The inhibin/activin signalling pathway in human gonadal and adrenal cancers

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ABSTRACT: The biological function of the inhibin-α subunit (INHA) in gonadal tumorigenesis is different in humans compared with mouse. The INHA subunit is up-regulated in most human ovarian and testicular cancers but knock-out studies in mice showed the INHA subunit is a tumour suppressor with gonadal and adrenal specificity. The INHA subunit is a component of the inhibin/activin signalling pathway, which includes activin receptors ActRIIA/IIB and intracellular Smads-2/3. To resolve the incongruity in function in humans versus mouse, we re-evaluated the inhibin/activin pathway in human gonadal and adrenal cancers using contemporary protein and mRNA expression data for multiple pathway components rather than INHA alone. We used an INHA antibody raised against the N-terminal domain to compare immunoreactivity with the more commonly used antibody raised against the C-terminal domain. This study also described, for the first time, a comprehensive protein expression profile of activin-βC in reproductive and adrenal cancers, and its effect on a human granulosa cell line, providing evidence for a role in ovarian, testis and adrenal tumour biology. Our data show reduced INHA expression at both protein and mRNA levels, and increased activin signalling in human testicular, ovarian and malignant versus benign forms of adrenal cancer. We also found that activin-C acts as an activin-A antagonist by binding to activin receptor subunits IIA and IIB and modulating the canonical Smad pathway. In conclusion, analysis of the inhibin/activin signalling pathway helps to explain discrepancies arising from studies of only one hormone or subunit and suggests that altered expression of the inhibin and activin subunits is associated with reproductive and adrenal cancer biology.

Key words: inhibin / activins / testis / ovary / adrenal / cancer

Introduction

Activin and inhibin are dimeric glycoprotein hormones with opposing actions on the hypothalamic–pituitary axis and gonads, like other members of the transforming growth factor (TGF-β) superfamily, and they exert a multitude of functions during embryogenesis, in tissue homeostasis and tumorigenesis. Activin is a disulphide-linked dimer of inhibin-β subunits (βA or βB), whereas inhibin is formed as a combination of the inhibin-α subunit with one of the β-subunits. Inhibins have largely an endocrine role, mainly produced by the gonads inhibin acts as a negative regulator of pituitary FSH synthesis and secretion from the anterior pituitary. Gonad specificity is evident in castrated animals where a reduction of circulating inhibin induces a rise in serum FSH. However, inhibin also acts locally on a number of tissues (Vale et al., 1990).

The importance of inhibin in reproductive biology has been clearly shown in mice with homozygous deletion of the inhibin-α subunit (INHA) (Matzuk et al., 1992). Matzuk et al. showed that the inhibin knock-out (INHAKO) mice develop gonadal sex cord stromal tumours within 6 weeks (granulosa cell tumours in female and Sertoli tumours in male), causing death in males and females in 13 and 17 weeks, respectively. These tumours are aggressive with 100% penetrance and are followed by a cachexia-like-wasting syndrome (Matzuk et al., 1994). Gonadectomy in these mice increases life expectancy but the development of adrenal tumours occurs, causing death at 36 and 33 weeks in males and females, respectively. This evidence suggested that the INHA gene was a tumour suppressor with gonadal and adrenal specificity in mice.

Despite the clear role of inhibin in mice, in recent years a strong paradox has emerged regarding its role in human cancer biology. This is primarily due to conflicting studies. Some reports described up-regulation of INHA in the tissue and serum of human ovarian cancers, Sertoli cell tumours, adrenocortical carcinomas and placental tumours (Cobellis et al., 2001; Risbridger et al., 2001; Robertson et al., 2004), whereas other studies reported its down-regulation (Gurusinghe et al., 1995; Yamashita et al., 1999). Additionally, further investigations failed to provide evidence of loss of heterozygosity in the chromosomal region harbouring the INHA region (Watson et al., 1995; Yamashita et al., 1997, 1999). Results...
from these studies led to further discrepancies with some findings, suggesting that no INHA was detected in serous epithelial ovarian cancers (Gurusinjhe et al., 1995; Yamashita et al., 1999), with other findings, suggesting the opposite (Arora et al., 1997; Rishi et al., 1997) and with the more recent study, suggesting that the stromal compartment of the tumour is the only source of INHA in this specific subset of cancers (Zheng et al., 2000).

Ball et al. (2004) in order to resolve the INHA dilemma in cancer biology proposed the hypothesis regarding the INHA gene that, like TGF-B1, it may have a dual role in carcinogenesis as both a tumour suppressor and pro-metastatic factor. Despite significant research efforts and clinical studies, conflicting data made it difficult to reconcile the demonstration of INHA as a tumour suppressor in mice versus an up-regulation in similar human cancers leading to considerable debate about the role of the INHA subunit in human gonadal and adrenal tumorigenesis.

Activins and inhibins are mutually antagonistic regulators of reproductive and other organs, and their temporal expression is a biological event of pivotal importance for normal cellular biology. The majority of studies conducted in the last decade exploring the role of inhibins and activins were based on the analysis of only one hormone or subunit, representing a considerable limitation to the understanding of the intricate relationship between inhibins, activins and their effect both in vitro and in vivo. A novel approach including the analysis of multi-pathway expression both at the mRNA and protein levels was therefore warranted.

