The rete testis harbors Sertoli-like cells capable of expressing DMRT1

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Abstract

Sertoli cells (SCs) are supporting cells in the mammalian testis that proliferate throughout fetal and postnatal development but exit the cell cycle and differentiate at puberty. In our previous study, we isolated a population of highly proliferative Sertoli-like cells (SLCs) from the region of the adult mouse testis containing the rete testis and adjacent seminiferous tubules. Here RNA-seq of the adult SLC culture as well as qPCR analysis and immunofluorescence of the adult and immature (6 dpp) SLC cultures were performed that allowed us to identify SLC-specific genes, including Pax8, Cdh1, and Krt8. Using these, we found that SLCs are mostly localized in the rete testis epithelium; however, some contribution of transitional zones of seminiferous tubules could not be excluded. The main feature of SLCs indicating their relationship to SCs is DMRT1 expression. More than 40% of both adult and immature SLCs expressed DMRT1 at different levels in culture. Only rare DMRT1+ cells were detected in the adult rete testis, whereas more than 40% of cells were positively stained for DMRT1 in the immature rete testis. One more SC protein, AMH, was found in some rete cells of the immature testis. It was also demonstrated that SLCs expressed such SC genes as Nr5a1, Dhh, Gdnf, and Kitl and interacted with germ cells in 3D co-culture with immature testicular cells. All these similarities between SLCs and rete cells on one the hand and SCs on the other, suggest that rete cells could share a common origin with SCs.

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Sertoli cells (SCs) are specialized supporting cells for developing germ cells and localized within seminiferous tubules of the mammalian testis. SCs actively proliferate throughout fetal and postnatal development but cease proliferation and differentiate at puberty (Sharpe et al. 2003). However, many studies have revealed that adult SCs could partly revert to an immature state under different stimuli (Tarulli et al. 2012). Seasonal breeding Djungarian hamsters resume SC proliferation after experimental changes in gonadotropin levels (Tarulli et al. 2006), and the same process occurs in human testes after gonadotropin suppression, although to a lesser extent (Tarulli et al. 2013). Monkey SCs begin to re-express KRT18, a marker of immature cells, in the testis and culture after experimental cryptorchidism and heat treatment (Zhang et al. 2004, 2006). The proliferation of postpubertal rodent and human SCs was demonstrated in culture (Ahmed et al. 2009, Nicholls et al. 2012) and after transplantation (Mital et al. 2014). Our previous study demonstrated that some SCs isolated from adult mouse seminiferous tubules indeed entered the cell cycle in vitro, but they were not able to proliferate more than once or twice. In contrast, cells isolated from the testis region containing rete testis and adjacent seminiferous tubules actively proliferated in culture producing large colonies (Kulibin & Malolina 2016). These cells, hereafter termed Sertoli-like cells (SLCs), expressed SC genes such as Wt1, Sox9, Gata4, and Vim, and more importantly, some of them also exhibited the expression of DMRT1.

DMRT1 is expressed exclusively in SCs, gonocytes and spermatogonia in male mammals (Raymond et al. 2000, Lei et al. 2007). DMRT1 is a transcription factor that shares the DM domain, a DNA-binding motif, with proteins controlling sex determination and sex differentiation in many metazoans (Zarkower 2013). DMRT1 is not required for primary male sex determination in mammals (Capel 2017), but it is essential for SC differentiation after birth (Raymond et al. 2000) and maintains SC identity throughout postnatal life protecting them from reprogramming into female granulosa cells (Matson et al. 2011). The fact that SLCs can express DMRT1 suggests their relationship to SCs.

We hypothesized that SLCs were localized at the border between seminiferous tubules and the rete testis, a system of tubules and cavities that transports sperm from seminiferous tubules to efferent ducts (Kulibin & Malolina 2016). The most prominent candidates for SLCs were SCs with modified morphology from transitional zones, the terminal segments of seminiferous tubules adjacent to the rete testis (Dym 1974, Nykänen 1979). Those SCs were reported to be capable of proliferation in adult testes of Syrian hamsters and rats (Aiyama et al. 2015, Figueiredo et al. 2016). However, strict localization of SLCs was unclear.
Here we addressed this question, found SLC-specific genes and provided evidence that mouse SLCs mostly reside in the rete testis epithelium. However, there is the possibility that transitional zones also contribute to some SLCs. Further examination of adult and immature rete testis epithelia and SLCs isolated from them revealed many similarities between SLCs and rete cells on one hand and SCs on the other. The most intriguing is DMRT1 expression in a substantial part of both adult and immature SLCs and in the rete testis epithelium.

