REVIEWS

The Rete Testis: Development and Role in Testis Function

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Abstract—The *rete testis* connects seminiferous tubules in which germ cells develop to the efferent ducts and the epididymis, where gametes mature and gain mobility. Several recent studies have thoroughly explored the morphogenesis of this structure in mice during embryonic and postnatal periods. A part of the *rete testis* has been shown to derive from the precursors of gonad somatic cells before sex determination. The other part forms from embryonal Sertoli cells of testis cords adjacent to the mesonephros. The transformation of Sertoli cells into *rete testis* cells is apparently not limited to the embryonic stage of development and continues during postnatal testis development. Recently, it was found that the *rete testis* participates in the formation and maintenance of specialized Sertoli cells in terminal segments of seminiferous tubules, transitional zones. Current views suggest that the transitional zones of the seminiferous tubules may represent a niche for spermatogonial stem cells, the site of the prolonged proliferation of Sertoli cells in the pubertal and postpubertal periods of testis development, and also could be a generator of spermatogenic waves. To sum up, the *rete testis* transports gametes from the testis to the epididymis, maintains pressure within seminiferous tubules, regulates the composition of the testicular fluid, and impacts the spermatogenic process itself.

Keywords: *rete testis*, Sertoli cell, embryonal development of the *rete testis*, transitional zone of seminiferous tubules, spermatogenesis

In most animals, except for the primitive forms, male gametes development (spermatogenesis) occurs in specialized organs, the testes. In mammals, their structural unit is the convoluted seminiferous tubules (Fig. 1a), in the spermatogenic epithelium of which germ cells develop. Differentiation begins with spermatogonial stem cells (SSCs) located on the basement membrane surrounded by somatic Sertoli cells (SCs), which carry out a niche-forming function and support the development of germ cells at all stages of spermatogenesis (Fig. 1b). The SSCs, upon entering differentiation, first undergo a series of mitotic divisions and then enter the meiosis prophase, in which the last round of DNA replication occurs before two consecutive divisions, culminating in the formation of haploid round spermatids. Meiotic germ cells at the pre-leptotene stage leave the basement membrane, cross the area of tight junctions between neighboring SCs, and move to the adluminal compartment (Fig. 1b), where they complete the meiosis process and enter the last stage of differentiation, spermiogenesis. During spermiogenesis, round spermatids are transformed into elongated spermatids through a series of morphological changes involving all cell organelles (Fig. 1b). The elongated spermatids lose their contacts with the spermatogenic epithelium and enter the lumen of the seminiferous tubule, becoming spermatozoa. The spermatozoa then leave the testis and are carried from the seminiferous tubules to the first section of the efferent system, the *rete testis* (Fig. 1c), with the flow of fluid formed by the SCs.

