Pachytene spermatocytes in co-culture inhibit rat Sertoli cell synthesis of inhibin β B-subunit and inhibin B but not the inhibin α -subunit

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Abstract

This study investigates the effects of spermatogenic germ cells on inhibin α -subunit and β B-subunit expression, and inhibin α -subunit and inhibin B production by rat Sertoli cells *in vitro*. Sertoli cells isolated from 19-day-old rats were cultured for 48 h at 32 °C, in the presence or absence of FSH (2.3–2350 mIU/ml), and in the presence of pachytene spermatocytes, round spermatids or cytoplasts of elongated spermatids purified from adult rat testis by elutriation and density gradient separation. Sertoli cell secretion of inhibin α -subunit and inhibin B, as measured by immunoassay, was dose-dependently stimulated by FSH (maximal stimulation 13- and 2-fold, respectively). Round spermatids or cytoplasts co-cultured with Sertoli cells had no effect on basal or FSH-induced secretion of inhibin α -subunit or inhibin B. When Sertoli cells were co-cultured with pachytene spermatocytes, inhibin α -subunit secretion was unaltered, while inhibin B secretion was suppressed in a cell concentration-dependent manner to reach a maximal suppression of 45% compared with Sertoli cells alone ($P < 0.01$). A similar suppression in inhibin B was still observed (64% of Sertoli cells alone)

when the pachytene spermatocytes were separated from Sertoli cells by a 0.45 μ m pore membrane barrier in bicameral chambers. Pachytene spermatocytes also suppressed FSH-induced inhibin B levels in Sertoli cell co-cultures and this suppression was attributed to a decrease in basal inhibin B production rather than a change in FSH responsiveness. Quantitation of Sertoli cell inhibin α - and β B-subunit mRNA by quantitative (real-time) PCR demonstrated that pachytene spermatocytes did not alter Sertoli cell α -subunit mRNA expression, but significantly ($P < 0.01$) suppressed basal and FSH-induced β B-subunit mRNA expression to a similar degree to that seen with inhibin B protein levels. It is concluded that pachytene spermatocytes *in vitro* suppress Sertoli cell inhibin B secretion via factor-mediated suppression of inhibin β B-subunit expression. These findings support the hypothesis that specific germ cell types can influence inhibin B secretion by the testis independent of FSH regulation.

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Introduction

Inhibin is a dimeric glycoprotein, consisting of an α - and a β -subunit, that suppresses the production and/or secretion of follicle-stimulating hormone (FSH) from the pituitary. The three major circulating forms of inhibin are free α -subunit, inhibin A and inhibin B. Inhibin B (consisting of a disulphide linked α - and β B-subunit) is the major circulating form of dimeric inhibin in men, and is the physiologically relevant inhibin in the male (Illingworth *et al.* 1996, Wallace *et al.* 1997). It is well established that the testes are the source of inhibin B, and it is likely that Sertoli cells are the primary site of inhibin B synthesis in the adult (see Burger & Robertson 1997 editorial for review), although Leydig cell

production of inhibin has been demonstrated (Risbridger *et al.* 1989).

Inhibin is known to be regulated by FSH (see Anderson & Sharpe 2000 for review), but there is now increasing evidence that germ cells contribute to the regulation of testicular inhibin production. This latter hypothesis may be of clinical relevance, since numerous studies have indicated a positive correlation between sperm output and inhibin B in men, and thus suggested that inhibin may be a marker for the degree of spermatogenesis and testicular function in infertile men (see Anderson & Sharpe 2000 for review). The demonstration that men with chemotherapy-induced spermatogenic arrest have low levels of inhibin B and elevated levels of circulating FSH and free α -subunit (Wallace *et al.* 1997) supports the

growing body of evidence that α -subunit production is FSH-dependent, but that inhibin B production may also be regulated by gonadotrophin-independent mechanisms. Although it has been hypothesised that the positive relationship between circulating inhibin B and sperm counts may be due to the fact that germ cells themselves may contribute to inhibin production (Andersson *et al.* 1998), it is also likely that this relationship is due to the influence of germ cells on Sertoli cell inhibin secretion.

