Three-dimensional analysis and in vivo imaging for sperm release and transport in the murine seminiferous tubule

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Abstract

In brief: Spermatozoa are released from Sertoli cells and flow in the seminiferous tubule to the rete testis. Our results suggest that the luminal flow in the tubules is repeatedly reversed and that this physical force helps spermatozoa release from the Sertoli cells.

Abstract: Spermatozoa released from Sertoli cells must be transported to the epididymis. However, the mechanism of the luminal flow in seminiferous tubules has remained unclear to date. Therefore, in this study, we investigated luminal flow and movements in the seminiferous tubules by three-dimensional analysis and in vivo imaging. Serial 5-μm-thick mouse testicular sections at 50-μm-intervals were prepared and stained by Periodic Acid-Schiff-hematoxylin. After three-dimensional reconstruction of the seminiferous tubules, the localization of the released spermatozoa and the stages observed in the sections were recorded in each reconstructed tubule. Luminal movements in the seminiferous tubules were observed by in vivo imaging using a fluorescent-reporter mouse and two-photon excitation microscopy system. Spermatozoa without contact to the seminiferous epithelium were not accumulated toward the rete testis. Additionally, such spermatozoa were found on their way not only to the most proximal rete testis but also to more distant rete testis from any stage VIII seminiferous epithelia. In vivo imaging demonstrated that the direction of the flagella of spermatozoa attached to the seminiferous epithelium was repeatedly reversed. The epithelium at the inner curve of the seminiferous tubule was shaken more actively and had fewer spermatozoa attached compared with the epithelium at the outer curve. Our results hence suggest that the luminal flow in the seminiferous tubules is repeatedly reversed and that this physical force helps spermatozoa to be released from Sertoli cells.

Introduction

Spermatozoa differentiate in the seminiferous tubules (STs) and are then released into the lumen. Because the released spermatozoa are not motile (Holstein et al. 2003), they must be transported to the epididymis, where they acquire motility (Soler et al. 1994). Before their transport to the epididymis, spermatozoa flow through the ST, tubuli recti, rete testis, and efferent duct of the testis. Therefore, the ST is the start of the male genital tract.

Spermatozoa do not possess active motility in the ST and obtain the motility in the epididymis. Additionally, testis-derived secreted factors, such as Nell2 (Neural EGFL Like 2), are necessary to regulate the development of the epididymal epithelium (Kiyozumi et al. 2020). Therefore, a mechanism to transport the spermatozoa and luminal fluid from the STs to the epididymis is required. Previous studies have focused on peritubular myoid cells (PMC). The transport of spermatozoa to the epididymis is induced by the peristaltic motion of the STs (Maekawa et al. 1996). Maekawa et al. (1996) reported that ‘the frequency of the pressure change in the seminiferous tubule is 0.17–0.23Hz, and is lower than that of epididymal ducts where several muscle layers and rich innervation are present’. The diameter of rat STs shows a 40% reduction when they contract (Losinno et al. 2016). When the function of PMC is disrupted, luminal flow in the STs is modulated, resulting in dilation of the STs (Uchida et al. 2020). Recently, unidirectional movement in the lumina of the STs was shown by an in vivo imaging method (Fleck et al. 2021). Motile cilia on the epithelial cells of the efferent tubules were also found to generate luminal flow using in vivo imaging (Yuan et al. 2019). Additionally, more than 90% of the luminal liquid from the testis is absorbed between the rete testis and efferent tubules (Clulow et al. 1994), and ligation of the efferent duct induces dilation of...
the ST and rete testis (Anton 1979). Therefore, the liquid absorbance also contributes to the luminal flow. Although the mechanism of transport of spermatozoa has recently been reported as above, luminal flow through an entire ST cannot be observed by current in vivo imaging techniques.

