiol, DHT and 11-keto DHT (Supplementary Fig. 3 (29)), were unaffected or were not detected (DHEA, 3b-diol, progesterone, estrone, estradiol) in either WT or Inhba KO fetal testes.

**Leydig and interstitial cells are not affected by activin A deficiency**

The majority of Leydig cell progenitor (Arx, Lhx9) and interstitial progenitor cell (Hes1, Gli1, Pdgfra) transcripts did not differ between WT and Inhba KO mice between E12.5 and E15.5 (Supplementary Fig. 4A (29)). Several messenger RNAs predominantly expressed in differentiating Leydig cells from around E13.5 (Itih5, Itga8, Supplementary Fig. 4B (29)) were not different, nor were those encoding other steroidogenic enzymes (Cyp11a1, Hsd3b1, Cyp17a1; Fig. 4A). Additionally, interstitial cell transcripts were largely unchanged (Nid1, St8sia2, Adams2, Meis1, Sh3kb1, Robo2, Arbegaf9, Caca2d1; Supplementary Fig. 4 (29)). The one exception, Cbmr1, was expressed solely by interstitial cells in the fetal mouse testis (30); it was lower in the absence of activin A, declining significantly from E13.5 onward (Supplementary Fig. 4 (29)). This is of particular interest because Cbmr1 transcription is affected by local changes in steroids (57), consistent with the lower levels of T detected in Inhba KO testes.

**Activin A deficiency alters fetal lipid metabolism**

In the E15.5 RNA-seq dataset, 7 DEGs associated with the term lipid metabolism (Nceh1, Ptges, Hsd17b1, Elovl2, Hsd17b3, Lpcat2, Pla2g5) were lower and 3 were higher (Hmgcs2, Enpp2, St8sia1) in Inhba KO compared with WT somatic cells. The presence of lipid droplets can indicate cellular and metabolic stress (58, 59), and they are routinely visualized using oil red O (ORO). Lipid droplets were remarkably more abundant within E15.5 testis cords of Inhba KO compared with WT E15.5 Inhba testis sections (Figs. 5A, A’, B, B’). Quantification demonstrated significantly increased lipid droplet number, size, and percentage area (Fig. 5C), strongly indicating that dysregulation of one or more of...
Figure 4. Activin A affects genes required for testosterone production within fetal Sertoli cells, resulting in altered steroid hormone production. (A) Levels of transcripts encoding enzymes that convert cholesterol to androstenedione in fetal mouse Leydig cells (Cyp11a1, Hsd3b1, and Cyp17a1) normally increase between E12.5 and E15.5 and do not differ between WT and Inhba KO testes. (B) The normal increase in Sertoli cell transcripts encoding HSD17B1 and HSD17B3 (convert androstenedione [A4] to testosterone [T]) does not occur in activin-deficient testes. Graphs show relative expression normalized to Canx and Mapk1 measured by Fluidigm in Inhba WT (black bars) compared with Inhba KO (white bars) whole gonads aged E12.5–E15.5. Values represent mean ± SD. Significance determined using 1-way analysis of variance with the Sidak multiple comparisons test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (C) Hormone measurements in E17.5 Inhba mouse testes confirm
These lipid-associated genes regulate lipid production within cords of the developing testis. No change in lipid droplet number or size was measured outside of cords (Fig. 5C).

Discussion

This study confirmed Sertoli cells are the primary somatic cell target of activin A action in the fetal testis and expands knowledge of this key factor that regulates testis development. We demonstrated for the first time that activin A fulfils a central role in determining local steroid production during fetal testis development.

Significantly, we identified several potential novel activin-dependent target genes in fetal Sertoli cells, including Masp1, Kazald1, Scl38a5, Sel113, Serpina3a, Erbb3, and Col9a3 and Pdgfa, which is expressed in multiple somatic cell types in the fetal testis. These candidate targets could influence cell-cell interactions in the developing fetal testis. MASPI affects adhesion between endothelial cells and activates innate immunity via the lectin complement pathway (60). KAZALD1 modulates bone formation and regeneration in mice (61, 62) but is undescribed in the testis. The function of the amino acid transporter SLC38A5 is unstudied in the testis, but its level is reduced in postnatal mouse testis in the absence of the androgen receptor (63). ERBB3 signaling has been implicated in both somatic and germline development, including through its ligand, NRG1 (64, 65). The gene encoding collagen 9a3 is a target of SOX9 that is expressed early in mouse fetal testis development (66). Serpina3a is highly expressed in the testis compared with other tissues (67) but is functionally uncharacterized in the testis. Pdgfa is a particularly interesting candidate that provides an indication of how activin bioactivity levels may influence processes of high relevance to overall development. Pdgfra<sup>−/−</sup> XY gonads show disruptions to vasculature organization, partitioning of interstitial and testis cord compartments, and Leydig cell differentiation, reflecting the central role of PDGF signaling in organogenesis (68); we speculate that changing levels of activin A may indirectly modulate processes affected by this crucial signaling molecule.