Various *in vivo* and *in vitro* studies have addressed germ cell regulation of testicular inhibin production in rodents (Pineau *et al.* 1990, Allenby *et al.* 1991, Klaij *et al.* 1994a, Guitton *et al.* 2000) and in primates (Foucault *et al.* 1994, Carreau 1995, Foppiani *et al.* 1999, Anderson & Sharpe 2000); however the regulatory role of each germ cell type remains unclear. Although there is a large amount of conflicting data arising from these studies which have used a variety of experimental models and methods to measure inhibin, the general consensus is that Sertoli cell inhibin production changes in response to the presence or absence of various germ cells. This is illustrated by the observed stage-specific expression of inhibin subunits (Kaipia *et al.* 1992, Klaij *et al.* 1994b), which cannot be accounted for by differential sensitivity to FSH across the various spermatogenic stages (Kaipia *et al.* 1994). The conflict in the literature surrounding the germ cell regulation of inhibin is likely a reflection of the degree of complexity of inhibin regulatory mechanisms in the testis. *In vivo* manipulation of germ cells such as in methoxyacetic acid (MAA) treatment (Allenby *et al.* 1991) and irradiation (Guitton *et al.* 2000) models have suggested that an absence of post-meiotic germ cells in rats is associated with a decline in inhibin, whereas irradiation models in monkeys suggest that a loss of pre-meiotic germ cells causes the initial decline in inhibin B (Foppiani *et al.* 1999). Such models may have direct effects on Sertoli cell function and/or produce changes in multiple germ cell types at any one time, and thus it is difficult to ascribe regulatory effects to a specific germ cell population. *In vitro* studies are more suited to the assessment of the influence of specific germ cell types on Sertoli cell inhibin production – however as yet no *in vitro* studies on germ cell regulation of inhibin B have been performed.

The availability of specific assays to measure inhibin B (Groome *et al.* 1996), together with quantitative PCR methods (Bustin 2000) to measure inhibin subunit mRNA levels, allows an examination of the regulation of Sertoli cell inhibin B production by specific germ cell types. This study aimed to investigate inhibin subunit (α and β B) expression and protein (α -subunit and inhibin B) secretion in immature rat Sertoli cell cultures. The regulation by FSH and specific germ cell types was studied in order to understand the factors that regulate inhibin B in the rat testis.

Materials and Methods

Animals and materials

Sprague Dawley rats were obtained from Monash University Central Animal Services. All animal experiments were approved by the Monash Medical Centre Animal Ethics Committee.

Dulbecco's modified Eagle's medium (DMEM) and McCoy's 5A medium, L-glutamine, sodium carbonate, non-essential amino acids and HEPES buffer were obtained from Trace Biosciences, Sydney, Australia. Penicillin, streptomycin and fungizone were obtained from CSL, Parkville, Australia. Lactic acid and pyruvic acid were obtained from Sigma, St Louis, MO, USA. Follicle-stimulating hormone preparation was Metrodin HP (highly purified urofollitrophin) from Serono, Sydney, Australia. The bicameral chambers used to separate cells during co-culture were Transwell plate inserts, with a pore size of 0.45 μ m (Coster, Corning Coster Corporation, Cambridge, MA, USA).

Sertoli cell isolation

Sertoli cells were isolated from 19-day-old Sprague Dawley rats based on procedures described by Dorrington *et al.* (1975) with modifications (Lampa *et al.* 1999). Using this procedure cell purity was 92%, with peritubular cells the major contaminant (Lampa *et al.* 1999). Viability was >96%, as determined by Trypan Blue exclusion.

