

# Regulated production of activin A and inhibin B throughout the cycle of the seminiferous epithelium in the rat

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## Abstract

Production and regulation of activin A and inhibin B during the cycle of the seminiferous epithelium were investigated in adult rats. Immunohistochemistry localised the activin  $\beta_A$ -subunit to the Sertoli cell cytoplasm, with much weaker expression in spermatocytes and spermatids. Both activin A and inhibin B, measured by ELISA were secreted by seminiferous tubule fragments over 72 h in culture. Activin A was secreted in a cyclic manner with peak secretion from tubules isolated at stage VIII. Tubules collected during stage VI produced the least activin A. Inhibin B secretion was highest from stage IX–I tubules and lowest from stage VII tubules. Addition of interleukin-1 $\beta$  (IL-1 $\beta$ ) had relatively little effect on activin A or inhibin B secretion in culture. In contrast, the peak secretion of activin A by stage VIII tubules

was blocked by co-incubation with an excess of human recombinant IL-1 receptor antagonist, whereas inhibin B secretion increased slightly. Dibutyryl cAMP stimulated activin A secretion by late stage VII and VIII tubules and stimulated inhibin B across all stages. These data indicate that activin A and inhibin B are cyclically regulated within the seminiferous epithelium, with endogenous IL-1 (presumably IL-1 $\alpha$  produced by the Sertoli cells), responsible for a peak of activin A production subsequent to sperm release at stage VIII. These data provide direct evidence that production of activin A and inhibin B by the Sertoli cell is locally modulated by IL-1 $\alpha$ , in addition to FSH/cAMP, under the influence of the developing spermatogenic cells.

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## Introduction

Previously, we have shown that Sertoli cells from immature (20-day-old) and adult rat testes respond to stimulation with interleukin-1 (IL-1) *in vitro* by secreting activin A, and this response is in turn regulated by follicle-stimulating hormone (FSH) through a cAMP-mediated mechanism (Okuma *et al.* 2005a, 2005b). Stimulation of IL-1 $\alpha$  production in Sertoli cells by the bacterial inflammatory agent, lipopolysaccharide (LPS), causes a corresponding stimulatory effect on Sertoli cell activin A production (Okuma *et al.* 2005b). This action of LPS is partially blocked by co-incubation with an excess of the IL-1 receptor antagonist (IL-1ra), indicating that activin A production in this inflammation model is driven by endogenous IL-1 $\alpha$  production (Gérard *et al.* 1991, Jonsson *et al.* 1999). Since both IL-1 $\alpha$  and activin A have regulatory effects on spermatogonial DNA synthesis, proliferation and survival (Mather *et al.* 1990, Hakovirta *et al.* 1993a, Meehan *et al.* 2000), such interactions might underpin the mechanisms driving cyclic spermatogonial development in the adult testis.

In stage-dissected adult rat seminiferous tubules, both IL-1 $\alpha$  mRNA and protein are produced at all stages throughout

the cycle of the seminiferous epithelium, with the exception of stage VII (Gérard *et al.* 1991, Söder *et al.* 1991, Jonsson *et al.* 1999). Studies suggest that this production is driven by the developing germ cells and residual bodies (Gérard *et al.* 1992, Wang *et al.* 1998, Jonsson *et al.* 1999). Significantly, spermatogonial DNA synthesis is lowest in isolated stage VII tubule fragments, but can be stimulated by addition of exogenous IL-1 $\alpha$  (Parvinen *et al.* 1991). Nonetheless, IL-1 $\alpha$  alone cannot account for the pattern of spermatogonial DNA synthesis across the entire seminiferous cycle. IL-1 $\alpha$  however, shows a relatively consistent production level at all stages except stage VII, DNA synthesis shows a more complex pattern, with two distinct peaks of synthesis at stages IV–V and at stages VIII–IX (Clermont & Harvey 1965, Parvinen *et al.* 1991). This points to the involvement of other regulators in addition to IL-1 $\alpha$ . As far as activin A is concerned, expression of the activin  $\beta_A$ -subunit mRNA is cyclic, with a peak at stages IX–XI (Kaipia *et al.* 1992, Buzzard *et al.* 2004). Most studies indicate that this is primarily due to the Sertoli cells, although other tubule cells have been implicated as an additional source of the  $\beta_A$ -subunit, including spermatocytes, spermatids and the peritubular cells (Kaipia *et al.* 1992, de Winter *et al.* 1994, Buzzard *et al.* 2004).

In a previous study (Okuma *et al.* 2005a), it was demonstrated that production of inhibin B, which comprises a heterodimer of an  $\alpha$ -subunit and homologous activin  $\beta_B$ -subunit, is regulated in the Sertoli cell by IL-1 in a reciprocal relationship with activin A production. Production of the inhibin B subunits ( $\alpha$  and  $\beta_B$ ) and the inhibin B protein is primarily under the control of circulating FSH, but also displays a cyclical pattern of production, with the highest levels of subunit mRNA at stages XIII–IV and lowest at stages VI–VIII (Bhasin *et al.* 1989, Kaipia *et al.* 1991, 1992, Buzzard *et al.* 2004). Measurement of inhibin protein using a RIA directed against the  $\alpha$ -subunit of inhibin confirmed that immunoreactive inhibin production mirrors the mRNA expression data with highest levels in tubule fragments from stages XIII–VI and the lowest content and secretion at stages VII–VIII, under both basal and FSH-stimulated conditions (Gonzales *et al.* 1989, Klajj *et al.* 1994). This cyclic pattern of inhibin production is consistent with the fact that germ cells have a direct influence on inhibin production mediated via secreted factors (Pineau *et al.* 1990, Clifton *et al.* 2002). While  $\beta_B$  mRNA and protein expression also have been localised to the germ cells, particularly pachytene spermatocytes and early spermatids in both the human and rat (Andersson *et al.* 1998, Marchetti *et al.* 2003, Buzzard *et al.* 2004), these cells do not appear to significantly express the  $\alpha$ -subunit and there is some controversy as to whether germ cell expression contributes to the formation of inhibin B *in vivo*. There is no assay for the  $\beta_B$  homodimer (activin B), hence nothing is known of its production or regulation in the testis at present.

