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Metabolic, Endocrine and Genitourinary Pathobiology

Activin C Antagonizes Activin A *in Vitro* and Overexpression Leads to Pathologies *in Vivo*

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Activin A is a potent growth and differentiation factor whose synthesis and bioactivity are tightly regulated. Both follistatin binding and inhibin subunit heterodimerization block access to the activin receptor and/or receptor activation. We postulated that the activin-β_C subunit provides another mechanism regulating activin bioactivity. To test our hypothesis, we examined the biological effects of activin C and produced mice that overexpress activin-β_C. Activin C reduced activin A bioactivity *in vitro*; in LNCaP cells, activin C abrogated both activin A-induced Smad signaling and growth inhibition, and in LβT2 cells, activin C antagonized activin A-mediated activity of an follicle-stimulating hormone-β promoter. Transgenic mice that overexpress activin-βC exhibited disease in testis, liver, and prostate. Male infertility was caused by both reduced sperm production and impaired sperm motility. The livers of the transgenic mice were enlarged because of an imbalance between hepatocyte proliferation and apoptosis. Transgenic prostates showed evidence of hypertrophy and epithelial cell hyperplasia. Additionally, there was decreased evidence of nuclear Smad-2 localization in the testis, liver, and prostate, indicating that overexpression of activin-β_C antagonized Smad signaling *in vivo*. Underlying the significance of these findings, human testis, liver, and prostate cancers expressed increased activin-βC immunoreactivity. This study provides evidence that activin-β_C is an antagonist of activin A and supplies an impetus to examine its role in development and disease. ***(Am J Pathol 2009, 174:184–195; DOI: 10.2353/ajpath.2009.080296)***

During development, activin A is a mesoderm-inducing factor,[1] and different concentrations of activin are involved in establishing the body plan *in vivo*.[2] In the adult, activin A is a potent regulator of cell division, differentiation, or death as exemplified in the testis, liver, and prostate.[3–7] Exposure of a cell to activin has profound consequences, and, as such, the synthesis and biological action of activin A must be tightly controlled *in vivo*.[8,9]

Activin bioactivity, or bioavailability, can be regulated in several ways including assembly of intracellular heterodimers such as inhibin A ($\alpha\beta_A$) that once secreted block activin binding to its receptors.[10] Follistatin-315 can bind to, and inactivate, activin A in the circulation, whereas cell surface-bound follistatin-288 diverts activin A to a lysosomal pathway for degradation.[11]

It is generally thought that the activin-β_C subunit is not a significant regulator of activin bioactivity. This is because of limited expression of activin-β_C mRNA and a lack of abnormalities in activin-β_C-null mice.[12] However, in the context of the null mouse, there may be functional redundancy with other transforming growth factor-β family members. If true, overexpression rather than underexpression is more likely to have physiological consequences.

We propose that activin C is an antagonist of activin A bioactivity. To test our hypothesis, we produced recombinant activin C and several lines of mice overexpressing the human activin-β_C subunit. Because activin A has tissue-specific patterns of expression and biological action, we examined tissues known to be responsive to

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activin A: testis, liver, and prostate.[13–15] In the testis, activin A is a paracrine regulator of Sertoli cell proliferation and spermatogonial maturation.[5] In the liver, activin A inhibits hepatocyte DNA synthesis[7] and induces apoptosis.[6] In the prostate, activin A is a negative growth regulator and involved in branching morphogenesis during development.[3]

Our results demonstrated activin C antagonized the effects of activin A *in vitro*, and testis, liver, and prostate phenotypes consistent with our hypothesis were observed *in vivo*. Additionally, we show an increase in activin-β_C immunoreactivity in human cancer tissue microarrays, thus demonstrating an analogous situation might occur in human pathology.

Materials and Methods

Transgenic (TG) Mice

Human activin-β_C (under the control of a CMV promoter)-overexpressing mice were produced at Mouseworks, Department of Physiology, Monash University, by standard methods. Three independent lines were established and crossed with wild-type (WT) C57BL/6 mice and heterozygous littermates to obtain single-heterozygous (SH) haploid, and double-heterozygous (DH) diploid, transgenes. Southern blot and semiquantitative polymerase chain reaction (PCR) were used to determine transgene copy number. SH1 had ~2 to 5 copies of the transgene, SH2, 5 to 10 copies, and, SH3, 20 to 30 copies (data not shown). A competitive genomic PCR screening strategy with primers specific for the incorporated human activin-β_C and endogenous mouse activin-β_C was used to identify SH- or DH-positive progeny and results were confirmed by breeding studies.

Tissue Collection

All animal handling and procedures were performed in accordance with National Health and Medical Research Council guidelines for the Care and Use of Laboratory Animal Act and according to the Animal Experimentation and Ethics Committee at Monash Medical Centre, Clayton, Australia. WT and TG mice were obtained from the same litters at age 14 to 16 weeks. Animals were euthanized by cervical dislocation after blood was collected by cardiac puncture. Organs were removed, wet weight recorded, and a portion immersion-fixed in Bouin's for histology or stored at −80°C for RNA and protein extraction.

Histology

Fixed tissues were processed and embedded in paraffin for histological analysis. Serial sections were cut and dried onto Superfrost Plus-slides (Menzel-Glazer, Braunschweig, Germany) before examination by investigators blind as to genotype and overall hypothesis.

Expression of Activin C

CHO cells were stably transfected with full-length human activin-β_C cDNA[16] in the pCI-neo vector or empty vector as a control. Serum-free conditioned media from these stable cells were collected and concentrated and 0.1% bovine serum albumin was added for stability. CHO cell-conditioned media was assessed under nonreducing conditions for activin C expression by Western blot. Conditioned media was also analyzed under reducing and nonreducing conditions with a specific activin A subunit antibody (clone E4; Oxford Bio-Innovations, Oxford, UK) and a follistatin antibody (clone H10[17]) to determine whether activin-β_A subunit proteins ($\beta_A\beta_A$, $\beta_A\beta_C$) or follistatin were detectable in the conditioned media. We could not detect activin-β_A subunit proteins or follistatin in the conditioned media (data not shown). Concentration of activin C in conditioned media was determined by running samples run under reducing and nonreducing conditions on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) along with known amounts of activin A (R&D Systems, Minneapolis, MN) followed by SYPRO Ruby staining (Bio-Rad, Hercules, CA).

Cell Culture and Activin Assays

The activin-responsive human prostate tumor cell line, LNCaP, was routinely cultured in RPMI (Gibco, Invitrogen, Grand Island, NY) with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) and antibiotics (100 IU/ml penicillin and 10 μg/ml streptomycin) (Gibco) in 75-cm^2 culture flasks at 37°C in a humidified atmosphere of 5% CO_2 in air. For growth assays LNCaP cells were plated at a density of 5000 cells/well, in RPMI/5% fetal calf serum into 96-well plates for 24 hours and allowed to attach. Medium was removed and replaced with RPMI/2% fetal calf serum containing 50 ng/ml of activin C, 40 ng/ml of follistatin, buffer, or empty vector controls. After 6 hours, 10 ng/ml of activin A or buffer controls were added and incubated for a total of 72 hours. The CellTiter Aqueous One Solution cell proliferation assay (Promega Corp, Sydney, Australia) was used to determine the number of viable cells after 72 hours of treatment and percent change in cell number was calculated relative to media control.