Accordingly, the first part of this study was designed to investigate the inhibin/activin signalling pathway in human tissues. We re-evaluated the protein expression of INHA and components of the inhibin/activin signalling pathway including activin receptors ActRIIA/IIIB, the activin antagonists: follistatin and activin-βC, and intracellular effectors Smads-2/3 as well as the non-canonical extracellular signal-regulated kinase 1/2 (ERK) signalling pathway. The mRNA expression profile of INHA was also investigated using the Oncomine database.

Considering the recent focus on the importance of the INHA N-terminal domain (Zhu et al., 2012), we used an INHA antibody raised against this domain to compare immunoreactivity with the more commonly used antibodies raised against the C-terminal domain. The second part of this study was focused on the effect of activins on the human COV434 granulosa cell line, clarifying the mechanism of action of activin C.

Materials and Methods

Human tissue samples

Samples of ovarian, adrenal and testicular cancers with normal controls, benign diseases and hyperplasia were purchased from US BIOMAX (microarrays ID: OV483, OV1002, 2314, TE808, AD2081). Samples were classified in categories in relation to the histopathological features of the tumour. Ovarian Cancers. Epithelial ovarian cancer (n = 35): Mucinous papillary cystadenocarcinoma, adenocarcinoma and serous papillary adenocarcinoma, sex cord stromal tumour (n = 30): thecoma and granulosa cell tumour, germ cell tumour (n = 35) included: yolk sac tumour, teratoma and dysgerminoma. Testicular Cancers. Germ cell tumour: seminoma (n = 34) and non-seminoma (n = 33), embryonal carcinoma, teratoma, yolk sac tumour and choriocarcinoma. Adrenal Cancers. Cancer of adrenal cortex (n = 89) included: adrenocortical carcinoma and adreno-cortical adenoma, cancer of the adrenal medulla (n = 63): neuroblastoma and pheochromocytoma. Each array also included normal controls ovary (n = 8), testis (n = 13) and adrenal (n = 16).

The antibodies used were activin-βA (H-120 Santa Cruz Biotechnology-50288), activin-βB (H-110 Santa Cruz Biotechnology-50287), activin-βC (C-20 Santa Cruz Biotechnology-6888), follistatin (H-110 Santa Cruz Biotechnology-30194), two different INHA antibodies were used to evaluate differences in cross-reactivity within the tissues analysed, as previous reports showed antibody-specific results (Risbrider et al., 2001): monoclonal inhibin-α (Serotec MCA91ST) and polyclonal inhibin-α (abcam 81234), activin receptor IIA and IIB (R&D System AF-340 and AF-339), Smad-2 (#3122 cell signalling) and Smad-3 (S1–1500 Invitrogen). Immunohistochemical chemistry was performed after microwave antigen retrieval (1000 Watts for 14 min) in buffers as follows: inhibin-alpha, activin Receptor IIA and IIB, smad-2 (10 mM Sodium Citrate Buffer pH 6.0), activin-βA, activin-βB, activin-βC, follistatin and Smad-3 (10 mM glycine buffer pH 4.5). After cooling, slides were washed three times in phosphate-buffered saline (PBS) and endogenous peroxidase activity was quenched by using the peroxidase-blocking solution (Dako Real S2023). Sections were treated with CAS blocking reagent (Invitrogen 00-B120). All the antibodies, except for the activin receptor IIA and IIB antibodies (detected using the secondary polyclonal rabbit anti-goat immunoglobulin-HRP Dako P0160) were detected with DAKO Real EnVision Detection System (DAKO K5007). Negative controls included secondary antibodies only or immunoglobulin matched to the primary antibody. To assess the specificity of the antibodies, evaluation of the staining was performed using appropriate positive controls and western blots were undertaken.

Immunohistochemical evaluation

The intensity and distribution patterns of staining were evaluated by two blinded, independent experienced observers, using a semi-quantitative, immunoreactive score (IRS) (Remmle and Stegner, 1987). The IRS was calculated by multiplication of the optical staining intensity (graded as 0 = no, 1 = weak, 2 = moderate and 3 = strong staining) and the percentage of positively stained cells (0 = no staining, 1 = <10% of the cells, 2 = 11–50% of the cells, 3 = 51–80% of the cells and 4 = >81% of the cells). Nuclear localization of smad-2/3 was estimated based on a method that allowed an unbiased semi-quantification of the percentage of positive cells. Random fields were systematically selected using the Spot software, version 3.5.4 (Spot Imaging Solution) and sampling was conducted using an unbiased 10 x 10 cm counting frame. Frame counting was performed on sections uniformly spaced throughout the tissue, 150 frames and 100 x magnification, with an average of 1000 cells counted per section using ImageJ (Gold et al., 2005).