The *rete testis* is a set of connected cavities and ducts lined with simple epithelium, through which sperm are transported from the gonad via the efferent tubules to the epididymis, in which they finally mature and gain mobility (Figs. 1a, 1c, 1d). While the biology of SSCs and SCs, as well as the entire process of male germ cell development, have been sufficiently studied and covered in many reviews (Griswold, 1998; Griswold, 2018; Hess and Renato de Franca, 2008; Kopera et al., 2010; Kubota and Brinster, 2018; de Rooij, 2017), the development of *rete testis* and understanding of its role in the functioning of the spermatogenic system are only beginning to gain clarity. Recently, a study (Imura-Kishi et al., 2021) revealed the role of the *rete testis* in maintaining the regional specialization of the SCs in the terminal sections (transitional zones) of the convoluted seminiferous tubules. Evidence suggests that the terminal sections of the seminiferous tubules are a niche for *Gfra1*^{+} SSCs (Aiyama et al., 2015), the site of the prolonged proliferation of SCs in the pubertal and postpubertal testis (Figueiredo et al., 2016, 2019; Malolina and Kulibin, 2017), and the site of spermatogenic wave generation that supports conti-
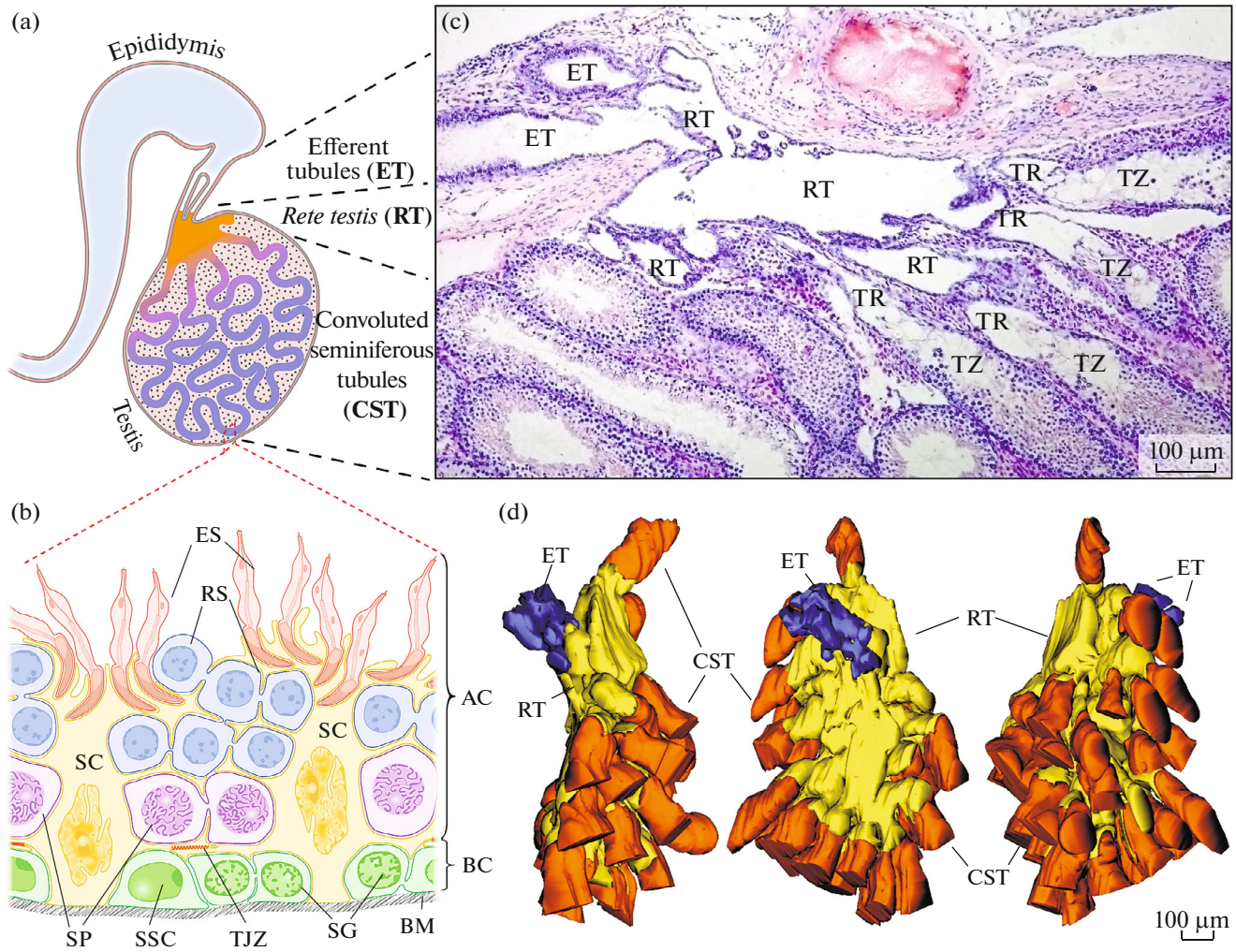
Fig. 1. The structure of the mouse testis, the rete testis, and the seminiferous epithelium of the convoluted seminiferous tubule. (a) a schematic diagram of the adult mouse testis, efferent ducts, and the epididymis. The convoluted seminiferous tubule represents an arc, both ends of which are connected to the rete testis. Seminiferous tubules are surrounded by interstitial tissue. (b) A schematic diagram showing a segment of the transverse section of the seminiferous epithelium. AC—the adluminal compartment of the seminiferous epithelium, BC—the basal compartment, BM—basement membrane, TJ—tight junctions between adjacent Sertoli cells, SC—Sertoli cell, RS—round spermatid, SG—differentiating spermatogonium, SP—spermatocyte, SSC—spermatogonial stem cell, ES—elongated spermatid. (c) A histological section of the adult mouse rete testis, hematoxylin-eosin staining. ED—efferent duct, RT—rete testis, TR—tubulus rectus, TZ—transitional zone. (d) 3D-reconstruction of the adult rete testis region with initial segments of the efferent ducts and seminiferous tubules, constructed from serial histological sections, several projections are presented.

nuity of male germ cell development (Perey et al., 1961). These findings, taken together, extend the functions of the *rete testis* beyond the simple transit of gametes from testis to epididymis, maintenance of intratesticular pressure, and regulation of the composition secreted by testes (Hess and Hermo, 2018), to involvement in the development of spermatogenic system and maintenance of spermatogenesis. In this review, we briefly overview recent findings concerning the development of the *rete testis* in mice, the structure and function of the transitional zones of the seminiferous tubules, and the involvement of the *rete testis* in their formation.