The hypothesis that interaction between endogenous IL-1 $\alpha$  and FSH/cAMP plays a role in the cyclic regulation of both activin A and inhibin B in the seminiferous epithelium was investigated using isolated fragments of staged seminiferous tubules from adult rats in culture. Although IL-1 $\alpha$  is the form of IL-1 produced by Sertoli cells, IL-1 $\beta$  is the form that is secreted by monocytes and macrophages during inflammation. Both forms of IL-1 bind to the same receptor with the same range of biological actions, including the stimulation of activin A production by Sertoli cells, and are inhibited by IL-1ra (Okuma *et al.* 2005a, 2005b). Inflammation in the testis involves production of both IL-1 forms (O'Bryan *et al.* 2005, Okuma *et al.* 2005b). Rather than duplicating these studies using both forms of IL-1, however, IL-1 $\beta$  was employed as the bioactive ligand for all the studies described herein. In contrast to earlier studies on the expression of activins and inhibins in the seminiferous epithelium, the use of specific two-site ELISAs permitted the measurement of the mature dimeric proteins, rather than just the individual subunits. In addition, the cellular distribution of the  $\beta_A$ -subunit of activin A was investigated using immunohistochemistry on whole testis sections. Immunohistological localisation of the inhibin B subunits in the testis of adult rats has been performed by several groups previously (Merchenthaler *et al.* 1987, Roberts *et al.* 1989, Shaha *et al.* 1989, Majdic *et al.* 1997) and was not repeated in the present study.

## Materials and Methods

### Animals

Adult (80–100-day-old) male Sprague–Dawley rats were obtained from the Central Animal House, Monash University, Victoria, Australia. All studies were approved by the Monash University Animal Ethics Committee and conformed to the Australian Code of Practice for the Care and Use of Animals for Experimental Purposes.

### Seminiferous tubule dissection and culture

Testes were decapsulated immediately post-mortem and individual tubules were isolated using fine forceps under sterile conditions in a Petri dish containing Dulbecco's modified Eagle's medium (DMEM), under an Olympus SZX12 transillumination inverted stereomicroscope (Olympus, Tokyo, Japan). Tubules were staged according to the 14-stage classification (I–XIV) for rat spermatogenesis (Leblond & Clermont 1952), using the transillumination identification criteria of Parvinen and colleagues (Parvinen & Ruokonen 1982) and cut into 2 mm long fragments.

In each culture experiment, ten fragments of each stage or group of stages were used to generate each single replicate. Freshly isolated tubule fragments were homogenised on ice using a glass hand-held homogeniser in 0.5 ml 0.1% Triton X-100 (Sigma) in Dulbecco's phosphate-buffered saline for the measurement of cell content. For *in vitro* production studies, tubule fragments were cultured in 0.5 ml DMEM and 0.1% BSA in 24-well Falcon culture plates (Becton–Dickinson, Bedford, MA, USA). The medium was supplemented with the appropriate test substances in triplicate wells: 40 U/ml recombinant human (rh)IL-1 $\beta$  (200 000 U/ $\mu$ g) (R & D Systems, Minneapolis, MN, USA), 1  $\mu$ g/ml rhIL-1 IL-1ra (Amgen, Thousand Oaks, CA, USA) and 500  $\mu$ g/ml dibutyryl cAMP (dbcAMP; Sigma). Tubules were cultured at 32 °C and the media were collected 72 h later and stored at –20 °C prior to analysis. All the experiments were carried out a minimum of twice to confirm the reproducibility of the results.

The use of 72 h cultures was found to be necessary, as short-term cultures (24 and 48 h) did not produce sufficient activin A to be consistently detectable in any except in the peak secretion stages. It has already been established that in 72 h cultures of seminiferous tubule fragments, there is an on-going progression of the cycle throughout the culture period (Clermont & Harvey 1965, Parvinen *et al.* 1991). Dissected tubule fragments collected in stage I, for example, will progress through stages II, III and IV during the culture period, so that the content of the culture medium is a summation of production at all these stages. Similarly, tubule fragments collected during stage VIII progress as far as stage XII during the same period and accumulation of secreted protein in these cultures corresponds to the production during all stages from VIII to XII.

### $\beta_A$ -subunit immunohistochemistry

Testes were fixed by whole-body vascular perfusion with Bouin's fluid, as previously described (O'Bryan *et al.* 2000), postfixed for 5 h and processed for embedding in paraffin by routine procedures. Sections (5  $\mu$ m thick) were cut and attached to Superfrost Plus Glass slides (BioLabs Scientific, Melbourne, Australia).