Ovarian metastatic granulosa cell line COV434

The COV434 cell line derived from a metastatic granulosa cell tumour obtained from a 27-year-old female (Zhang et al., 2000) was purchased from Sigma-Aldrich and tested to exclude mycoplasma contamination. These cells retain the morphological appearance of luteinized granulosa cells and are gonadotrophin responsive. COV434 cells were cultured in Dulbecco’s modified eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Invitrogen, Australia) in 75-cm<sup>2</sup> culture flasks at 37°C with 5% CO<sub>2</sub> in air. To determine the effect of activins A (cat #338-AC-010 R&D), B (cat #659-AB-005 R&D), C (produced using Chinese hamster ovary cells stably transfected with full-length human activin-βc (Gold et al., 2009)) and follistatin (cat #4889-FN-025 R&D), on cell viability, three independent growth assays were performed. COV434 cells were plated at a density of 10,000 cells/well in DMEM-5% FBS for 24 h and allowed to attach. Medium was replaced with DMEM-2% FBS containing follistatin.

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(200, 400 ng/ml) or activin-C (100 ng/ml), buffer or empty vector controls. After 4–6 h, 10 ng/ml of activin A, activin B or buffer control were added and incubated for a total of 72 h. Cell viability was measured with the CellTiter96 Aqueous Assay (Promega). All experiments were conducted between passage numbers 12 and 20.

**co-Immunoprecipitation and Western blotting**

The Pierce Co-Immunoprecipitation (co-IP) Kit (cat. #26149 Thermo Scientific) was used according to the manufacturer’s instructions. Briefly, two columns were used: one containing the activated AminoLink Plus Coupling resin and the other containing the agarose control (cross-linked 4% beaded agarose) as a negative control. Thirty micrograms of affinity-purified antibody (Inhibin β-C C-20 cat # sc-6888 Santa Cruz Biotechnology, Inc.) was used for antibody immobilization. COV434 were seeded as previously described and when 70% confluency was achieved medium was replaced with DMEM-2% FBS containing 100 ng/ml of activin C for 24 h. After 24 h, the cell suspension was centrifuged at 1000 g for 5 min to pellet the cells. After 2 washes in cold PBS, ice-cold IP Lysis/Wash Buffer, provided with the kit, was used for protein extraction. The lysate was incubated on ice for 5 min with periodic mixing and protein concentration determined as previously described. A total of 1 mg/ml of protein was used to perform the co-IP elution step and the resulting pull-down washes were evaluated to exclude protein content in the final wash solution. The flow-through was analysed for protein content and samples were prepared for the sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis. Briefly, 5 × sample buffer was added to samples to a 1 × final concentration. Samples were heated at 95 – 100 °C and applied to a 12% polyacrylamide gel, run in denaturing conditions, and blots incubated using antibodies raised against the activin receptors IIA (human activin RII A cat # AF340 R&D), IIB (human activin RIIB cat # AF340 R&D) and activin C (Inhibin β-C C-20 cat # sc-6888 Santa Cruz Biotechnology, Inc.). IRDye 800CW Donkey Anti-Goat IgG, H + L dilution 1:15 000 (Li-cor cat # 926-32211) was used as a secondary antibody. Blots containing positive controls with receptors only or activin C were used to demonstrate antibody capture specificity.

**In-Cell Western assay**

The in-cell western (ICW) assay was performed to analyse signalling effectors in response to pathway stimulation (Cooevoets et al., 2009). We investigated potential cross talk between the Smad pathway (p-Smad2/Smad3, p-Smad3-Smad3 and Smad-4) and the non-Smad pathway p-ERK1/2, ERK1/2 to compare the effect of activin A and C on the downstream signalling in COV434 cell line. COV434 cells were plated as described above for 24 h and allowed to attach. After 24 h, medium was replaced with DMEM-2% FBS containing activin C (100 ng/ml). After 4–6 h, 10 ng/ml of activin A or buffer control were added and incubated for a total of 24 h. After 24 h, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then permeabilized using 70% ethanol and 0.1% Triton X-100 in PBS. After permeabilization 30 µl of blocking solution (Odyssy Blocking Buffer Licor # 927-40000) was added to each well and cells were incubated for 1 h at room temperature with gentle shaking. After an hour, the blocking solution was removed and diluted primary antibodies were added to each well (Smad-2 abcam # 47083, p-Smad2 abcam # 53100, Smad-3 abcam # 40854, p-Smad3 abcam 51451, p-ERK 1/2 abcam # 47339, ERK 1/2 cell signalling # 4695 and Smad-4 cell signalling # 9515) except for the negative control wells (all the antibodies used were tested for specificity and validated by western blots). After an overnight incubation at 4 °C, secondary antibodies were added (Li-Cor # 926-32211, # 32350). DRAQ5 (abcam # 108410) was used to normalize for differences in cell number in each well. Secondary antibodies were detected at 800 nm and DRAQ5 at 700 nm. Plates were scanned using the Odyssey Li-Cor Infrared Imaging System (Li-Cor) and signal intensities quantified using the Odyssey application software (version 3.0). All values were adjusted for background using the Odyssey application software.

