

Differential marker protein expression specifies rarefaction zone-containing human A_{dark} spermatogonia

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Abstract

It is unclear whether the distinct nuclear morphologies of human A_{dark} (Ad) and A_{pale} (Ap) spermatogonia are manifestations of different stages of germ cell development or phases of the mitotic cycle, or whether they may reflect still unknown molecular differences. According to the classical description by Clermont, human dark type A spermatogonium (Ad) may contain one, sometimes two or three nuclear 'vacuolar spaces' representing chromatin rarefaction zones. These structures were readily discerned in paraffin sections of human testis tissue during immunohistochemical and immunofluorescence analyses and thus represented robust morphological markers for our study. While a majority of the marker proteins tested did not discriminate between spermatogonia with and without chromatin rarefaction zones, doublesex- and mab-3-related transcription factor (DMRT1), tyrosine kinase receptor c-Kit/CD117 (KIT) and proliferation-associated antigen Ki-67 (KI-67) appeared to be restricted to subtypes which lacked the rarefaction zones. Conversely, exosome component 10 (EXOSC10) was found to accumulate within the rarefaction zones, which points to a possible role of this nuclear domain in RNA processing.

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Introduction

Human spermatogonial stem cells (SSCs) are assumed to represent a subpopulation of type A spermatogonia that reside on the basement membrane of the testicular seminiferous tubules intermingled with differentiating type B spermatogonia. However, our knowledge concerning their molecular identity and the mechanisms that regulate their transition from quiescence to proliferation and from self-renewal to differentiation is still limited *in vivo* and *in vitro*. Based on morphological criteria, human spermatogonia have been classified as dark type A (Ad), pale type A (Ap) and type B that differ in their nuclear morphology (Clermont 1963, 1966, 1972). Ad spermatogonia have nuclei showing a homogeneous, dark stainable chromatin and one or more 'clear cavities' of vacuole-like appearance (Clermont 1963). Ap spermatogonia are characterised by an ovoid nucleus containing a uniformly pale grey granular chromatin, and no 'cavities'; type B spermatogonia have nuclei that, in addition to a fine chromatin granulation, contain heavily stained heterochromatin and no 'cavities' as well. Additional morphological subtypes have been discerned by electron microscopy (Rowley *et al.* 1971, Schulze 1978). The ultrastructural

analyses revealed that the 'clear cavities', also called the 'vacuoles', represented chromatin rarefaction zones which lacked a limiting membrane and were devoid of chromatin fibrils, but were filled with a faint fibrillar matrix (Tres & Solari 1968, Rowley *et al.* 1971, Paniagua *et al.* 1986).

There is ongoing discussion about whether and how the diverse nuclear morphology of Ad and Ap spermatogonia may correlate with different stem cell characteristics (for review, see de Rooij & Russell (2000), Amann (2008), Dym *et al.* (2009) and Hermann *et al.* (2010)). Clermont (1972) claimed that the Ad and Ap spermatogonia represent 'reserve' and 'active' stem cells respectively. Others proposed that the low mitotic index of the Ad spermatogonia is indicative of a 'true' stem cell phenotype (for review, see Dadoune (2007)) while the regularly dividing Ap spermatogonia may represent 'renewing progenitors' (compare Ehmcke & Schlatt (2006)). Different from these suggestions, a study in the rhesus monkey indicated that the Ad and Ap nuclear phenotypes of primate spermatogonia may rather correlate with different stages of the cell cycle (i.e. G0 vs G1/S/G2/M) than with diverse stem cell fates (Hermann *et al.* 2009, 2010). This links to earlier

proposals that the Ad and Ap spermatogonia do not represent different cell generations with diverse fates (Roosen-Runge & Barlow 1953, Schulze 1978). Yet, BrdU-labelled Ad spermatogonia exhibiting a classical nuclear phenotype (dense and homogeneous chromatin) have been described in juvenile monkeys (for review, see Plant (2010)).

Considering the obvious differences in nuclear morphology, it is conceivable that Ad and Ap spermatogonia may exhibit differences at the molecular level. However, apart from varying proliferative activity such differences have rarely been studied. In the adult rhesus monkey, a considerable proportion of Ad and Ap spermatogonia shared a similar molecular phenotype with respect to the consensus rodent stem cell markers glial cell line-derived neurotrophic factor receptor α (GFRA1) and promyelocytic leukaemia zinc finger (PLZF; Hermann *et al.* 2009). A previous report suggested that GFRA1 expression was heterogeneous among human type A spermatogonia, but the study did not discriminate between Ad and Ap morphological subtypes (Grisanti *et al.* 2009). A molecular analysis of human repopulating SSCs identified various surface markers suitable for the enrichment of these cells (Izadyar *et al.* 2011); unfortunately, information about how these markers might correlate with the distinct nuclear morphology of Ad and Ap spermatogonia was not provided.

The expression of spermatogonial markers is conserved from rodents to primates to a remarkable degree. However, species differences have also been reported, including the succession of markers and their correlation with the differentiation state of spermatogonia (see reviews by Dym *et al.* (2009) and Hermann *et al.* (2010)). Moreover, a quiescent stem cell compartment, comparable to the Ad spermatogonia, has not been described in non-primate mammals. Thus, molecular markers suitable to identify rodent SSCs may not be well suited to discriminate the Ad and Ap spermatogonial subtypes in the human, and exploration of novel markers is highly desirable to provide information about their stem, progenitor and differentiating characteristics. Indeed, B-cell-associated transcription factor POU2F2/OCT2 was recently proposed to discriminate between human Ad and Ap spermatogonia (Lim *et al.* 2011).

Using microarray analysis, we previously listed more than 200 potential candidates for human spermatogonia-specific markers (von Kopylow *et al.* 2010). These may be expected to comprise additional markers suitable to discriminate between Ad and Ap morphological subtypes. In this study, we used selected markers to further dissect the molecular phenotypes of human spermatogonia using immunohistochemistry and immunofluorescence procedures. The combined differential expression of these markers, including PLZF (ZBTB16), undifferentiated embryonic cell transcription factor 1 (UTF1), survival time-associated PHD finger in ovarian cancer 1

(SPOC1; PHF13), GFRA1, fibroblast growth factor receptor 3 (FGFR3), c-Kit receptor tyrosine kinase (KIT) and doublesex- and mab-3-related transcription factor (DMRT1) revealed a remarkable degree of heterogeneity among human type A spermatogonia. Most notably, we show that EXOSC10, a highly conserved 3'-5' exonuclease involved in RNA processing, localises to the chromatin rarefaction zones in human Ad spermatogonial nuclei, indicating a functional role of this poorly characterised subnuclear structure.

Results

Spermatogonia with and without nuclear rarefaction zones are immunopositive for PLZF, UTF1, SPOC1, GFRA1 and FGFR3

We first compared the nuclear morphology of the germ cells residing immediately at the basement membrane of the seminiferous tubule following various fixations and embedding protocols (Fig. 1). These included the Zenker-formol fixed and haematoxylin-stained testis as described by Clermont (1963, 1966). In the latter, a quantity of germ cells presented faint zones of $\sim 3 \mu\text{m}$ in diameter inside their nuclei while others lacked these zones (Fig. 1A). Nuclei with rarefaction zones frequently also had darker chromatin. Fixation in glutaraldehyde/osmium, in combination with Epon embedding, semithin sectioning and toluidine staining, likewise exhibited spermatogonial nuclei with and without rarefaction zones (Fig. 1B) although the different processing protocols sometimes led to varying diameters of the faint zones. In addition, in the semithin sections the position of the nucleoli became apparent, allowing discrimination of the types Ad and Ap spermatogonial nuclei. Fixation in modified Davidson's fluid (mDF; Fig. 1C and D) gave similar results as seen for the Zenker-formol fixed testis, and rarefaction zones were likewise visible in a subfraction of dark-stained spermatogonial nuclei. This was substantiated by fluorescent DNA staining by DRAQ5 (Fig. 1D) or 4',6-diamidino-2-phenylindole (DAPI; not shown).