In order to detect the  $\beta_A$ -subunit of activin, sections were de-paraffinised and rehydrated prior to antigen retrieval in 50 mM glycine-HCl buffer, pH 3.5, in a microwave oven (1350 W) for 2–3 min at the high-power setting, followed by 7–8 min on the defrost setting. Sections were allowed to cool down to room temperature and washed twice with Tris-buffered saline (TBS: 10 mM Tris, 150 mM NaCl, pH 7.6), which was used for all subsequent washing steps. Pretreatment with 3% H<sub>2</sub>O<sub>2</sub> was performed for 5 min to inactivate endogenous peroxidases. Sections were incubated in a blocking solution consisting of 5% normal rabbit serum, 0.1% BSA diluted in TBS for 20 min and then incubated at 4 °C with a mouse monoclonal antibody (E4) directed toward the  $\beta_A$ -subunit of activin (Knight *et al.* 1996), diluted to 10  $\mu$ g/ml in blocking solution. Localisation was detected using biotinylated sheep anti-mouse serum (Silenus, Hawthorn, Vic, Australia) diluted to 1.28  $\mu$ g/ml in blocking solution as secondary antibody (1 h), followed by incubation using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) for 30 min. Staining was visualised with 3,3'-diaminobenzidine tetrahydrochloride (Sigma; 2.13 mg/ml in TBS) as chromogen. The sections were counterstained with Harris' haematoxylin (Sigma) and mounted under DPX (BDH Laboratory Supply, Poole, UK). Mouse IgG2b (isotype matched to the E4 antibody) was used in place of the primary antibody for negative controls.

Immunohistochemistry results were assessed using an Olympus BX 50 microscope, attached to a 3CCD MTI video camera (Dage-MTI, Michigan City, IN, USA) coupled to a Pentium computer. Photoshop version 5.5 (Adobe Systems) was used to generate photomicrographs. Cross-sections of seminiferous tubules were staged according to the cell association and morphological criteria using the 14-stage classification for rat spermatogenesis (Leblond & Clermont 1952).

### Activin A and inhibin B ELISA

Activin A was measured using a specific ELISA (Knight *et al.* 1996) according to the manufacturer's instructions (Oxford Bio-Innovations, Oxfordshire, UK) with some modifications. This assay measures total activin A and is not affected by the presence of the activin-binding protein, follistatin. The recombinant activin A standard (Robertson *et al.* 1992) and samples were diluted in culture medium. A 6% SDS solution in PBS was added (3% final concentration) followed by boiling for 3 min. The samples were allowed to cool before the addition of H<sub>2</sub>O<sub>2</sub> (2% final concentration) and subsequent 30 min incubation. Duplicate samples were added to the E4

(anti- $\beta_A$ -subunit) monoclonal antibody-coated plate and incubated overnight at room temperature. The plates were washed and the second detection antibody (biotinylated-E4) was added for 2 h at room temperature. After washing, alkaline phosphatase linked to streptavidin was added to the wells and incubated at room temperature for 1 h. After further washes, the alkaline phosphatase activity was detected using an amplification kit (ELISA Amplification System; Invitrogen) wherein the substrate was incubated for 1 h at room temperature. This assay has an intraplate coefficient of variation (CV) of 7.4% and an interplate CV of 6.3%, with a mean assay detection limit of 10 pg/ml ( $n = 12$  assays).

Inhibin B was measured using a specific ELISA (Groome *et al.* 1996) according to the manufacturer's instructions (Oxford Bio-Innovations) with some modifications. The standard used was WHO 96/784 inhibin B reference standard (NIBSC, Potter's Bar, Herts, UK). Standard and samples were diluted in unconditioned culture media containing the same additives as were used in the culture and treated as per the manufacturer's protocol. Duplicates were added to the plates and incubated overnight at room temperature. The plates were washed and the second detection antibody (R1 coupled to alkaline phosphatase) was added for 3 h at room temperature. After washing, the alkaline phosphatase activity was detected using an amplification kit (ELISA Amplification System; Invitrogen) wherein the substrate was incubated for 2 h at room temperature. This assay has an intraplate CV of 8.6% and an interplate CV of 7.9%, with a mean assay detection limit of 12 pg/ml ( $n = 7$  assays).

### Statistical analyses

All the data were analysed using one- or two-way ANOVA following appropriate transformations to normalise data and equalise variance, where necessary. Mean values were compared using either Student–Newman–Keuls multiple range test or Dunnett's test for multiple comparisons with a control (Sigmastat version 1.0; Jandel Scientific Software, San Rafael, CA, USA). Differences between responses were considered statistically significant at the  $P < 0.05$  level.

## Results

### Expression of activin A during the cycle of the seminiferous epithelium

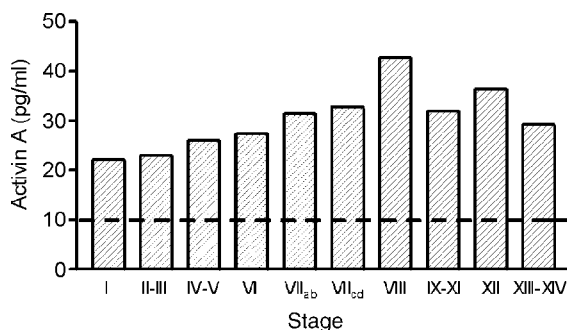
In routine preparations using ten fragments/dissected stage, activin A levels in extracts of tubule homogenates were either very close to or below the defined sensitivity of the assay, i.e. < 10 pg/ml (data not shown). In order to establish the presence of activin A in tubule fragments from all stages of the cycle prior to the start of the cultures, tubule fragments from ten separate experiments were combined to give pooled extracts containing 100 fragments/stage. In these combined extracts, activin A was detectable in homogenates of tubules

collected at all stages of the cycle (Fig. 1), tending toward the highest levels around stages VIII–XII.

Strong immunostaining for the  $\beta_A$ -subunit of activin A protein was observed within the cytoplasm of Sertoli cells at all stages of the cycle of the seminiferous epithelium, with much weaker staining associated with the cytoplasm of all meiotic and postmeiotic stages of germ cell development (Fig. 2). The staining within the Sertoli cell cytoplasm appeared distinctly granular and this was particularly noticeable around stages IX–XI (stage XI is shown as an example in Fig. 2d), where at least some of the staining may have been associated with residual bodies. In the interstitial tissue, very strong staining was observed in the cytoplasm of resident immune cells (macrophages and mast cells), with significant albeit weaker staining in most Leydig cell clusters. Neither the peritubular cell nor vascular endothelial cell cytoplasm showed detectable  $\beta_A$  labelling in these studies.