Germ cell isolation

Testicular germ cells were isolated from adult rats (>90 days old) as described by Meistrich *et al.* (1981) with modifications (Loveland *et al.* 1993). Briefly, decapsulated testes from 8 rats were subjected to serial collagenase–trypsin digests followed by a germ cell isolation procedure utilising elutriation centrifugation. Round spermatids, pachytene spermatocytes and cytoplasts of elongated spermatids were further purified by centrifugation through a linear Percoll density gradient (Pharmacia, Uppsala, Sweden). Refractive indices for upper and lower limits of the germ cell collection fractions for round spermatids were 1.3379–1.3394; pachytene spermatocytes, 1.3381–1.3400; cytoplasts of elongated spermatids, 1.3377–1.3393. On average, 6 million, 12 million and 16 million pachytene spermatocytes, round spermatids and cytoplasts of elongated spermatids respectively, were obtained per rat. Germ cell viability was >99% (Trypan Blue exclusion) prior to co-culture, and this percentage viability remained unchanged after 48 h of co-culture with Sertoli cells. Germ cells pellets collected following the culture period were fixed in Bouins, embedded in methacrylate, sectioned (10 μ m), stained with periodic acid–Schiff's reagent, and quantitated using stereology (refer to

O'Donnell *et al.* 2001 for details). Briefly, 25–30 counting frames ($793 \mu\text{m}^2$) were superimposed randomly over each section, cell nuclei within these frames were scored according to their morphological characteristics, and purity determined as a percentage of the total number of nuclei scored. The round spermatid fraction, consisting predominantly of early step 1–2 spermatids, was 96% pure, with pachytene (0.4%) and preleptotene (0.4%) spermatocytes as the major contaminants. The pachytene spermatocytes were 89% pure, secondary spermatocytes (3.7%) and early spermatids (2.7%) were the major contaminants. Due to the lack of distinguishing morphological characteristics (no nucleus), the cytoplasts of elongated spermatids preparation was not assessed; however the only contaminant observed was late stage spermatids.

Germ cell–Sertoli cell co-cultures

Sertoli cells were added (2.5 million cells/ml DMEM/well; $1.2 \text{ million cells/cm}^2$) to 24-well culture plates (Costar, Oneonta, NY, USA) pre-coated with Matrigel (1.95 mg/ml in DMEM; Collaborative Research, Waltham, MA, USA), and cultured for 72 h at 37°C in 95% O_2 /5% CO_2 . The cells were then exposed to a hypotonic shock solution (10% DMEM in water) for 90 s to remove residual germ cells (Galdieri *et al.* 1981). Sertoli cells were allowed to recover for 24 h in DMEM supplemented with 0.1% bovine serum albumin (BSA) prior to the addition of treatments/cells in McCoy's 5A/0.1% BSA for a period of 48 h at 32°C . At the end of the treatment/co-culture period, medium containing germ cells was removed, centrifuged and cell pellet and supernatant snap frozen in dry ice prior to analysis. Sertoli cell monolayers were washed once with McCoy's 5A medium and snap-frozen prior to mRNA analysis. For each culture treatment, wells were in triplicate, and each culture/experiment repeated three times.

Immunoassays

α C Immunofluorometric assay (α C IFMA) This two-site assay detects all dimeric and monomeric forms containing the α C region of inhibin α -subunit and has been applied previously to rat Sertoli cell culture medium (Lampa *et al.* 1999). The standard used was a human recombinant 30 kDa inhibin A calibrated in terms of the human inhibin A reference preparation (WHO 91/624). Assay sensitivity was 40 pg/well, with within- and between-assay variations of 14% and 19% respectively.

Inhibin B ELISA Inhibin B levels in culture medium were determined based on the method of Groome *et al.* (1996) using a kit provided by Oxford Bio-Innovations Ltd (Upper Heyford, Oxon, UK). Culture medium sample or standard (100 μl) diluted in culture medium, assay buffer (100 μl), and 6% hydrogen peroxide (20 μl)

were added to the inhibin B microtitre plates. The inhibin B ELISA was then conducted as described in the manufacturer's instructions without SDS or boiling pre-treatments. Parallelism between the Bio-Innovations kit standard (human follicular fluid preparation) and a Sertoli cell culture medium preparation was assessed by comparing the slope values following log–log transformation of the respective dose–response curves. Slope values of 0.81 ± 0.05 and 1.12 ± 0.05 respectively (mean $\pm$ s.d. for 3 assays) were significantly different ($P < 0.001$), indicating non-parallelism. Consequently, a pool of conditioned culture medium from control and FSH-stimulated Sertoli cells was used as a reference standard with a defined arbitrary unitage of 1 unit/ μl of culture medium standard. This non-parallelism was attributed to differences in affinity between human and rat inhibin B preparations under the assay conditions employed. Within- and between-assay variations of 11% and 17% respectively were obtained.

