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Regulation of epithelial function, differentiation, and remodeling in the epididymis

Sylvie Breton¹, Ye Chun Ruan¹,², Yoo-Jin Park¹, Bongki Kim¹

The epididymis is a single convoluted tubule lined by a pseudostratified epithelium. Specialized epididymal epithelial cells, the so-called principal, basal, narrow, and clear cells, establish a unique luminal environment for the maturation and storage of spermatozoa. The epididymis is functionally and structurally divided into several segments and sub-segments that create regionally distinct luminal environments. This organ is immature at birth, and epithelial cells acquire their fully differentiated phenotype during an extended postnatal period, but the factors involved in this complex process remain incompletely characterized. In the adult epididymis, the establishment of an acidic luminal pH and low bicarbonate concentration in the epididymis contributes to preventing premature activation of spermatozoa during their maturation and storage. Clear cells are proton-secreting cells throughout the epididymis, but principal cells have distinct acid/base transport properties, depending on their localization within the epididymis. Basal cells are located in all epididymal segments, but they have a distinct morphology depending on the segment and species examined. How this structural plasticity of basal cells is regulated is discussed here. Also, the role of luminal factors and androgens in the regulation of epithelial cells is reviewed in relation to their respective localization in the proximal versus distal regions of the epididymis. Finally, we describe a novel role for CFTR in tubulogenesis and epithelial cell differentiation.

Keywords: basal cells; clear cells; principal cells; pseudostratified epithelia; transepithelial transport

INTRODUCTION

After being produced by the testis, spermatozoa acquire their capacity to reach and fertilize an oocyte while in the epididymis, a small organ located downstream of the testis.¹⁻⁵ Epididymal sperm maturation is, therefore, essential for the establishment of male fertility. However, this process is often overlooked, and between 40% and 50% of male infertility cases diagnosed in the clinic are still labeled “idiopathic.”⁶ The epididymis is a long and single tubule that is organized into four major segments, the initial segment (IS), the caput, the corpus, and the cauda epididymidis, which are further divided into intra-segmental regions that are delineated by connective tissue septa and express a distinct set of genes.⁷⁻⁹ Epithelial cells with specific functions and morphological characteristics are located in these regions; they form the so-called blood-epididymis barrier,¹⁰,¹¹ and establish a unique luminal microenvironment for the concentration, maturation, and storage of spermatozoa.²,¹²,¹³

The pseudostratified epithelium that lines the epididymal tubule includes principal, narrow, clear, and basal cells.¹⁻³ Principal and basal cells are present throughout the epididymis, but narrow cells are located exclusively in the initial segment while clear cells are present in the caput, corpus, and cauda epididymidis. An elaborate communication network between these cells contributes to the regulation of various transport mechanisms in the epididymis.⁴,¹⁴ This review focuses on some aspects of epithelial cell function in the epididymis, including luminal acidification and regulation of the proximal epididymis by luminal factors, and describes a novel role for CFTR in the regulation of epithelial cell proliferation and differentiation.

LUMINAL ACIDIFICATION IN THE EPIDIDYMIS

Early micropuncture studies revealed that the luminal environment of the epididymis was remarkably distinct from blood.¹³,¹⁵,¹⁶ In particular, an acidic luminal pH and low bicarbonate concentration contribute to maintaining spermatozoa in a dormant state during their maturation and storage in the epididymis.⁴,¹⁷⁻²¹ During ejaculation and transit to the female reproductive tract, elevations of pH and bicarbonate concentration induce sperm capacitation, which involves modulation of key ion channels, activation of the sAC/cAMP-signaling pathway, and protein phosphorylation events in spermatozoa.²²,²³