Spermatids are transformed into mature spermatozoa via 16 and 19 steps referred to as spermiogenesis in mice and rats, respectively (Russell et al. 1990, Nakata et al. 2015a). During these steps, spermatids need to be attached to Sertoli cells by adhesion structures, such as the ectoplasmic specialization and tubulolobular complex (Lie et al. 2010). Sertoli cells also need to form specific cytoskeleton networks inside them during spermiogenesis (O’Donnell et al. 2011, Yan Cheng & Mruk 2015). These structures and molecules, including focal adhesion kinase, are tightly regulated during spermiogenesis, and therefore, sperm release called spermiation is induced via these regulations. Disruption of this regulation leads to the failure of spermiation, resulting in the persistence of mature spermatozoa in the seminiferous epithelia even after stage IX (Wen et al. 2018). In contrast, when the adherent molecule Nectin 2 or Nectin 3 required in ectoplasmic specialization is deleted specifically in the adherents junctions in the testis of mice, the number of adherent spermatids on the seminiferous epithelia is reduced in these Nectin2−/− and Nectin3−/− mice (Nakata et al. 2015a).

The three-dimensional (3D) reconstruction method is useful for investigating the entire structure of STs. Nakata et al. (2017) revealed the 3D structure of STs, including the distribution of the stages of the seminiferous epithelia. Furthermore, a high dose of busulfan, which is an alkylating antineoplastic agent that inhibits DNA replication and impairs spermatogenesis in the STs (Bucci & Meistrich 1987), increased the length but not the number of impaired STs on 3D analysis of the testis of mice (Nakata et al. 2020).

In the present study, to estimate the sperm flow from Sertoli cells to the rete testis, the localization of the released spermatozoa in each ST was investigated using a 3D reconstruction model. Furthermore, luminal movement in the ST was observed by in vivo imaging using a fluorescent-reporter mouse and a two-photon excitation microscopy system. We herein report that luminal flow is repeatedly reversed in the STs, possibly contributing to the bidirectional transport of spermatozoa and that physically shaking the seminiferous epithelia possibly contributes to the release of spermatozoa.

Materials and methods

Animals

The animal experiment using 3D analysis was approved by Kanazawa University (study approval no.: AP-153636) and was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals in Kanazawa University. Male C57BL/6 mice were purchased from Nippon SLC, Inc., reared under standard 12h light:12h darkness conditions with free access to standard food and water, and used at the age of 90 days.

Pax2-LynVenus male mice were produced by crossing Pax2-Cre mice (Ohyama & Groves 2004) and R26R-Lyn-Venus mice (Abe et al. 2011) at Kyoto University. All the animal experiments for in vivo imaging were approved by the local ethics committee for animal experimentation (MedKy 19090 and 20081) and were performed in compliance with the guide for the care and use of laboratory animals at Kyoto University. For histological analysis using Pax2-LynVenus mice, mice were maintained in a specific pathogen-free facility at Tokyo Medical University. The mice were housed in a room at 22–24°C and 50–60% relative humidity, on a 12h light:12h darkness cycle. This study was approved by the Tokyo Medical University Committee (approval no.: R2-0042).

Histological analysis

Male C57BL/6 mice were sacrificed by cervical dislocation. The testis and epididymis were dissected out en bloc, fixed by immersion in Bouin’s solution overnight, dehydrated in a graded ethanol series, and embedded in paraffin. Serial 5-µm-thick sections at intervals of 50 µm were made from three testes by cutting the specimen longitudinally in parallel to the plane comprising both the testis and epididymus using a microtome, and then mounted on glass slides. The sections were treated with Periodic Acid-Schiff-hematoxylin (PAS-H) to stain the basement membrane of the STs, as previously described (Nakata & Iseki 2019). The sections were digitized using a whole-slide scanner (Nanozoomer 2.0-HT; Hamamatsu Photonics) with 20-fold objective lens. The resulting digital images of the sections were visualized with viewing software (NDDview2; Hamamatsu Photonics). The number of STs’ cross-sections, the number and position of spermatozoa without contact with the seminiferous epithelium of the ST, and the stage of the seminiferous epithelium within the images were recorded. Furthermore, the number of step 16 spermatozoa attached to the seminiferous epithelium in the curved ST at stages VII/VIII were counted within an area of 100 µm around the vertex of the inner and outer curves.

