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Differential expression profiles of conserved Snail transcription factors in the mouse testis

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Abstract

Snail transcription factors are key regulators of cellular transitions during embryonic development and tumorigenesis. The closely related SNAI1 and SNAI2 proteins induce epithelial-mesenchymal transitions (EMTs), acting predominantly as transcriptional repressors, while the functions of SNAI3 are unknown. An initial examination of *Snai2*-deficient mice provided evidence of deficient spermatogenesis. To address the hypothesis that Snail proteins are important for male fertility, this study provides the first comprehensive cellular expression profiles of all three mammalian Snail genes in the postnatal mouse testis. To evaluate Snail transcript expression profiles, droplet digital (dd) PCR and *in situ* hybridisation were employed. *Snai1*, *2*, and *3* transcripts are readily detected at 7, 14, 28 days postpartum (dpp) and 7 weeks (adult). Unique cellular expression was demonstrated for each by *in situ* hybridisation and immunohistochemistry using Western blot-validated antibodies. SNAI1 and SNAI2 are in the nucleus of the most mature germ cell types at postnatal ages 10, 15, and 26. SNAI3 is only detected from 15 dpp onwards, and is localised in the Sertoli cell cytoplasm. In the adult testis, *Snai1* and *Snai2* transcripts are detected in spermatogonia and spermatocytes, while *Snai3* is in both germ and Sertoli cells. SNAI1 protein is evident in nuclei of spermatogonia, spermatocytes, round spermatids, and elongated spermatids (Stages IX-XII). SNAI2 is present in the nuclei of spermatogonia and spermatocytes, with a faint signal detected in round spermatids. SNAI3 was detected only in Sertoli cell cytoplasm, as in the juvenile testes. Additionally, co-localisation of SNAI1 and SNAI2 with previously

identified key binding partners, LSD1 and PRC2 complex components, provides strong evidence that these important functional interactions are conserved during spermatogenesis to control gene activity. These distinct expression profiles suggest that each Snail family member has unique functions during spermatogenesis.

Keywords

Snail transcription factors – Postnatal mouse testis - Germ cells – Spermatogenesis - Cycle of the seminiferous epithelium

Introduction

Spermatogenesis is characterized by a series of essential cellular transitions that are required for the production of functional spermatozoa. Each spermatogenic stage involves changes in gene expression that are tightly regulated, and this study addresses the hypothesis that Snail transcription factors contribute to the correct progression of germline development.

Snail proteins belong to a large superfamily of zinc-finger transcription factors that play crucial roles in transcriptional regulation, cellular migration, chromatin remodelling, cell signalling and impact on many developmental processes (Batlle, Sancho et al. 2000, Kalluri and Weinberg 2009, Lin, Dong et al. 2014), including mesoderm formation and neural crest cell migration during embryogenesis (Alberga, Boulay et al. 1991). Additionally, Snail family members are overexpressed in various cancers where they are major regulators of cell survival, proliferation, and invasion (Turner, Broad et al. 2006, Uygur and Wu 2011).

The mammalian Snail family has three members, SNAI1, SNAI2, and SNAI3. Each features a highly conserved carboxy- (C) terminal region and a more divergent amino (N) terminal region, particularly for SNAI3 (Kataoka, Murayama et al. 2000, Nieto 2002). A conserved DNA-binding domain within the C-terminus contains four to six zinc fingers which directly interact with target genes to regulate their activity. The N-terminal region is composed of the SNAG transactivation domain, which interacts both directly and indirectly with co-factor proteins to form multi-molecular structures that drive target gene repression (Chiang and Ayyanathan 2013). Snail co-factors are chromatin remodelling enzymes. These include:

LSD1, HDAC1/2, EZH2, G9a and SUV39H1 (Lin, Dong et al. 2014), and several of these have critical roles in the reprogramming of the transcriptome during spermatogenesis, promoting both spermatogonial differentiation (Myrick, Christopher et al. 2017), and progression of meiosis (Mu, Starmer et al. 2014).

Spermatogenesis is a complex process by which mature spermatozoa are produced through a series of cellular divisions and maturation steps, each featuring a unique transcriptome (Ahmed and de Rooij 2009, Shalet 2009). Germ cells are strictly organised within the seminiferous epithelium formed by Sertoli cells, so that each generation of spermatogonia, spermatocytes and spermatids develops in parallel with subsequent generations, in a highly ordered, cyclic manner, termed Stages (Leblond and Clermont 1952). The cycle of the seminiferous epithelium of the adult mouse testis is considered as divided into 12 Stages (I-XII) (Oakberg 1956, Hess and Renato de Franca 2008). In mice, the first complete cycle of spermatogenesis commences at 7 days post-partum (dpp) and yields spermatozoa by 42 dpp. . Key time points corresponding to the first appearance of a particular germ cell type have been identified during the first wave of spermatogenesis. At 7 dpp, the mouse seminiferous cords contain only Sertoli cells and spermatogonia. At 14 dpp, spermatocytes are present within the tubules which form when post-mitotic Sertoli cells secrete fluids in an apical directions. As a result of meiotic divisions, round spermatids are first formed at 28 dpp. These haploid cells undergo morphological changes to first produce elongated spermatids at 30 dpp, and functional spermatozoa by 35 dpp (Fig. 1 a) (Kramer and Erickson 1981). The transition from one spermatogenic cell type to the next is governed by tight control of transcription (Eddy 1998). This progressive emergence of each more mature germ cell type during the first wave of spermatogenesis in juveniles provides a good opportunity to visualise how the dynamic changes in gene expression are coordinated by transcriptional regulators.

The Snai-deficient mice for which a testicular phenotype has been reported are those lacking *Snai2* which exhibit pigmentation, epithelial and hematopoietic defects (Cobaleda, Perez-Caro et al. 2007). The testis was only analysed in these *Snai2* deficient mice at six week of age; they display testicular atrophy, resulting from an apparent reduction in germ cell number (Perez-Losada, Sanchez-Martin et al. 2002), although this has not been quantitated. In addition, *Snai2* knockout mice are reported to be subfertile. Fertility was analysed in other Snai-deficient mouse models. Specifically, the complete absence of *Snai3* does not affect the testicular phenotype (Bradley, Norton et al. 2013), implying that SNAI3 is not necessary for spermatogenesis. However mice lacking both *Snai2* and *Snai3* have been reported to be

infertile (Pioli, Dahlem et al. 2013), suggesting that SNAI2 and SNAI3 are functionally redundant in the testis. This result is surprising given the structural differences between SNAI2 and SNAI3; this potential has not been investigated further. A different study, examining ubiquitin ligase *β-TrCP* knockout mice, provided indirect evidence that elevated SNAI1 levels can impair spermatogenesis. In this strain, the prominent nuclear SNAI1 immunostaining in spermatogonia suggested that SNAI1 was not targeted for proteasomal degradation and resulted in spermatogonial cell loss (Kanarek, Horwitz et al. 2010). However, analysis of the testes in both of these mouse models was limited. In the present study, ddPCR, *in situ* hybridisation and immunohistochemistry are used to document the cellular expression profiles of all three Snail family members in the normal postnatal mouse testis, including during pre- and post-pubertal development. The findings reveal distinct cellular expression profiles for the Snail mRNAs and proteins that are concordant with developmental switches during cellular differentiation. Importantly, we provide evidence that these co-localise with previously identified Snail co-factors, thereby providing a platform for understanding the potential functions of Snail family members during the cellular transitions of spermatogenesis.

