Contractions transport exfoliated epithelial cells through the neonatal epididymis

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Abstract

Contractions of the adult epididymal duct are well known in the context of sperm transport. Some reports also describe contractions of the epididymal duct during development, but data about their character, regulation and function are sparse. In the foetal human epididymis we found luminal cells and could identify them as exfoliated epithelial cells originating from the epididymis and not from testis by using antibodies against neutral endopeptidase as an epithelial epididymal duct marker. Exfoliated cells were also found in the epididymal duct after birth. Time-lapse imaging revealed directional transport of luminal cells in the neonatal rat epididymis interrupted by pendular movement. Spontaneous contractions were discovered in the neonatal epididymis and an association between these contractions and the transport of the luminal cells could be observed. Both, transport and spontaneous contractions, were affected significantly by substances known to contract (noradrenaline) or relax (the phosphodiesterase 5 inhibitor sildenafil) smooth muscle cells. Immunohistochemistry showed staining for the proliferation marker proliferating-cell-nuclear-antigen (PCNA) in cells of the ductal lumen of the neonatal rat epididymis indicating the extrusion of cells also during proliferation. Our data showed spontaneous contractions of the immature epididymal duct associated with the transport of exfoliated luminal cells before the first occurrence of sperm cells. Results suggest an important role including both (i) a mechanical place holder function of exfoliated luminal cells (ii) together with a novel idea of organized waste disposal of these cells during development.

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Introduction

The epididymis plays an important role in the male reproductive tract. Transit through the epididymis is necessary for spermatozoa to completely mature and to acquire their motility and fertilizing capacity (Robaire & Hinton 2014). The epididymis also stores and protects sperm (Robaire *et al.* 2006). During development, failed elongation or incorrect coiling of the epididymal duct leads to poor sperm maturation and male infertility in adulthood (Robaire *et al.* 2006, Hinton *et al.* 2011). Furthermore, defects of ductal development could cause aberrations of the caput region, the cauda or vas deferens (Chen *et al.* 2012, Robaire & Hinton 2014).

Contractility of ductal smooth muscle cells in the adult epididymis ensures transport of immotile spermatozoa through the duct during their maturation (Elfgen *et al.* 2018). The activity of epididymal smooth muscle cells is predominately regulated by hormonal and paracrine mechanisms (Knight 1972, 1974, Hib & Caldeyro-Barcia 1974, Cosentino & Cockett 1986, Elfgen *et al.* 2018). Compared to the development of

the epididymal epithelial cells (Sun & Flickinger 1979, Hermo *et al.* 1992), data about the development of the epididymal smooth muscle cells are sparse. A limited number of publications propose contractions of prenatal and postnatal epididymal smooth muscle cells (Battaglia 1958, van de Velde & Risley 1963), but there is no knowledge about their character, regulation or function. By immunohistochemical analyses, we could show that polysialylated neural cell adhesion molecule (NCAM) polysialic acid (polySia), known to prevent cell adhesion, was localized to the smooth muscle cells surrounding the epithelial layer before puberty, but not in adulthood (Simon *et al.* 2013, 2015). As reduced cell adhesion between smooth muscle cells could disturb their coordinated contractions, this observation speaks rather against than for a considerable contractile activity of the postnatal epididymal duct.

This study aims to reveal whether neonatal epididymal smooth muscle cells are able to contract in an *ex vivo* environment and if systematic contractions of smooth muscle cells are essential for a transport function during the development of the epididymal duct.

Materials and methods

Human tissue and animals (ethical approval)

Foetal (gestation week 29–39) human paraffin-embedded tissue (testis with epididymis, $n=6$) from stillbirths provided by Dr Davor Ježek, archive of Dept. of Histology and Embryology, School of Medicine University of Zagreb, Croatia. Their provision was approved by the Ethics Committee of the University of Zagreb Medical School (no.: 04-1130-2006). According to the Articles 232 (4), 235 (2) and 236 (2) of the Law on Health Care (Republic of Croatia), a written patients' tissue use consent was not necessary.

