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Claudin-11 expression and localisation is regulated by androgens in rat Sertoli cells *in vitro*

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Abstract

Claudin-11 and occludin are protein components in tight junctions (TJs) between Sertoli cells which are important for the maintenance of the blood–testis barrier. Barrier formation occurs during puberty, with evidence suggesting hormonal regulation of both claudin-11 and occludin. This study aimed to investigate the regulation of claudin-11 and occludin mRNA expression by testosterone (T) and FSH and their immunolocalisation at rat Sertoli cell TJs *in vitro*, and to correlate any steroid regulation with the functional capacity of TJs. Sertoli cells formed functional TJs within 3 days as assessed by transepithelial electrical resistance (TER). Both T and dihydrotestosterone significantly ($P<0.01$) increased TER twofold and claudin-11 mRNA two- to threefold within 3 days. FSH partially stimulated TER and claudin-11 mRNA, but estradiol had no effect. T also promoted claudin-11 localisation into extensive inter-cellular contacts. In contrast to claudin-11, T and FSH did not change occludin mRNA expression, however, T promoted localisation of occludin at cell contacts in a similar manner to claudin-11. Addition of flutamide to T-stimulated cells caused a twofold decrease in both TER and claudin-11 mRNA expression, and resulted in the loss of both proteins from cell contacts. This effect was reversible following flutamide removal. It is concluded that androgens i) co-regulate claudin-11 mRNA expression and TER, implicating claudin-11 in TJ formation and ii) promote the localisation of claudin-11 and occludin at Sertoli cell contacts. Hence, the ability of androgens to maintain spermatogenesis *in vivo* is partly via their effects on TJ proteins and regulation of the blood–testis barrier.

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Introduction

The inter-Sertoli cell junctional complex forming the blood–testis barrier is located around the basal aspect of the seminiferous epithelium and is comprised of various types of cell junctions, including tight junctions (TJs), adherens junctions, desmosome-like junctions, gap junctions and a testis-specific actin-containing junctional structure known as the ectoplasmic specialisation (Russell & Peterson 1985, Pelletier & Byers 1992, Mulholland *et al.* 2001, Lui *et al.* 2003*b*, 2003*c*, Toyama *et al.* 2003, Mruk & Cheng 2004). TJs maintain a selectively permeable barrier separating the seminiferous epithelium into basal (outside the blood–testis barrier) and adluminal compartments (inside). This barrier prevents the bidirectional passage of molecules (Russell & Peterson 1985) and creates a specialised microenvironment required for germ cell meiosis and maturation in the adluminal compartment that is biochemically and immunologically distinct from the remainder of the testis (Maddocks & Setchell 1990). It is well established that disruption of TJ function leads to germ cell atresia, cessation of spermatogenesis and the potential exposure of sequestered antigens to immunological attack (Russell & Peterson 1985).

Several transmembrane proteins involved in TJs in various epithelia have been described, including claudins, occludin and junction adhesion molecules (JAMs; for reviews, see Gonzalez-Mariscal *et al.* 2003, Ebnet *et al.* 2004, Feldman *et al.* 2005, Koval 2006). Testicular TJs in mice and rats contain occludin (Saitou *et al.* 1997, Moroi *et al.* 1998, Cyr *et al.* 1999), some members of the claudin family (Morita *et al.* 1999*a*) including claudin-11 (Morita *et al.* 1999*b*, Hellani *et al.* 2000) and claudin-3 (Meng *et al.* 2005), and JAM-A and JAM-C (Aurrand-Lions *et al.* 2001, Gliki *et al.* 2004). The claudin-11 knockout male mouse is infertile and lacks functional Sertoli cell TJs and mature spermatozoa (Gow *et al.* 1999, Mitic *et al.* 2000), whilst the occludin knockout mouse testis contains a normal germ cell complement in animals of reproductive age (Saitou *et al.* 2000). However, the suppression of rat testicular

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occludin function caused the loss of germ cells *in vivo* and partially disrupted formation of Sertoli cell TJs *in vitro* (Chung *et al.* 2001), suggesting that occludin may contribute to TJ function. The JAM-A knockout is fertile (Cera *et al.* 2004), and the blood–testis barrier remains functional in JAM-C knockout mice (Gliki *et al.* 2004). Thus, evidence from these models suggests that claudin-11 is a major component involved in both the formation and function of Sertoli cell TJs, with additional input from occludin.

Although not well understood, an association exists between blood–testis barrier function and endocrine status. For example, Sertoli cell TJs and the resultant impermeable barrier first appear in the pubertal (15–20 days old) rat testis as follicle stimulating hormone (FSH) and luteinizing hormone increase (Russell & Peterson 1985, Russell *et al.* 1989), and TJ formation can be delayed (Vitale *et al.* 1973) or prevented in the absence of these hormones (Bressler 1976). Similarly, treatment of hypogonadotrophic hypogonadal men with gonadotrophins converts Sertoli cell TJs from a pre-pubertal phenotype to the mature junctional structure (de Kretser & Burger 1972).

Direct links between Sertoli cell TJ proteins and androgens have recently been established. Androgens upregulate claudin-11 mRNA expression in primary cultured mouse and rat Sertoli cells *in vitro* (Gye 2003, Florin *et al.* 2005), and also upregulate claudin-3 mRNA and protein in the Sertoli-like TM4 cell line (Meng *et al.* 2005). The regulation of occludin in these models is unclear, although *in vivo* treatment of adult rats with the androgen antagonist, flutamide, caused an ~40% decrease in testicular occludin mRNA expression (Gye & Ohsako 2003). Despite these studies, the mechanism(s) by which hormones (androgens, FSH) regulate both Sertoli cell TJ proteins and function remains to be clarified. Recently, we demonstrated that FSH is pivotal for the formation of two of the other junctional types in the inter-Sertoli cell junctional complex (adherens junctions and ectoplasmic specialisations), and that androgens were without apparent effect (Sluka *et al.* 2006). In the current study, we have used a well-established *in vitro* model of Sertoli cells in bicameral culture (Hadley *et al.* 1987, Handelsman *et al.* 1989, Onoda *et al.* 1990, Janecki *et al.* 1991*a*, Djakiew & Onoda 1993) to study the effects of androgens and FSH on TJs. TJ function in this culture system is readily monitored by the determination of transepithelial electrical resistance (TER; Janecki *et al.* 1991*a*, 1991*b*, 1992, Chung *et al.* 1999, Fanning *et al.* 1999, Chung & Cheng 2001, Li *et al.* 2001, Lui *et al.* 2003*a*, Siu *et al.* 2003). Testosterone and FSH, either alone or in combination, are known to increase rat Sertoli cell TER two to threefold in this model (Janecki *et al.* 1991*a*, 1991*b*, Steinberger & Klinefelter 1993) indicating that Sertoli cell TJ function can be regulated by gonadotrophins. Hence, the aim of this study was to further elucidate the hormonal regulation of key TJ components (claudin-11, claudin-3, occludin) by correlating the effects of steroid (T, DHT and estradiol (E2)) treatment and FSH with both the functional capacity of TJs (by monitoring TER), and with TJ protein mRNA expression and immunolocalisation.