Pax2-LynVenus male mice were anesthetized using 2% isoflurane and euthanized by cervical dislocation. The testis and epididymis were collected and flash-frozen in Optimal Cutting Temperature compound (Sakura, Finetek, Japan) with liquid nitrogen. The frozen blocks were cut into 5-µm-thick sections using a Cryostar NX70 cryostat (Thermo Fisher Scientific) and fixed in 10% formalin in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature. After washing in 0.1 M phosphate buffered saline (pH 7.4) containing 0.5% Tween 20 (PBST), the sections were incubated with peanut lectin (PNA) conjugated to Alexa Fluor 647 (L32460, Thermo Fisher Scientific, 1:2,000) and 4’,6-DAPI (340-07971, DOJINDO, 1:1,000) for 1 h at room temperature. PNA staining was performed for staging of the STs (Nakata et al. 2015a). After washing with PBST, the sections were coverslipped in FluorSave (Merck Millipore) and observed using an LSM700 (Zeiss).
Three-dimensional analysis

Three-dimensional reconstruction was performed as previously described (Nakata et al. 2017). Briefly, digital images were extracted from serial sections of the PAS-H-stained basement membrane representing the outline of the STs and then converted into grayscale in JPEG format using Adobe Photoshop 2020 software (Adobe Systems). Using Amira 6.3.0 software (Visage Imaging), the inside of the basement membrane of the selected tubule was filled with a particular color using threshold processing and traced from section to section. This procedure was repeatedly applied to all STs, and then 3D images were reconstructed. The core lines of all reconstructed STs were also drawn with the same software, and the number of STs was recorded from the cranial side. The counted spermatozoa were placed on the core line, and their distance from the nearest rete testis was recorded. The core lines were segmented according to the stage of the seminiferous epithelium, which was divided into three groups, that is stages I–VI, VII/VIII, and IX–XII.

In vivo imaging

For intravital imaging, male mice were anesthetized with 1.5% vaporized isoflurane using an inhalation device (TK-7, Biomachinery). An incision in the murine scrotum and the parietal layer of the tunica vaginalis was made by surgical scissors, and the testis was gently pulled out. The mouse was laid in the prone position on an electric heat pad (no. MATS-U52AXKA26-A13L, Tokai Hit, Fujinomiya, Shizuoka, Japan) maintained at 37°C, and the testis was set on a glass coverslip (no. C024501, Matsunami Glass Ind., Kishiwada, Osaka, Japan) attached to the heat pad for inverted observation. The testis was incubated in atmospheric air, and the temperature of the mouse body and testis was not monitored during the observation. Then, a two-photon excitation microscopy was performed using an inverted microscope system (FV1200MPE-IX83, Olympus), equipped with the ×30 silicone-immersion lens (NA = 1.05, WD = 0.8 mm, UPLSAPO30XS, Olympus). Time-lapse images were acquired at 945 nm excitation wavelength (InSight DeepSee, Spectra-Physics) with the emission filter (BA520-560, Olympus). STs with obvious sperm flagella in the lacuna were observed. Images were taken at 2.71- or 4.90-s intervals, with a scanned field of 512 × 512 (pixel size: 0.828 μm) or 640 × 640 pixels (pixel size: 0.662 μm), respectively. In the obtained images, the distance moved by the germ cells in the seminiferous epithelium during the observation period was calculated by ImageJ software (version 4.1.2 (2021-11-01)). Values of the inner and outer curves were compared by the pairwise Wilcoxon rank-sum test, and a P-value of less than 0.05 was considered to indicate a statistically significant difference between groups.

Results

Number of spermatozoa in the sections

We analyzed 34,668 ST cross-sections in three testes (Table 1). Spermatozoa, which were not in contact with the seminiferous epithelium, were counted as released spermatozoa (Fig. 1A, B, C, D and E). Because the luminal fluid contains proteins (Hinton & Keefer 1983), Bouin’s fluid can fix the fluid into a jelly-like substance, which still contains the spermatozoa, and the spermatozoa in the lacuna are expected not to be washed out during the histological procedures. Indeed, we observed sperm heads in focus at different focus heights under the microscope, showing that the spermatozoa in the lacuna of the STs were not washed out even if the sperm head itself was not attached to the slide. As a result, approximately 5% of the ST cross-sections contained spermatozoa without contact to the seminiferous epithelium (Table 1). These spermatozoa were mainly scattered in the ST lumen (Fig. 1B, C, and D). In total, 1–3 × 10^3 released spermatozoa were observed (Table 1), and therefore, the number of such spermatozoa in a whole testis was estimated to be about 1–3 × 10^4 by multiplying by 10.