Neonatal epididymal tissues were obtained from 4-day-old to 6-day-old Wistar rats ($n=15$) and as an example of mouse development from 10-day-old C57BL/6J mice (Black6) ($n=2$) housed in the animal facility of Justus-Liebig-University (JLU) Giessen, Germany. Housing, animal care and all procedures were conducted according to the guidelines for animal care and approved by the committee for laboratory animals of Justus-Liebig-University Giessen, case number JLU Nr. 527_AZ (rats) and JLU Nr. 543_M (mice).

Time-lapse imaging

Epididymal tissues from rats and from mice, as an example for the development in another rodent model, were removed immediately after the killing of the animal and kept in

minimal essential medium (MEM; Gibco, Invitrogen) at 4°C less than 1 h. Under a binocular microscope, one epididymis of each animal was isolated. Epididymides ($n=2$) were either filmed in total (Video 1) without further preparation or were divided into the three epididymal regions (caput $n=4$, corpus $n=4$, cauda $n=5$) (Fig. 1, Video 2 and an example for mice Video 3). The corpus epididymidis could be embedded in its entirety in collagen without any further preparation. Imaging of the denser and tightly packed caput and cauda was possible only by incising the tunica. In each collagen-embedded epididymis, the same regions of the upper caput, the middle corpus and the lower cauda of the epididymal duct were filmed (Fig. 1).

Video 1

Contractions and transport visualized in an entire epididymis of the neonatal rat. Movie shows the corpus of an entire collagen-embedded epididymis. This video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-19-0617/video-1>) is available from the online version of the article at <https://doi.org/10.1530/REP-19-0617>.

For time-lapse imaging, we used an Olympus BX50WI microscope (Olympus) and a T.I.L.L. Photonic camera combined with the software 'TILLvision' (T.I.L.L. Photonics, Gräfeling, DE). To obtain a sharp and stable image, prepared

Figure 1 Preparation of the 6-day-old rat epididymis for time-lapse imaging. After isolation of epididymis and testis in their entirety (A), testis and fat tissue were removed. The epididymis was either filmed in total (B, Video 1) or divided in caput, corpus and cauda without further preparation (C). All parts were then used for time lapse imaging (C, Video 2 result section). In each experiment the foci of the movies were upper caput (red circle), middle corpus (blue circle) and lower cauda (green circle), (1 frame/sec) (D, E and F).

epididymal tissues were kept in place using polymerized collagen. The epididymal duct segment was placed in the middle of the dish and care was taken to cover the duct segment with the collagen. After polymerization for 30 min at 34°C in an incubator, the wells were covered with 1 mL MEM at 34°C. Preparation of collagen stock solution was described in detail in a previously published paper (Mietens *et al.* 2014). Movies were captured at 1 Frame/s for a cycle time of 1200 frames (20 min). To test the responsiveness of the epididymal ducts to a smooth muscle relaxant, we added sildenafil (Pfizer) in a final concentration of 5 μ M (Mietens *et al.* 2012, 2014) after the first 400 frames. Sildenafil as a specific phosphodiesterase 5 inhibitor increases cGMP levels by inhibiting its degradation and leads to a decrease of smooth muscle contractions (Elfgen *et al.* 2018). In each case, after application of sildenafil for another 400 frames, noradrenaline (10 μ M) (Sigma-Aldrich) was added to observe the effect of a stimulatory compound and to additionally confirm viability of the tissue.