Materials and Methods

Animals

Male outbred Sprague–Dawley rats at 19–21 days of age were obtained from Monash University Animal Services, Monash University (Melbourne, Australia). Rats were killed by CO_2 asphyxiation and testes were removed immediately for the isolation of Sertoli cells. The use of animals for this study was approved by the Monash Medical Centre Animal Ethics Committee.

Preparation of Sertoli cells for culture experiments

Primary Sertoli cells were isolated from 19–21-day-old Sprague–Dawley rats as previously described (Perryman *et al.* 1996, Sluka *et al.* 2006). Freshly isolated Sertoli cells were suspended in serum free Dulbecco's Modified Eagle's Medium (DMEM)/Hams F12 medium (1:1) supplemented with L-glutamine (1 mM, Trace Scientific, Melbourne, Australia), non-essential amino acids (1:100 dilution of 100× stock, Trace), $NaHCO_3$ (1.4 mM, Trace), BSA (1% (w/v), Sigma Chemical Co.), HEPES (10 mM, Trace), insulin (5 μg/ml Novo-Nordisk, Sydney, NSW, Australia), transferrin (5 μg/ml, Sigma), sodium selenite (50 ng/ml, Sigma) and penicillin (200 U/ml)-streptomycin (200 μg/ml)-fungizone (0.5 μg/ml; CSL, Melbourne, Australia). Cells were plated at a density of 1.25×10^6 cells/cm^2 into either 24-well culture plates (Nunc, Nalge Nunc International, Denmark) for total RNA isolation, or into Millicell PCF bicameral chambers (12 mm diameter, 0.4 μm pore size, 0.6 cm^2 surface area; Millipore, Bedford, MA, USA) for measurement of TER and immunocytochemistry. All cell culture surfaces (24-well plates, bicameral chambers) were pre-coated 4 h before use with Matrigel (BD BioSciences, Bedford, MA, USA) at a 1:8 dilution in DMEM/F12 medium. Cells were then incubated at 37 °C in a humidified atmosphere of 95% air–5% CO_2 (v/v) for periods of 5–13 days, with the day of isolation designated as day 0. Media were routinely replaced every 2 days. On day 3, cells were hypotonically shocked with 10% culture medium in water for 45 s to lyse the contaminating germ cells (Galdieri *et al.* 1983), after which the cells were washed once with DMEM/F12 and returned to the incubator for the remaining culture period. Sertoli cell cultures prepared by this method were typically of 92% purity, with the remaining cells being peritubular cells and residual germ cells (Perryman *et al.* 1996, Lampa *et al.* 1999).

Hormonal treatments were added at the following concentrations on day 0 unless otherwise stated: testosterone (T), 28 μg/ml (Sigma); human recombinant FSH, 2.35 IU/ml (Puregon, Organon, Oss, The Netherlands); DHT, 28 ng/ml (Sigma); E2, 28 ng/ml (Sigma); flutamide, 27.6 μg/ml (Sigma). Steroids were prepared from stock solutions in ethanol, and diluted in DMEM/F12 immediately prior to use, while an equivalent volume of ethanol (0.26% v/v final) was added to all other culture wells not receiving steroid treatments.

Measurement of TER

To assess the assembly of inter-Sertoli cell TJs, TER across the Sertoli cell epithelia was quantified using a Millipore Millicell-electrical resistance system (Millipore), with measurements taken daily commencing from the day of plating (day 0). To enhance the reproducibility of measurements, cultured cells were allowed to equilibrate at room temperature for 30 min, prior to the measurement of TER. The final resistance readings were calculated by subtracting the mean TER of bicameral chambers coated with Matrigel but containing no cells, and correcting for the surface area of the PCF bicameral chamber (0.6 cm^2) to produce a value expressed as Ω/cm^2. All TER values were calculated from triplicate culture wells.

Isolation of total RNA and RT

Cells were removed at specified time points for extraction of total RNA using a total RNA extraction kit (Qiagen) according to the manufacturer's instructions. Any contaminating DNA was removed using the DNAse-free kit (Ambion, Austin, TX, USA), and samples were stored at −80 °C. Total RNA concentrations were determined using the Ribogreen fluorescence RNA assay (Molecular Probes, Eugene, OR, USA) as described elsewhere (Sluka *et al.* 2002).

RT was performed on 500 ng total RNA/sample using AMV- reverse transcriptase (8 U; Roche), random hexamer primers (200 ng; Amersham Biosciences), dNTPs (20 nmol each; Roche), RNasin (20 U; Promega) and 5× reaction buffer (Roche) in a final volume of 20 μl for 90 min at 46 °C, after which samples were heated for 2 min at 95 °C before storage at −20 °C.

