

A complex containing $\alpha_6\beta_1$ -integrin and phosphorylated focal adhesion kinase between Sertoli cells and elongated spermatids during spermatid release from the seminiferous epithelium

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Abstract

Spermiation is the final step of spermatogenesis and culminates in the disengagement (release) of elongated spermatids from Sertoli cells into the seminiferous tubule lumen. Spermiation failure, wherein spermatids are retained by Sertoli cells instead of releasing, occurs after hormone suppression. The mechanisms involved in spermatid disengagement and retention are not well understood. We previously showed that β_1 -integrin is associated with spermatids until the point of disengagement, but the ectoplasmic specialisation junction (ES) is not. The aims of this paper are to further characterise the complex that is present immediately prior to spermatid disengagement by identifying the α -integrin form dimerised with β_1 -integrin, localising focal adhesion kinase (FAK) and determining if microtubules are involved. Adult Sprague–Dawley rats received testosterone and oestradiol implants and an FSH antibody for 7 days to suppress testicular testosterone and FSH and induce spermiation failure.

Control rats were treated with saline. Immunohistochemical analysis showed that α_6 -integrin and a phosphorylated form of FAK (FAK-Tyr³⁹⁷) are present between late spermatids and Sertoli cells after ES removal, until the point of disengagement, and both proteins remain associated with retained spermatids after spermiation failure induced by hormone suppression. Using dual-label immunofluorescence, tubulins (and thus microtubules) were observed to co-localise with ES, but were neither associated with elongated spermatids just prior to release nor with retained spermatids following hormone suppression. These results suggest that microtubules are not involved in the final release of spermatids from Sertoli cells. We conclude that spermatid release during spermiation is mediated by a 'disengagement complex' containing $\alpha_6\beta_1$ -integrin and phospho-FAK, the function of which can be affected by gonadotrophin suppression.

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Introduction

Spermiation is the final step of spermatogenesis and is the process whereby elongated spermatids undergo their final maturation and release from the supporting Sertoli cells into the seminiferous tubule lumen. Morphological studies have shown that spermiation is a multi-step process commencing at the beginning of stage VII and ending during stage VIII in the rat, and includes: (1) the removal of excess cytoplasm from around the spermatid to form the streamlined spermatozoa, (2) extension of the spermatid into the tubule lumen, (3) the removal of specialised adhesion junctions, including the ectoplasmic specialisation junctions (ES), (4) formation and degradation of tubulobulbar complexes, (5) retraction of the Sertoli cell cytoplasm from around the spermatid until it only contacts the dorsal surface of the spermatid head and (6) the final disengagement of the spermatid from the Sertoli cell (reviewed by Russell (1993)).

Spermiation is vulnerable to disruption via the administration of reproductive toxicants (Huang & Marshall 1983, Chapin *et al.* 2001) or the suppression of gonadotrophins (Russell & Clermont 1977, Saito *et al.* 2000, O'Donnell *et al.* 2001). This results in spermiation failure, wherein spermatids are not released, but instead retained and phagocytosed by Sertoli cells (Russell & Clermont 1977, Russell 1991). The acute suppression of testosterone and FSH in adult rats for 1 week results in 50% of spermatids failing to be released (Saito *et al.* 2000), suggesting that spermiation failure is an important determinant in the acute onset of spermatogenic suppression after hormone withdrawal. Subsequent studies in monkeys (O'Donnell *et al.* 2001) and men (McLachlan *et al.* 2002b) have confirmed this observation.

The molecular mechanisms involved in normal spermiation and spermiation failure are largely unknown. Morphological analysis has showed that spermiation failure induced by gonadotrophin suppression occurs as a result of a defect in the

final release or disengagement of spermatids, and not defects in earlier spermiation processes (Beardsley & O'Donnell 2003). In particular, removal of adhesion junctions such as the ES, retraction of the Sertoli cell cytoplasm and removal of spermatid cytoplasm occur normally during gonadotrophin suppression (Beardsley & O'Donnell 2003). Prior to, and during the early part of spermiation, spermatids are attached to the Sertoli cells via the apical Sertoli cell ES, which begin to develop at step 8 of spermiogenesis (Russell 1977, Vogl *et al.* 2000). Importantly, previous studies showed that this adhesion junction is removed from elongated spermatids ~30 h before disengagement, suggesting that a hitherto uncharacterised adhesion junction is probably involved in spermatid release (Beardsley & O'Donnell 2003). Various cell adhesion molecules and associated kinases are present between spermatids and Sertoli cells during the spermiation process (Chapin *et al.* 2001); however, the composition of the adhesion complex that mediates spermatid disengagement is unknown.

β_1 -integrin is a probable component of this latter junction, as it is present during spermiation, after ES removal and until the point of spermatid release on the dorsal side of the spermatid, and in addition is seen opposite retained spermatids after hormone suppression in adult rats (Beardsley & O'Donnell 2003). α_4 - (Chapin *et al.* 2001) and/or α_6 -integrin (Salanova *et al.* 1995, 1998) are the probable β_1 -integrin partners as both have been immunolocalised to the site of spermiation.

A number of regulatory kinases associate directly or indirectly with β_1 -integrin-containing dimers to regulate adhesion processes (Hynes 1992, Sanchez-Mateos *et al.* 1996, Hannigan & Dedhar 1997). Integrin-linked kinase (ILK) and focal adhesion kinase (FAK) are two kinases that have known effects on β_1 -integrin-mediated cell adhesion (Hannigan & Dedhar 1997) and are present in the testis (Mulholland *et al.* 2001). Despite the demonstration of ILK co-immunoprecipitating with β_1 -integrin from the seminiferous epithelium (Mulholland *et al.* 2001), we recently demonstrated that ILK is associated with spermatids early in spermiation, but is removed along with the ES before disengagement (Beardsley & O'Donnell 2003). Therefore, ILK is not likely involved in spermatid disengagement. Immunolocalisation studies have suggested that FAK is not present at the site of spermiation (Chapin *et al.* 2001, Mulholland *et al.* 2001), however, a recent study using antibodies against specific phosphorylated forms of FAK (P-FAK) suggests that P-FAK is indeed present around spermatids during spermiation and immunoprecipitates with β_1 -integrin from whole testis extracts (Siu *et al.* 2003). Given that this kinase is likely to be important in regulating integrin-mediated adhesion (Wehrle-Haller & Imhof 2002, Parsons 2003), it is important to determine whether P-FAK is present at the time of spermatid disengagement and is likely to participate in the regulation of adhesion during spermatid release.

Microtubules are the structural support elements of Sertoli cells and localised mostly within the stalk of the Sertoli cell

cytoplasm (Vogl *et al.* 1993), however, a direct involvement in disengagement is not known. Microtubules, apart from providing structural support, play an important role in the translocation of elongated spermatids from deep within Sertoli cell crypts up to the tubule lumen at the onset of spermiation (Amlani & Vogl 1988, Russell *et al.* 1989, Redenbach *et al.* 1992, see Vogl *et al.* 1993 for review). Microtubules are attached via motor proteins (kinesin and dynein) to the endoplasmic reticulum side of the ES in Sertoli cells (Hall *et al.* 1992, Miller *et al.* 1999, Guttman *et al.* 2000). Immunohistochemical localisation of α -tubulin also suggests that microtubules may be present during spermiation (Wenz & Hess 1998), however, whether microtubules are present at the time of spermatid release or are involved in spermatid retention during spermiation failure is unclear.