From all experiments ($n=20$), the epididymides containing luminal cells ($n=15$, 75% of all cases) were further analysed using Fiji ('Fiji is just ImageJ', Image Processing and analysis in Java, National Institutes of Health, USA). The contractile activity was evaluated using the 'Reslice' function of Fiji allowing the contractions to be counted within a defined period of time as already described by Mietens *et al.* (2014). For each time-lapse movie, after a waiting period of 60 s,

spontaneous contractions were counted for 2 min. After the addition of sildenafil and noradrenaline, contraction frequencies were assessed correspondingly. To analyse the transport of the luminal cells, movies were evaluated using five defined categories as shown in Fig. 4A ranging from 'no movement within the observed time frame' ('0') to 'directional flow through the field of view without pendular movement' ('4').

Histological analyses

Paraffin-embedded neonatal rat and foetal human tissues were cut into serial sections of 5 μ m and deparaffinized in xylene, followed by descending ethanol series.

For immunohistochemistry of neonatal rat tissue rabbit monoclonal anti-PCNA (Abcam) (1:500) was used in PBS (pH 7.4) supplemented with 0.1% NaN_3 and 0.2% BSA. For visualization the Envision Kit (DAKO) was used followed by the nickel-glucose oxidase approach (Davidoff *et al.* 1997).

For immunofluorescence staining of foetal human tissue we used mouse monoclonal anti-NEP (Novocastra, Newcastle upon Tyne, UK) (1:200) as primary antibody and goat-anti-mouse Alexa 488 (Roche) (1:500) as secondary antibody. Sections were counterstained with DAPI (Roche) or haematoxylin (Merck).

All images were taken with a Zeiss Axioplan 2 imaging microscope and Axiovision LE Software (Carl Zeiss).

Figure 2 Luminal cells in the epididymal duct of fetal human. (A and B) Paraffin sections of fetal human epididymal tissue stained with antibodies against NEP (green) and counterstained with DAPI (blue). Arrows indicate luminal cells marked by NEP. Arrowheads mark the NEP staining of the apical part of epithelial cells of the duct. (C) No immunofluorescence staining for NEP was detectable in the foetal testis. (D and E) Sections of foetal human epididymis stained with haematoxylin and antibodies against NEP. Pictures of the same sections were taken by fluorescence microscopy (D) and bright field illumination (E). Arrows indicate luminal cells. Arrowheads mark the apical part of epithelial cells of the duct.

Statistical analysis

The functional experiments conducted (transport of luminal cells, contractions of the epididymal duct, $n=15$ each) were evaluated by a one-way ANOVA with matched samples followed by Tukey's multiple comparisons test. Normal distribution was verified by the Kolmogorov–Smirnov test.

GraphPad (GraphPad Prism 5, version for Windows, GraphPad Software, www.graphpad.com) was used to analyse all data, differences were considered significant if $*P<0.05$, $**P<0.01$, $***P<0.001$ and non-significant (ns) if $P>0.05$.

Results

Luminal cells in the foetal human epididymis

In order to understand the functional relevance of foetal and neonatal smooth muscle cell contractions of the epididymal duct we investigated the human foetal epididymal duct and found an abundant amount of isolated cells within the lumen (Fig. 2).

Identification of luminal cells in the foetal human epididymis

To clarify the origin of these cells, we used antibodies against neutral endopeptidase (NEP) which is known

to localize to the luminal surface of epithelial cells within the human epididymal duct (Thong *et al.* 2014). We found NEP staining in the luminal cells in addition to the expected staining of the apical surface of epididymal epithelial cells (Fig. 2A, B and D, E). Since no cells within the testis showed any NEP immunofluorescence (Fig. 2C) we were able to identify the luminal cells as exfoliated cells originating from the epididymal epithelium.

Luminal cells in the neonatal rat epididymis

The occurrence of exfoliated cells was also detected in the epididymal duct of rats at neonatal day 6 in caput, corpus and cauda (Fig. 3, Video 2). Comparable results were found in young mice as shown here at day 10 after birth (Video 3). The abundant amount of cells increased from caput towards cauda (Fig. 3D, E, F and Video 2).