**RT–PCR analysis**

Total RNA was extracted from mouse testis, ovary and COV434 cell pellet using Trizol and the RNasea Mini Kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed using the Superscript III reverse transcriptase (Life Technologies). INHA mRNA expression was analysed with primers that amplified mouse and human mRNA. β-actin (mouse-specific primers) and β2 microglobulin (human-specific primers) were used as housekeeping genes for block PCR. Mouse tests and ovary were used as positive controls. Primers sequences (all 5‘–3’) were designed using Primer-BLAST: INHA, forward: CGCCCCTGCTAGATCTTCGT, reverse: CAAA AACAGGGCTGAACCG; β-actin, forward GCCCTCCTCGTTGGTA TGG, reverse: CAGCTCAGTAACAGTCGCCG; β2 microglobulin, forward CCGTGTGAACCATGTGACCT, reverse: CAAACATGGAGACAGCACG. The PCR programme was 94°C for 5 min; 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C; 72°C for 7 min. All PCR products were run on 1.5% (w/v) agarose gels.

**Oncomine database interrogation**

The Oncomine cancer microarray database was used to analyse INHA mRNA expression profiles (Rhodes et al., 2004). Differential INHA mRNA expression was investigated in cancer tissues versus normal controls (filtering criteria) or across cancer subsets (filtering criteria). A gene centric analysis with graphical representation of the microarray data was exported from Oncomine™ (Compendia Bioscience, Ann Arbor, MI, USA). The following datasets were used: Korkola Seminoma (Korkola et al., 2006), Hendrix Ovarian (Hendrix et al., 2006), Bittner Ovarian (GSE2109) and Giordano adrenal (Giordano et al., 2003).

**Statistical analysis**

The IRS for each tissue was treated as ranked data and analysed with Mann–Whitney U-test for two independent groups using the IBM SPSS 20 statistic software; fold change was calculated based on the mean IRS of disease versus normal control. Percentage positive Smad-2/3 nuclei and ICW results were analysed with Student’s t-test (GraphPad Prism version 5). Cell proliferation assays were analysed using ANOVA with Tukey’s post hoc test. Significant differences were assumed at P ≤ 0.05.

**RESULTS**

**Activin-βC expression in human ovarian, adrenal and testicular cancer**

Within the ovarian cancer specimens activin-βC expression was confined to the epithelial cells and papillary structures of the tumour (epithelial cancers), to the germ cell characteristic of the tumour (granulosa cell tumours), to the connective tissue and cytoplasm of the theca cells (thecoma cell tumours) and to the cystic structures characteristic of the germ cell tumours; in normal ovarian controls staining in primordial follicles and stromal areas was also recorded (Supplementary data, Figs S1A and S2). Within the testicular specimens activin-βC was confined to primary spermatocytes and spermatogonia in a limited number of samples (n = 8, seminoma samples); within the morphologically heterogeneous non-seminoma cancers immunostaining was detected in the solid sheets of large undifferentiated cells (embryonal carcinoma and
yolk sac tumour) and in the tubular and papillary structures of the tumours (embryonal carcinoma).

In normal testicular tissues activin-BC staining was also confined to the germ cells: round spermatids and spermatogonia, with less staining in interstitial cells (Supplementary data, Figs S1B and S3).

Staining within the adrenocortical carcinoma was predominately concentrated in the connective tissue with less staining in malignant cells. The area stained in the pheochromocytoma was the medulla, whereas the staining was extended to the cells aggregated in the rosette shape features characteristic of the neuroblastoma cancers. Within the normal adrenal tissue activin-BC staining was mainly distributed in the intranuclear spaces with minimal extension to the nuclei (Supplementary data, Figs S1C and S4).

**Immunoreactive score**

**Ovary**

Positive immunostaining for activin-βA, activin-βB, activin-βC, follistatin, ActRIIA/ActRIIB and Smad-2/3 was apparent in all the specimens analysed. Inhibin-α subunit was not detected in all specimens, including normal tissue controls, using the monoclonal INHA antibody (Supplementary data, Fig. S5); however, staining with the polyclonal INHA antibody was evident in all the specimens analysed, with less staining in the cancer tissues versus normal control (Supplementary data, Fig. S6). The immunostaining for activin-βA and activin-βC (except for the thecoma cell tumour) was increased in all ovarian cancers. ActivinRIA immunoreactivity was increased in thecoma and granulosa cell tumours (Table I A).

**Testis**

Positive immunostaining for activin-βA, activin-βB, activin-βC, INHA subunit, follistatin, ActRIIA/ActRIIB and Smad-2/3 was detectable in all the specimens analysed. The immunostaining for activin-βA and activin-βC was increased in seminoma and non-seminoma specimens. A decrease in the INHA subunit staining (with no difference of staining recorded using the monoclonal versus polyclonal INHA antibodies) was detected in all the cancer tissues (Supplementary data, Fig. S7). The number of positive cell nuclei stained with smad-2/smad-3 antibodies was significantly increased in seminoma and non-seminoma cancers (Table I B).

Despite an increase in the antagonists activin-βC (increased in all ovarian and testicular cancer analysed except for the thecoma cell tumour) and follistatin, the activin signalling cascade was activated as shown by the increase in the number of positive cell nuclei stained with Smad-2/3. However, the IRS represents an overall tissue staining assessment; therefore, based on data published by our group (Gold et al., 2013), where over-expression of activin-βC modulated Sertoli and granulosa cell tumours in mice, we assessed the activin-βC/activin-βA expression ratio where the two subunits were co-expressed in the same cell type, and as expected a negative correlation between these two variables was evident ($r = -0.7075, P < 0.0001$ (Fig. 1 G); $r = -0.7324, P < 0.0001$ (Fig. 1 H)) with activin-βC antagonizing activin-βA signalling as indicated by a reduction in Smad-2 positive nuclei (Fig. 1).