Materials and Methods

Animal Ethics

All procedures involving animal samples were approved by the Monash Animal Research Platform (MARP) Ethics Committee and conformed to the National Health and Medical Research Council (NHMRC) of Australia's Code of Practice for the Care and Use of Animals for Experimental Purposes.

Mouse tissues and histological sections

For histology, C57Bl/6J mice were obtained from (MARP). Testis sections at various ages including 10, 15, 26 days post-partum (dpp) and adult (60-90 dpp) were first fixed in Bouin's or 4% PFA for 3-5 hrs, then paraffin-embedded using the Shandon Histocentre 3 (Thermo Electron Corporation) at the Monash University Histology Facility. Sections of 5 µm were dried onto Superfrost 2 glass slides (Menzel-Glaser, Braunschweig, Germany). *Snai2* knockout tissue was obtained from the *Snai2*^{LacZ} mouse line, generated by replacing the zinc

finger coding region of the *Snai2* gene with the β- galactosidase gene (Jiang, Lan et al. 1998). PFA-fixed adult mouse testis samples (*Snai2* $^{+/+}$, n=1; and *Snai2* $^{-/-}$, n=1) were kindly provided by Professor Donna Frances Kusewitt (MD Anderson, Smithville, Texas) to verify the specificity of the SNAI2 antibody.

Droplet digital PCR

Total RNA was extracted from four decapsulated Swiss mouse testes at postnatal days 7, 14, 28 and adult (Week 7) using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, USA). Genomic DNA contamination was eliminated by DNase treatment using DNase-free kit (Ambion, Thermo Fisher Scientific, Waltham, USA). cDNA was synthesised using 100 U Superscript III reverse transcriptase (Invitrogen, Thermo Fisher Scientific, Waltham, USA) with 2.5 µM random hexamer oligonucleotides (Roche, Basal, Switzerland). Methods were performed according to manufacturers' instructions. cDNA was diluted 1:10 with filtered MilliQ water and quantified using the QIAxpert system (QIAGEN, Hilden, Germany).

The cDNAs from enriched preparations of spermatogonia (> 95% pure; n = 3), spermatocytes (> 85% pure; n = 3) and round spermatids (> 88% pure; n = 2) were used in previous studies and supplied at a 1:10 dilution for these experiments. Spermatogonia had been isolated from 8 days old mouse testes, while 8 week old mouse testes were used for isolation of spermatocytes and round spermatids. Samples were collected from a 2-4% continuous bovine serum albumin (BSA) gradient, as previously described (McIver, Stanger et al. 2012, Bromfield, Aitken et al. 2017).

Digital droplet (dd) PCR was performed using the BioRad QX100/200 system. The ddPCR reaction contained 12.5 µl of 2 x ddPCR Supermix for probes (BioRad, Hercules, USA), 1.25 µl of 20 x target probes with a FAM dye label (final concentration 250 nM each), 2 µl of 1:10 template dilution, and filtered MilliQ water to a final volume of 25 µl. Twenty µl of ddPCR reaction followed by 70 µl of droplet generator oil for probes (BioRad, Hercules, USA) were loaded into a DG8^{TM} cartridge for the QX100/200 droplet generator (BioRad, Hercules, USA). Droplets produced were transferred into a 96 well plate (Eppendorf, Hamburg, Germany) and a two-step thermocycling protocol [(enzyme activation at 95°C x 10 mins); 40 cycles x (denaturation at 94°C x 30s, annealing at 59°C x 60s, enzyme deactivation at 98°C X 10 mins), ramp rate set at 2.5°C/s] was performed in a thermalcycler (BioRad C1000 Touch). The plate was then placed in a QX100/200 Droplet Reader (BioRad,

Hercules, USA). Data were analysed using the QuantaSoft analysis software (BioRad, Hercules, USA). Age series values and isolated germ cell data were not normalised. Data were reported as the means ± standard error of the means (SEM).

Probes, supplied by IDT (Coralville, USA), were: *Snai1* (MmPT. 58.43057042); *Snai2* (MmPT. 58.5576027); *Snai3* (MmPT. 58.43894205).

DIG-labelled RNA probes and in situ hybridisation

Primer sequences used to generate *in situ* hybridisation probes are listed in Table 1. The following PCR parameters were used to amplify target transcript regions: 95°C for 3 mins; 40 cycles of 95°C (30s), 61°C (30s), 72°C (30s); 72°C for 7 mins using 1 µl cDNA. Templates used were adult mouse testis (to amplify *Snai1* and *Snai3*) and 6 dpp (to amplify *Snai2*). PCR products were purified (QIAquick PCR Purification Kit, QIAGEN, Hilden, Germany), cloned in pGEM T-Easy vector (Promega, Madison, USA) following the manufacturer's guidelines and sequenced (Big Dye Terminator v3.1 Cycle Sequencing Kit, ABIPRISM 377 DNA Sequencer, Applied Biosystems) by the Gandel Charitable Trust Sequencing Centre, Hudson Institute of Medical Research, Clayton, VIC, Australia. PCR amplification of these plasmids using pBS forward and reverse primers produced products that included T7 and Sp6 polymerase binding sites which were used as templates for *in vitro* transcription to generate antisense and sense cRNA probes (Itman, Wong et al. 2011).