Real-time PCR

Quantitative real-time PCR analysis was performed using the Roche Lightcycler (Roche) and the FastStart DNA Master SYBR-Green 1 system (Roche). Oligonucleotide primers for claudin-11 (forward 5′-TTAGACATGGG-CACTCTTGG-3′, reverse 5′-ATGGTAGCCACTTGCCTTC-3′), occludin (forward: 5′-CTGTCTATGCTCGTCATCG-3′, reverse 5′-CATTCCCGATCTAATGACGC-3′) and claudin-3 (forward 5′-CGTTAGCGTGCTCCGTCCAT-3′, reverse 5′-CCCGAAGGCTGCCAGTAGGATA-3′) were obtained from published sources (Chung *et al.* 2001, Lui *et al.* 2001) or were designed using the Oligo program (version 6; Molecular Biology Insights, Cascade, CO, USA). For PCR analysis, sample cDNA was diluted 1:20 to 1:150 fold and PCR conditions including Mg^{2+} and primer concentrations, anneal time and extension time are summarised in Table 1. Standard curves for PCR analyses were generated using dilutions of an adult rat testicular cDNA preparation of arbitrary unitage. Unless otherwise noted, PCR of all samples was performed using triplicate reactions for 38 cycles, after which a melting curve analysis was performed to monitor PCR product purity (see Table 1). In initial experiments, amplification of a single PCR product was confirmed by agarose gel electrophoresis and DNA sequencing.

Immunocytochemistry

For immunocytochemical analyses, Sertoli cell monolayers on PCF filters were pre-extracted on ice with ice-cold PBS (PBS; 10 m 0.01 M PBS with 154 mM NaCl) containing 0.2% (v/v) Triton X-100 for 2 min and then fixed in 3% paraformaldehyde in PBS for 30 min at room temperature. Cells were then permeabilised with 0.05% (v/v) Triton X-100 in PBS for 5 min on ice and washed in PBS. Non-specific binding sites were blocked with CAS block (Zymed, San Francisco, CA, USA) containing 10% normal serum (sheep or rabbit) for 20 min. Primary antisera used were affinity-purified rabbit anti-occludin (2.5 μg/ml, Zymed) or rabbit anti-claudin-11 (1:400, CovalAb) diluted in PBS/bSA and were incubated with cells overnight at room temperature. Cell monolayers were then washed and secondary antisera (goat anti-rabbit Alexafluor-488 1:100 dilution; Molecular Probes or biotinylated sheep anti-rabbit

Table 1 Primer-specific LightCycler conditions used for PCR amplification of genes examined.

Name	Product size (bp)	Primer concentration (pmol)	Mg^{2+} Concentration (mM)	Anneal temperature (°C)	Extension time (s)	Data acquisition temperature (°C)[a]	PCR product melting temperature (°C)[b]
Claudin-11	624	40	3.0	68	25	85	91.7
Occludin	294	40	4.0	64	20	85	89.2
Claudin-3	436	40	2.5	64	23	72	93.0

[a]Refers to the temperature at which the fluorescence of the PCR product was quantified during LightCycler analysis.
[b]As determined by melting temperature analysis on the LightCycler.

immunoglobulin G (IgG) 1:100 dilution) were applied for a period of 1 h at room temperature. Claudin-11 immunostaining was then visualised with streptavidin – Alexafluor 488 (1:100, 30 min, Molecular Probes), and monolayers were counterstained with the fluorescent stains DAPI (90 μM) and TO-PRO-3 iodide (10 μm in PBS/bSA; Molecular Probes) for 5 min to allow nuclear visualisation. Monolayers were then rinsed thrice with PBS/bSA and mounted on glass slides with FluorSave reagent (Calbiochem, San Diego, CA, USA).

Confocal analysis was performed using an Olympus Fluoview FV300 confocal system equipped with Fluoview version 4.2 software (Olympus) and attached to an Olympus IX 81 inverted microscope. A 60× water immersion lens was used, with confocal aperture set to 2, laser intensities set to 5%, software zoom set to ×2, and software filter mode set to Kalman with 4 scans. Excitation of Alexa Fluor 488 (green) was performed using the 488 nm line of the Argon laser, while TO-PRO-3 iodide (red) was excited using the 633 nm line of the red HeNe laser. Sertoli cell monolayers were initially visualised by epifluorescence microscopy, and an optic plane that contained inter-Sertoli cell junctions was selected for image capture. Confocal scanning of double label experiments was performed sequentially to prevent bleed-through between red and green output channels.

Immunoblot analysis for occludin and claudin-11 used the same antibodies as for immunocytochemistry, with cell extracts prepared in hot non-reducing sample buffer (125 mM Tris–HCl, 2.5% (w/v) SDS, 10% (w/v) glycerol, pH 6.8), electrophoresed on 10% polyacrylamide gels (Laemmli 1970) and blotted onto a PVDF membrane (Immobilon-P, Millipore). Detection was with a horseradish peroxidise conjugate in conjunction with an ECL western blot detection system (Amersham Biosciences).

Statistical analysis

TER values ($n=3$ wells/treatment) and PCR data ($n=3$ PCR estimations from total RNA pooled from two wells) were calculated as mean ± S.D. from a single culture. Each study was repeated 2–3 times with representative experiments presented in the Results section. Statistical analyses were performed using GB Stat (Dynamic Systems Inc., Silver Spring, MD, USA) with an initial assessment of homogeneity of variance for all groups. Homogeneous groups were assessed using one-way ANOVA, followed by the Newman–Keuls *post hoc* multiple comparisons test. When groups were non-parametric, data were $\log_{10}$ treated, and the above tests repeated. Remaining non-parametric data were analysed using the Kruskal–Wallis test followed by the Newman–Keuls analogue test (equal ns).

Results

Androgens stimulate Sertoli cell TER

When Sertoli cells were cultured on Matrigel-coated bicameral chambers in serum-free DMEM/F12, TJs typically formed within the first 2–3 days post-plating as assessed by the measurement of TER. Under control conditions, TER reached a plateau of 60–80 Ω/cm^2 shortly after junction formation (days 2–3, Fig. 1) and increased slightly thereafter (up to day 7; Fig. 1), (or up to day 13 (see Fig. 4A)) reaching a maximum of ∼130 Ω/cm^2. A significant ($P<0.01$) increase in TER was observed for Sertoli cells cultured with added T (28 ng/ml) compared with control wells, when a 1.5- to 2-fold stimulation of TER was observed after day 3 (Fig. 1, see also Fig. 4A and B). Similar findings were observed for Sertoli cells cultured with both T (28 ng/ml) + FSH (2.35 IU/ml), with twofold differences to control which is evident after day 5 (Fig. 1). FSH alone produced a minor but significant ($P<0.05$) increase in TER, but this was not observed until days 5 and 7 of culture (Fig. 1). E2 (28 ng/ml) alone had no effect on TER (data not shown). Cells treated with DHT (28 ng/ml) responded in a similar manner to T-treated cultures (data not shown). These results show that T, and to a lesser extent FSH, stimulates Sertoli cell TER.