EMBRIONIC DEVELOPMENT OF THE *RETE TESTIS* IN MICE

In mice, gonad development begins at around embryonic day 9.0 (E9.0), when $Sf1^+$ (Steroidogenic factor 1) and $Wt1^+$ (Wilms' tumor protein 1) cells of the coelomic epithelium located on the ventral side of the mesonephros begin to actively proliferate and invade its parenchyma, forming by E9.5 small thickenings (genital ridges) inward the coelomic cavity. At E10.0, the ridges begin to populate the germ cells coming from the hindgut (Nef et al., 2019). At this point in development, the gonads are arranged similarly in both sexes, and the constituent $Sf1^+$ somatic cell pre-

cursors are bipotential and can differentiate into ovarian or testicular cells.

The choice of the pathway of further development, i.e., sex determination, is triggered by a complex cascade of gene expression. For most mammals, including mice, the key factor determining the development of testes is the *Sry* (Sex-determining region Y) gene of the Y chromosome. In the absence of the *Sry* expression, the *Rspo1* (R-spondin 1) and *Wnt4* (Wnt family member 4wingless-type protein 4) genes guide the development of bipotential gonads into ovaries (Svingen and Koopman, 2013). *Sry* triggers expression of the *Sox9* gene (SRY-box transcription factor 9) in progenitor cells, which activates the SC development program. In response, these cells begin to highly express the SCs marker genes *Amh* (Anti-Mullerian hormone) and *Dmrt1* (Doublesex and mab-3 related transcription factor 1), actively proliferate, and by E12.5 form an irregular network of testis cords with germ cells located in the center (Cool et al., 2012).

The testis cords are the precursors of the convoluted seminiferous tubules, while the mesonephric duct and mesonephric tubules provide most of the testis efferent system, the epididymis, and the efferent tubules, respectively. The *rete testis* can be seen as a connecting structure between these two primordia (Fig. 2a). Such a location of the *rete testis* determines the features of its formation. Most recent data (Kulibin and Malolina, 2020; Omotehara et al., 2020, reviewed in detail in Major et al., 2021) demonstrate that *rete testis* can develop as two separate primordia. The first part of *rete testis* origin even at the bipotential gonad stage at E10.5 from a small group of $Sf1^+$ progenitor cells located between the mesonephros tubules at the anterior end of the gonad (Omotehara et al., 2020, Fig. 2b1). By E12.5, these cells form a network of tubules extending from the anterior end of the gonad to the posterior end and contacting the mesonephros tubules on one side and the testis cords on the other (Fig. 2b2).

After E13.5, the second part of the *rete testis* develops from the end sections of the testis cords connected into a network, facing the mesonephros (Figs. 2b3, 2b4), i.e., from embryonic SCs (Kulibin and Malolina, 2020). As they transform into *rete testis* cells, SCs begin to express *Pax8* (Paired bBox 8), a marker of *rete testis* cells (Ozcan et al., 2011; Malolina and Kulibin, 2019), and lose *Amh* expression, albeit partially maintaining *Dmrt1* expression (Fig. 2B4). This process begins at the anterior end of the gonad and spreads to its posterior end, culminating by E16.5 in the formation of separate Amh^+ testis cords that are interconnected with the $Pax8^+$ rete cords (Fig. 2b5). Such a complex bipartite *rete testis* formation appears to be necessary for the proper connection of all ends of the testis cords, the number of which varies between 10 and 11 at E13.5-14.5 (Nel-Themaat et al., 2009), to the ends of the four mesonephric tubules.

We can summarize that both reports (Kulibin and Malolina, 2020; Omotehara et al., 2020) present evidence supporting the hypothesis of the "gonadal" origin of the *rete testis* (Combes et al., 2009) in contrast to the assumption of its "mesonephric" origin (Zamboni and Upadhyay, 1982; Wrobel, 2000; Joseph et al., 2009).