In situ hybridisation was performed on Bouin's fixed, paraffin embedded, adult mouse testis sections to determine the cellular sites where *Snai1, Snai2* and *Snai3* transcripts are synthesised using standard procedures (Itman, Wong et al. 2011). Briefly, slides were dewaxed and rehydrated. To permeabilise the tissue, sections were incubated with Proteinase K solution (Roche, Basal, Switzerland) at 37°C for 30 mins. Tissue sections were incubated with Prehybridisation buffer (50% v/v DIF, 14% v/v 20 x SSC, 33% v/v phosphate buffer, 4% v/v 50 x Denhardts solution) for 2 hrs at 50 - 55°C in a hybridisation oven within a covered humid chamber. Hybridisation was performed with 150 ng/100 µl probe per slide at 50 – 55°C overnight, followed by washes of 15 mins from 2 x SSC to 0.1 x SSC at the hybridisation temperature. Bound DIG-labelled cRNA probes were detected by anti-DIG antibody (1:1000 dilution, Roche, Basal, Switzerland) and visualised by alkaline phosphatase developer (BCIP/NBT, Thermo Fisher Scientific, Waltham, USA). Slides were counterstained with Harris Hematoxylin (diluted 1:3 v/v) and mounted under coverslips in

GVA aqueous solution (Genemed Biotechnologies, South San Francisco, USA). In situ hybridisation was performed three times using adult testis sections from two different mice. Slides were imaged on a Zeiss imager A.1 microscope with an AxioCam MRc5.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Dias, Rajpert-De Meyts et al. 2009). Immunostaining with anti-SNAI1, anti-SNAI2, anti-EED, anti-EZH2, and anti-LSD1 was performed on PFA-fixed testis sections, whereas SNAI3 immunostaining was performed on Bouin's fixed testis sections. Slides were dewaxed and rehydrated. Antigen retrieval was performed by heating slides for 10 mins in 10 mM citrate buffer (pH 6.0) using a 1000 W Pressure Cooker (Tefal). Slides were then treated with 0.3% hydrogen peroxide for 5 min at RT and washed 2 x 5 min at RT in tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.5) (TBS). Blocking solution and antibody diluent consisted of 5% (v/v) normal serum diluted in TBS/0.1% BSA [(Sigma Aldrich, St Louis, USA) (to detect SNAI3)] or CAS block [(Invitrogen, Thermo Fisher Scientific, Waltham, USA) (to detect SNAI1 and SNAI2)] added for 1 hr at RT in a humid chamber. Sections were incubated overnight with primary antibody at RT in a humid chamber. Antibodies used to detect SNAI1 (GeneTex, GTX125918; 1:100), SNAI2 (abcam, ab27568, 1:100), SNAI3 (Santa Cruz Biotechnology, sc10439, 1:100), EED (R&D system, #AF5827, 1:200), EZH2 (Cell Signaling Technology, #5246, 1:200), LSD1 (abcam, ab17721, 1:200). Anti-SNAI1, anti-SNAI2, anti-EZH2, and anti-LSD1 were detected using biotinylated anti-rabbit secondary antibody (Invitrogen, #656140, 1:500). SNAI3 was detected using biotinylated anti-goat secondary antibody (DAKO, #E0466, 1:500), whereas biotinylated anti-sheep secondary antibody was used for EED (Thermo Scientific, #618640, 1:500). Signal was amplified with Vectastain Elite ABC kit reagents according to the manufacturer's instructions (Vector Laboratories, Burlingame, USA) followed by detection with DAB (3,3-diaminobenzidine tetrahydrochloride, DAKO, Steinheim, USA) to produce a brown precipitate. Sections were counterstained with Harris hematoxylin (Sigma Aldrich, St Louis, USA), dehydrated and mounted using DPX (Sigma Aldrich, St Louis, USA). Control sections were incubated in the absence of primary antibody to check for non-specific reactivity. Slides were imaged on a Zeiss imager A.1 microscope with an AxioCam MRc5.

The EED antibody used in this study was validated on *Eed* knockout samples (Prokopuk, Stringer et al. 2017). The specificity of EZH2 antibody is evident over 75 publications,

248 including the chromatin immunoprecipitation (ChIP) application (Snitow, Li et al. 2015). The
249 specificity of LSD1 antibody is evident over 99 publications, including the ChIP application
250 (Muralidharan, Khatri et al. 2017). The SNAI1 antibody was previously validated on *Snai1*
251 knockout tissue (Horvay, Jarde et al. 2015). Western blots were performed in this study to
252 validate the SNAI1, SNAI2 and SNAI3 antibodies using mouse testis lysates (see
253 Supplementary Materials and Methods). The specificity of the SNAI2 antibody was further
254 validated on *Snai2* knockout testis samples.

255

256 **Results**

257 **Snail mRNAs are expressed in distinct cell populations during postnatal testis**
258 **development**

259 To determine the pattern of Snail mRNA and protein expression through mouse testis
260 development, we performed ddPCR and immunohistochemistry, respectively. *Snai1*, *Snai2*
261 and *Snai3* transcripts were measured by ddPCR using RNA extracted from postnatal ages 7,
262 14, 28 and adult (week 7) whole mouse testes. These ages correspond to germ cell population
263 shifts that occur during the first postnatal wave of male germ cell development (Fig. 1 a). At
264 day 7, the only cells present in the seminiferous tubule are undifferentiated and differentiated
265 spermatogonia, and immature Sertoli cells. At day 14, spermatogonia and early
266 spermatocytes are present, Sertoli cell proliferation has just arrested and tight junctions have
267 begun to form between adjacent Sertoli cells to create the epithelial barrier required to
268 support post-mitotic spermatogenesis. At day 28, spermatogonia, spermatocytes, round
269 spermatids, and mature Sertoli cells are present. In the adult testis, all germ cell subtypes are
270 present, including elongated spermatids, in addition to mature Sertoli cells. Droplet digital
271 (dd) PCR revealed that all Snail family member genes were expressed at every postnatal age
272 examined in the testis (Fig. 1 b-d). The *Snai1* transcript was detected at similar levels at each
273 age (Fig. 1 b), suggesting that *Snai1* mRNA is present in multiple cell types within the testis.
274 The *Snai2* transcript was most abundant at day 7, then significantly declined and was
275 consistent through to adulthood (Fig. 1 c). The *Snai3* transcript was present at relatively low
276 and consistent levels in all ages, but most abundant at day 7 (Fig. 1 d). This implies that
277 *Snai2* and *Snai3* transcripts are enriched within the cells present prior the transition of mitotic
278 to meiotic cells.

279

Snail transcription factors display distinct mRNA profiles in the adult mouse testis and in isolated germ cells

We next conducted a detailed analysis of Snail transcription factors in the adult mouse testis. This delineated the specific germ cell types in which Snail mRNAs are expressed, as the progression of germ cells between each differentiation state to form mature spermatozoa can be readily observed. To determine the cellular expression profiles of Snail transcripts, *in situ* hybridisation was performed on fixed wild-type adult mouse testis sections. *Snai1* was predominantly detected in spermatogonia and spermatocytes, with a faint signal apparent in round spermatids (Fig. 2 a, d). Similarly, the *Snai2* signal was strong in spermatogonia and spermatocytes, but not detectable in haploid germ cells (Fig. 2 b, e). *Snai3* showed a less restricted expression profile, with a signal evident in the cytoplasm of all germ cells and Sertoli cells (Fig. 2 c, f). *In situ* hybridisation results were verified by ddPCR which provided measurements of Snail transcript levels in isolated germ cells. Consistent with the *in situ* hybridisation data, *Snai1* and *Snai2* levels were higher in spermatogonia (Fig. 2 g) and spermatocytes (Fig. 2 j), but drastically lower in round spermatids (Fig. 2 k). In comparison, *Snai3* levels were highest in spermatogonia (Fig. 2 g), but significantly lower in spermatocytes and round spermatids (Fig. 2 j, k).