Defective luminal acidification in the epididymis results in male infertility owing to the inability of spermatozoa to move through the female reproductive tract and fertilize an oocyte.²⁴⁻²⁶ Luminal acidification depends on several processes, including bicarbonate reabsorption and proton-secretion that occur in different cell types, depending on their location in the epididymis. Bicarbonate reabsorption is achieved by principal cells in the initial segment,²⁰,²⁷ and proton-secretion occurs in clear cells, which become progressively more abundant in the more distal regions, especially the cauda epididymidis and the proximal vas deferens.²⁸ Clear cells express...
the proton pump V-ATPase in their apical membrane and sub-apical recycling vesicles (Figure 1). The V-ATPase is a complex enzyme that is composed of several subunits. A particular set of V-ATPase subunit isoforms, including the transmembrane α2 and the cytoplasmic subunits β1, A and E2, is highly enriched in clear cells.29,30 Foxi1 is a master regulator of V-ATPase subunit expression, and Foxi1 KO male mice have abnormally elevated epididymal luminal pH and are infertile, illustrating the importance of the V-ATPase in male fertility.31,32

REGULATION OF LUMINAL ACIDIFICATION BY LUMINAL FACTORS

Proton-secretion by clear cells is regulated via recycling mechanisms.4 The activation of the cAMP and cGMP pathways in clear cells induces the redistribution of V-ATPase from sub-apical vesicles to the apical membrane, leading to an increase in proton-secretion.4,20,31 This process is accompanied by the formation and extension of numerous V-ATPase-rich apical membrane extensions. From their appearance revealed by transmission electron microscopy (TEM) and immunofluorescence, these membrane protrusions were initially named “microvilli,” but recent advances in high-resolution helium ion microscopy techniques have revealed the presence of membrane “ruffles” or “microplicae” that increase in number and become more numerous when clear cells are activated32 (Figure 2). These protrusions are analogous to the “leaf-like” structures previously described by the Hamilton group’s scanning electron microscopy (SEM) study.33

Clear cells respond to changes in their luminal environment, such as an increase from the physiological pH of 6.6 to the alkaline 7.8, by accumulating V-ATPase in their apical membrane, which increases proton-secretion.34,35 Depolymerization of the cortical actin cytoskeleton, via inhibition of RhoA and its effector ROCKII, favors the recruitment of V-ATPase from sub-apical vesicles to the apical membrane in clear cells.36 V-ATPase apical accumulation also requires intracellular calcium and is phospholipase C-dependent.34

While clear cells are involved in the maintenance of an acidic luminal pH in the steady state cauda epididymidis, principal cells have the ability to secrete bicarbonate upon basolateral stimulation.37–42 The subsequent increase in luminal bicarbonate concentration is proposed to “prime” spermatozoa before ejaculation.39,43 Bicarbonate secretion by principal cells is mediated by CFTR, and it induces an alkalization of the luminal environment.39,43,44 However, a sustained elevation of luminal pH and bicarbonate concentration may be considered detrimental to spermatozoa, by maintaining them in a preactivated state during storage. This should be prevented by subsequent stimulation by luminal bicarbonate of proton-secretion in clear cells, via the bicarbonate-activated soluble adenylyl cyclase (sAC) and activation of the cAMP/PKA pathway.35,36

In addition to mediating bicarbonate secretion, CFTR participates in ATP secretion in principal cells.45 Luminal ATP and its hydrolysis product adenosine act as extracellular messengers that activate proton-secretion in clear cells via principal cell/clear cell crosstalk.46 Several purinergic receptors are expressed in epididymal epithelial cells isolated by laser cut micro-dissection (LCM).47 These include the P1 A2B receptor, which activates the cAMP pathway upon adenosine stimulation47 and the P2X4 receptor, which is activated by alkaline pH and triggers an elevation in intracellular calcium.48 Whether these receptors are involved in the activation of clear cells will require additional studies.

Taken together, these results indicate that activation of proton-secretion in clear cells occurs via crosstalk with principal cells and involves a dual mechanism that depends on ATP and bicarbonate secretion by principal cells. We are currently investigating the potential role of principal cells as direct modulators of luminal pH in the cauda epididymidis, in addition to their indirect role in the modulation of clear cells.

