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Effects of Spermatogenic Cycle on Stem Leydig Cell Proliferation and Differentiation

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Abstract

We reported previously that stem Leydig cells (SLC) on the surfaces of rat testicular seminiferous tubules are able to differentiate into Leydig cells. The proliferation and differentiation of SLCs seem likely to be regulated by niche cells, including nearby germ and Sertoli cells. Due to the cyclical nature of spermatogenesis, we hypothesized that the changes in the germ cell composition of the seminiferous tubules as spermatogenesis proceeds may affect tubule-associated SLC functions. To test this hypothesis, we compared the ability of SLCs associated with tubules at different stages of the cycle to differentiate into Leydig cells in vitro. SLCs associated with stages IX-XI were more active in proliferation and differentiation than SLCs associated with stages VII-VIII. However, when the SLCs were isolated from each of the two groups of tubules and cultured in vitro, no differences were seen in their ability to proliferate or differentiate. These results suggested that the stage-dependent local factors, not the SLCs themselves, explain the stage-dependent differences in SLC function. TGFB, produced in stage-specific fashion by Sertoli cells, is among the factors shown in previous studies to affect SLC function in vitro. When TGFB inhibitors were included in the cultures of stages IX-XI and VII-VIII tubules, stage-dependent differences in SLC development were reduced, suggesting that TGFB may be among the paracrine

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factors involved in the stage-dependent differences in SLC function. Taken together, the findings suggest that there is dynamic interaction between SLCs and germ/Sertoli cells within the seminiferous tubules that may affect SLC proliferation and differentiation.

Keywords

Stem Leydig Cells; Spermatogenic Cycles; Testosterone; TGFB

1. Introduction

In rats and other mammals, germ cell development is classified into stages defined by the associations of the germ cells with each other as they develop over time in a sequential and cyclic manner. Testosterone, produced by Leydig cells in the interstitial compartment of the testis, is integrally involved in the regulation of spermatogenesis. Altered testosterone production can block the differentiation of specific germ cells at specific stages of the cycle (Kerr et al. 1993), and can affect blood–testis barrier formation, meiosis, spermiogenesis, and spermiation (De Gendt et al., 2004; Meng et al., 2005; Chang et al., 2004; Sun et al., 1990; Saito et al., 2000). There also is evidence that Leydig cell functions can be affected by the surrounding tubules (Parvinen and Huhtaniemi 1990; Jauregui et al., 2018; Syed et al., 1985; Vihko et al., 1989; Bergh and Damber 1984; Bergh 1985; Gizang-Ginsberg and Wolgemuth 1985; Parvinen et al., 1984). Parvinen et al (1990) reported that testosterone levels varied with the stage of the cycle, highest at Stages VII–VIII. Other studies have found that changes in the morphology (Bergh and Damber 1984; Bergh 1985) or gene expression (Jauregui et al., 2018) of Leydig cells occurred in a stage-specific manner, suggesting that there is a paracrine relationship between Leydig cell function and the seminiferous epithelium.

Adult Leydig cells (ALCs) arise from undifferentiated stem cells. These cells, referred to as stem Leydig cells (SLCs), were first identified in the early postnatal rat testis and shown to have the ability to self-renew or to differentiate into testosterone-producing cells (Ge et al., 2006; Chen et al., 2009; 2010; 2017). Numerous studies have shown that the elimination of Leydig cells from the adult testis by a single intraperitoneal injection of ethanedimethanesulfonate (EDS) is followed by Leydig cell repopulation within 6–10 weeks thereafter, suggesting that there also are stem cells in the adult testis (Kerr et al., 1985; Jackson et al., 1986; Morris et al., 1986). Indeed, there now is compelling evidence that there are SLCs situated around both seminiferous tubules and blood vessels of the interstitial compartment of the testis that are capable of giving rise to Leydig cells after the elimination of the preexisting Leydig cells by EDS treatment (Chen et al., 2016; Stanley et al., 2012; Odeh et al., 2014; Li et al., 2016; Davidoff et al., 2004; O’Shaughnessy et al., 2008).

The functions of stem cells, including their renewal and differentiation, have been shown to be affected by both extracellular and intracellular signals (Li and Xie 2005; David and Scadden 2006). As yet, however, little is known about the mechanisms involved in the regulation of adult SLC quiescence, proliferation and differentiation. We showed that the culture of isolated seminiferous tubules with LH alone resulted in the differentiation of

tubule-associated SLCs into testosterone producing ALCs by 4 weeks (Stanley et al., 2012; Odeh et al., 2014; Li et al., 2016), but that isolated SLCs, when cultured with LH alone, did not differentiate (Li et al., 2016). These observations have led us to hypothesize herein that the functions of SLCs may be influenced by signals from local Sertoli and germ cells of the seminiferous tubules with which the SLCs are associated.