Snail transcription factors display unique protein expression profiles during postnatal testis development

To gain further understanding of the potential function of Snail transcription factors, we analysed Snail protein localisation in the postnatal mouse testis during the first wave of spermatogenesis. Germ and Sertoli cells were identified by their size, shape and chromatin morphology. SNAI1 displayed nuclear immunostaining in spermatogonia at day 10 (Fig. 3 a), in spermatogonia and spermatocytes at day 15 (Fig. 3 b) and in spermatogonia, spermatocytes and round spermatids at day 26 (Fig. 3 c). At postnatal days 10 and 15, SNAI2 was detected in the nucleus of spermatogonia, but was in the cytoplasm of spermatocytes at day 15 (Fig 3 e). At day 26, SNAI2 was localised in the nucleus of spermatogonia, spermatocytes, round and elongated spermatids (Fig. 3 g), but in the adult mouse testis, SNAI2 appeared to be restricted in the nucleus of spermatogonia and spermatocytes, with a faint signal only in round spermatids (Fig. 3 h, Fig. S1). These data suggest that SNAI1 and SNAI2 are active during major cellular transitions. During the first wave of spermatogenesis, SNAI3

immunostaining was not detectable at day 10 (Fig. 3 i). A signal restricted to the Sertoli cell cytoplasm was observed at postnatal ages 15 (Fig. 3 j), 26 (Fig. 3 k), and in the adult (Fig. 3 l, Fig S2). However, the SNAI3 protein profile was different to that of its transcript, detected by in *situ* hybridisation in both germ and Sertoli cells in the adult mouse testis (Fig. 2 c, f). This suggests that there is tight regulation of SNAI3 transcript and protein synthesis.

Western blots were performed using lysates from adult mouse testis, isolated germ cells and whole mouse embryo (e11.5) to assess antibody specificity (Fig. S3). A single band at ~ 24 kDa for SNAI1 was detected in 30 µg lysates from whole adult mouse testis and isolated germ cells. Additionally, two bands of expected sizes for SNAI3 were observed at ~ 45 and ~47 kDa in 30 µg lysates from whole embryo at e11.5 and adult mouse testis. SNAI2 could not be readily detected by Western blot. To determine specificity of antibody, immunohistochemistry was performed on adult testis from a *Snai2* knockout mouse (Fig. S3 d). Compared to control, SNAI2 was absent from spermatogonia and spermatocytes. Non-specific signal was detected in the cytoplasm of elongated spermatids.

Overall these results demonstrate that the expression pattern of Snail transcription factors is dynamic, suggesting that each Snail family member has a unique role in the developing mouse testis.

SNAI1 subcellular localisation in the adult mouse testis is Stage-specific

Snail transcription factors are required to be nuclear in order to function. We investigated the subcellular localisation of SNAI1 in adult mouse testis by immunohistochemistry using an antibody that was previously used for detecting nuclear SNAI1 in intestinal cells (Horvay, Jarde et al. 2015). Interestingly, SNAI1 appeared to be nuclear in germ cells at specific Stages of the seminiferous epithelium cycle. At Stages I-VIII, strong signal was seen in the nucleus of spermatogonia, pachytene spermatocytes, and round spermatids (Fig. 4 a-c). At Stages IX-XII, some spermatogonia, pachytene and meiotically dividing spermatocytes displayed an intense nuclear staining; signal was absent from leptotene and zygotene spermatocytes (Fig. 4 d, e). The localisation of SNAI1 in elongated spermatids is particularly intriguing, as it changed. At Stages IX-XII, SNAI1 was in the nucleus of elongating spermatids (Fig. 4 d, e, g-j), but the signal became cytoplasmic at Stages I-VII (Fig. 4 a-c). This indicates that SNAI1 may have a specific role during the period of global transcriptional repression in elongating spermatids that occurs at Stages IX-XII.

SNAI1 and SNAI2 share protein localisation patterns with co-repressors in the adult mouse testis.

Snail family members need to recruit co-factors to mediate transcriptional repression. To understand the mechanism of Snail-mediated transcriptional regulation, we investigated the expression of EED and EZH2, PRC2 complex components, and LSD1 in the adult mouse testis, each of which is known to co-operate functionally with SNAI1 and SNAI2 (Mu, Starmer et al. 2014, Myrick, Christopher et al. 2017). By immunohistochemistry, EED and EZH2 displayed nuclear localisation in spermatogonia, leptotene, zygotenes, pachytene and meiotically dividing spermatocytes, and in Sertoli cells at Stages I-XII (Fig. 5). These proteins were detected in round spermatid nuclei at Stages I-VI (Fig. 5 a, b, f, g), while these signals were restricted to the cytoplasm of round spermatids at Stage VII-VIII (Fig. 5 c, h).

LSD1, lysine-specific histone demethylase 1, showed subcellular localisation profiles identical to SNAI1 in the adult mouse testis, with nuclear signal in spermatogonia, spermatocytes, round spermatids and Sertoli cells (Fig. 6). Like SNAI1, nuclear LSD1 was observed in elongated spermatids in a Stage-specific manner. At Stages IX-XII, LSD1 was nuclear in elongating spermatids (Fig. 6 d, e), but restricted to the cytoplasm of elongated spermatids following nuclear condensation at Stages I-VII (Fig. 6 a-c). Overall, these results identify potential partners required for Snail-mediated transcriptional regulation during spermatogenesis.

Discussion

Snail factors are of central importance to transcriptional regulation, cellular migration, signal transduction and chromatin remodelling in many developmental and disease systems, but their functional relevance to male fertility are poorly understood. As an initial approach to addressing this knowledge gap, we identified the cellular production sites of Snail transcription factors in the postnatal mouse testis. We show here for the first time that each Snail family members' transcript and protein has a distinct, developmentally regulated expression profile in cells of the postnatal mouse testis. This strongly suggests that each Snail family member has a distinct function during spermatogenesis.