ROLE OF LUMINAL FACTORS IN THE MAINTENANCE AND REMODELING OF EPITHELIAL CELLS IN THE INITIAL SEGMENT

The epididymis is an immature organ at birth, and epithelial cells continue to develop and differentiate over an extended postnatal period.49–54 Failure of IS epithelial cells to differentiate results in male infertility.56,54–60 The IS epithelium receives luminal testicular factors from its junction with the efferent ducts. The establishment

Figure 1: Expression of the water and solute channel aquaporin 9 (red) in principal cells, and the proton pumping V-ATPase (green) in clear cells of the cauda epididymidis. In this segment, numerous clear cells express the V-ATPase in their apical membrane and sub-apical vesicles (arrows). Nuclear and spermatozoa are visualized in blue with DAPI. Scale bar: 20 μm, Inset: 5 μm.

Figure 2: Helium Ion Microscope (HIM) imaging of the apical surface of the epididymal tubule. (a) Low-magnification image of a control rat cauda epididymidis, after removal of spermatozoa by in vivo luminal perfusion. Darker clear cells of various sizes (arrows) are interspersed among principal cells, which appear brighter owing to their apical stereocilia. The inset shows the apical surface of a rat cauda epididymidis immunolabeled for the V-ATPase (green). The arrow indicates a large clear cell. (b–e) High-magnification images of the apical surface of rat cauda epididymidis clear cells perfused under control conditions (b and d), and in the presence of the permeant cAMP analog, cpt-cAMP (c and e). Under control conditions, the clear cell shows a few microvilli and relatively short microplicae, and a large part of the membrane appears smooth and devoid of protrusions. cAMP induced the formation of numerous microplicae and the apical membrane did not show any smooth surface. In b and c, brighter principal cell stereocilia surrounding clear cells are evident. Scale bars: (a) 50 μm, Inset: 15 μm; (b and c): 4 μm; (d and e) 1 μm. Modified from Paunescu et al. Mol Hum Reprod 2014.
and maintenance of the IS epithelium require both androgen and lumicrine factors,\textsuperscript{57–65} which include several hormones, ligands, and unknown factors that contribute to maintaining the MAPK pathway in an activated state in this segment\textsuperscript{66} (Kim \textit{et al.} unpublished). Another protein that is potentially activated by luminal factors is the orphan receptor tyrosine kinase ROS1, which was originally described as an oncogene receptor, but was later identified as a key player in the initiation of epithelial cell differentiation.\textsuperscript{67} In the epididymis, ROS1 expression is very low at birth, but it becomes activated in the IS at the onset of epithelial cell differentiation.\textsuperscript{67,68} Male mice KO for the c-ROS1 oncogene 1 (c-ros1 KO), or ROS1\textsubscript{KMKM} mice carrying a kinase-dead allele of ROS1, the protein that is encoded by the c-ros1 gene, have an undifferentiated IS and are infertile, but have normal spermatogenesis.\textsuperscript{26,67} Impaired differentiation of IS epithelial cells in the ROS1\textsubscript{KMKM} mice was demonstrated by a reduction of expression of AQP9, a “terminal differentiation” protein (Figure 3), and a reduction in epithelial height, a hallmark of immature cells in this segment.\textsuperscript{67}

The ligand for ROS1 remains unknown, but an unbiased phosphoproteomics approach (Bio-Plex) has shown that the lack of ROS1 kinase activity in ROS1\textsubscript{KMKM} mice induces a decrease in the phosphorylation of MAPK targets, including phosphoMEK1/2 (Ser217, Ser221), phosphoERK1/2 (Thr202/Tyr204), phosphoCREB (Ser133), phospho90RSK (Ser380), and phosphoSTAT-3 (Ser727), but does not affect the AKT, p38 MAPK, or Src pathways.\textsuperscript{67} As the MAPK pathway is constitutively activated in the adult IS\textsuperscript{39,66} (Kim \textit{et al.} unpublished), these data indicate that ROS1 participates, with other effectors, in maintaining MAPK activation. Spermatooza from both c-ros1 KO mice and ROS1\textsubscript{KMKM} mice display hairpin morphology, and CASA showed significantly reduced progressive and total sperm motility in ROS1\textsubscript{KMKM} males (Figure 4).\textsuperscript{26,67} A pharmacological approach with Crizotinib during the prepubertal period (postnatal days 17–28) has shown that transient inhibition of ROS1 kinase activity delays, but does not permanently inhibit IS differentiation.\textsuperscript{67} However, when administered to adult animals for 7 or 14 days, Crizotinib has no effect on the architecture of the IS epithelium and fertility, and it