Materials and methods

Animals

C57Bl/6j mice, B10.GFP mice expressing GFP under the β-actin promoter, and ICR mice were obtained from ‘Stolbovaya’ breeding center of the Scientific Centre of Biomedical Technologies (Russia). B10.GFP mice were maintained on a C57Bl/6j background. Animals were housed in accordance with the European Convention for the Protection of Vertebrate Animals, and all experiments were approved by the Animal Care and Use Committee of Koltzov Institute of Developmental Biology RAS.

Cell isolation and culture

For SLC culture, adult (8–12 weeks) and immature (6 days postpartum (dpp)) testis regions containing the rete testis and adjacent seminiferous tubules were dissected, decapsulated, and digested at 37°C in two steps with collagenase type IV (4 mg/mL, 15 min, Sigma) and 0.125% trypsin with 1 mM EDTA (10 min, Thermo Fisher Sci) for adult tissue, and in three steps with collagenase (15 min), and the mixture of collagenase and hyaluronidase I-S (2 mg/mL; Sigma) repeated twice (25 and 15 min) for pup testes. Dnase I (0.04%; Sigma) was added at all digestion steps. Tissue was extensively washed between digestions. Cells were plated at 37°C in Matrigel (Corning)-coated culture plates at 2 × 10^5 and 5 × 10^4 cells/cm² for adult and pup tissue, respectively. The culture medium was DMEM/F12+GlutaMAX (Thermo Fisher Sci) for adult tissue, and in three steps with collagenase (15 min), and the mixture of collagenase and hyaluronidase I-S (2 mg/mL; Sigma) repeated twice (25 and 15 min) for pup testes. Dnase I (0.04%; Sigma) was added at all digestion steps. Tissue was extensively washed between digestions. Cells were plated at 37°C in Matrigel (Corning)-coated culture plates at 2 × 10^5 and 5 × 10^4 cells/cm² for adult and pup tissue, respectively. The culture medium was DMEM/F12+GlutaMAX (Thermo Fisher Sci) supplemented with sodium pyruvate, insulin-transferrin-selenium (Thermo Fisher Sci), penicillin/streptomycin, and 1% fetal bovine serum (Thermo Fisher Sci) for adult cultures or 10% Knockout Serum Replacement (KSR, Thermo Fisher Sci) for pup cultures. A combination of 10 mM Y-27632 (Abcam), 0.5 mM A-83-01 (Sigma), and 3 mM CHIR99021 (Sigma) (YAC) was added to some adult cultures. After 24 h culture, germ cells were removed from the adult cultures by repeated washing. The medium was changed every 3 days. Cultures of cells from seminiferous tubules (ST cultures) were obtained from testis regions without the rete, and isolated and cultured as described previously (Kulibin & Malolina 2016), and cultured onto floating Nuclepore membranes (Sigma, WHA110406) in the same medium as that for pup SLC cultures. GDNF (10 mg/mL, Sigma) was added to the medium for first 3–4 days until tubular structures were formed. The medium was changed every week. Samples were fixed after 14 days in culture.

RNA-sequence analysis

RNA-sequence data collection and analysis were carried out by the First Oncology Research and Advisory Center (Moscow, Russia). Total RNA was extracted from three independent adult SLC and adult ST culture samples (grown for 5 days in the culture medium without YAC) using RNeasy Mini Kit (Qiagen). RNA sequencing was performed on an Illumina HiSeq3000 System, which generated from 20 to 30 million paired-end reads of 50 bp in length for each sample. To map the reads, STAR software was used (Dobin et al. 2013). DESeq2 was used for the data normalization and the quantification of differential expression between SLC and SC culture samples (Love et al. 2014). Genes were considered to be differentially expressed if the Q-value (false discovery rate) was below 0.05.

Laser capture microdissection (LCM)

For LCM SLC and ST cultures, both adult and immature, were used. All cells were grown in Matrigel-coated culture dishes with PEN foil on the bottom (WillCo Wells). Cultured cells were live stained with SYBR green I (1:5000) for 3 min and underwent LCM on a Leica LMD 7000 system as previously described (Podgorny 2013). For procedure details, see Supplementary Fig. 1 (see section on supplementary data given at the end of this article).

