Segment-specific regulation of epididymal gene expression

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Abstract

The epididymis is necessary for post-testicular sperm maturation. During their epididymal transit, spermatozoa gain ability for progressive movement and fertilization. The epididymis is composed of several segments that have distinct gene expression profiles that enable the establishment of the changing luminal environment required for sperm maturation. The epididymal gene expression is regulated by endocrine, lumicrine, and paracrine factors in a segment-specific manner. Thus, in addition to its importance for male fertility, the epididymis is a valuable model tissue for studying the regulation of gene expression. This review concentrates on recent advances in understanding the androgen, small RNA, and epigenetically mediated regulation of segment-specific gene expression in the epididymis.

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Introduction

The epididymis is a long (approximately 1.2 m in mice and 6 m in humans) convoluted tubule connecting the efferent ducts to vas deferens. It is derived from the Wolffian duct (WD), with the exception of the most proximal part in rodent epididymis, which is suggested to form from the mesonephric tubules along with the efferent ducts (Reviewed in [Robaire et al. 2006](#), [Shaw & Renfree 2014](#)). In adults, the epididymal epithelium lining the tubule is a pseudostratified columnar epithelium composed of different cell types, namely principal, basal, clear/narrow, and apical cells. The tissue is divided into three main segments: caput, corpus, and cauda, which are further divided into several subsegments by connective tissue septae ([Abou-Haila & Fain-Maurel 1984](#)). Further, in rodents, the most proximal part of the epididymis, initial segment (IS), can be separated from the rest of the caput and forms the fourth main segment. In each of the segments, the morphology of the epithelium and the diameter of the lumen differ from other segments. In the IS, the epithelium is tall, and the luminal diameter is small, and from thereon, toward more distal epididymal regions, the epithelial cell height diminishes and the luminal diameter increases ([Fig. 1](#)).

In addition to the morphology of the epithelium, the epithelial cells are functionally different from epididymal segments that are regulated by a very tightly controlled gene expression. Many genes exhibit cell type and segment-specific expression patterns, where expression can be restricted to one particular subsegment. Additionally, several gene families, for example, prostate and testis expression ([Pate, Turunen et al. 2011](#))

and wingless-type MMTV integration site family (*Wnt*, [Koch et al. 2015](#)), display distinct expression patterns along the tissue; certain family members are restricted to specific epididymal regions, whereas other members are present in the whole tissue ([Fig. 2](#)). Moreover, segment-specific gene expression is regulated differentially in distinct segments, for example, most caput-specific genes are under androgen regulation, whereas most IS-specific genes require additional lumicrine factors (i.e. factors coming from testis along with the luminal fluid) for full expression ([Sipilä et al. 2006](#)). Furthermore, some genes are regulated differentially in different epididymal regions. For example, for gamma-glutamyl transpeptidase (*Ggt*), multiple mRNAs are produced in the epididymis and regulated in a segment-specific manner. *Ggt* mRNA IV expressed in the IS, corpus, and cauda is regulated by lumicrine factors in the IS, but not in the corpus and cauda, whereas mRNAs II and III expressed in all epididymal regions are under the control of androgens ([Palladino & Hinton 1994](#)). This precise control of epididymal gene expression is thought to provide the basis for epididymal sperm maturation. In each of the epididymal segments, a specific set of proteins is expressed and secreted into the luminal fluid creating the changing luminal environment required for sperm maturation in a sequential manner.

Spermatozoa produced in the testis need to go through epididymal maturation to obtain the ability to move progressively forward and full fertilization capacity. During this post-testicular maturation, the interaction with luminal proteins secreted by epididymal epithelial cells results in constant remodeling of the sperm cell