Addressing the interactions between SLCs and local testicular cells in vivo is complicated by the cyclical nature of spermatogenesis and thus the changes over time in the local environment of the SLCs. In the present study, we have taken advantage of the in vitro system that we developed in which SLCs in association with seminiferous tubules can differentiate in response to LH in the absence of other factors and influences (Stanley et al., 2012; Odeh et al., 2014; Li et al., 2016). Our objective was to determine whether the association of the SLCs with tubules containing germ cells at different stages of the cycle, and thus potentially with differing signals from the germ and/or Sertoli cells, would differentially influence the proliferation and differentiation of SLCs into testosterone-producing Leydig cells.

2. Materials and Methods

2.1. Chemicals

The culture media (M-199, DMEM/F12) and Click-iT EdU (5-ethynyl-2'-deoxyuridine) imaging kit were purchased from Invitrogen (Carlsbad, CA). Smoothed Agonist (SAG) was from EMD Bioscience. Testosterone was obtained from Steraloids Inc. (Newport, RI). (1,2,6,7,16,17-³H [N])-Testosterone was obtained from PerkinElmer (Boston, MA). Ethane dimethanesulfonate (EDS) was synthesized according to the method described by Jackson and Jackson (Jackson and Jackson 1984). CellTiter-Glo® Luminescent Cell Viability Assay kit was from Promega (Madison, WI). Rabbit anti-CYP11A1 antibody and non-immune control rabbit IgG were from MyBiosource. HRP-conjugated secondary antibodies for Western blots were from Amersham Pharmacia Biotech. Alexa Fluor secondary antibodies for immune-fluorescent staining were from Thermo Fisher Scientific. All other reagents, including 4',6-diamidino-2-phenylindole (DAPI), ITS cell culture supplement solution, rabbit anti-ACTA2, and mouse monoclonal anti-GAPDH were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Animals and treatment

Adult male Brown Norway rats, 3 to 5 months of age, were supplied by Harlan Sprague-Dawley, Inc. (Indianapolis, IN) through the NIA animal resource program. The rats were housed in animal facilities of the Johns Hopkins Bloomberg School of Public Health under controlled light:dark cycle (14L:10D) and with free access to water and rat chow. All animal procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals, according to protocols approved by the Johns Hopkins Animal Care and Use Committee. To eliminate Leydig cells from the testes, rats were injected with a single dose of EDS (i.p., 80 mg/kg of BW) dissolved in a mixture of DMSO:PBS (1:3). Testes were collected 4 days after EDS treatment, by which time all adult Leydig cells had been eliminated (Kerr et al., 1985; Jackson et al., 1986; Morris et al., 1986).

2.3. Isolation and culture of seminiferous tubules

Seminiferous tubules were mechanically separated from the interstitium with fine forceps under a transillumination dissection microscope (Kotaja et al. 2004). Tubules at stages VII-VIII and IX-XI were identified by published criteria (Kotaja et al. 2004). Equal lengths (5 cm) of isolated tubules were cultured in 24-well plates in a 1:1 mixture of DMEM/F-12 and Medium 199 supplemented with 0.1% BSA, 15 mM HEPES, 2.2 mg/ml sodium bicarbonate, penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively), insulin/transferrin/selenium (ITS), and LH (10 ng/ml) for up to 4 weeks at 34°C and 5% CO₂. Duplicate wells were used for each treatment, and each experiment was repeated at least three times. At the end of experiments, media were collected and frozen (−20°C) for testosterone measurement, and tubules were dried for cytochemical staining of 3β-hydroxysteroid dehydrogenase/delta(5)-delta(4) isomerase (HSD3B), or immediately fixed in either 2.5% glutaraldehyde for Epon plastic embedding or 10% formalin for paraffin embedding. Sections of paraffin-embedded tissue were used for immunofluorescent staining of CYP11A1 and ACTA2. Some tubules were frozen and stored at −80°C for Western blots. To assay cell viability, the total ATP contents of cultured tubules were assayed by CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI), according to the manufacture's instructions.