In order to begin to understand the molecular complexes that control spermatid release, the failure of the disengagement process during gonadotrophin suppression and to elucidate the complexes present on retained spermatids during gonadotrophin suppression, we sought to further characterise the adhesion junction present around spermatids immediately prior to their release. We previously showed that this adhesion junction contains β_1 -integrin, but neither ILK nor ES-associated proteins such as espin (Beardsley & O'Donnell 2003). The present study aimed to: (1) identify whether α_4 - or α_6 -integrin associates with β_1 -integrin at the time of spermatid release, (2) determine if P-FAK (specifically FAK-Tyr³⁹⁷) is part of this β_1 -integrin containing complex at disengagement and (3) investigate whether microtubules associate with this complex during spermatid disengagement. The immunolocalisation of these proteins was examined in normal testes, and in testes where spermiation failure had been induced by hormone suppression, in order to pinpoint which adhesion systems may be involved in spermatid retention. We also examined the hormonal regulation of FAK and P-FAK proteins in seminiferous tubules from normal and gonadotrophin-suppressed rats.

Materials and Methods

Animals

Sixteen adult male Sprague–Dawley rats (age 70–90 days) were obtained from Monash Central Animal House and housed under 12 h light:12 h darkness photoperiod with access to food and water *ad libitum*. The study was approved by the Monash Medical Centre Animal Ethics Committee, Australia.

Experimental design

Animals in each of the experiments were assigned into control or treatment groups ($n=8$ per group). Under isoflurane inhalation anaesthesia, animals in the treatment group received three 8 cm testosterone implants inserted *s.c.* along

the dorsal surface for 1 week to suppress circulating luteinizing hormone, but maintain spermatogenesis as previously described (O'Donnell *et al.* 1994, Saito *et al.* 2000). After 7 days, the implants were removed and replaced with a 3 cm testosterone implant and 0.4 cm oestradiol implant (TE treatment) to induce the suppression of testicular testosterone (O'Donnell *et al.* 1994, 1996). During TE treatment, animals also received daily s.c. injections of rat FSH polyclonal antibody (raised in sheep; 2 mg/kg per day in sterile 0.154 M NaCl), which has been previously shown to immunoneutralise over 90% circulating FSH in adult male rats (Meachem *et al.* 1998). These animals are referred to as the $\alpha_6\beta_1$ -testosterone/FSH group. This $\alpha_6\beta_1$ -testosterone/FSH regime has previously been shown to cause failure of 50% of the spermatids in the testis to spermiate, in the absence of major changes to earlier germ cell populations (Saito *et al.* 2000). The control group were given daily injections of non-immunised sheep immunoglobulin (ConAb; 2 mg/kg per day in sterile 0.154 M NaCl) for 1 week.

At the end of the treatment regime, four control and four $\alpha_6\beta_1$ -testosterone/FSH-treated rats were killed by CO₂ overdose, one testis was removed, weighed and snap-frozen in liquid N₂ for immunohistochemistry. The remaining testis was retained for use in another study. The remaining four control and four treated animals were killed by CO₂ overdose and one testis from each rat was removed, weighed and immersion-fixed in Bouin's fixative for 5 h to be used for morphological analysis and immunohistochemistry. The other testis was removed, weighed and placed into warm PBS (0.01 M PBS, 0.154 M NaCl, pH 7.4, no sodium azide) for seminiferous tubule isolation (see below).

Tissue preparation for immunohistochemistry

For immunohistochemical analysis on frozen tissue, 10 μ m sections of frozen testes were cut on a cryostat set at -20°C and collected onto slides coated with 2%, 3-aminopropyltriethoxysilane (AAS; Sigma). Sections were then post-fixed in either acetone at -20°C , or ice-cold Bouin's fixative for 8 min, rinsed in either cold PBS or cold 100% ethanol respectively, and dried overnight at 4°C .

Wedges of Bouin's-fixed testes were embedded in a low-melting-point-ribboning polyester wax (BDH, Poole, Dorset, UK) as previously described (Oke & Suarez-Quian 1993, O'Donnell *et al.* 2000). Sections of 10 μ m were cut on a cryostat set at 0°C , floated onto a waterbath set at 32°C , collected onto AAS-coated slides and allowed to dry for 48–72 h at 4°C .

Immunohistochemistry and double-label immunofluorescence

All antibodies used for immunohistochemistry and Western blot analysis are detailed in Table 1. The immunohistochemical detection protocol for light microscopy has been described in detail previously (O'Donnell *et al.* 2000); biotinylated secondary antibodies, a streptavidin–horseradish

peroxidase complex (ABC complex; Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) and a pink chromogenic substrate (VIP; Vector) were used to detect the antigen of interest. The signal for FAK-Tyr³⁹⁷ was amplified by employing a tyramide signal-amplification kit (TSA; NEN Life Science products, Boston, MA, USA) as described previously (Beardsley & O'Donnell 2003).

All antibodies were tested for specificity by substituting the primary antibody for an appropriate negative control serum or IgG on the same slide. All incubations were carried out at room temperature in a humidified chamber. Based on a series of optimisation experiments, tubulins and espin were detected in Bouin's-fixed, polyester wax-embedded tissue; α_6 -integrin was detected in frozen sections post-fixed in acetone and FAK-Tyr³⁹⁷ was detected in frozen sections post-fixed in Bouin's fixative.

The sections were observed under a 40 \times objective or a 100 \times oil-immersion objective on an Olympus BX50 microscope. Images were captured using a FujixHC-2000 high-resolution digital camera (Fujifilm, Tokyo, Japan) and Analytical Imaging Station software (Imaging Research, Inc., St Catherines, ON, Canada). Images were then compiled and labelled using Adobe Photoshop 5.5 (Adobe).

Dual-label immunofluorescence was performed using previously described methods (O'Donnell *et al.* 2000) and used to co-immunolocalise microtubules and espin. The sections were incubated with the primary antibody overnight at room temperature in a humidified chamber. Following PBS washes, they were incubated in either goat anti-rabbit-546 or goat anti-mouse-488 Alexa fluorophores (Molecular Probes, Eugene, OR, USA) for 1 h in the dark. They were counterstained with Mayer's hematoxylin (Sigma) and washed in PBS. Coverslips were mounted with Fluorosave (Calbiochem, San Deigo, CA, USA) and were viewed on a confocal microscope (Fluoview FV300, Olympus Australia, Mt Waverley, Vic., Australia). Images from 1 μ m optical sections throughout the tissue were collected and compiled using Adobe Photoshop 5.5.