SNAI1, SNAI2 and SNAI3 have similar molecular structures, however there are differences in their biological actions. SNAI1 functions as a potent survival factor (Vega, Morales et al. 2004) and is essential to the preservation of stem cell function (Miller and Gauthier-Fisher 2009) (Horvay, Jarde et al. 2015). Gene repression is best understood to be its mechanism of mediating these outcomes (De Craene, van Roy et al. 2005). In the testis, SNAI1 was previously suggested to be associated with spermatogonial stem cell maintenance; indirect upregulation of SNAI1 resulted in disruption of adherens junctions within the seminiferous epithelium and loss of spermatogonia (Kanarek, Horwitz et al. 2010). The present study describing SNAI1 mRNA and protein expression in the postnatal and adult mammalian testis provides new information that highlights the potential for SNAI1 to function at specific stages of spermatogenesis. Analyses by ddPCR and immunohistochemistry show that SNAI1 mRNA and protein are present at every age in the postnatal testis (Fig. 1, Fig. 3). Intriguingly, SNAI1 protein is present in the nucleus of the most mature germ cell type present at each age, suggesting that SNAI1 is involved in the regulation of developmental switches during the cellular transitions of spermatogenesis. This function associated with SNAI1 is well-described during the epithelial to mesenchymal transition (EMT), in which nuclear-localised SNAI1 drives epithelial cells to acquire mesenchymal characteristics via the reprogramming of gene expression; key outcomes are cytoskeletal reorganisation and changes in cell behaviour (Wang, Shi et al. 2013). In the adult mouse testis (Fig. 2, Fig. 4), *in situ* hybridisation and ddPCR identify *Snai1* predominantly in spermatocytes, although a less intense signal is also detected in spermatogonia and round spermatids. At a post-translational level, our analysis reveals a Stage- and cell-specific profile for SNAI1 protein in the adult mouse testis. SNAI1 is confined to the nucleus of a restricted number of spermatogonia, and of pachytene and diplotene spermatocytes, suggesting it could regulate gene transcription in both mitotic and meiotic germ cells. The two domains of Snail that are required for interaction with downstream targets and for recruitment of co-factors may be central to determining its diverse functions during spermatogenesis. In the post-meiotic phase, SNAI1 localises within spermatid nuclei at Stages VIII-XII (Fig. 4), during the interval of extensive morphological changes that includes chromatin reorganisation and compaction. In the nucleus, histones are gradually replaced first by histone variants and transition proteins (Stages VIII-XII), then finally by protamines (Stages I-VII). As DNA is packaged, transcription is down-regulated (Hermo, Pelletier et al. 2010). Major contributors to transcriptional repression in spermatids are chromatin remodelling enzymes, capable of methylating or demethylating histones at specific residues (Rathke, Baarends et al. 2014).

Histone lysine demethylases 1 (LSD1) mediates transcriptional repression via demethylation of H3K4 (Gu and Lee 2013), and in the adult mouse testis LSD1 is observed in spermatid nuclei at Stages VIII-XII (Fig. 6). Consistent with the capacity of SNAI1 to recruit chromatin remodelling complexes at the SNAG domain, our results suggest that SNAI1 may interact with LSD1 to potentially mediate DNA packaging and promote repression of gene transcription.

Like SNAI1, SNAI2 regulates transcription and mediates the EMT (Villarejo, Cortes-Cabrera et al. 2014), however each induces common and distinct gene expression outcomes, as shown in epithelial cells (Moreno-Bueno, Cubillo et al. 2006). Specific SNAI2 functions in the testis are unknown, although it appears to be important for male fertility. Histological sections from *Snai2*-deficient mice were interpreted as displaying reduced germ and somatic Leydig cell numbers in the testis, though this outcome was not further quantified. This was hypothesised to arise from the disruption of the stem cell factor (SCF)/KIT signalling pathway, of known critical importance to spermatogonial cell migration, proliferation and differentiation. Droplet digital PCR indicates that the SNAI2 transcript and protein are present throughout postnatal testis development (Fig. 1, Fig. 3). In the adult testis, *in situ* hybridisation and ddPCR identified *Snai2* as more highly expressed in spermatocytes than in spermatogonia (Fig. 2). Consistent with the mRNA data, SNAI2 protein was detected in the nucleus of spermatogonia, and spermatocytes with a faint signal also observed in spermatids, thus SNAI2 may be involved in the events relating to mitotic and meiotic germ cell progression. Interestingly, EZH2 and EED, components of the PRC2 complex, co-localise with SNAI2 in nuclei of spermatogonia, spermatocytes and round spermatids (Fig. 5). Known for its unique roles in maintaining the undifferentiated spermatogonial pool through regulating SSC self-renewal, and in progression of meiosis (Mu, Starmer et al. 2014), the PRC2 complex represents a strong candidate to initiate SNAI2-mediate transcriptional repression. Because PRC2 controls many targets, it is not surprising that its spermatogenic phenotype, featuring the absence of post-meiotic cells, is more severe than that of *Snai2* knockout.

SNAI1 and SNAI2 are known to compensate for each other's loss by binding to the same target gene regulatory sequences (Chen and Gridley 2013). In the postnatal testis, we demonstrated that both SNAI1 and SNAI2 localise in the nucleus of spermatogonia and spermatocytes, where they may be functionally redundant. However SNAI3 displayed a

distinct expression profile. SNAI3 is structurally divergent and only recruits target genes with specific E-box sequences to its zinc finger domain. SNAI3 represses gene activity via its SNAG domain and an additional non-zinc finger region (Kataoka, Murayama et al. 2000). Although *Snai3* transcript is highly expressed in the skeletal muscle and thymus of adult wildtype mice (Kataoka, Murayama et al. 2000), its function is unknown. In this study, we demonstrate that *Snai3* is present at low levels at every age in the postnatal mouse testis (Fig. 1), indicating that it is restricted to a selected population of cells. In the adult mouse testis, *in situ* hybridisation reveals that *Snai3* is expressed in Sertoli and all germ cells (Fig. 2). The *Snai3* expression profile in germ cells was confirmed by ddPCR, with *Snai3* levels higher in spermatogonia and spermatocytes, but lower in round spermatids (Fig. 2). In the developing postnatal testis, SNAI3 protein was visible in the Sertoli cell cytoplasm from 15 dpp, but not earlier, until adulthood (Fig. 3), indicating that SNAI3 might have a specific role during the formation of Sertoli-germ cell adherens junctions, Sertoli-Sertoli tight junctions, which contribute to the blood-testis barrier. It is intriguing that the major components of the adherens and tight junctions are E-Cadherin (Tanwar, Zhang et al. 2011), occludins and claudins, essential for blood-testis barrier integrity (Morrow, Mruk et al. 2010) and well-known Snail downstream targets (Nieto 2002, Ikenouchi, Matsuda et al. 2003). The absence of a SNAI3 protein signal in any germ cells may reflect a high turnover or lack of synthesis in the spermatogenic lineage. To our knowledge, there is no evidence for alternative isoforms.

In summary, this study has provided evidence that synthesis of each of the three mammalian Snail transcriptions factor is tightly regulated during mouse spermatogenesis. The cellular distribution of Snail transcripts and proteins in the postnatal mouse testis indicates their likely sites of function, including in relationship to the regulatory mechanisms that mediate developmental changes during cellular transitions in spermatogenesis and during Sertoli cell development. These data provide a foundation for defining what regulates Snail levels and target genes, many of which are implicated in key processes in other biological systems.