2.4. SLC proliferation in vivo

To measure SLC proliferation in vivo, rats (N=4) were injected with EdU (i.p., 50 mg/kg BW) dissolved in a mixture of DMSO:PBS (1:3) two days after injection of the rats with EDS. Twenty-four hours after EdU injection, testes were fixed in formalin (6 hrs) and then embedded in paraffin. Dividing nuclei were labeled in sections by the Click-iT procedure. The tissues were co-stained with the DNA dye DAPI. To quantify the positive cells, labeled nuclei on the surfaces of stage VII-VIII and IX-XI tubules were counted. A total of 40 tubules were counted for each group from 4 individual animals.

2.5. SLC proliferation and differentiation in vitro

To measure proliferation, dividing cells on the surfaces of cultured tubules were labeled with the Click-iT EdU Imaging Kit. In brief, seminiferous tubules of stages VII-VIII and IX-XI were cultured for 5 days and then labeled with EdU (10 µM) for 24 h. Click-iT positive cells on the tubule surfaces were visualized using either whole mounted tubules or tubule sections. For detection of the dividing cells in relationship to the tubule surface, sections were stained for ACTA2 to localize myoid cells and co-stained with EdU to localize the dividing cells. To measure the differentiation of Leydig cells, SLCs on whole-mounted tubules or tubule sections were stained for HSD3B or CYP11A1. To quantify the positive cells, EdU or CYP11A1 stained cells were counted with at least 40 sections that came from three different experiments. Additionally, testosterone levels were determined in the media by radioimmunoassay (RIA). The sensitivity and intra-assay and inter-assay coefficients of variation of the RIA were 13 pg/tube and 8.9 and 13.6%, respectively.

2.6. Purification and culture of stem cells by flow cytometry

The peritubular SLCs were stained for CD90 protein (Li et al., 2016). To isolate CD90+ cells, the tubules were digested with 1 mg/ml collagenase-D in DMEM/F12 medium at 34°C

for 30 min with slow shaking (90 cycles/min). After allowing seminiferous tubules to settle, the dispersed cells were filtered through a 50µm pore nylon mesh and stained for CD90, and then sorted by flow cytometry. CD90 antibodies were conjugated with the fluorochromes PE. Cells were incubated with CD90 antibody (1:100) in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS (0.5% BSA, 5µM EDTA) for 45 min on ice. After washing 3 times, the cells were suspended in 1 ml HBSS (0.5% BSA and 5 mM EDTA) for flow cytometric sorting (MoFlo Sorter, Beckman-Coulter, Brea, CA). To compare the ability of SLCs isolated from different stages (VII-VIII vs IX-XI) to form Leydig cells, SLCs were first expanded in DME/12 medium containing 2.5% FBS, 10ng/ml FGF2 and 10ng/ml PDGFBB. When the cells reached 80% confluence, they were switched into M199 medium containing LH (10 ng/ml) for a week. Then the cells were treated with LH plus desert hedgehog (DHH) agonist SAG (0.5µM) for 2 weeks. By the end of 3 weeks, the differentiation of the cells was determined by assessing their ability to produce testosterone in response to LH (24 hours), or the cells were stained for HSD3B.

2.7. Staining of CYP11A1 and HSD3B

Seminiferous tubules or tubule sections were washed with Ca^{2+} and Mg^{2+} free HBSS (0.5% BSA) and then incubated with primary antibody (CYP11A1 or ACTA2, 1:100) for 1 hour followed by incubation with conjugated secondary antibody (1:1000) for another hour. As a negative control, sections were stained with non-immune control rabbit IgG in place of CYP11A1 primary antibody, which resulted in no staining (Fig S1A). For some studies, tubules or sections were first reacted with Click-iT EdU before the primary antibody. After washing 3 times, tissues reacted with ACTA2 primary antibodies were then treated with fluorescent secondary antibodies (Alexa-conjugated anti-rabbit or anti-mouse IgG, 1:1000) for 1h. After 3 washes, the tissues were examined with a Nikon Eclipse 800 microscope. To validate the specificity of the Click-iT reaction, tubules or sections were not exposed to EdU before the reaction, resulting in absence of labeling (Fig S1B). For enzymatic staining of HSD3B, the whole mounted tubules were dried at room temperature for 30 minutes. The samples were then stained for 45 min with a solution contained 0.4 mM 5β-androstan-3β-ol-17-one steroid substrate, 1 mg/ml NAD, and 0.2mg/ml tetranitro blue tetrazolium, as being described in a previous publication (Stanley et al., 2012).