Epigenetic mechanisms

Epigenetic control of gene expression refers to the regulation of gene activity that does not involve alterations in the DNA sequence. The most studied epigenetic mechanisms are histone modifications and DNA methylation. Methylation and demethylation of histone 3 lysines affect the accessibility of chromatin and thus transcription (Zhou *et al.* 2011). Even though widely studied in many other organs, the involvement of histone modifications controlling epididymal gene expression has not been studied. However, a recent knock out of absent, small, or homeotic discs-1 like (*Ash1l*) gene implied that this epigenetic mechanism is an important controller also in the epididymis (Brinkmeier *et al.* 2015). ASH1L is a histone methyltransferase shown to associate with Hox genes, with knock down causing reduced histone 3 lysine 4 (H3K4) trimethylation at *Hoxa10* gene *in vitro* (Gregory *et al.* 2007). Knocking out *Ash1l* led to segment-specific down-regulation of Hox gene expression and morphological transformation from corpus to caput and from vas deferens to cauda, similar to that seen in *Hoxa10* and *Hoxa11* knockout mice (Brinkmeier *et al.* 2015). The lack of *Ash1l* could cause a reduction in activating histone mark H3K4me3 and repression of target genes. Nonetheless, further studies are required to demonstrate the role of histone modifications in the epididymis.

DNA methylation is an important regulator of fundamental biological processes such as X-chromosome inactivation and genomic imprinting. In DNA methylation, a methyl group is covalently attached to the C5 position of a cytosine residue, forming 5-methylcytosine (5mC) by DNA methyltransferase (DNMT) enzymes (Kohli & Zhang 2013, Sadakierska-Chudy *et al.* 2015). Generally, DNA methylation is associated with transcriptional repression. However, for example, genome-wide analysis of human DNA methylation in different tissues revealed a group of intragenic tissue-specific methylation sites, where methylation was positively correlated with the expression of the host gene (Straussman *et al.* 2009). Previously, DNA methylation was considered a stable and persistent mark; nevertheless, recent findings have demonstrated that methylation is a reversible and dynamic process (Sadakierska-Chudy *et al.* 2015). DNA demethylation occurs via passive process during replication but also actively via oxidation by ten-eleven translocation (TET) family enzymes (Kohli & Zhang 2013, Sadakierska-Chudy *et al.* 2015). For further regeneration of unmodified cytosine residues, three putative pathways have been suggested: passive dilution during replication, removal of the oxidized substituent, or the modified nucleotide by DNA repair-mediated mechanisms. The establishment of DNA methylation patterns has a significant contribution in the development and differentiation of different tissues and thus in controlling tissue and cell-specific expression patterns.

Epididymal DNA methylation

DNA methylation in the epididymis has been analyzed only in a few studies. In the newborn and 7-day-old rat epididymis, cyclin D1 promoter methylation was found to correlate with expression levels; in newborns with high cyclin D1 expression, the promoter area was unmethylated, whereas in 7-day-old rats, cyclin D1 expression levels came down and methylation of the promoter area increased (Darwanto *et al.* 2008). However, at day 7, there is a marked difference in cyclin D1 expression levels between different epididymal segments, with high expression levels in the cauda epididymis (Wang & Kumar 2012), which was not taken into account in the study by Darwanto *et al.* (2008). As the epididymal cyclin D1 expression patterns change during postnatal development, and result in a segment-specific expression in adults (Wang & Kumar 2012), it would be interesting to study the promoter methylation in a more detailed manner.

A study on male piglets discovered one intragenic CpC site whose methylation status correlated with estrogen receptor 1 (ESR1) expression. The epididymis with high ESR1 expression levels had 200% higher methylation levels of that specific CpC site than heart tissue with low ESR1 expression levels (Fürst *et al.* 2012). Interestingly, that CpC site harbored a putative transcriptional repressor TG-interacting factor 1 (TGIF) binding site. TGIF has been previously shown to interact with histone deacetylase 1 (HDAC1) leading to chromatin condensation and repression of transcription (Fürst *et al.* 2012). Accordingly, a reduced histone H3 presence at the specific intragenic, highly methylated CpC site in the epididymis was confirmed by chromatin immunoprecipitation, suggesting a loose chromosomal structure around that area (Fürst *et al.* 2012).