Number of spermatozoa in an intact ST

We next investigated the number and localization of spermatozoa without contact to the seminiferous epithelium in each intact ST using the 3D reconstruction model. After all the STs were reconstructed, a simple

<table>
<thead>
<tr>
<th>Table 1</th>
<th>The number of counted seminiferous tubules (ST) and spermatozoa without contact to the epithelium in each testis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counted ST (A)</td>
<td>ST containing spermatozoa without contact to the epithelium (B)</td>
</tr>
<tr>
<td>No.1</td>
<td>10,776</td>
</tr>
<tr>
<td>Stage VII/VIII</td>
<td>1285</td>
</tr>
<tr>
<td>No.2</td>
<td>10,772</td>
</tr>
<tr>
<td>Stage VII/VIII</td>
<td>1467</td>
</tr>
<tr>
<td>No.3</td>
<td>13,120</td>
</tr>
<tr>
<td>Stage VII/VIII</td>
<td>2563</td>
</tr>
</tbody>
</table>

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In vivo imaging of luminal movement

Based on the above results, we hypothesized that not all spermatozoa released from the same stage VIII seminiferous epithelium were swept out to the same rete testis. In other words, we predicted that luminal flow can be reversed in an ST. Therefore, we next tried to observe the luminal movement by in vivo imaging using a two-photon excitation microscope system. The fluorescence reporter mouse line, Pax2-LynVenus, which expresses the yellow-fluorescent protein, Venus, on the cell membrane of sperm flagellum, was created (Fig. 3). The reporter mouse was anesthetized and placed on the warmed stage of the microscope after its testis was pulled out from the scrotum (Fig. 4A). The cell membrane of germ cells after meiosis, and the flagella of spermatozoa attached to the seminiferous epithelium in the STs were found to be positive for the Venus protein (Fig. 4B, C, D, E, F, G, H, I, and J). On time-lapse imaging, changes in the direction of flagella were observed, and flagella were found to change their direction repeatedly reversed in the STs. Directions were changed every few tens of seconds, although the period between the direction changes was not the same among observations (Fig. 4 and video 1).

Video 1

Representative time-lapse imaging of a seminiferous tubule in the Pax2-LynVenus mouse at 2.7-s intervals. The direction of flagella on the seminiferous epithelium is repeatedly reversed during the observation. This video (http://movie-usa.glencoesoftware.com/video/10.1530/REP-21-0400/video-1) is available from the online version of the article at https://doi.org/10.1530/REP-21-0400.

Analysis in the curved region of the STs

When focusing on the various ST structures in the cross-sections, we found characteristic attachment of spermatozoa on the seminiferous epithelia, particularly in the curved STs. The STs were all highly twisted, consistent with previous 3D analyses of STs (Nakata et al. 2015b, 2017). We were therefore able to analyze some longitudinal cross-sections of the curved STs. A small number of spermatozoa were found to be attached to the seminiferous epithelium in the inner curve (Fig. 5A). Spermatids at step 8, which usually localize beneath spermatozoa at step 16 at stage VIII, were observed even at the vertex of the epithelium of the inner curve (Fig. 5A'). We found 23 curves of STs at stage VII/VIII and counted the spermatozoa (step 16) attached to the seminiferous epithelia within 100 μm around the vertex of the curve. There were significantly fewer spermatozoa attached to the inner curve than to the outer curve (Fig. 5B).

Figure 1 Representative pictures of spermatozoa without contact to the seminiferous epithelium in PAS-stained paraffin testicular sections. (A, B, C, and D) A few seminiferous tubules containing spermatozoa that are not in contact with the epithelium (red circles) are observed in section (A) and their magnified images are shown in (B, C, and D). Arrowheads indicate spermatozoa without contact to the seminiferous epithelium. Bars = 1 mm (A), 50 μm (B, C, and D).

ST with no or few branching points was each chosen from the cranial, middle, and caudal regions of three testes. The localizations of the spermatozoa without contact to the seminiferous epithelium were plotted on the reconstructed STs (Fig. 2A and B). Some regions contained a large number of spermatozoa. Such areas did not differ between the cranial–caudal and dorsal–ventral axis. Therefore, the stage of the STs was next recorded.