Testosterone stimulates claudin-11 mRNA but not occludin or claudin-3 mRNA expression

The expression of three TJ transmembrane proteins, claudin-11, occludin and claudin-3 were examined using real-time RT-PCR. Under control conditions, claudin-11 mRNA expression showed a slight but significant increase ($P=0.015$) over the 7-day culture period (Fig. 1B), whereas T stimulated claudin-11 mRNA expression significantly ($P<0.01$) within 3 days, and was two- to threefold higher than control at days 5–7. Cells treated with T plus FSH responded in a similar manner to T alone (Fig. 1B), while FSH alone elevated claudin-11 mRNA expression approximately twofold greater than control wells between days 3–7 (Fig. 2B). E2 had a marginal effect on claudin-11 expression with a significant increase only evident after 7 days of treatment (Fig. 1B), whereas DHT increased claudin-11 mRNA expression in a similar manner to T (data not shown).

In contrast to claudin-11, occludin mRNA expression levels remained relatively stable throughout the culture in all treatment groups (Fig. 1C), with no evidence for hormonal stimulation of mRNA expression. Although recent data suggest the presence of claudin-3 mRNA expression in newly forming Sertoli cell TJs in the mouse (Meng *et al*. 2005), we were unable to detect this protein by PCR at any time in this rat Sertoli cell culture system (data not shown). Subsequent studies showed positive claudin-3 immunostaining in the interstitium of the adult

rat testis, but not in the seminiferous epithelium (Stanton unpublished observations).

These results show that T and FSH stimulate claudin-11 mRNA but not occludin mRNA expression in rat Sertoli cells whilst claudin-3 is not expressed in rat Sertoli cells.

Claudin-11 mRNA expression correlates with TER

Correlations were performed between occludin and claudin-11 mRNA expressions and inter-Sertoli cell TJ function as measured by TER to assess whether these variables were related. No significant relationship ($r=0.212$, $n=32$, ns) was observed between occludin mRNA expression and TER (data not shown). In contrast, a significant correlation ($r=0.534$, $n=32$, $P<0.01$) was observed between claudin-11 mRNA expression and TER for pooled data from all treatment groups (Fig. 2A), which was maintained within the T-treated group ($r=0.790$, $n=8$, $P<0.02$; Fig. 2B).

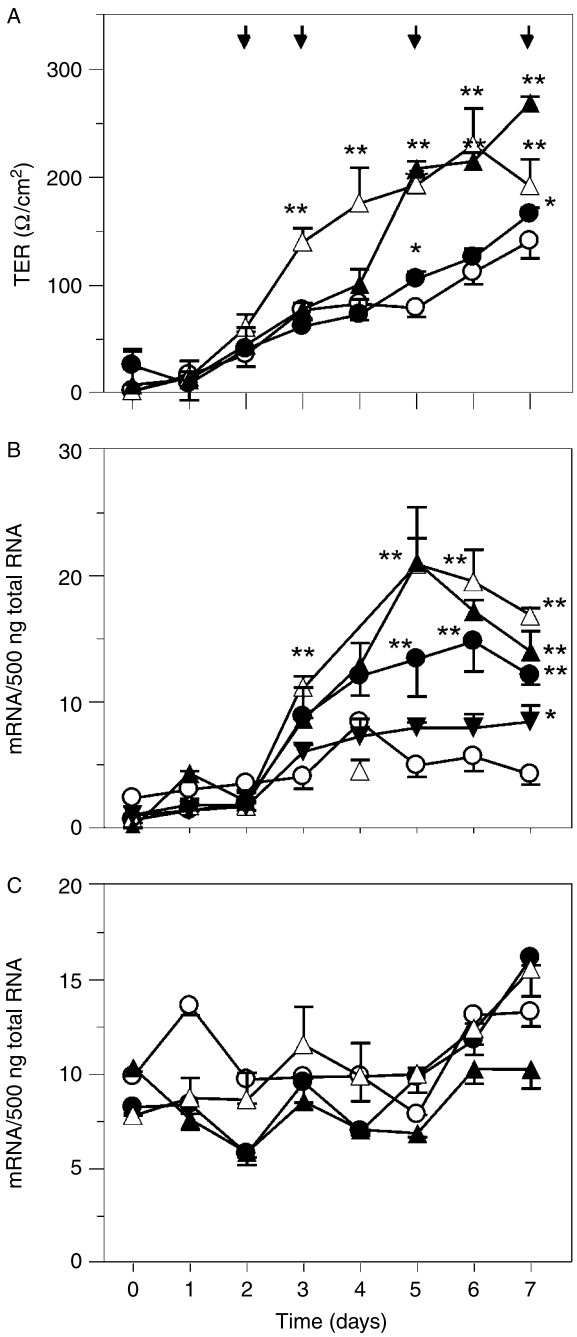

Figure 1 Effect of hormonal treatments on inter-Sertoli tight junction permeability and tight junction protein mRNA expression *in vitro*. (A) Transepithelial electrical resistance (TER) across the Sertoli cell epithelia was measured at daily intervals, beginning at day 0. Sertoli cells (1.25×10^6 cells/cm^2) were cultured in DMEM/F12/1% BSA on Matrigel-coated bicameral chambers for a period of 7 days under basal conditions (medium alone, ○) or in the continuous presence of FSH (2.35 IU/ml; ●), testosterone (T; 28 ng/ml; △) or T (28 ng/ml) + FSH (2.35 IU/ml; ▲), with medium and treatments replaced at 2-day intervals as indicated by the arrows (↓). On day 3, all cells were treated with a hypotonic solution to remove contaminating germ cells. Results are expressed as mean ± S.D. of triplicate wells from a single culture, and a representative from three cultures is shown. Asterisks indicate significant differences from basal cultures for each particular day by one-way ANOVA and Newman-Keuls *post hoc* analysis test (*$P<0.05$, **$P<0.01$). (B) Claudin-11 mRNA expression, and (C) occludin mRNA expression as measured by real-time PCR in Sertoli cells cultured (1.25×10^6 cells/cm^2) in Matrigel-coated plastic 24-well culture plates with treatments as described in (A). Data are also included for the addition of estradiol (E2, 28 ng/ml, ▼) in (B). Results are expressed as mean ± S.D. of triplicate wells from a single culture, and a representative culture is shown.

