Analysis of activin/TGFB-signaling modulators within the normal and dysfunctional adult human testis reveals evidence of altered signaling capacity in a subset of seminomas

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Abstract

Activin is a pleiotropic growth factor belonging to the transforming growth factor- β (TGFB) superfamily of signaling molecules. Regulated activin signaling is known to influence several steps in rodent male gamete differentiation. TGFB ligand isoforms, TGFB1–B3, also influence germ cell survival in the rodent testis at the onset of spermatogenesis and around the time of puberty. Given the importance of regulated activin and TGFB signaling in testis development and function, we sought to investigate the cellular production sites of activin/TGFB-signaling modulators in normal and dysfunctional adult human testes samples. Signaling transducers phosphorylated SMAD2/3, and signaling modulators SMAD6, MAN-1, inhibin α (INHA), and β -glycan were detected in Bouins fixed, paraffin-embedded adult human testis sections using immunohistochemistry. Additional samples examined were from testicular cancer patients and from normal men subjected to gonadotropin suppression with androgen-based contraceptives. Our findings identify distinct differences between normal and gonadotropin-deprived human testis in the expression and cellular localization of activin/TGFB-signaling modulators. The presence of a nuclear phosphorylated SMAD2/3 signal in all analyzed seminoma specimens indicated active activin/TGFB signaling. Moreover, a subset of seminoma specimens exhibited selective enhanced expression of β -glycan (4 out of 28 seminoma tumors), INHA (6 out of 28), and MAN-1 (6 out of 28), highlighting potential functional differences between individual tumors in their capacity to regulate activin/TGFB signaling. Within the heterogeneous nonseminomas, expression of signaling modulators was variable and reflected the degree of somatic differentiation. Thus, synthesis of activin and TGFB-signaling modulators may be affected by spermatogenic disruption and altered hormone levels in the testis.

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Introduction

The transforming growth factor- β (TGFB) superfamily of pleiotropic growth factors exert a broad range of effects on the differentiation, proliferation, and function of numerous cell types, including those in the mammalian testis (Loveland & Robertson 2005). Among these, signaling by the activin ligand in particular has been shown to play pivotal roles in the development and function of both germ and Sertoli cells in the juvenile and adult rodent testis (Mather *et al.* 1990, Matzuk *et al.* 1992, 1995, Boitani *et al.* 1995, Brown *et al.* 2000, Meehan *et al.* 2000, Fragale *et al.* 2001, reviewed in Itman *et al.* (2006)). All three TGFB ligand isoforms, TGFB1–B3, also act *in vitro* to regulate rodent gonocyte, pubertal spermatogonia, and spermatocyte numbers by inducing germ cell apoptosis (Olaso *et al.* 1998,

Konrad *et al.* 2006). Expression of two activin and TGFB modulators at distinct stages of rodent spermatogenesis has been previously documented, indicating the presence of regulated TGFB superfamily signaling during germ cell development (Meehan *et al.* 2000, Loveland *et al.* 2003). In the newborn rat testis, the membrane-bound pseudoreceptor *Bambi* mRNA and activin antagonist follistatin mRNA and protein are up-regulated in gonocytes at day 3 of postnatal testis development, around the time when gonocytes are differentiating into spermatogonia, indicating that downregulation of activin signaling is required for germ cell maturation in rodents (Meehan *et al.* 2000, Loveland *et al.* 2003).

TGFB superfamily signaling is initiated at the cell surface by ligand interaction with type I (ACVR1B and ACVR1C for activin, and ACVRL1 and TGFBR1 for TGFB

isoforms) and type II (ActRIIA, ActRIIB, and TGFBR2) transmembrane receptors, each with intrinsic serine/threonine kinase activity (Chang *et al.* 2002, Shi & Massague 2003, Mazerbourg *et al.* 2005, Lin *et al.* 2006). Ligand binding enables formation of a heterotetrameric receptor complex between type I and type II receptors, which leads to the selective phosphorylation and activation of the activin/TGFB-specific intracellular signal transducers, SMAD2 and SMAD3. These interact with the common SMAD, SMAD4, and translocate into the nucleus to initiate transcription of activin/TGFB response genes (Chang *et al.* 2002, Shi & Massague 2003, Lin *et al.* 2006).

Regulation of activin and TGFB signaling has been identified at each level of their signaling cascades (Harrison *et al.* 2004, 2005). At the cell surface, inhibin and its co-receptor β -glycan interfere with signaling by competing with activin for binding to type II activin receptors (Lewis *et al.* 2000, Esparza-Lopez *et al.* 2001). Expression of β -glycan also confers sensitivity to all three TGFB ligand isoforms and to the bone morphogenetic protein (BMP) ligands, BMP2, 4, 7 and GDF5, by acting as a co-receptor to promote ligand interaction with serine/threonine kinase receptors and facilitate signal transduction (Lopez-Casillas *et al.* 1993, Kirkbride *et al.* 2008). Inside the cell, the inhibitory SMADs, SMAD6 and SMAD7, prevent activation and signal propagation by receptor-regulated SMAD proteins in the cytoplasm and negatively regulate gene transcription in the nucleus. Among the inhibitory SMADs, SMAD6 preferentially inhibits BMP signaling and only weakly inhibits TGFB/activin signaling, whereas SMAD7 ubiquitously inhibits TGFB, activin, and BMP signaling (Itoh *et al.* 1998, Ishisaki *et al.* 1999, Hanyu *et al.* 2001, Goto *et al.* 2007). Moreover, expression of inhibitory SMADs is induced by TGFB superfamily signaling, creating a negative feedback loop to modulate the magnitude and duration of TGFB/activin and BMP signaling (Nagarajan *et al.* 1999, Denissova *et al.* 2000, Ishida *et al.* 2000). At the inner nuclear membrane, MAN-1 interacts with phosphorylated SMAD2/3 and target DNA sequences to inhibit transcriptional activation of activin/TGFB response genes (Imamura *et al.* 1997, Bai & Cao 2002, Lin *et al.* 2005, Pan *et al.* 2005, Caputo *et al.* 2006).