Inhibin α - and β B-subunit mRNA assay

Inhibin subunit mRNA was reverse transcribed and quantitated using the Roche Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany). The standard used in both assays was a cDNA preparation generated from a pool of isolated Sertoli and germ cells and given an arbitrary unitage.

RNA extraction and reverse transcription Frozen Sertoli cells in monolayers were suspended in Dulbecco's phosphate buffered saline (CaCl_2 and MgCl_2 free; Life Technology, Melbourne, Australia) (4°C , 100 μl /well) using a rubber policeman and total RNA was extracted using a commercial kit (High Pure RNA Isolation kit; Roche). Contaminating DNA was removed using a DNA-free kit (Ambion, Austin, TX, USA). Purity was assessed by 260/280 nm ratio, and agarose gel electrophoresis. cDNA was synthesised using AMV reverse transcriptase (7.5 units, Roche), sample RNA (0.3 μg), deoxynucleoside triphosphates (2.5 mM, Roche), [pd(N)6] random hexamers, RNasin (20 units, Promega, Madison, WI, USA). Preparations were incubated at 46°C for 1.5 h, 95°C for 2 min and stored at -20°C .

Inhibin α -subunit mRNA assay Inhibin α -subunit primers were designed from the rat α -subunit sequence described by Esch *et al.* (1987). The sense and antisense primers correspond to base pairs (bp) 223–240 (5'-GTG TGGGCTCCCTGTCGT-3') and 693–712 (5'-ATAGC TCTAGGCCCTGC-3'), with a product size of 490 bp. Each capillary (reaction tube) contained standard/sample cDNA, 4 mM MgCl_2 , 30 pmol sense and antisense primer (Sigma Genosys, Castle Hill, Australia) and Faststart sybr green (Roche). Following initial denaturation at 95°C for 7 min, cDNA was amplified for 36 cycles: denatured at

95 °C for 15 s; annealed at 70 °C for 5 s; extended at 79 °C for 25 s. Sample fluorescence was read at 79 °C. The melting curve (95 °C–50 °C at 0.2 °C/s) gave a single peak at approximately 89 °C, indicating production of a single product, which was later confirmed on an agarose gel. α -Subunit mRNA levels were quantitated by Light Cycler (Roche). All samples were assayed in one run with a within-assay variation of 11%. All samples required dilution to be in the range of the assay. The working range of the assay was from 3 to 100 units/capillary.

Inhibin β B-subunit mRNA assay Inhibin β B-subunit primers were designed from the rat β B-subunit sequence described previously (Drummond *et al.* 2000). Sense primers corresponded to 540–461 bp (5'-TCTTCATCG ACTTTCGGCTCAT-3'), and antisense to 822–843 bp (5'-AGGAGTGTGGCTGCGCCTGAC-3') with a product size of 304 bp. Each capillary contained standard/sample cDNA, 4 mM MgCl₂, 50 pmol sense and antisense primer, and Faststart sybr green. Following initial denaturation at 95 °C for 7 min, cDNA was amplified for 35 cycles: denatured at 95 °C for 15 s; annealed at 55 °C for 5 s; extended at 72 °C for 14 s; read at 85 °C. The melting curve (95 °C–45 °C at 0.2 °C/s) gave a small primer dimer peak at 77.5 °C and a larger product peak at 89 °C. Fluorescent readings were taken at 85 °C to eliminate detection of non-specific primer dimer fluorescence (data not shown). β B-Subunit mRNA levels were quantitated by Light Cycler (Roche). All samples were assayed in one run with a within-assay variation of 15%. All samples required dilution to be in the range of the assay (3 to 100 units/capillary).