#### Regulation of activin A and inhibin B secretion

In 72 h cultures of seminiferous tubules, activin A showed a very distinctive cyclical secretion into the culture medium under basal conditions, with a significantly higher production in tubules collected at stage VIII (Fig. 3a). Activin A levels also appeared to be elevated in some cultures of tubules dissected at stages VII<sub>cd</sub> (compare Figs 3a and 4e). Production from all other stages was lower, but still detectable. The content of the media collected at the end of the culture for all stages was consistently greater than the levels of activin A present in the tubule fragments at the start of the culture, indicating that activin A was synthesised as well as secreted in the cultures. Addition of IL-1 $\beta$  did not alter the pattern or production substantially (Fig. 3c), however, addition of dbcAMP did extend the duration of the statistically significant peak of secretion to include both stages VII<sub>cd</sub> and VIII tubule cultures (Fig. 3e). Addition of an excess of IL-1ra completely abrogated the peak of activin A secretion and production across the entire cycle appeared to be reduced relative to cultures under basal conditions, falling below the sensitivity of the assay at stages I–V in particular (Fig. 3g).



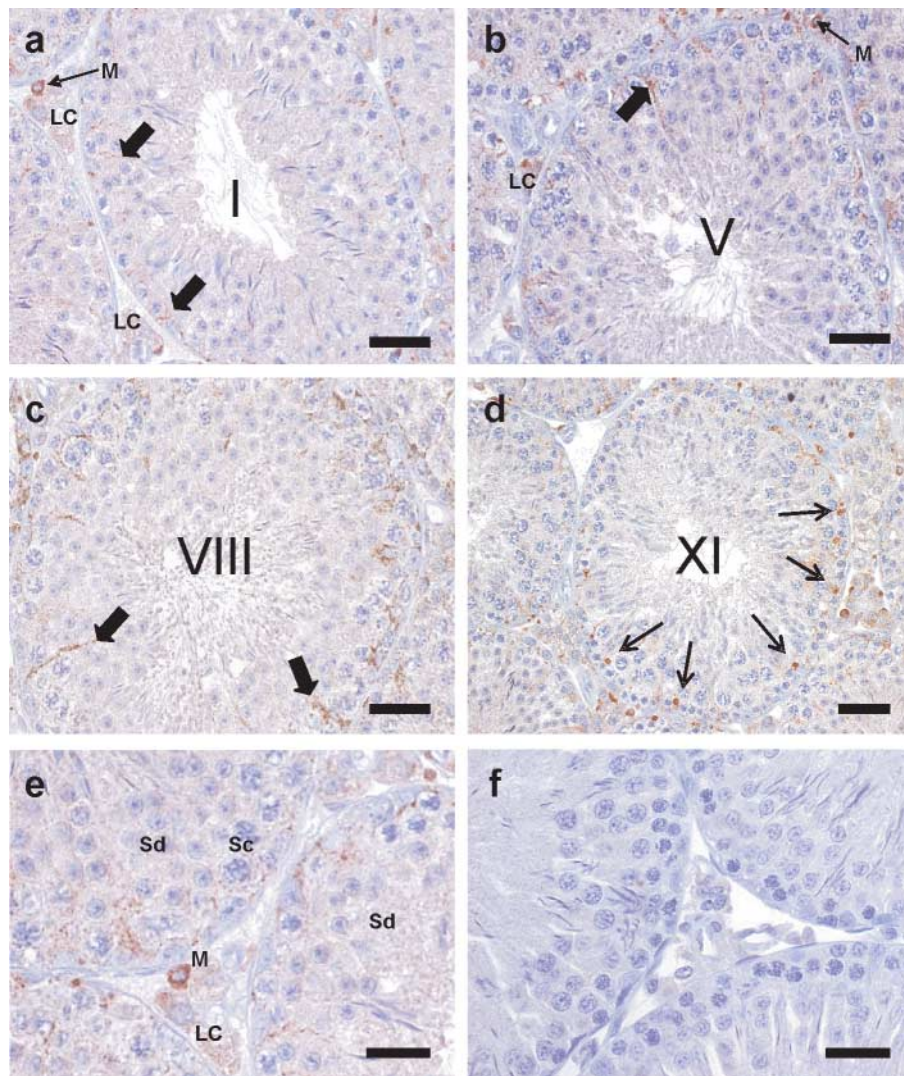
**Figure 1** Activin A content measured in homogenates of isolated staged tubules. Values are for homogenates of pooled 100 fragments from each stage in a final extract volume of 0.5 ml. The broken line indicates the detection limit of the assay.

Inhibin B also displayed a significant cyclic variation in the secretion in culture, with higher levels in tubules collected at stages XI–I and slightly lower levels between stages IV and VII (Fig. 3b). This cyclic secretion was not evident in cultures with IL-1 $\beta$ , although a considerable group variability observed in these cultures would have obscured any subtle difference (Fig. 3d). dbcAMP stimulated inhibin B production at all stages, although the cyclic pattern observed under basal conditions was maintained (Fig. 3f). Addition of IL-1ra had relatively little apparent effect on the cyclic pattern of inhibin B secretion (Fig. 3h).

