Sertoli–immature spermatids disengagement during testis regression in the armadillo

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Abstract

In nature, mammalian seasonal breeders undergo spermatogenetic arrest during the non-breeding season. In the large hairy armadillo *Chaetophractus villosus*, testis regression initiates with immature post-meiotic germ cells sloughing into the tubule lumen and continues with the death of the remaining spermatocytes. At the end of the regression period, only spermatogonia and Sertoli cells persist in the seminiferous epithelium. It has been suggested that cell sloughing is determined by changes in the adhesion complexes between Sertoli cells and spermatids, which are mediated by low intra-testicular testosterone levels. By immunofluorescence and Western blotting we studied key proteins of the N-cadherin/N-cadherin and A6B1-integrin/laminin interlocks that contribute to the complex Sertoli/spermatid adhesion system throughout the eight stages of the seminiferous epithelium cycle in the comparison between active and regressing testes. In active testis, B1-integrin, laminin G3, N-cadherin, B-catenin, P-B-catenin-Tyr⁶⁵⁴, FAK, P-FAK-Tyr³⁹⁷, SRC, P-SRC-Tyr⁴¹⁶ proteins present a spermatogenetic cycle-dependent localisation pattern, unmaintained in regressing testes. In the latter, quantitative variations and changes in the phosphorylation state of protein FAK, SRC and B-catenin contribute to the disassembly of the N-cadherin/N-cadherin and A6B1-integrin/laminin interlocks, thus promoting the massive release of immature spermatids.

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Introduction

Seasonal reproduction in the non-equatorial regions of the planet is an adaptive strategy aimed at assuring newborns the best environment for survival. During the non-reproductive season, block of the ovarian function and reduction or interruption of the testis activity determines the inability to produce offspring (for a review [Jimenez et al. 2015](#)). Spermatogenetic arrest has been described in several mammalian species including the Iberian mole ([Dadhich et al. 2010, 2013](#)), the hamster ([Bex & Bartke 1977](#)), the black bear ([Tsubota et al. 1997](#)), the white-footed mouse ([Young et al. 1999](#)) and the large hairy armadillo *Chaetophractus villosus* whose seminiferous epithelium undergoes, once a year, rapid regression ([Luaces et al. 2013, 2014](#)).

In *C. villosus*, two subsequent waves of germ cell loss lead to the sole presence of spermatogonia and Sertoli cells in fully regressed testes. During the first wave, live immature spermatids exfoliate and drop into the

tubule lumen, where they initiate apoptosis. Then, in a second wave, residual germ cells complete apoptosis and are eliminated by the phagocytic activity of Sertoli cells ([Luaces et al. 2014](#)). Reduction in the extension of vimentin intermediate filaments and altered localisation of the integral membrane nectin-3, N-cadherin and the adaptor β -catenin proteins suggest that the dissociation of adherens junctions (AJ) at the Sertoli–spermatids interface is involved in this massive shedding of immature post-meiotic germ cells ([Luaces et al. 2014](#)). Similarly, in the Iberian mole, altered localisation of the Sertoli germ cell adhesion proteins B-catenin, N-cadherin and E-cadherin was associated to germ cell desquamation leading to testis regression during the non-reproductive season ([Dadhich et al. 2013](#)).

In both the large hairy armadillo and the Iberian mole, the rapid decrease of intra-testicular and serum testosterone levels correlate with massive post-meiotic germ cell depletion ([Dadhich et al. 2013, Luaces et al. 2014](#)). In the rat, testosterone withdrawal experiments

have shown a causal link between hormone levels and the detachment of immature spermatids from the seminiferous epithelium by compromising the apical ectoplasmic specialisation (ES) (O'Donnell *et al.* 2000, Zhang *et al.* 2005, for a review Lui & Lee 2009). Apical ES are testis-specific anchoring junctions present at the Sertoli–spermatid (step 8 and beyond in the mouse, Russell & Malone 1980) interface, formed by multiple types of cell junctions such as AJ (N-cadherin/B-catenin and nectins/afadin complexes), focal contacts (A6B1-integrin/laminin 333 complexes) and tight junctions (e.g. Jam-C) (for a review Berruti & Paiardi 2014). In the rat, when testosterone levels are experimentally reduced, integrins of the A6B1-integrin/laminin G3 complex dissociate from the focal adhesion kinase (FAK)/c-SRC complex present in the Sertoli cell cytoplasm, while B1-integrin protein levels increase together with Tyr phosphorylation of both FAK and of SRC (P-FAK and P-SRC; for a review Wong *et al.* 2008). The increment of the P-FAK/SRC complex levels likely activates the extracellular signal-regulated kinase pathway, which favours the activity of the matrix metalloprotease 2 (MMP2). In testosterone-suppressed testis, dissociation of the N-cadherin/N-cadherin interlocks also correlates to germ cell loss (Zhang *et al.* 2005). Compromised integrity of the cadherin/cadherin interlocks is mediated by phosphorylation of B-catenin at Tyr⁶⁵⁴ (P-B-catenin) (Zhang *et al.* 2005), the latter a peripheral adaptor protein of the actin-linked N-cadherin/B-catenin complexes. Increase in Tyr phosphorylation of B-catenin determines the loss of protein–protein interactions between N-cadherins and B-catenins and between B-catenin and SRC (which binds the cadherin/catenin complex via catenin, Lee & Cheng 2005) contributing to AJ disruption (Zhang *et al.* 2005).

Overall, these studies on testosterone-depletion model animals indicate that the quantity and phosphorylation state of key proteins of the cell-anchoring junctions are critical for the stability of the apical ES, whose loss of integrity leads to the release of immature spermatids. This knowledge is lacking for the large hairy armadillo, a seasonal reproducer in which, during the circannual cycle of its testis functional arrest, a seminiferous epithelium regression occurs under a 12-fold decrease of the intra-testicular-testosterone level (Luaces *et al.* 2013).