![Figure 3: Impairment of terminal differentiation of principal cells in the initial segment of ROS1\textsubscript{KMKM} mice. (a) Strong immunofluorescence labeling for AQP9 is detected in the initial segment (IS) and efferent ducts (ED) of an ROS1\textsuperscript{KMKM} normal mouse. (b) AQP9 is not detectable in the corresponding segment “IS,” while AQP9 expression is not affected, in an ROS1\textsuperscript{KMKM} mouse. Scale bars: 100 μm. Modified from Jun \textit{et al.} Endocrinology 2014.](image)

![Figure 4: ROS1 kinase mutant spermatozoa exhibit abnormal morphology and reduced motility. Top panels: Spermatooza isolated from the cauda epididymis of a WT mouse or an ROS1\textsuperscript{KMKM} mouse and suspended in Biggers, Whitten and Whittingham (BWW) medium containing polyvinyl alcohol (PVA). The ROS1\textsuperscript{KMKM} spermatooza shows flagellar angulation compared with the WT spermatooza. Bottom panels: Computer-aided sperm analysis (CASA) showed decreases in the percentage of spermatozoa with total motility (left) and progressive motility (right) in ROS1\textsuperscript{KMKM} mice compared with WT mice. Experiments were performed on three independent animals/genotype. Bars represent mean ± s.e.m. (*P < 0.05 by t-test), n = 3 mice per group. Scale bar: 10 μm. Modified from Jun \textit{et al.} Endocrinology 2014.](image)

![Figure 5: Three-dimensional reconstruction of a mouse initial segment by laser scanning confocal microscopy. BCs are labeled for keratin 5 (KRT5; green) and TJs are labeled for ZO1 (red). A BC with an intercellular projection that has crossed the TJs and is in contact with the luminal content is visible (arrow). Nuclei and spermatozoa are labeled with DAPI (blue). Scale bar: 5 μm. Modified from Kim \textit{et al.} Biol Reprod 2015.](image)

![Figure 6: Effect of EDL on the number of BCs with intercellular projections in the mouse initial segment. BCs were labeled for KRT5 (green). (a) Numerous BCs with a long intercellular projection are visible in a control mouse. (b) No BC projections are detectable in a mouse initial segment 5 days after EDL. Scale bars: 15 μm. Modified from Kim \textit{et al.} Biol Repro 2015.](image)
was concluded that sustained ROS1 inhibition in the ROS1<sup>EMK1/EMK1</sup> mice is required to induce a permanent undifferentiated IS infertility phenotype.

In the IS epithelium, principal cells are the more-studied and better-characterized cell type in the IS, and they require lumicrine factors for their survival and differentiation.<sup>70,71</sup> However, very little is known of the regulation of narrow cells and basal cells (BCs) by luminal factors in this segment. In the mouse IS, BCs send a narrow body projection between adjacent epithelial cells in the direction of the lumen.<sup>72–74</sup> Labeling with the tight-junction protein ZO1 shows that BC projections, identified by their positive labeling for keratin 5, have the ability to cross the blood-epididymis barrier, so that the tip of the projection is in contact with the luminal content (Figure 5). The presence of BCs with intercellular projections in the mouse IS is compatible with their luminal sensory role, which has been shown in the rat epididymis.<sup>71</sup> The formation of BC projections is segment-specific and species-dependent. In the rat, BC projections are present in the distal corpus and proximal cauda regions but are absent from the IS.<sup>71,75</sup> In the mouse, they are exclusively present in the IS.<sup>70,71</sup>