The data presented in each panel of Fig. 3 were obtained from three separate experiments comprising the entire range of stages under a single treatment; hence, it was not possible to directly compare the responses to the various treatments relative to one another. In order to establish the secretion of activin A and inhibin B as regulated by IL-1, cAMP and IL-1ra, the effects of these treatments were re-evaluated by direct comparisons in cultures of specific stages around the peak of activin A secretion. These studies indicated that the addition of IL-1 $\beta$  caused a small, but a significant increase in activin A secretion in cultures of stage VIII (Fig. 4g), but had no effect at the other stages examined (stages VI–VII; Fig. 4a, c and e). It also was confirmed that cAMP caused a significant stimulation of activin A in cultures of late stages VII and VIII (Fig. 4e and g). Addition of IL-1ra inhibited activin A secretion at stage VIII (Fig. 4g), but no significant effect at the earlier stages studied (VI and VII) was observed (Fig. 4a, c and e). As expected, inhibin B production was stimulated by dbcAMP at all the stages examined, but IL-1 $\beta$  appeared to have no effect (Fig. 4b, d, f and h). Addition of IL-1ra had a small, but significant stimulatory effect on inhibin B secretion at stage VIII (Fig. 4h). These data were entirely consistent with the results obtained in the cultures comparing all the stages of the cycle (Fig. 3).

#### Discussion

The data in the present study show that activin A protein is present at all stages of the cycle of the seminiferous epithelium in the adult rat testis. Activin A is particularly associated with the Sertoli cells, but meiotic and postmeiotic germ cells also appear to contain activin  $\beta_A$  immunoreactivity. Staining of  $\beta_A$  in both Sertoli cells and in spermatogenic cells previously has been identified using rabbit polyclonal antibodies raised against  $\beta_A$  synthetic peptides on frozen sections of adult rat testes (Roberts *et al.* 1989, Shaha *et al.* 1989), while  $\beta_A$  mRNA has been localised to Sertoli cells, spermatocytes and spermatids (Kaipia *et al.* 1992, Buzzard *et al.* 2004). Both the expression of mRNA in these earlier studies and the release of activin A by tubule cultures observed in the present study were cyclic. Curiously, activin A was not detectable by immunohistochemistry in the peritubular cells of the adult testis, even though these cells from immature testes secrete activin A *in vitro* (de Winter *et al.* 1994, Buzzard *et al.* 2003,