For *Rhox5* homeobox gene, it was shown that CpG islands in the *Rhox5* proximal promoter were differentially methylated in the epididymal segments of mice. Further, methylation correlated with the expression pattern of *Rhox5* during epididymal development. CpG islands in the promoter area were hypermethylated in caput of 11- and 25-day-old animals when *Rhox5* is hardly expressed and were demethylated as *Rhox5* expression increased at 30 days of age. In contrast, in corpus and cauda where *Rhox5* is not expressed, the methylation levels were not reduced during postnatal development (Bhardwaj *et al.* 2012). The data also suggested that androgen receptor (AR) occupancy at the androgen response element (ARE) sites in the *Rhox5* promoter region was dependent on DNA methylation status in that region (Bhardwaj *et al.* 2012). On the other hand, recent genome-wide methylation status analysis from rats did observe differentially methylated genomic areas in distinct epididymal regions, but the methylation status of genes did not correspond to the expression pattern of those genes. In addition, the rat

Rhox5 promoter was not found to be methylated in that study (Chu *et al.* 2015). Thus, further studies are required to obtain a better understanding of the species differences and the overall role of segment-specific DNA methylation in the epididymis.

Small RNAs

The majority of the mammalian genome is transcribed, ranging from 63% in mice (Carninci *et al.* 2005) to a staggering estimate of 93% of the human genome that may be transcribed (ENCODE Project Consortium *et al.* 2007). Furthermore, it was estimated that less than half of transcribed RNA code proteins and the rest are the so called non-coding RNAs (ncRNA; Carninci *et al.* 2005). ncRNAs include, for example, long ncRNAs and small RNAs such as microRNA (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and PIWI-interacting RNAs (piRNAs). High-throughput small RNA sequencing studies have identified piRNA and miRNA in the epididymis (Yan *et al.* 2011, Li *et al.* 2012). Although the existence of piRNAs in mammalian somatic cells is still questionable (Ross *et al.* 2014), the presence of miRNAs and their functions in the cells have been studied extensively and are currently quite well understood.

miRNAs

miRNAs are transcribed by RNA polymerase II and cleaved by the RNase III enzyme, DROSHA, producing individual pre-miRNAs. EXPORTIN 5 exports pre-miRNAs into the cytoplasm, where another RNase III enzyme, DICER1, cleaves the loop structure, producing mature miRNA. One miRNA strand is loaded into the RNA-induced silencing complex (RISC) where it directs translational repression or cleavage of mRNA targets (Fig. 3, reviewed recently in Hammond 2015). miRNAs mainly target 3'-untranslated regions (3'-UTRs) of mRNAs. As the target sequence is often not perfectly complementary to the miRNA sequence, each miRNA potentially targets hundreds of genes/mRNAs and each mRNA has several regulatory miRNAs (Selbach *et al.* 2008). Thus, the miRNAs form a regulatory network providing yet another level of complexity to the regulation of gene expression.

After the discovery of miRNAs, an atlas of miRNA expression patterns in different tissues of several different species was generated (Landgraf *et al.* 2007). Using the data generated in the miRNA Atlas, it was further shown that the human and non-human primate-specific X-chromosomal *miR-888* cluster of six miRNAs is predominantly expressed in the epididymis and other male reproductive tissues (Li *et al.* 2010). Comparison of epididymal miRNA profiles between humans (Belleannee *et al.* 2012a,b), mice (Nixon *et al.* 2015a,b), and rats (Chu *et al.* 2015) revealed a

Figure 3 Interactions of androgen and miRNA regulatory pathways. Ligand-activated androgen receptor (AR) moves into nucleus where it binds to the regulatory areas of target genes and miRNAs. Mature miRNAs control AR activity by targeting mRNAs of AR target genes. ARE, androgen response element; HSF, heat-shock factor; RISC, RNA-induced silencing complex; T, testosterone.