Estimation of the percentage of tubules with elongated spermatids immunostained with adhesion and structural proteins

The estimation of the percentage of tubules during spermiation immunostained with particular antigens is described in detail elsewhere (Beardsley & O'Donnell 2003). Briefly, tubules were scored based on the presence or the absence of spermatids lined along the luminal edge of the tubules, which are termed adluminal spermatids (i.e. beginning of spermiation to disengagement). Tubules with adluminal spermatids were then further classified as to whether spermatids were associated with immunostaining of the antigen of interest (i.e. a positive tubule) or few (< 10) or no spermatids with immunostaining (i.e. a negative tubule). The percentage of tubules during spermiation that showed immunostaining associated with the antigen of interest was then calculated. Data were analysed using one-way ANOVA

Table 1 Primary antibodies, types and sources

	Antibody type	Catalogue no.	Source ^a	Use ^b	Dilution
Antigen					
α_4 -Integrin	Monoclonal	MR494422 Sc-14003 MAB1383 MAB1396Z	Antigenix America Santa Cruz Chemicon Chemicon	IHC, WB	N/A
α_6 -Integrin	Polyclonal	Sc-6597	Santa Cruz	IHC	1:50
FAK-Tyr ³⁹⁷	Polyclonal (serum)	07012	Upstate bio- technologies	IHC, WB	1:200, 1:1000
FAK	Monoclonal	610087	BD transduction labs	WB	1:2000
α -Tubulin	Monoclonal	T5168	Sigma	IHC, IF	1:2000, 1:500
β -Tubulin	Monoclonal	T5293	Sigma	IHC, IF	1:1000, 1:200
Espin	Polyclonal	N/A	*	IF	1:200
Actin	Monoclonal	69100	MP Biomedicals	WB	1:2000

*Espin antibody kindly provided by Prof J R Bartles, Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL, USA.
^aAntigenix America, Huntington, NY, USA; Santa Cruz, Santa Cruz, CA, USA; Chemicon, Temecula, CA, USA; Upstate Biotechnologies, Lake Placid, NY, USA; BD Transduction Labs, Franklin Lakes, NJ, USA; Sigma, St Louis, MO, USA; MP Biomedicals, Irvine, CA, USA.
^bIHC, immunohistochemistry; IF, immunofluorescence; WB, Western blot.

and appropriate *post-hoc* comparisons using Prism 3.0 software (GraphPad software, San Diego, CA, USA).

Isolation of seminiferous tubules during spermiation

Seminiferous tubules were isolated based on modifications of previously published methods (Chapin *et al.* 2001). From each of the four control and four — testosterone/FSH animals, one testis was bisected on the longitudinal axis and the contents placed into 20 ml warm digestion media (1 mg/ml collagenase, catalogue no. 103586; 1 mg/ml trypsin, catalogue no. 109819, 0.5 mg/ml hyaluronidase, catalogue no. 106500; Roche; in PBS) and shaken (180 r.p.m.) for 15 min at 32 °C. The enzyme solution was decanted and the tissue rinsed with PBS, followed by PBS⁺Mg/Ca. The solution was decanted to ~15 ml and incubated with ~150 kUnitz DNase (DN-25, Sigma) for 3 min at 32 °C with gentle agitation (100 r.p.m.). The tubules were rinsed with PBS, excess PBS was removed by aspiration and seminiferous tubules snap-frozen on dry ice.

Western blot analysis

Protein from whole seminiferous tubules was extracted in a buffer containing 20 mM PO₄, 150 mM NaCl, 50 mM NaF, 0.5 mM Na₃VO₄, 2% v/v Triton-X-100 and complete mini protease inhibitor, EDTA-free (Roche; Chapin *et al.* 2001). An aliquot was taken at this point to determine the protein concentration using the BCA protein assay (Pierce, Rockford, IL, USA). The supernatant was diluted in a ratio of 1:3 in sample buffer (5 ml 0.5 M Tris-HCl (pH 6.8), 5 ml glycerol, 5 ml 10% w/v SDS, 0.5 ml β -mercaptoethanol and 24.5 ml MilliQ water). Twenty micrograms protein per sample were separated by SDS-PAGE (7.5% agarose) and

transferred onto nitrocellulose membranes for Western blotting. The membranes were blocked in 5% skim milk in PBS overnight at 4 °C. FAK-Tyr³⁹⁷ was detected using an Infrared 680-conjugated goat anti-rabbit secondary antibody (catalogue no. A21076; Molecular Probes). FAK and actin were detected using an Infrared 800-conjugated goat anti-mouse secondary antibody (catalogue no. 610132121; Rockland Immunochemicals, Inc., Gilbertsville, PA, USA). The membranes were scanned on an infrared imaging system (Odyssey IR imaging scanner; Li-Cor Biosciences, Lincoln, NE, USA) at a resolution of 169 μ m and a laser intensity of 5. Results were quantitated using densitometry software provided with the imaging system and data normalised to actin.

Results

Immunohistochemical localisation of spermiation-associated proteins

Integrins At the beginning of spermiation, when elongated spermatids were lined along the luminal edge (stage VII), intense α_6 -integrin immunostaining was seen around the heads of step 19-elongated spermatids (Fig. 1A). The localisation of α_6 -integrin around these spermatids was concentrated around the dorsal aspect of their heads (Fig. 1A and B) and persisted until the spermatids were released from Sertoli cells during mid-stage VIII, since all stage VIII tubules with elongated spermatids showed α_6 -integrin immunostaining. Immunostaining was also seen in focal points of developing ES junctions opposite step 8 round spermatids (Fig. 1B). However, obvious immunostaining was not observed in association with steps 9–18 elongating and elongated spermatids (data not shown). After spermatid disengagement, α_6 -integrin was not seen along the luminal