Statistical analysis

Differences between treatments within cultures and from multiple cultures were analysed by two-way ANOVA followed by Fisher's Least Significant Difference post-hoc comparison. Data were log transformed prior to statistical analysis. Statistics were performed using GB-STAT v5.30 (Dynamic Systems Inc, Silver Spring, MD, USA).

Results

Inhibin α -subunit and inhibin B produced by Sertoli cell–germ cell co-cultures

The addition of human FSH (2.3–2350 mIU/ml) to immature Sertoli cells in culture caused a dose-dependent increase in inhibin α -subunit (13-fold) and inhibin B (2-fold peaking at 39 mIU/ml) in the culture medium over a 48-h period (Fig. 1).

The effects of germ cells on the basal secretion of α -subunit and inhibin B were investigated by co-culture, either in direct contact with Sertoli cells or on bicameral

Figure 1 Levels of (A) inhibin α -subunit forms and (B) inhibin B in Sertoli cell (SC) culture medium following 48-h incubation with FSH. Data presented are means $\pm$ S.D. ($n=3$ cultures). Different letters denote a significant difference ($P<0.05$) between FSH doses.

chamber inserts placed in medium above the Sertoli cell monolayers. Bicameral chambers had no effect on basal or FSH-stimulated α -subunit or inhibin B production by Sertoli cells alone (data not shown).

When pachytene spermatocytes at various cell concentrations were co-cultured in contact with Sertoli cells there was no effect on α -subunit secretion (Fig. 2A), but there was a cell concentration-dependent suppression of basal inhibin B secretion with a maximal suppression to 45% of control at the highest cell concentration (Fig. 2B). A similar response was seen when the pachytene spermatocytes were co-cultured on bicameral chamber inserts with the Sertoli cells, with a maximum inhibin B suppression to 64% of controls; however this did not achieve statistical significance.

Figure 2 Secretion of (A) inhibin α -subunit forms and (B) inhibin B by Sertoli cells (SC) co-incubated (48 h) with various concentrations of pachytene spermatocytes (PSC). PSC were incubated in direct contact with SC (solid boxes), or were physically separated in bicameral chambers (open boxes). Data presented are means $\pm$ S.D. ($n=3$ cultures). Different letters denote a significant difference ($P<0.05$) with PSC addition. No significant differences between contact and non-contact models were observed.

In the presence of FSH, co-incubation of Sertoli cells with a fixed concentration of pachytene spermatocytes (2 million/well) did not affect inhibin α -subunit secretion (Fig. 3A and C) but significantly suppressed basal and FSH-induced inhibin B secretion (Fig. 3B and D). Inhibin B suppression occurred in both the contact (Fig. 3B) and non-contact models (Fig. 3D); however in the non-contact model, significance was not achieved. A combination of data from eight cultures of pachytene spermatocytes (2 million/well) in bicameral chamber inserts co-cultured with Sertoli cells showed a significant decrease (58% of control) in inhibin B (113 ± 56.1 units/ml vs 65.0 ± 32.3 units/ml, $P=0.0012$).

The co-culture of round spermatids or cytoplasts of elongated spermatids in contact or on inserts with Sertoli cells did not alter basal α -subunit or inhibin B secretion (Fig. 4A,B and Fig. 5A,B respectively). No effect on FSH-induced α -subunit or inhibin B secretion in either the contact or non-contact models (data not shown) was observed with these cells/organelles.

Inhibin α - and β B-subunit mRNA levels in Sertoli cells in co-culture with pachytene spermatocytes

FSH significantly stimulated Sertoli cell α - and β B-subunit mRNA expression by 4.5-fold and 1.3-fold respectively (Fig. 6). Sertoli cell α -subunit mRNA expression was not altered by co-culture with pachytene spermatocytes (2 million/well) on bicameral chamber inserts (Fig. 6A). However, pachytene spermatocytes suppressed basal and FSH-induced inhibin β B-subunit mRNA levels to 52% and 53% respectively of Sertoli cells alone ($P<0.05$, Fig. 6B).