**Adrenal**

Positive immunostaining for activin-βA, activin-βB, activin-βC, INHA subunit, follistatin, ActRIIA/ActRIIB and smad-2/3 was detectable in all the specimens analysed. Activin-βA subunit was increased in adrenocortical carcinoma and pheochromocytoma. Activin-βB subunit staining was decreased in neuroblastoma and adrenocortical adenoma. Staining for INHA (with no difference of staining recorded using monoclonal versus polyclonal inhibin-α antibodies) subunit was increased in adrenocortical adenoma, adrenocortical hyperplasia and normal tissue adjacent to the cancer (Supplementary data, Fig. S8). Follistatin immunostaining was reduced in both adrenocortical carcinoma and neuroblastoma (Table I C).

**Effects of activins on the human COV434 granulosa cell line and INHA expression**

Activin-A (10 ng/ml) and activin-B (10 ng/ml) increased COV434 cell number by 18% (activin-A $P < 0.01$ versus media control) and 22% (activin-B $P < 0.01$ versus media control), whereas activin-C (100 ng/ml) alone reduced cell number by 33% ($P < 0.0001$ versus media control). Activin-A in the presence of follistatin (400 ng/ml) or activin-C (100 ng/ml) did not increase cell number; cell viability was reduced by 24% ($P < 0.0001$ follistatin versus activin A) and 41% ($P < 0.0001$ activin C versus activin A), respectively. Similarly, activin B in the presence of follistatin (200 ng/ml) or activin C (100 ng/ml) reduced cell viability by 21% ($P < 0.01$ follistatin versus activin A) and 52% ($P < 0.0001$ activin C versus activin B), respectively; indicating that both activin C and follistatin antagonized the growth-promoting effect of activin A and B in the COV434 cell line (Fig. 2A). RT–PCR analysis revealed no expression of INHA mRNA in the COV434 cancer cell line, whereas positive expression was revealed as expected in mouse testis and ovary (Fig. 2B).

**Activin C binds to activin receptors IIA and IIB**

When COV434 cells were incubated with activin C and flow-through analysed for protein content, results showed that the activin receptor IIA (ActRIIA) directly interacted with activin C (activin receptor IIA can be visualized at 58 kDa and activin C at 38 kDa (pre-protein) and 17 kDa (reduced form of the protein). A similar interaction between activin C and the activin receptor IIB (ActRIIB) was also observed. Flow-through from negative control and positive control for ActRIIA/IIB and activin C confirmed specificity of the antibodies used (Fig. 3).

**Effect of activin C on downstream signalling**

Increased Smad-2 ($P < 0.0001$ versus media control) phosphorylation was evident in COV434 cells treated with activin A versus media control, with no effect on Smad-3, ERK 1/2 phosphorylation or total Smad-4 (Fig. 4A and B). Treatment with activin C alone did not affect any of the analysed pathways (Fig. 5A and B). Pretreatment of COV434 cells with activin C followed by activin A abolished the activin A mediated Smad-2 phosphorylation and also reduced Smad-3 phosphorylation ($P < 0.001$) (Fig. 6A and B).

**Oncomine database**

Oncomine interrogation for the Hendrix ovarian cancer database showed that INHA is down-regulated in ovarian epithelial samples relative to normal controls (Fig. 7A). Next, we looked at INHA expression in a variety of ovarian cancer subsets available on the Bittner ovarian database where the expression was analysed among the different subtypes of cancer and the majority of cancers showed a down-regulation of INHA expression except for a cohort of borderline ovarian mucinous tumours and malignant ovarian granulosa cell tumours (Fig. 7B). Interrogation of
Table I  IRSs for immunohistochemical evaluation of the activin/inhibin signalling pathway in human ovarian, testicular and adrenal tissues.

<table>
<thead>
<tr>
<th></th>
<th>Activin-βA</th>
<th>Activin-βB</th>
<th>Activin-βC</th>
<th>Inhibin-α Serotec</th>
<th>Inhibin-α AbCam</th>
<th>Follistatin</th>
<th>ActRIIA</th>
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<td>Epithelial ovarian cancer, n = 35</td>
<td>+3.07***</td>
<td>−1.56*</td>
<td>+3.18***</td>
<td>Not detected</td>
<td>−2.41***</td>
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<td>ns</td>
<td>ns</td>
<td>+2.75**</td>
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<td>+2.77*</td>
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<td>+2.67***</td>
<td>+2.65***</td>
<td>ns</td>
<td>+1.85*</td>
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<td>+1.84*</td>
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<td>+3.34***</td>
<td>Not detected</td>
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<td>+2.71**</td>
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<td>Seminoma, n = 34</td>
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<td>+2.28***</td>
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<td>Adrenocortical carcinoma, n = 13</td>
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<tr>
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<td>+1.83***</td>
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Fold difference (shown in table) calculated on the mean IRS or percentage of positive cells (Smad-2 and Smad-3) of the disease ovary (A), testis (B) and adrenal (C) was compared with the normal controls: ovary (n = 8), testis (n = 13) and adrenal (n = 16). ActRIIA: activin receptor IIA, ActRIIB: activin receptor IIB. The IRS for each tissue was treated as ranked data and analyzed with Mann–Whitney U-test for two independent groups.