Transient transfections were performed in the pituitary gonadotrope cell-line, LβT2. Cells were plated 1 day before transfection in 24-well plates and transiently transfected with LipoFectamine Plus (Invitrogen, Carlsbad, CA) for a 338rFSHβ promoter construct[18] or in Opti-MEM (Invitrogen) with a CAGA (12)-lux, a luciferase reporter encoding 12 copies of the CAGA canonical Smad DNA-binding sequence.[19] All experimental treatments were performed in phenol-free, serum-free media. Empty vectors of reporter and expression plasmids were used as controls and to balance DNA where necessary. Cells were treated with control media, activin A (10 ng/ml), increasing concentrations of CHO cell-expressed activin C (25 to 200 ng/ml), or matched volumes of empty vector

control CHO cell-conditioned media (62.5 to 500 μl). Cells were lysed and luciferase activity measured for 30 seconds using an AutoLumat (Berthold Technologies Co., Oak Ridge TN) as previously described.[18,19] At least three independent experiments were performed in triplicate.

RNA Purification and RT-PCR

Total RNA was extracted from testis and liver using the RNeasy mini kit (Qiagen Pty. Ltd., Victoria, Australia). Genomic DNA contamination was removed and total RNA reverse-transcribed using Superscript II reverse transcriptase (Roche Diagnostics, Basel, Switzerland). Real-time PCR was performed using the Roche LightCycler and SYBR Green protocol (Roche Diagnostics). Transgene expression was quantified by expressing cDNA (human activin-β_C, mouse activin-β_C, mouse activin-β_A) as a ratio of β-actin.

Serum Assays

Circulating activin A concentration was measured using a specific enzyme-linked immunosorbent assay (Oxford Bio-Innovations) as previously described.[20] The intra- and interassay coefficients of variation were 5.0% and 10.9%, respectively, and the limit of detection for the assay was 10 pg/ml (four assays). Plasma concentrations of follicle-stimulating hormone (FSH) were measured by radioimmunoassay using mouse FSH as the standard. The assay sensitivity was 1.3 ng/ml, the mean ED50 was 6.4 ng/ml, and the intra- and interassay coefficients of variation were 7.2% and 5.4%, respectively (five assays). Follistatin was measured by radioimmunoassay, as previously described.[21] The assay sensitivity was 1.2 ng/ml, the mean ED50 was 10.1 ng/ml, and the intra- and interassay coefficients of variation were 9.2% and 7.9%, respectively (four assays). The inhibin radioimmunoassay used iodinated 31-kDa bovine inhibin (no. 1989) as tracer and a rat ovarian extract as a standard.[22] No significant cross-reactivity is detected with activin A, however, the assay cross-reacts with pro-αc. Consequently, inhibin levels are reported as immunoreactive total inhibin. Therefore, concentrations need to be interpreted as including cross-reacting inhibin species, which may not be biologically active. The assay sensitivity was 0.12 ng/ml, the mean ED50 was 1.13 ng/ml, and the intra- and interassay coefficients of variation were 10.6% and 6.0%, respectively (four assays).

SDS-PAGE and Western Blot

Serum activin-β_C subunit protein was detected by reducing SDS-PAGE and Western blotting with a specific monoclonal activin-β_C subunit antibody (clone 1)[13–15] at 0.5 μg/ml. Equal volumes of urea-treated (8 mol/L), albumin-stripped (Blue Sepharose; Amersham Biosciences, Uppsala, Sweden) serum were loaded onto 12.5% gels and Western blot was performed as previously described.[14,23] Treated LNCaP cells were lysed in RIPA buffer containing protease and phosphatase inhibitors, and the BCA assay was used to determine total protein concentration. Thirty μg of treated LNCaP supernatant were assessed for phosphorylated Smad-2 activity (no. 3101; Cell Signaling Inc., Beverly, MA), Smad-2 (no. 3122, Cell Signaling), and Smad-4 (no. 9515, Cell Signaling), Total Smad-2 or GAPDH (ab9484; Abcam, Cambridge, UK) was assessed as a loading control and intensity of bands was assessed with Scion Image (National Institutes of Health, Bethesda, MD).

Liver Enzymes

Serum albumin was assayed in duplicate using a 96 well protocol (Bio-assay Systems CA, USA) and serum L-alanine:2-oxogluterate aminotransferase (ALT) was analyzed using an Infinity ALT liquid stable reagent (Thermo Electron Corp., Waltham, MA).

Immunohistochemistry

Proliferating cell nuclear antigen (PCNA) (PC10; DAKO, Kingsgrove, Australia), activated caspase-3 (Asp175, Cell Signaling), activin-β_C (clone-1[15]), and Smad-2 (no. 3122, Cell Signaling) were detected using the DAKO Autostainer universal staining system (DAKO A/S, Glostrup, Denmark). Microwave antigen retrieval for PCNA was performed in 0.01 mol/L citrate buffer (pH 6.0), activin-β_C in 0.1 mol/L glycine buffer (pH 4.5), caspase-3, and Smad-2 in 0.01 mol/L sodium citrate buffer (pH 6.0). Sections were treated with CAS blocking reagent (DAKO, Kingsgrove, Australia), antibodies detected with avidin-biotin complex (ABC) and color-reacted with diaminobenzidine (DAKO, Kingsgrove Australia).

Fertility Assessment

Male mice (WT, SH, and DH) of varying ages (6 to 30 weeks) were cohabited with 10- to 12-week female C57BL/6 WT mice (1:1). Females were checked daily for the presence of copulatory plugs. Females with positive plugs were separated from males, and the number of pups per plug was recorded. Daily sperm production (DSP) was determined using a frozen left testis from WT, SH, and DH mice by a procedure that has been previously described.[24]

Sperm Motility Analyses

Sperm were collected and capacitated in modified Tyrode's medium without sodium pyruvate or sodium lactate. Sperm from 23 mice were collected at 14 to 16 weeks (n = 10 WT, 5 SH2, and 9 DH2). Both caudae epididymidis were dissected from the mice and a small slit was made in each before transfer to a 1.5-ml tube containing 1 ml of pre-equilibrated modified Tyrode's medium and incubation in 5% CO_2 in air at 37°C for 10 minutes to allow the sperm to swim out from the tissue. After incubation the caudae were removed and a 10-μl aliquot of the sperm suspension placed into an 80-μm

chamber (Hamilton-Thorne Biosciences, Beverly, MA). Replicates (two per mouse) were analyzed using a Hamilton-Thorne IVOS computer-assisted sperm analyzer (Hamilton-Thorne Biosciences). At least 1000 sperm were analyzed per replicate. Analyses were performed using the recommended settings for mouse sperm. Data were arcsin-transformed and then subjected to general linear analysis, and the difference between means was determined by Tukey's HSD test (SPSS for Windows; SPSS, Chicago, IL).

In Situ *Detection of DNA Fragmentation in Testis Sections*

Apoptosis in testis sections was analyzed by the ApopTag *in situ* apoptosis detection kit (Chemicon Int., Temecula, CA).[25]

Testis Stereology

Slides were masked before quantitation to facilitate unbiased counting. PCNA-positive cell types were identified based on their location within the tubule, their size, and the shape of the cell nucleus. Apoptotic cells were identified by deep brown nuclear staining and included spermatogonia, spermatocytes, and spermatids. Two sections per mouse were examined. Each tubule cross section was classified in one of three stage groupings (XII to IV, V to VIII, and IX to XI).

Cell Number Estimates

The optical dissector (*sic*) stereological method[26] was used to determine the total number of cell nuclei per testis. All measurements were performed using a ×100 objective on an Olympus BX-50 microscope (Olympus, Tokyo, Japan). A microcator (D 8225; Heideinhain, Traunreut, Germany) that monitored scanned depth was attached to the microscope stage. Fields were selected by a systematic uniform random sampling scheme as previously described[26] with the use of a motorized stage (Multicontrol 2000; ITK, Lahnau, Germany).