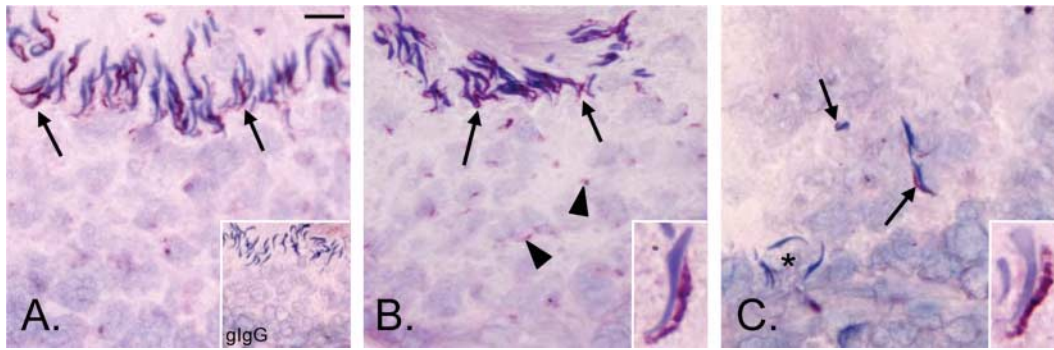


Figure 1 Immunohistochemical analysis of α_6 -integrin in a control rat testis during (A) early spermiation (stage VII), (B) late spermiation (stage VIII) and (C) on retained spermatids in stage IX after hormone suppression. In all micrographs, the seminiferous tubule lumen is positioned at the top and the basement membrane is along the bottom. α_6 -integrin staining was confined to the dorsal curvature step 19 elongated spermatids (arrows) during early (A) and late (B) spermiation. Immunostaining is also seen around developing junctions opposite step 8 round spermatids (arrowheads). (C) α_6 -integrin immunostaining during spermiation failure in a late stage VIII tubule from a $-$ testosterone/FSH testis, where immunostaining is seen around the dorsal curvature of retained spermatids within the epithelium (arrows). Retained spermatids along the basement membrane generally were not associated with immunostaining (*). Scale bar in (A) = 10 μ m with (B) and (C) taken at the same magnification. Inset in (A) shows the negative control where the α_6 -integrin antibody was substituted with goat IgG. Inset in (B) and (C) are magnified views of step 19 spermatids in stage VIII and a retained spermatid respectively.

edge of late stage VIII–early IX tubules (not shown). α_6 -integrin immunostaining was occasionally very faintly associated within the basal compartment consistent with an inter-Sertoli cell junction localisation (not shown). In $-$ testosterone/FSH animals, the localisation of α_6 -integrin was identical to that seen in control animals in all the tubules examined, i.e. immunostaining was seen until the point of spermatid disengagement. α_6 -integrin was also seen around many retained spermatids within the epithelium (Fig. 1C), and particularly prominent around retained spermatids in the adluminal portion of the seminiferous epithelium. Staining intensity was reduced and eventually not seen as spermatids were translocated down to the basement membrane and digested by the Sertoli cell (Fig. 1C). The localisation of α_6 -integrin mirrors previous reports seen for β_1 -integrin (Beardsley & O'Donnell 2003).

Despite previous reports of immunostaining in the testis (Chapin *et al.* 2001), α_4 -integrin could not be detected using four different antisera (see Table 1) and a variety of fixation and detection protocols. In addition, a specific signal was not observed in seminiferous tubule lysates by Western blotting using the same antibodies (not shown). Thus, we cannot make any assessment of α_4 -integrin reactivity in the seminiferous epithelium.

FAK-Tyr³⁹⁷ Prior to spermiation in stages IV–VI, FAK-Tyr³⁹⁷ localisation was seen in discrete focal bands associated with clusters of steps 17–18–elongated spermatids within Sertoli cell crypts (data not shown). During spermiation, FAK-Tyr³⁹⁷ was localised around the dorsal curvature of step 19–elongated spermatids in stages VII and VIII (Fig. 2A and B), consistent with previous reports (Siu *et al.* 2003). This

localisation persisted until they were released, as all tubules in stage VIII with step 19 spermatids showed FAK-Tyr³⁹⁷ localisation to elongated spermatids (Fig. 2B) similar to the localisation of α_6 -integrin. FAK-Tyr³⁹⁷ was also present in developing ES opposite steps 8 and 9 spermatids in both control and $-$ testosterone/FSH rats (Fig. 2B and C). In $-$ testosterone/FSH rats, the localisation of FAK-Tyr³⁹⁷ was observed until spermatid release as seen in control animals. Furthermore, FAK-Tyr³⁹⁷ immunostaining was seen on many retained spermatids in the adluminal portion of the epithelium (Fig. 2C).

FAK The immunolocalisation of total FAK was attempted using the same antibody as that used for Western blot analysis, however, this antibody failed to yield any specific staining above background levels.

Microtubules The tubulins (both α and β) were immunolocalised in the seminiferous epithelium during spermiation, most prominently within the central stalk of the Sertoli cell cytoplasm (Fig. 3A). They were also evident in the spermatid flagella that were in the tubule lumen throughout the spermiation process and until spermatids were released (not shown). They were also apparent around the heads of elongated spermatids at the beginning of spermiation in stage VII (Fig. 3A). During stage VIII, however, tubulins became more diffusely associated with the heads of elongated spermatids and, immediately prior to spermatid disengagement, there was no immunolocalisation of tubulins associated with spermatid heads (Fig. 3B). Instead, tubulin staining was visible in central Sertoli cell cytoplasm and step 8 round spermatid manchettes (opposite developing ES junctions;

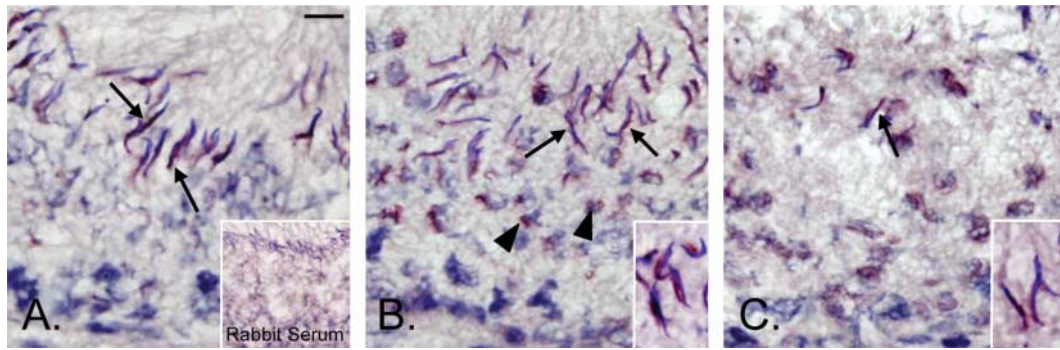


Figure 2 Immunohistochemical localisation of FAK-Tyr³⁹⁷ in control animals during (A) early (stage VII) and (B) late spermatid (stage VIII) and (C) on retained spermatids in stage IX after hormone suppression. FAK-Tyr³⁹⁷ is associated with step 19 elongated spermatids during spermatidiation (arrows in (A) and (B)). Immunostaining during stage VIII is also seen associated with developing ES junctions opposite step 8 round spermatids (arrowheads in (B)). (C) FAK-Tyr³⁹⁷ immunostaining in a -- testosterone/FSH rat testis where focal immunostaining is seen associated with elongated spermatids that are retained within the epithelium (arrows). Scale bar in (A) = 10 μm with (B) and (C) taken at the same magnification. Inset in (A) shows the negative control where the FAK-Tyr³⁹⁷ antibody was substituted with rabbit serum. Inset in (B) and (C) are magnified views of step 19 spermatids in stage VIII and a retained spermatid respectively.

Fig. 3B). In -- testosterone/FSH rats, the immunolocalisation pattern of tubulins was identical to that seen in control testes. In addition, tubulins did not appear to be immunolocalised around retained spermatids (Fig. 3C), but rather were located within the Sertoli cell cytoplasm.