The increase in the levels of α -subunit mRNA following FSH (147 mIU/ml) treatment was fivefold higher than the corresponding increase in the β B-subunit mRNA.

Sertoli cell α -subunit mRNA levels were positively correlated with inhibin α -subunit protein after 48 h in culture ($r=0.86$, $P<0.001$, Fig. 7A) and to a lesser extent with inhibin B protein ($r=0.67$, $P<0.01$, data not shown). Sertoli cell β B-subunit mRNA showed a strong positive correlation ($r=0.83$, $P<0.001$) with inhibin B secreted into the medium (Fig. 7B).

Discussion

This study investigated whether germ cells have a direct effect on rat Sertoli cell inhibin production *in vitro* in order to understand whether germ cells, in addition to FSH, can influence the levels of circulating inhibin B in the male. We show that pachytene spermatocytes in culture suppress Sertoli cell expression of the β B-subunit but not the α -subunit, and consequently decreased both basal and FSH-stimulated production of inhibin B by immature Sertoli cells. Since round spermatids and cytoplasts had no effect on Sertoli cell inhibin (α -subunit or inhibin B) production this effect was deemed specific to pachytene spermatocytes. These studies provide evidence for a factor(s) produced by pachytene spermatocytes that specifically suppresses β B-subunit expression in Sertoli cells.

In a similar study using immature rat Sertoli cells in culture, Pineau *et al.* (1990) investigated the regulation of inhibin by pachytene spermatocytes, round spermatids and cytoplasts of elongated spermatids. In contrast to our findings, Pineau and colleagues found that the co-culture of Sertoli cells with germ cells increased the secretion of inhibin. This increase was attributed to a stimulatory effect

Figure 3 The effect of pachytene spermatocytes (PSC) on FSH-induced inhibin α -subunit forms and inhibin B secretion. Sertoli cells (SC) were co-incubated for 48 h with PSC (2 million/well) in the presence of various concentrations of FSH. PSC were placed in direct contact with the SC or in bicameral chamber inserts to physically separate them from the SC monolayer (non-contact). Inhibin α -subunit (A and C) and inhibin B (B and D) levels in the medium were measured in contact (A and B) and non-contact (C and D) co-cultures. Data presented are means $\pm$ S.D. ($n=3$ cultures). Different letters denote significant difference within each treatment model ($P < 0.05$). * $P < 0.05$ and ** $P < 0.01$ represent significant differences between SC and SC+PSC cultures.

of round spermatids on basal or dibutyryl cyclic AMP-stimulated inhibin secretion, although the data suggested that pachytene spermatocytes also had a weak stimulatory effect (Pineau *et al.* 1990). The conflicting data between our study and those of Pineau and colleagues is not readily explained but probably involves methodological differences related to culture and cell plating conditions, and assay methods used between studies.

The possible physiological role of pachytene spermatocyte-induced down-regulation of inhibin β B-subunit expression and inhibin B protein secretion by immature Sertoli cells may be to facilitate the pubertal rise in FSH. In the male rat, pachytene spermatocytes are the predominant germ cell seen between days 20 and 30 of age (Hilscher & Hilscher 1976, Russell *et al.* 1987, Zhengwei *et al.* 1990) which is the time of the pubertal peak in circulating FSH (Ackland & Schwartz 1991, Sharpe *et al.* 1999, Guitton *et al.* 2000). This pubertal peak in FSH is thought to be important for the survival of developing

germ cells during the first wave of spermatogenesis (Russell *et al.* 1987). Studies by Sharpe *et al.* (1999) showed that in the developing rat, circulating inhibin B levels peaked at day 20 and then declined sharply between days 25 and 40 to reach adult levels. This decline in inhibin B after day 20 was shown to coincide with an increase in plasma FSH, which reached a peak at day 30 (Sharpe *et al.* 1999). It is thus reasonable to speculate that the appearance of pachytene spermatocytes in the pubertal testis signals to developing Sertoli cells to down-regulate inhibin B secretion, which allows FSH levels to rise. Earlier studies, in which immunoneutralisation of circulating α -subunit-containing forms of inhibin in male rats aged between 10 and 24 days caused an elevation of circulating FSH (Rivier *et al.* 1988), support our contention that the pubertal decrease in inhibin B can influence circulating FSH levels. Identification of the factor responsible for the suppression of the β B-subunit will allow the physiological relevance of this matter to be explored. It is