In the following, mDF was used as a substitute for Zenker-formol, as it was compatible with the immunostaining procedures below. While chromatin staining was too variable to be a useful marker during these procedures, the rarefaction zones were repeatedly observed in tissue sections from all subjects studied and following various processing procedures. Thus, they are unlikely to result from degeneration or processing artefacts. The expression of chromatin-associated PLZF, UTF1 and SPOC1, as well as of membrane receptors GFRA1 and FGFR3 was subsequently studied by immunoperoxidase staining and immunofluorescence methods (Fig. 2). Validating and extending upon previously documented antibody specificities, PLZF, UTF1 and SPOC1 were restricted to the nuclei of

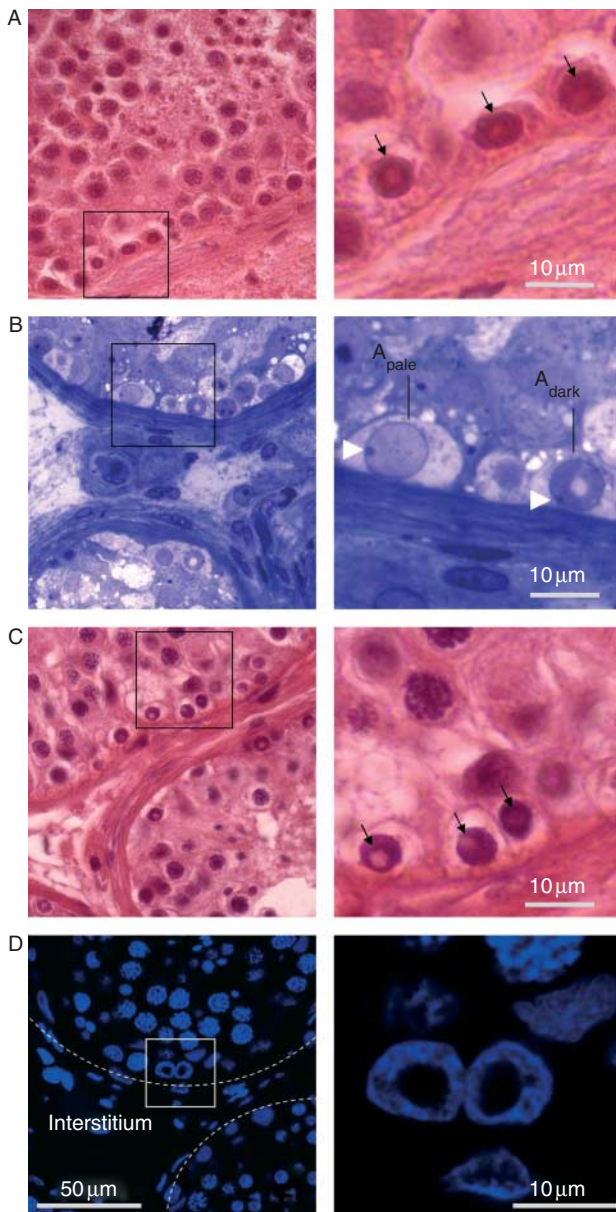


Figure 1 Histology of the normal adult human testis employing different protocols of tissue fixation, embedding and staining. (A) Bright-field microscopy of tissue section following fixation in Zenker-formol, paraffin embedding and staining with haematoxylin. Right panel shows enlargement of boxed area; arrows point at nuclei with rarefaction zones. (B) Bright-field microscopy of tissue section following glutaraldehyde/osmium tetroxide fixation, Epon embedding, semithin sectioning and toluidine blue staining. Right panel shows enlargement of boxed area, allowing detection of peripheral nucleoli (white arrowheads) and discrimination of types A_{dark} and A_{pale} spermatogonia. (C) Bright-field microscopy following fixation in modified Davidson's fluid (mDF), paraffin embedding and staining with haematoxylin. Right panel shows enlargement of boxed area; arrows point at nuclei with rarefaction zone. (D) Fluorescence microscopy of tissue section following fixation in mDF, paraffin embedding and DNA staining by DRAQ5. Dashed lines mark positions of basement membrane. The boxed area singles out a pair of nuclei with large chromatin rarefaction zones; enlargement of this area is shown in right panel.

spermatogonia which resided near the basement membrane of the seminiferous tubules (Fig. 2A–C, F and G) and were observed in subtypes with and without rarefaction zone (Fig. 2A–C). In parallel experiments, an antiserum against stem cell marker POU5F1 (Oct3/4) did not yield nuclear staining of spermatogonia and thus was not further analysed.

Testicular expression of GFRA1 and FGFR3 was likewise restricted to the spermatogonia (Fig. 2D, E and H), while in the paraffin sections GPR125 antibodies showed inconsistent labelling of various cell types (data not shown). The GFRA1⁺ and FGFR3⁺ spermatogonia often emerged as small cell cohorts and, such as the chromatin-associated markers described earlier, included nuclei with and without rarefaction zones. GFRA1 antibodies revealed typical cell surface staining, characteristic of transmembrane receptors (Fig. 2D). Much of the FGFR3 protein, in comparison, appeared to be internalised within cytoplasmic vesicles. To exclude any unspecific staining, confocal microscopy was conducted using two different primary antibodies against the receptor protein. Both antibodies showed near congruent staining, confirming the main subcellular distribution of the FGFR3 protein within cytoplasmic vesicles (Fig. 2H). The immunostaining patterns described here were only observed when primary and secondary antibodies were used together, and were absent when negative control antisera were used. During confocal microscopy, signals were not due to bleed-through between channels. In summary, the above-described markers were shared by various spermatogonial subtypes, including the subtype with visible rarefaction zones; their potentially differential expression in human types A and B spermatogonia was not specifically assessed here. The staining patterns, however, were heterogeneous for the different markers, and the overlap between spermatogonial subtypes was apparently not complete. Still, none of the markers above was sufficiently specific to distinguish them.

KIT and DMRT1 are undetectable in human spermatogonia with nuclear rarefaction zones

To establish potential molecular differences between spermatogonial subtypes, KIT and DMRT1 were tested in immunohistochemistry and immunofluorescence. KIT was not among the human spermatogonia-specific markers identified by microarray analysis (von Kopylow *et al.* 2010) because it is highly expressed also in interstitial cells of the human testis, most probably the mast cells. Still, in tissue sections, the receptor tyrosine kinase was clearly present on the surface of cohorts of germ cells near the basement membrane. Rarefaction zones were only discerned in the nuclei of KIT[−] spermatogonia (Fig. 3; arrows). To monitor the proliferating cell fraction, co-labelling with the KI-67 proliferation marker was conducted (Fig. 3E); to specifically