To further understand the localisation pattern of tubulins during spermatidiation, dual-label immunofluorescence was performed with espin, a marker for the ES junction, which is removed approximately 30 h before spermatids disengage from the Sertoli cells (Beardsley & O'Donnell 2003). At the beginning of spermatidiation in stage VII, espin surrounded the entire head of step 19-elongated spermatids and tubulins

co-localised with espin around the dorsal aspect of the spermatid heads (Fig. 4A–D). In stage VIII tubules (just prior to spermatid disengagement), espin was no longer associated with the heads of elongated spermatids. While tubulins did not co-localise with espin during this stage, they were also not associated with elongated spermatids and rather localised to the round spermatids manchettes and within the Sertoli cell cytoplasm (Fig. 4E–H). In -- testosterone/FSH rats, the same immunolocalisation pattern was seen as in control animals until disengagement, and neither tubulin nor espin were associated with retained spermatids within the epithelium (Fig. 4I–L).

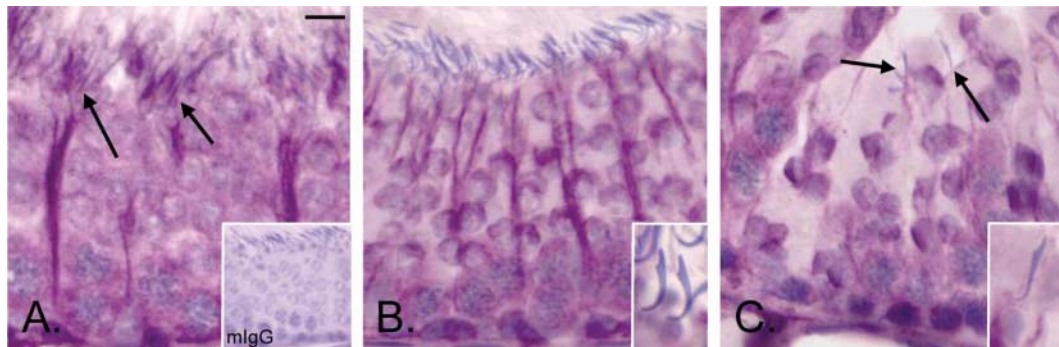


Figure 3 Tubulin localisation during (A) early (stage VII), (B) late spermatid (stage VIII) and (C) during spermatidiation failure (in stage IX). Immunohistochemical localisation of α and β -tubulin yielded identical results, therefore, representative micrographs are shown. (A) Tubulin localisation during early spermatidiation (stage VII) in control animals is seen in the central Sertoli cell stalk (seen here as vertical 'stripes'), extending up around the heads of the elongated spermatids (arrows). (B) Tubulin immunostaining prior to sperm release (in stage VIII) is faint or absent from the area surrounding the elongated spermatid heads, however, it is visible in developing ES opposite step 8 spermatids and within the central Sertoli cell cytoplasm. (C) Tubulin immunostaining during spermatidiation failure in -- testosterone/FSH rats is noted within the Sertoli cell cytoplasm and developing ES, but is not directly associated with retained spermatids (arrows). Scale bar in (A) = 10 μm with (B) and (C) taken at the same magnification. Inset in (A) shows the negative control where the tubulin antibody was substituted with mlgG. Inset in (B) and (C) are magnified views of step 19 spermatids in stage VIII and a retained spermatid respectively.