Liver and Prostate Proliferation and Apoptosis

The incidence of proliferation (PCNA)- and apoptosis (activated caspase-3)-positive cells in tissue sections was estimated based on a method that allowed an unbiased semiquantitation of the percentage of positive cells in TG and WT samples. Masked tissue sections were mapped at ×10 magnification to define tissue boundaries. Random fields were systematically selected by CAST V1.10 software and sampling was conducted using an unbiased counting frame. Frame counting was performed on sections uniformly spaced throughout the tissue, 150 frames, and ×100 magnification per section, n = five triplicate sections with an average of 1000 cells counted per section.

Localization of Smad-2

Tissue sections were masked and the incidence of nuclear localization of total Smad-2 in testis, liver, and prostate sections was estimated as described above. Frame counting was performed on five to eight duplicate sections, 150 frames, ×40 magnification, with an average of 1000 cells counted per section.

Cancer Tissue Microarrays

Activin-β_C subunit protein was assessed in normal human and cancer tissue arrays with one example of each tissue and tumor type on each array (n = 2; SuperBioChips Laboratory, Seoul, Korea) using a specific monoclonal antibody (clone 1) as previously described.[15]

Statistical Analysis

TG and WT littermate controls were compared using analysis of variance with Dunnett's posthoc test and the significance threshold used at a level of 5% (GraphPad Software, Inc., San Diego, CA).

Results

Activin C Antagonized the Growth Inhibitory Effects of Activin A in Vitro

CHO cells were stably transfected with full-length human activin-β_C cDNA in the pCI-neo vector or empty vector (EV) control. CHO cell-conditioned media containing activin C (50 ng/ml) was added to the activin-responsive LNCaP prostate cell line and cell growth-assessed (Figure 1A). Empty vector conditioned media or media only served as controls. Empty vector or activin C alone did not alter cell number compared to media control, indicating that activin C alone had no effect on cell growth. As expected, activin A (10 ng/ml) decreased cell number by 60% in both media and EV controls ($P < 0.001$), whereas activin A only reduced growth by 30% in the presence of activin C-conditioned media ($P < 0.01$ versus media and EV + activin A controls), indicating that activin C antagonized the growth inhibitory effects of activin A. Again, as expected, follistatin, a well-characterized activin binding protein, antagonized the growth inhibitory effects of activin A with cell numbers returning to 80% of control. Addition of follistatin and activin C together attenuated this effect with values rising to 110% of media control ($P < 0.01$ versus media + activin A + follistatin and EV + activin A + follistatin), which implies that antagonism of activin A is likely to be via different mechanisms.

To determine the mechanism by which activin C-conditioned media antagonized the growth inhibitory effects of activin A, we assessed the activin signaling molecules, Smad-2, phosphorylated Smad-2, and Smad-4 in treated LNCaP cells. As expected, activin A (10 ng/ml) increased phosphorylation of Smad-2 ($P < 0.01$ versus media and EV controls) and follistatin (40 ng/ml) decreased activin-

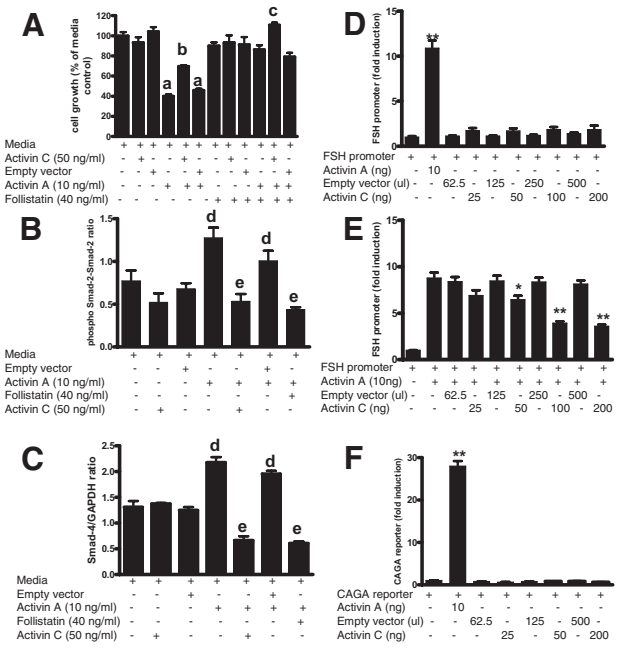

Figure 1. Effects of activin C *in vitro*. **A:** LNCaP cells were treated with 100 μl of RPMI/2% fetal calf serum containing 50 ng/ml of activin C-conditioned media, 40 ng/ml of follistatin (FS), buffer, or empty vector (EV) controls. After 6 hours, either 10 ng/ml of activin A or buffer controls were added, and the CellTiter assay was used to determine the number of viable cells after 72 hours of treatment. Percentage change in cell number was calculated relative to media control. Results are mean ± SD from triplicate wells in three independent experiments. **a** = $P < 0.001$, **b** = $P < 0.01$ versus media + activin A and EV + activin A, **c** = $P < 0.01$ versus media + follistatin + activin A and EV + follistatin + activin A. **B:** Levels of phosphorylated Smad-2 relative to total Smad-2 in LNCaP cells after treatment with activin A (10 ng/ml), follistatin (40 ng/ml), activin C-conditioned media (50 ng/ml), media only, or empty vector (EV) control. Results are mean ± SD in three independent Western blots assessed using Scion software (National Institutes of Health). **d** = $P < 0.01$ versus media and EV controls, **e** = $P < 0.001$ versus media + activin A and EV + activin A. **C:** Levels of Smad-4 relative to GAPDH in LNCaP cells after treatment with activin A (10 ng/ml), follistatin (40 ng/ml), activin C-conditioned media (50 ng/ml), media only, or empty vector (EV) control. Results are mean ± SD in three independent Western blots assessed using Scion software (National Institutes of Health). **d** = $P < 0.01$ versus media and EV controls, **e** = $P < 0.001$ versus media + activin A and EV + activin A. **D:** LβT2 cells were transiently transfected with a rat FSH-β promoter construct and treated with activin A (10 ng/ml), CHO cell-expressed activin C-conditioned media (25 to 200 ng/ml), or an equal volume of empty vector control. Twenty-four hours later, luciferase activity was assessed. Results are mean ± SD in three independent assays. **$P < 0.001$. **E:** LβT2 cells were transiently transfected with a rat FSH-β promoter construct and treated with activin A (10 ng/ml) plus CHO cell-expressed activin C-conditioned media (25 to 200 ng/ml) or an equal volume of empty vector control. Twenty-four hours later luciferase activity was assessed. Results are mean ± SD in three independent assays. *$P < 0.05$, **$P < 0.01$ versus activin A alone. **F:** LβT2 cells were transiently transfected with CAGA (12)-lux, a luciferase reporter encoding for 12 copies of the CAGA canonical Smad DNA-binding sequence and treated with activin A (10 ng/ml) CHO cell-expressed activin C-conditioned media (25 to 200 ng/ml) or an equal volume of empty vector control. Twenty-four hours later luciferase activity was assessed. Results are mean ± SD in three independent assays. **$P < 0.001$.

mediated phosphorylation of Smad-2 × 50 to 60% ($P <$ 0.001) versus media and EV + activin A (Figure 1B). Activin C- conditioned media (50 ng/ml) decreased activin A-induced Smad-2 phosphorylation by 50% ($P <$ 0.001 versus media and EV + activin A), indicating that activin C antagonism of activin A-induced signaling is as potent as follistatin. Results are presented relative to total Smad-2 (Figure 1B). There was no significant difference in total Smad-2 protein levels with treatment, indicating that the decreases in phosphorylated Smad-2 were not related to total Smad-2 levels. Activin A (10 ng/ml) increased Smad-4 protein expression ($P < 0.01$ versus media and empty vector controls. Activin C (50 ng/ml) or empty vector-conditioned media did not alter Smad-4, however, activin C-conditioned media (50 ng/ml) decreased activin A-mediated Smad-4 protein expression ($P < 0.01$) as effectively as follistatin (40 ng/ml, Figure 1C).