Directional movement of luminal cells

Movies revealed the transport of these luminal cells in the caput, corpus and cauda region (Video 2A, B and C). The quality of the cell movements could be divided into five categories from category 0 'no movement within

Figure 3 Luminal cells in the epididymal duct of the neonatal rat. (A, B and C) Screenshots of time-lapse movies from 6-day-old rats in the caput, corpus and cauda epididymidis prepared as shown in Fig. 1 of the material and methods section. (D, E and F) Enlarged details of time-lapse movies (1 frame/s, Video 2) show luminal cells in different regions of the rat epididymis. Some of these cells are marked by arrows.

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Figure 4 Categorisation of the transport quality of luminal cells in the neonatal rat epididymis and changes of the transport by sildenafil and noradrenaline. (A) The quality of the transport of luminal cells was described by the use of five defined categories. Examples for categories 1, 2 and 3 are shown in **Video 2**. (B and C) The graphs illustrate cell transport without ('untreated') and with ('+sildenafil', '+noradrenaline') treatment. Addition of sildenafil diminished luminal transport significantly ($P < 0.0001$) and noradrenaline significantly increased the transport again ($P = 0.0002$). Data of the single experiments ($n = 15$) are shown by coloured lines in (C). In (B), data $\pm$ s.d. are shown. (***) ($P < 0.001$) (one-way ANOVA with matched samples followed by Tukey's multiple comparisons test).

the observed time frame' to category 4 'directional flow through the field of view without pendular movement' (Fig. 4A). In all regions of the untreated rat and mouse epididymis, movies revealed a directional transport of the luminal cells from proximal to distal (Videos 2 and 4) with more or less pendular movements (categories 1–3).

Video 2

Directional movement of exfoliated cells in the epididymal duct of the neonatal rat with examples for the categories 1–3. The videos show directional transport of the exfoliated epididymal epithelial cells in the caput (A), corpus (B) and cauda region (C) of a 6-day-old rat epididymis. Video A serves as an example for category 1 ('predominant pendular movement within the field of view with marginal net flow'). Video B corresponds

to category 2 ('directional flow within the field of view interrupted by pendular movement'). Finally, Video C represents an example for category 3 ('directional flow to the margins of the field of view interrupted by pendular movement'). No correlation was found between the different categories and the investigated parts of the epididymal duct. This video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-19-0617/video-2>) is available from the online version of the article at <https://doi.org/10.1530/REP-19-0617>.

Video 3

Directional movement of exfoliated cells in the epididymal duct of the neonatal mouse. The videos show directional transport of exfoliated epididymal epithelial cells in the caput (A), corpus (B) and cauda region (C) of a 10-day-old mouse epididymis. This video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-19-0617/video-3>) is available from the online version of the article at <https://doi.org/10.1530/REP-19-0617>.

Video 4

Effects of sildenafil and noradrenaline on contractions and transport functions of neonatal rat epididymis (cauda). This video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-19-0617/video-4>) is available from the online version of the article at <https://doi.org/10.1530/REP-19-0617>.

Substances known to contract or relax smooth muscle cells affected the quality of the transport (Video 4). The relaxing drug sildenafil significantly diminished the transport of the luminal cells, thereby shifting it to a lower category (Fig. 4B). In 66% of cases, sildenafil led

Figure 5 Effects of substances known to contract or relax smooth muscle cells on the spontaneous contractility of the neonatal rat epididymis. (A and B) Contractions per minute in the neonatal epididymis are affected by sildenafil and noradrenaline. The graphs show contractions without treatment ('untreated') as well as sildenafil- and noradrenaline-induced effects. The addition of sildenafil diminished spontaneous contractions significantly ($P = 0.0002$). In all cases, noradrenaline significantly increased the sildenafil-diminished frequency of contractions ($P < 0.0001$). Data of the single experiments ($n = 15$) are shown by coloured lines in (B). In (A), data $\pm$ s.d. are shown. (***) ($P < 0.001$) (One-way ANOVA with matched samples followed by Tukey's multiple comparisons test).