surprisingly low conservation of miRNAs across species (Fig. 4). Intriguingly, a majority of conserved miRNAs were found to be expressed throughout the epididymis, indicating a general housekeeping function of miRNA-mediated regulation (Nixon *et al.* 2015a,b). Comparable analyses of epididymal miRNA and mRNA profiles have been performed to identify miRNA target genes in the different regions of the human and rat epididymis at different ages (Zhang *et al.* 2010, Wang & Ruan 2010a,b, Belleannee *et al.* 2012a,b). Those studies identified several putative targets known to affect fertility. In the human epididymis, claudin 10 (*Cldn10*) and estrogen-related receptor Gamma (*Esrrg*) were confirmed to be the targets of *miR-145* and *miR-892b* *in vitro* using a luciferase reporter system (Belleannee *et al.* 2012a,b), whereas RAS p21 protein activator 1 (*Rasa1*) and β -catenin were shown to be *miR-335* and *miR-200a* targets in rats respectively (Wang & Ruan 2010a,b, Wu *et al.* 2012). In mouse epididymis, *in vitro* reporter assays confirmed that mitogen-activated protein kinase 14 (*Mapk14*) is a target for *miR-200c* and forkhead box O1 (*Foxo1*) for *miR-486* (Nixon *et al.* 2015a,b).

The overall importance of miRNAs to the epididymal functions and sperm maturation was shown by specific deletion of *Dicer1* in the proximal mouse epididymis (Björkgren *et al.* 2012, 2015). In the *Defb41*Cre;*Dicer1*^{fl/fl}

Figure 4 Segmental expression of miRNAs in the mouse epididymis. Pie charts from caput, corpus, and cauda showing percentages of segmental miRNAs from total miRNAs present in that given segment. The shading marks represent the percentage of miRNAs that are conserved across mice, rats, and humans. Due to different analysis methods used, the two mature miRNAs, -3p and -5p, were not distinguished. Note that all the conserved miRNAs showing segment specificity in the mouse epididymis are uniformly expressed in all the epididymal regions of rat and human epididymis. For the full data sets, readers are referred to the following original articles: mice, [Nixon et al. \(2015a,b\)](#); rats, [Chu et al. \(2015\)](#); humans, [Belleannee et al. \(2012a,b\)](#).

mouse model, the lack of *Dicer1* caused dedifferentiation of the epithelium with imbalanced sex steroid receptor expression and defects in lipid homeostasis in the

epithelium, subsequently causing changes in the lipid constitution of the sperm membrane and male infertility ([Björkgren & Sipilä 2015](#)). The direct effects of individual epididymal miRNAs have been assessed for two miRNAs. *miR-7578* was shown to regulate both mRNA and protein levels of early growth response 1 (*Egr1*) gene and thus acts as a negative regulator of inflammatory responses ([Zhang et al. 2013](#)). *miR-29a* was shown to regulate epididymal cell proliferation during postnatal epididymal development in rats by targeting nuclear autoantigenic sperm protein (*Nasp*, [Ma et al. 2012](#)), which is known to control cell cycle progression via binding to histones and affecting chromatin assembly ([Wang et al. 2008](#)). Further, it was shown that *miR-29a* is directly regulated by androgens and it was suggested to form a regulatory circuit with AR by targeting two known AR regulators, insulin-like growth factor 1 (IGF1) and p53 ([Ma et al. 2013](#)). Transgenic mice overexpressing *miR-29a* had significantly smaller epididymides, which was suggested to be due to repressed AR signaling ([Ma et al. 2013](#)). However, as *miR-29a* was previously shown to repress cell proliferation ([Ma et al. 2012](#)), the observed phenotype could be due to a proliferative defect of the epithelium.