The function of activin and TGFB signaling and the mechanisms by which it is regulated in the human testis have not yet been defined. Cell-specific expression analysis of activin antagonists in the normal and dysfunctional human testis is largely limited to the inhibin α -subunit (INHA), which is produced by Sertoli cells within the seminiferous epithelium and by interstitial Leydig cells in fetal and adult testes. Both INHA and β -glycan are produced by Leydig cells in the fetal testis (Vliegen *et al.* 1993, Majdic *et al.* 1997, Roberts 1997, Andersson *et al.* 1998, Anderson *et al.* 2002, Marchetti *et al.* 2003), indicating regulated TGFB

superfamily signaling features during normal human fetal testis development and that gonocytes do not make these proteins. In addition, RNase protection assays and immunohistochemical analysis have identified the INHA subunit mRNA and protein in a subset of testicular germ cell tumors (TGCTs), indicating that individual tumors differ in their responses to activin and TGFB signaling (van Schaik *et al.* 1997, Cobellis *et al.* 2001).

Testicular cancer accounts for 1% of neoplasms in men and is the most common malignancy in males aged between 15–35 years (Bergstrom *et al.* 1996). TGCTs arise from a preinvasive precursor cell called carcinoma *in situ* (CIS), first described by Skakkebaek (1972) as atypical spermatogonia in testicular biopsies of patients who subsequently developed testicular cancer. CIS cells are thought to originate during fetal life due to an arrest in the differentiation program of fetal gonocytes or primordial germ cells (Rajpert-de Meyts & Høie-Hansen 2007). Comparative analyses of protein and RNA expression levels underpin this hypothesis, as seen by shared production of POU5F1 (OCT3/4), NANOG, AP2 γ , KIT and PDPN (podoplanin/M2A) between CIS cells and human gonocytes (Sonne *et al.* 2006, Rajpert-de Meyts & Høie-Hansen 2007). Later in life, usually after puberty, CIS cells transform to overt germ cell tumors, either seminomas or nonseminomas, presumably in response to the changing hormonal environment at this time. Seminomatous tumors proliferate along the germ cell lineage, while nonseminomas retain embryonic features such as embryonal carcinoma cells that can differentiate into somatic (teratomas) and extraembryonic (yolk-sac tumors and choriocarcinomas) lineages.

Our previous findings identified spermatogonia and Sertoli cells as the predominant targets of activin signaling in the adult human testis, based on the selective production of three activin receptor subunits ALK2, ACVR1B and ActRIIB in these cells (Dias *et al.* 2008). We also described upregulation of a fourth activin receptor subunit, ActRIIA, as preinvasive CIS cells progress to malignant seminomas, indicating the capacity for aberrant activin signaling to occur in germ cell neoplasms (Dias *et al.* 2008). Enhanced production of ActRIIA relative to normal testes was also detected in gonadotropin-deprived human testis samples, obtained following spermatogenic suppression in men treated with testosterone and depot progestin (McLachlan *et al.* 2002, Dias *et al.* 2008). The increased synthesis of ActRIIA in both seminomas and gonadotropin-deprived samples suggests the potential for activin signaling to be affected by changes in hormone levels. This could result in altered signaling within specific cells of the dysfunctional human testis by activin/TGFBs and other TGFB superfamily ligands, BMPs and Nodal, which also utilize ActRIIA for signal transduction.

In this study, we set out to define the cellular sites of production of activin and TGFB-signaling modulators in the normal, gonadotropin-deprived, and

neoplastic adult human testis. These include the activin/TGFB-signaling transducers, phosphorylated SMAD2/3 and activin antagonists, INHA, β -glycan, SMAD6, and MAN-1. Phosphorylation of SMAD2/3 is an indication of activin/TGFB-signaling activity (reviewed in Shi & Massague 2003), INHA and β -glycan are activin-signaling antagonists normally restricted to somatic cells of the testis (Vliegen *et al.* 1993, Majdic *et al.* 1997, Andersson *et al.* 1998, Anderson *et al.* 2002, Marchetti *et al.* 2003), and SMAD6 and MAN-1 have yet to be assessed in the contexts of normal and dysfunctional adult human testis. Our findings demonstrate a nuclear phosphorylated SMAD2/3 signal in all seminoma tumors, indicating active activin/TGFB signaling. Our results also identify INHA, β -glycan, and MAN-1 to be present in selected seminoma tumors, indicating that these factors may limit the bioavailability of activin and TGFBs in a subset of testis tumors.