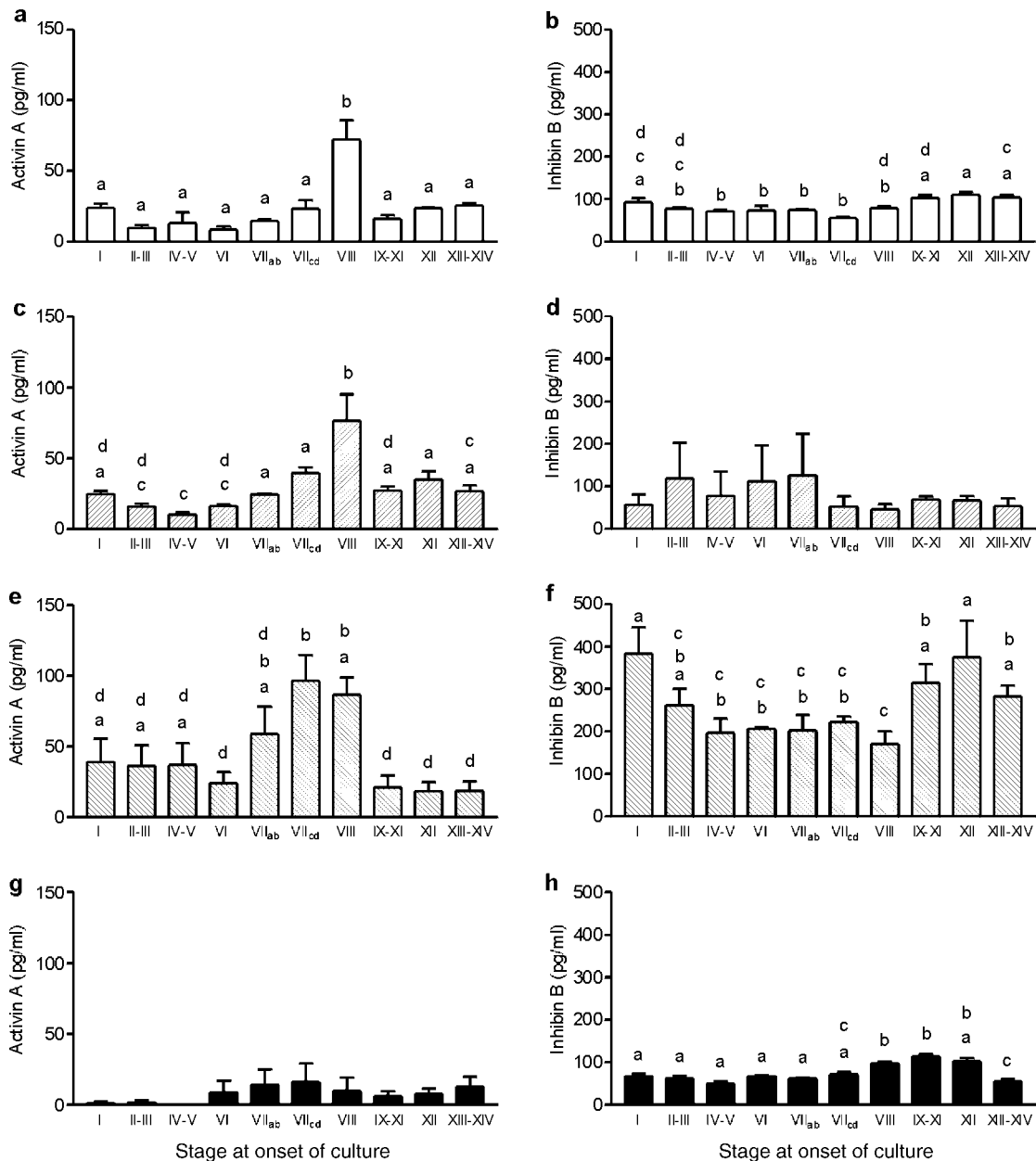


**Figure 2** Localisation of the  $\beta_A$ -subunit of activin A by immunohistochemistry in adult rat testis. (a) Micrograph showing tubule cross-section at stage I of the cycle of the seminiferous epithelium; (b) stage V; (c) stage VIII; (d) stage XI. Bar inset in (a)–(d)=50  $\mu$ m. (e) High power micrograph showing seminiferous epithelium of several adjacent tubules and interstitial tissue; (f) negative control. Bar in (e) and (f)=20  $\mu$ m. Within all tubule cross-sections, strong immunostaining was observed in the Sertoli cell cytoplasm (large arrows), with significant, but much less intense staining over spermatocyte (Sc) and spermatid (Sd) cytoplasm at all stages of their development (compare (e) with negative control section (f) at same magnification). Note that the Sertoli cell-staining pattern appears as distinct large granules in the basal cytoplasm at stage XI (narrow arrows). In the interstitial tissue, strong staining was associated with macrophages (M), while Leydig cell (LC) clusters showed a consistently weaker staining profile. There was no appreciable immunostaining associated with peritubular cells, basally located spermatogonia or vascular endothelial cells.

Okuma *et al.* 2005a) and expression of mRNA has been detected in these cells in the adult rat testis by *in situ* hybridisation (Buzzard *et al.* 2004). These data suggest that either transcription or storage of activin A by the peritubular cells in the adult testis may be greatly reduced compared to other cell types.

When interpreting the data presented in this study, it is important to recognise that there is an on-going progression

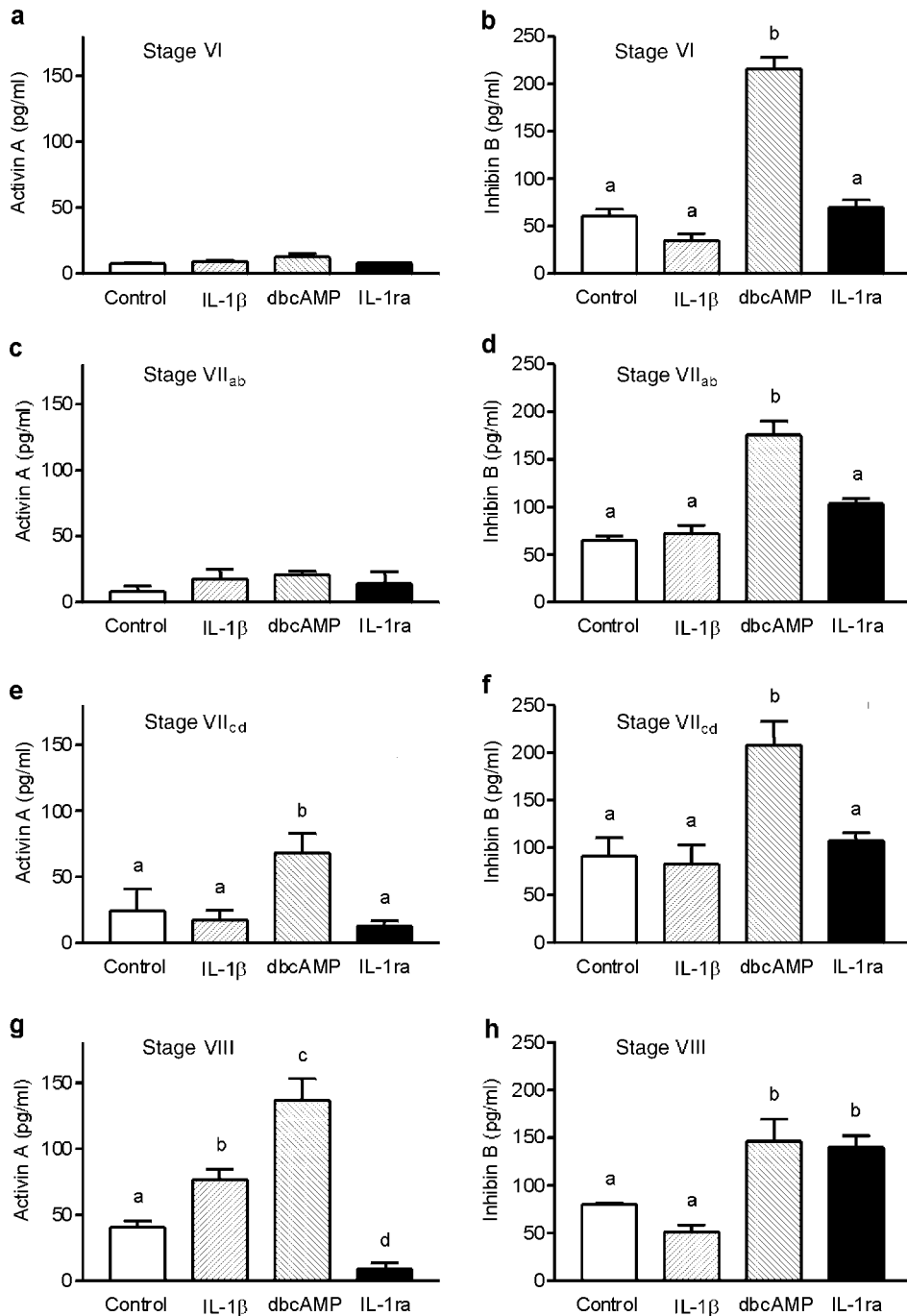
of the cycle during the culture period in long-term (72 h) cultures of seminiferous tubule fragments. Consequently, dissected tubule fragments collected at stage VIII can progress as far as stage XII over 72 h in the culture (Clermont & Harvey 1965, Parvinen *et al.* 1991). This means that the peak of activin A production *in vitro*, which is associated with the stage VIII tubules, overlaps with the peak of  $\beta_A$  mRNA expression at stages IX–XI, previously reported by