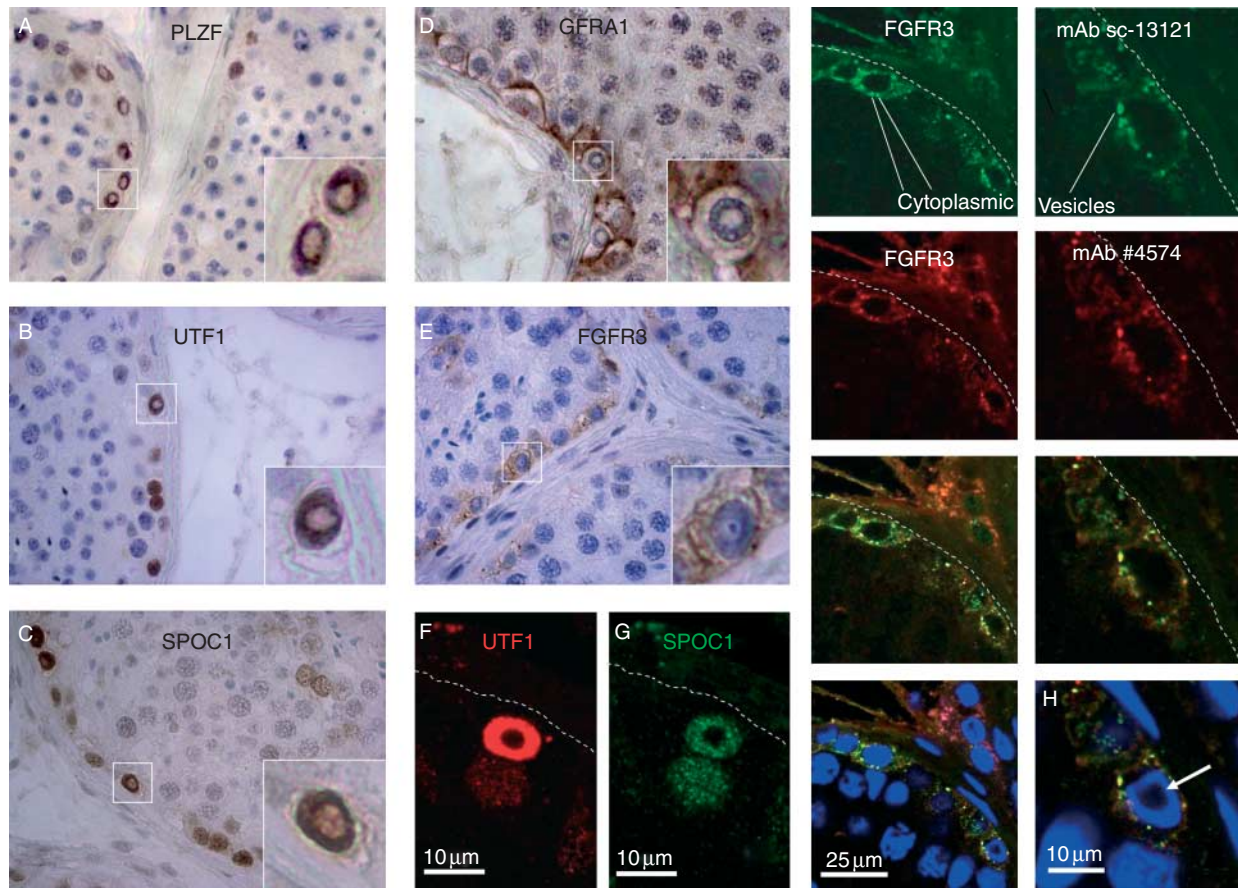


Figure 2 Immunolocalisation of PLZF (A), UTF1 (B and F), SPOC1 (C and G), GFRA1 (D) and FGFR3 (E and H) in cross-sections of normal adult human testis. The insets show single immunopositive spermatogonia with nuclear rarefaction zones. Note that the rarefaction zones are clearly discerned during bright-field microscopy using a hemalaun counterstain (A–E) as well as during confocal fluorescence microscopy using antibodies against chromatin-associated markers UTF1 (F) and SPOC1 (G) and DNA staining by DRAQ5 (H). Note nucleolus-like structure within rarefaction zone (E). While GFRA1 antibodies showed plasma membrane labelling of spermatogonia (D), two FGFR3 mAbs generated in different species showed congruent labelling of cytoplasmic vesicles in FGFR3⁺ spermatogonia (H); arrow points to chromatin rarefaction zone. Dashed lines mark positions of basement membrane.

discern the spermatogonial nuclei, SPOC1 antibodies were additionally used (see above). Overlap of KIT membrane staining and KI-67 nuclear staining suggested that many of the KIT⁺ spermatogonia were mitotically active. KI-67 was often localised in the nucleoli, but also stained discrete foci of heterochromatin throughout the nucleoplasm. Confirming the immunohistochemical staining results above, spermatogonia with visible chromatin rarefaction zones were KIT⁻/KI-67⁻ (Fig. 3E; arrows).

Nuclear DMRT1 immunostaining was observed in different cell types of the human testis, but was likewise absent in nuclei with rarefaction zones (Fig. 4). As described in rodents (Lei *et al.* 2007, 2009), the nuclei of human Sertoli cells were also immunopositive for DMRT1, but they were distinct from the spermatogonial nuclei by their shape and weaker staining. Interestingly, during the metaphase of mitosis, when the nuclear membrane had disappeared, the dividing cell remained DMRT1⁺ (Fig. 4C). Co-labelling with KI-67 and SPOC1

antibodies confirmed that spermatogonia presenting a chromatin rarefaction zone were DMRT1⁻ (Fig. 4D–F). In contrast, strong DMRT1 staining was seen especially in the nuclei of the proliferating, i.e. KI-67⁺ spermatogonia. Overlap of DMRT1 and KI-67 nuclear staining, however, was highly variable, depending on cell cycle stage. Also, DMRT1⁻/KI-67⁺ spermatogonial nuclei were occasionally observed (Fig. 4F).

To further classify the DMRT1⁻ and DMRT1⁺ spermatogonial subtypes, the phosphoprotein C23 (nucleolin) was used as a marker of the nucleoli, again in combination with the chromatin-associated SPOC1 (Fig. 5). The nucleoli of the spermatogonia and also of the Sertoli cells were brightly stained by the C23 antibody; additionally, the chromatin of later germ cell stages showed a fuzzy staining. The peripheral and central locations of C23 nucleolin (compare Hartung *et al.* (1990)) suggested that a subfraction of both types Ap and B spermatogonia were DMRT1⁺. This is consistent with the DMRT1/KI-67 co-staining patterns (compare