*P value < 0.05, **P < 0.01, ***P < 0.001.
the Korkola testicular seminoma database showed a significant downregulation of INHA expression in all the cancers analysed compared with normal controls (Fig. 8). Interrogation of the Giordano adrenal cortex database showed comparable INHA expression between adrenal cortical adenoma, carcinoma and hyperplasia versus normal control (Fig. 9).
Although INHA acts as a tumour suppressor in mice, discrepant results from the human studies have emerged creating a dilemma in the understanding of inhibin/activin signalling in human cancer biology. Therefore, a study to clarify the expression of INHA, activins and the signalling cascade in human cancers was warranted.

Our data showed reduced INHA protein and mRNA expression in the majority of human gonadal cancers and malignant forms of adrenal cancers. Our investigation, integrating protein and mRNA data with the use of two INHA antibodies raised against the C and N-terminal domain, showed the advantage derived from a full comprehensive pathway analysis compared with single molecule studies to elucidate the function of INHA/activin(s) in cancer biology. Additionally, within this study we described for the first time the activin-βC expression profile in human ovaries, testis and adrenals and the effect of recombinant activin C in vitro on a human granulosa cell line.

Inhibin-α in ovarian, testicular and adrenal cancer

Re-evaluation of INHA in ovarian specimens showed reduced protein expression in cancers versus normal tissues. Reduced INHA mRNA expression was also evident in patients with epithelial ovarian cancers versus normal control (Hendrix Ovarian database). Interrogation of the Bittner database for the INHA expression profile revealed elevated mRNA levels in a cohort of patients with borderline ovarian mucinous tumours and malignant granulosa cell tumours. This is likely due to the patients age, in fact when a dataset filter was used for the interrogation (dataset detail: grouped by age) the mRNA expression profile levels were elevated in the cohort of patients (age 30–40 years; n = 10) compared with the cohort of post-menopausal women (age 40–50 years; n = 35 and 50–60 years n = 67). It is in fact known that the inhibin expression profile in post-menopausal women greatly reduced compared with younger women (Healy et al., 1993) and this was also evident in this study. Within the same subsets of cancers, our study noted reduced INHA protein levels in the cohort of patients (age 49.67 years ± 3.60; n = 15) versus controls (age 21.50 years ± 2.96; n = 8). The difference in age between the disease and control group could represent a contributing factor to the difference in these findings.

Discrepant results within our study using a polyclonal antibody generated against the synthetic peptide corresponding to a region within N terminal amino acids 109–158 of human INHA (NP_002182) and a monoclonal antibody generated against the synthetic peptide composed of amino acids 1–32 of human INHA represents an important problem to consider when assessing INHA expression in the ovary and may explain discrepancies from previous studies. Recent work suggested the importance of the INHA N-terminal region regarding its ability to antagonize activin function and FSH production (Zhu et al., 2012). Therefore, evaluation of human INHA subunit in cancers using an antibody produced against the N-terminal is likely to be more useful and indicative of INHA activity in particular activin/FSH antagonism, which has been shown to be associated with gonadal cancer development and
progression (Coerver et al., 1996). Findings from our study agree with previous reports assessing INHA protein expression and using antibodies raised against the amino terminus domain of the INHA subunit (Gurusinghe et al., 1995; Yamashita et al., 1999) but differ from those using a C-terminal antibody (Arora et al., 1997; Zheng et al., 1997).

The role of INHA in human testicular cancer is unclear. Studies aimed to investigate SNPs in inhibin and activin pathway genes failed to provide any evidence of causal SNPs (Purdue et al., 2008). Some reports described strong INHA expression in testicular germ cell tumours (Cobellis et al., 2001; Taniyama et al., 2001) but Dias et al. (2009)

Figure 4 Effects of activin-A on the Smad and ERK1/2 pathway. Ninety-six well microplates were loaded with COV434 cells seeded at a density of 20,000 cells/well and treated with activin-A for 24 h. Signal from p-Smad-2/Smad2, p-Smad-3/Smad3, p-ERK 1-2/ERK1-2 and Smad-4 appear as green fluorophores, 800 nm. Signal from cell dyes (cell number normalization) appears as red fluorophores, 700 nm (A). Quantification of signals (K. Counts Integrated Intensity) are shown in (A). Normalized values are expressed as relative intensities (800 nm channel/700 nm channel). Groups were compared using the Student’s t-test. Results are mean ± SD from replicates of 4; ns = P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ****P ≤ 0.0001 (B).
described reduced INHA protein expression in a limited number of seminoma and non-seminoma samples using an antibody raised against the C terminal domain. Within our study the levels of INHA were low in seminoma and non-seminoma cancers compared with normal testis controls and in the INHA mRNA expression profile reported in the Oncomine Korkola seminoma database.