Results

Western blot verification of anti-MAN-1 antibody

Target recognition by the goat polyclonal anti-MAN-1 (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was determined by western blot analysis on human NTERA cell lysates. A single band of the expected size (82 kDa) was detected, indicating selective detection of the MAN-1 protein (Fig. 1).

Figure 1 Western blot validation of anti-MAN-1. A single band of the expected band size of 82 kDa for MAN-1 was detected in human NTERA cell lysates. Protein loading was indicated by blotting for α -tubulin.

Figure 2 Cellular localization of activin and TGFB-signaling modulator proteins within normal human testis. Low magnification (A–E) and high magnification (A'–E') images of immunohistochemical detection for activin/TGFB signal transducing phosphorylated SMAD2/3 (A and A'), and activin/TGFB regulators SMAD6 (B and B'), MAN-1 (C and C'), INHA (D and D'), and β -glycan (E and E') in adult human testis sections. All antigens were detected in association with Sertoli cells. In addition, phosphorylated SMAD2/3 signal was present in the nucleus of all germ cell types, SMAD6 in the nucleus of spermatogonia and spermatocytes, and MAN-1 at the nuclear periphery and cytoplasm of spermatogonia and occasional spermatocytes and round spermatids. Right-hand panels represent negative control sections lacking each primary antibody. White arrow, spermatogonial cell staining; hatched arrowhead, spermatocyte staining; black arrow, Sertoli cell staining. pSMAD2/3, phosphorylated SMAD2/3. Scale bars in panel A and panel A' are representative of panels A–E and A'–E' respectively indicating 50 μ m.

Activin and TGFB-signaling modulators within the normal adult human testis

Within all sections ($n=3$) of phenotypically normal adult human testis, immunostaining for activin/TGFB-signaling modulators was consistently detected in Sertoli cells. Specifically, phosphorylated SMAD2/3 was detected within Sertoli cell nuclei, SMAD6 within Sertoli cell nuclei and cytoplasm, MAN-1 within the cytoplasm and nuclear periphery of occasional Sertoli cells, and INHA and β -glycan within the Sertoli cell cytoplasm (Fig. 2). Leydig cells exhibited immunostaining for all antigens. Within the seminiferous tubules, phosphorylated SMAD2/3 was localized to the nucleus of all germ cell types, SMAD6 to the nucleus of spermatogonia and spermatocytes, and MAN-1 to the nuclear periphery and cytoplasm of most spermatogonia and occasional spermatocytes and round spermatids (Fig. 2 and Table 1). INHA and β -glycan were not detected in association with germ cells. These results identify Sertoli cells and spermatogonia as the common sites of production of both transducers and modulators of activin and TGFB signaling within the seminiferous epithelium.

Activin and TGFB-signaling modulators within gonadotropin-deprived testis

To examine the effects of gonadotropin and intratesticular testosterone suppression on the expression of activin/TGFB-signaling modulators, human testis sections were obtained from a previous study investigating spermatogenic failure induced by androgen-based contraceptive treatments (McLachlan *et al.* 2002). As the immunostaining patterns following 12 weeks of testosterone or 12 weeks of testosterone plus depot medroxyprogesterone acetate (DMPA) treatment were

indistinguishable for all antigens, only staining of the former is shown (Fig. 3). Immunostaining for phosphorylated SMAD2/3 exhibited a nuclear signal in all germ cell types and Sertoli cells in all testosterone or testosterone plus DMPA-treated samples (Fig. 3 and Table 1). Interestingly, the intensity of this nuclear signal was markedly stronger in all 12-week testosterone or testosterone plus DMPA-treated samples ($n=4$ for each treatment group) than in untreated testis samples from the same study ($n=3$). Testis samples from men subjected to testosterone or testosterone plus DMPA treatment for 12 weeks demonstrated SMAD6 protein in the nucleus and cytoplasm of Sertoli cells, spermatogonia, and spermatocytes (Fig. 3 and Table 1). Relative to staining in the normal untreated testis samples, SMAD6 exhibited a distinct cytoplasmic signal in spermatocytes within gonadotropin-deprived samples (Fig. 3). Immunostaining for MAN-1 labeled the nuclear periphery and cytoplasm of most spermatogonia, Sertoli cells, and occasional spermatocytes in untreated and 12-week gonadotropin-deprived testis samples (Fig. 3 and Table 1). In men treated for 12 weeks with testosterone or testosterone plus DMPA, strong immunostaining for INHA and β -glycan was detected in association with Sertoli cells (Fig. 3 and Table 1). In contrast to the normal untreated adult human testis specimens ($n=3$), β -glycan protein was also localized to round spermatids in all sections from men subjected to 12 weeks of gonadotropin suppression ($n=4$ for each treatment group, Fig. 3). These results identify specific changes in the localization and expression patterns of activin/TGFB-signaling modulators in gonadotropin-deprived human testes samples, providing evidence that changes in activin and TGFB-signaling regulation occur in response to spermatogenic suppression.