Factors that regulate this segmental regulation of BC plasticity are for the most part unknown. Flutamide treatment reduces the number of BCs with projections in the rat epididymis,<sup>72</sup> but not in the mouse epididymis.<sup>70</sup> In the mouse IS, BC projections start to appear relatively soon after birth, at postnatal week 3,<sup>76</sup> but in the rat epididymis BC projection formation is coincident with puberty.<sup>74</sup> These results suggest a role for androgens in the formation and elongation of BC projections in the rat but not in the mouse. Mouse epididymal BCs progressively lose their projections after efferent duct ligation (EDL), a procedure that blocks luminal fluid entry to the epididymis without affecting blood flow (Figure 6).<sup>76</sup> These results indicate the role of luminal factors in the maintenance of BC projections in the mouse IS.

Epithelial cell proliferation and apoptosis are very low in the control epididymis. However, EDL triggers a wave of apoptosis in BCs and principal cells with a maximum effect observed 1 day after EDL, followed by a progressive decrease in the number of apoptotic cells, 2 and 5 days after EDL.<sup>43,70</sup> Treatment with the anti-androgen flutamide (without EDL) does not induce apoptosis but it further reduces the low proliferative activity of BCs.<sup>73</sup> A subset of the BCs that survive EDL in the mouse epididymis show an increase in proliferation 2 days after EDL, followed by a return to a low proliferating state after 5 days. Flutamide treatment prevents the increase in BC proliferation induced 2 days after EDL, indicating the involvement of androgens in the maintenance and survival of this cell type. In contrast, EDL (Kim et al. data not shown) or castration<sup>74</sup> does not affect narrow cell morphology, proliferation, and apoptosis. Thus, androgens may have a pro-proliferative effect on mouse epididymal BCs, similar to their well-recognized role in principal cell proliferation.<sup>75</sup> We observed similar proliferative indices of IS epithelial cells in ROS1<sup>EMK1/EMK1</sup> and WT mice, indicating that ROS1 is not directly involved in the control of cell proliferation.<sup>67</sup>

**REGULATION OF PRE- AND POST-NATAL EPIDIDYMAL DEVELOPMENT – AN UNEXPECTED ROLE OF CFTR**

During tubulogenesis, epithelial cells receive signals that induce a switch between proliferation and differentiation. This switch depends on cell density and is partially initiated at the cell-cell junctions. Upon tight junction (TJ) formation, the TJ-associated protein ZO1 participates in the retention of a transcription factor (ZO1 nucleic acid binding protein; ZONAB) outside of the nucleus in TJs.<sup>76</sup> ZONAB controls the expression of a subset of genes involved in cell growth and differentiation,<sup>77–79</sup> and its relocation to TJs decreases cell proliferation and promotes differentiation. We recently made the unexpected observation that CFTR was located in epididymal TJs, in addition to its being expressed in the apical membrane of principal cells (Figure 7).<sup>60</sup> CFTR is a cAMP-activated anion channel previously implicated in anion secretion, followed by water transport, in several organs, including the epididymis.<sup>81–86</sup> CFTR contains a PDZ-binding domain in its C-terminus,<sup>86</sup> and we have shown that this motif participates in the interaction between CFTR and ZO1, which contains three PDZ domains.<sup>86</sup> Using the epididymal cell culture DC2, developed in the laboratory of Marie-Claire Orgebin-Crist,<sup>67</sup> we have shown that the absence of, or mutations in, CFTR reduces the stability of ZO1, leading to TJ disassembly and the release of ZONAB from TJs to nuclei.<sup>80</sup> This promotes the transcription of the proliferation-associated gene cyclin D1 (CCND1)<sup>79</sup> and represses the transcription of the differentiation-associated gene ErbB2.<sup>87</sup> When DC2 cells are cultured on a matrigel layer, they form well-established tubular structures (Figure 8 Left). In contrast, a disorganized cell growth is induced after CFTR knockdown (KD) (Figure 8 Right) or inhibition,<sup>80</sup> supporting the notion that CFTR plays an important role in tubulogenesis.