During the development of ovaries, at the boundary between the gonad and mesonephros, a structure homologous to the *rete testis*, the *rete ovarii*, is formed (Byskov and Lintern-Moore, 1973). Omotehara et al. (2020) showed that it forms in embryonic development in a similar way, from $Sf1^+$ cells at the stage of the indifferent gonad. Two works (Kulibin and Malolina, 2020; McKey et al., 2021) showed that in the *rete ovarii* in the vicinity of the gonad, cells are co-expressing *Pax8* and the granulosa cell marker *FoxL2* (Forkhead box L2). The presence of such cells suggests that granulosa cells, like embryonic SCs, participate in the formation of the *rete ovarii*. The reverse process of transformation of *rete ovarii* cells into ovarian cells is also not excluded. Thus, there is evidence that *rete ovarii* cells can become theca cells (Smith et al., 2014; Liu et al., 2015). The final answer to this question can be obtained only after lineage-tracing studies.

Little is known concerning regulatory mechanisms of *rete* development, but we can assume that the transcription factor *Pax8*, which participates with *Pax2* in mesonephros development (Sharma et al., 2015), also takes an essential role in *rete* formation in both sexes. Thus, according to Omotehara et al. (Omotehara et al., 2020), a part of the *rete testis* and *rete ovarii* arises from $Sf1^+$ cells that start expressing *Pax8* between E11.5-12.5 (Kulibin and Malolina, 2020) during the rete tubule formation. In addition, the presence of cells with an intermediate phenotype $Pax8^+/Amh^+$ (Figs. 2b4, 2b5) at the boundary between the newly formed *rete testis* and the testis cords (Kulibin and Malolina, 2020) indicates the participation of the *Pax8* in the transformation of SCs into *rete testis* cells.

Omotehara et al. (2020) noted that the connection of mesonephric tubules to the rete testis could be regulated by Notch signaling pathway. Additionally, morphogenetic factors produced by mesonephric tubule cells can likely support the formation of the rete testis and its connection to the efferent system. Retinoic acid (RA) produced by the mesonephros spreads from the anterior end to the posterior end of the gonad and may also be involved in the morphogenesis of the *rete testis*. Thus, artificially increasing the concentration of RA in the embryonic testes leads to increased expression of *Nr0b1* (nuclear receptor subfamily 0 group B member 1 dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1), which suppresses the expression of SCs marker genes, including *Amh* (Bowles et al., 2018). After E12.5, *Nr0b1* is known to be highly expressed in the *rete testis*
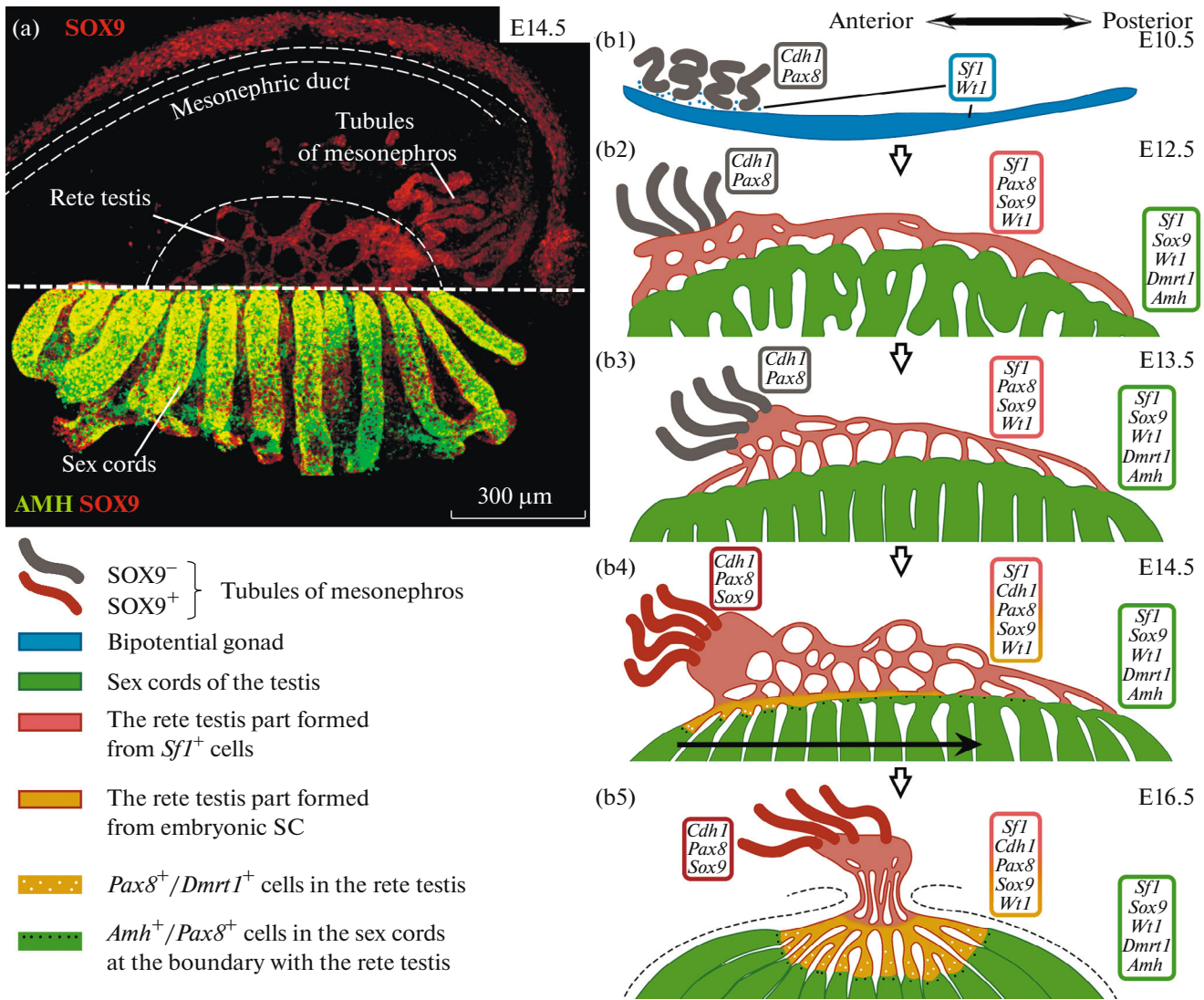
Fig. 2. Embryonic development of the mouse rete testis. (a) A representative image of a gonad/mesonephros complex stained for Sox9 and Amh at E14.5. A maximal projection of serial optical sections obtained by confocal microscopy, taken from (Kulibin and Malolina, 2020) and presented here with minor changes. (b1–5) A diagram of the rete testis development in mice. (b1–2) According to the data from (Omotehara et al., 2020). (b3–5) A diagram was partly taken from (Kulibin and Malolina, 2020) and presented here with minor changes. An arrow in B4 indicates the direction of the formation of the rete region originating from embryonic Sertoli cells. Marker genes of mesonephric tubules, rete testis, and testis cords at different embryonal stages are indicated in rectangular insets.