Table 1 Summary of activin and transforming growth factor- β (TGFB)-signaling modulator protein immunostaining levels in the normal adult human testes, testicular carcinomas, and gonadotropin-deprived human testes.

	Normal (untreated) testes	Gonadotropin-deprived human testes		Testicular carcinomas			
		Testosterone 12 weeks	Testosterone + DMPA 12 weeks	Normal tubules in CIS	CIS	Seminoma	Non seminoma
Sample number	($n=3$)	($n=4$)	($n=4$)	($n=7$) ^a	($n=14$) ^a	($n=28$) ^b	($n=9$) ^a
Staining intensity	+++ ^c	+++	+++	+++ ^c	+++	See below	+++
Phosphorylated SMAD2/3	Sc, SG, SC, R	Sc, SG, SC, R	Sc, SG, SC, R	Sc, SG, SC, R	Sc, CIS	+++ ^d Sem	V
SMAD6	Sc, SG, SC	Sc, SG, SC	Sc, SG, SC	Sc, SG, SC	Sc, CIS	+++ Sem	V
MAN-1	Sc, SG, and some SC	Sc, SG, and some SC	Sc, SG, and some SC	Sc, SG, and some SC	Sc, CIS	+/- ^e Sem	V
INHA	Sc	Sc	Sc	Sc	Sc	+/- ^e Sem	V
β -Glycan	Sc	Sc, R	Sc, R	Sc	Sc	+/- ^f Sem	V

Samples scored according to subjective, semiquantitative analysis. +++, strong staining intensity; ++, moderate staining intensity; +, low staining intensity; -, no staining. Testosterone; DMPA, depot medroxyprogesterone acetate; SG, spermatogonia; SC, spermatocyte; R, round spermatid; Sc, Sertoli cell; Sem, seminoma cell; V, various cell types; N/A, not tested.

^a $n=4$ for SMAD6; ^b $n=6$ for SMAD6; ^c+++ signal for phosphorylated SMAD2/3; ^dThree out of 28 samples with +/- signal; ^eSix out of 28 samples with ++ signal; ^fFour out of 28 samples with +++ signal.

Activin and TGFB-signaling modulators within testicular neoplasms

Expression of activin/TGFB-signaling modulators was examined within a range of testicular cancer specimens consisting of CIS, seminomas, and nonseminomas. Immunohistochemistry signals in tubules with retained complete spermatogenesis within CIS specimens provided controls for comparison with normal testes samples as shown in Fig. 4. The premalignant CIS cells exhibited nuclear localization of phosphorylated SMAD2/3 ($n=14$) and SMAD6 ($n=4$), lacked INHA ($n=14$) and β -glycan ($n=14$) proteins, and exhibited a cytoplasmic signal for MAN-1 ($n=14$) (Fig. 4 and Table 1). All five antigens were detected within Sertoli cells of CIS tubules. In seminoma tumors, SMAD6 ($n=6$) and phosphorylated SMAD2/3 ($n=28$) proteins localized

to the nuclei of seminoma cells, although 3 out of the 28 tumors tested showed a much weaker phosphorylated SMAD2/3 nuclear signal (Fig. 5A and Table 1). Similarly, MAN-1 ($n=28$), INHA ($n=28$), and β -glycan ($n=28$) expression was heterogeneous, with a subset of tumors exhibiting a comparatively strong signal in seminoma cells across majority of the section for MAN-1 (6 out of 28 seminoma tumors; Fig. 5h), INHA (6 out of 28; Fig. 5D), and β -glycan (4 out of 28; Fig. 5E; Table 1). Among the subset of tumors that displayed heterogeneous staining for phosphorylated SMAD2/3, MAN-1, INHA, and β -glycan, three individual seminoma tumors displayed intriguing correlations in their altered expression of activin and TGFB-signaling transducers and antagonists. One seminoma tumor exhibited a particularly strong signal for both INHA and β -glycan, and a corresponding weak nuclear signal for phosphorylated SMAD2/3 (Fig. 5A–E), while the other two tumors demonstrated a comparatively weak signal for INHA and β -glycan, and a very strong signal for phosphorylated SMAD2/3 in the nucleus of seminoma cells (one of these tumors depicted in Fig. 5F–J). All three tumors demonstrated a strong SMAD6 signal and a weak to absent MAN-1 signal in seminoma cells (Fig. 5B, G and C, H depicts SMAD6 and MAN-1 localization respectively in two of these three tumors). These results suggest there are distinct differences in the response of these three tumors to activin and TGFB signaling. Within the morphologically heterogeneous nonseminoma samples, phosphorylated SMAD2/3,