In addition to controlling epididymal functions in a given cell, miRNAs have been proposed to mediate intercellular communication in a paracrine-like fashion via transfer by epididymosomes, membrane vesicles that are released from epididymal epithelial cells via apocrine secretion ([Hermo & Jacks 2002](#)). Distinct miRNA populations have been found from the epididymosomes from different epididymal regions of the bovine and human epididymis ([Belleannee et al. 2013a,b](#)). Studies on bovine demonstrated that miRNAs present in the epididymosomes differ from those of the surrounding epithelial cells, suggesting that miRNAs are selectively released into epididymosomes ([Belleannee et al. 2013a,b](#)). Furthermore, epididymosomes obtained from the proximal epididymis were shown to bind to the epithelial cells from distal epididymis *in vitro* ([Belleannee et al. 2013a,b](#)). Similar mechanisms have been demonstrated in other tissues as well, suggesting a general mode of intercellular regulation (reviewed in [Belleannee 2015](#)). As epididymosomes have been shown to transfer proteins and lipids to the spermatozoa present in the epididymal lumen ([Sullivan & Saez 2013](#)), it is conceivable that miRNAs needed in the zygote may be transferred into spermatozoa during the epididymal maturation. This hypothesis is supported by three recent studies. Exciting work by [Sharma et al. \(2016\)](#) demonstrated that the tRNA fragment (tRF) content of spermatozoa changes during epididymal maturation. The tRF load of epididymosomes from the cauda epididymis correlated with that of caudal sperm, and further, purified caudal epididymosomes were able to deliver cauda-enriched tRFs to sperm isolated from the caput region. Sperm tRFs were suggested to be the epigenetic

factor transferring intergenerational inheritance (Chen *et al.* 2016, Sharma *et al.* 2016). Moreover, Nixon *et al.* (2015a,b) showed that also the miRNA profile of mouse spermatozoa changes during epididymal transit from caput to cauda, and in a study by Johnson *et al.* (2015), long (>100 nt) RNA content of spermatozoa was shown to comprise extracellular-vesicle-associated RNA, possibly incorporated into spermatozoa during epididymal transit.

Regulation via steroid hormones

Steroid hormones transmit their signal via nuclear receptors. In the absence of ligand, the receptors are located in the cytoplasm bound to heat-shock proteins. Upon ligand binding, the receptors undergo a conformational change facilitating recruitment of coactivator proteins and the ligand–receptor complex is shuttled to the nucleus. In the nucleus, classical steroid receptors form homodimers and bind to the hormone response elements of target genes to regulate gene transcription (Fig. 3). Steroid hormones known to regulate epididymal gene expression are androgens and estrogens. Further studies are needed to reveal the significance of recent findings of active glucocorticoid signaling in the rat epididymis (Silva *et al.* 2011) and vitamin D nuclear receptor and its metabolizing enzymes in the human epididymis (Blomberg Jensen *et al.* 2010) for epididymal gene expression.

Testosterone enters to the epididymis via blood circulation but also through the efferent ducts. Inside the epididymal epithelial cells, testosterone is reduced to more potent dihydrotestosterone (DHT) by 5 α -reductases (Robaire & Hamzeh 2011). The main source of estrogen in males is the testis; however, the additional presence of P450 aromatase in spermatozoa provides a local source of estrogen in the epididymis (Reviewed in Hess 2014). As the estrogen action in the epididymis was recently reviewed (Hess *et al.* 2011, Hess 2014), this review will focus on androgen regulation. Of note, however, is the notion that the correct balance between androgen and estrogen levels might be of importance for maintaining epididymal functions. Observed imbalance in androgen–estrogen signaling might partly explain the epithelial cell phenotype, diminished cell height, increased proliferation, and regression to an undifferentiated state, in the mouse model lacking DICER1 in the proximal epithelium (Björkgren *et al.* 2012). The importance of correct hormonal balance has been noted in the prepubertal development of the reproductive tract in male rats, where changes in the testis and reproductive tract morphology induced by exposure to estrogens were shown to result from a combination of high estrogen and low androgen action (Williams *et al.* 2001). Furthermore, low androgen:estrogen ratio seems to be important for Leydig tumor cell proliferation and in regulating immune and inflammatory responses (Cutolo *et al.* 2002, Maris *et al.* 2015).