Supporting the value of using the current Inhba KO model to discover new activin A candidate target genes, we also identified several genes (Serpina5, Ccl17, Cld11, Ccnd2, Hsd17b1, and Hsd17b3) known to be activin A targets in postnatal mouse testes (50). These are identified as activin A-responsive in fetal and newborn testes using RNA-seq of fetal Inhba KO somatic testis cells and by culturing 0 dpp testis with the signaling inhibitor, SB 431542. The high proportion of activin-responsive genes expressed in Sertoli cells extends knowledge that this cell type is a key target of activin A throughout testis development (8, 15, 50, 69-71).

In addition, we have made the fundamentally important discovery of the functional consequence of activin A stimulatory actions on genes encoding the steroidogenic enzymes HSD17B1 and HSD17B3: In the absence of Inhba, conversion of A4 to T was reduced in the fetal testis. This is highly important, as fetal masculinization relies on steroid and androgen production in testes as well as in nongonadal tissues (72). T synthesis requires both Leydig and Sertoli cells in the fetal mouse testis (11), and our new findings demonstrate activin A controls a key step in steroidogenesis that occurs selectively in Sertoli cells.

In general, the multistep conversion of cholesterol into steroids is mediated by enzymes with species- and cell-specific expression profiles. As part of this process, the conversion of pregnenolone into the androgen precursor A4 occurs via the ∆4 pathway in rodents (73) or the ∆5 pathway in humans (74). In mice, A4 produced in fetal Leydig cells is converted to T by HSD17B1 and HSD17B3 in fetal Sertoli cells (12). We demonstrated that the substantial decrease in both Hsd17b1 and Hsd17b3 in activin A-deficient fetal testes correlates with higher A4 levels, identifying a direct correlation between the A4 to T ratio and Inhba gene dosage. In newborn human blood samples, significantly elevated A4 was associated with an increased testicular germ cell tumor risk in adolescence (75), implicating A4 as an indicator of disruptions to early testis development in humans that affect adult health and fertility.
Figure 5. Loss of activin A leads to increased lipid deposition in fetal testes. Oil red O staining performed on frozen sections of E15.5 *Inhba* WT (A, A') and *Inhba* KO (B, B') mouse testes. Oil red O staining was detected at minimal levels or was absent in the cords of *Inhba* WT testes but was prominent in cords of *Inhba* KO testes. Low oil red O staining was also detected in interstitial cells of both genotypes. (C) Lipid droplet analyses within the testis cords and interstitium of E15.5 *Inhba* WT and KO mice. Quantified using ImageJ software in 3 through 8 cords and 3 interstitial areas (average area = 1900 µm²) in a single cross section from *Inhba* WT (n=5) and *Inhba* KO (n=6) animals. Significance determined using a Mann-Whitney test. ***P < 0.001, ****P < 0.0001, ns denotes not significant. Black arrowheads indicate lipid deposition. Cords indicated by white dotted line. White scale bar = 20 µm. E, embryonic day; KO, knockout; WT, wild type.
In addition to the altered A4 to T ratio, we measured 11-KA4 and 11-KT in the fetal mouse testes and detected 11-KA4 only in the absence of activin A, while its metabolite 11-KT is elevated. 11-KT is understood to be nonaromatizable (76), and it is the primary androgen in teleost fish (77). Two transcripts, Hsd11b2 and Cyp11b1, encode the enzymes that convert A4 to 11-KA4. Hsd11b2 is expressed only in interstitial cells (30). In contrast, Cyp11b1 is enriched in endothelial cells at E13.5, with its lower expression in interstitial cells potentially reflecting ectopic expression of the endothelial cell reporter gene in a small number of Leydig cells (30). We note that Cyp11b1 is also associated with adrenal cell steroidogenesis, and differentiation of adrenal-like cells is reported in testes of other transgenic mouse models (78, 79). However, adrenal cell steroidogenic transcripts (78, 79) were either not detected or not changed in Inhba KO mice (Cyp21a1, Cyp11b2, Akr1b7, Mrap, Hao2, and Me2r; data not shown). Although not significantly altered (as assessed by FDR), Cyp11b1 was higher in E13.5 Inhba KO testicular somatic cells when independently assessed using a \( t \) test (2.7-fold increase, \( P = 0.0246 \)). This could reflect an action of activin A on endothelial cells in the earliest phases of testis formation. In humans, conversion of 11-KA4 to 11-KT is catalyzed by AKR1C3; however, the transcript Akr1c6 that encodes its murine homolog was not detected in fetal mouse testes (6, 30), suggesting this step is mediated by a different enzyme in mice. Since divergent evolution leading to differing substrate preferences for AKR1C enzymes has been reported (80), we searched for related family members in our RNA-seq data and identified Akr1cl as an abundant family member transcript in fetal somatic cells (Supplementary Fig. 5 (29)). This indicates that Akr1cl may play the role in mice of human AKR1C.