In every testis and reconstructed ST, more than half of the spermatozoa without contact with the seminiferous epithelium were found in STs of stages VII/VIII (Tables 1 and 2). To clarify their localization in the ST, the distance from the rete testis to these spermatozoa and the end of each stage VIII was recorded. The distance from the end of each stage VIII to the rete testis was 25.3 ± 164 μm (mean ± s.d.) in nine STs. Although there were more released spermatozoa at stage VII/VIII than in the other stages, they were scattered in the STs (Fig. 2C). On the other hand, clustered spermatozoa were also observed in one or two regions each from the nine analyzed STs (Fig. 2D). At first, we speculated that the spermatozoa released from some seminiferous epithelia in stage VIII would be accumulated toward the rete testis in the ST. In other words, we expected that spermatozoa without contact with the seminiferous epithelium would be found more frequently near the rete testis. However, increased accumulation of spermatozoa toward the rete testis was not observed (Fig. 2C). Furthermore, spermatozoa without contact with the seminiferous epithelium were found on their way to not only the most proximal rete testis but also a more distant rete testis from any stage VIII seminiferous epithelia.
Figure 2 Representative localization of spermatozoa without contact to the seminiferous epithelium in a seminiferous tubule (no. 2-ST5). (A and B) A reconstructed seminiferous tubule in the cranial view and ventral view, respectively. Red dots indicate the connection to the rete testis. Each black node indicates the position of spermatozoa without contact to the seminiferous epithelium. Each color represents the stage of the seminiferous epithelium (green: stage I–VI, magenta: stage VII/VIII, blue: stage IX–XII). (C) Histogram of the number of spermatozoa without contact to the seminiferous epithelium, within a reconstructed seminiferous tubule. Each color bar represents the length of the seminiferous tubule at each stage (green: stage I–VI, magenta: stage VII/VIII, blue: stage IX–XII). Red dots represent the rete testis, and each narrow horizontal line connecting each bar represents the branching point. Arrowheads indicate spermatozoa on their way to a more distant rete testis from any stage VIII seminiferous epithelia. (D and E) A representative picture of clustered spermatozoa and a spermatozoon (arrowhead) located on their way to a more distant rete testis from any stage VIII seminiferous epithelia in the PAS-stained paraffin section, respectively. Bars = 1 mm (A and B), 50 μm (D and E).
We next observed curved STs clearly containing sperm flagella by in vivo imaging. Few flagella of the spermatozoa were also observed on the top of the seminiferous epithelium at the inner curve, resulting in a vacant space in the inner lumen (Fig. 5C, D, E, F, G, H, and video 2). Flagella showed a change in direction also in the curved region, and germ cells in the seminiferous epithelia also moved together with the flagella. Furthermore, we found that the seminiferous epithelium was periodically shaken, and this shaking was most prominent in the inner curve. In fact, we tracked germ cells in the seminiferous epithelium of the inner and outer curves during their reciprocating movements and found that the traveling path of germ cells in the inner curve was significantly longer than that in the outer curve (Fig. 5I and J).

Video 2

Representative time-lapse imaging of a curved seminiferous tubule at 4.90-s intervals. The direction of flagella on the seminiferous epithelium is repeatedly reversed during the observation. The epithelium in the inner curve of the seminiferous tubule moved more actively and had fewer spermatozoa attached compared with the epithelium in the outer curve. This video (http://movie-usa.glencoesoftware.com/video/10.1530/REP-21-0400/video-1) is available from the online version of the article at https://doi.org/10.1530/REP-21-0400.

Table 2  The number of counted spermatozoa without contact to the epithelium in each seminiferous tubule.

<table>
<thead>
<tr>
<th>Spermatozoa without contact to the epithelium (A)</th>
<th>Length (mm) (B)</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>Stage VII/VIII</td>
<td>Total</td>
</tr>
<tr>
<td>No.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST2</td>
<td>138</td>
<td>(59)</td>
</tr>
<tr>
<td>ST6</td>
<td>113</td>
<td>(86)</td>
</tr>
<tr>
<td>ST9</td>
<td>209</td>
<td>(103)</td>
</tr>
<tr>
<td>No.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST2</td>
<td>35</td>
<td>(17)</td>
</tr>
<tr>
<td>ST5</td>
<td>207</td>
<td>(137)</td>
</tr>
<tr>
<td>ST10</td>
<td>187</td>
<td>(105)</td>
</tr>
<tr>
<td>No.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST2</td>
<td>442</td>
<td>(291)</td>
</tr>
<tr>
<td>ST5</td>
<td>342</td>
<td>(238)</td>
</tr>
<tr>
<td>ST10</td>
<td>143</td>
<td>(92)</td>
</tr>
</tbody>
</table>