Figure 4 Secretion of (A) inhibin α -subunit forms and (B) inhibin B by Sertoli cells (SC) co-incubated (48 h) with various concentrations of round spermatids (RST). RST were incubated in direct contact with SC (solid boxes), or were physically separated in bicameral chambers (open boxes). Data presented are means $\pm$ S.D. ($n=3$ cultures). No significant differences were observed between treatment groups or models.

proposed in future studies to investigate germ cell regulation of inhibin B production by adult rat Sertoli cells *in vitro* (Lampa *et al.* 1999), to explore whether there are age-dependent differences in inhibin B regulation as suggested previously (Andersson & Skakkebaek 2001).

It is also worth noting that Sertoli cell inhibin production may be regulated by spermatogonia. Studies in irradiated male monkeys showed that inhibin B disappeared from the circulation within 1 week of irradiation (Foppiani *et al.* 1999). Given that irradiation causes a rapid depletion of differentiating spermatogonia, the authors suggested that the loss of these cells might cause a suppression of Sertoli cell inhibin B production (Foppiani *et al.* 1999). This could explain the maintenance of

Figure 5 Secretion of (A) inhibin α -subunit forms and (B) inhibin B by Sertoli cells (SC) co-incubated (48 h) with various concentrations of cytoplasts of elongated spermatids (CES). CES were incubated in direct contact with SC (solid boxes), or were physically separated in bicameral chambers (open boxes). Data presented are means $\pm$ S.D. ($n=3$ cultures). No significant differences were observed between treatment groups or models.

detectable inhibin B in men given a contraceptive (see Anderson & Sharpe 2000 for review) where early forms of spermatogonia are present (Zhengwei *et al.* 1990), whereas irradiation or chemotherapy eradicates spermatogonia and causes inhibin B in circulation to fall to undetectable levels (Wallace *et al.* 1997, Petersen *et al.* 1999). Whether spermatogonia can influence inhibin subunit expression and production by Sertoli cells in our co-culture system warrants further investigation.

Studies in pre-pubertal irradiated rats suggested that the decline in serum inhibin B correlated with the disappearance of spermatogonia and spermatocytes and possibly spermatids from the testis (Guitton *et al.* 2000), suggesting that pre- and post-meiotic germ cells may influence inhibin B levels in the circulation. Therefore,

Figure 6 Effects of pachytene spermatocytes (PSC) on basal and FSH-induced inhibin α - and β B-subunit mRNA expression in Sertoli cell (SC) cultures. PSC (2 million cells/well) were co-incubated with SC for 48 h, physically separated from the SC by bicameral chambers, and co-cultured in the absence/presence of FSH (147 mIU/ml). RNA was extracted from SC monolayers and assayed for inhibin α - and β B-subunits mRNA (A and B respectively). Data presented are means $\pm$ S.D. ($n=3$ cultures). Different letters denote a significant difference ($P<0.05$) between groups.

the demonstration that inhibin is decreased and/or FSH increased when pachytene spermatocytes are depleted from the testis by irradiation (Guitton *et al.* 2000) or MAA treatment of adult rats (Allenby *et al.* 1991) is at odds with our finding of a negative regulation of inhibin B by these cells. However, it is interesting that hypophysectomy is known to increase the testicular expression of the β B-subunit (Feng *et al.* 1989a, Shaha *et al.* 1989) and to cause an arrest of spermatogenesis to the early spermatocyte phase (Russell & Clermont 1977). It is of interest to note that serum inhibin B levels showed little change at a time when round spermatids were heavily depleted in pubertal