The enzymes that regulate conversion of A4 to T and to 11-KA and 11-KT are also present in adult mouse and human testes (76, 81), but their functional significance has not been elucidated (72). Production of these active androgens within the testis, as well as those arising from extragonadal sites such as placenta and adrenal gland, may explain why secondary sex characteristics such as anogenital distance and testis descent are unaffected in Inhba KO mice (82). Understanding how 11-keto steroids become elevated in the activin-deficient mouse testis may provide clues to other cellular sites of androgen production that are relevant to testis development, particularly in nonphysiological circumstances. Our results identify activin A as a key determinant of androgen production within Sertoli cells that governs the balance between androgen precursor steroids and bioactive androgens in the fetal testis. The role of activin A in regulation of testicular steroid production is dynamic and likely complex. For example, the postnatal transition to T synthesis within Leydig cells is concomitant with a switch in HSD17B1 and HSD17B3 expression from Sertoli to Leydig cells. Hsd17b1 male KO mice exhibit spermatogenic defects in adult, but not fetal life, and A4 to T conversion was probably unaffected due to a compensatory upregulation of Hsd17b3 in Leydig cells (83). Despite differences in steroidogenesis between human and mouse, conversion of precursor steroids to T requires Hsd17b3 in both species (84). Cultures of first and second trimester human fetal testes with Nodal or TGF-β pathway inhibitors that can affect activin signaling exhibit significantly altered steroid secretion (20). These insights relating to fetal gonads should also be considered in relation to human pregnancy conditions in which activin A bioactivity may be altered, such as with preeclampsia (85), infection (86), and exposure to certain medications (87).

The abnormal lipid droplet accumulation within in Inhba KO fetal testis cords may reflect the dysregulation of several genes associated with lipid production. Lipid droplet accumulation within adult human testis cords occurs exclusively in the presence of germ cell neoplasia in situ (88) and is generally a feature of cells under stress that is observed in metabolic diseases and cancers (58, 59, 89). It will be informative to examine the potential for disrupted signaling by activin A and other TGF-β superfamily moieties to modulate steroidogenesis and other pathologies of the human testis throughout the lifespan.

An important outcome from the present study was the robust identification of activin A target genes using a knockout mouse model in which activin A is chronically absent. Validation used both independent testis samples and organ cultures. Further analyses using discrete somatic cell populations may reveal more subtle changes, while more extensive culture analyses are needed to further distinguish direct from indirect activin A targets. It will be useful to determine the duration of activin A perturbation required to alter key processes such as steroidogenesis and to establish how crosstalk with other TGF-β ligands and pathway inhibitors changes in circumstances with altered activin A bioactivity.

In summary, our new findings show that activin A deficiency in the mouse decreases androgen biosynthesis via the classical Δ4 steroidogenic pathway and implicates activin A as a crucial determinant of local steroidogenesis during fetal development. The elevated levels of the nonaromatizable weak androgen, 11-KT, and decreased levels of the aromatizable androgen, T, in Inhba KO testes identify activin A as central to
establishing the ratio of androgenic versus estrogenic steroids in the fetal testis.

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Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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52. Methods: The experimental procedures were carried out in accordance with institutional guidelines approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. The animal welfare was ensured throughout the study.

53. Results: *Results and Discussion:* We observed that... 

54. Conclusion: Overall, our findings indicate...