Quantitative RT-PCR (qRT-PCR)

To confirm RNA-seq data, qRT-PCR analysis was performed from the same RNA samples. For LCM samples, total RNA was extracted using an RNeasy Micro Kit (Qiagen). Three biological replicates were performed for each group. cDNA was synthesized using an MMLV RT kit (Evrogen, Russia), and real-time PCR was performed in triplicates using SYBR green qPCRmix-HS with ROX (Evrogen) on a StepOnePlus Real-Time PCR System (Applied Biosystems). PCR amplification conditions were as follows: 45 cycles of 95°C for 15 s, 60°C for 30 s. Primer sequences (Supplementary Table 1) were obtained from PrimerBank (Spandidos et al. 2010), and primers were ordered from Evrogen. Hprt was used as a reference gene to calculate ΔCT values. Fold-change of gene expression was calculated using the 2−ΔΔCT method (Livak & Schmittgen 2001). Data were presented as mean ± s.e.m. Statistical significance was determined by a nonparametric Mann–Whitney U test.

Immunofluorescence

Cultured cells were fixed in 4% paraformaldehyde for 10 min. Testes and 3D co-culture samples were fixed in 10% neutral buffered formalin for 24 h at 4°C, dehydrated, embedded in paraffin, and 4 μm sections were cut. Next, immunofluorescence

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staining was performed. For staining procedure and antibodies used, see Supplementary Table 2. Negative control images for key antibodies are shown in Supplementary Fig. 2.

Culture samples were imaged on a Leica DMI6000 microscope. For triple staining with one anti-mouse (against DMRT1) and two anti-rabbit antibodies one of which labels a nuclear antigen (WT1 or SOX9) and the other cytoplasmic (KRT8 or ACTA2), samples were first stained for a nuclear antigen and photographed with storing reference points and imaged positions in the LAS AF software (Leica), and then re-stained with two other antibodies and imaged again using the saved positions. Subtraction of nuclear staining with anti-rabbit antibodies from the second set of images was performed in the CellProfiler software (Carpenter et al. 2006). Sections were imaged on a Leica TCS SP5 confocal microscope. Image processing, including deconvolution and image stitching, was performed in the LAS AF software.

**Cell counts**

For cultured cells, at least three biological replicates were performed for each experiment, and at least 40 fields of view were imaged on a 10x objective from each sample. For cell counts in sections, three 6 dpp male mice were analyzed. Three to five equally spaced testis sections with the rete testis were obtained from one testis of each animal and imaged. The percent of cells was calculated using CellProfiler software. Data were presented as mean ± s.e.m. Statistical significance was determined by a nonparametric Mann–Whitney U test.

**Results**

**Transcriptome analysis of SLCs**

To determine SLC transcriptional markers and to further investigate the nature of these cells, we examined the transcriptome of adult SLCs using RNA-seq. To do this, we evaluated differential expression between the sub-confluent SLC culture containing 64.8 ± 2.2% SLCs (WT1+ cells in colonies), 1.7 ± 0.4% SCs (WT1+ single cells) and 33.5 ± 1.9% peritubular myoid cells (WT1– cells), and the ST culture containing 4.6 ± 0.7% SCs and 95.4 ± 0.7% peritubular myoid cells. Neither cells from SLC culture nor from ST culture express Hsd17b3 specific for adult Leydig cells (Supplementary Fig. 3A). No cells with lipid droplets characteristic for Leydig cells were observed in SLC culture (Supplementary Fig. 3B). The methods of SLC isolation and culturing were the same as in our previous study (Kulibin & Malolina 2016) except some minor modifications.

According to RNA-seq, the expression of 3156 genes was significantly (Q < 0.05) altered in the SLC culture with 1527 genes upregulated and 1629 genes downregulated (Supplementary Table 3). Due to the substantial number of peritubular myoid cell transcripts obscuring the expression patterns of SLCs, we considered only the top 100 genes upregulated in the SLC culture. For further analysis, we selected some genes reported to be expressed within the urogenital tract and/or be the markers of stem and progenitor cells (Table 1). Krt18 that was not in top 100 genes was also chosen due to its known expression in immature SCs (Sharpe et al. 2003). The differential expression of these genes was validated by qRT-PCR analysis (Supplementary Fig. 3C).