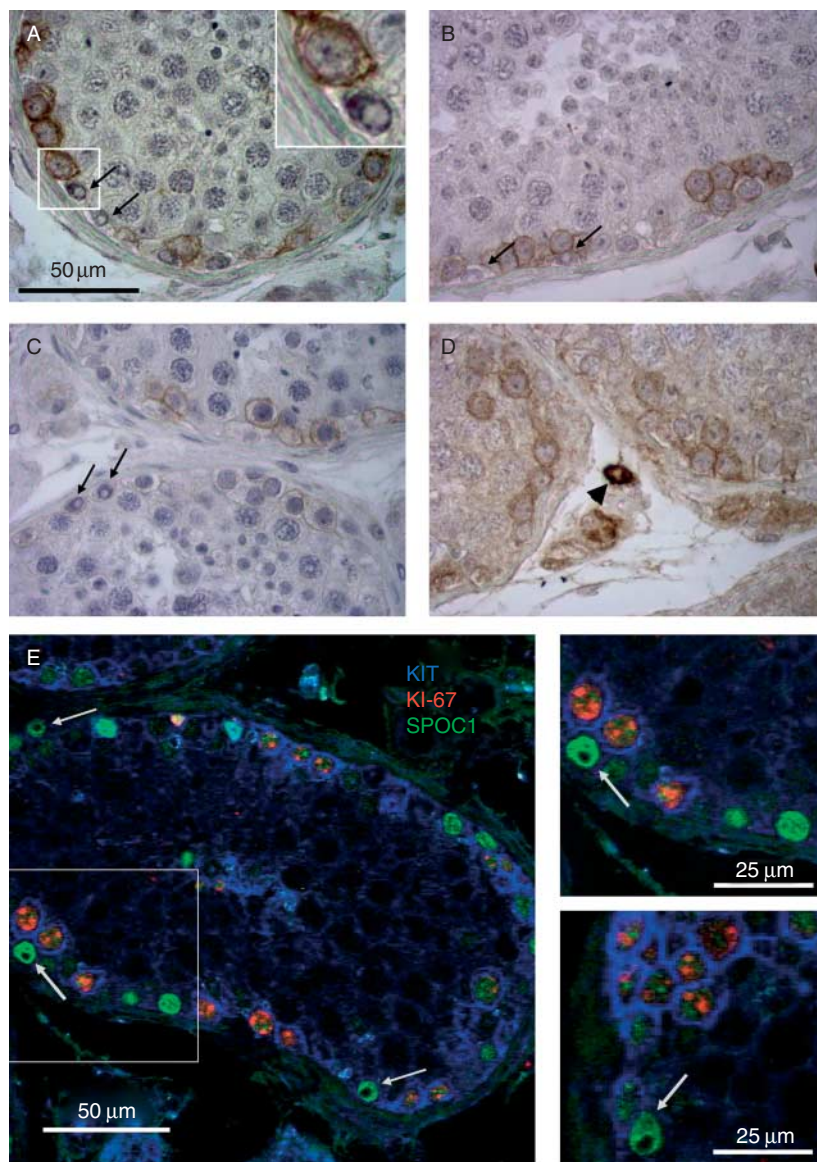


Figure 3 Immunolocalisation of KIT in cross-sections of normal adult human testis. (A–D) Immunoperoxidase staining revealed plasma membrane staining of different subtypes of spermatogonia as well as strongly immunopositive mast cells in the interstitium (see arrowhead in D). Hemalaun counterstain illustrates that spermatogonia exhibiting plainly visible rarefaction zones are KIT⁻ (highlighted by black arrows). The insert in A shows details of neighbouring KIT⁺ and KIT⁻ spermatogonia with and without nuclear rarefaction zones respectively. (E) Co-immunofluorescence of KIT (blue), KI-67 (red) and SPOC1 (green). Overview of seminiferous tubule shows substantial overlap of KIT and KI-67 labelling. White arrows point at spermatogonia with chromatin rarefaction zones. Boxed area is enlarged in upper right panel, exhibiting nuclei with and without rarefaction zone, respectively, by SPOC1 chromatin staining. Lower right panel shows additional detail of honeycomb-like neighbouring KIT⁺/KI-67⁺ and a single KIT⁻/KI-67⁻ cell.

Fig. 4D–F) where much of the KI-67 protein seemed to be located within the peripheral and central nucleoli of these subtypes respectively. Spermatogonia with chromatin rarefaction zones had peripheral C23-positive nucleoli as well, but were DMRT1⁻, in comparison (Fig. 5). Triple immunofluorescence employing DMRT1, UTF1 and SPOC1 antibodies revealed an unexpected molecular heterogeneity of human spermatogonial nuclei including the nuclei with rarefaction zones (Supplementary Figure 1, see section on supplementary data given at the end of this article). The variable nuclear staining intensities, although not a quantitative measure, possibly reflected varying proportions of nuclear protein contents and showed no simple correlation with the nuclear morphology. However, chromatin rarefaction zones were not observed in the subpopulation of DMRT1⁺/SPOC1⁺/UTF1⁻ spermatogonia, while some

‘UTF1⁺-only’ nuclei were scattered near the basement membrane, which contained a nuclear rarefaction zone (Supplementary Figure 1).

In summary, both KIT and DMRT1 were absent from the spermatogonia with chromatin rarefaction zones which were at the same time KI-67⁻, which is in keeping with their reportedly low proliferative activity. In addition to this, both KIT and DMRT1 were primarily expressed in spermatogonia that contained little or no nuclear UTF1 (compare Supplementary Figures 1 and 2, see section on supplementary data given at the end of this article). Combining KIT and DMRT1 antibodies in co-staining experiments resulted in a high background as both antibodies had been generated in the same species. Still, there seemed to be overlap in KIT and DMRT1 expression in both human types A and B spermatogonia (data not shown).