Activin C Antagonized Activin A-Stimulated Activity of the Rat FSH-β Promoter

To confirm results in an independent activin A assay, we assessed the effects of activin C- or empty vector-conditioned media on a rat FSH-β promoter. Activin C-conditioned media (25 to 200 ng/ml) or an equal volume of empty vector control (62.5 to 500 μl) had no effect in the FSH-β promoter assay, whereas activin A (10 ng/ml) increased rat-FSH-β promoter activity ($P < 0.001$, Figure 1D). Addition of activin C-conditioned media (25 to 200 ng/ml) plus activin A (10 ng/ml) significantly decreased activin A-stimulated rat-FSH-β promoter activity (50 ng/ml, $P < 0.05$; 100 to 200 ng/ml, $P < 0.01$), whereas an equal volume (62.5 to 500 μl) of empty vector control had no effect (Figure 1E).

CAGA Smad DNA-Binding Sequence

To confirm that activin C had no independent effect on Smad signaling we assessed if activin C-conditioned media activated or antagonized an extremely sensitive CAGA Smad DNA-binding sequence.[18] As expected, activin A (10 ng/ml) activated the CAGA reporter ($P <$ 0.001), whereas increasing concentrations (25 to 200 ng/ml) of activin C or matched volumes of empty vector control-conditioned media (62.5 to 500 μl) had no effect (Figure 1F), thus indicating that activin C had no independent effect on the CAGA reporter.

Overexpression of Activin-β_C in Vivo *Causes an Age-Related Decrease in Male Fertility*

Based on previous reports on the expression of activin-β_C in the testis[13,14] and the known importance of activin A for male fertility,[5] we examined testis histology and fertility of TG mice compared to age-matched WT littermates. Hypospermatogenesis, characterized by the presence of normal and abnormal testis tubules in close proximity, was evident in TG testis sections (Figure 2A). Similar histology was recorded in all TG lines.

Figure 2, B to D, shows a significant decrease in litter size when double-heterozygous (DH) or single-heterozygous (SH) TG male mice were mated with WT females, compared with WT-WT matings. Significant decreases in number of pups per plugged mating were evident in all DH lines ($P < 0.01$ within 10 to 12 weeks). SH1 showed a significant decrease in litter size compared with WT age-matched males from 19 to 21 weeks ($P < 0.01$, Figure 2B); SH2 from 13 to 15 weeks ($P < 0.01$, Figure 2C) and SH3 from 16 to 18 weeks ($P < 0.01$, Figure 2D).

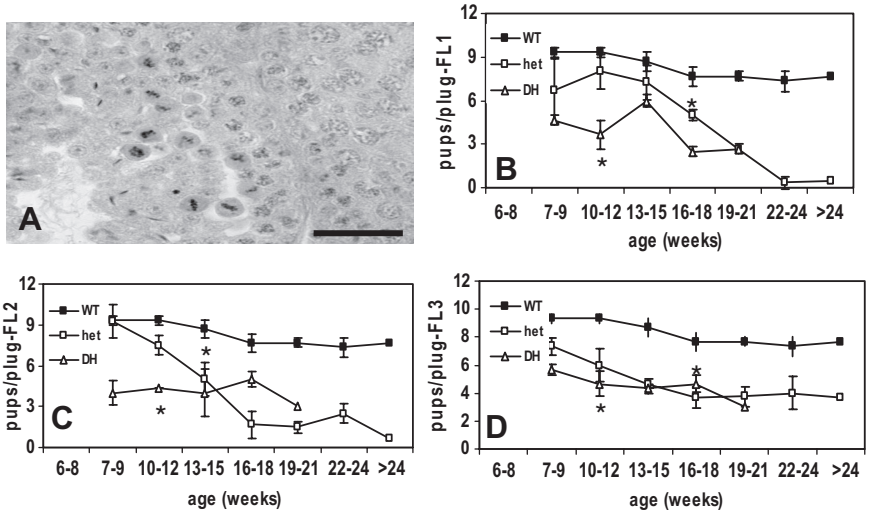

Figure 2. Testis histology and pups per copulatory plug in activin-β_C-overexpressing mice. **A:** An example of histology in a representative TG testis section. **B–D:** Pups per copulatory plug in WT, SH (het), and DH activin-β_C subunit-overexpressing founder lines (FL) 1 (**B**), 2 (**C**), and 3 (**D**). In all lines of TG mice, a consistent age-dependent decrease in fertility was evident when mated with WT females, resulting in reduced litter size. Mean ± SD. $^*P < 0.01$ versus WT, data were analyzed by analysis of variance, n = six WT, six to nine SH1 to SH3, and four to six DH1 to DH3. Scale bar = 100 μm.

Mechanisms Underlying Decreased Litter Size DSP

To examine the mechanism underlying decreased litter size DSP was assessed in mice 14 to 16 weeks of age. Overexpression of activin-β_C decreased DSP by 31%, 59%, and 39% in DH1, DH2, and DH3 and by 58%, 63%, and 19% in SH lines 1, 2, and 3, respectively (Figure 3A).

Stage-Specific Increase in Apoptosis

To determine whether reduced sperm output was a result of alterations in germ cell proliferation and/or survival we quantified apoptosis and proliferation in stage-specific cross-sections of testis tubules in DH2 mice 14 to 16 weeks of age compared to age-matched WT littermates. DH2 mice were chosen because there were no confounding serum FSH effects (see serum proteins below). The percentage of tubules containing apoptotic germ cells (TUNEL-positive) was elevated by 180% and 130% in stages V to VIII (P = 0.001) and IX to XI (P = 0.04) (Figure 3B) in DH2 males, respectively, compared with age-matched WT littermates. In contrast to apoptosis, there was no significant change in percentage of proliferating (PCNA-positive) germ cells (data not shown).

To determine whether the alteration in apoptosis affected a specific germ cell type, we used stereological methods to quantitate the number of germ cells and Sertoli cells in tubular cross sections from DH2 mice compared to age-matched WT littermates. The number of Sertoli and germ cells remained at control levels; although it was noted that pachytene spermatocytes were lower compared to WT controls (66%, P = 0.069; see Supplementary Table S1 at *http://ajp.amjpathol.org*).