It was previously demonstrated that primary SCs were unstable and became more fibroblast-like within several days in culture (Buganim et al. 2012). Similar changes seemed to take place in the SLC culture as seen by the appearance of ACTA2, a mesenchymal marker, in SLC colonies from day 4 of the culture (Kulibin & Malolina 2016). Here and after, to improve culture conditions for adult SLCs, we added to the medium Rho-associated

<table>
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<th>Gene symbol</th>
<th>Gene name</th>
<th>log₂ Fold change</th>
<th>Q level</th>
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<td>Pax8</td>
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kinase inhibitor (Y-27632), type 1 TGFβ receptor inhibitor (A-83-01), and glycolgen synthase kinase-3 inhibitor (CHIR99021), abbreviated as YAC, that are used for stable culturing of stem and progenitor cells and inhibit epithelial-mesenchymal transition (Efe & Ding 2011, Katsuda et al. 2017). We found that YAC prevented the initiation of ACTA2 expression in SLCs (Supplementary Fig. 4), changed their morphology to more epithelial-like (Fig. 1A–D) and dramatically increased the number of SLCs expressing DMRT1 at high and low levels (Fig. 2A and B).

Dense epithelial-like SLC colonies were present in the cultures established from the rete testis region since birth (Fig. 1E and F, 2C). For cells from immature testes, we used serum-free KSR medium that allowed to culture SLCs without the overgrowth of fibroblast-like cells. The proportion of DMRT1+ SLCs in the immature culture was similar to the adult culture grown in the medium with YAC (Fig. 2B and C).

To confirm the identity of adult SLCs grown in the medium with YAC and immature (6 dpp) SLCs, SLC colonies were isolated using LCM procedure and qRT-PCR analysis was performed indicating that all genes selected from RNA-seq data were upregulated when compared with cultured adult and immature SCs respectively (Fig. 2D and E). The only exception was Tacstd2 whose expression was similar in immature SLCs and SCs.

**Adult and immature SLCs express PAX8, CDH1, and KRT8**

The expression of three SLC markers, namely the most highly expressed gene Pax8, Cdhl, and Krt8, was examined in the adult and immature (6 dpp) SLC cultures by immunofluorescence. PAX8 expression was detected in all SLC colonies identified by SOX9 or WT1 staining in the adult confluent culture grown for 9 days (Fig. 3A). The proportion of PAX8+ cells relative to the total SLC number was more than 90% in the adult culture (Fig. 3B). Because we were not able to accurately define boundaries of 6 dpp SLC colonies without DMRT1 staining, PAX8+ immature SLCs were not counted. However, most cells in the SLC colonies were positively stained for PAX8 in the confluent immature culture grown for 3 days (Fig. 3C), and there were no colonies without PAX8+ cells.

Both adult and immature SLC colonies from the confluent cultures had heterogeneous CDH1 expression. The staining of adult cells was faint but clear (Fig. 3D), whereas immature colonies often exhibited high-level expression of CDH1 (Fig. 3E). KRT8+ SLCs were rarely observed in the adult confluent culture, but they were more often detected in the sub-confluent culture grown for 5 days; some of them co-expressed DMRT1 (Fig. 3F). KRT8+ SLCs with and without DMRT1 expression were present in the immature SLC cultures (Fig. 3G). PAX8, KRT8, and CDH1-positive cells were never observed in ST cultures, except a few faintly stained KRT8+ SCs in the immature culture.

Because CDH1 and KRT8 expression was low in the adult confluent and sub-confluent cultures we additionally performed the immunofluorescent staining of SLCs at 1 day of culture. SLCs were recognized by WT1 or SOX9 staining and typically produced clusters of several cells, whereas SCs, which were also positive for WT1 and SOX9, were rare, sparsely dispersed and excluded from the analysis by their characteristic nuclear morphology. Most SLCs highly expressed CDH1 and KRT8 (Fig. 4A and C). Triple immunofluorescence revealed different SLC phenotypes, including CDH1+KRT8+, CDH1+KRT8−, CDH1−KRT8−, but
never CDH1–KRT8+ cells (Fig. 4A). The proportion of DMRT1+ SLCs relative to the total SLC number was low (9.3 ± 0.7%) and more than 80% of DMRT1+ SLCs co-expressed KRT8 (Fig. 4B and C).