**Figure 3** Cyclic secretion of activin A and inhibin B by staged tubules over 72 h in culture, under basal conditions (a and b), in the presence of interleukin-1 $\beta$  (c and d), dibutyryl cAMP (e and f) and IL-1 receptor antagonist (IL-1ra) (g and h). Each panel shows the means  $\pm$  S.E.M. of three separate cultures and each test substance was assessed in a separate culture. All values with same letter superscript are not significantly different ( $P > 0.05$ ). Note that no significant difference in activin A production was detected between the stages in the presence of IL-1ra (g).

Kaipia and colleagues (1992). Thus, peak activin A synthesis and secretion are found in those stages immediately following the release of sperm into the tubule lumen, which occurs during stage VIII and coincides with the phagocytosis of the residual cytoplasm (summarised in Fig. 5). Studies have shown that, while all germ cells induce IL-1 $\alpha$  production by the Sertoli cells *in vitro*, the residual bodies are particularly potent

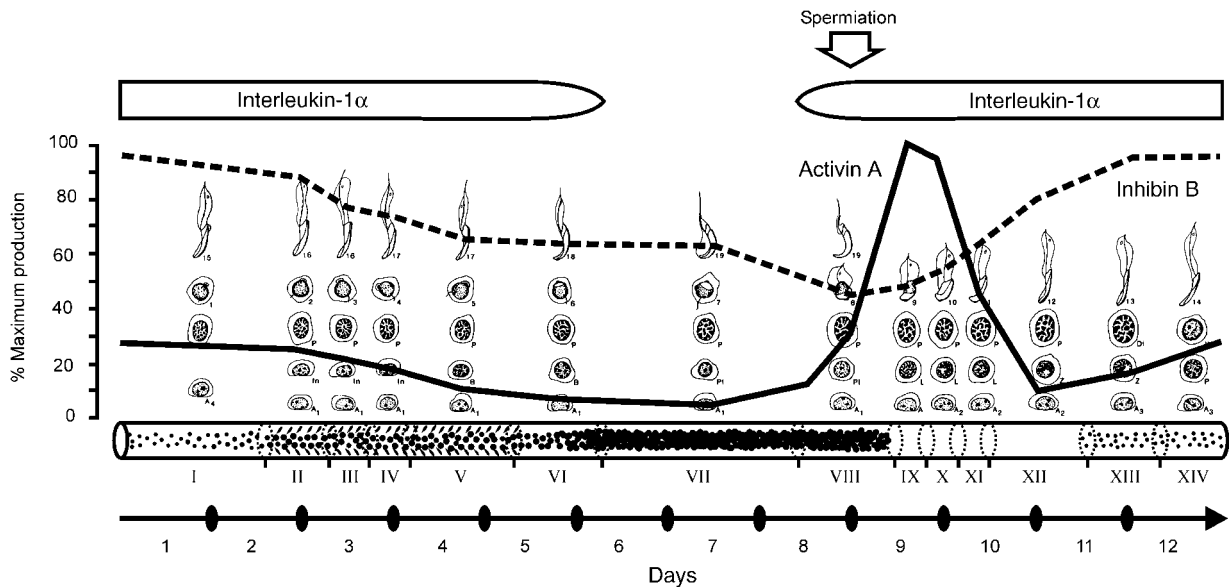
stimuli (Gérard *et al.* 1992, Syed *et al.* 1995, Wang *et al.* 1998). In studies on the cyclic expression of IL-1 $\alpha$ , production is very low at stage VII and returns to maximum during stage VIII (Söder *et al.* 1991, Gérard *et al.* 1992, Syed *et al.* 1995, Wang *et al.* 1998, Jonsson *et al.* 1999). This recovery may be due to the presence of the residual bodies. Peak stimulation of activin A production occurs at this time as well, suggesting



**Figure 4** Stage-specific secretion of activin A and inhibin B by tubules at stage VI (a and b), stage VII<sub>ab</sub> (c and d), stage VII<sub>cd</sub> (e and f) and stage VIII (g and h), under basal conditions and in the presence of interleukin-1β, dibutyrylcAMP and IL-1 receptor antagonist over 72 h in culture. Data shown are means ± s.e.m. of triplicate wells from a single representative experiment. All values with same letter superscript are not significantly different ( $P > 0.05$ ).

that the surge of activin A production might be driven by the endogenous rise in IL-1α. This was confirmed in the present study using IL-1ra to block the secretion *in vitro*. However, since IL-1α continues to be expressed in the seminiferous

epithelium after the peak activin A production has finished, it appears that the Sertoli cell may become less sensitive to stimulation by IL-1α, at least as far as activin A production is concerned, before the end of stage XI.



**Figure 5** Summary diagram showing the predicted pattern of activin A and inhibin B production, derived by extrapolation of the data in Fig. 3, corresponding with the production of IL-1 $\alpha$  and events of the cycle of the seminiferous epithelium. It is important to recognise that the sampling duration of 72 h and resulting overlap of sampling time-points means that the placement of some events, such as the exact timing of the commencement of the activin A surge and its subsequent decline, are approximations.