2.8. Light microscopy of plastic embedded tissues

Some of the cultured tubules were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in cacodylate-buffered 1% osmium tetroxide, and embedded in Epon. Sections (1µm) were mounted on glass slides and stained with toluidine blue.

2.9. Western blot analysis

Tissues or cells were lysed with Tris-HCl buffer (100 mM Tris, 0.1% Triton X-100, 50mM dithiothreitol, 1× Sigma protease inhibitor cocktail, pH 6.8) for 30 mins on ice. After centrifugation (18,000g, 10 min), the supernatant was mixed with 3X SDS loading buffer (New England BioLab, Ipswich, MA). Equal amounts of total protein (about 30 µg) from each sample were separated by 10% SDS-PAGE, and then transferred onto a nitrocellulose membrane. After incubation with primary antibody (CYP11A1, 1:1000) and horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000), the signals were detected by the enhanced chemiluminescence Western blot kit from Pierce (Rockford, IL). The bound

antibodies on the membranes were stripped by Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), and the membranes were re-probed with GAPDH antibody.

2.10. Statistical analyses

Data are expressed as the mean $\pm$ SEM. For the comparisons between two groups, the unpaired t-test was used. For the comparisons among multiple groups, one-way ANOVA was used. If the means of group differences were revealed by ANOVA to be $P < 0.05$, differences between individual groups were determined with the Student-Neuman-Kuels test, using SigmaStat software (Systat Software Inc., Richmond, CA). Values were considered significant at $P < 0.05$.

3. Results

3.1. Effects of spermatogenic cycle on the development of peritubular Leydig cells

We showed previously that culture of seminiferous tubules physically separated from the interstitium of the rat testis can result in the differentiation of the stem cells on the tubule surfaces into testosterone producing Leydig cells (Stanley et al., 2012; Odeh et al., 2014; Li et al., 2016). In the conduct of these in vitro studies, we noted variations in testosterone production by different tubule preparations. We hypothesized that a reason for the variation might be that different seminiferous tubule preparations contained tubules at different stages of the cycle of the seminiferous epithelium, and therefore that there might be different germ/Sertoli cell influences on the tubule-associated stem cells. To test this, we separated tubules of specific stages (VII-VIII vs IX-XI). These two sets of stages were easily identifiable by their dark (VII-VIII) and light (IX-XI) appearances with transillumination under a dissection microscope (Fig 1A). The light appearance of stages IX-XI tubules results from the release of mature sperm at stage VIII (Kotaja et al. 2004). When equal lengths (5 cm) of tubules were cultured separately with LH for 4 weeks, the light (stages IX-XI) tubules produced almost 5 times more testosterone than the dark (stages VII-VIII) tubules (Fig 1B). Sections of the tubules after their culture for 4 weeks revealed a thicker layer of cells on the surfaces of stages IX -XI (Fig 1C) than stages VII-VIII (Fig 1D) tubules. At higher magnification, the cell layer generated on the stages IX-XI tubules contained many round cells with typical mature Leydig cell characteristics (Fig 1E), while such cells were largely missing from the stages VI-VII tubules (Fig 1F). This result is consistent with the testosterone production results, and suggests that peritubular SLCs associated with tubules of stages IX-XI are more active in generating Leydig cells than are the cells associated with tubules of stages VII-VIII.

To confirm that the cells associated with cultured stages IX-XI tubules in fact are Leydig cells, the tubules were stained for the Leydig cell marker proteins HSD3B (Fig 2A, 2B) or CYP11A1 (Fig 2C, 2D). There were more HSD3B positive cells formed on the tubules at stages IX-XI (Fig 2A) than stages VII-VIII (Fig 2B). That also was the case of cells stained for CYP11A1 (red, Figs 2C, 2D). The CYP11A1-stained cells (red) are seen above the ACTA2-stained (green) myoid cell layer. Quantification of the CYP11A1 positive cells revealed that stages IX-XI tubules had about 5 times more Leydig cells than stages VII-VIII tubules (Fig 2E). Western blot analyses of the CYP11A1 content of the cultured tubules of

stages IX-XI and VII-VIII were consistent with the analyses of testosterone production and with staining; the cells of stages IX-XI had significantly higher levels of CYP11A1 protein than cells of stages VII-VIII (Fig 2F).