Figure 6 PCNA staining of luminal cells in the epididymal duct from neonatal rats. Paraffin sections of 6-day-old rat caput (A), corpus (B) and cauda (C) epididymides were stained with antibodies against PCNA. Arrows indicate PCNA⁺ epithelial cells. Asterisks mark the epididymal lumen with exfoliated PCNA stained cells and arrowheads show some negative cells.

to a complete stop of the transport (category 0) (Fig. 4C). Noradrenaline significantly increased the sildenafil-diminished luminal transport (Fig. 4B). In 40% of cases, this resulted in an enhanced directional flow of the luminal cells without any pendular movement during the observed time (category 4) (Fig. 4C).

Contractions of the epididymal duct

In addition, the movies showed contractions of the epididymal duct coinciding with the observed transport of luminal cells (Videos 1, 2, 3 and 4). Systematic analyses of the epididymal duct identified the occurrence of spontaneous contractions in all regions investigated (caput, corpus and cauda). As demonstrated before for the transport of luminal cells, these spontaneous contractions were significantly decreased by sildenafil and significantly increased again after additional treatment with noradrenaline (Fig. 5). The transport of exfoliated cells and the contractions of the epididymal duct showed a correlating response to sildenafil and noradrenaline (Figs 4B and 5A).

Extrusion of cells during proliferation into the lumen

Since a large number of epithelial cells of the duct are known to proliferate the proliferation marker PCNA was used to verify the extrusion of epithelial cells during proliferation into the ductal lumen. The entire epididymis showed a high proliferation rate visible by PCNA staining (Fig. 6). A positive staining was found in the ductal epithelium and the surrounding interstitial tissue. In agreement with the extrusion of also proliferating epithelial cells into the lumen, luminal PCNA⁺ cells were detected as well. The amount of luminal cells increased from caput (Fig. 6A) via corpus (Fig. 6B) to cauda (Fig. 6C) in accordance with the movies.

Discussion

Within the epididymal duct of the foetal human and neonatal rodent tissues (rat and mice), we could visualize exfoliated epithelial cells. In human and rodent (data not shown) tissue, we could authenticate these cells as originating from the epididymal epithelium and not from the testis. These exfoliated epithelial cells were transported by directional movement interrupted by pendular movements. An association between the transport of the luminal cells and the spontaneous contractions of the epididymal duct could be observed and both were affected by substances known to contract or relax smooth muscle cells.

We demonstrated the occurrence of cells in the lumen of the epididymal duct in pre- and postnatal tissue of humans and rodents, respectively. At the investigated time points these epithelial cells are still undifferentiated (Sun & Flickinger 1979, Hermo *et al.* 1992), therefore, it is not possible to identify a specific epididymal epithelial cell type. To our knowledge, there are no data about the occurrence of luminal cells in the epididymal duct before the first immature germ cells reach the epididymis on day 20 (Picut *et al.* 2015, Parker & Picut 2016) or before the first mature spermatozoa are visible at day 49 in the rat (Robaire & Hinton 2014).

The epididymis and the epididymal epithelium are known as highly proliferating tissues during development

and in adulthood (Clermont & Flannery 1970, Sujarit & Jones 1991). We could demonstrate PCNA-stained cells in the lumen of the neonatal rat epididymis. Thus, the highly proliferating epithelium of the epididymis might extrude cells which are still proliferating into the lumen to create more space. This has been suggested by others before (Clermont & Flannery 1970). We assume that these extruded cells are not only removed as waste (see below) but also act as a matrix in order to maintain an organized ductal structure (Luschnig & Uv 2014). Luminal release of proliferating cells has previously been described in adult seasonal breeding animals like bats (Beguelini *et al.* 2015, Campolina-Silva *et al.* 2019). We found such proliferating cells also in the ductal lumen of the adult roe deer epididymis (seasonal breeder) (data not shown).