Overall, the data in the present study suggest that IL-1 $\alpha$  may be driving activin A production by the seminiferous tubules at all stages, where IL-1 $\alpha$  is produced, but the ability of the Sertoli cells to respond to IL-1 $\alpha$  is changing throughout the cycle as well. This may involve a change in IL-1 receptor expression or signalling. Alternatively, since IL-1 stimulates IL-1ra secretion by cultured mouse Sertoli cells (Zeyse *et al.* 2000), regulation may involve the induction of endogenous IL-1ra. Regardless of the mechanisms involved, the trigger for synthesis and release of activin A that occurs during stage VIII is followed very quickly by a downregulation of the response, resulting in a transient surge of the production of activin A around stages IX–XI. This would seem to suggest an important role for activin A at this time in the cycle, in particular. However, activin A may be retained by the Sertoli cells across several stages of the cycle and may be able to diffuse along the tubule as well, so that activin A produced at one stage of the cycle may be able to influence other stages. Support for this concept of ‘action at a distance’ comes from the observation that activin A protein levels in the tubule homogenates and tissue sections across the entire cycle of the seminiferous epithelium did not vary as dramatically as the synthesis data would seem to indicate.

Inhibin B also showed a cyclic pattern of production (Fig. 5). The nadir of inhibin B production corresponded with the commencement of the activin A surge, consistent with an inhibitory effect of IL-1 $\alpha$  on inhibin B production at this time. In contrast, both cytokines displayed a parallel decline from the levels seen at stage XIV throughout stages I–VI. At present, it is unclear what might be driving the changes in the

production of inhibin B and activin A during this period, although interactions between IL-1 $\alpha$ , FSH and possibly even testosterone must be presumed to be involved.

The actual functional role of the activin A surge requires elucidation. There is evidence that IL-1 stimulates both mitosis and meiosis by the spermatogonia (Pöllänen *et al.* 1989, Parvinen *et al.* 1991, Hakovirta *et al.* 1993b) and activin A has been shown to regulate spermatogonial proliferation as well, with both stimulatory and inhibitory effects observed in different culture systems (Mather *et al.* 1990, Hakovirta *et al.* 1993a, Meehan *et al.* 2000). Consequently, it is reasonable to hypothesise that the activin A released in response to upregulation of IL-1 $\alpha$  during stage VIII is involved in modulating the proliferative response of spermatogonia to IL-1 $\alpha$ . Moreover, since activin A blocks both IL-1 and IL-6 responses in other cell systems (Phillips *et al.* 2001), it is possible that activin A modulates the response of spermatogonia to both the cytokines. In turn, activin A is likely to be regulated by its binding protein, follistatin, which is produced by both Sertoli cells and spermatogenic cells within the seminiferous epithelium throughout the cycle, but with peak production at and immediately after the time of the peak production of activin A (Kaipia *et al.* 1992, Meinhardt *et al.* 1998).

In contrast to the principal effects of FSH and cAMP on isolated, immature or adult Sertoli cells in culture (Okuma *et al.* 2005a, 2005b), cAMP was found to stimulate activin A production specifically in cultures of tubule fragments from stages VII and VIII. This observation is consistent with the fact that the  $\beta_A$ -subunit promoter contains both IL-1-signalling pathway and cAMP-responsive elements

(Tanimoto *et al.* 1996, Ardekani *et al.* 1998, Yoshida *et al.* 1998). The data also suggest that the reciprocal relationship between the actions of IL-1 and FSH on Sertoli cell, activin A and inhibin B, secretion is modified by the presence of developing spermatogenic cells, consistent with previous observations (Clifton *et al.* 2002). It should be noted that co-incubation of the stage VIII tubules with IL-1 $\alpha$  caused a reduction in activin A secretion and a corresponding increase in inhibin B, providing further evidence for the reciprocal regulation of two proteins by IL-1 $\alpha$  at this time. Taken together, the data highlight the considerable complexity of the regulatory interactions involved.

The data suggest that the presence of germ cells also affects the Sertoli cell response to IL-1 $\beta$ . In contrast to large upregulation seen in isolated Sertoli cells without significant germ cell contamination (Okuma *et al.* 2005a, 2005b), exogenous IL-1 $\beta$  significantly affected activin A production by stage VIII tubules only, indicating that germ cells may inhibit the response to inflammatory IL-1 in a stage-specific manner. Alternatively, the fact that IL-1 $\beta$  does not appear to have major effects on activin A in tubules with intact spermatogenesis may be due to the production of endogenous IL-1 $\alpha$  by the tubule fragments.

Finally, although not the focus of this study, it was observed that the Leydig cells and interstitial immune cells (macrophages and mast cells) also contain activin  $\beta_A$  immunoreactivity. Whether this is due to the synthesis of activin A by these cell types themselves or uptake of activin A secreted from the Sertoli cells or from the circulation remains to be determined. Previous studies have not reported significant interstitial tissue  $\beta_A$  staining in the adult testis (Roberts *et al.* 1989, Shaha *et al.* 1989, Majdic *et al.* 1997), but it should be noted that both macrophages and mast cells produce activin A in other tissues (Erämaa *et al.* 1992, Cho *et al.* 2003) and may be important sources in the testis as well.

In conclusion, the data clearly show that, in addition to hormonal control via FSH/cAMP, both activin A and inhibin B are under continuous regulation throughout the cycle of the seminiferous epithelium and that endogenous IL-1 $\alpha$  is involved. It should be noted, however, that the cycle of the seminiferous epithelium appears to operate in mice lacking the IL-1 receptor and is hence insensitive to either IL-1 $\alpha$  or IL-1 $\beta$  (Cohen & Pollard 1998). It remains to be discovered whether this means that a link between IL-1 and activin in spermatogenesis is dispensable, or that alternative mechanisms operate in animals that lack the ability to signal through the IL-1 receptor.

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