Figure 7 Regression analyses comparing (A) inhibin α -subunit levels and inhibin α -subunit mRNA levels and (B) inhibin B and inhibin β B-subunit mRNA levels produced by Sertoli cells in culture.

irradiation models (Guitton *et al.* 2000) which would support our findings of a lack of an effect of round spermatids on inhibin B production by immature Sertoli cells *in vitro*. *In vivo* observations on inhibin B suppression at a time when spermatogonia are depleted following irradiation in the rat (Guitton *et al.* 2000) and monkey (Foppiani *et al.* 1999) suggest that spermatogonia may play a stimulatory role in the process. It is thus not unreasonable to suspect that spermatogonia and spermatocytes may have independent and possibly opposing roles on Sertoli cell

inhibin B synthesis. Thus data from *in vitro* and *in vivo* studies in pubertal and adult models suggest that inhibin B regulation is complex and is likely to involve multiple factors. Our studies indicate that while immature Sertoli cell expression of β B-subunit is decreased by pachytene spermatocytes, α -subunit expression and production is not influenced by meiotic and post-meiotic germ cells. This is in contrast with other reports on *in vitro* co-cultures where germ cells stimulated α -subunit production (Pineau *et al.* 1990) and could again be related to methodological differences. Depletion of pachytene spermatocytes by MAA caused an increase in the circulating levels of immunoreactive α -subunit; however this could be due to a direct effect of MAA (Allenby *et al.* 1991).

Differential regulation of α - and β B-subunit expression in the testis has been noted in numerous studies (Feng *et al.* 1989b, Roberts *et al.* 1989, Shaha *et al.* 1989, Pineau *et al.* 1990, Klaij *et al.* 1994a, Tena-Sempere *et al.* 1999), leading to the speculation that the α -subunit is primarily under the control of FSH whereas β B-subunit expression is less sensitive to FSH and is regulated by other factors, such as germ cells. Analysis of α - and β B-subunit mRNA expression by real time PCR showed that both were significantly increased by FSH; however FSH stimulation caused a more marked increase in α -subunit mRNA compared with β B-subunit mRNA, thereby leading to an excess of α -subunit. The high levels of α -subunit mRNA and protein produced in response to FSH treatment, together with the less pronounced effect of FSH on β B-subunit expression and inhibin B dimer, suggest that expression of the β B-subunit is the primary determinant of immunoreactive inhibin B secreted into the culture medium by immature Sertoli cells. This proposition is supported by the fact that inhibin B protein showed a strong positive correlation with the levels of β B-subunit mRNA, but a weaker correlation with the α -subunit mRNA.

Our studies confirm other studies that have shown that immature Sertoli cell α -subunit mRNA expression and protein production is highly sensitive to FSH stimulation, but that β B-subunit expression and inhibin B formation is less so. Previous studies have shown that α -subunit mRNA was stimulated by FSH-induced cAMP (Toebosch *et al.* 1988, Klaij *et al.* 1990, Pineau *et al.* 1990) presumably via a CRE in the promoter region of this gene (see Mather *et al.* 1990 for review), while other studies have suggested that inhibin B is either not regulated by FSH (Pineau *et al.* 1990) or that FSH causes a doubling of β B-subunit mRNA expression (Najmabadi *et al.* 1993). The β B-subunit promoter does not appear to contain a functional CRE; however FSH effects may be mediated by a putative AP2 site (Najmabadi *et al.* 1993).

In summary, we show that pachytene spermatocytes in culture specifically suppress inhibin β B-subunit expression, leading to a reduction in the secretion of inhibin B by immature Sertoli cells. The fact that pachytene spermatocytes

suppressed Sertoli cell β B-subunit expression and inhibin B production when separated from the Sertoli cell monolayer by a 0.45 μ m barrier suggests that pachytene spermatocytes in culture secrete a factor(s) that suppresses β B-subunit expression. This mechanism may have physiological relevance by promoting a pubertal decrease in inhibin B that is important for the peak in circulating FSH after day 20 in the rat.

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