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Author Contributions: DJM, KLL, HEA, GRH designed experiments, interpreted data and wrote the manuscript. DJM performed experiments. EAM provided materials for the study.

Compliance with ethical standards

Conflict of interest: The authors have no conflicts of interest in presenting information and material described in this paper.

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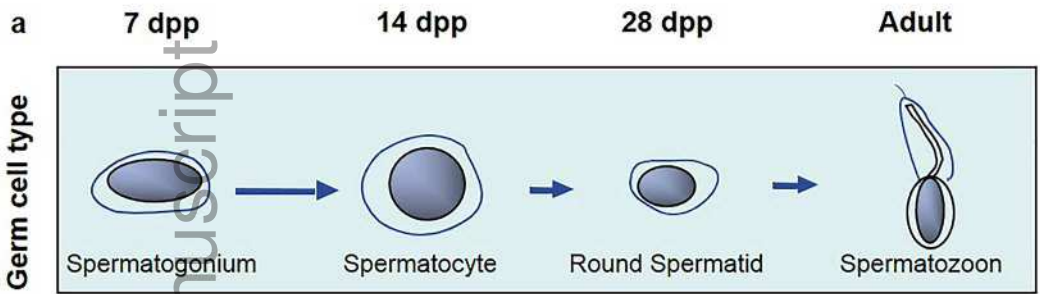
Gene	Accession number	Forward (5'-3')	Reverse (5'-3')	Region amplified
Snai1	NM_011427.3	TTCAGGCCACCTTCTTTGAG	TGAAACAGGTGTCACCAGGA	1178-1353
Snai2	NM_011415.2	TGATGCCTGGTTGTCATCAG	GACACGCACCAGGAATGTTT	1462-1666
Snai3	NM_013914.2	TCTGCTGGACCTGTTCCAAA	GGGGCAGGAGAAAATGTGTC	1159- 1373

Table 1. Primer sequences used for generation of in situ hybridisation probes to detect mouse transcripts.