Our study was aimed at understanding whether the disengagement of immature post-meiotic cells occurring at the beginning of the seminiferous epithelium regression associates with qualitative/quantitative and localisation changes of key proteins of the N-cadherin/N-cadherin and A6B1-integrin/laminin 333 complexes. To this end, following immunofluorescence, B1-integrin, laminin G3 (at the A6B1-integrin/laminin 333 interlocks), N-cadherin, B-catenin, P-B-catenin-Tyr⁶⁵⁴ (at the N-cadherin/N-cadherin interlocks), FAK, P-FAK-Tyr³⁹⁷, SRC and P-SRC-Tyr⁴¹⁶ proteins were

localised throughout the eight stages of the seminiferous epithelium cycle and the 13 steps of spermatid differentiation in the comparison between active and regressing testes. Their quantitative variations were evaluated by immunoblotting. We report that massive post-meiotic germ cell sloughing correlates with: (1) *in situ* altered localisation of B1-integrin, laminin G3, FAK, P-FAK, N-cadherin, B-catenin, P-B-catenin, SRC and P-SRC proteins; (2) decreased quantity of B-catenin, FAK and SRC proteins whereas (3) their phosphorylated forms and B1-integrin levels increased.

Materials and methods

Testes collection and histology

Armadillos were caught in the area of Loma Verde (35°16'S; 58°23'W; <http://g.co/maps/sswm>, Province of Buenos Aires, Argentina) during several trapping campaigns from 2008 to 2012. Permissions were given by the Ministerio de Asuntos Agrarios de la Provincia de Buenos Aires. Animal handling was carried out in strict accordance with UK Animals Scientific Procedures Act and approved by the local Comité Institucional para Cuidado y Uso de Animales de Laboratorio (CICUAL-UM).

From each trapped male, the left testis was withdrawn under anaesthesia (Luaces *et al.* 2013). Prior to fixation, the tip of the testes was cut and frozen at –80°C for subsequent protein extraction (see 'Immunoblotting analysis' section). Then, the tunica albuginea was carefully and rapidly perforated in different regions with a thin needle and testes dropped in 10% buffered formalin for 20 min to let them harden for subsequent cut into transversal slices (about 3 mm thick) with a sharp razor blade to fasten fixation. Testis slices were kept in the same fixative for further 24 h, washed in tap water, dehydrated through an ascending ethanol series and separately included in paraffin wax. For each animal, three 5 µm transverse cross-sections from a testis slice were analysed. Testis sections with a diameter larger than 20 mm were halved and each half was laid onto different slides. Sections were stained with periodic acid-Schiff (PAS)-hematoxylin reaction, useful to visualise the morphology of the developing acrosome (Leblond & Clermont 1952), as follows: following deparaffinisation, sections were treated with periodic acid (1% in distilled water, 6 min at room temperature, r.t.), rinsed in distilled water, stained with Schiff's reagent (5 min, r.t.), washed in running tap water (4 times, 5 min each), counterstained with Harris haematoxylin (8 min, r.t.), washed in running tap water (5 min), differentiated in 1% acidic alcohol (2 dips), washed in running tap water (1 min), blued in 0.2% ammonia water (1 min), washed in running tap water (5 min), immersed in 95% alcohol (10 dips), in Xylene (1 min) and mounted in Eukitt. Then, sections were analysed to determine the activity of the spermatogenetic process (Luaces *et al.* 2013, 2014) in order to select active and regressing testes. Twenty-seven testes were chosen, 13 of which were fully active whereas 14 were in regression. Table 1 reports, for each sample, the animal identification number, the testis activity status and the type of analysis performed.

Table 1 Animal identification codes, phase of testis activity, and sets of analyses carried out for each individual.

Animal number	Testis phase*	Histology	Immuno-fluorescence	Immuno-blotting
1024-5	Active	X	X	
1024-1	Active	X	X	X
1044-1	Active	X	X	X
1060-1	Active	X	X	
1060-3	Active	X	X	
1029	Active	X		X
984b	Active	X		X
984a	Active	X		X
1026	Active	X		X
978	Active	X		X
030	Active	X	X	
014	Active	X	X	
013	Active	X	X	
025	Regressing, I	X	X	
001	Regressing, I	X	X	
029	Regressing, I	X	X	
016	Regressing, I	X	X	
1051-1	Regressing, I	X		X
1050-3	Regressing, I	X		X
1051-3	Regressing, I	X		X
011	Regressing, I/II	X		X
007	Regressing, I/II	X	X	
028	Regressing, I/II	X	X	
965	Regressing, I/II	X		X
960	Regressing, II	X		X
002	Regressing, II/III	X	X	
1050-1	Regressing, III	X		X

*Roman numbers refer to the regression phase of the seminiferous epithelium (Luaces *et al.* 2014).

Immunofluorescence

As for the histology, 5 µm transverse cross-sections were cut from slices of eight active and seven regressing testes (Table 1). One transverse cross section was mounted onto each slide. Testis sections with a diameter larger than 20 mm were halved and laid onto two slides. For each testis and for each primary antibody (Table 2), immunofluorescence was separately performed onto two independent slides. Antigen retrieval was performed in 10 mM sodium citrate (product no. S4641; Sigma) and 0.05% Tween 20 (product no. P9416; Sigma), pH 6.0 at 95°C for 20 min (Shi *et al.* 1993). Then, sections were incubated with 1% v/v Fetal Bovine Serum (FBS, product no. 10270-106; Gibco) in 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄) for 30 min at 37°C to prevent non-specific binding of the primary antibody. Primary antibodies were applied at the dilutions reported in Table 2 in 1% v/v FBS/1× PBS and incubated overnight at 4°C, with the exception of B-catenin and SRC antibodies that were incubated at 37°C for 1 h. After three washes in 1× PBS, sections were incubated with goat AlexaFluor 555-conjugated anti-rabbit IgG (product no. A21428, Thermo Fisher Scientific) or with sheep Cy3-conjugated anti-mouse (product no. C2181, Sigma) or goat AlexaFluor 633-conjugated anti-rabbit (product no. A-21070, Thermo Fisher Scientific) secondary antibodies diluted 1:400 in 1% v/v FBS/1× PBS for 30 min at 37°C; then slides were washed again, counterstained with 0.1 µg/mL 4',6'-diaminido-2-phenylindole (DAPI; product no. D8417,

Sigma) and mounted with VECTASHIELD Mounting Medium (product no. H-1000; Vector Laboratories). In negative controls, primary antibodies were omitted.