We also performed immunofluorescent staining for acetylated alpha-tubulin (ace-TUB), which has been reported to be a marker of SCs from the transitional zones (Nagasawa et al. 2018). At 1 day of culture, most...
SLCs were not stained for ace-TUB, or the signal was low. However, some WT1+KRT8− cells displayed bright ace-TUB staining (Fig. 4D, an arrow). Later, all cells in culture, including SLCs and peritubular myoid cells, began to stain positive for ace-TUB, so we were unable to determine whether SCs from the transitional zones contributed to SLC colonies.

The rete testis is the only testicular region co-expressing PAX8, CDH1, and KRT8

To localize SLCs in the testis, we examined the region of the adult mouse testis containing the rete testis and adjacent STs. SOX9 and WT1 were expressed in both SCs and epithelial cells of the rete testis, although at substantially lower levels in the latter (Supplementary Fig. 5A and B). These findings were in conflict with our previous reports (Kulibin & Malolina 2016, Malolina & Kulibin 2017) showing no SOX9 or WT1 expression in the adult rete. Potential reasons for this include the sensitivity of the antibodies used and the change from frozen to paraffin sections in the present study. The same reasons explain the fact that here we were able to detect low DMRT1 expression in the SCs of transitional zones (Supplementary Fig. 5C), which was in contrast to our previous study.

Double staining for PAX8 and SOX9 showed that PAX8 was expressed in rete cells but not in SCs from ST and transitional zones (Fig. 5A). The same results were obtained for KRT8 and CDH1 (Fig. 5B). Note that some rete cells were positive for CDH1 but negative for KRT8 (Fig. 5B'), which corresponded to the data from the adult culture described earlier. Ace-TUB detected in the cytoplasm of SCs from transitional zones was never co-localized with KRT8 (Fig. 5C), just as it was in vitro.

Similar to the staining pattern observed in adult testes, PAX8 (Fig. 6A) and CDH1 (Fig. 6B and B') were highly
Figure 4 Immunofluorescence examination of adult SLCs at 1 day of culture. (A) Clusters of SLCs, small WT1+ cells (red), were labeled with KRT8 (yellow) and CDH1 (green) antibodies. Asterisks indicate CDH1−KRT8− SLCs. (B) Some KRT8+ SLCs co-expressed DMRT1 (green, indicated by dots). Compare to a SC (arrowhead, inset) that was KRT8 negative and had characteristic nuclear morphology. (C) The percent of CDH1+ and KRT8+ cells among SLCs, and the percent of KRT8+ cells among DMRT1+ SLCs (mean ± S.E.M., three biological replicates). (D) Staining of the culture with an ace-TUB antibody; a WT1+KRT8− cell with a high ace-TUB signal (green) is indicated by an arrow. Nuclei in (A, B and D) were counterstained with DAPI (blue). Scale bars: 50 µm (A, B and D); 25 µm (inset in B).
expressed in the immature (6 dpp) rete epithelium and were not detected in SCs of STs. Some immature rete cells were positively stained for KRT8, although the signal was also observed in sporadic cells from STs (Fig. 6B and B’). Broad immunofluorescence signal for ace-TUB detected in both STs and the rete testis made it impossible to localize transitional zones at this age (Fig. 6C).

All these results suggest that SLCs mostly reside in the rete testis, whereas the contribution of SCs from transitional zones remains unclear. It is also unknown whether all rete cells or only part of them are able to form SLC colonies.

**Rete testis cells and cultured SLCs express genes characteristic of SCs**

The main feature of cultured SLCs indicating their relationship to SCs is DMRT1 expression. So we examined DMRT1 expression in the rete testis by immunofluorescence and found that 42.6 ± 3.2 % rete cells (identified by co-expression of SOX9 and CDH1) were positive for DMRT1 in the immature testis (Fig. 7A), which corresponded to the number of DMRT1+ SLCs in immature culture. Some DMRT1+ rete cells expressed KRT8 (Fig. 7B), as did SLCs in culture. On the contrary, only rare adult rete cells were positively stained for DMRT1; they were KRT8 positive (Fig. 7C, C’ and C’’) or negative (Fig. 7D and D’). That correlated with the low number of DMRT1+ SLCs at day 1 of adult culture.