cells (Swain et al., 1996; Ikeda et al., 2001). It is conceivable that after E13.5, the concentration of RA at the mesonephros-testis interface is high enough to increase the expression of $Nr0b1$ in the SCs of the testis cords and suppress the Amh expression in them, which together with the $Pax8$ expression would lead to the transformation of the SCs into *rete testis* cells, but further studies are needed to confirm this hypothesis.

On the other hand, the cells of the developing *rete testis* may, together with the gonad cells, produce factors supporting the survival and proliferation of the mesonephric tubule cells. One such factor, as noted by de Mello Santos and Hinton (2019), could be the epi-

dermal growth factor (EGF), which prevents cell death in the developing kidney (Carev et al., 2008).

DEVELOPMENT OF A MICE *RETE TESTIS* IN THE POSTNATAL PERIOD

The development of the *rete testis*, which began in the embryonic period of development, lasts in the postnatal period. Meanwhile, the *rete testis* cells actively proliferate and form a cavity lined by a simple epithelium, the height of which varies in different sections from squamous to cuboidal-shaped (Major et al., 2021; Malolina and Kulibin, 2017). Outside, the *rete testis* is surrounded by a layer of peritubular myoid

cells, as well as interstitial tissue permeated by a network of blood and lymphatic capillaries (see Fig. 3, Rebourcet et al., 2014Hess and Hermo, 2018; Figueiredo et al., 2016). The proliferation of *rete testis* cells in mice in vivo during the postnatal developmental stage ends at approximately the same time as the proliferation of SCs in the convoluted seminiferous tubules, i.e., after 18 days (Malolina and Kulibin, 2017). Nevertheless, the *rete testis* cells obtained from adult mice, unlike the SCs of seminiferous tubules, maintain the ability to proliferate actively and form colonies in cell culture (Malolina and Kulibin, 2018; Malolina and Kulibin, 2019).