3.2. Effects of spermatogenic cycle on the proliferation of peritubular stem Leydig cells in vitro

Our previous studies had shown that tubule-associated SLCs in the adult testis proliferate during the first week of tubule culture with LH, and then differentiate to testosterone-producing cells during weeks 2–4 (Odeh et al., 2014; Li et al., 2016). Having shown (Fig. 1) that over a 4-week culture period, stages IX-XI tubules produced significantly more testosterone than stages VII-VIII tubules, we hypothesized that factors from tubules at these different stages might have differing effects on the proliferation of the tubule-associated SLCs, the differentiation of testosterone-producing Leydig cells, or both. To examine the effect on cell proliferation, we labeled the dividing cells with EdU during the first week (day 5) of culture. The tubules of stages IX-XI (Fig 3A) appeared to have more EdU-positive cells than those of stages VII-VIII (Fig 3B). To quantify the EdU-positive SLCs, cultured tubules were sectioned and the myoid cells were stained for ACTA2 (green, Figs. 3C, 3D). Labeled cells were evident both within the tubules and outside the myoid layer (Fig 3C). The positive cells (pink) located on the outer surfaces of the myoid cell layer were counted, revealing that stages IX-XI tubules had about three times more dividing cells (SLCs) than stages VII-VIII tubules (Fig. 3E).

To examine the possible effect of in vitro culture conditions on cell viability, the ATP contents of the cultured tubules were assayed over a 3 week culture period (Fig 3F). There was little change in ATP content during the first week (15% reduction without statistical significance). Significant reductions in ATP were noted during the second week. It should be noted that there was no difference in cell viability between the two groups of staged tubules at any assessed time point.

3.3. Effects of spermatogenic cycle on the proliferation of peritubular stem Leydig cells in vivo

Having shown that the spermatogenic cycle apparently affects SLC proliferation in cultured tubules, we wished to determine whether such effects also occur in vivo. SLCs have been shown to divide 2–3 days after EDS treatment (Chen et al., 2010). Consequently, 2 days after EDS treatment, the dividing cells were labeled with EdU for 24h. EdU-positive cells were seen outside the tubules (Fig 4). Significantly greater numbers of such cells were seen in tubules of stages IX-XI than VII-VIII (Fig. 4), as had also been seen in cultured tubules of those stages.

3.4. Do peritubular SLCs differ per se among different spermatogenic stages?

We had shown previously that the peritubular SLCs express the protein CD90 (Li et al., 2016). To examine whether the spermatogenic cycle affects the distribution of SLCs along the tubular surfaces, freshly isolated tubules were stained for CD90 (Figs 5A, 5B). There was no apparent difference in the number or distribution of CD90+ cells between stages IX-XI (Fig 5A) and VII-VIII (Fig 5B). To compare these cells at the two stages, the CD90+

cells were digested from the tubules and then isolated by flow-cytometry from the pooled stages IX-XI (Fig 5D) or VII-VIII (Fig 5E) tubules. Unstained cells are seen in Figure 5C. The percentages of the CD90+ positive cells (cells on top of the diagonal line) from the two groups were similar, at about 0.5% of all the cells (Fig 5D vs. Fig 5E). As illustrated in Figures 5F (stages IX-XI) and 5G (stages VII-VIII), the numbers and the sizes of the cells also appeared to be similar. Figure 6 shows the results of culturing isolated CD90+ SLCs from stages VII-VIII and IX-XI with LH alone or with LH plus SAG. When cultured for 3 weeks with LH alone, the cells, whether from stages VII-VIII or IX-XI, did not become HSD3B positive (Fig. 6A) and did not produce testosterone in response to subsequent LH stimulation for 24 hours (Fig. 6D). When cultured with LH plus SAG (0.5 μ M) for 3 weeks, the cells from both sets of stages (Figs. 6B, 6C) became HSD3B positive. These cells were then stimulated with LH for 24 hours to assay their ability to produce testosterone (Fig 6D). No differences were seen between the stages from which the cells had been isolated. These observations suggest that factors from the tubules and not intrinsic differences among the SLCs themselves explain the stage-dependent differences in SLC differentiation.