Figure 3 Cellular localization of activin and TGFB-signaling modulator proteins within gonadotropin-deprived human testis. Immunohistochemistry on testis sections from untreated men (A–E) and normal men that had received hormone treatment for 12 weeks with testosterone (F–J) for phosphorylated SMAD2/3 (A and F), SMAD6 (B and G), MAN-1 (C and H), INHA (D and I), and β -glycan (E and J). In untreated and gonadotropin-deprived samples, phosphorylated SMAD2/3 signal (A and F) localized to the nucleus of Sertoli cells and all germ cell types, and the signal is relatively stronger in the 12-week testosterone-treated samples. SMAD6 staining (B and G) was present within the nucleus and cytoplasm of Sertoli cells, spermatogonia, and spermatocytes. The SMAD6 signal was stronger in the spermatocyte cytoplasm in 12-week testosterone-treated samples relative to untreated sections (panel G left-hand insert indicates high magnification of spermatocyte cytoplasmic signal). MAN-1 protein (C and H) was detected in the nuclear periphery and cytoplasm of spermatogonia, Sertoli cells, and occasional spermatocytes within untreated and gonadotropin-deprived samples (panel H left-hand insert indicates high magnification of stained spermatogonium). INHA (D and I) and β -glycan (E and J) signals were detected in association with Sertoli cells in untreated and 12-week testosterone-treated samples, while β -glycan protein also localized to round spermatids in 12-week testosterone-treated sections (panel J left-hand insert indicates high magnification of round spermatid). Right-hand panels represent negative control sections lacking each primary antibody. White arrow, spermatogonial cell staining; hatched arrowhead, spermatocyte staining; white arrowhead, round spermatid staining; black arrow, Sertoli cell staining. Testosterone; pSMAD2/3, phosphorylated SMAD2/3. Scale bar in panel A indicates 50 μ m and is representative of all other panels.

SMAD6, MAN-1, INHA, and β -glycan were detected in association with a variety of cell types (Fig. 5K–O and Table 1). Specifically, phosphorylated SMAD2/3 exhibited minimal to absent immunostaining in the embryonal carcinoma component, but demonstrated positive staining in stromal fibroblasts/fibrocyte-like cells and in a subset of cells in the somatically differentiated component. Anti-SMAD6 protein showed weak staining in the embryonal carcinoma cells (Fig. 5L) and strong staining in a subset of cells within glandular epithelium, and in some large cells resembling adipocytes. The inner nuclear membrane protein MAN-1 localized to scattered round mesenchymal-like cells. This staining was also moderate in embryonal carcinoma cells and was

heterogeneously positive in glandular epithelial cells. Immunostaining for INHA was predominantly detectable in stromal fibroblasts/fibrocyte-like cells and in round lymphocyte-resembling cells, with heterogeneous staining in glandular epithelium and weak staining in a subset of embryonal carcinoma cells. Strong immunostaining for β -glycan was evident in squamous epithelium and vascular endothelium, including lymphatic vessels, and in a subset of glandular epithelium, while moderate staining was detected in the embryonal carcinoma component in a subset of samples (Fig. 5O). These findings demonstrate expression of activin/TGFB-signaling modulators persists in the testis following transformation of normal germ cells to the malignant non-germ cell phenotype of nonseminomas.

Discussion

This study has defined for the first time the cellular production sites of several activin and TGFB-signaling modulators within the normal adult human testis seminiferous epithelium and in its dysfunctional and neoplastic counterparts. Common sites of expression identify Sertoli cells and spermatogonia as the predominant cellular sites of activin/TGFB signal transduction and regulation (Fig. 6). These results are in accordance with our previous findings, which identify activin receptors in spermatogonia and Sertoli cells of the normal adult human testis (Dias *et al.* 2008). Our findings also identify a subset of tumors in which expression of activin/TGFB transducers and antagonists is distinctly altered in a trend that correlates with either increased or decreased signaling. Comparison of normal and gonadotropin-deprived human testis samples demonstrate changes in the cellular localization and expression patterns of activin/TGFB modulators, indicating that activin and TGFB-signaling regulation is sensitive to fluctuations in testis hormone levels.

Figure 4 Cellular localization of activin and TGFB-signaling modulator proteins within CIS specimens. Immunohistochemistry on testis sections from CIS patients with some tubules containing preserved spermatogenesis (A–E), and patients with only CIS tubules (F–J) for phosphorylated SMAD2/3 (A and F), SMAD6 (B and G), MAN-1 (C and H), INHA (D and I), and β -glycan (E and J). Within tubules containing ongoing spermatogenesis in CIS samples, phosphorylated SMAD2/3 signals (A) were evident within the nucleus of Sertoli cells and all germ cell types; SMAD6 (B) within the nucleus of Sertoli cells, spermatogonia, and spermatocytes; MAN-1 (C) within the nucleus and cytoplasm of Sertoli cells, spermatogonia, and some spermatocytes and rounds; and INHA (D) and β -glycan (E) in association with Sertoli cells. In CIS tubules, phosphorylated SMAD2/3 (F) and SMAD6 (G) signals were present in the nucleus of CIS cells; while INHA (I), and β -glycan (J) proteins were not detected in CIS cells. White arrow, spermatogonium or CIS cell staining; hatched arrowhead, spermatocyte staining; black arrow, Sertoli cell staining. CIS, carcinoma *in situ*; pSMAD2/3, phosphorylated SMAD2/3. Scale bar in panel A indicates 50 μ m and is representative of all other panels.