A recent study (Malolina and Kulibin, 2019) revealed the marker genes of the *rete testis* cells, such as the already mentioned *Pax8*, as well as *Krt8* (Keratin 8), and *Cdh1* (Cadherin 1, Aiyama et al., 2015). Notably, on day 6 of postnatal development, a significant part of the *rete testis* cells also expresses the SCs marker gene *Dmrt1*, the expression of which then decreases and persists only in single cells of the rete in adult animals. Besides, there are many $Amh^+/Dmrt1^+$ SCs in the *rete testis* on day six after birth in the regions connecting to the convoluted seminiferous tubules as well as cells expressing SCs markers and *rete testis* cells simultaneously: $Amh^+/Sox9^+/Cdh1^+$, $Amh^+/Sox9^+/Pax8^+$, and $Amh^+/Dmrt1^+/Cdh1^+$ cells. The presence of such cells in the *rete testis* during this period probably explains a large amount of $Dmrt1^+$ cells in it and suggests that the recruitment of cells to the rete due to SCs can continue in the postnatal period of development of the structure. The decrease in *Dmrt1* expression in *rete testis* cells in adult animals compared to day 6 of postnatal development can be considered as a discrepancy in the differentiation pathways between the SCs of the seminiferous tubules and the *rete testis* cells. This assumption is supported by PCR analysis data showing that the level of expression of SCs marker genes in *rete testis* cells in 6-day-old animals is more consistent with that in SCs of the same age than in *rete testis* cells and SCs obtained from adult animals (Malolina and Kulibin, 2019).

STRUCTURE AND FUNCTION OF TERMINAL SEGMENTS OF SEMINIFEROUS TUBULES

The *rete testis* forms special protrusions, *tubuli recti* (Fig. 1c), through which it connects to the terminal segments of the seminiferous tubules, the so-called transitional zones (TZ) (Hess and Hermo, 2018; Roosen-Runge and Holstein, 1978). Fig. 3 presents a scheme and histological section of the terminal segment of the mouse seminiferous tubule. As can be seen from the figure, there is a gradual decrease in the tubule diameter in TZ due to the vanishing of germ cells from it. As a result, the terminal part of TZ maintains only few spermatogonial cells and SCs possessing altered morphology, according to electron micro-

scopic studies (Perey et al., 1961; Dym, 1974; Nykänen, 1979; Wrobel et al., 1986). These modified SCs, lacking connection with the germ cells, form cytoplasmic protrusions occupying almost the entire lumen and forming at the end a kind of valve (Hermo and Dworkin, 1988), which allows only a one-way flow of fluid and spermatozoa from the convoluted seminiferous tubules to the *rete testis* and then to the efferent tubules (Fig. 3).

As reported by Nykänen (1979), the nuclei of the TZ SCs have the usual multiple lobule shape but with a more significant number of peripheral heterochromatin blocks (Fig. 3), which makes them similar to undifferentiated SCs of immature animals. Indeed, the SCs from TZ are not completely differentiated. Thus, according to Figueiredo et al. (Figueiredo et al., 2016), in sexually mature rats, only some of TZ SCs express such essential markers of SCs differentiation as the transcription factor *Gata 4* (GATA Binding binding protein 4) and androgen receptor (*Ar*, Androgen Receptor). The expression of the transcription factor *Dmrt1* is reduced in TZ SCs (Kulibin and Malolina, 2016; Malolina and Kulibin, 2017; Malolina and Kulibin, 2019), which is necessary for proper differentiation of SCs during puberty and maintaining germ cell development (Raymond et al., 2000; Kim et al., 2007; Matson et al., 2011; Minkina et al., 2014). At the same time, Aiyama et al. (Aiyama et al., 2015) reported increased expression levels of the SSCs proliferation regulator *GDNF* (Glial cell line-derived neurotrophic factor, Meng et al., 2000) in TZ SCs. Finally, Imura-Kishi et al. (2021) showed that RA-degrading enzyme *Cyp26a1* (cytochrome P450 family 26 subfamily A member 1) is highly expressed in TZ SCs. RA is crucial for maintaining the proliferation and differentiation of spermatogonial cells and their entry into meiosis (Gewiss et al., 2020); the high expression of the enzyme destroying RA explains the absence of differentiating germ cells in TZ. Meanwhile, the increased expression of GDNF contributes to the maintenance of the $Gfra1^+$ population of SSCs and niche formation (Aiyama et al., 2015). Since SSCs niches are found along the entire length of the convoluted seminiferous tubules (Kubota and Brinster, 2018; de Rooij, 2017) and maintain the balance between SSCs proliferation and differentiation in the norm, the "terminal" niches in the TZ can play the role of a reserve.