3.5. Involvement of niche factors in the stage-dependent differences in SLC differentiation

As yet, little is known as to the nature of the niche factors that are capable of regulating SLC function. It is well known that in addition to the changes in germ cell composition at different stages of the cycle, Sertoli cell functions also change in response to the germ cells. We reported that the Sertoli cell products PDGF, FGF2, activin, DHH and TGFB had apparent regulatory effects on SLC proliferation and differentiation (Li et al. 2016). In a previous study that compared the transcriptional changes across the spermatogenic cycle (Johnston et al., 2008), TGFB was among the candidates that held promise as potentially involved in the stage-specific regulation of SLC functions, for the following reasons: First, there is stage-specific expression of the three major members of this family, with the highest expression levels at stages VII-VIII and lowest levels at stages IX-XI (Fig S2A, Johnston et al., 2008). This expression pattern is consistent with the negative effects of TGFB on SLC proliferation and differentiation (Li et al., 2016), and in particular with the differences in SLC functions between stages VII-VIII and IX-XI. Second, we found that the expression levels of the receptors for members of this family are highly expressed by SLCs, and down-regulated during the differentiation and maturation of Leydig cells from their progenitors (Fig S2B, Stanley et al., 2011). These data suggest that tubule-derived TGFB may play roles in stage-dependent SLC development. In a preliminary study designed to address this possibility, we cultured the tubules of the two stages with TGFB inhibitors, reasoning that if the TGFB family members play a role in the stage-dependent differences in SLC development, the inhibitors should block the regulatory effects of the tubule-derived factors, and therefore reduce the stage-dependent differences. Adding the three inhibitors during the 4 weeks of culture indeed increased Leydig cell testosterone production in all groups (Fig 7A). The increases (expressed as percent increase from controls) were more extensive in the dark (VII-VIII) stages than the light (XI-IX) stages (Fig 7B). Thus including TGFB inhibitors resulted in smaller stage-dependent differences, suggesting that members of the TGFB family may indeed play a role in the stage-dependent differences in SLC development.

4. Discussion

Following the elimination of the Leydig cells of the adult rat testis with EDS, the ALC population is restored within two months. It has been shown that the division and differentiation of SLCs (Kerr et al., 1985; Jackson et al., 1986; Morris et al., 1986), situated both on the surfaces of the seminiferous tubules and around blood vessels of the interstitial compartment, give rise to the new ALCs (Chen et al., 2016; Stanley et al., 2012; Odeh et al., 2014; Li et al., 2016; Davidoff et al., 2004; O'Shaughnessy et al., 2008). We demonstrated that SLCs associated with the seminiferous tubules were able to proliferate and differentiate when the tubules were cultured with LH, and thus give rise to testosterone-producing ALCs in vitro (Stanley et al., 2012; Odeh et al., 2014; Li et al., 2016). However, culturing SLCs isolated from the tubules with LH alone did not result in their differentiation (Li et al., 2016). When the seminiferous tubules and interstitium were encapsulated separately in alginate and implanted subcutaneously into castrated rats, Leydig cells formed on the surfaces of the implanted tubules, and this resulted in increased serum testosterone. The tubules, when removed from the implanted rats and incubated with LH ex vivo, produced testosterone (Chen et al., 2016). With implanted interstitial tissue, no Leydig cells were formed. However, co-culture of interstitium with tubules in vitro resulted in the formation of Leydig cells by both compartments (Chen et al., 2016). These results suggested that the seminiferous tubules contain factors required for stem cells to proliferate and/or differentiate whether the cells are associated with the tubules or distant from them.

There is in vivo evidence that the content of the seminiferous tubules affect the regeneration of Leydig cells in EDS-treated rats. It has been reported, for example, that seminiferous tubule damage induced by cryptorchidism, x-irradiation, or busulfan at the time of EDS treatment accelerates Leydig cell regeneration (Kerr and Donachie 1986; O'Leary et al., 1986). More rapid restoration of Leydig cells has been reported around atrophic tubules than germ cell-filled tubules in EDS-treated rats (Sharpe et al., 1990). This is consistent with the findings in unilaterally cryptorchid animals in which more rapid Leydig cell regeneration occurred after EDS in the cryptorchid testis than in the contralateral scrotal testis (Kerr and Donachie 1986; O'Leary et al., 1986).

We hypothesized herein that seminiferous tubules at different stages of the cycle, and thus containing different complements of developing germ cells, may affect SLC function differently. To test this hypothesis, we used an in vitro system that we established in which culturing the tubules with LH resulted in the proliferation and differentiation of the SLCs and ultimately in the formation of ALCs (Li et al., 2016). Using this system, we compared SLC development when associated with tubules of stages VII-VIII versus tubules of stages IX-XI. After 4 weeks in culture, stages IX-XI tubules generated 5 times more Leydig cells than stages VII-VIII. Similarly, stages IX-XI tubules produced 5-times more testosterone than stages VII-VIII. The expressions of CYP11A1 and HSD3B supported the conclusion that peritubular SLCs associated with stages IX-XI tubules are more active in producing Leydig cells than those associated with stages VII-VIII.