Compromised Sperm Motility and Function

Sperm was collected from the caudae epididymides and motility characterized using a computer-assisted sperm analyses system. Sperm from TG mice showed a significant reduced percentage of motile sperm compared to age-matched WT littermates (DH2, 54.2%; SH2, 49.3%; WT, 69.2%; $P < 0.05$ versus WT). Further, of those sperm that were motile, a significant percentage dis-

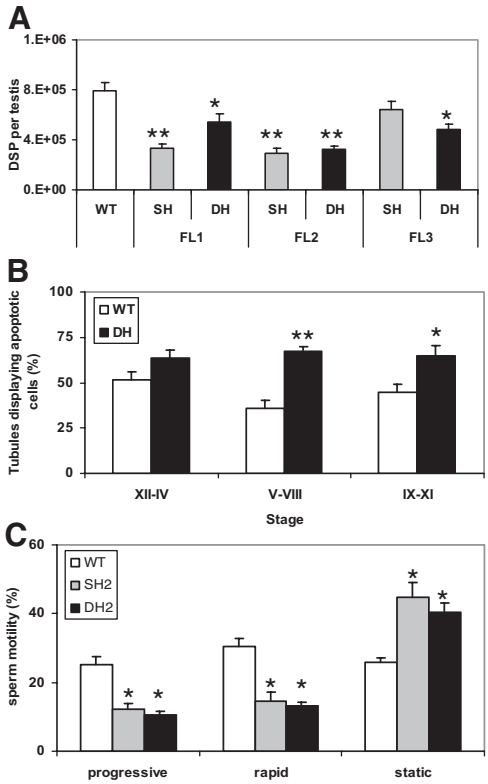

Figure 3. DSP, apoptosis, and sperm motility in activin-β_C-overexpressing TG mice. **A:** DSP per testis in WT and three independent activin-β_C founder lines (FLs). Overexpression of activin β_C subunit decreased DSP. Mean ± SD. $^{**}P < 0.005$, $^*P < 0.05$ versus WT, data were analyzed by analysis of variance, n = 12 WT, 6 to 9 SH1 to SH3, and 4 to 6 DH1 to DH3. **B:** Apoptosis in WT and DH2 testis sections was analyzed by the ApopTag *in situ* apoptosis detection kit. Overexpression of activin-β_C caused a stage-specific increase in apoptosis. Mean ± SD. $^{**}P < 0.005$, $^*P < 0.05$ versus WT, data were analyzed by analysis of variance, n = five per group. **C:** Sperm from 10 WT, 5 SH2, and 9 DH2 were collected from the caudae epididymides and motility parameters assessed by IVOS, a computerized assisted sperm analyses system. Data were subjected to general linear analysis and the difference between means was determined by Tukey's HSD test. Mean ± SD. $^*P < 0.05$ versus WT.

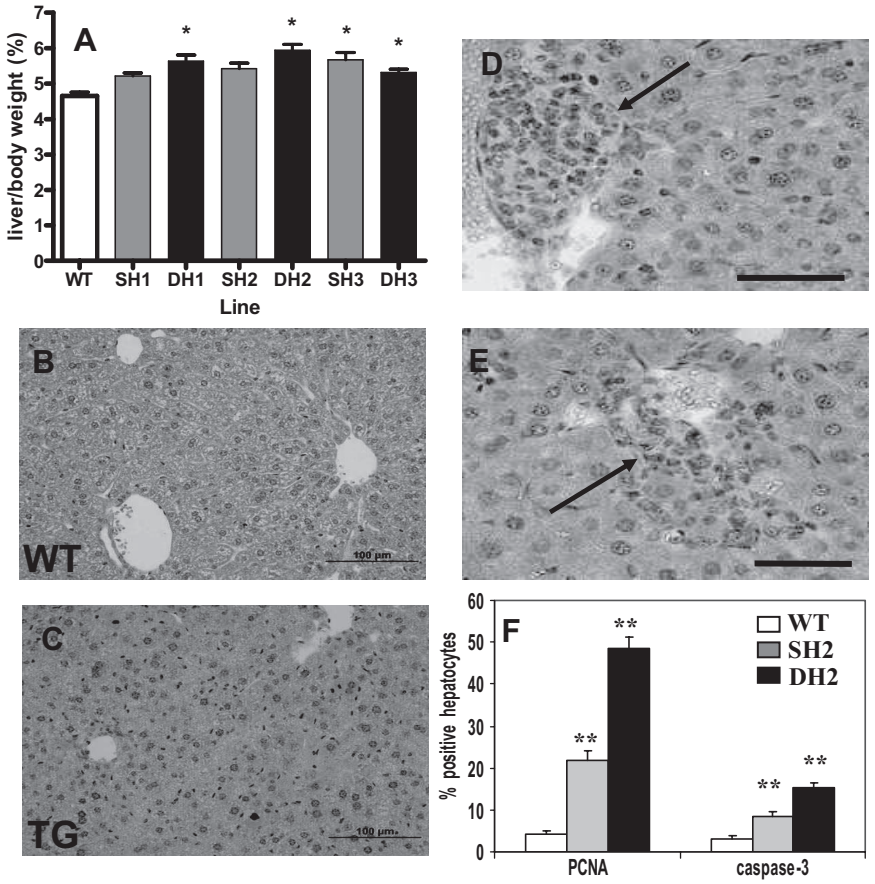

Figure 4. Liver phenotype in activin-β_C-overexpressing mice. Fresh liver weight was recorded and a small portion of tissue was immersion-fixed in Bouin's fixative. The incidence of proliferation (PCNA-positive) and apoptosis (caspase-3-positive) in hepatocytes was estimated based on a method that allowed an unbiased semiquantitation of the percentage of positive cells. **A:** Overexpression of activin-β_C increased liver weight as a percentage of body weight in SH3 and all DH lines. Mean ± SD. $^*P < 0.05$ versus WT, data were analyzed by analysis of variance, n = five to eight per group. **B** and **C:** a low-power example of WT and TG H&E-stained liver sections. **D:** An example of inflammation in activin-β_C-overexpressing liver (**arrow**). **E:** An example of piece-meal necrosis in activin-β_C-overexpressing liver (**arrow**). **F:** Stereological assessment of liver sections in WT and line 2 TG mice. Overexpression of activin-β_C increased hepatocyte proliferation and apoptosis. Mean ± SD. $^{**}P < 0.005$ assessed by analysis of variance, n = five triplicate sections in five to eight animals per group. Scale bars = 100 μm.

played reduced sperm velocity and beat cross frequency (Figure 3C; see Supplementary Table S2 at *http://ajp.amjpathol.org*).

Liver Inflammation in Activin-β_C-Overexpressing Mice

Overexpression of activin-β_C increased total liver weight and percentage of body weight in DH1 to DH3 and SH3 mice (Figure 4A). A low-power example of WT and TG liver section is presented in Figure 4, B and C. In all TG livers, foci of inflammatory cells were observed primarily in zones 1 and 2, which occasionally extended into zone 3. Infiltration consisting primarily of neutrophils and macrophages indicated regions of acute inflammatory response (Figure 4D). There was occasional Kupffer cell activation and regions of necrosis (Figure 4E), without evidence of hepatic stellate cell activation or fibrosis. There was increased evidence of larger hepatocyte nuclei and dividing hepatocytes in livers of all TG lines.

Increased Hepatocyte Turnover

Histological assessment of PCNA- and caspase-3-stained liver sections in TG mice showed increased hepatocyte proliferation and apoptosis compared to WT littermate controls (Figure 4F). The combined effect of these two parameters (evidenced by analysis of the ratio of proliferation to apoptosis) indicated activin-β_C overexpression influenced hepatocyte proliferation to a greater extent than apoptosis. This observation was most apparent in DH2 mice; the ratio of proliferation to apoptosis in WT was 1.3, SH2 was 2.6, and DH2 was 3.1.