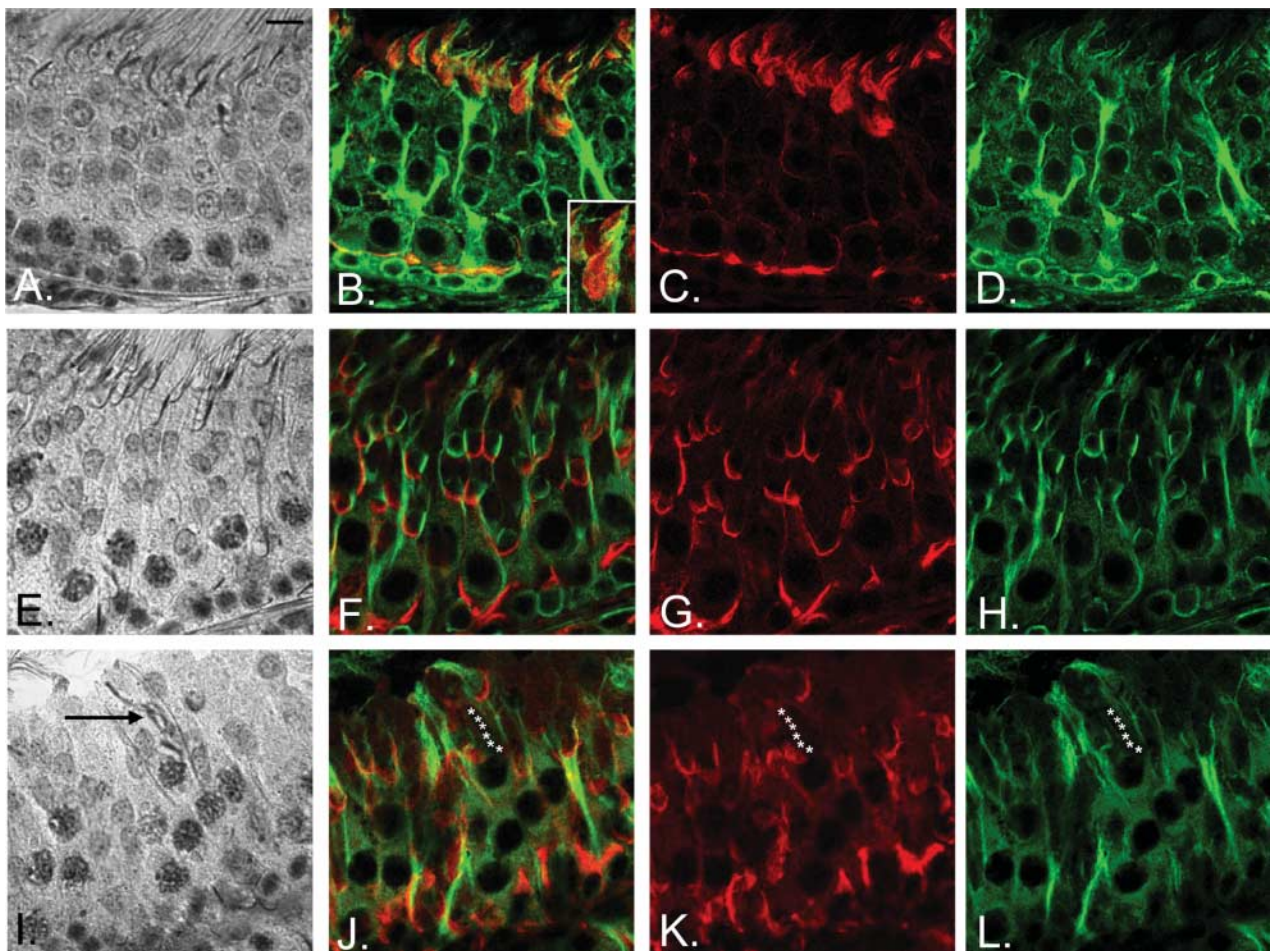


Figure 4 Dual-label immunofluorescence of espin (red) and tubulins (green) in the seminiferous epithelium: (A), (E) and (I) are the light micrographs associated with the fluorescence images; (B), (F) and (J) show co-localised espin and tubulin; (C), (G) and (K) show espin alone; and (D), (H) and (L) show tubulin alone. (A–D) Espin and tubulins at the beginning of spermiation during stage VII. Espin is seen around the heads of the elongated spermatids and tubulin is seen predominantly within the Sertoli cell stalk. Co-localisation is seen along the dorsal aspect of the spermatid head (inset in B). (E–H) Espin and tubulin localisation prior to spermatid release in a stage VIII. Both espin and tubulin are present, but not co-localised in the Sertoli cell cytoplasm, however, neither are associated with elongated spermatids. (I–L) Espin and tubulin immunolocalisation in —testosterone/FSH testis in early stage IX. Neither espin nor tubulin associate with retained spermatids (arrow in I; * in J–L). Tubulin immunostaining is evident in the flagella of retained spermatids (J, L, see photomicrograph in I). Scale bar in (A) = 10 μ m with (B)–(L) taken at the same magnification.

Estimation of the percentage of tubules with immunolabelled spermatids

The percentage of spermiation tubules with immunolabelled spermatids was determined in order to compare the time in which the proteins of interest were present during spermiation (Beardsley & O'Donnell 2003). During spermiation, α_6 -integrin was associated with spermatids in $94 \pm 1.05\%$ (mean \pm S.E.M.) of tubules (see Table 2); this is comparable to that seen previously for β_1 -integrin ($93 \pm 0.55\%$ of tubules (Beardsley & O'Donnell 2003)). FAK-Tyr³⁹⁷ associated with spermatids in $92 \pm 0.25\%$ of tubules and is comparable to the integrins. Both integrins and FAK-Tyr³⁹⁷ remained associated with spermatids for a

longer period of time than the ES protein espin ($P < 0.05$ compared with α_6 -, β_1 -integrin and FAK-Tyr³⁹⁷). It was noted that unlabelled tubules probed for α_6 -integrin and FAK-Tyr³⁹⁷ were seen during early (stage VII) rather than late spermiation prior to disengagement, whereas tubules that were negative for espin were noted during late spermiation (stage VIII).

During stages VII and VIII, α - and β -tubulin were immunolocalised to spermatid heads in $80 \pm 1.32\%$ and $76 \pm 1.04\%$ of tubules respectively (Table 2). It was noted that the majority of unlabelled tubules (20 and 24% for α - and β -tubulin respectively) were seen during stage VIII. Tubulins remained associated with step 19–elongated spermatid heads for the same time as espin as there was no significant

Table 2 Percentage of seminiferous tubules during spermiation (stages VII–VIII), which shows immunostaining of antigens around step 19 elongated spermatids

Antigen	Percentage tubules during spermiation with immunostained spermatids
α_6 -Integrin	93.95 ± 1.05*
β_1 -Integrin ^a	92.85 ± 0.55*
FAK-tyr ³⁹⁷	92.42 ± 0.25*
Espin	76.46 ± 1.04 [†]
α -Tubulin	79.98 ± 1.32 [†]
β -Tubulin	75.83 ± 1.63 [†]

Stages VII–VIII tubules were selected on the basis of the presence of elongated spermatids positioned along the luminal edge. Data are means ± s.e.m. ($n = 4$ testes).

*, [†]Different symbols denote significant difference between antigens ($P < 0.05$).

^aPreviously published data shown for comparison (Beardsley & O'Donnell 2003).

difference between the percentage of espin and α/β -tubulin-positive tubules.

Western blot analysis

Since FAK-tyr³⁹⁷ immunolocalisation remained associated with retained spermatids in stage IX seminiferous tubules from –testosterone/FSH rats, it was hypothesised that FAK phosphorylation may be hormone regulated. Both phosphorylated (FAK-Tyr³⁹⁷) and FAK were detected at the expected molecular mass of 125 kDa in seminiferous tubules from control rats and in tubules from –testosterone/FSH rats. Comparative Western blots and densitometric analysis revealed that there was no significant change in either FAK-Tyr³⁹⁷ or FAK protein levels in seminiferous tubules during spermiation failure compared with controls (Fig. 5).

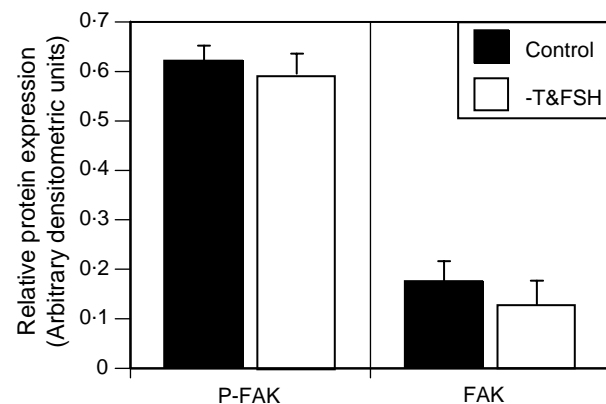


Figure 5 FAK-tyr³⁹⁷ and FAK protein levels in whole seminiferous tubules from four control and four –testosterone/FSH animals. Western blot analysis was performed and analysed by densitometry. The densitometric values for each band were normalised to actin. Data shown as means ± s.e.m. $n = 4$, corrected for actin.

Discussion

Results from this study show that spermatid disengagement is likely to be mediated by an adhesion complex that contains α_6 -integrin and FAK-Tyr³⁹⁷. The demonstration of these proteins between Sertoli cells and elongated spermatids until the time of disengagement, and on retained spermatids during spermiation failure, suggests that they play a key role in the release of spermatids during spermiation. These results extend previous findings in which β_1 -integrin also forms part of this complex (Beardsley & O'Donnell 2003). Thus, spermatid disengagement, and in particular the failure of this process during gonadotrophin suppression, may be hormonally regulated via an integrin/phosphorylated FAK-containing complex. Furthermore, we show that microtubules associate neither with this complex, nor with retained spermatids after phagocytosis by the Sertoli cell. We conclude that spermatid release at the end of spermiation is mediated by a 'disengagement complex' containing $\alpha_6\beta_1$ -integrin and phosphorylated FAK.