There have been very few published reports concerning the expression of INHA in human adrenal tumours. One study showed that INHA was among the genes which had discriminatingly low expression in adrenocortical adenomas compared with normal adrenal tissue and INHA mRNA expression in the cancer subsets analysed versus normal tissue in the Giordano adrenal database showed no significant changes between adrenocortical

INHA as a suitable immunohistochemical marker in the histological evaluation for the differentiation from non-adrenocortical tumours, such as pheochromocytomas or renal cell carcinomas (Cho and Ahn, 2001). Our data indicated no significant changes in INHA expression in adrenocortical adenomas, neuroblastoma and pheochromocytoma but up-regulation in adrenocortical carcinoma agreed with the results previously reported in micro-array studies of human adrenocortical tumours (de Fraipont et al., 2005). INHA mRNA expression in the cancer subsets analysed versus normal tissue in the Giordano adrenal database showed no significant changes between adrenocortical

**Figure 5** Effects of activin-C on the Smad and ERK1/2 pathway. Ninety-six-well microplates were loaded with COV434 cells seeded at a density of 20,000 cells/well and treated with activin C for 24 h (**A**). Refer to Fig. 4 for experimental details. Results are mean ± SD from replicates of 4; *P ≤ 0.05; **P ≤ 0.01; ****P ≤ 0.0001 (**B**).
adenoma, carcinoma and hyperplasia; however, the number of samples analysed in this database was very limited due to the rarity of these tumours.

Additionally, INHA mRNA expression conducted in the human COV434 cell line revealed no INHA expression consistent with the hypothesis of INHA being down-regulated in advanced stages of ovarian cancer.

In summary, our findings indicate that INHA expression is reduced in the majority of human gonadal and malignant adrenal cancers leading to unopposed activin expression and signalling.

**Activins in ovarian, testicular and adrenal cancers**

Inhibin and activin have an intricate relationship in human cancer biology and elevated activins are evident in ovarian granulosa cells, follicular fluid (DePaolo et al., 1991), Sertoli and Leydig cells (Anderson et al., 1998). Activin-βA and βB subunit mRNA and protein have been detected in germ cell tumours, granulosa cell tumours, sex cord tumours, Sertoli cell and Leydig cells tumours (Roberts et al., 1993; Anderson et al., 1998). This study found increased levels of activin-βA in all the ovarian cancers.
and testicular cancers analysed, while activin-βB subunit expression was increased in thecoma cell tumours only and down-regulated in epithelial ovarian cancers. These data confirm previous reports describing high secretion of activin-βA in ovarian and testicular cancers (Lambert-Messerlian et al., 1999; Cobellis et al., 2004). Using the COV434 granulosa cell line we showed that activin-βA and activin-βB are growth promoting, demonstrating the importance of activins in human granulosa cell tumours.

Hofland et al. (2006) reported decreased levels of activin-βA subunit in adrenocortical carcinoma compared with normal and hyperplastic adrenal cortex. However, our assessment showed increased expression in adrenocortical carcinoma and pheochromocytoma and decreased expression of activin-βB in neuroblastoma and adrenocortical adenoma versus normal tissue.

Our results therefore validated the importance of activins in all the subsets of cancers analysed, supporting the hypothesis that these tumours secrete high levels of activin. Heterodimerization of the INHA subunit with the activin-β subunits decreases the level of homodimeric activins; therefore, reduced expression of INHA in these cancers is likely to be a major mechanism leading to increased activins and may represent an early event, resulting in increased proliferation.
Both follistatin and activin C are activin A antagonists. Activin C was recently identified as an activin A antagonist both in vitro and in vivo (Gold et al., 2009). Some studies described more pronounced follistatin expression in ovarian clinical conditions, such as cancer (Ren et al., 2012), endometriosis (Florio et al., 2009) and testicular tumours (non-seminoma and spermatocytic seminomas) (van Schaik et al., 1997), but once again a significant discrepancy emerges from the literature with other studies describing its down-regulation (Chan et al., 2009). Follistatin expression in adrenal glands has been well described (Suzuki et al., 2004) and its analysis in a variety of adrenal cancers showed down-regulation versus normal tissues (Hofland et al., 2006). Our histological assessment found follistatin overexpression in thecoma cell tumours only and reduced expression in adrenocortical carcinoma and neuroblastoma, confirming data previously reported by Hofland et al. (2006).

This study is the first description of the expression patterns of activin-βC in human ovary, testis and adrenal (previous studies were focused on the expression profile of activin-C in rat and mouse) (Gold et al., 2004; Lau et al., 2000; Welt et al., 1997). The presence of activin-βC in normal ovary, testis and adrenal and altered expression in cancers implies a role in maintenance of tissue homeostasis. Decreased activation of the activin signalling cascade was evident in cells over-expressing both activin-βA and activin-βC providing evidence of a role for activin-βC as an activin A antagonist and therefore its importance in gonadal tumours. Further evidence of activin-βC antagonism was described in the COV434 cell line where activin C (Gold et al., 2009, 2013) had a significant inhibitory effect on cell growth and antagonized the growth-promoting effects of activin A and B.