Prostate Hypertrophy and Epithelial Cell Hyperplasia with Activin-β_C Overexpression

A significant increase in anterior prostate weight (AP) was evident in TG mice compared to WT litter-mate controls (SH3, 130% compared to WT, $P < 0.05$; DH1, 148%, $P < 0.01$; and DH2, 130%, $P < 0.05$). An increase in ventral prostate (VP) weight was evident (DH1, 165%, $P < 0.01$; DH2, 162%, $P < 0.01$). Lateral prostate (LP) weight increased (SH3, 167%, $P < 0.0$); DH2, 157%, $P < 0.01$; and DH3, 155%, $P < 0.01$). There was no change in dorsal prostate (DP) weight. WT VP sections were mostly composed of a single layer of epithelial cells lining a lumen (Figure 5A). Diffuse luminal epithelial cell hyperplasia, characterized by increased stratification in the form of tufting and papillary in-folding and a reduction in luminal size, was evident in all DH lines (Figure 5B). No atypical nuclei were observed in SH TG mice; however, there was evidence of atypical nuclei in DH VP sections; including nuclear enlargement (one to three per high-power field), increased prominence of nucleoli (one to three per high-power field), and cribriform structures

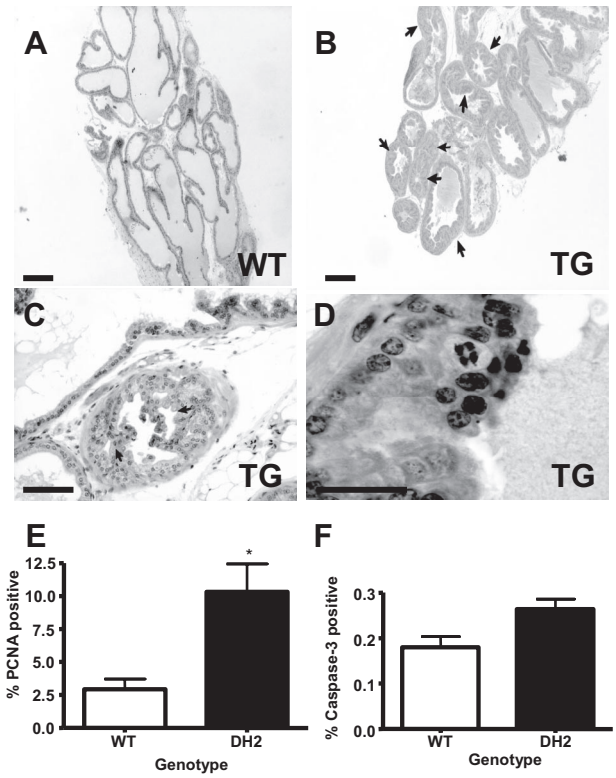

Figure 5. Prostate histology and proliferation. The incidence of proliferation (PCNA-positive) and apoptosis (caspase-3-positive) was estimated based on a method that allowed an unbiased semiquantitation of the percentage of positive cells in TG and WT samples. **A** and **B:** Example of a typical WT (**A**) and TG (**B**) ventral prostate, note a single layer of epithelial cells lining the lumen in WT and increased in-folding and multiple layers of epithelial cells (**arrows**) in TG VP sections. **C:** Abnormal pathology (cribriform structure) in a TG VP. **D:** An example of a mitotic epithelial cell in TG VPs. **E:** Overexpression of activin-β_C increased luminal epithelial cell proliferation whereas there was no significant difference in apoptotic luminal epithelial cells (**F**). Mean ± SD in triplicate sections in n = five mice per group with an average of 1000 cells counted per section. Scale bars = 100 μm. *$p < 0.01$.

(Figure 5C). Epithelial cell mitosis was more frequently observed in DH VP sections (one to four cells per high-power field, Figure 5D), whereas mitotic cells were not observed in WT VP sections. Assessment of the percentage of proliferating (PCNA-positive) and apoptotic (activated caspase-3-positive) epithelial cells in 14- to 16-week-old WT and DH2 TG mice showed a significant increase in PCNA-positive cells (Figure 5E) and no difference in epithelial cell apoptosis (Figure 5F).

Reduced Nuclear Localization of Total Smad-2 in Activin-β_C-Overexpressing Mice

To determine whether phenotypes observed with activin-β_C overexpression in the testis, liver, and prostate were related to reduced Smad-2 signaling *in vivo* we quantified nuclear localization of total Smad-2 in TG and WT littermate controls. Significant reductions in nuclear localization of total Smad-2 were evident in the testis, liver, and prostate of activin-β_C-overexpressing mice ($P < 0.01$; Figure 6, A–C). Figure 6, D to F, shows representative examples of Smad-2 staining in WT and TG testis, liver, and ventral prostate, respectively.

Activin Subunit Expression

To quantify TG gene expression and to determine whether phenotypic changes were related to changes in endogenous mouse activin-β_C or activin-β_A gene expression, real-time RT-PCR was performed. Transgene (human activin-β_C) mRNA expression was evident in the testis, liver, and prostate of all TG mice. Founder line 2 mice showed the highest total activin-β_C mRNA expression in the testis and VP and line 3 in the liver (Figure 7A). Overexpression of human activin-β_C did not alter endogenous mouse activin-β_C mRNA expression (Figure 7B). Transgene expression did not alter activin-β_A mRNA levels in the testis or prostate; however, a significant increase in activin-β_A mRNA expression was evident in the liver (Figure 7C). Therefore tissue-specific phenotypes were not related to changes in endogenous activin-β_C mRNA expression or a reduction in activin-β_A mRNA. Consistent with the elevated activin-β_C mRNA levels an increase in activin-β_C subunit protein was evident in the serum of TG mice (Figure 7D).

Serum Proteins in Activin-β_C-Overexpressing Mice

Activin A

To determine whether overexpression of activin-β_C lowered synthesis of activin A ($\beta_A\beta_A$ homodimer), serum levels of activin A were measured by enzyme-linked immunosorbent assay. All TG mice overexpressing activin-β_C showed lower serum activin A (Table 1) compared to age-matched WT littermates, reaching significance in SH1 (60% of WT), and DH2 (48%), and DH3 (43%).

FSH

We investigated if increased activin-β_C subunit expression altered FSH secretion. Serum FSH levels were significantly decreased in DH1 and SH1 (DH1, 69%; and SH1, 73% of control; $P < 0.05$) and 3 (DH3, 69%; and SH3, 75%; $P < 0.05$) (Table 1). There was a significant increase in luteinizing hormone (LH) compared to age-matched WT controls in DH1 (186%, $P < 0.001$) and DH2 lines (143%, $P < 0.01$) (data not shown).

Inhibin and Follistatin

Total serum inhibin increased in TG lines DH1 to DH3 × 191%, 157%, and 142%, respectively, and SH3 × 134% compared to age-matched WT controls (Table 1, $P < 0.005$). Follistatin levels were significantly increased in lines DH2 (162%) and SH3 (156%, $P < 0.005$) when compared to WT controls (Table 1).

Liver Enzymes

Total serum albumin, a marker of liver function, decreased by 18% in all DH lines ($P < 0.05$), whereas ALT levels, a marker of liver inflammation, increased twofold