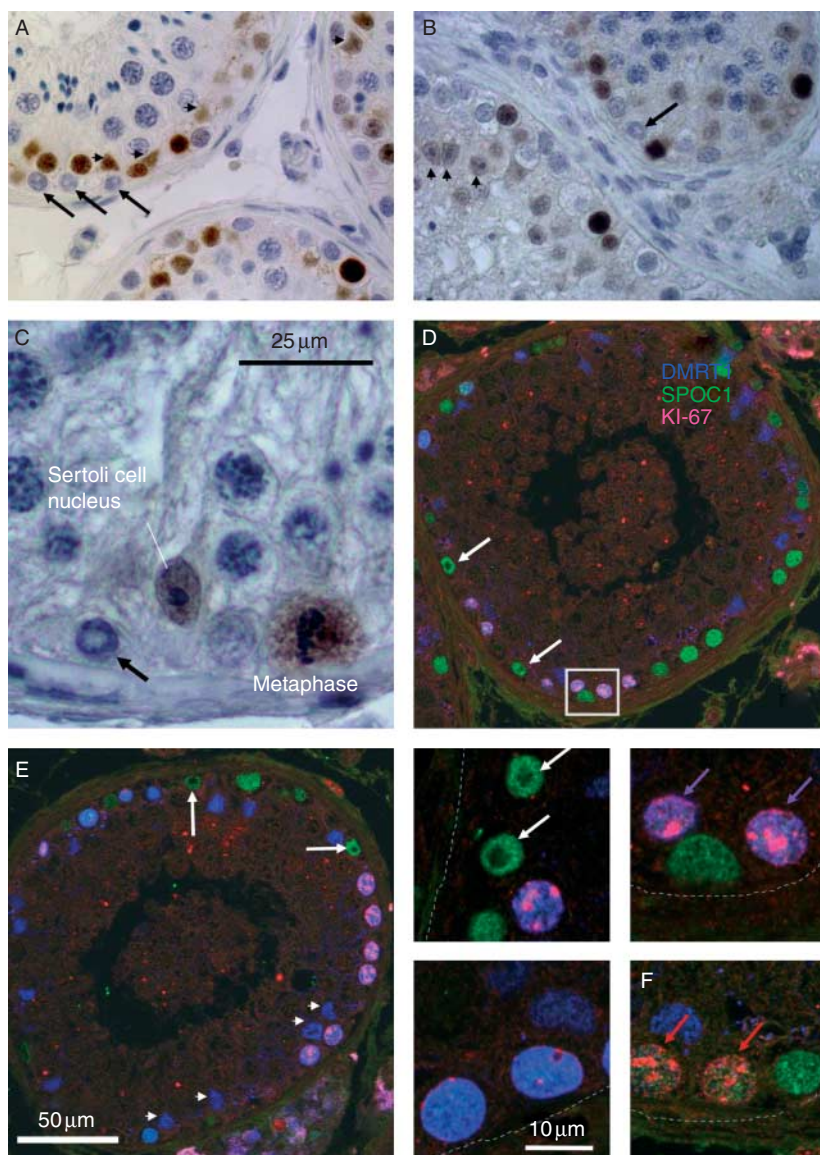


Figure 4 Localisation of DMRT1 in cross-sections of normal adult human testis. (A–C) Immunoperoxidase staining reveals strong DMRT1 labelling of spermatogonial nuclei and weak labelling of Sertoli cell nuclei (arrowheads). Spermatogonia with plainly visible rarefaction zones are DMRT1⁻ (marked by black arrows). Note a DMRT1⁺ metaphase cell in C. (D and E) Triple immunofluorescence combining DMRT1 (blue), KI-67 (red) and SPOC1 (green) shows extensive overlap in spermatogonial nuclei of DMRT1 and KI-67 labelling (bright magenta). Nuclei with plainly visible rarefaction zones (green, as revealed by chromatin-associated SPOC1) are invariably DMRT1⁻/KI-67⁻ (highlighted by white arrows). (F) Details of different spermatogonial subtypes. Upper left, pair of DMRT1⁻/KI-67⁻/SPOC1⁺ nuclei with plainly visible chromatin rarefaction zone (arrows), most probably of Ad subtype, next to DMRT1⁺/KI-67⁺ nucleus; upper right (detail from D), DMRT1⁺/KI-67⁺ spermatogonial nuclei (arrows; note localisation of magenta-stained nucleoli); bottom left, strongly DMRT1⁺/KI-67⁺ nuclei, probably of Ap subtype (note peripheral nucleoli); bottom right, DMRT1⁻/KI-67⁺ nuclei, probably of B type (arrows). Dashed lines mark positions of basement membrane.

EXOSC10 localises to the chromatin rarefaction zone

We used an antibody against EXOSC10 (also known as the polymyositis/scleroderma PM/Scl-100 autoantigen) that was previously characterised as an independent marker of human nucleoli (Targoff & Reichlin 1985). As opposed to the above tested nucleolar markers, i.e. C23 and KI-67, the EXOSC10 antibody showed a conspicuous labelling of material that accumulated inside the nuclear rarefaction zones (Fig. 6). Co-staining of DNA with DRAQ5 suggested that all visible rarefaction zones were indeed strongly EXOSC10⁺. This was also true for spermatogonial nuclei showing multiple rarefaction zones (Fig. 6D). Since anti-PM/Scl antibodies reacted specifically with the granular component of the human nucleolus (Reimer *et al.* 1986), co-labelling with the nucleolar marker C23 was conducted. Overlap of the immunostaining patterns confirmed that EXOSC10 was

indeed a constituent of the peripherally located nucleoli of type A spermatogonia (Fig. 7).

The varying patterns of EXOSC10⁺ spermatogonial nuclei above already suggested that the protein was associated with multiple nuclear compartments. Closer examination confirmed that these compartments were either related to the chromatin rarefaction zones or associated with the C23-positive nucleoli located at the nuclear periphery of both spermatogonial subtypes with and without rarefaction zones. As opposed to that, co-staining of EXOSC10 and KI-67 resulted in non-overlapping patterns (Fig. 8). Again, strong EXOSC10 immunostaining was associated with the chromatin rarefaction zones. In addition, immunopositive dots representing the nucleoli were visible at the nuclear periphery (compare Fig. 8A and B). Although a specific nuclear staining, most probably of the nucleoli, was observed with the EXOSC10 as well as the KI-67

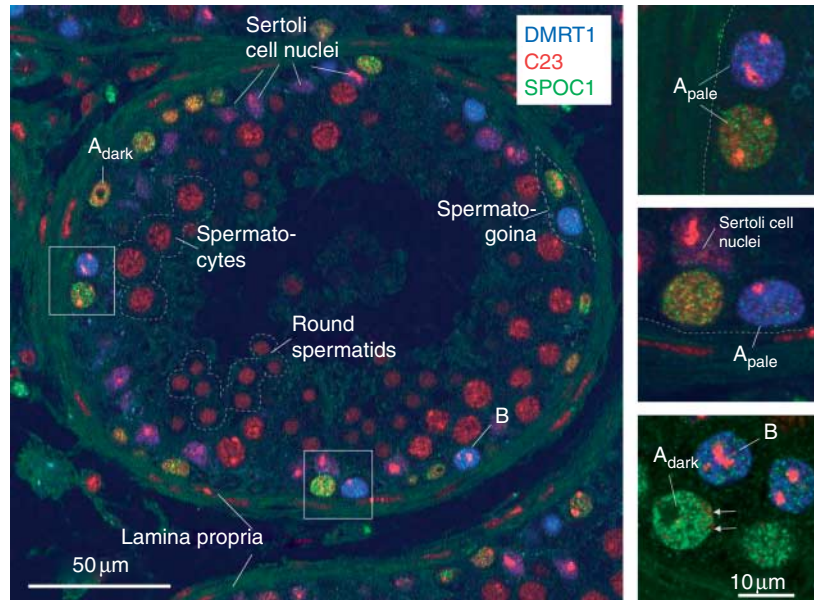


Figure 5 Triple immunofluorescence of DMRT1 (blue), C23 nucleolin (red) and SPOC1 (green) allows discrimination of spermatogonial subtypes. Overview of tubular cross-section (overlay) shows nuclei of different germ cell types and of Sertoli cells (left). Spermatogonial nuclei are either DMRT1⁺ (blue) or DMRT1⁻ (green); correspondingly, the C23-labelled nucleoli are observed in magenta or orange. Nuclei (green or blue) with peripheral nucleoli are probably of the A type while type B nuclei have central nucleoli. The DMRT1⁺ Sertoli cell nuclei are dark blue with a brightly stained, large central nucleolus. In spermatocytes and round spermatids, the entire chromatin shows fuzzy C23 staining (dark red). Boxed areas highlight doublets of spermatogonia that are enlarged in right panels (upper and middle). Note that type A spermatogonia often appear to be arranged in pairs or doublets at the basement membrane. Remarkably, pairs of Ap nuclei, as judged from the peripheral nucleoli, were not identical in their DMRT1 expression, but rather were either DMRT1⁺ or DMRT1⁻. The Ad nuclei (green, lower panel) as characterised by their chromatin rarefaction zone and condensed peripheral nucleoli (two nucleoli highlighted by arrows) are DMRT1⁻. Dashed lines highlight different germ cell types.

antibodies, both antibodies did not label identical nuclei. Rather, they showed alternate staining in neighbouring spermatogonia.

Discussion

We asked whether specific marker proteins would reveal differences in the molecular characteristics of human spermatogonial subtypes. To this end, morphological criteria – the chromatin rarefaction zone and the location of the nucleoli – have been used to classify them. According to Clermont (1963), ‘one, sometimes two or three clear cavities’ can be seen in the dense chromatin mass of human Ad spermatogonia. Thus, although our visual inspection of the rarefaction zones may not have covered the entire population of Ad spermatogonia in tissue sections of the human testis, it was sufficient to discern at least a proportion and to distinguish them from the other subtypes. This was supported by the fact that the DNA-intercalating DRAQ5 as well as chromatin-associated proteins reproducibly spared the rarefaction zones also during immunofluorescence procedures. In addition, various nucleolar markers were used which revealed the peripheral or central location of the nucleoli within human spermatogonial nuclei and facilitated our efforts to discriminate human spermatogonial subtypes (Tres & Solari 1968, Paniagua *et al.* 1986, Hartung *et al.* 1990).

In conclusion, the nuclear rarefaction zones were characteristic of a distinct subtype of human spermatogonia to which Clermont’s (1963, 1966) definition of the Ad spermatogonium was applicable, although we cannot exclude the existence of Ad nuclei that lack this zone.