Figure 5 Cellular localization of activin and TGFB-signaling modulator proteins within testicular neoplasms. Immunohistochemistry on two seminoma tumors that exhibited distinct correlations in their altered expression of activin and TGFB-signaling modulator proteins (A–J), a third seminoma tumor that demonstrated an enhanced MAN-1 signal (h), and on nonseminoma tumors (K–O) for phosphorylated SMAD2/3 (A, F and K), SMAD6 (B, G and L), MAN-1 (C, H, h and M), INHA (D, I and N), and β -glycan (E, J and O). One seminoma tumor (A–E) displayed a strong INHA (D) and β -glycan (E) signal and a corresponding weak phosphorylated SMAD2/3 (A) signal. A second seminoma tumor (F–J) displayed a comparatively weak INHA (I) and β -glycan (J) signal and a strong phosphorylated SMAD2/3 (F) signal. Both tumors demonstrated a strong SMAD6 (B and G) signal and a weak to absent MAN-1 (C and H) signal in seminoma cells. An example of a third seminoma tumor that exhibited a comparatively strong signal across majority of the section for MAN-1 (h). In the heterogeneous nonseminoma tumors (K–O), all antigens were detected within a variety of cell types. pSMAD2/3, phosphorylated SMAD2/3. Scale bar in panel A indicates 50 μ m and is representative of all other panels.

The activin/TGFB-signaling transducers phosphorylated SMAD2/3, and antagonists, MAN-1, INHA and β -glycan, exhibited heterogeneous expression patterns within and between seminoma tumors. INHA and β -glycan are normally produced by somatic cells in the adult testis; their synthesis in a subset of germ cell-derived seminomas may allow selected tumors to gain independence from Sertoli cell-derived factors as they

gain the capacity to antagonize activin signaling in an autocrine manner and thereby limit activin bioactivity. Our data illustrating a differential expression of the INHA subunit between individual seminoma specimens (6 out of 28) is consistent with a previous report documenting the presence of *INHA* mRNA in a subset of seminomas (three out of nine) using RNAase protection assays (van Schaik *et al.* 1997). In the normal testis, production of the INHA subunit is confined to Sertoli and Leydig cells in species, including the human and mouse (Roberts 1997, Majdic *et al.* 1997, Barakat *et al.* 2008). Mice lacking the INHA subunit develop Sertoli cell tumors 3–4 weeks after birth, due to excessive activin production and signaling (Matzuk *et al.* 1992). The absence of Sertoli cells in germ cell-derived tumors may also reflect excessive activin signaling, as the ActRIIA subunit is consistently up-regulated in seminomas (Dias *et al.* 2008). The synthesis of INHA by a subset of seminomas may therefore engender in cells in these tumors the capacity to suppress activin signaling, with implications for the growth of seminoma cells (Fig. 6).

In addition to inhibiting activin, β -glycan facilitates signaling by all three TGFB (TGFB1–B3) ligands and enhances BMP signaling by directly binding BMP2, 4, 7 and GDF5 to augment their binding to type I BMP receptors (Lopez-Casillas *et al.* 1993, Kirkbride *et al.* 2008). Therefore, the synthesis of β -glycan protein by a subset of seminomas would confer responsiveness to several additional TGFB superfamily signaling molecules. In other human cancers, such as breast, ovary, lung, pancreas, and prostate carcinomas, loss of β -glycan mRNA and protein has been linked to enhanced migration and invasion by tumor cells (Dong *et al.* 2007, Hempel *et al.* 2007, Turley *et al.* 2007, Finger *et al.* 2008, Gordon *et al.* 2008), indicating a tumor suppressive function of β -glycan. If β -glycan functions in a similar manner in TGCTs, its detection within particular specimens may reflect a tendency for these tumors to remain confined to their site of origin.

The integral inner nuclear membrane protein MAN-1 inhibits activin signaling by interfering with signal transduction via SMAD2/3 proteins and restricting transcriptional activation of activin/TGFB response genes (Pan *et al.* 2005, Lin *et al.* 2005, Caputo *et al.* 2006). Mutations in MAN-1 have been linked to a number of human diseases characterized by increased bone density, implicating MAN-1 as a negative regulator of bone formation by inhibiting TGFB superfamily signaling (Helleman *et al.* 2004). To date, neither the expression nor function of MAN-1 in human neoplasms has been investigated. The presence of MAN-1 in both Sertoli and germ cells of the normal adult human testis indicates that it is directly involved in the function and/or development of both cell lineages. The enhanced MAN-1 protein signal detected in a subset of seminomas may reflect an additional level of TGFB superfamily signaling regulation that would lead to altered

Figure 6 Schematic illustration of normal spermatogenesis, the pathogenesis of CIS, its progression to malignant neoplasms, and the cellular localization of activin/TGFβ-signaling modulators within these. In the normal context, gonocytes undergo mitosis and differentiate into spermatogonia that progress through different stages of spermatogenesis to give rise to mature sperm (top). Within the normal adult testis, phosphorylated SMAD2/3 were expressed by all germ cell types, SMAD6 by spermatogonia and spermatocytes, MAN-1 by spermatogonia and occasional spermatocytes, while all tested signaling modulators were expressed by Sertoli cells. In the neoplastic state, an arrest in the differentiation program of fetal germ cells causes gonocytes to transform to premalignant CIS cells, which progress to malignant seminomas or nonseminomas after puberty (bottom). Phosphorylated SMAD2/3, SMAD6, and MAN-1 are produced by CIS cells. All seminoma tumors exhibited phosphorylated SMAD2/3 and SMAD6 production, while MAN-1, INHA, and β-glycan were up-regulated in a subset of seminomas, highlighting the capacity for changed activin/TGFβ-signaling modulation to occur in response to spermatogenic disruption.

expression of activin/TGFβ target genes in these testicular tumor cells (Fig. 6).