Image analysis

Histological sections staining with PAS were examined with an Olympus BX60 epifluorescence microscope equipped with a Tango motorised Z-axis (Märzhäuser Wetzlar, Wetzlar, Germany) and digital images were captured (under 100× objective) using an Olympus DP72 digital camera controlled by Olympus CellSens Dimension 1.4.1 software.

Antibody immunofluorescence localisation of SRC, P-SRC, FAK, P-FAK, laminin G3 and P-B-catenin was analysed with a Leica TCS SP5 II confocal microscope equipped with lasers for AlexaFluor 555, Cy3 and DAPI fluorochromes. Antibody immunofluorescence localisation of N-cadherin, B-catenin and B1-integrin was analysed with a Zeiss LSM800 confocal microscope equipped with lasers for AlexaFluor 555, AlexaFluor 633 and DAPI fluorochromes. Stacks were obtained with axial distances of 0.5 µm.

Immunoblotting analysis

Seven active and seven regressing testis samples (Table 1) were homogenised in RIPA buffer with protease inhibitors (150 mM NaCl, 5 mM EDTA, pH 8.0, 50 mM Tris-HCl, 1.0% v/v NP-40 (product no. 28324, Thermo Fisher Scientific), 0.5% sodium deoxycholate, 0.1% SDS (product no. 71725, Sigma), 5 µg/mL Protease Inhibitor Cocktail (product no. P2714, Sigma)). Protein suspension was centrifuged for 10 min at 13,000g and concentration of proteins in the supernatant was measured using the Bradford's method (Bradford 1976). Ten microgram per sample were mixed with gel loading buffer (2% w/v SDS in Tris-HCl (pH 8), 25% glycerol (product no. G7757, Sigma), 1% v/v 2-mercaptoethanol (product no. M3148, Sigma) and 0.1% w/v bromophenol blue (product no. B8026, Sigma)). Samples were then denatured for 5 min at 95°C and separated onto 8% v/v (P-FAK, FAK, B1-integrin, N-cadherin, laminin G3, actin and GAPDH) or 12.5% v/v (P-B-catenin, B-catenin, P-SRC, SRC, MMP2 and actin) SDS-polyacrylamide mini-gels. After gels were dipped in the transfer buffer (25 mM Tris-HCl, 192 mM glycine (product no. G8898, Sigma) and 20% v/v methanol (product no. 322415, Sigma)) for 30 min, proteins were blotted on a nitrocellulose membrane (product no. 162-0145, Bio-Rad Laboratories). Membranes containing the proteins were blocked for 30 min at room temperature with 3% bovine serum albumin (BSA, product no. A3311, Sigma) in TBS-T buffer (2 mM Tris-HCl, 13.6 mM NaCl and 0.1% v/v Tween-20 (pH 7.6)), in gentle agitation, and then incubated for 2 h at 37°C with the primary antibodies, diluted in TBS-T buffer (for antibody concentration see Table 1). After washing with TBS-T buffer, the membranes were incubated with goat HRP conjugated anti-rabbit (product no. A9169, Sigma) (for B1-integrin, P-FAK, FAK, B-catenin, P-B-catenin, SRC, MMP-2, N-cadherin, laminin G3 and GAPDH) or goat HRP-conjugated anti-mouse (product no. F21453, Molecular Probes; for p-SRC) IgG antibodies for 30 min at 37°C, using a 1:25,000 dilution in TBS-T buffer, in gentle agitation, and then washed

Table 2 Primary antibodies used, their source, application and dilution.

Antibody	Source	Catalogue number	Application and dilution
Rabbit anti-human integrin-B1	Merck Millipore	1952	IF (1:100) IB (1:5000)
Rabbit anti-human FAK	Cell Signalling	3285	IF (1:100) IB (1:5000)
Rabbit anti-human phospho-FAK (Tyr397)	Cell Signalling	3283	IF (1:50) IB (1:5000)
Rabbit anti-human SRC	Cell Signalling	2108	IF (1:200) IB (1:5000)
Mouse anti-avian phospho-SRC (Tyr416)	Merck Millipore	05-677	IF (1:50) IB (1:5000)
Rabbit anti-human MMP2	Abcam	79781	IB (1:5000)
Rabbit anti-human B-catenin	Abcam	32572	IF (1:250) IB (1:5000)
Rabbit anti-human N-cadherin	Abcam	12221	IF (1:100) IB (1:2000)
Rabbit anti-human phospho-B-catenin (Tyr654)	ECM Biosciences	4021	IF (1:50) IB (1:5000)
Mouse anti-mouse laminin G3	Santa Cruz Biotechnology	517444	IB (1:2000)
Rabbit anti-human laminin G3	Thermo Fisher Scientific	PA5-38939	IF (1:200)
Goat anti-human actin, HRP conjugated	Santa Cruz Biotechnology	1615-HRP	IB (1:10,000)
Rabbit anti-human GAPDH	GeneTex	100118	IB (1:5000)

IB, immunoblotting; IF, immunofluorescence microscopy.

thoroughly. Chemiluminescent detection was performed using the Westar Supernova (product no. XLS3, Cyanagen), suitable for low femtogram samples, according to the manufacturers' instructions that recommend a primary antibody dilution between 1:5000 and 1:100,000.

For each antibody used, species cross-reactivity was determined by Western blot comparison between armadillo protein extracts and those obtained from the species to which antibodies were raised or cross-react (as detailed in the manufacturers' datasheets).

Since immunoblotting of GAPDH and actin proteins did not show any different quantitative expression in the comparison between active and regressive testes ($n=3$ for each condition, data not shown), we used the latter protein as loading control. Moreover, being equally expressed ($P=0.787$) in both active and regressing animals, actin was used to normalise the expression of target proteins. The chemiluminescent blots were imaged with ChemiDoc XRS system (Bio-Rad) and acquired with the Quantity One software (Bio-Rad). Densitometric intensities of the bands were determined with ImageJ software (Schneider *et al.* 2012).

Statistical analysis

Statistical analyses were carried out using Sigma Stat 3.0 software. After immunoblotting, ImageJ software was used to quantify the intensity of protein bands, and data were expressed as arbitrary units of pixel density following normalisation on actin. For each protein, at least five animals per active or regressing group were analysed.