Figure 3  Representative pictures of stained seminiferous tubules in a snap-frozen cryosection from a Pax2-LynVenus reporter mouse. Venus fluorescence is found on the cell membrane of germ cells, including the flagella of spermatozoa (arrowhead), in the seminiferous tubules. The negative control for PNA demonstrates no fluorescence in the acrosome (G). Roman numerals indicate the stage of the seminiferous tubule. Bars = 50 µm.
Sperm release and transport in the testis

Discussion

The present study investigated the luminal flow in STs by 3D analysis and in vivo imaging. Investigating the localization of released spermatozoa in serial ST sections demonstrated that released spermatozoa were present on their way to a more distant rete testis from any stage VIII. Furthermore, we found that the movement of flagella was repeatedly reversed when observed by time-lapse in vivo imaging. These results suggest that the luminal flow can be changed in the STs.

Spermatozoa are thought to be transported to the epididymis by a peristaltic motion of the STs, and defects in PMC development induce the dilation of the rete testis after puberty (Uchida et al. 2020). In this study, we found that the orientation of flagella was repeatedly reversed when observed by in vivo imaging. Because spermatids during or immediately after spermatogenesis show no or little motility (Inaba 2011), this change in flagella orientation is probably caused by movements in the luminal flow, suggesting that the luminal flow can be repeatedly reversed. This change in luminal flow may be induced by contraction and relaxation of PMCs. Fleck et al. (2021) recently observed unidirectional bulk movements of the luminal content in the STs. This discrepancy is possibly owing to the different observation methods and magnifications used. Imaging in the present study was performed in a narrow region using two-photon excitation microscopy, in contrast to the previous study that observed a vast area using brightfield microscopy. Therefore, they were able to observe the macroscopic transport of the spermatozoa to the rete testis, whereas we were able to observe local luminal movements.

Indeed, we found released spermatozoa at the most distal region of the seminiferous tubule to the rete testis in the 3D analysis. This observation also

Figure 4 Representative time-lapse imaging of a seminiferous tubule from the Pax2-LynVenus mouse at 2.7-s intervals. (A) The Mouse in the prone position with its testis withdrawn from the scrotum is placed on a warmed stage during the observation. (B, C, D, E, F, G, H, I, and J) The direction of flagella on the seminiferous epithelium was repeatedly reversed during the observation. Orange arrows and asterisks indicate the direction (B, D, E, F, and J) and the period between transitions (C, G, H, and I), respectively. Bars = 50 μm.
suggests that the liquid flow in the STs is not in a constant direction. Of course, all spermatozoa will finally reach the rete testis. Therefore, spermatozoa found in such a distal region will also eventually be transported to the rete testis. A possible mechanism for the transport of spermatozoa to the rete testis is the water pressure difference between the STs and rete testis. Because more than 90% of the luminal fluid is absorbed by the epithelial cells in the efferent tubules (Clulow et al. 1994), luminal fluid, which has high pressure in the STs, is probably pulled to the rete testis and efferent tubules, which have luminal fluid with lower pressure. Further investigation of what induces this gradient of luminal fluid pressure through the STs is necessary.

Because Bouin’s fluid probably fixes the luminal fluid in the STs, which contains proteins (Hinton & Keefer 1983), spermatozoa in the lacuna cannot be washed out during the histological procedures. Additionally, Bouin’s fluid is recommended in the evaluation of testicular specimens to reduce artifacts, such as luminal sloughing of cells (Cerilli et al. 2010). Therefore, we estimated the total number of released spermatozoa in a whole testis to be $1–3 \times 10^4$. Daily sperm production

![Figure 5 Analysis of a curved seminiferous tubule.](https://rep.bioscientifica.com)