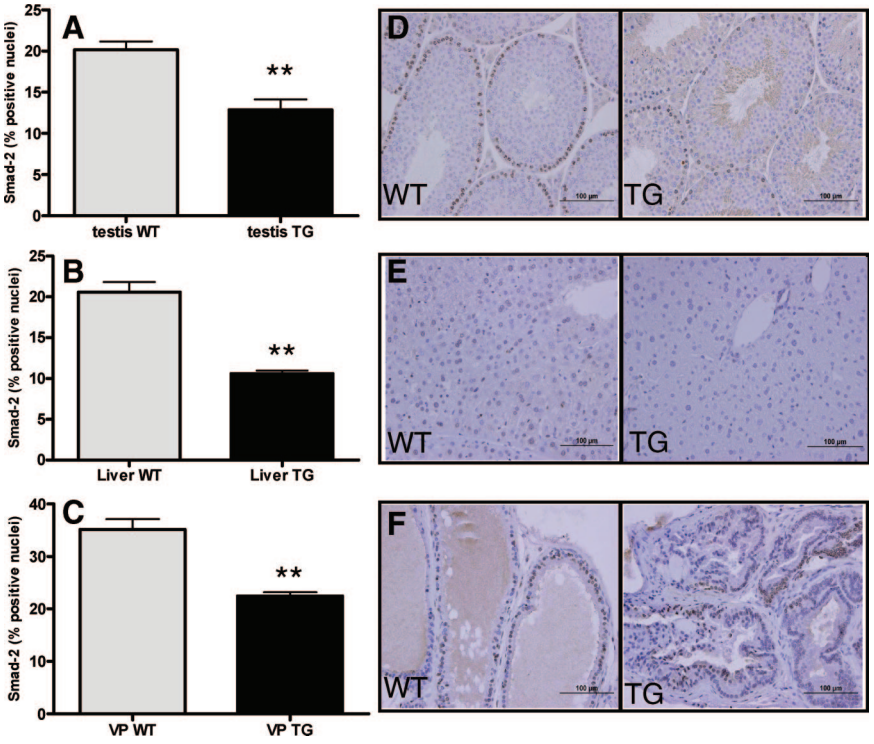

Figure 6. Nuclear localization of total Smad-2 in TG tissues. Total Smad-2 was detected using the DAKO Autostainer universal staining system. Tissue sections were masked and the incidence of nuclear localization of total Smad-2 in testis, liver, and prostate sections was estimated by standard stereology methods. Frame counting was performed on five to eight duplicate sections, 150 frames, ×40 magnification, with an average of 1000 cells counted per section. The percentage of positive nuclei are presented in the testis (**A**), liver (**B**), and ventral prostate (**C**). $^{**}P < 0.01$ versus WT littermate controls. Examples of WT and TG Smad-2 staining patterns in the testis, liver, and ventral prostate are presented in **D–F**, respectively. Scale bars = 100 μm.

to threefold in DH1 to DH3 and SH3 ($P < 0.005$) compared to WT controls (Table 1).

Up-Regulation of Activin-β_C Subunit Immunoreactivity in Human Testis, Liver, and Prostate Cancer

To determine whether activin-β_C subunit proteins were altered in human disease, we assessed activin-β_C subunit protein expression in normal and cancer tissue arrays with one example of each tissue and tumor type on each array (n = two) using a specific activin-β_C monoclonal antibody.[15] Increased activin-β_C immunoreactivity was evident in a range of human cancers including testis (two of two), liver (two of two), and prostate (two of two) when compared to normal controls (Figure 8, A, C, and E, normal testis, liver, and prostate, respectively; Figure 8B, testis seminoma; Figure 8D, hepatocellular cancer; and Figure 8F, prostate cancer). Nuclear localization of activin-β_C subunit protein was evident in normal testis, testis seminoma, and hepatocellular cancer. Nuclear localization of activin-β_C has previously been shown[14,27] and may indicate a novel regulatory mechanism that warrants further study.

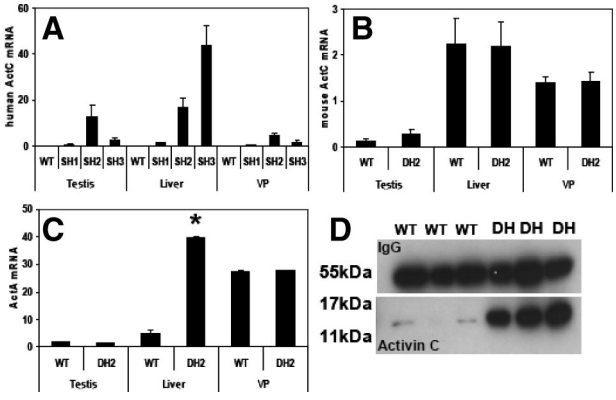

Figure 7. Gene expression in activin-β_C-overexpressing TG mice. **A:** Transgene mRNA expression. mRNA expression was assessed by real-time RT-PCR in n = five WT and TG mice. Human activin-β_C mRNA expression was highest in SH2 testis and VP, whereas the SH3 line showed the highest expression in liver. **B:** Mouse endogenous activin-β_C expression levels: There was no significant difference in mouse activin-β_C mRNA expression. **C:** Activin-β_A mRNA expression levels. Activin-β_A mRNA expression did not significantly change in TG testis or prostate however there was an increase in the liver of DH2 mice. *Significantly increased $^{*}P < 0.05$ versus WT. **D:** Circulating activin-β_C subunit protein. Urea treated (8 mol/L) albumin stripped reduced serum samples from three WT and three DH2 TG mice were assessed by SDS-PAGE and Western blotting with a specific activin-β_C subunit antibody, increased activin-β_C subunit protein was evident in the serum of TG mice. Mouse IgG heavy chain 55 kDa is provided as a loading control.

Discussion

This study provides the first compelling evidence that activin-β_C is a regulator of activin A bioactivity. CHO cell-expressed activin C-conditioned media antagonized activin A in two independent assays *in vitro*. When overexpressed in tissues that are known to be regulated by activin A, pathologies become evident as exemplified in the liver, testis, and prostate of the activin-β_C-overexpressing mice. Pathologies were associated with reduced Smad-2 nuclear localization, suggesting that the mechanism of action is antagonism of activin A signaling *in vivo*. The potential significance of activin-β_C overexpression in human disease is implied by its up-regulation in human liver, testis, and prostate cancer.

Activin is involved in development and is a potent growth and differentiation factor in the adult. Several means of regulating bioactivity are known to occur.

Table 1. Serum Assessment in WT and Activin-β_C Subunit Transgenic Male Mice Aged 14 to 16 Weeks

	Activin A (pg/ml)	FSH (ng/ml)	Total inhibin (ng/ml)	Follistatin (ng/ml)	Albumin (mg/dl)	ALT (U/L)
WT	58 ± 19	15.2 ± 3.2	0.67 ± 0.12	3.2 ± 0.89	2.34 ± 0.18	13.8 ± 6.6
DH1	37 ± 16	11.2 ± 1.0*	1.28 ± 0.14*	3.4 ± 0.3	1.95 ± 0.20†	34.5 ± 8.7*
DH2	28 ± 5*	12.4 ± 1.2	1.05 ± 0.26*	5.2 ± 1.6*	2.15 ± 0.09†	33.6 ± 8.5*
DH3	25 ± 4*	11.5 ± 1.2†	0.95 ± 0.28†	3.9 ± 0.6	1.95 ± 0.17†	29.9 ± 1.7*
SH1	35 ± 4*	10.5 ± 1.0†	0.74 ± 0.13	2.4 ± 0.5	2.49 ± 0.43	20.9 ± 11.6
SH2	43 ± 12	13.8 ± 2.3	0.58 ± 0.22	3.0 ± 0.2	2.88 ± 0.55	21.7 ± 7.7
SH3	44 ± 15	10.5 ± 1.1†	0.90 ± 0.24†	5.0 ± 0.5*	2.56 ± 0.13	34.5 ± 9.8*

Serum activin A, FSH, total inhibin, follistatin, and the liver enzymes albumin and ALT were assessed in WT, DH, and SH activin-β_C subunit overexpressing transgenic mice. Mean ± SD.
*$P < 0.01$; †$P < 0.05$ versus WT, $n = 16$ WT, 4 to 6 DH, and 7 to 9 SH male mice in each line.

Based on our findings, activin C should be regarded as another important regulator of activin A. Although there are some similarities to other means of regulating activin, there are more notable differences, thus adding further evidence of the significance of activin-β_C to activin biology.