In the present study, we investigated the effect of activin-A on the canonical TGF-β/activin signalling pathways focusing on the effect mediated by pretreatment of COV434 cell line with activin C. We also investigated a non-canonical pathway. Of the mitogen-activated protein kinase molecules, we investigated the effect of treatments on the ERK 1/2 as they are expressed in the granulosa cells and have

**Figure 8** INHA mRNA expression in Korkola Seminoma database exported from the Oncomine platform. Gene centric analysis with graphical representation of the cancer microarrays versus normal controls. 0. No value (6); 1. Choriocarcinoma (2); 2. Embryonal Carcinoma, NOS (15); 3. Mixed Germ Cell Tumour, NOS (45); 4. Seminoma, NOS (12); 5. Teratoma, NOS (16); 6. Testicular Seminoma with High Mitotic Index (1); 7. Yolk Sac Tumour, NOS (10). Cancer Res 2006/01/15 mRNA Human Genome U133A Array, Human and genome U133B Array 107 Samples, 17.779 measured genes.

**Figure 9** INHA mRNA expression in Giordano adrenal database exported from the Oncomine platform. Gene centric analysis with graphical representation of the cancer microarrays versus normal controls. 0. No value (10); 1. Adrenal Cortex Adenoma (22); 2. Adrenal Cortex Carcinoma (33). Clin cancer Res 2009/01/15 mRNA Human Genome U133 Plus 2.0 Array, 65 Samples, 19.574 measured genes.
been most closely associated with the regulation of these cells (Moore et al., 2001). As expected activin A induced activation of Smad-2, whereas activin C had no effect on the canonical and non-canonical pathways. When activin C was used in combination with activin A, it antagonized the Smad pathway having no effect on the non-canonical ERK pathway nor on total levels of Smad-4. The same results were obtained after 2, 6 and 12 h of stimulation with activin A, activin C and activin C and A (data not shown). Based on these data and on previous work (Gold et al., 2009) we predict that activin C reduced cell growth by antagonizing the high levels of activin A secreted by the COV434 by binding to activin receptors and antagonizing the activation of the Smad pathway.

Data obtained from a co-IP assay showed for the first time that activin A can bind to the activin receptors IIA and IIB, thus clarifying the antagonistic effect on COV434. By binding to the receptor and therefore antagonizing activin A binding, activin C reduced Smad-2 activation. Additionally, based on data previously published by our group, a further mechanism of activin C antagonism is based on the ability of activin-βC subunit to form heterodimers with the activin-βA subunit to form Activin AC with a concomitant reduction in the formation of activin A; this mechanism may have also been active in the human cancers when both the activin-βA and activin-βC subunits were expressed within the same cell type.

**Activin receptors and Smad-2/3**

Previous reports analysing activin receptor expression in the ovary and ovarian cell lines reported down-regulation of these receptors suggesting a role in the pathogenesis of ovarian cancer (Ramachandran et al., 2009). However, our study found increased ActRIIA expression in thecoma and granulosa cell tumours, in agreement with studies conducted by Fuller et al. (2002). Dias et al. (2009) described increased ActRIIA expression in seminoma samples versus normal, but in our study we found no statistically significant difference. The only study analysing activin receptor mRNA in human adrenal cancers reported reduced expression of ActRIIA mRNA in carcinomas compared with normal tissue (Hofland et al., 2006), whereas our study did not record any significant differences. However, the detection of ActRIIA and IIB in the cancers and normal tissues reported herein indicate full potential for activin signalling in these tissues (Hofland et al., 2006).

Smad-2 and Smad-3 are important downstream effectors of the activin receptor pathway. Enhancement of Smad-2 in ovarian cancers and ovarian cancer cell lines suggests activin may stimulate the production of the smad-2 protein in order to enhance its own effect in ovarian cancers (Ito et al., 2000). Our data support this hypothesis, in fact we recorded increased Smad-2 positive nuclei in all ovarian cancers analysed versus normal tissues. Our assessment in seminoma and non-seminoma cancers showed increased Smad-2 and Smad-3 positive nuclei confirming the involvement of Smad-2/3 signalling in the pathogenesis of gonadal tumorigenesis (Li et al., 2007). To the best of our knowledge our study represented the first assessment of Smad-2 and Smad-3 activity in a variety of adrenal cancers where we did not find any statistical differences versus normal controls.

**Conclusions and future prospective**

Most of the effects of inhibin are associated with its antagonism of activin formation and signalling (Fuller and Chu, 2004; Walton et al., 2012). Therefore, to understand the role of inhibin in cancer biology its expression cannot be considered in isolation. Based on this study we suggest reduced expression of INHA in gonadal and aggressive forms of adrenal cancers led to unopposed activin expression and signalling. Additionally, these data provide compelling evidence to consider activin-βC as a regulator of gonadal and adrenal tissue homoeostasis. This study also demonstrates the importance of temporal expression of activin(s) and inhibin, rather than the actions of any one component, for final biological outcome.

**Supplementary material**

Supplementary material is available at http://molehr.oxfordjournals.org/ online.

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**Authors’ roles**

F.E.M. designed the study, carried out data collection, data analysis and wrote the manuscript. E.G. designed the study and wrote the manuscript. G.R. designed the study and revised the manuscript. All authors have read and approved the final version.

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**Conflict of interest**

The authors declare there is no conflict of interest relating to this work.

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