(A) A representative picture of the curved region of a seminiferous tubule in a PAS-stained paraffin section. Few spermatozoa are attached to the seminiferous epithelium in the inner curve (black arrow). A spermatid is observed at step 8 in the seminiferous epithelium in the inner curve (A’). (B) The number of spermatozoa adhering to the seminiferous epithelium within 100 µm around the vertex of the curve is significantly lower in the inner curve compared with in the outer curve. (C, D, E, F, G, and H) Representative time-lapse imaging of a seminiferous tubule in the Pax2-LynVenus mouse at 4.90-s intervals. The direction of the flagella is repeatedly reversed during the observation. Orange arrows indicate the direction of the flagella, and the transitional period (asterisks) is shown in panels (D) and (G). Some areas in the inner side had few flagella (red arrows). The position of germ cells in the seminiferous epithelium is altered according to the direction of flagella, and their movement is more extensive in the inner curve (magenta arrowheads) than the outer curve (blue arrowheads). (I) A representative picture showing the path of travel of germ cells in the seminiferous epithelium during a reciprocating movement (inner curve: green, red, yellow; outer curve: magenta, blue, cyan). (J) The distance of travel of germ cells in the seminiferous epithelium during a reciprocating movement is significantly longer in the inner curve than in the outer curve. Bars = 100 µm (A), 50 µm (C, D, E, F, G, H, and I).
has been reported to be $4 \times 10^7$ per gram of testis (Hess & de Franca 2009), and the weight of a mouse testis is about 0.1 g (Suto 2008), suggesting that about $4 \times 10^8$ spermatozoa pass through the STs toward the rete testis per day. From these data, transport of whole released spermatozoa to the rete testis may be repeated at least $1.3 \times 10^7$ times/day. In other words, released spermatozoa may reach the rete testis in an average of 2.4 to 11.1 min. The distance from the end of stage VIII to the rete testis was about 25 mm, so the estimated average velocity of spermatozoa through an ST is 38.3 to 173.3 μm/sec. Although no data has been reported to date on the transport speed of released spermatozoa in the ST, this time span appears possible because the length of a mouse spermatozoon is about 126 μm (Albrechtová et al. 2014). Furthermore, we were unable to observe the increased accumulation of released spermatozoa toward the rete testis, suggesting that the released spermatozoa reach the rete testis very quickly. Further studies are needed to address this hypothesis, after establishment of the method to trace spermatozoa from their release to reaching the rete testis.

We found that there were fewer spermatozoa attached to the seminiferous epithelium on the outer side of the curved ST compared with on the outer side and that epithelia on the outer side were more rigorously shaken. These observations suggest that shaking the seminiferous epithelium helps to release spermatozoa into the lumen. Alternatively, the ability of the Sertoli cell to adhere to spermatozoa may differ between the outer and inner curves. One factor affecting the function of Sertoli cells physically is shear force. Cells that are subjected to shear force react to resist the force, by actin polymerization to strengthen their cytoplasm (Lee et al. 2006). Actin remodeling is necessary for the ectoplasmic specialization and tubulobulbar complex during spermiogenesis, both of which are known as adherent structures between the spermatozoon and Sertoli cell (Lie et al. 2010, O’Donnell et al. 2011, Yan Cheng & Mruk 2015). We found that spermatids at step 8 were located on the seminiferous epithelium, even in the inner curve. Although spermatids after step 8 are assumed to be attached tightly to Sertoli cells by testis-specific adherens junctions, which is called an ectoplasmic specialization (Yan Cheng & Mruk 2015), the spermatids are probably released between steps 9–16 in the inner curve. The shear force may be different between the inner and outer curves, and the different strengths of the actin filaments may cause malfunction of the adherent structures between the Sertoli cells and spermatozoa, resulting in the earlier release of spermatozoa in the inner curve of the STs.

The present study focused on the release and transport of spermatozoa from a morphological point of view. We propose that luminal flow in the STs can be repeatedly reversed and that shaking of the seminiferous epithelium contributes to the release of spermatozoa. Because early spermatozoa released from the inner curve may not have fully completed their spermiogenesis, they may have relatively low fertility. This point leads to the hypothesis that the more active movements of the PMCs or external pressure may impair fertility owing to the earlier release of the spermatozoa, even after the emergence of the ectoplasmic specialization. The present study provides a new possibility that changing the movements of the seminiferous epithelium and luminal flow can affect male fertility and may contribute to comprehending the release and transport of spermatozoa in the STs.

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

Y K, T O, and H N performed the histological and 3D analyses. T H created the Pax2-LynVenus reporter mouse and performed the in vivo imaging. All authors wrote and reviewed the manuscript.

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