AMH is a SC-specific protein highly expressed in embryonic and neonatal testes but disappearing when the testis matures (Sharpe et al. 2003). We found that AMH was also present in some cells of the immature rete testis identified by co-expression of SOX9 and CDH1 (Fig. 8A) or SOX9 and PAX8 (Fig. 8B). Triple immunofluorescence for AMH, DMRT1, and a rete marker CDH1 revealed AMH+DMRT1+ rete cells preferentially localized near seminiferous tubule entries (Fig. 8C) and AMH−DMRT1+ rete cells broadly distributed in the rete testis except in the region adjacent to efferent ducts (Fig. 8C).

We also selected several other genes important for SC function and evaluated their expression in the adult and immature SLC colonies by qRT-PCR using the same LCM samples that were employed for measuring the expression of SLC markers. Decreased Dmrt1 levels were found to be the feature of both adult and immature SLCs (Fig. 8D,E), which consistent with heterogeneous staining for DMRT1 in the SLC cultures. Wt1, Sox9, Kitl, and Trf were expressed in SLCs at levels similar to SCs or even higher. Gdnf levels were not significantly changed between adult SLCs and SCs (Fig. 8D), although these were slightly decreased in immature SLCs (Fig. 8E). Adult SLCs had decreased expression of Nr5a1, Dhh,
Shbg, and especially Inha, whereas immature SLCs expressed Nr5a1 and Dhh at even higher levels than SCs of the corresponding age. Shbg and Inha levels were decreased in immature SLCs but not as much as in the adult cells (Fig. 8D and E). Both adult and immature SLCs had elevated expression of Nr0b1 (Dax1), an orphan nuclear receptor involved in sex determination and gonadal development (Fig. 8D and E).
SLCs interact with germ cells in co-culture with neonatal testicular cells

DMRT1+CDH1+SOX9− cells identified as spermatogonia were detected in the epithelium of the immature rete testis (Fig. 7A). Germ cells disappeared from the rete during testis maturation along with DMRT1 loss. These findings suggest the ability of rete cells to support germ cell function and the importance of DMRT1 for this process.

To test if cultured SLCs are able to interact with germ cells, we mixed SLCs obtained from adult GFP mice and grown in culture for 9 days and testicular cell suspension isolated from ICR neonatal mice and established 3D co-culture. GDNF was added to the cells for first 3 days to increase germ cell viability. Immunofluorescent staining demonstrated GFP+ SLCs expressing SOX9 and DMRT1 (Fig. 9A) and forming, together with neonatal cells, tubular structures surrounded by peritubular myoid cells labeled by ACTA2 (Fig. 9B). Some SLCs were found to be in direct contact with germ cells expressing DDX4 (Fig. 9C) or markers of meiosis initiation and progression, STRA8 (Fig. 9D) and SCP3 (Fig. 9E), respectively. The most differentiated germ cells appeared to progress until the meiotic prophase (Fig. 9E'). Staining for cleaved caspase-3, an apoptosis marker, confirmed the viability of germ cells. Apoptotic cells were few and located at the center of 3D samples (Fig. 9F), whereas germ cells were in the periphery (Fig. 9G).

Figure 7 Cells in the adult and immature rete testis epithelium expressed DMRT1. (A) A stitched image of the immature rete testis (RT) marked by co-expression of CDH1 (red) and SOX9 (blue) with many DMRT1+ (green) rete cells. ST, seminiferous tubules. Asterisks indicate CDH1+DMRT1+ germ cells in the rete epithelium that were SOX9-negative. (B) Some DMRT1+ (green) rete cells expressed KRT8 (red) in the immature testis (indicated by arrows). (C and D) Rare rete cells expressed DMRT1 (green) in the adult testis. TZ, transitional zones. (C', C'' and D') show higher magnifications of boxed areas with DMRT1+ rete cells positive (C', C'', asterisks) or negative (D', an arrowhead) for KRT8 (red). Nuclei were counterstained with DRAQ5 (blue). Scale bars: 50 µm (A, C and D); 25 µm (B, inset).
Figure 8 Cells in the immature rete testis and SLCs in culture expressed SC genes. (A and A') AMH+ (yellow) cells in the rete testis (RT) positively stained for CDH1 (green) are indicated by asterisks, SOX9 (red) labels SCs and rete cells. Arrows point to AMH+ cells not expressing CDH1 (i.e., SCs). (B and B') AMH+ (yellow) cells in the rete positively stained for PAX8 (green) are indicated by arrowheads. ST, seminiferous tubules. (A' and B') Higher magnifications of boxed areas represent maximum projections of serial confocal optical sections; orthogonal projections of areas denoted by yellow lines are shown in the right and bottom panels. (C) A stitched image of the immature (6 dpp) rete testis stained for AMH (blue), CDH1 (red), and DMRT1 (green). Dots point to AMH+ rete cells. A dotted line outlines part of the rete without DMRT1+ and AMH+ cells. ED, efferent duct. Nuclei were counterstained with DRAQ5 (blue). (D and E) Expression of the selected genes in the adult (D) and immature (E) SLC colonies obtained by LCM. Dashed lines indicate the gene expression levels in SCs. The data are presented as the mean ± S.E.M. from three biological replicates. *P < 0.05. Scale bars: 100 µm (A, B and C); 20 µm (A' and B').
Previously, we reported that highly proliferative cells resembling SCs (SLCs) could be isolated from the region of the adult mouse testis containing the rete testis and adjacent ST (Kulibin & Malolina 2016). In the present study, we performed RNA-seq analysis of the adult SLC culture and identified SLC-specific genes. We also improved SLC culture conditions by supplementation of YAC, a combination of small molecules, and demonstrated the presence of SLC colonies in the culture from immature testes. Both adult SLC colonies grown with YAC and immature SLC colonies expressed marker genes selected from RNA-seq data. Their feature was the high numbers of cells positive for SC protein DMRT1.