Well-characterized regulators of activin biology are the inhibin α-subunit and follistatin. The testis phenotypes in mice overexpressing the inhibin-α subunit or follistatin differ to that of the activin-β_C-overexpressing mice. Whereas overexpression of activin-β_C resulted in a progressive age-related decrease in male fertility, a stage-specific increase in germ cell apoptosis, decreased sperm production, and poor motility in those sperm that were produced, overexpression of inhibin-α decreased testis size because of a reduction in seminiferous tubule volume. Inhibin-α-overexpressing males were fertile, despite a 50% reduction in sperm number and there were no abnormalities in sperm motility.[28,29] In follistatin-overexpressing mice, Leydig cell hyperplasia, decreased Sertoli cell number and seminiferous tubular degeneration were evident.[30]

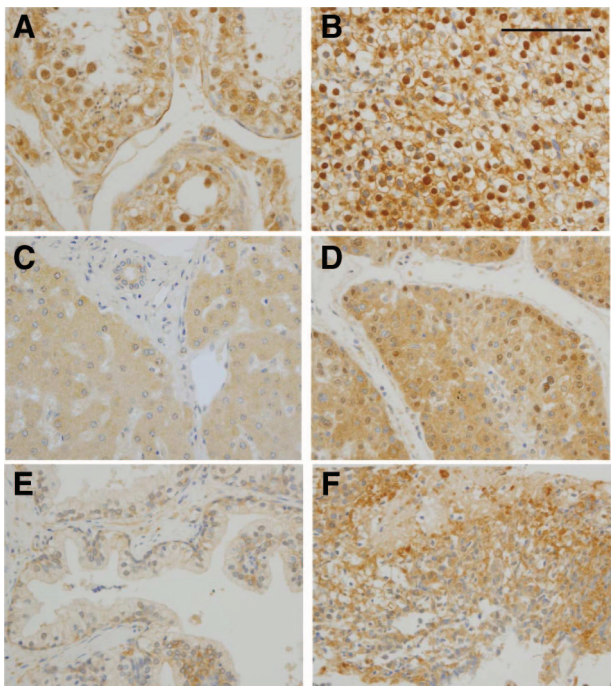

Figure 8. Activin-β_C subunit immunoreactivity increased in human testis, liver, and prostate cancers. Activin-β_C subunit protein expression was assessed in two normal human and cancer tissue arrays (SuperBioChips Laboratory, Seoul, Korea). **A**, **C**, and **E:** Normal testis, liver, and prostate. **B:** Testis seminoma. **D:** Hepatocellular cancer. **F:** Prostate cancer. Scale bar = 100 μm.

The BK mouse, in which the activin-β_B allele is knocked into the β_A locus, is another model that has been used to study the consequences of perturbing activin A levels *in vivo*. In this model activin-β_B functions as a hypomorphic version of β_A, making it similar to the mouse model described herein. BK mice survive to adulthood and display a delayed onset of fertility in males.[31] Whether or not BK mice have compromised DSP or sperm motility is not known. However, all models underscore the importance of activin A for normal male fertility. The activin-β_C TG mice described herein identify a novel regulatory mechanism in the testis, which has not been previously appreciated.

In liver, activin-β_C overexpression induced liver inflammation, which was observed microscopically and confirmed by increases in serum ALT. Synthetic function of the liver was decreased as determined by serum albumin production. These changes were associated with hyperplastic livers attributable to an alteration in hepatocyte cellular turnover that favored proliferation. No liver phenotypes were reported in the inhibin-α- or follistatin-overexpressing mice, but when follistatin is overexpressed in inhibin knockout mice, inflammation in the liver also occurs.[32] This is the only feature in common with the activin-β_C-overexpressing mice and concurs with our view that a decline in activin A is associated with liver inflammation.

Activin family members are known to significantly influence hepatic homeostasis. Activin A is an inhibitor of hepatocyte DNA synthesis[6] and is involved in hepatic pathologies such as viral hepatitis[33] and fibrosis.[34] Activin-β_C mRNA is down-regulated after partial hepatectomy in the rat, suggesting that it may be a negative regulator of liver cell growth.[35,36] However, the role of activin-β_C in the liver is controversial, with studies suggesting the activin-β_C subunit inhibits DNA synthesis of hepatic cells *in vivo*[37] and *in vitro*.[38] Wada and colleagues[39,40] demonstrate that the activin-β_C homodimer is growth promoting *in vitro* and *in vivo*, and we found increased activin-β_C subunit immunoreactivity associated with mitotic hepatocytes in regenerating rat liver.[23] In our mouse model, overexpression of activin-β_C increased hepatocyte cell proliferation to a greater extent

than apoptosis, indicating activin-β_C is a positive growth regulator in the liver, thus adding further evidence to our hypothesis that the activin-β_C subunit antagonizes the growth inhibitory effects of activin A in the liver.

Prostate hypertrophy was evident in the activin-β_C-overexpressing mice and has not been reported in the inhibin-α- or follistatin-overexpressing mice. Activin is however an important regulator of prostate growth and differentiation, particularly during development, and is involved in branching morphogenesis.[3] Thus it is perhaps not surprising that if activin-β_C is a functional antagonist of activin A, that prostate pathologies develop. Prostate hypertrophy, epithelial cell hyperplasia, increased evidence of activin-β_C subunit immunoreactivity in human prostate cancer, and antagonism of the growth inhibitory effects of activin A in the LNCaP prostate cell line implicate a role for the activin-β_C subunit in maintenance of tissue homeostasis in the prostate.

Our results in relation to the bioactivity of activin C differ from a previous study, which showed activin C did not antagonize activin A in LNCaP cells.[16] The preparations of activin C arise from different cell lines; the current study uses a mammalian cell line (CHO cells), whereas the earlier work used Noctuidae insect larvae infected with a recombinant baculovirus.[41] We believe these data might demonstrate the importance of expressing proteins in a mammalian cell line that has the capability to correctly process the final product.

The activin-β_C knockout mouse shows no overt phenotype even when partial hepatectomy was performed.[12] Lack of phenotype in a knockout model may reflect compensation by other growth factors.[12,42] This has previously been demonstrated for another transforming growth factor-β superfamily member, GDF15/MIC-1. Like activin-β_C, GDF-15/MIC-1 mRNA is predominately expressed in the liver and is up-regulated after liver injury, yet homozygous null mice show no abnormalities even when subjected to liver insult. These data lead to the conclusion that GDF-15 does not appear to be essential or that loss of GDF15 may be compensated by other secreted growth factors.[43] Recently, GDF15/MIC-1 was shown to be low in normal tissues and up-regulated in cancers, and an overexpression mouse model demonstrated GDF15/MIC-1 is a central regulator of appetite and weight and is a potential target of cancer-associated weight loss as well as obesity.[44]

We have shown in independent assays that activin C antagonizes activin A *in vitro*. Pathologies in activin A-responsive tissues and a reduction in nuclear localization of Smad-2 with activin-β_C overexpression *in vivo* indicates antagonism of activin signaling also occurs *in vivo*. Therefore, our study shows for the first time that overexpression of activin-β_C *in vivo* alters testis, liver, and prostate tissue homeostasis and strongly suggests that the mechanism of action is antagonism of activin A signaling. Further mechanisms by which the activin-β_C subunit antagonizes activin A, for instance the production of an activin AC heterodimer,[16] will be the focus of future investigations. The relevance of our results to human pathology is implied by evidence of increased activin β_C-subunit immunoreactivity in human cancer arrays. The results of our study provide sufficient justification to warrant efforts to obtain new reagents to determine which activin-β_C ligands form *in vivo* and their precise biological functions. Our results will have profound implications for activin biology and should provide stimulus for a new body of work that are relevant to the biology of mice and men.

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