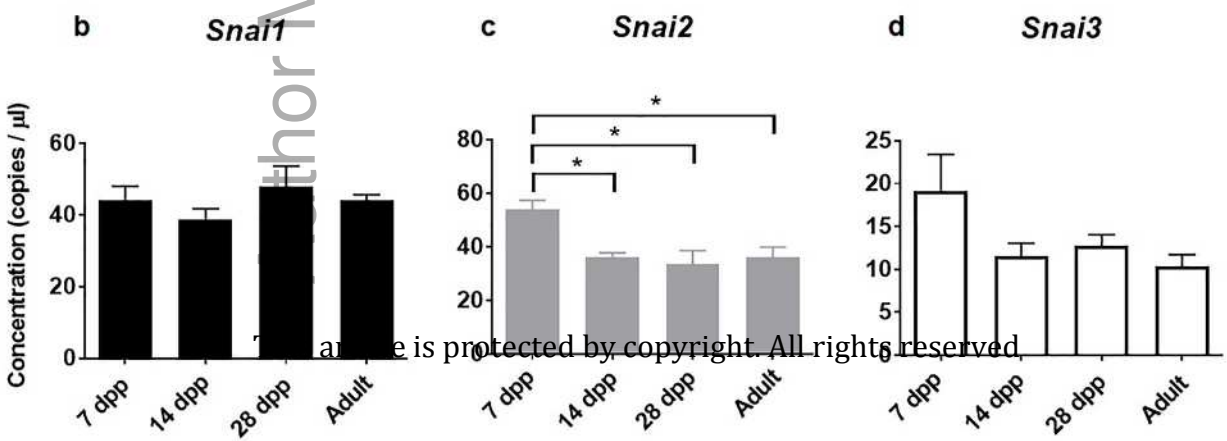


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Fig. 2

andr_12465_f2.pdf

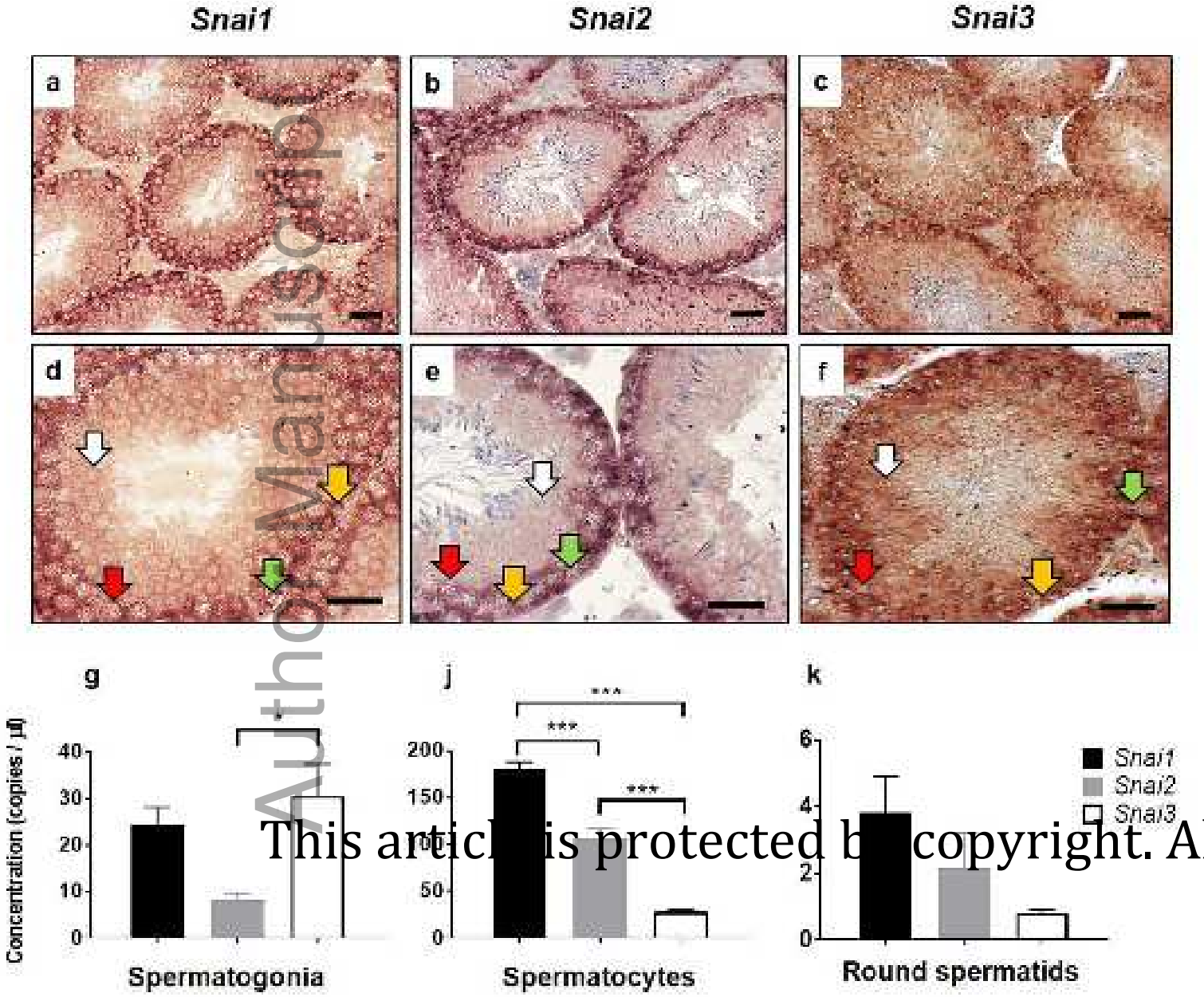

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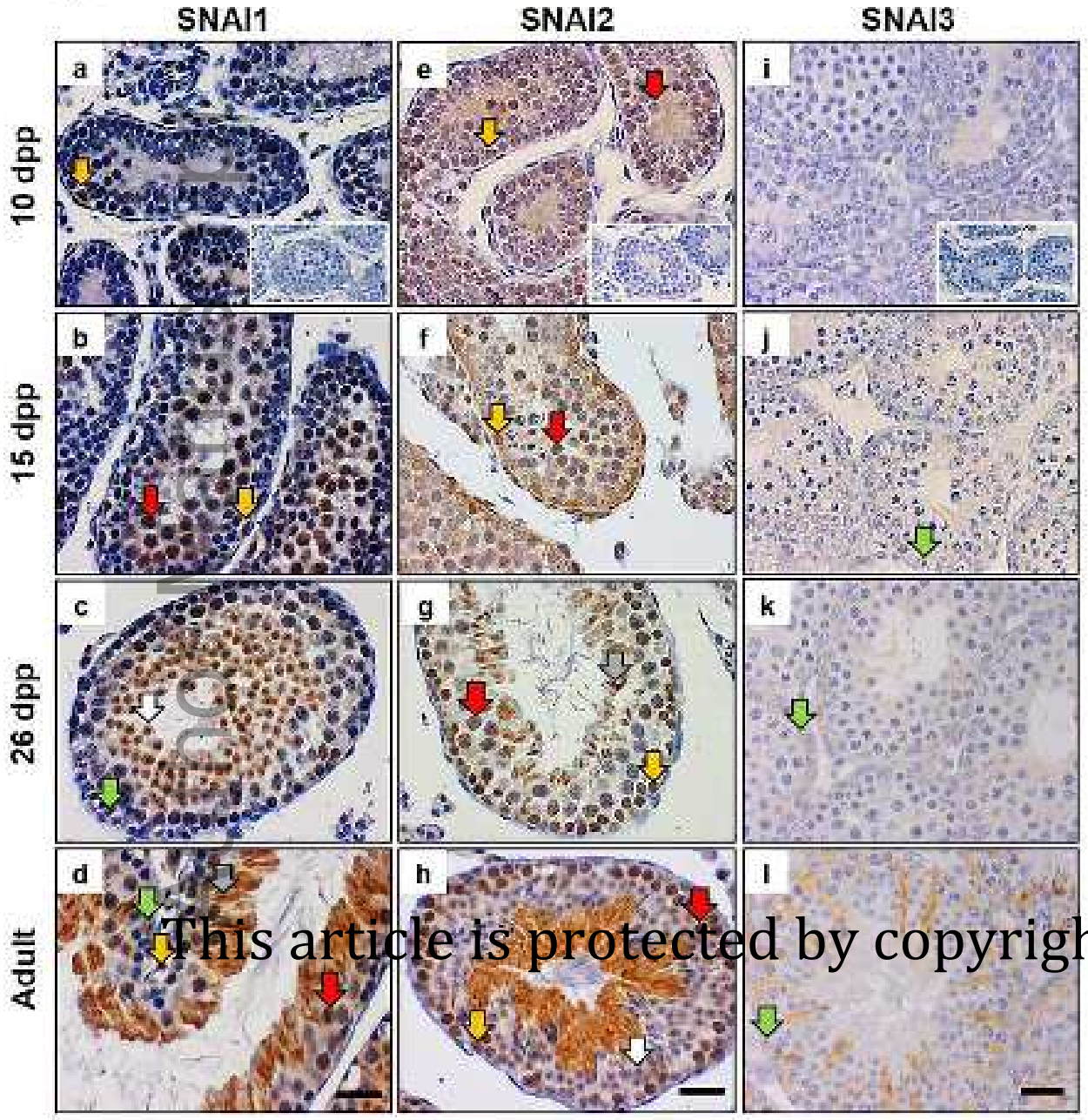
Fig. 3