The one-way ANOVA was used for intra- and inter-group comparisons when the values of protein expression were normally distributed. The non-parametric Mann-Whitney test was used when protein expression values were not normally distributed. Non-parametric data are expressed as the median

and first and third quartiles (25% and 75%). For each target protein, fold-change (FC) variations of the mean of normalised values in regressing compared to active testes were determined.

Results

Following the criteria for stage identification described earlier in Luaces *et al.* (2013), B1-integrin, laminin G3, N-cadherin, FAK, P-FAK-Tyr³⁹⁷, SRC, P-SRC-Tyr⁴¹⁶, B-catenin and P-B-catenin-Tyr⁶⁵⁴ proteins were first localised in active testes throughout the eight stages of the cycle of the seminiferous epithelium. Then, the same proteins were mapped in the seminiferous epithelium of regressing testes.

B1-integrin and laminin G3

The A6B1-integrin/laminin 333 interlocks are among the multiprotein complexes present at the apical ES-associated AJ structures. B1-integrin is an integral membrane protein of the Sertoli cells that associates, on the cytoplasm side, with the FAK/c-SRC complex to make a cell matrix focal adhesion-like structure (Siu *et al.* 2011). In the extracellular compartment, it binds to the laminin 333 (A3, B3 and G3 chains) complex present on the elongating/elongated spermatids membrane (Siu & Cheng 2004, Yan & Cheng 2006). In the armadillo, B1-integrin and laminin G3 immunofluorescence (Fig. 1) localise at the head of round (Fig. 1k–kⁱ and s–sⁱ, respectively; arrowheads) and elongating (Fig. 1i–jⁱ and n–xⁱ) spermatids, until when mature spermatids are released into the tubule lumen (stage V) (Fig. 1m–mⁱ and u–uⁱ). Stage I and II round spermatids are negative (Fig. 1i–jⁱ and q–rⁱ).

FAK and P-FAK-Tyr³⁹⁷

FAK is a cytoplasmic non-receptor protein kinase expressed by both Sertoli and germ cells, mainly localised at the blood–testis barrier (BTB) near the basal membrane, and a modulator of the tight junctions (TJ) integrity. The downregulation or upregulation of its expression, together with its phosphorylation status at Tyr⁴⁰⁷, timely regulates the dismantlement or reconstitution of the blood–testis barrier (BTB) (Siu *et al.* 2009, Lie *et al.* 2012). In the seminiferous

epithelium of *C. villosus*, FAK immunosignals localise in the basal compartment with a clear diffused pattern around spermatogonia (Fig. 2a–hⁱ, empty arrowheads), throughout the eight stages of the cycle, in accordance with its presence in the TJ at the BTB.

Next, we examined the localisation of the FAK phosphorylated form at Tyr³⁹⁷ (P-FAK). FAK autophosphorylation is involved in the separation of the FAK–integrin complex at the A6B1-integrin/laminin G3 interlocks and contributes to the disassembly of the apical ES (Beardsley & O'Donnell 2003, Beardsley *et al.* 2006,

Figure 1 Periodic acid-Schiff-hematoxylin (PAS, a–h) of cross-sections depicting the eight stages (annotated with Roman numeral) of the cycle of the seminiferous epithelium in active testes. The eight stages are defined by characteristic cell-specific associations, the progressive changes of spermatid nuclei shape and the acrosome formation (magenta staining) (Luaces *et al.* 2013). During spermiogenesis, spermatid nuclei acquire a characteristic spoon-like shape, very large in size and extremely thin (Cetica *et al.* 1998). The loosely packed chromatin (Luaces *et al.* 2013) within a large and flat nucleus results in very dim DAPI fluorescence (red pseudocolor). Confocal images of anti-β1-integrin (i–p) and anti-laminin G3 (q–x) immunofluorescence staining; nuclei are counterstained with DAPI (red pseudocolor) and merged with the corresponding immunofluorescence images (iⁱ–pⁱ) and (qⁱ–xⁱ). The signal is present, as a small rod, at stage III round spermatid heads (k–kⁱ and s–sⁱ, respectively; arrowheads). Then, it progressively extends and, at stage VIII, a thin thread outlines the anterior-lateral head region of elongating spermatids (p–pⁱ, x–xⁱ, arrows). The same pattern is maintained until when mature spermatids are released into the tubule lumen (stage V) (m–mⁱ, u–uⁱ, arrows). Stage I and II round spermatids were negative (i–jⁱ; q–rⁱ, arrowheads). The signal is also positive at metaphase spermatocytes (p–pⁱ, x–xⁱ, arrowheads). Spermatogonia, empty arrowheads. Spermatocytes, double arrowheads. round spermatids, arrowheads; elongating spermatids, arrows. Bar, 20 μm.

Lie *et al.* 2012, Wan *et al.* 2014). In the seminiferous epithelium of *C. villosus*, P-FAK immunolabeling is restricted to stages III–V (Fig. 2k–mⁱ), where it is diffused in both basal and adluminal compartments with higher intensity at step 13 elongated spermatids (Fig. 2m–mⁱ, arrows), the latter ready to be released into the tubule lumen.

N-cadherin, B-catenin and P-B-catenin-Tyr⁶⁵⁴

In the testis, N-cadherin is a calcium-dependent cell–cell integral membrane adhesion glycoprotein that associates with its cytoplasmic interacting partner, B-catenin, to form the actin-linked N-cadherin/B-catenin complex of the cadherin/cadherin interlocks (Zhang *et al.* 2005, Lui & Lee 2009). These interlocks contribute to the AJ stability present at both apical and basal ES (Salanova *et al.* 1995, Ozaki-Kuroda *et al.* 2002, Lee *et al.* 2003, Siu *et al.* 2003, 2011, Gliki *et al.* 2004).

In the armadillo, using double immunolabeling with anti-N-cadherin and anti-B-catenin antibodies, fluorescence signals were always found to co-localise at spermatogonia (Fig. 3a–hⁱⁱ, empty arrowheads), spermatocytes (double arrowheads) and elongating/elongated spermatids (arrows) throughout the eight stages of the seminiferous epithelium cycle. Both signals co-localise also at pre-leptotene (stage VI, Fig. 3f–fⁱⁱ, empty arrows) and leptotene (stage VII, Fig. 3g–gⁱⁱ, empty arrows) spermatocytes and, from stage III, at round spermatids (Fig. 3e–eⁱⁱ, arrowheads).