While the ES junction is well known to mediate adhesion between spermatids and Sertoli cells, this junction is removed prior to the final disengagement of spermatids at the end of spermiation (Beardsley & O'Donnell 2003). Previous studies have proposed various molecules and molecular pathways at the spermatid–Sertoli cell junctional complexes during the entire spermiation process occurring in stages VII and VIII of the rat spermatogenic cycle (Wine & Chapin 1999, Chapin *et al.* 2001, Mulholland *et al.* 2001) and reviewed by Lui *et al.* (2003b), Lee & Cheng (2004), Siu & Cheng (2004), Wong & Cheng (2005) and Zhang *et al.* (2005); however, little effort has focused on identifying the molecules and processes involved specifically after the removal of ES. In addition, there are limited studies investigating the hormonal regulation of spermiation and disengagement, reviewed by Russell (1991) and McLachlan *et al.* (2002a). Spermatid release is the first process to show morphological signs of damage after hormone suppression in rodents (Russell & Clermont 1977, Saito *et al.* 2000) and failure of this process contributes to acute and chronic sperm count suppression in contraceptive-treated monkeys and men (Zhengwei *et al.* 1998, O'Donnell *et al.* 2001, McLachlan *et al.* 2002b).

Previously, we and other researchers have shown that β_1 -integrin is present during spermiation at the Sertoli cell–spermatid junction (Palombi *et al.* 1992, Salanova *et al.* 1995, Chapin *et al.* 2001, Mulholland *et al.* 2001, Beardsley & O'Donnell 2003, Siu *et al.* 2003). Furthermore, β_1 -integrin persists along the dorsal curvature of the spermatid (which is the site where the Sertoli cell remains in contact with the spermatid after it has retracted away from the rest of the cell), until the point of disengagement and present on retained spermatids during spermiation failure (Beardsley & O'Donnell 2003). Both α_4 - and α_6 -integrin have been immunolocalised to the spermatid–Sertoli cell junction during spermiation (Salanova *et al.* 1995, Chapin *et al.*

2001); however, the persistence of these proteins until disengagement and their presence on retained spermatids had not been investigated. Despite previous reports of α_4 -integrin in the testes (Chapin *et al.* 2001), we could not detect α_4 -integrin immunoreactivity using multiple antisera; thus, we cannot further assess the role of α_4 -integrin in spermiation.

Although the localisation of α_6 -integrin to the site of spermiation has been previously demonstrated (Salanova *et al.* 1995, Chapin *et al.* 2001), the present study extends previous findings to show that this integrin, along with β_1 -integrin, persists after the ES has been removed and until disengagement, indicating that $\alpha_6\beta_1$ -integrins are part of the disengagement complex that mediates spermatid release. The fact that both α_6 and β_1 -integrin associate with newly retained spermatids after hormone suppression-induced spermiation failure supports this proposition.

The molecular mechanisms regulating the adhesive function of the $\alpha_6\beta_1$ -integrin dimer during spermiation are unknown. Various adhesion-related signalling molecules have been either immunolocalised to the site of spermiation or co-immunoprecipitated with integrins and other ES-related molecules during the spermiation process (Chapin *et al.* 2001, Siu *et al.* 2003, Lui *et al.* 2003a, Lee *et al.* 2005). In particular, protein phosphorylation seems to be important in spermatid disengagement (Chapin *et al.* 2001) and thus, signalling cascades are presumably involved in regulating the function of the $\alpha_6\beta_1$ -integrin dimer. Studies using an *in vitro* culture of spermiation showed that spermatid release decreased when serine/threonine kinase activity was inhibited, yet was increased with the addition of okadaic acid, a serine/threonine phosphatase inhibitor (Chapin *et al.* 2001), supporting the concept that spermatid release is controlled by protein phosphorylation. Consistent with this, antibodies raised against phosphorylated serine, threonine and tyrosine proteins immunostain the Sertoli cell–spermatid junction during spermiation (Chapin *et al.* 2001, Mulholland *et al.* 2001).

Focal adhesion kinase is a key regulator of integrin-mediated adhesion, reviewed by Giancotti & Ruoslahti (1999). Previously, FAK was reported not at the site of spermiation, but rather towards the basal aspect of the epithelium (Mulholland *et al.* 2001). More recent studies confirmed this, but also showed that phosphorylated FAK forms Tyr³⁹⁷ and Tyr⁵⁷⁶ were present between spermatids and Sertoli cells during spermiation, leading the authors to conclude that phosphorylated FAK is a component of the ES (Siu *et al.* 2003). FAK is auto-phosphorylated on its tyrosine³⁹⁷ residue following an association with the cytoplasmic tail of β_1 -integrin (Giancotti & Ruoslahti 1999). The present study shows that FAK-Tyr³⁹⁷ co-localises with $\alpha_6\beta_1$ -integrin not only at the site of the ES between spermatids and Sertoli cells early in spermiation, but that this complex persists after ES removal until spermatid disengagement. Although it has been previously demonstrated that FAK-Tyr³⁹⁷ is present in ES junctions (Siu *et al.* 2003) and likely to be involved in junction formation and turnover

(Wong & Cheng 2005), the present data strongly suggest that FAK-Tyr³⁹⁷ is present in association with spermatids immediately before they are released, and therefore not solely a part of ES junctions. Moreover, the demonstration of FAK-Tyr³⁹⁷ immunostaining on retained spermatids during spermiation failure supports the hypothesis that spermatid release and retention involves an $\alpha_6\beta_1$ -integrin/FAK-Tyr³⁹⁷ complex.

De-phosphorylation of FAK on the tyrosine³⁹⁷ residue is associated with FAK-exiting adhesion sites and the subsequent disassembly of integrin-mediated adhesion complexes (Kabir *et al.* 2002). It is therefore plausible to speculate that FAK de-phosphorylation is a key mediator of spermatid disengagement. The observation that FAK-Tyr³⁹⁷ associated with retained spermatids during spermiation failure supports this hypothesis. In order to investigate changes in FAK phosphorylation, protein levels of FAK and FAK-Tyr³⁹⁷ were investigated in seminiferous tubules. Given the persistence of FAK-Tyr³⁹⁷ on retained spermatids, we expected to see an increase in FAK-Tyr³⁹⁷ protein in seminiferous tubules from gonadotrophin-suppressed rats. The fact that no changes were seen may reflect that changes in FAK-Tyr³⁹⁷ at other stages of the cycle may mask any change after disengagement, or an induction of a 50% spermiation failure rate is not enough to see any changes in the overall protein levels.

Results from this study, together with our previous study (Beardsley & O'Donnell 2003), suggest that spermatid disengagement and the retention of spermatids after gonadotrophin suppression is mediated by an adhesion complex containing $\alpha_6\beta_1$ -integrin and FAK-Tyr³⁹⁷. This complex is separate from the ES, and thus we use the term 'disengagement complex' to distinguish between the adhesion complex present between Sertoli cells and spermatids at the time of disengagement, from the better-known ES junction, which is present between Sertoli cells and spermatids from mid-spermiogenesis until mid-spermiation (Fig. 6). While the ES has a distinct ultrastructure, reviewed by Vogl *et al.* (2000), Mruk & Cheng (2004), the disengagement complex between spermatids and Sertoli cells just prior to the release lacks ultrastructural features (Beardsley & O'Donnell 2003). However, molecularly, the disengagement complex resembles a focal adhesion complex (FAC), which is a well known adhesion system involved in cell migration and anchorage (Martin *et al.* 2002, Wehrle-Haller & Imhof 2002). Integrin-containing FACs are dynamic junctions involved in rapid adhesion and loss-of-adhesion events. That the disengagement complex is a type of FAC is supported by the localisation of $\alpha_6\beta_1$ -integrin and phosphorylated FAK, as well as the localisation of other FAC-associated proteins to the site of spermiation, for example, paxillin, cortactin, Ras, RalA and Rac1 (Wine & Chapin 1999, Chapin *et al.* 2001). This disengagement complex is likely assembled at the beginning of spermiation, when the $\alpha_6\beta_1$ -integrin molecules cluster on the Sertoli cell plasma membrane opposite the dorsal curvature of the step 19 spermatid, and persists after ES removal (Beardsley & O'Donnell 2003 and present study).