Another exciting feature of TZ SCs in rodents has been highlighted in several recent studies carried out in Syrian hamsters, rats, and mice (Aiyama et al., 2015; Figueiredo et al., 2016, 2019; Malolina and Kulibin, 2017). This is prolonged, in comparison with SCs from convoluted seminiferous tubules, proliferation of TZ SCs in postnatal development of the testes. As Figueiredo et al. (2016, 2019) point out, this phenomenon may occur widely in other mammals but was not previously observed by researchers because they did not study the proliferation of SCs in the TZ. A recent paper by the authors (Figueiredo et al., 2019)
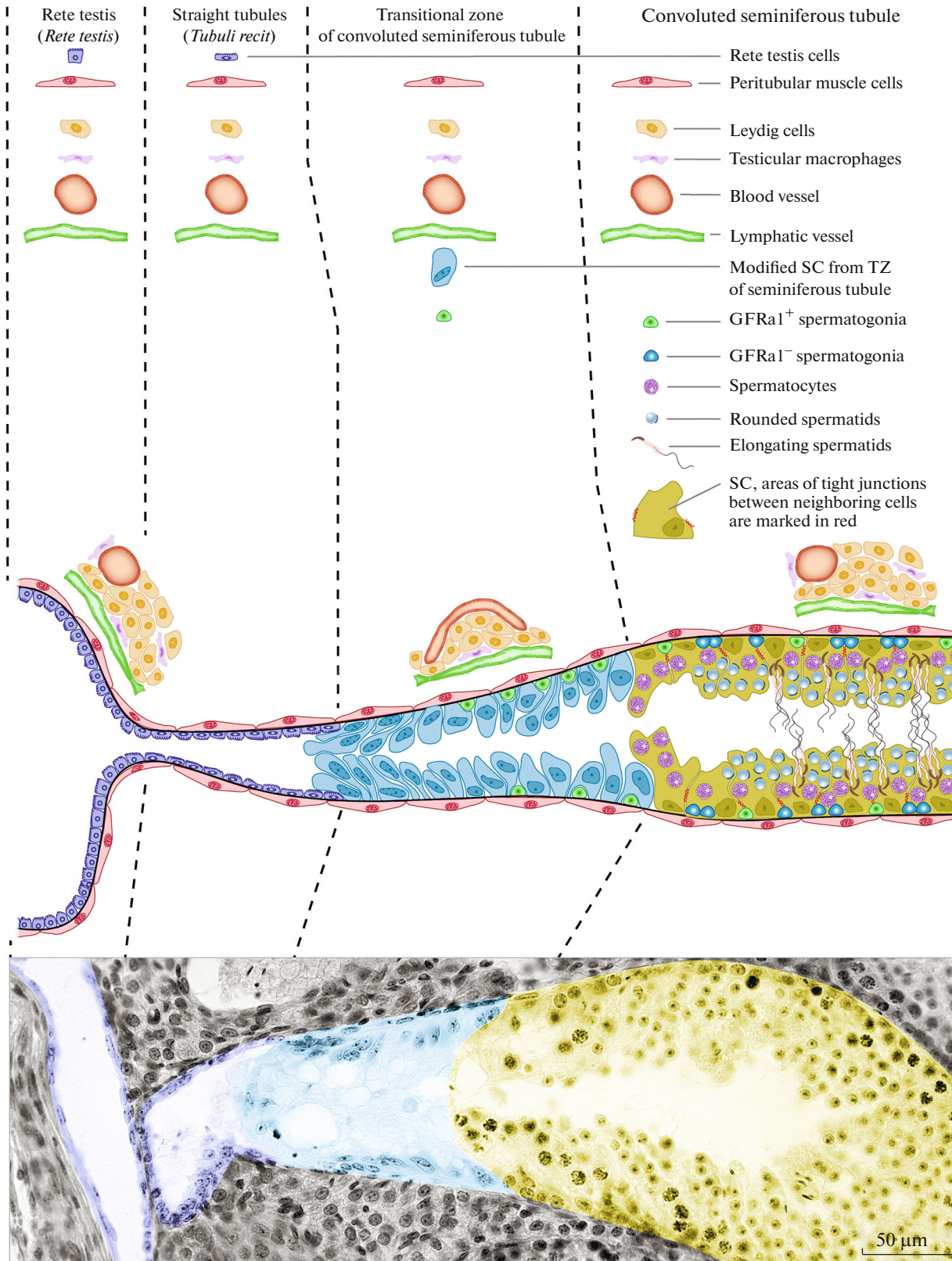
Fig. 3. A schematic diagram showing the transitional zone of the convoluted seminiferous tubule and the rete testis from an adult mouse. A representative section of the terminal segment of the seminiferous tubules is presented at the bottom, hematoxylin-eosin staining. The same regions on the section and the diagram have the same color.

outlined an experimental study of 160-day-old rats, undergo addition of the T3 hormone synthesis inhibitor PTU (goitrogen 6-n-propyl-2-thiouracil) after 21 days of postnatal development when the SCs in the seminiferous tubules are already differentiated and not capable of proliferation. In these animals, the transient hypothyroidism leads to an increase in testes mass, SCs number, and sperm production compared to intact controls due to the proliferation of SCs in the TZ. The results of this work indicate that the spermatogenic system, in theory, can compensate, through an additional proliferation of SCs in the TZ, for their deficiency in the seminiferous tubules in case of developmental disorders and, thus, provide the production of mature gametes sufficient for fertility.