Since a surge of Tyr⁶⁵⁴ phosphorylation of B-catenin (P-B-catenin), likely induced by the SRC kinase activity (Zhang *et al.* 2005), has been associated, in the rat, with loss of N-cadherin-B-catenin bonds followed by germ cell detachment (Zhang *et al.* 2005), the localisation of this phosphorylated form was performed to verify its presence at the Sertoli cell/spermatid junctions. P-B-catenin immunostaining is negative at round spermatids (Fig. 3b–jⁱ, arrowheads) and at stage VI elongating spermatids (Fig. 3l–lⁱ); then, it becomes positive at the tip of stage VII elongating spermatids (Fig. 3n–nⁱ, arrows) and, progressively, it decorates the elongating spermatid heads (Fig. 3b–jⁱ, arrows) until when, at stage VI, elongated spermatids are released into the tubule lumen.

SRC and P-SRC-Tyr⁴¹⁶

SRC, a non-receptor protein tyrosine kinase member of the SRC family, is a binding partner of FAK at the A6B1-integrin/laminin G3 interlocks (Yan & Cheng 2006) and of catenin at the cadherin/catenin complexes (Lee & Cheng 2005), where it modulates the phosphorylation status of several proteins (Zhang *et al.* 2005, Lie *et al.* 2010).

In the active large hairy armadillo testis, SRC diffuse signals localise within the Sertoli cell cytoplasm and brightly contour germ cells in both the basal and adluminal compartments throughout the eight stages

of the cycle of the seminiferous epithelium (Fig. 4a–hⁱ). Since SRC autophosphorylation contributes to the detachment of the activated FAK/c-SRC complex from integrins (Wong *et al.* 2008), we analysed its phosphorylated form at Tyr⁴¹⁶ (P-SRC). P-SRC showed a stage-specific distribution, stages IV (Fig. 4l–lⁱ) and V (Fig. 4m–mⁱ), with signals localised at elongated spermatids (arrows).

After having defined the localisation profiles of these proteins in active testes, we analysed individuals at the initial phases of testis regression, when premature post-meiotic cells are detached, but still present, in the tubule lumen (Luaces *et al.* 2014).

Apical ES dynamics in the seminiferous epithelium of regressing testes

Armadillo testis regression entails four phases of progressive cell depletion, which initiates with round spermatids prematurely detached (phase I) and released into the lumen leading to massive desquamation (phase II). Then, spermatocytes are eliminated through apoptosis (phase III) and regression ends with the sole Sertoli cells and spermatogonia remaining in the seminiferous epithelium (phase IV) (Luaces *et al.* 2014).

Proteins were localised in the seminiferous epithelium during the desquamation process occurring at phases I and II (Figs 5, 6, 7 and 8) and their quantitative expression was compared in regressing vs active testes (Fig. 9).

B1-integrin and laminin G3

At both regression phase I (Fig. 5a–c) and II (Fig. 5d), diffused B1-integrin signals are visible at both basal and antral compartments. Thin immunofluorescent threads decorate the head of elongated spermatids (arrows) from stage I to V at regression phase I (Fig. 5e–fⁱ) and at regression phase II (Fig. 5h and hⁱ). The same pattern was detected when using an anti-laminin G3 antibody (Fig. 5i–lⁱ).

When compared to active, B1-integrin expression was 7.06-fold upregulated in regressing testes (Fig. 9B), showing a significantly ($P=0.022$) higher value (median value, $M=2.87$ and $M=0.48$, regressing vs active testes) (Fig. 9A). Laminin G3 was also 1.97 upregulated in regressing (median value, $M=1.5$) vs active (median value, $M=0.79$) testes, although without statistical significance ($P=0.062$; Fig. 9A and B). Since, in the rat, a surge of MMP2 has been described at the time of spermiation, and it has been found to co-localise with laminin G3 at the apical ES (Siu & Cheng 2004), we questioned whether its quantitative level might increase during the massive release of immature post-meiotic cells in regressing testes. In fact, significantly ($P=0.010$) higher levels of proteins were measured in regressing ($M=0.42$) compared to active ($M=0.14$) testes (Fig. 9A), with a FC increase of 3.69 (Fig. 9B).

Figure 2 Confocal images of anti-FAK (a–h) and anti-P-FAK (i–p) immunofluorescence staining on cross-sections representative of the eight stages (annotated with Roman numeral) of the cycle of the seminiferous epithelium in active testes. Nuclei are counterstained with DAPI (red pseudocolor) and merged with the corresponding immunofluorescence images (a'–h' and i'–p'). FAK signals are present at the basal compartment throughout the eight stages of the seminiferous epithelium cycle. Post-meiotic cells are always negative (arrows and arrowheads). P-FAK-Tyr³⁹⁷ is temporally detected only at stages III–V (k–m'). Signals are visible at the basal compartment (empty arrowheads), at spermatocytes (double arrowheads) at stages IV and V and at round spermatids (m–m', arrowheads) at stage V. The signals contour elongated spermatids (arrows) with higher intensity at stage V (m–m'). Cells at stages I–II (i–j') and VI–VIII (n–p') are negative to the immunoreaction.

FAK and P-FAK

At regression phase I, FAK immunofluorescence showed diffused signals localised at the basal compartment at

the BTB region from stage I to VIII (Fig. 6a–c', empty arrowheads) spreading towards spermatocytes only at stages VI–VIII (Fig. 6c–c', double arrowheads). Both round and elongated spermatids were always negative