Testosterone stimulates localisation of claudin-11 and occludin at inter-Sertoli cell contacts in vitro

Claudin-11 protein was detected as a major band of 27 kDa in rat Sertoli cells (Fig. 3H) as reported elsewhere (Hellani *et al.* 2000, Florin *et al.* 2005). Under control conditions, punctate immunostaining of claudin-11 at Sertoli cell contacts and in Sertoli cell cytoplasm was apparent at day 1 (data not shown) and day 5 (Fig. 3A), while more extensive contacts containing claudin-11 were apparent in extended cultures at days 9 and 13 (Fig. 3B and C). In contrast, claudin-11 immunostaining was markedly upregulated in cells treated with T for 5 days (Fig. 3E), and was present in both the cytoplasm of the Sertoli cells and in extensive contacts between Sertoli cells. This pattern of immunostaining was preserved in cells continuously treated with T for 9 and 13 days (Fig. 3F and G).

The occludin protein was observed as a major band of immunoreactivity at ~57 kDa in rat Sertoli cells (Fig. 3L) consistent with published data (Furuse *et al.* 1993, Sakakibara *et al.* 1997), although other minor bands (87, 42 and 36 kDa, Fig. 3L) were also observed. Under control conditions, punctate occludin immunostaining was observed at Sertoli cell contacts at both day 1 (data not shown) and day 7 (Fig. 3I). In contrast, Sertoli cells treated with T for 7 days exhibited a marked increase in occludin

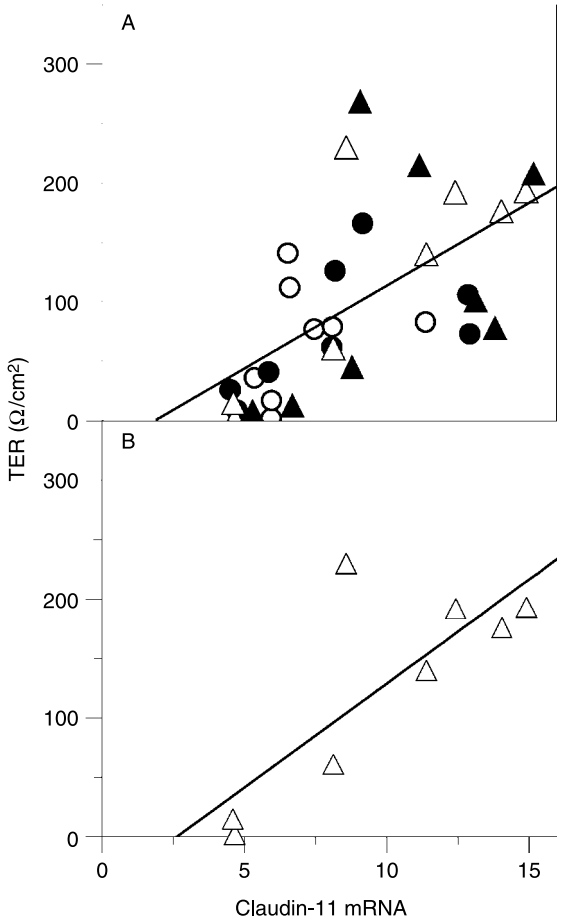

Figure 2 Relationship between TER and claudin-11 mRNA expression. Correlations between (A): TER established by Sertoli cells and mRNA expression data for all treatment groups (medium alone (○), FSH (●),T (△),FSH +T (▲))and (B): T alone (△) for days 0–7 were constructed. A significant correlation was observed for the entire data set (A) (r=0.534, P<0.01, n=32), and also for the T-treated group (B) (r=0.790, P<0.02, n=8).

localisation at inter-Sertoli cell contacts (Fig. 3J) compared with control cultures. Limited non-specific staining was observed for claudin-11 within the cell nuclei (Fig. 1D), however, non-specific staining was not observed for occludin (Fig. 1K) when the primary antibodies were substitituted for a non-specific rabbit IgG.

Flutamide antagonises testosterone-stimulated TER and claudin-11 mRNA expression in Sertoli cells

Sertoli cells were allowed to form TJs for 5 days in medium alone, after which the androgen receptor antagonist, flutamide, was added for 4 days. Under control conditions (Fig. 4A), a twofold decrease in TER was observed following the addition of flutamide by day 9 of culture compared with cells maintained in medium alone. Similarly, a significant (P<0.01) greater than threefold decrease in TER to the same level as non-stimulated cells was observed when flutamide was added to Sertoli cells pre-stimulated with T for 5 days (Fig. 4B). This effect was reversible, as replacement of flutamide on day 9 of culture with either medium alone (Fig. 4A), or with T (Fig. 4B) restored TER to pre-treatment levels within 2 days. The TER of Sertoli cells constantly treated with T for 13 days continued to increase to ∼350 Ω/cm^2 and did not appear to reach a maximum, although this increase may have been biphasic with a plateau around days 3–5 (Fig. 4B).

Claudin-11 mRNA expression was also measured in cells treated with the same protocol as described above for TER (Fig. 4C). In accord with earlier experiments (see Fig. 1), claudin-11 mRNA expression in Sertoli cell monolayers continuously stimulated with T was significantly (P<0.05) elevated compared with its respective control (medium alone – open circles) at days 5, 9 and 13. When T was removed at day 5 and replaced with flutamide (closed squares), a significant (P<0.01)> threefold decrease in claudin-11 mRNA expression was observed at day 9, with claudin-11 mRNA expression not different from medium-alone cells similarly treated with antagonist (closed circles). Additionally, no difference in claudin-11 mRNA expression was detected between the medium-alone and medium-alone plus antagonist treated group between days 5 and 9. Following removal of flutamide from the cells at day 9 and replacement with either medium alone or testosterone, no significant changes in claudin-11 mRNA expression were observed at day 13.