Changes in the expression and cellular localization of activin signaling regulators in the 12-week gonadotropin-deprived testis sections suggest that testicular responsiveness to activin/TGFβ stimulation is modulated by spermatogenic disruption and altered testicular hormone levels and highlight the potential of TGFβ superfamily signaling to influence testicular physiology. The administration of testosterone or testosterone plus DMPA to healthy adult men causes significant impairment in the transition of type B spermatogonial cells to early spermatocytes and profound inhibition of sperm release (McLachlan *et al.* 2002). β-Glycan protein induction in haploid germ cells in gonadotropin-deprived testis would be predicted to enhance suppression of activin signaling and thereby increase TGFβ and BMP ligand responsiveness. SMAD6 has been shown to preferentially antagonize BMP signaling (Ishisaki *et al.* 1999, Goto *et al.* 2007). The increased SMAD6 signal in these spermatocytes may reflect circumstances in which activin signaling is enhanced due to reduced competition with BMPs for signal transducing machinery. Collectively, the enhanced TGFβ ligand signaling conferred by β-glycan synthesis in round spermatids and enhanced activin signaling caused by preferential SMAD6 inhibition of BMP signaling may account for the particularly strong nuclear phosphorylated SMAD2/3 signal within 12-week testosterone or testosterone plus DMPA samples. These results are in agreement

with our previous findings of enhanced ActRIIA protein production within the gonadotropin-deprived testis that could allow for enhanced activin and TGFβ ligand signaling (Dias *et al.* 2008).

The progression of CIS to malignancy is associated with puberty, as seen by a sharp rise in the age-specific incidence of germ cell tumors following puberty (Rajpert-De Meyts & Skakkebaek 1993). The dramatic change in testicular hormone production is a likely trigger for this malignant transformation. The induction of β-glycan protein in a subset of samples displaying neoplastic transformation of germ cells and also following hormonal manipulation of the testis highlights the potential of β-glycan to either influence or be effected by spermatogenic disruption. We hypothesize further that β-glycan synthesis by germ cells is influenced by changes in testicular testosterone levels. Differences in β-glycan protein production between seminoma specimens may reflect differences in the hormonal environment between individual testis tumors.

The molecular events associated with CIS progression to malignancy remain largely unknown. Our results identify distinct differences between individual TGCTs in their potential response to and modulation of activin and TGFβ signaling. The production of INHA and β-glycan by somatic cells in the human fetal testis (Majdic *et al.* 1997, Anderson *et al.* 2002) at a time when germ cells are sensitive to transformation to premalignant germ cells highlights the importance of regulated activin/TGFβ signaling during fetal germ cell

development. Therefore, the aberrant expression of somatically expressed activin antagonists by a subset of seminomas may lead to altered activin bioactivity and change germ cell tumor behavior (Fig. 6). Given the findings of this study, further investigations into the role of activin/TGF β signaling and their regulation in testicular tumor development and their potential effect on clinical outcomes are warranted.

Materials and Methods

Samples

Histological analysis was performed on Bouins fixed, paraffin-embedded sections of normal adult human testis, TGCTs, and gonadotropin-deprived testes. Testicular cancer samples consisting of CIS with or without areas of morphologically normal spermatogenesis, seminomas, and nonseminomas were obtained from pathology departments in the greater Copenhagen area. All tumor samples were assessed by experienced pathologists and routinely stained with placental like alkaline phosphatase (PLAP) to identify CIS cells. Permission for their collection and use in immunohistochemical studies was obtained from the Regional Committee for Medical Research Ethics in Denmark.

All other sections were obtained from biopsy samples collected from healthy adult volunteers. Samples of gonadotropin-deprived testis were obtained following treatment with either testosterone or testosterone plus a depot progestin (testosterone + DMPA) for 12 weeks samples from McLachlan *et al.* (2002). Testes samples from control men not subjected to testosterone or testosterone + DMPA treatment were also analyzed. Sample collection was approved by the Human Research and Ethics Committee of the Monash Medical Centre, and informed consent was obtained from each subject. The numbers of normal, neoplastic, and gonadotropin-deprived testes samples examined are listed in Table 1.