Figure 3 Confocal images of anti-N-cadherin (green), anti-β-catenin (red) and anti-P-β-catenin-Tyr⁶⁵⁴ (white) immunofluorescence staining on active testis sections. Nuclei are counterstained with DAPI (white, aⁱⁱ–hⁱⁱ, or red, iⁱ–pⁱ, pseudocolors) and merged with the corresponding immunofluorescence images. N-cadherin and β-catenin signals always co-localise. At stages I (a–aⁱⁱ) and II (c–cⁱⁱ), immunofluorescent signals are detectable at spermatogonia (empty arrowhead), spermatocytes (double arrowheads) and elongating spermatids (arrows) whereas they are undetectable at round spermatids (arrowheads). At stage III (e–eⁱⁱ), the signals mark both the Sertoli cell stalks at round (arrowheads) and elongating (arrows) spermatids. The same pattern is present at stage IV (g–gⁱⁱ). At stage V (i–iⁱⁱ), the signal remains strong at the basal compartment, marking spermatogonia (empty arrowhead), spermatocytes (double arrowheads), round (arrowheads) and elongated spermatids (arrows). At stages VI (k–kⁱⁱ) and VII (m–mⁱⁱ), immunofluorescence is delineating both pre-leptotene (empty arrows, bottom left) and leptotene (empty arrows) spermatocytes. At stage VIII (o–oⁱⁱ), co-localised signals are present at MI and MII spermatocytes (double arrowheads). P-β-catenin-Tyr⁶⁵⁴. Round (b–jⁱ, arrowheads), from stage I to stage V, and elongating spermatids (l–lⁱ) at stage VI are negative to the immunostaining. At stage VII, the head tips of step 7 elongating spermatids (n–nⁱ, arrows) become brightly decorated. At stage VIII, a thread-like signal extends from the anterior tip of the spermatid heads (p–pⁱ, arrows) and elongates towards the opposite pole (b–jⁱ) until when, at stage V, elongated spermatids are ready to be released into the lumen. The stages of the cycle of the seminiferous epithelium are annotated with Roman numeral. Bar, 20 μm.

(Fig. 2a–cⁱ). At regression phase II, the basal compartment was evidenced by diffused dim fluorescence (Fig. 6d–dⁱ, empty arrowheads). A 3.07-fold downregulation (Fig. 9B) was measured in regressing testes that had significantly ($P=0.035$) less relative protein expression ($M=0.19$) compared to active ($M=0.42$) testes (Fig. 9A).

Instead, P-FAK protein level was higher in regressing ($M=1.08$) compared to active ($M=0.50$) testes ($P=0.001$) (Fig. 9A), with a FC of 2.46 (Fig. 9B). When mapped on histological sections, at regression phase I, P-FAK dim signals were diffused at the basal compartment from stage I to stage V (Fig. 6e–fⁱ, empty

Figure 4 Confocal images of anti-SRC (a–h) and anti-P-SRC (i–p) immunofluorescence staining on active testis section. Nuclei are counterstained with DAPI (red pseudocolor) and merged with the corresponding immunofluorescence images (aⁱ–hⁱ and i–pⁱ). From stage I (a–aⁱ) to stage VIII (h–hⁱ) diffused SRC immunofluorescence is visible from the basal compartment to the lumen, located in the Sertoli cells cytoplasm surrounding spermatocytes (double arrowhead), round (arrowheads) and elongating (arrows) spermatids. At stages V (e–eⁱ) and VI (f–fⁱ) the immunosignal stains spermatogonia (empty arrowheads); at stage VII leptotene (g–gⁱ, empty arrowheads) and at stage VIII zygotene (h–hⁱ, empty arrowhead) spermatocytes. P-SRC-Tyr⁴¹⁶. Stages I–III (i–kⁱ) and VI–VIII (n–pⁱ) are negative to the immunostaining. At both stages IV (l–lⁱ) and V (m–mⁱ), fluorescence is localised at the adluminal compartment, surrounding the head of elongated spermatids (arrows) and protruding towards the lumen of the tubule with a rod-like shape. The stages of the cycle of the seminiferous epithelium are annotated with Roman numeral. Bar, 20 μ m.

arrowheads). At stages VI–VIII, bright signals localise at the tip of elongating spermatids (Fig. 6g–gⁱ, arrows). At regression phase II, P-FAK immunofluorescence is negative (Fig. 2h–hⁱ).

N-cadherin, B-catenin and P-B-catenin-Tyr⁶⁵⁴

At regression phase I, N-cadherin and B-catenin signals co-localise at spermatocytes (Fig. 7a–cⁱⁱ, double

Figure 5 Testis section stained with Periodic acid-Schiff-hematoxylin (PAS, a–d). At the onset of the process, regression phase I, epithelium dysregulation and cell detachment determine alterations of the typical cell associations that impede the precise identification of each stage of the cycle (annotated with Roman numeral). For this reason, the eight stages were grouped in representative categories (a–c). At regression phase II (d), consistent cell sloughing made stage assignment impossible. Confocal images of anti- β 1-integrin (e–hⁱ) and anti-laminin G3 (i–lⁱ) immunofluorescence staining on testis sections during regression phase I (a–c) and II (d). Nuclei are counterstained with DAPI (red pseudocolor) and merged with the corresponding immunofluorescence images (e–h and i–l). At both regression phase I and II, β 1-integrin and laminin G3 confocal images show diffused signals from the basal to the adluminal compartment. Only elongated spermatid heads (arrows, squared nuclei) are still decorated with a thin fluorescence thread. Nuclei are counterstained with DAPI (red pseudocolor). Spermatocytes, double arrowheads; round spermatids, arrowheads; elongating spermatids, arrows. Bar, 20 μ m.

arrowheads) throughout the stages of the seminiferous epithelium cycle. They also co-localise at elongating spermatids from stage I to stage V (Fig. 7a–bⁱ, arrows) and at round spermatids at stages IV–V (Fig. 7b–bⁱ, arrowheads). Spermatids at stages VI–VIII are negative (Fig. 7c–cⁱⁱ, arrows). Compared to active testes (Fig. 5), the pattern of N-cadherin localisation is more diffused at stages I–III (Fig. 7a and aⁱⁱ). At regression phase II, N-cadherin and B-catenin co-localise at leptotene and pachytene spermatocytes (Fig. 7d–dⁱⁱ, empty arrows and double arrowheads, respectively) and at elongated spermatocytes (Fig. 7d–dⁱⁱ, arrows). Desquamated round spermatocytes are also positive (arrowheads). Positive P-B-catenin signals mark the elongating spermatid heads at both regression phase I and II (Fig. 7e–hⁱ, arrows).

Both N-cadherin and B-catenin protein levels decrease (–1.4 and –2.67-folds, respectively) in regressing ($M=0.12$ and $M=0.45$, respectively) compared to active ($M=0.18$, $M=1.21$, respectively) testes, although those of N-cadherin were not significantly different ($P=0.057$; Fig. 9A and B). Instead, P-B-catenin protein level was 3.05-fold increased in regressing testes ($M=0.13$ and $M=0.07$ in regressing and active testes, respectively, $P=0.035$).