In conjunction with the decreases in claudin-11 mRNA, flutamide treatment of T-stimulated cells also caused a marked loss of claudin-11 immunostaining at inter-Sertoli cell contacts at day 9 of culture, but some cytoplasmic staining remained apparent (compare Figs 3F with 4D). A similar action of flutamide on claudin-11 immunostaining in control cultures was also observed (data not shown). This effect appeared reversible, as replacement of the flutamide with T for a further 4 days (to day 13) caused the reappearance of claudin-11 immunostaining at inter-Sertoli cell contacts (Fig. 4E). A similar regulation of occludin immunostaining at cell contacts was also observed (data not shown).

Discussion

This study has established that testosterone and DHT significantly increased claudin-11 mRNA expression two- to threefold during TJ formation by rat Sertoli cells *in vitro*. This increase was androgen dependent as it was not reproduced by E2, but was inhibited by the non-steroidal androgen-receptor antagonist, flutamide. Androgens, and to a lesser extent FSH, also increased the 'tightness' of Sertoli cell TJs as quantitated by TER from a basal value of ∼80 to ≥150 Ω/cm^2. A significant correlation between TER and claudin-11 mRNA expression suggests that claudin-11 plays a major role in Sertoli cell TJ formation. Androgens also increased

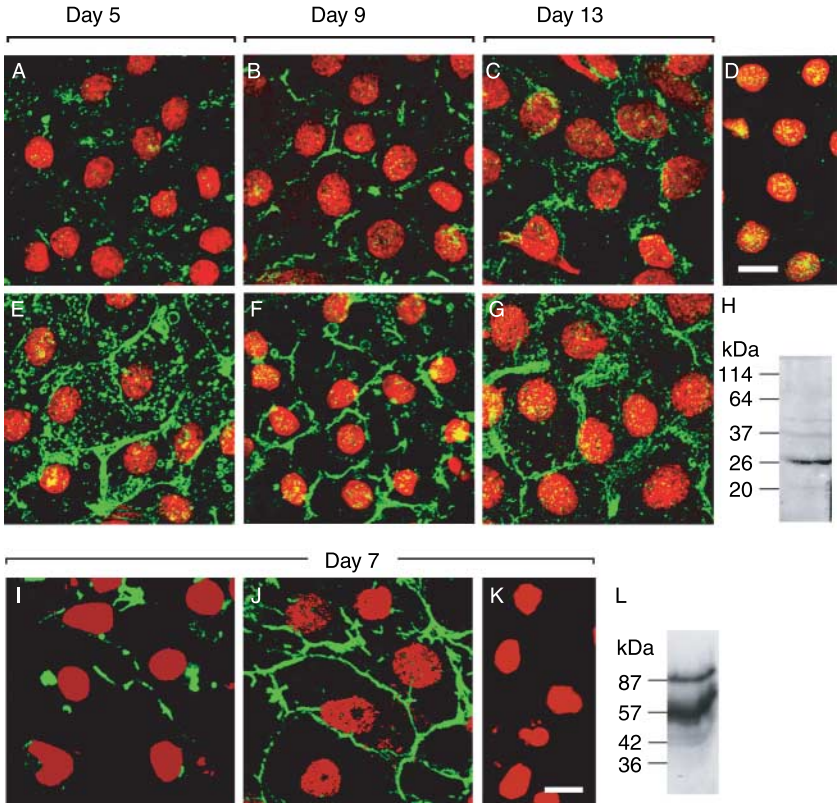

Figure 3 Effect of androgen treatment on the localisation of claudin-11 and occludin at inter-Sertoli cell contacts *in vitro*. Sertoli cells were cultured on Matrigel-coated bicameral chambers under either basal conditions (medium alone), (panels A–C and I) or with added testosterone (28 ng/ml, Panels E–G and J) for time periods (days) as shown. Confocal immunofluoresence staining was then carried out for claudin-11 (panels A–G) or occludin (Panels I–J; green staining) with the nuclear counterstain TO-PRO-3 iodide (red-orange). Non-specific binding (panel D for claudin-11, and K for occludin) was assessed by substituting an equivalent concentration of non-specific rabbit IgG for each primary antibody. Bar=10 μm. Western blots for claudin-11 (panel H) or occludin (panel L) were probed with the same primary antibodies used for immunohistochemistry with extracts from cells cultured in the presence of testosterone for 5–7 days.

both the cytoplasmic staining of claudin-11 in Sertoli cells and its localisation to extensive inter-Sertoli cell contacts *in vitro.* In contrast, Sertoli cell occludin mRNA expression was not regulated by hormones (T, E2, FSH), although T did promote localisation of occludin to inter-Sertoli cell contacts in a similar manner to claudin-11. Hence, these studies show that androgens promote the synthesis and/or localisation of two key TJ proteins to regions of inter-Sertoli cell contact *in vitro.*

Our study demonstrates that rat Sertoli cell TJ function and TJ proteins (claudin-11 and occludin) are regulated in a biphasic manner *in vitro,* with a basal component and a second androgen-regulated component. In medium alone, TJs formed with an average TER of ~80 Ω/cm^2, during which time both claudin-11- and occludin- mRNA expression were also evident, with punctate claudin-11 and occludin protein localisation at inter-Sertoli cell contacts. Hence, we propose that this level of expression and localisation comprises a basal component of Sertoli cell TJ function. Androgens (T or DHT) significantly increased both TER and claudin-11 mRNA two- to threefold compared with controls, and increased both cytoplasmic claudin-11 staining and incorporation of both proteins into extensive intercellular contacts. We therefore propose this to be an androgen-regulated component of TJ function. Addition of the androgen receptor antagonist flutamide to T-stimulated cells inhibited this component of claudin-11 mRNA expression, with a marked decrease in cell surface localisation of claudin-11 and occludin, and a concomitant decrease in TER to basal levels. This effect was partially reversible, as replacement of the antagonist with T restored both TER and junctional immunostaining of both proteins; however, no significant increase in claudin-11 mRNA expression was observed. While this result could indicate an adverse or toxic effect of flutamide in this study, we note that the antagonist concentration used (~100 μM) was mid-range compared with other *in vitro* rat Sertoli cell studies which have employed concentrations of 0.5 μM (Swift & Dias 1988), 1 μM (Lyng *et al.* 2000) or 3 mM (Gorczynska & Handelsman 1995).