Finally, as has long been known, spermatogenic waves originate from the TZ of the seminiferous tubules. The spermatogenic wave, first described by Perey et al. (1961), is a series of adjacent segments of the seminiferous tubule containing all possible sets of differentiating germ cells: stages of the spermatogenic epithelium cycle (there are 12 in mice, Oakberg, 1956; and 14 in rats, Leblond and Clermont, 1952). If we trace the spermatogenic wave from the TZ, the further we go away from the *rete testis*, the earlier stages of the cycle we will encounter. Spermatogenic waves determine the spatial and temporal organization of spermatogenesis; without that, the joint development of numerous generations of germ cells throughout the testis would be impossible. The mechanism of wave formation is unknown, but TZs are probably involved in this process.

FUNCTIONS OF THE ADULT MOUSE *RETE TESTIS*

For a long time, it was believed that the main functions of the *rete testis* are the transit of gametes from the convoluted seminiferous tubules to the efferent tubules as well as the regulation of the protein and salt composition of the seminal fluid (discussed in detail in the review by Hess and Hermo, 2018). In addition, in the case of obstruction of various parts of the genital ducts or impaired fluid absorption in the efferent tubules, the *rete testes* serves a protective function and regulates the intratesticular pressure in the testis, significantly increasing in volume (Lupien et al., 2006; Nanjappa et al., 2016; Hess and Hermo, 2018; Major et al., 2021; Cao et al., 2021).

In a recent study, Imura-Kishi et al. (2021) revealed that the *rete testis* could participate in the regional specialization of the SCs in TZs. The researchers transplanted labeled SCs obtained from the convoluted seminiferous tubules of mice (C57BL/6-R26-H2B-mCherry) into the testes of recipient mice (C57BL/6-Tg AMH-Treck, Shinomura et al., 2014), whose SCs were previously destroyed by injection of diphtheria toxin. At 45 days after transplantation, donor SCs formed new TZs in the terminal segments of the recipient's seminiferous tubules, stained positive for this zone markers p-AKT (RAC-alpha serine/threonine-protein kinase, Protein kinase B alpha, Imura-Kishi et al., 2021) and ace-Tub (acetylated form of tubulin, Aiyama et al., 2015; Imura-Kishi et al., 2021) and highly expressed GDNF, as is the norm.

The results suggest that TZ SCs specialization is region-specific and is determined by factors emanating from the *rete testis*. *FGF*, the fibroblast growth factors, can be such factors. By RNA-sequencing Imura-Kishi et al. (2021) showed that *rete testis* cells highly express FGF9, and receptors to this growth factor are present on the TZ SCs. FGF signals from the *rete testis* were found to induce constitutive activation of serine/threonine protein kinase (AKT), a key enzyme of the phosphoinositide-3-kinase signaling pathway (PI3K/AKT) in TZ SCs. In contrast to TZ SCs, AKT activity in the SCs of the seminiferous tubules changes cyclically, achieving a maximum at stages II-VI of the spermatogenic epithelial cycle and a minimum at stages IX-XII. Thus, phosphorylated (p-AKT) is a molecular marker of the TZ region, as is the phosphorylated signal transducer and activator of transcription 3 (p-STAT3, Nagasawa et al., 2018) reported previously.

The authors found that the p-AKT first emerges in the terminal segments of the mouse seminiferous tubules on day 7 of postnatal development, indicating the beginning of TZ region formation. Since the PI3K/AKT signaling pathway is active in most cell types and one of its main functions is to block apoptosis and stimulate proliferation (Chen et al., 2001), we can assume that constitutive AKT activity in the TZ region is responsible for the proliferative activity of SCs in the terminal parts of the tubules described above. Whether this is true or not remains to be determined, but the results obtained in this work make it clear that the *rete testis* is not just a part of the excretory system but also an important structural element involved in the development of the spermatogenic system.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interests.

In this study, humans and animals were not used as objects.

AUTHOR CONTRIBUTIONS

The authors made the same contribution to the preparation of the article.

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