Immunohistochemistry

Immunohistochemistry was performed as previously described on dewaxed sections blocked with 5% (v/v) normal serum diluted in Tris-buffered saline (TBS) with 0.1% BSA before the addition of primary antibody (Loveland *et al.* 1999). Briefly, antigen retrieval was performed at 90 °C and maintained for 8 mins in 50 mM glycine pH 3.5 for phosphorylated SMAD2/3, SMAD6 and INHA, 0.01 M Citrate pH 6.0 for β -glycan, and testosterone-EG (10 mM Tris, 0.5 mM EGTA) pH 9.0 for MAN-1. Samples were blocked in 5% (v/v) normal serum (normal sheep serum for phosphorylated SMAD2/3, SMAD6, and INHA and normal rabbit serum for MAN-1 and β -glycan), diluted in TBS with 0.1% BSA for 1 h. Primary antibodies were then applied: phosphorylated SMAD2/3 at 1:150 dilution, SMAD6 at 1.7 μ g/ml, MAN-1 at 2.7 μ g/ml, INHA at 65.5 μ g/ml, and β -glycan at 1.25 μ g/ml, diluted in TBS with 0.1% BSA for overnight incubation at room temperature. All washes between incubations were performed at room temperature with TBS. Primary antibody binding was detected using a biotinylated secondary antibody: sheep anti-rabbit (Chemicon,

Temecula, CA, USA) for phosphorylated SMAD2/3 and SMAD6, rabbit anti-goat (DAKO, Botany, NSW, Australia) for MAN-1 and β -glycan, and sheep anti-mouse (Chemicon) for INHA (each diluted 1:500 in TBS/0.1% BSA, 1 h). The Vectastain Elite ABC kit was then applied according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Antibody binding was detected as a brown precipitate following development with 3,3'-diaminobenzidine tetrahydrochloride against a Harris hematoxylin counterstain. Germ cell types were identified primarily on the basis of their nuclear morphology and position within the seminiferous epithelium, as described in McLachlan *et al.* (2002). Immunohistochemistry was performed at least in duplicate with identical results between experiments for each sample. The number of individual samples tested from each treatment or sample group for each antibody is listed in Table 1. Immunostained sections were scored blinded by relative staining intensity as high, medium, low, and negative by subjective semiquantitative analysis. To account for variations in staining intensity between different immunohistochemistry runs, adult human testis samples were included in each run; these consistently demonstrated identical results. Controls for nonspecific binding of the secondary antibody were performed in all experiments by omission of primary antibody, and these consistently yielded no signal within the seminiferous epithelium or in the interstitial space.

Antibody validation and western blot analyzes

Selective target recognition by the mouse monoclonal INHA R1 antibody (Groome N) has been previously established (Groome & O'Brien 1993, Arora *et al.* 1997). Western blot validation of the rabbit polyclonal anti-phospho-SMAD2/3 (Cell Signaling Technology, Danvers, MA, USA) antibody was performed on immature day 6 mouse Sertoli cell lysates (the amino acid sequences of both SMAD2 and SMAD3 proteins share >95% sequence homology between mouse and human), and yielded a single band of the expected size of 58 kDa (Itman *et al.* 2009). Manufacturer's specifications indicate this antibody will detect both phosphorylated SMAD2 and SMAD3 in fixed tissues due to sequence similarity between these two proteins. The rabbit polyclonal anti-SMAD6 antibody (Zymed Laboratories, San Francisco, CA, USA) has also been used on western blots of mouse testis lysates to detect a single band of 60 kDa (Itman & Loveland 2008; amino acid sequences of human and mouse SMAD6 share ~95% sequence homology). An individual sample of the goat polyclonal anti-human TGFBR3 antibody (R&D Systems, Minneapolis, MN, USA) was previously verified by immunofluorescence analysis on a β -glycan null mouse line (Sarraj *et al.* 2007; amino acid sequence of human and mouse β -glycan shares ~90% sequence homology).

Target recognition by the goat polyclonal anti-MAN-1 (N-20; Santa Cruz Biotechnology) antibody was determined by western blot analysis on human NTERA cell lysates. Protein loading was indicated by blotting for α -tubulin (Sigma Chemical Co). Briefly, lysates were prepared by homogenization at 4 °C in RIPA buffer (150 mM sodium chloride, 1% Nonidet P-40, 0.5% Tween-20, 0.1% SDS, 1 mM

ethylenediaminetetraacetic acid) in the presence of protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, San Diego, CA, USA), as previously described (Loveland *et al.* 1999). Protein concentration was determined using the BioRad DC protein assay (Bio-Rad). A total of 30 µg of human NTERA cell protein lysate was loaded per lane onto 12% SDS-PAGE gels with protein size standards (Page-Ruler Prestained Protein Ladder, Fermentas, Burlington, Ontario, Canada). Following electrophoresis, the proteins were transferred to Hybond-C Nitrocellulose membrane (Amersham Biosciences) and all subsequent incubations were performed at room temperature unless otherwise stated. The membranes were blocked for 1 h with Odyssey buffer:PBS (1:2) (LICOR Biosciences, Lincoln, NE, USA). Primary antibody incubations were carried out at 4 °C in blocking buffer plus 0.1% (v/v) Tween. MAN-1 and α -tubulin antibodies were used at dilutions or concentrations of 0.6 µg/ml and 1:5000 respectively. Membranes were washed with PBS plus 0.1% Tween (v/v), then incubated with Alexa Fluor 680-coupled secondary antibodies (Molecular Probes, Eugene, OR, USA) at 1:10 000 dilution in blocking solution with 0.1% (v/v) Tween and 0.01% (v/v) SDS for 1 h. Bound antibody was detected using the LI-COR Odyssey System (LI-COR Biosciences).

Declaration of interest

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

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