By combining the inspection of morphological criteria, i.e. occurrence of a rarefaction zone and the position of the nucleoli, and various immunostaining procedures we found that the expression of PLZF, UTF1, SPOC1, GFRA1 and FGFR3 was similar in spermatogonial subfractions with and without rarefaction zones while the expression of KIT, KI-67 and DMRT1 was not. The shared expression of the well-characterised nuclear factors and cell surface receptors by morphologically different subtypes supports the notion that types Ad and Ap spermatogonia exhibit overlapping, albeit not congruent molecular phenotypes (Hermann *et al.* 2009) which may go along with common stem cell fates. Differences between types A and B spermatogonia concerning the expression of these markers were not specifically addressed here, but became apparent when nucleolar markers were additionally used. KIT and DMRT1 appeared to be specifically excluded from the spermatogonia that exhibited a rarefaction zone, i.e. from at least a subfraction of the Ad spermatogonia. Instead, they were expressed by subsets of both human types Ap and B spermatogonia. This result corresponded to the restriction of proliferation marker KI-67 that in our

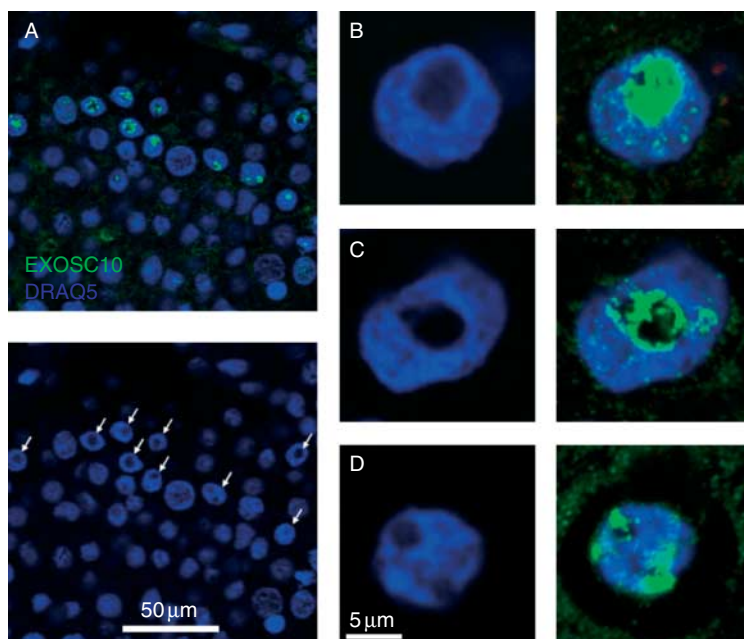


Figure 6 Immunolocalisation of EXOSC10 (green) in DRAQ5 (blue)-counterstained tissue sections of normal adult human testis. (A) Tangential section parallel to the tubular surface showing outermost layer of the seminiferous tubule with numerous EXOSC10-positive spermatogonia (upper panel) that exhibit a rarefaction zone (see arrows in lower panel). (B, C and D) Details of selected spermatogonial nuclei are shown, illustrating accumulation of EXOSC10 within chromatin rarefaction zones.

study appeared to be excluded from the subtype with nuclear rarefaction zones as well. There is, however, still lack of consensus concerning the cell cycle characteristics of Ad spermatogonia in primates, and additional studies are needed to confirm whether this cell type is indeed quiescent.

KIT expression has been described to mark the transition from undifferentiated to differentiating spermatogonia in rodents (Schrans-Stassen *et al.* 1999) and non-human primates (Hermann *et al.* 2009). Moreover, it promotes cell cycle progression in spermatogonia (Feng *et al.* 2000). By analogy, it may be assumed that the KIT⁻ spermatogonial subfraction contained 'undifferentiated' and non-cycling cells also in the human testis. This may specifically apply to the spermatogonial subtype with a visible rarefaction zone which was KIT⁻/DMRT1⁻/KI-67⁻. In this study, DMRT1 was likewise absent from the spermatogonia with rarefaction zones, but it was observed in both types Ap and B nuclei, mostly comprising proliferating KI-67⁺ spermatogonia. In rodents, DMRT1 was reported to be highly expressed in undifferentiated spermatogonia, less abundantly expressed in KIT⁺ differentiating spermatogonia, and absent in preleptotene spermatocytes (Matson *et al.* (2010), for review, see Don *et al.* (2011)). In the human, mutations of the *DMRT1* gene are implicated in male infertility, testicular dysgenesis and cancer (Ottolenghi & McElreavey 2000, Krentz *et al.* 2009, Turnbull *et al.* 2010), mainly spermatocytic seminomas (Looijenga *et al.* 2006). This might reflect species differences concerning the correlation of DMRT1 expression with varying differentiation states of spermatogonia. The combined application of three nuclear markers, DMRT1, UTF1 and SPOC1, revealed highly heterogeneous patterns of human spermatogonial nuclei, including those with a

rarefaction zone. Whether such intrinsic variation in nuclear factors may contribute to cell fate heterogeneity is currently unknown. In this context, our observation of 'UTF1⁺-only' spermatogonia, together with a subfraction of DMRT1⁺/UTF1⁻ spermatogonia may be of specific interest. Our study thus characterised human spermatogonia with rarefaction zones as KIT⁻/DMRT1⁻/KI-67⁻ cell type which strongly expressed the consensus stem cell markers PLZF and GFRA1 (at least in subfractions) as well as SPOC1, FGFR3 and UTF1, the latter being associated with an 'undifferentiated' state in rodents (van Bragt *et al.* 2008). However, this expression signature was not absolutely specific, but occasionally was shared by other spermatogonial subtypes.

The unexpected finding that EXOSC10 protein (PM/Scl-100 autoantigen) strongly accumulated inside the rarefaction zones and thus represented a marker for this nuclear domain, may shed light on a specific role in RNA processing during the development of spermatogonia. The PM/Scl complex is the human counterpart of the exosome complex, which is an RNA-processing complex capable of degrading messenger RNAs and non-coding RNAs (for review, see Brouwer *et al.* (2001)). Nucleolar localisation of the exosome (Targoff & Reichlin 1985) suggested a role in ribosome biosynthesis, and, indeed, it was found that Rrp6, yeast homologue of PM/Scl-100, is involved in the processing of 5.8S ribosomal RNA (Briggs *et al.* 1998). Moreover, Rrp6 is required for the elimination of meiosis-specific non-coding RNAs in vegetatively growing budding yeast (Lardenois *et al.* 2010). The stability and molecular function of the mammalian Rrp6 orthologue EXOSC10 in the rodent and the human male germline are being investigated (C Kirchhoff, H Scherthan & M Primig, unpublished observation).