As indicated, these studies were conducted in vitro, using cultured tubules. We realized that there was likely to be degeneration of cells within the tubules over the course of the 4 weeks

of culture, potentially making it difficult to interpret results. Little change occurred in the ATP content of the tubules during the first week of culture when SLC proliferation occurred, but significant reduction in ATP content occurred thereafter. These results indicate that cells inside seminiferous tubules did lose viability (degenerate), though not until weeks 2–3, after the period of cell proliferation. Interestingly, the changes in ATP content over the course of the culture period were the same regardless of the stages of the tubules. Additionally, we conducted in vivo studies designed to determine whether the stage-related effects on cell proliferation seen during the first week in vitro also occur in vivo. To this end, dividing cells were labeled with EdU in vivo after EDS treatment of the rats. Significantly greater numbers of dividing cells were seen in vivo on the surfaces of tubules of stages IX–XI than VII–VIII, consistent with the results obtained using cultured tubules.

In studies designed to determine whether the SLCs associated with stages VII–VIII and IX–XI themselves differ, the SLCs were isolated and their abilities to proliferate and differentiate, in the absence of tubule-derived factors, were determined. There was no difference in either proliferation or differentiation, supporting the contention that it is the supporting niche cells associated with specific seminiferous tubule stages that define the stage-dependent difference in SLC development. How might seminiferous tubules with different germ cell contents affect Leydig cell regeneration? It is possible that the differences are due to the inhibitory effect of stage VII–VIII tubules or, alternatively, to the stimulatory effects of stage IX–XI tubules. Because cells within the seminiferous tubules do not physically contact SLCs on the tubule surface or elsewhere in the interstitial compartment, any effect of seminiferous tubule cells must be through paracrine factors. There are signaling molecules that have been shown to affect Leydig cell differentiation, including DHH, Notch, PDGF, FGF2, SCF, IGF1, LIF and TGFB (Ge et al., 2006; Li et al., 2016; Tony et al., 2013; Clark et al., 2000; Pierucci-Alves et al., 2001). It is well known that Sertoli cells secrete most of these factors. However, how these factors change with the spermatogenic cycle is largely unknown. Through carefully examining previously published array data, we identified the TGFB signaling family of particular interest. First, all its major members change with the spermatogenic cycle, with significant reductions during the transition from stages VII–VIII to IX–XI (Johnston et al., 2008). Second, we found that TGFB receptors were highly expressed by SLCs, and decreased significantly as soon as the cells committed to the Leydig lineage (PLC and onward) (Stanley et al., 2011). To test whether TGFB family members in fact play a role in the stage-dependent changes in SLC proliferation and differentiation, three TGFB inhibitors were included in the cultures of tubules. As expected, all three inhibitors had effects on testosterone production at 4 weeks that were stage-specific, with significantly greater effects seen at stages VII–VIII tubules than at stages IX–XIs. Indeed, in the presence of TGFB inhibitors, the differences between the two sets of tubules became much less severe, suggesting strongly that TGFB may play a role in the stage-dependent effects found in this study. How and whether other regulatory factors are involved is currently under study.

The significant losses of germ cells after the first week of tubule culture make it difficult to interpret apparent stage-dependent effects on SLC differentiation over time. Moreover, it is possible that factors ultimately affecting SLC differentiation in fact act during the first week, before significant loss of germ cells or altered Sertoli cell function, thus providing the

triggering signals for subsequent differentiation. Another possibility could be that the surviving cells, most likely Sertoli and myoid cells, are able to maintain their ability to signal SLCs even after the loss of the germ cells. These possibilities currently are under study.

In summary, we found that peritubular SLCs associated with stages IX-XI tubules have greater potential to divide and differentiate than SLCs associated with stages VII-VIII tubules. These differences are most likely due to the differences in the niche environment created by cells in the seminiferous tubules rather than to differences in the SLCs per se. Previous studies suggest that Sertoli cell products, in particular, are of importance. Among the latter, TGFB family members apparently play important roles, though the mechanism by which they do so is not known. These findings provide in vitro and in vivo evidences supporting the contention that the continuous, cyclical changes that characterize mammalian spermatogenesis play significant roles in regulating the changing functions of the associated testicular stem cells that give rise to Leydig cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Stem Leydig cell (SLC) functions are affected by the spermatogenic cycle.
2. SLCs associated with stages IX-XI are more active in proliferation and differentiation than those of stages VII-VIII.
3. TGFB may be involved in the stage-dependent regulation of SLCs.