The intraluminal cells showed directional movement within the neonatal duct. This transport was interrupted by pendular movement. Oil droplets injected into the lumen of the adult epididymis demonstrated such a phenomenon in the context of sperm transport (Talo *et al.* 1979, Jaakkola & Talo 1982). The intraluminal cells were transported from caput to cauda, which resulted in an increase in the number of luminal cells towards cauda. In line with these experiments, we were able to show that the directional movement of physiological content occurs in the lumen of the neonatal duct.

The transport of the cells was associated with spontaneous contractions of the smooth muscle layer in all three regions of the epididymis. Such an association has also been described in the adult epididymis (Jaakkola & Talo 1983) where spontaneous contractions ensure the transport of immotile spermatozoa through the duct during their maturation (Robaire & Hinton 2014).

Our results are consistent with the contractions of proximal epididymal duct previously described in pre- and postnatal rats (van de Velde & Risley 1963). In contrast we also found neonatal contractions in cauda epididymidis. In this study, substances known to contract and relax smooth muscle cells were tested and both, noradrenaline and sildenafil, respectively, affected contractility of the neonatal epididymal duct as expected. We could thus demonstrate that mechanisms regulating contractile activity of the epididymal smooth muscle cells and luminal cell transport are already functional at a very early age. In addition, the response to these treatments shows the susceptibility of epididymal smooth muscle to drugs interfering with smooth muscle function. This finding points towards a prolonged window of vulnerability during which the development and maturation of the epididymis may be affected by pre- or neonatal exposure to drugs.

The combination of (i) the movement of exfoliated cells in the immature duct, (ii) its correlating contractile pattern and (iii) its regulation by contracting and relaxing agents points to a novel mechanism of epididymal development and organized waste disposal of the high amount of extruded epithelial cells. The luminal cells could be important for maintaining a lumen and an

organized epithelial structure during the formation of the epididymal duct. The contractions of smooth muscle cells could be necessary to avoid agglutination of the luminal content and thereby ensure patency of the epididymal duct. In mice, the formation of a lumen in the Wolffian duct as the predecessor of the epididymal duct occurs at gestation days 8–9 (Bouchard *et al.* 2002) and at day E13 a lumen with a circumference of 20 epithelial cells can be observed (Dyche 1979). Interrupting the luminal flow by ligating the epididymal duct after birth between caput and corpus leads to a narrowed lumen and an unorganized thickened epithelium of the epididymal duct (Abe *et al.* 1984), suggesting an important role for luminal flow in maintaining the patency of the epididymal lumen during development and maturation of the organ. Another factor for proper duct formation is the mechanical resistance from the tissue surrounding the epithelium (Hirashima 2014) which constrains the luminal diameter of the duct. However, it is not clear, how the duct drives the formation of its 3D structure while also maintaining a constant luminal diameter at the same time. Therefore, we suggest that the exfoliated luminal cells of the epididymal duct are important for maintaining the lumen and the luminal diameter in terms of a mechanical place holder. In different organisms, for example *Drosophila*, the cell lining of tubular tissue requires the support of a solid material to maintain its organization (Luschnig & Uv 2014). Interestingly, the luminal dimensions stay consistent throughout the size maturation process of the organ (Luschnig & Uv 2014).

In summary, we discovered exfoliated luminal cells originating from the epididymis, demonstrated their movement within the lumen of the immature duct and investigated their association to contractions of the smooth muscle layer of the epididymal duct. Our data suggest a mechanical function of exfoliated cells together with a novel idea of organized waste disposal of the high amount of extruded epithelial cells in the epididymal duct during development.

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

D W performed the experiments, contributed to the statistical analyses, wrote the manuscript and designed the study. A M wrote the manuscript. B S wrote the manuscript. D J provided

the human foetal material. G S contributed valuable advice to the redaction of the manuscript. R M wrote the manuscript, designed the study and directed the project.

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