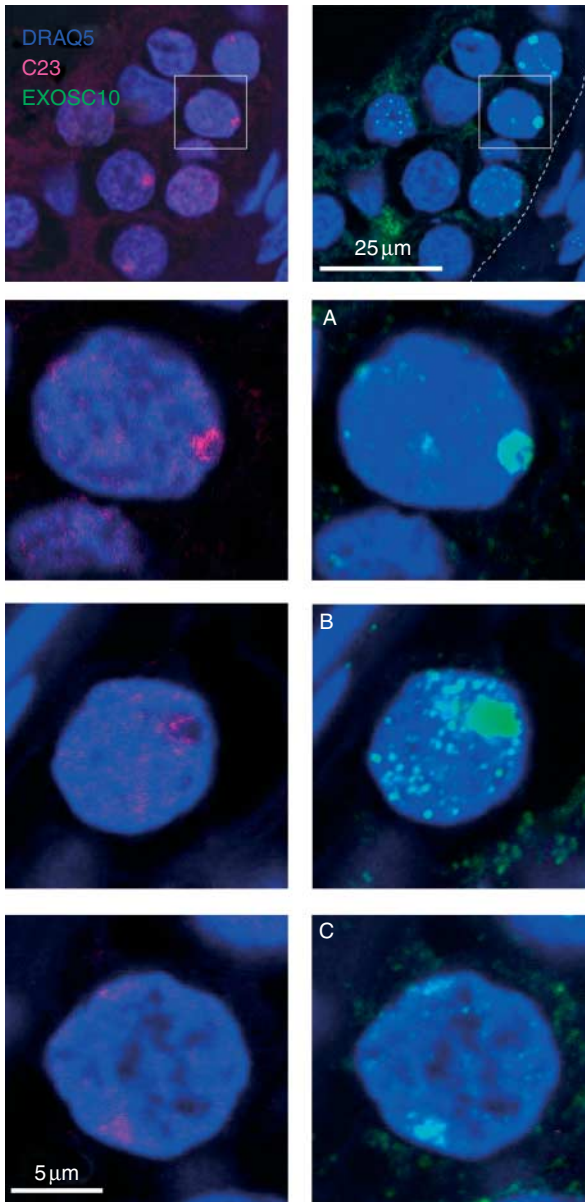


Figure 7 Co-immunolocalisation of EXOSC10 (green) and C23 (red) in DRAQ5-counterstained spermatogonial nuclei (blue). (A) A group of spermatogonial nuclei is positive for both EXOSC10 and C23 showing largely congruent staining of nucleoli, albeit with different staining intensities. The dashed line marks the position of basement membrane. Boxed areas of upper panels are enlarged in panel A to better illustrate congruent EXOSC10 and C23 (nucleolin) staining. B and C show additional aspects of EXOSC10 and C23 staining of peripheral spermatogonial nucleoli.

In the human testis, EXOSC10 protein was found predominantly inside the chromatin rarefaction zones and in the peripheral nucleoli of KI-67⁻ type A spermatogonia. Although its expression in other cell types was not precluded, from our results it seems that the KI-67⁺, i.e. proliferating spermatogonia, did not contain much EXOSC10 protein. Considering that KI-67 is a constituent of the nucleoli in proliferating cells,

strong EXOSC10 immunostaining of either chromatin rarefaction zones and/or nucleoli may instead be characteristic of rarely proliferating or long cycling type A spermatogonia. Currently, it is unclear how this observation relates to the finding that the fly counterpart dRrp6 is important for cell cycle progression (Graham *et al.* 2009). From the highly variable EXOSC10 staining patterns of human spermatogonia, it may be hypothesised that the protein is redistributed between chromatin rarefaction zones and nucleoli, possibly accompanying transformation of one spermatogonial subtype into another. In this context, it seems of interest that Ad spermatogonia can transform into Ap spermatogonia when Ap spermatogonia are diminished, and only after this transition they will start to proliferate (van Alphen & de Rooij 1986). Also, the co-expression of EXOSC10 in chromatin rarefaction zones and nucleoli is suggestive of a functional relationship between these compartments as proposed earlier (compare Paniagua *et al.* (1986)). Indeed, nucleolus-like structures are seen within some rarefaction zones (compare Fig. 2E and description by Clermont (1963)).

First described nearly 90 years ago by Branca (1924), the role of the chromatin rarefaction zone or ‘vacuole’ of human Ad spermatogonial nuclei is still obscure. Eukaryotic nuclei contain numerous morphologically and functionally distinct domains, e.g. nucleoli, Cajal bodies, PML nuclear bodies and nuclear speckles, which are collectively called the nuclear bodies (for review, see Mao *et al.* (2011)). These non-chromatin compartments range in size from tenths of a micrometre to several micrometres, and can be discerned immunologically with antibodies against specific marker proteins. The ‘vacuole’ represents an apparently primate-specific nuclear domain emerging in human spermatogonia only after birth (for review, see Ong *et al.* (2005)); there are, however, scant reports of similar structures in ‘undifferentiated’ spermatogonia of the neonatal mouse testis (Dettin *et al.* 2003) and also of juvenile Utp14b^{jsd} mutant mice (Bolden-Tiller *et al.* 2007, Chiarini-Garcia & Meistrich 2008). Our finding that a highly conserved component of the eukaryotic RNA-degradation machinery, EXOSC10, specifically accumulates within the chromatin rarefaction zones suggests that this long-known nuclear structure may function as a nucleolus-related nuclear RNA-processing body characteristic of the rarely proliferating or long cycling spermatogonia.

The classical view of the Ad spermatogonia as a ‘reserve stem cell’ in primates (Clermont 1972) has recently been subjected to re-examination. One proposal was that the Ad and Ap spermatogonia are the same cell type, and that the distinct difference in their nuclear morphology corresponds to whether the cell is proliferating or in G0 phase respectively (Hermann *et al.* 2010). Categorical classification of ‘undifferentiated’ primate spermatogonia as either Ad or Ap may not always be possible (for discussion, see Plant (2010)). The rarefaction zone or ‘vacuole’,

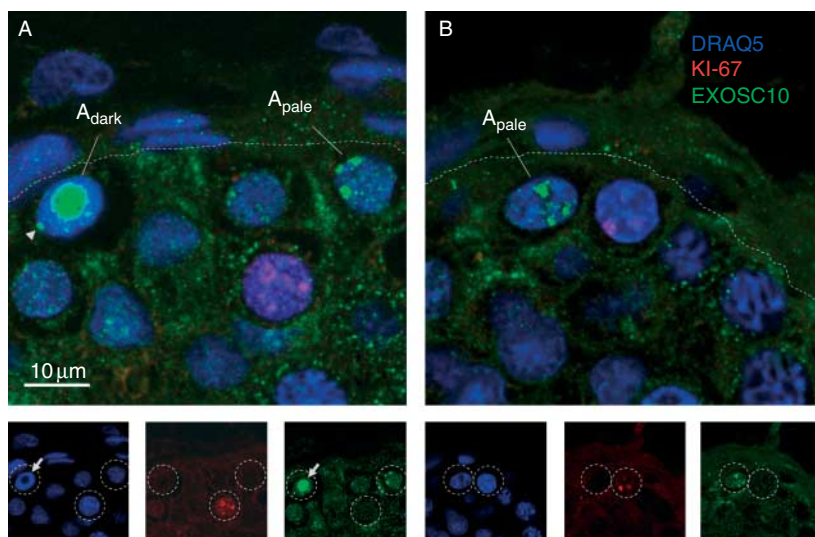


Figure 8 Co-immunolocalisation of EXOSC10 and KI-67 in DRAQ5-counterstained spermatogonial nuclei. (A) Spermatogonia of Ad and Ap subtype show different nuclear localisations of EXOSC10; still, in both subtypes the peripheral nucleoli are EXOSC10⁺ (single nucleolus of Ad nucleus marked by arrowhead). KI-67, in comparison, labels different spermatogonial nuclei, here probably of type B. Single channel images of DRAQ5 (left most), KI-67 (middle) and EXOSC10 (right) are shown below the overlay to illustrate alternate labelling of spermatogonia. Note that KI-67⁺ spermatogonial nucleus appears to be EXOSC10⁻. (B) Neighbouring spermatogonial nuclei, at least the left judged as Ap from the peripheral nucleoli, show alternating EXOSC10⁻ and KI-67-labelling. Single channel images of DRAQ5 (left most), KI-67 (middle) and EXOSC10 (right) are shown below. Dashed lines mark position of basement membrane.

however, was a discriminatory criterion that allowed us to reliably discern during the immunostaining procedures at least a proportion of the human Ad nuclei. The manifestations of the EXOSC10 nuclear staining suggested that the peculiar nuclear morphology of these Ad spermatogonia may reflect a unique nuclear RNA metabolism that is linked to a quasi-quiescent and 'undifferentiated' state by an as yet unknown mechanism.