Data from mice with the conditional knockout of the Sertoli cell androgen receptor (SCARKO) confirm our *in vitro* findings, by showing a significant 40% reduction in claudin-11 mRNA expression in both adult (Tan *et al.* 2005) and pubertal (Wang *et al.* 2006) mice. Other *in vitro* (Gye 2003, Florin *et al.* 2005) studies have also demonstrated that testosterone upregulates claudin-11 mRNA expression in Sertoli cells, although our study is the first to show a direct link between claudin-11 mRNA and TER, thereby suggesting that claudin-11 is a major contributor to Sertoli cell TJ function (Koval 2006). An additional androgen-regulated claudin, claudin-3, has recently been localised at the mouse blood–testis barrier, where it is potentially involved in newly formed TJs (Meng *et al.* 2005). Our inability to detect claudin-3 mRNA expression in the rat Sertoli cell culture system at

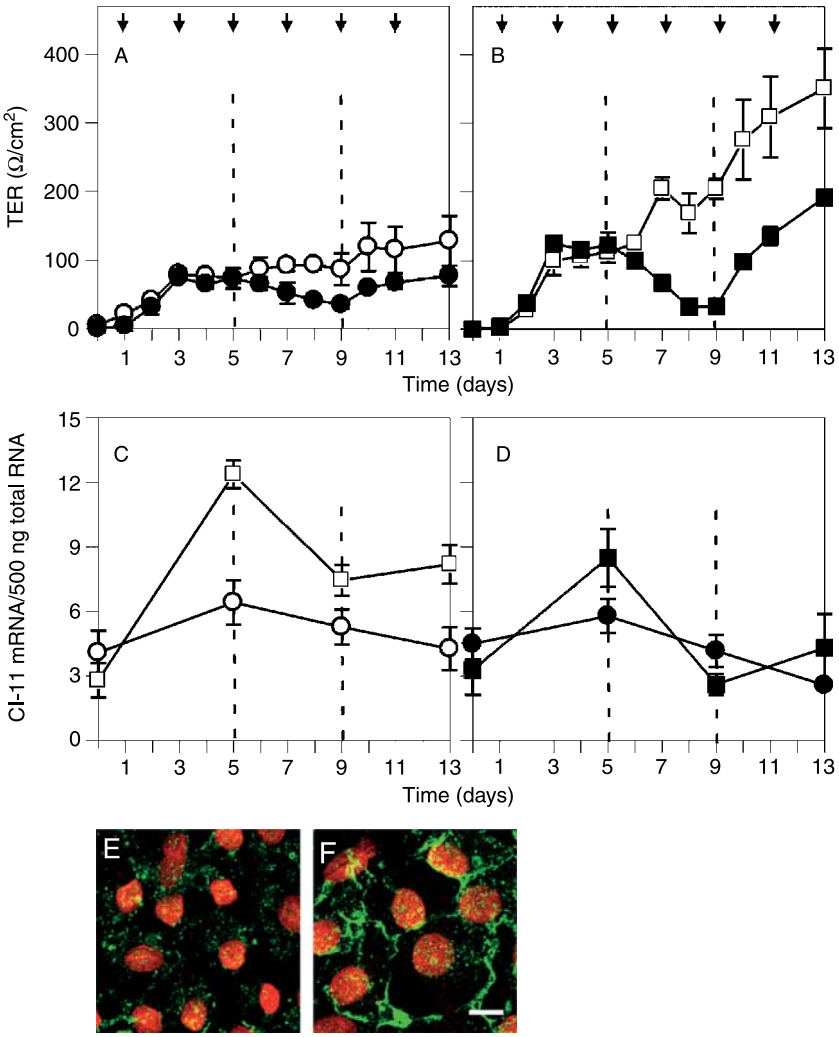

Figure 4 Effect of the androgen receptor antagonist, flutamide, on TER and claudin mRNA expression and protein localisation. Sertoli cells were cultured in the presence of medium alone (A) or testosterone (28 ng/ml, B) as described in the legend to Fig. 1 and TER measurements were taken daily. On day 5, cells from each group were washed and placed in medium containing flutamide (28 μg/ml; ● or ■) for 4 days as shown by the vertical dashed lines. Open symbols (○, □) represent cells which remained in medium alone or medium plus testosterone and did not receive flutamide. On day 9, flutamide was removed and cells were returned to medium alone, or medium plus testosterone (28 ng/ml) until end of culture on day 13. Routine medium replacements were undertaken as shown by the arrows. At days 0, 5, 9 and 13 cells were removed and total RNA extracted for RT and real-time PCR. Panel C presents claudin-11 mRNA expression data for cells continuously treated either with medium alone (○) or testosterone (□), and data for control (●) or testosterone-treated cells (■), which received flutamide between days 5 and 9. Data are presented as mean ± S.D. ($n=3$ PCR estimations) from a single representative culture, and asterisks indicate significant differences compared with respective controls as determined by one-way ANOVA and Newman–Keuls *post hoc* analysis tests (*$P<0.05$; **$P<0.01$, ns, not significant). a versus b; $P<0.01$, c versus d; ns. Immunohistochemistry for claudin-11 was carried out as described in Fig. 2 legend on testosterone-treated cells cultured with flutamide and sampled at day 9 (Panel D, equivalent to ■ at day 9 in panel B), or on cells following testosterone replacement and sampled at day 13 (panel E, equivalent to ■ at day 13 in panel B).

any time during junction formation, or by immunohistochemical localisation in the adult rat testis, does not support a role for this protein in the rat testis.