SRC and P-SRC

At regression phase I, SRC immunofluorescence contours spermatogonia (Fig. 8a–cⁱ, arrows), spermatocytes (Fig. 8a–cⁱ, double arrowheads), round

Figure 6 Confocal images of anti-FAK (a–d) and anti-P-FAK (e–h) immunofluorescence staining of testis sections during regression phase I and II. Nuclei are counterstained with DAPI (red pseudocolor) and merged with the corresponding immunofluorescence images (aⁱ–dⁱ and eⁱ–hⁱ). At regression phase I, FAK immunostaining is positive at the basal compartments marking spermatogonia (empty arrows). Diffused signals projects also towards spermatocytes (c–cⁱ, double arrowheads) at stages VI–VIII. At regression phase II, very dim signals are present at the basal compartment (d–dⁱ). At regression phase I, dim P-FAK immunofluorescence is present at the basal (e–f, empty arrowheads) and, more dispersed, at the adluminal (e–f, double arrowheads) compartment at stages I–V; whereas very bright signals are visible at the tip of elongating spermatid heads (arrows, g–gⁱ). P-FAK immunostaining is negative at regression phase II (h–hⁱ). The stages of the seminiferous epithelium cycle are annotated with Roman numeral. Bar, 20 μ m.

(Fig. 8a–bⁱ, arrowheads) and elongated spermatids (Fig. 8a–cⁱ, arrows), cells throughout the cycle stages. The same pattern is maintained at regression phase II (Fig. 8d–dⁱ), although with more diffused signals. Furthermore, the expression of this kinase is lower in regressing ($M=0.20$) compared to active ($M=0.37$) testes ($P=0.014$; $FC=-2.11$) (Fig. 9A). Instead, the level of its phosphorylated form (P-SRC) is 4.11-fold (Fig. 9B) higher in regressing ($M=1.18$) compared to active ($M=0.36$) testes ($P=0.008$) (Fig. 9A). At regression phase I, P-SRC immunofluorescence (Fig. 8e–gⁱ) has a very similar distribution of its non-phosphorylated form. At regression phase II, the signal becomes much brighter and diffused (Fig. 8h–hⁱ).

Discussion

In seasonal reproducers, the mechanisms regulating the process of germ cell desquamation during testis

regression is still poorly known. These wild animals have strong hormonal response to the natural succession of breeding and non-breeding seasons, showing, during the non-breeding season, very low circulating and intra-testicular testosterone levels (for a review, Jimenez *et al.* 2015). Both testosterone and gonadotropins are known to regulate the expression of cell adhesion proteins in the testis (Xia *et al.* 2005, Tarulli *et al.* 2008, McCabe *et al.* 2010) and, in testosterone-suppressing experiments in the rat, it has been shown that dissociation of the adhesion complexes at the apical ES contributes to the massive sloughing of immature spermatids (Zhang *et al.* 2005). Similarly, in armadillo regressing testes, immature spermatid release occurs under low testosterone levels (Luaces *et al.* 2013). In the present study, we provide evidence that the localisation and quantity of proteins of adhesion complexes present at the apical ES change during testes regression, likely compromising the integrity of the apical ES, resulting

Figure 7 Confocal images of anti-N-cadherin (green), anti-B-catenin (red) and anti-P-B-catenin (white) immunofluorescence staining during regression phase I and II. Nuclei are counterstained with DAPI (white, aⁱⁱ–dⁱⁱ, or red, eⁱ–hⁱ, pseudocolors) and merged with the corresponding immunofluorescence images. At both regression phase I and II, N-cadherin and B-catenin signals always co-localise. At regression phase I with positive signals at spermatocytes (a–cⁱⁱ, double arrowheads), at elongating spermatids from stage I to stage V (a–bⁱ, arrows) and at round spermatids at stages IV–V (b–bⁱ, arrowheads). At regression phase II, N-cadherin and B-catenin co-localise at leptotene and pachytene spermatocytes (d–dⁱⁱ, empty arrows and double arrowheads, respectively) and at elongated spermatocytes (d–dⁱⁱ, arrows). Desquamated round spermatocytes (right to the dashed line) are also positive. Elongating spermatid heads are positive to the P-B-catenin immunosignals at both regression phase I and II (e–hⁱ, arrows). At the latter regression stage, the positive spermatids are present in the tubule lumen (right to the dashed line). The stages of the seminiferous epithelium cycle are annotated with Roman numeral. Bar, 20 μm.

in Sertoli cells/immature spermatids disengagement. Specifically, while in active testes, proteins of the A6B1-integrin/laminin 333 and N-cadherin/B-catenin adhesion complexes localise in the seminiferous epithelium with a spermatogenic cycle-dependent pattern, this specific pattern is lost in regressing testes. In the latter, the relocation of B1-integrin, laminin G3, FAK, P-FAK, SRC, P-SRC, N-cadherin, B-catenin and P-B-catenin proteins, as conceptualised in Fig. 10, may reflect the dissociation of the A6B1-integrin/laminin 333 and N-cadherin/B-catenin interlocks, thus contributing to immature post-meiotic germ cell release from Sertoli cells. Dissociation of these interlocks might be mediated

by changes of protein levels and of the phosphorylation state of FAK, SRC and B-catenin (see below).

In the active testis, the localisation of the protein components of A6B1-integrin/laminin 333 and N-cadherin/B-catenin adhesion complexes throughout the eight stages of the seminiferous epithelium cycle allowed to highlight the formation of these interlocks in relation to the progression of differentiating germ cell across the epithelium. A6B1-integrin/laminin 333 and N-cadherin/N-cadherin interlocks formation was marked by the presence of small laminin G3, B1-integrin and of N-cadherin signals associated with round spermatids at stage III of the cycle of the seminiferous epithelium.