Materials and Methods

Patients and testicular biopsies

Testis tissue was obtained from patients presenting at the Department of Andrology, University Hospital Hamburg-Eppendorf, Germany, between August 2008 and

May 2011. Tissue samples were taken simultaneously for therapeutic testicular sperm extraction and diagnostic purposes as described previously (Jezek *et al.* 1998, Feig *et al.* 2007, Spiess *et al.* 2007). Informed consent and ethics committee approval by the Ärztekammer Hamburg was obtained (WF-007/11), and the study conducted in accordance with the ethics principles laid down in the Declaration of Helsinki. For this study, tissue samples from a total of 20 normogonadotrophic patients presenting with obstructive azoospermia were selected which showed qualitatively and quantitatively normal spermatogenesis.

Tissue fixation, embedding and histological evaluation

Small fragments of testis tissue (~30 mg) were taken at surgery (left and right sides of the testis) and immediately immersed in

Table 1 Antibodies used in immunohistochemistry and immunofluorescence.

Antigen	References	Company	Code	Species	Clonality	Dilution
C23 nucleolin		Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	sc-8031	Mouse	Monoclonal	1:100
KIT	Schrans-Stassen <i>et al.</i> (1999)	Dako, Hamburg, Germany	A4502	Rabbit	Polyclonal	1:200
DMRT1	Looijenga <i>et al.</i> (2006)	Atlas Antibodies AB, Stockholm, Sweden	HPA027850	Rabbit	Polyclonal	1:1200
EXOSC10	Lardenois <i>et al.</i> (2010)	Abcam, Cambridge, UK	ab50558	Rabbit	Polyclonal	1:50/1:100
FGFR3	Juul <i>et al.</i> (2007)	Santa Cruz Biotechnology	sc-13121	Mouse	Monoclonal	1:10
FGFR3		Cell Signalling Technology, Danvers, MA, USA	#4574	Rabbit	Monoclonal	1:100
GFRA1	Grisanti <i>et al.</i> (2009)	R&D Systems, Minneapolis, MN, USA	AF714	Goat	Polyclonal	1:20/1:40
GPR125	He <i>et al.</i> (2010)	Abcam	ab51705	Rabbit	Polyclonal	1:10–1:100
KI-67 (clone MIB-1)	Cattoretto <i>et al.</i> (1992)	Dako	M7240	Mouse	Monoclonal	1:10
Rabbit Ig fraction		Dako	X0936	Rabbit		1:500
OCT3/4		Santa Cruz Biotechnology	sc-8629	Goat	Polyclonal	1:10–1:100
PLZF	Buaas <i>et al.</i> (2004) and Costoya <i>et al.</i> (2004)	Calbiochem/Merck, Darmstadt, Germany	OP128	Mouse	Monoclonal	1:20/1:50
PGP9.5		Dako	Z5116	Rabbit	Polyclonal	1:500
SPOC1	Mohrmann <i>et al.</i> (2005), Kinkley <i>et al.</i> (2009) and Bördlein <i>et al.</i> (2011)	Dr Elisabeth Kremmer, Munich, Germany		Rat	Monoclonal	Undiluted–1:10
UTF1	Kristensen <i>et al.</i> (2008)	Chemicon, Temecula, CA, USA	MAB4337	Mouse	Monoclonal	1:200

mDF (Latendresse *et al.* 2002), Zenker-formol (Clermont 1963) or 5.5% glutaraldehyde and 1% OsO₄ (Jezek *et al.* 1998). Paraffin embedding was performed by a Thermo Fisher Scientific Shandon Excelsior tissue processor (Thermo Scientific, Karlsruhe, Germany); sectioning was performed on a Leica RM2255 automated rotary microtome (Leica Microsystems, Bensheim, Germany). Epon embedding was performed by a Leica EM TP tissue processor; blocks were cut by a diamond knife (Diatome AG, Biel, Switzerland), using a Reichert ultramicrotome (Leica). Haematoxylin/eosin staining was carried out on deparaffinised and rehydrated 5 µm tissue sections. Semithin sections (section thickness 1 µm) were stained with Toluidine Blue/Pyronine as described (Jezek *et al.* 1998).

Immunohistochemistry

Sections (5 µm) were prepared from mDF-fixed, paraffin-embedded fragments of testis tissue. After deparaffinisation, antigen retrieval was achieved by heat-induced epitope retrieval techniques in a microwave oven employing 0.05 M boric acid (pH=8.5) or Target Retrieval Solution (Dako, Hamburg, Germany). Blocking of endogenous peroxidases was conducted using Dual Endogenous Enzyme Block (Dako) according to the manufacturer's protocol. Blocking of unspecific binding sites was performed by 2% normal goat or 2% normal donkey serum in 1× TBS buffer containing 0.1% Tween (1 h, room temperature). Primary antibodies at optimum dilutions (as listed in Table 1) were applied overnight. Immunoperoxidase staining was achieved by a two-step (Envision plus polymer System, Dako) and/or three-step staining procedure (ABC Staining System sc-2017, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). For each positive antibody, tissues from at least three patients were tested. Specificity of immunostaining was confirmed by both omission of primary antibody and staining of parallel sections with control antibodies. Specifically, rabbit polyclonal PGP9.5 (Z5116, Dako) served as positive and rabbit immunoglobulin fraction (X0936 Dako) as negative control. Stained sections were evaluated by bright-field microscopy (Axiovert 100, Carl Zeiss AG, Oberkochen, Germany) and images captured with an AxioCam ICc3 digital camera (Carl Zeiss AG).

Immunofluorescence and confocal laser scanning microscopy

Sections (5 µm) were prepared from mDF-fixed, paraffin-embedded testis tissue. Immunofluorescence was performed on the sections that had been deparaffinised and rehydrated as described in the previous paragraph. Antigen detection was conducted using the appropriate combination of Alexa Fluor 488, 555 and 647 secondary antibodies (1:200 or 1:300; Invitrogen) and/or DNA stain DRAQ5 (Alexis Biochemicals, San Diego, CA, USA) or DAPI (AppliChem, Darmstadt, Germany). Specificity of staining was again confirmed on parallel sections by omission of primary antibodies and staining with control antibodies. Slides were evaluated on a Zeiss Confocor 2 confocal microscopy system based on the Axiovert 200 M inverted microscope, using the LSM/FCS software

(Carl Zeiss). The microscope was equipped with Plan-Neofluar 10×, 20×, 40× and Plan Apochromat 63× objectives. Visualisation of green fluorophore (Alexa Fluor 488) was achieved by an Argon/2 laser (LASOS Lasertechnik GmbH, Jena, Germany), of red fluorophore (Alexa Fluor 555) by a helium/neon 1 laser (543 nm; LASOS) and of blue fluorophore (Alexa Fluor 647 and DNA dye DRAQ5) by a helium/neon 2 laser (633 nm; LASOS). The pinhole for each channel and the respective objective was set 1 airy unit for captured confocal images. For controls, identical photomultiplier and pinhole settings were used. Confocal images were captured with a Zeiss AxioCam (Carl Zeiss).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0290>.

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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