Figure 1.

Effects of stages of the spermatogenic cycle on peritubular stem Leydig cells in vitro. A: Transition from stage VIII (dark) to stage IX (light). B: Testosterone production by stage VII-VIII and stage IX-XI tubules after 4 weeks in culture. The experiments were repeated 5 times with tubules isolated from different animals. Data expressed as mean $\pm$ SEM.

*Significantly different from stage VII-VIII at $P < 0.05$. C-F: Leydig cells (arrows) generated on the surfaces of the stage IX-XI (C, E) and VII-VIII (D, F) tubules by 4 weeks of culture. (Scale bars: A, 200 μ m; C, D, 20 μ m; E, F, 10 μ m)

Figure 2.

Effects of spermatogenic stages on the expression steroidogenic enzymes after 4 weeks in culture. A, B: HSD3B staining of cells associated with tubules of stages IX-XI (A) and VII-VIII (B). C, D: CYP11A1 staining (red) of stages IX-XI (C) and VII-VIII (D) tubules. Myoid cells are stained for ACTA2 (green). E. Quantification of CYP11A1 positive cells of stages VII-VIII and IX-XI tubules. F: Western blots of CYP11A1 expression by stages VII-VIII and IX-XI tubules. The experiments were repeated 3 times with tubules isolated from

different animals. Data are expressed as mean $\pm$ SEM. *Significantly different from stages VII-VIII at $P < 0.05$. (Scale bars: 20 μ m)

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Figure 3.

Effects of spermatogenic cycle on SLC proliferation. A, B: EdU-labeled cells associated with whole-mount tubules of stages IX-XI (A) and VII-VIII (B). C,D: EdU-labeled cells associated with sections of tubules of stages IX-XI (C) and VII-VIII (D). The myoid cell marker ACTA2 appears green. White arrows indicate dividing SLCs. E: Quantification of EdU positive cells in sections from 3 different experiments. *Significantly different from stages VII-VIII tubules at $P < 0.05$. F: Viability of seminiferous tubular cells maintained in

vitro for 3 weeks. Data are expressed as mean $\pm$ SEM of 3 replicate experiments.

*Significantly different from day one cultures at $P < 0.05$. (Scale bars: 20 μ m)

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Figure 4.

Effects of spermatogenic cycle on SLC proliferation in vivo. A: Labeling of cells by Click-iT reaction (green) with DAPI co-staining (blue). Arrows indicate labeled peritubular SLCs of stages VII-VIII and IX-XI. B: Quantification of labeled peritubular SLCs of stages VII-VIII and IX-XI. *Significantly different from stage VII-VIII at $P < 0.05$. (Scale bars: 20 μ m)

Figure 5.

Distribution of CD90+ SLCs in relationship to the stage of the cycle. A, B: CD90+ cells associated with stages IX-XI (A) and VII-VIII (B) tubules. Unstained (C) and CD90-stained cells were isolated from stages IX-XI (D) and VII-VII (E) tubules by flow-cytometry. The percentages of the CD90+ positive cells are cells on top of the diagonal line. F, G: Final collected cells from stages IX-XI (F) and VII-VIII (G). (Scale bars: A, B, 20 μ m; F, G, 50 μ m)

Figure 6.

Differentiation of CD90+ cells in vitro. A-C: CD90+ cells were cultured in vitro for 3 weeks with LH (A) or LH+DHH agonist SAG (B, C). In the absence of SAG, no HSD3B positive cells were formed whether cells were from stages VII-VIII or IX-XI (A). In the presence of SAG, equal percentages of HSD3B positive cells were differentiated from cells of stages IX-XI (B) and VII-VIII (C) tubules. D: Quantification of testosterone production by CD90+ cells isolated from stages VII-VIII and IX-XI tubules and incubated with LH alone or LH + SAG. The experiments were repeated 3 times with the tubules isolated from different animals. Data are expressed as mean $\pm$ SEM. (Scale bars: 30μm)

Figure 7.

Effects of TGFB inhibitors on the differentiation of SLCs. Stages VII-VIII and IX-XI tubules were cultured in the presence or absence of 3 TGFB inhibitors for 4 weeks. A: Testosterone production by VII-VIII and IX-XI tubules. The experiments were repeated 3 times using tubules isolated from different animals. Data are expressed as mean $\pm$ SEM. *Significantly different from the controls of corresponding stages at $P < 0.05$. B: Expression of data in A as percent of the corresponding controls.