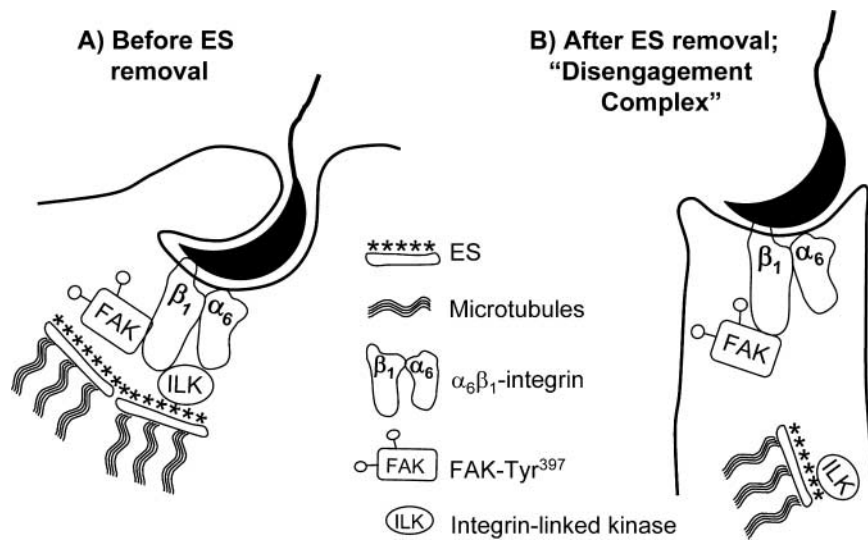


Figure 6 Schematic representation of a model of spermatid disengagement and some of the molecules involved. (A) During stage VII, the ES junction is present along with associated microtubules. $\alpha_6\beta_1$ -integrins on the Sertoli cell plasma membrane associate with the spermatid on the dorsal curvature and phosphorylated FAK and integrin-linked kinase (ILK) are present, presumably via an interaction with β_1 -integrin. (B) Prior to spermatid disengagement in stage VIII, the ES junctions have been removed, along with ILK (Beardsley & O'Donnell 2003). Instead a 'disengagement complex' containing $\alpha_6\beta_1$ -integrins and phosphorylated FAK is present between the spermatid and the Sertoli cell. Microtubules appear to be present within the Sertoli cell cytoplasmic stalk, but are not associated with the spermatid at this time.

The degree of integrin clustering in well-developed FACs is proportional to junctional strength (Giancotti & Ruoslahti 1999, Shemesh *et al.* 2005) and the heavy integrin immunostaining around the dorsal curvature of the spermatid would suggest that the disengagement complex has a strong adhesive function. Consistent with this hypothesis is the fact that, for at least a day prior to release, elongated spermatids are extended well out into the tubule lumen and thus presumably encountering considerable shear forces from seminiferous tubule fluid flow, with the Sertoli cell only contacting its the dorsal curvature (Russell 1993, Beardsley & O'Donnell 2003). The ES, which is well known to have considerable adhesive strength (Vogl *et al.* 2000, Wolski *et al.* 2005), is not present at this time (Beardsley & O'Donnell 2003) and thus the disengagement complex must be of sufficient strength to prevent premature sperm release. Furthermore, the demonstration of the disengagement complex on retained spermatids suggests that it is the dysfunction of this complex during gonadotrophin suppression that underlies spermiation failure.

Tubulobulbar complexes (TBCs) are well known to be formed and degraded throughout the spermiation process, reviewed by Russell (1993), both before and after ES removal. These structures are thought to participate in spermatid head shaping, adhesion of the spermatid to the Sertoli cell during spermiation and the removal of ES junctions (Romrell & Ross 1979, Tanii *et al.* 1999, Guttman *et al.* 2002, 2004). The immunostaining of proteins involved in TBCs is clearly

localised to Sertoli cell cytoplasm in the inner ventral curvature of the spermatid head during spermiation (Guttman *et al.* 2004), as TBCs predominantly arise in this area (Russell 1993). This staining pattern (Guttman *et al.* 2002) is clearly distinct from the localisation of integrins and FAK-Tyr³⁹⁷ on the outer dorsal curvature of the spermatid head in the present study. Thus, the disengagement complex as described here is likely to be structurally and functionally distinct from TBCs.

Lastly, this study investigated whether the Sertoli cell microtubule network was involved in sperm release. It is well known that microtubules have a structural role as well as a transport role within the Sertoli cell (Russell *et al.* 1989, Vogl *et al.* 1993). Microtubules attach to the Sertoli cell ES and assist in the translocation of elongated spermatids prior to spermiation (Amlani & Vogl 1988, Russell *et al.* 1989, Vogl *et al.* 1993, Guttman *et al.* 2000) and are involved in the extension of the Sertoli cell stalk into the tubule lumen during spermiation (Russell 1993). Given their translocation and structural functions within the seminiferous epithelium, we hypothesised that microtubules may be involved in spermatid release and/or retention after hormone suppression. The immunolocalisation of tubulins, co-localised with the ES protein espin, was employed to map the localisation of tubulins during spermiation. The results presented here suggest that microtubules, while assisting in spermatid translocation, do not appear to play a direct role in spermatid release, since tubulins did not associate with spermatids at the

time of disengagement. Likewise, microtubules did not appear to be involved in the initial retention of spermatids during spermiation failure. However, given their abundance and role in transport within the Sertoli cell, reviewed in Vogl *et al.* (1993), they may well be involved in the translocation of the retained spermatids down to the basement membrane after they have been phagocytosed by the Sertoli cell. We did not see any difference in the immunolocalisation patterns between controls and gonadotrophin-suppressed seminiferous tubules prior to, or after spermatid release, suggesting that microtubule localisation is not hormonally regulated.

In summary, this study provides further insights into mechanisms of spermatid release during spermiation and the retention of spermatids after gonadotrophin suppression-induced spermiation failure. The microtubule network, while may be providing structural and movement roles, does not appear to be directly involved in the disengagement or in the retention of spermatids. However, a disengagement complex containing $\alpha_6\beta_1$ -integrin and FAK-Tyr³⁹⁷ is present between Sertoli cells and elongated spermatids during spermiation until spermatids are released from the epithelium. The results support the contention that a defect in the function of the disengagement complex is involved in spermatid retention during gonadotrophin suppression.

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