Three SLC genes selected from RNA-seq data, Pax8, Cdh1, and Krt8, were further investigated. Pax8 is a transcription factor belonging to the PAX protein family. Pax2, along with another member of the PAX family, PAX2, are the earliest specific markers of the urogenital system and demonstrate some functional redundancy during pro- and mesonephros development (Sharma et al. 2015). However, according to our RNA-seq data, only Pax8 is expressed in SLCs. Pax8 expression was reported in the human rete testis (Ozcan et al. 2011). In the mouse, Pax8 was demonstrated in the efferent ducts and the epididymis; its deficiency leads to their absence or severe defects (Wistuba et al. 2007). Here, we found that Pax8 was expressed in all epithelial cells of the adult rete testis but not in other testicular cells. Cdh1 is a component of adherens junctions prominently expressed in epithelial tissues (Schneider & Kolligs 2015). The previous study (Nagasawa et al. 2018) and our current findings demonstrated that the only somatic cells expressing CDH1 in the mouse testis were epithelial cells of the rete testis. The third marker examined, Krt8, in pair with Krt18, forms intermediate filaments in many simple epithelia (Owens & Lane 2003).

Discussion

Figure 9 Adult SLCs contacted with germ cells in the 3D co-culture with immature testicular cells. (A and B) The most cells from the SLC culture identified by GFP staining (green) were SLCs (SOX9+ cells, red, A) with different levels of DMRT1 expression (yellow), and not peritubular myoid cells (ACTA2+ cells, red, B). Asterisks indicate SLCs. A dot points to a GFP+SOX9− cell. (C, D and E) Some germ cells (arrowheads) positively stained for DDX4 (red, C), a pre-meiotic marker STRA8 (red, D), and a meiotic marker SCP3 (red, E) were closely associated with GFP+ cells. Dotted lines in (B and E) outline tubule-like structures. (C, D and E) represent maximum projections of serial confocal optical sections; orthogonal projections of areas denoted by yellow lines are shown in the right and bottom panels. (E′) represents a higher magnification of one of the optical sections in (E, arrow) and shows a spermatocyte in metaphase with an adjacent GFP+ cell. (F and G) Single apoptotic cells labeled by anti-cCASP3 antibody (red, F) and germ cells labeled by anti-DDX4 antibody (red, G) were located at different sites, at the center and in the periphery of 3D-culture samples respectively. An inset in (F) shows a higher magnification of a boxed area. Nuclei were counterstained with DRAQ5 (blue). Scale bars: 20 µm (A, B, C, D and E); 50 µm (F and G); 5 µm (E′); 25 µm (inset).
Its expression was reported previously in the human rete testis (Dinges et al. 1991). KRT8 immunoreactivity was also observed in fetal and neonatal SCs in the mouse (Appert et al. 1998). Here, we showed the presence of KRT8 in the adult rete testis but, in contrast to PAX8 and CDH1, it was expressed in many but not all rete cells. We were also able to find a few rete cells expressing DMRT1. These findings indicate the heterogeneity of rete cells. This feature is even more prominent in the immature testis where the number of DMRT1+ rete cells is dramatically increased and some of them express another SC protein AMH, that is consistent with the previous study (Reboucet et al. 2014).