Figure 8 Confocal images of anti-SRC (a–d) and of anti-P-SRC (e–h) immunofluorescence staining of testis sections during regression phase I and II. Nuclei are counterstained with DAPI (red pseudocolor) and merged with the corresponding immunofluorescence images (aⁱ–dⁱ and eⁱ–hⁱ). Both SRC and P-SRC contour spermatogonia (empty arrowheads), spermatocytes (double arrowheads), round (arrowheads) and elongating (arrow) spermatids. At regression phase II, P-SRC (h–hⁱ) is very bright. The stages of the seminiferous epithelium cycle are annotated with Roman numeral. Bar, 20 μ m.

Then, the signals progressively decorate the spermatid heads until mature sperm are released into the tubule lumen at stage VI. This stage-specificity of the signals might reflect the timing of these adhesion complexes formation and dismantlement.

Interestingly, in the large hairy armadillo, apical ES formation seems to precede spermiation, differently from the rat, where the two events occur simultaneously. Apical ES may either replace desmosomes and gap junctions earlier than in rodents or coexist until they become the only anchoring structure conferring adhesion and polarity to spermatids (Mruk & Cheng 2004, Wong *et al.* 2008, Vogl *et al.* 2008, Cheng & Mruk 2010).

Similarly to B1-integrin, laminin G3 and N-cadherin the two non-receptor protein tyrosine kinases FAK and its binding partner SRC show specific localisation. While FAK is present near the basement membrane at the BTB, its phosphorylated form at Tyr³⁹⁷ is detectable at elongating spermatids at stages that just precede spermiation suggesting that, also in the armadillo, FAK phosphorylation is associated with the disassembly of the integrin/laminin G3 interlocks at the apical ES (Lie *et al.* 2012). This latter event appears to be mediated also by

the presence of its binding partner, SRC, suggesting SRC-mediated phosphorylation in the process of spermatid release (Wang *et al.* 2000, Lee & Cheng 2005, Xiao *et al.* 2014). To this regard, the presence of P-SRC-Tyr⁴¹⁶ has been detected at mature spermatids, lining up at the lumen of the tubule, only at stages IV and V. It has been suggested that c-SRC is a mediator of the disassembly of the apical ES and of the short-lived apical tubulobulbar complex, facilitating spermatid release (for a review see Chojnacka & Mruk 2015).

B-catenin, a peripheral adaptor protein of the cadherin–cadherin interlocks, co-localises with N-cadherin at spermatogonia, spermatocytes, round and elongating spermatids, marking their presence at the junction sites between Sertoli and germ cells. Instead, P-B-catenin-Tyr⁶⁵⁴ brightens at the head of elongating spermatids suggesting that the balance between the phosphorylated and non-phosphorylated form might be involved in the maintenance of both their polarisation (Chang *et al.* 2011) and back-and-forth changes of localisation (for a review Xiao *et al.* 2014). It is worth reminding that phosphorylation of B-catenin decreases its affinity for E-cadherin reducing cell

Figure 9 (A) Box plot of relative protein expression in active (A) or regressing (R) testes. Each box comprises 50% of the data (including second and third quartiles) and is bisected by the median value. The error bar indicates the min and max values recorded. The non-parametric Mann–Whitney test (see ‘Results’ section for details) revealed, for each protein, statistically significant differences when comparing the expression levels between A and R testes. For each target protein, representative immunoblotting images are reported for both A and R testes. Actin was used as quantitative reference marker. (B) Fold-change expression of mean quantitative protein values in the comparison between A and R testes. Protein quantitation is based on five blots, from five different animals, for each protein analysed, with the exception of laminin G3 that was quantified on three blots from three different animals.

adhesion (Miravet *et al.* 2003, Lilien & Balsamo 2005), thus contributing to the maintenance of a dynamic state of attachment/release of these junctions at the apical ES.

With the beginning of the non-reproductive season, occurring in autumn (May–June), when males display consistent drop of gonadal testosterone levels (Luaces *et al.* 2014), the quantitative pattern of expression of these adhesion proteins and their localisation changes significantly. The absence of B1-integrin/laminin G3 signals at round spermatids suggests possible failure in the formation of these interlocks, at the time when, in the active testis, they are built (stage III). This lack of signal at round spermatids and the thin fluorescence

at elongating spermatids is paralleled by a major relocation of B1-integrin, SRC and P-SRC proteins within the Sertoli cell cytoplasm and of P-FAK at spermatids at the beginning of their elongation (stages VI–VIII). The observed relocation may reflect A6B1-integrins uncoupling from their FAK/c-SRC cytoplasmic partner complex mediated by the increased levels of the phosphorylated forms of the two kinases. Similarly, the different localisation profile and the increased quantity of P-B-catenin-Tyr⁶⁵⁴ indicate that also the cadherin–catenin complex might be dissociated, likely contributing to the disassembly of the cadherin/catenin complex at the apical ES. This scenario is supported

Figure 10 Schematic drawing of the histology of the seminiferous epithelium showing germ cells at different stages of their differentiation. In active testes the proteins of the A6B1 integrin/laminin G3 and N-Cadherin/N-Cadherin interlocks, which are part of the apical ectoplasmic specialisation are localised (red mark) at elongating and elongated spermatids. In regressing testes their quantity, localisation (red mark) and phosphorylation profile (purple mark) changes in correlation to cell desquamation.

by previous findings using testosterone-suppressed testes showing that the Src-mediated phosphorylation of the catenin/cadherin complex at both B-catenin and N-cadherin determines the disruption of their binding (Lilien & Balsamo 2005) and that of B-catenin, A-catenin and c-SRC thus contributing to the disassembly of the apical ES (Zhang *et al.* 2005, Lui & Lee 2009). However, functional experiments are needed to identify those biomolecule(s) involved in the regulation of FAK, p-FAK, SRC and p-SRC signalling proteins and their role in determining the release of immature spermatids from Sertoli cells in regressing testes.

In summary, the use of a number of key proteins of the A6B1-integrin/laminin 333 and N-cadherin/B-catenin interlocks contributed to fill in a gap of knowledge on the Sertoli/germ cell adhesion complexes during the cycle of the seminiferous epithelium in active testes and at the time of regression, as it occurs in nature during the circannual cycle. The extension of the taxonomic sampling to a Xenarthra species, one of the earliest offshoots among placentals (Asher *et al.* 2009), advances the present knowledge on how cell desquamation is executed at the beginning of the seminiferous epithelium regression in seasonal breeders.

Declaration of interest

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

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