andr_12465_f3.pdf



Micati et al 2017
Fig. 4

andr_12465_f4.pdf

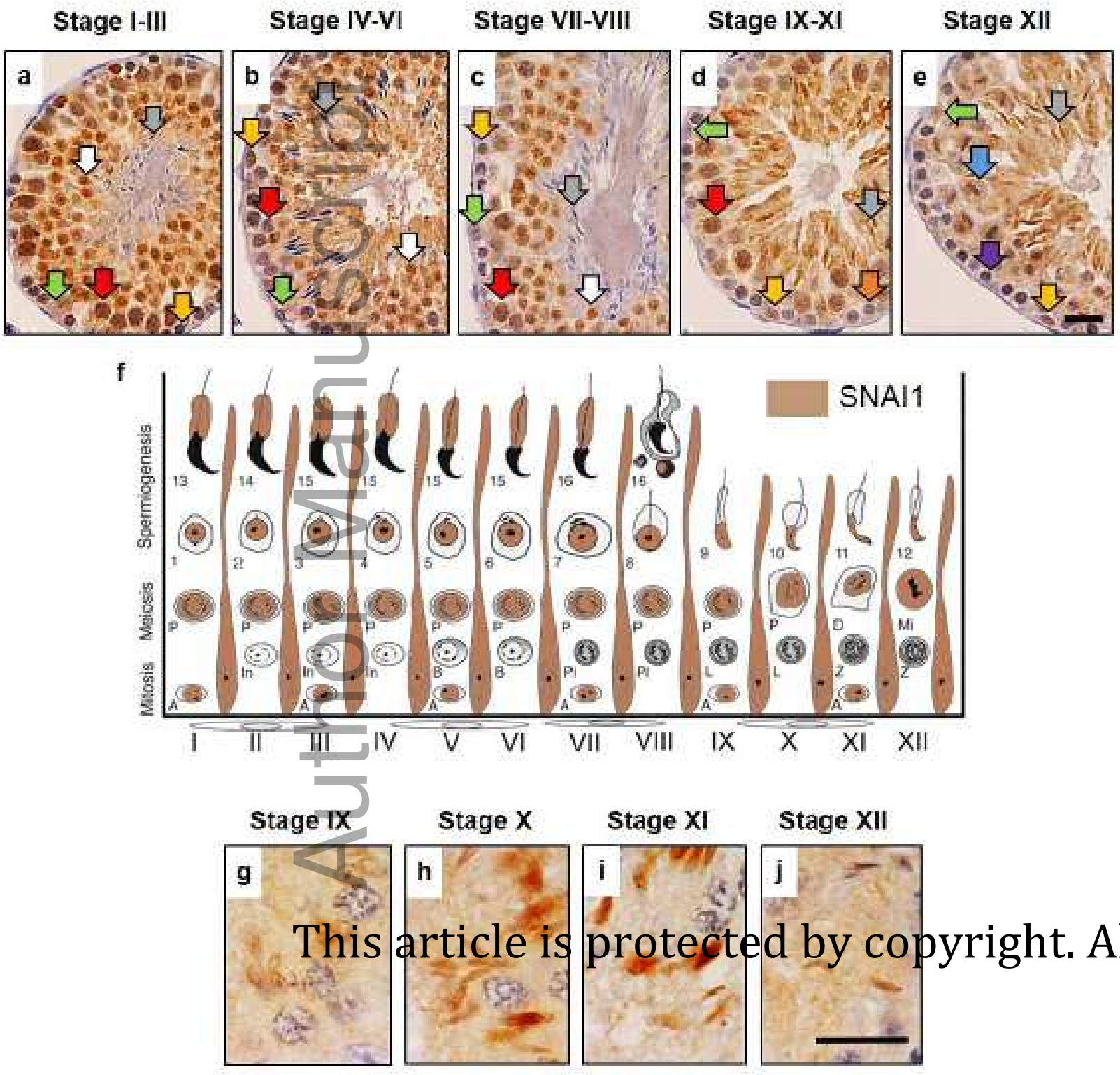


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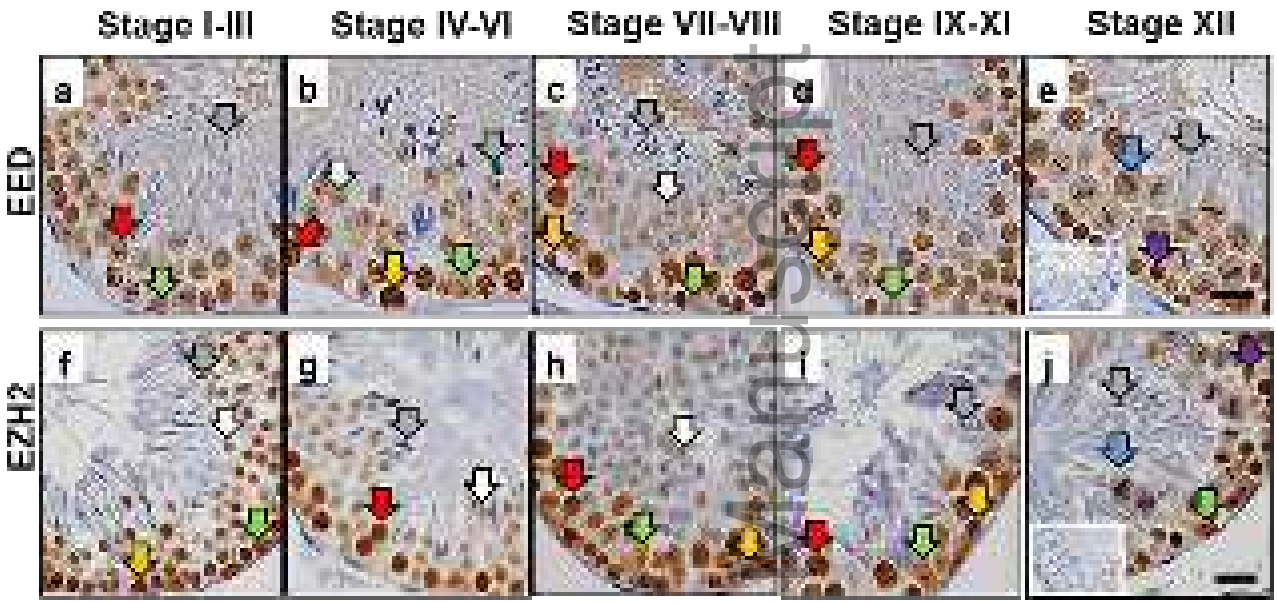
Fig. 5

andr_12465_f5.pdf



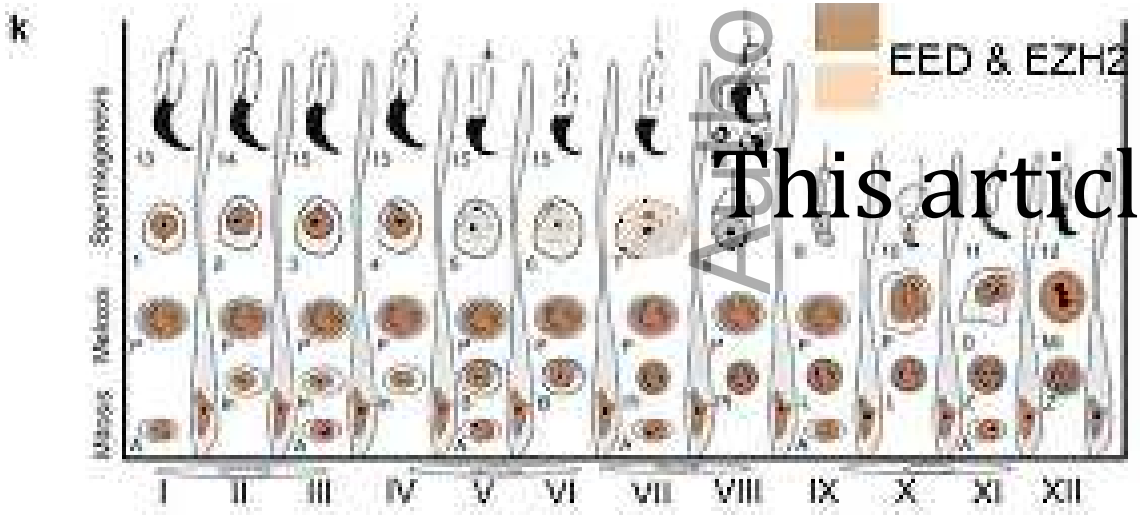


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Fig. 6