The extent to which the 'tightness' of Sertoli cell TJs can be regulated by androgens as observed here, and elsewhere (Janecki *et al.* 1991*a*, Gye 2003), is of interest in understanding how these junctions function. Under basal conditions, rat Sertoli cells at similar cell plating densities typically exhibit TERs of 60–100 Ω/cm^2 (this study, Janecki *et al.* 1991*a*, 1991*b*, Chung & Cheng 2001, Li *et al.* 2001, Lui *et al.* 2001, Siu *et al.* 2003), which is greater than 'leaky' TJs formed by cells from mammalian kidney proximal tubules (6–7 Ω/cm^2; Fanning *et al.* 1999), but less than kidney collecting ducts (300 Ω/cm^2) or the very tight epithelium of the bladder (≥6000 Ω/cm^2; Fanning *et al.* 1999). After T stimulation for 5–7 days, TER values increased from 150 to 250 Ω/cm^2; however, values ∼350 Ω/cm^2 were observed after continuous culture for 13 days, and it was evident that a plateau had not been reached. This suggests that optimal conditions for the establishment of TJs in Sertoli cell cultures have not been attained, which is supported by up to tenfold higher TERs (∼800 Ω/cm^2) observed elsewhere (Janecki *et al.* 1991*a*, 1991*b*).

The role of FSH in Sertoli cell TJ regulation *in vitro* remains an issue. Our study demonstrated that while FSH could upregulate claudin-11 mRNA expression, TER was not altered to the same extent. Other studies have demonstrated a greater upregulation of TER by FSH (Janecki *et al.* 1991*a*), or an inhibitory effect of FSH on claudin-11 mRNA expression (Hellani *et al.* 2000), leading us to presume that differences between *in vitro* culture methods can lead to altered FSH dose–response characteristics. It has also been speculated that FSH may transiently stimulate protease activity to alter TER (Janecki *et al.* 1991*a*, Chung & Cheng 2001).

Several lines of evidence suggest that gonadotrophins are important for the formation and function of Sertoli cell TJs *in vitro* (this study, Janecki *et al.* 1991*a*, 1991*b*, Gye 2003) and *in vivo* (Vitale *et al.* 1973, Bressler 1976, Russell & Peterson 1985, Bergmann 1987, Russell *et al.* 1989, Gye & Ohsako 2003). In several species of hamster (Bergmann 1987, Bergmann *et al.* 1989) and mink (Pelletier 1988), TJs comprising the blood–testis

barrier undergo a cyclic breakdown and reappearance in association with photoperiod and changes to circulatory gonadotrophins. We recently demonstrated an extensive disruption of the localisation of two TJ-associated proteins, claudin-11 and ZO-1, in the adult short-day Djungarian hamster (Tarulli *et al.* 2006) where serum gonadotrophins are low and there is a lack of functional TJs (Bergmann 1987). Exogenous FSH restored the organisation of these proteins to resemble the localisation observed in the functional TJs of the long-day hamster (Tarulli *et al.* 2006). In the mouse, ablation of the SCARKO resulted in an increased permeability of the blood–testis barrier to a biotin tracer (Meng *et al.* 2005), confirming a role for androgen in this species. In contrast, available data from the rat indicate that TJs remain morphologically present following short-term (6–8 days) androgen withdrawal by the selective Leydig cell toxicant, ethane dimethansulphonate (Kerr *et al.* 1993), or long-term (41 days) gonadotrophin withdrawal following hypophysectomy (Franca *et al.* 1998), although blood–testis barrier functionality was not tested in these models. It would be of interest to examine whether rat Sertoli cell TJs *in vivo* become 'leaky' following gonadotrophin or selective androgen withdrawal. Such studies may well be of importance in understanding why there is a non-uniform induction of azoospermia in men undergoing hormonal contraception following suppression of serum gonadotrophins (World Health Organization 1990, 1996).

During spermatogenesis, germ cells must translocate through the blood–testis barrier into the adluminal compartment. While the mechanism of this translocation is unknown, it stands to reason that remodelling of Sertoli cell TJs must occur (Russell & Peterson 1985, Pelletier & Byers 1992). In addition to the stimulatory effects of androgen presented in this study, rat Sertoli cell TER can be negatively regulated by a number of testicular cytokines and growth factors including TGF-β3 (Lui *et al.* 2001, 2003*a*, Siu *et al.* 2003) and TNFα (Lui *et al.* 2003*c*, Siu *et al.* 2003; for reviews see Wong & Cheng 2005, Xia *et al.* 2005), with TNFα also capable of downregulating occludin and claudin-11 mRNA expression (Hellani *et al.* 2000, Siu *et al.* 2003). Androgen receptor levels are highest in stages VII–VIII of spermatogenesis in the rat (Bremner *et al.* 1994), which precedes primary spermatocyte translocation into the adluminal compartment (Russell & Peterson 1985). Hence, Sertoli cell TJ function and TJ protein expression can be both up and down regulated by hormones and local factors *in vitro*.

In addition to these changes, our data also suggest that a TJ protein endocytosis or recycling process may be contributing to TJ function in rat Sertoli cells, as demonstrated by the depletion of claudin-11 and occludin from cell contacts after flutamide treatment. The removal of TJ structures, including claudin-3 (Matsuda *et al.* 2004) by endocytosis-related processes in various epithelial cells is well recognised (for review see Ben-Shaul & Ophir 2001); however, the extent to which Sertoli TJs proteins and function are regulated in this manner *in vivo* remains to be determined.

In summary, this study demonstrates that claudin-11 plays an important role in the establishment and function of Sertoli cell TJs *in vitro*, and that androgens promote the expression and localisation of claudin-11 to inter-Sertoli cell contacts. In addition, while not directly regulating occludin mRNA expression, this study demonstrates that androgens promote the localisation of occludin protein at inter-Sertoli cell contacts. In contrast to recent *in vivo* studies in the mouse, no evidence for a role for claudin-3 in the formation of rat Sertoli cell TJs *in vitro* could be found. Collectively, these data show that the ability of androgens to maintain spermatogenesis *in vivo* is partly via their effects on Sertoli cell TJ proteins and regulation of the blood–testis barrier.

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