These results revealed a novel connection between CFTR and epithelial morphogenesis and differentiation. Mutations in CFTR cause dysfunction of tubular organs, resulting in cystic fibrosis (CF),

![Figure 7: Co-localization of CFTR with ZO1 in TJs of the mouse initial segment. (Left panels) Confocal laser scanning microscope images showing CFTR (green) and ZO1 (red) labeling in TJs. (Right panels) Higher magnification images of the regions delineated in the boxes in the left panels. Co-localization of CFTR with ZO1 is indicated by the yellow/orange labeling in the merged panels. Scale bars: Left – 10 μm, Right – 2.5 μm. Modified from Ruan et al. J Cell Sci 2014.](image-url)
Epididymal epithelial cell regulation
S Breton et al

the most common autosomal recessive disease in the Caucasian population. Almost all male CF patients have congenital bilateral absence of the vas deferens (CBAVD) or absence or atrophy of the epididymis. Defects in chloride, bicarbonate and water secretion, leading to increased viscosity of luminal fluid and blockage of Wolffian duct elongation, have been proposed as the primary cause of CF and CBAVD. However, this concept is challenged by the absence of luminal obstruction in the vas deferens and epididymis of human CF fetuses; they have normal microscopical morphology, with obvious lumen formation. CFTR expression precedes ZO1 in the TJs of the Wolffian duct, consistent with the participation of CFTR during embryonic development and tubulogenesis. We have, therefore, proposed that impairment of the ZO1/ZONAB interaction, secondary to the absence of or mutations in CFTR, affects the switch between proliferation and differentiation that occurs during development (Figure 9). The most common CF-associated mutation - deletion of phenylalanine at position 508 (ΔF508) - induces the retention of CFTR in the endoplasmic reticulum. According to our model, this would prevent its interaction with ZO1 in TJs and induce the nuclear translocation of ZONAB. Indeed, we have shown an increased level of ZONAB in nuclear extracts from mice expressing ΔF508-CFTR. We have also shown that the epididymis of adult CFTR KO mice has a lower expression of ZO1, AQ99 (in principal cells) and V-ATPase (in clear cells), indicating impaired epithelial cell differentiation in the absence of CFTR.

At 26 weeks of gestation (which corresponds to the initiation of epithelial differentiation and apical localization of CFTR), destruction of the epithelial wall and infiltration of inflammatory cells was detected in a human CF fetus. In another CF-affected organ, the trachea, reduced airway caliber and reduced cross-sectional area were observed in newborn CFTR KO pigs and young CF patients. Of note, CFTR has recently been shown to regulate TJ-associated epithelial functions in a variety of organ systems, including the small intestine, colon, and testis. Taken together, these results are consistent with the hypothesis that CFTR mutations affect TJs and restrict epithelial development and differentiation, leading to progressive deterioration of tissues that become more susceptible to stress and injury.

CONCLUSION
The epididymis, located downstream of the testsis, is a complex organ that plays a crucial role in the maturation and storage of spermatozoa. However, only a few laboratories in the world study the posttesticular reproductive tract. Male infertility is often associated with dysfunctional spermatozoa that are produced in normal numbers, indicating impairment of the male excurrent duct, including the epididymis. The generation of transgenic mice and the development of novel imaging microscope modalities have allowed a better understanding of how the different cell types that line the epididymid tubule establish the unique environment, in which spermatozoa mature and are stored, and how their differentiated characteristics are regulated. However, much remains to be elucidated on how the epididymis operates in health and how it is affected in disease states.

COMPETING FINANCIAL INTERESTS
The author declared no competing interest.

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Epididymal epithelial cell regulation S Breton et al.