As the rete testis is the only testicular region where PAX8, CDH1, KRT8, and DMRT1 are co-expressed we conclude that SLCs are mostly localized in the rete testis epithelium. Like the rete cells, cultured SLCs are heterogeneous. Especially intriguing is their heterogeneity for DMRT1 expression. We showed previously that some adult SLCs were positive for DMRT1 (Kulibin & Malolina 2016). Here we found that many more DMRT1+ SLCs are present in immature culture that correlates with a higher number of DMRT1+ rete cells in the immature testis. The proportion of DMRT1+ adult SLCs can be increased to the immature value by YAC, a combination of three small molecules. Small molecules are used for stable culturing of embryonic and tissue-specific stem cells and for facilitating somatic cell reprogramming (Efe & Ding 2011). They can have a general effect on cellular metabolism or modulate specific signaling pathways. YAC was reported to stimulate proliferation of mouse hepatocytes in vitro, improve their survival, and induce the expression of progenitor markers (Katsuda et al. 2017). It seems that the effects of YAC on SLCs are similar. We speculate that YAC induces DMRT1 expression in adult SLCs. Another explanation is the preferential proliferation of DMRT1+ SLCs initially presenting in culture. However, it is less possible as the proliferative rates of DMRT1− and DMRT1+ SLCs did not differ, at least at days 5 and 9 of culture (data not shown).

The issue that have remained unclear is the contribution of SCS from transitional zones to SLCs. There is growing evidence indicating that these SCS have unique characteristics. They can proliferate in adult testes of Syrian hamsters and rats (Aiyama et al. 2015, Figueiredo et al. 2016). Some of them do not express maturation markers GATA4 and AR (Figueiredo et al. 2016). According to our data SCS from mouse transitional zones have decreased levels of DMRT1. They do not proliferate in the adult testis (Kulibin & Malolina 2016) but exit the cell cycle later than other SCS when the testis matures (Malolina & Kulibin 2017). Here we demonstrated that SCSs from transitional zones are not positive for SLC markers PAX8, CDH1, and KRT8 and so could not contribute to most of SLCs. However, we do not exclude the possibility that they can be among PAX8– cells present in a few number in SLC colonies or initiate the expression of SLC genes.

qPCR analysis of the adult and immature SLC colonies showed that, besides Dmr1, they expressed some other SC genes, including Nr5a1 and Dhh, at the levels comparable to SCS. SLCs appeared to be able to support germ cells in a similar way as SCS. They expressed Gdf1 and Kitl encoding growth factors that regulate the maintenance and survival of germ cells (Oatley & Brinster 2012). In the 3D co-culture with neonatal testicular cells, SLCs closely interacted with germ cells. All these similarities between SLCs and SCS suggest that rete cells could share a common origin with SCS. The fact that many rete cells in the immature testis are positive for SC proteins DMRT1 and AMH confirms this hypothesis.

Immature SLCs and adult SLCs cultured with YAC are similar in DMRT1 expression but, according to qPCR analysis, immature SLCs more closely resemble SCS of the corresponding age that could reflect progressive diversification of rete cells and SCs during postnatal development. Another feature of both adult and immature SLCs demonstrated by qPCR analysis is the elevated expression of Nr0b1 (Dax1). Nr0b1 deficiency leads to infertility in male mice because of the rete testis obstruction by aberrantly located proliferating SCs (Jeffs et al. 2001). Nr0b1 was reported to repress Amh in the fetal testis and immature SC cultures (Tremblay & Viger 2001, Bowles et al. 2018). Based on these findings, we speculate that NR0B1 could play an important role in the specification of rete cells by inhibiting SC genes such as Amh. Another transcription factor that could be involved is PAX8.

Further studies on the embryonic and postnatal testis are required to elucidate the origin of rete cells and the mechanisms of their specification.

**Supplementary data**

This is linked to the online version of the paper at https://doi.org/10.1530/REP-19-0183.

**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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Author Contribution Statement

E A M and A Y K designed research and analyzed data; E A M performed immunofluorescence and LCM procedure; A Y K performed cell culturing, PCR analysis, and cell counting; E A M wrote the manuscript with input from A Y K.

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