Androgens

It has long been known that the development and maintenance of the epididymis is dependent on androgens. During embryonal development, androgens are necessary for WD stabilization and further development. However, it was recently shown using conditional *Ar* knockout mouse models that androgen signaling via AR in the WD epithelium is not required for stabilization nor subsequent elongation and coiling of WD/epididymis. In contrast, AR in the mesenchyme surrounding the WD epithelium is essential for the above-mentioned processes (Murashima *et al.* 2011). Epithelial–mesenchymal interactions are a requisite for several developmental processes (Ribatti & Santoiemma 2014). The above-mentioned results, along with earlier studies, for example, on leucine-rich repeat-containing G protein-coupled receptor 4 (*lgr4*, Mendive *et al.* 2006) and inhibin beta-A (*Inhba*, Tomaszewski *et al.* 2007) demonstrate the need for epithelial–mesenchymal interactions in the development of WD derivatives as well. However, a considerable amount of research is needed before the reciprocal signaling between the WD epithelium and the surrounding mesenchyme is better characterized. The final differentiation of the epididymis, formation of epithelial cell types, and segmentation occurs after birth and in contrary to the embryonal development requires epithelial AR (Krutskikh *et al.* 2011, Murashima *et al.* 2011, O'Hara *et al.* 2011).

Previous studies have reported a wide variety of epididymal androgen-regulated genes in different species (reviewed in Belleannee *et al.* 2012a,b). These early studies revealed interesting patterns of androgen regulation of the epididymal gene expression, but did not provide any explanations for the segment-specific nature of the regulation. Specificity of spatiotemporal androgen regulation in different tissues is achieved by differential usage of coregulators, collaborating transcription factors and pioneer factors. Currently, there are over 160 AR coregulators known, some of which act as coactivators or corepressors, and some can act as both activators/repressors depending on cellular context (Heemers & Tindall 2007). Several known androgen coregulators were identified in the mouse epididymal array data. Some coregulators show segment enrichment in their expression, suggesting that these factors may contribute to the segment-specific responses (Sipilä *et al.* 2011).

Of all AR coregulators, only the role of forkhead box A2 (*Foxa2*) transcription factor in mediating androgen action in the epididymis has been studied in detail. *Foxa2* is expressed in the corpus and cauda, where it was found to suppress androgen activation of lipocalin 5 (*Lcn5*). The low levels of *Foxa2* in the caput allowed *Lcn5* transcription to be activated by AR (Yu *et al.* 2005, 2006). However, more recent research in prostate cancer cell lines has demonstrated that there are three distinct

classes of androgen-responsive genes, independent of FOXA1, activated by FOXA1 and repressed by FOXA1 (Sahu *et al.* 2011). Thus, FOXA2 is expected to have a more multifaceted function in the androgen regulation of epididymal gene expression as well. The development of the chromatin immunoprecipitation technology combined with sequencing (ChIP-seq) has enabled genome-wide analyses of actual AR binding to the chromatin. *In vivo* ChIP-seq studies in mouse caput epididymis demonstrated AR binding to several known ARE sites on epididymal genes. However, the previously published *in vitro* androgen receptor binding (ARB) data on *Lcn5*, reproductive homeobox 5 (*Rhox5*), and cysteine-rich secretory protein 1 (*Crisp1*) promoter areas was not corroborated in ChIP-seq or ChIP-qPCR experiments, suggesting that these *in vitro* identified binding sites do not have a biological relevance *in vivo* (Hu *et al.* 2010). Moreover, a comparison of ARBs and expression of androgen-regulated genes between three androgen-regulated tissues, prostate, kidney, and caput epididymis, revealed that AR cistromes and respective androgen-regulated transcription patterns are highly tissue specific, and the three tissues shared only 7–16% of ARBs and only 21 genes were androgen regulated in all tissues (Pihlajamaa *et al.* 2014). Interestingly, *de novo* motif analyses showed that transcription factor AP-2 gamma (TFAP2c) binding sites were adjacent to 25% of ARBs in the epididymis. Further *in vivo* ChIP-seq analyses confirmed TFAP2c binding to those sites. Finally, *in vitro* experiments in immortalized epididymal epithelial cell line, mE-Cap28, showed that depletion of *Tfap2c* significantly reduced AR binding to the ARBs shared with AR and TFAP2c. This suggests that TFAP2c may function as a pioneer factor priming the chromatin for subsequent AR binding in the epididymis (Pihlajamaa *et al.* 2014).

Conclusions

Due to its highly organized structure and strictly regulated gene expression, the epididymis is an especially suitable model tissue for studying the regulation of gene expression *in vivo*. Being regulated by endocrine, lumicrine, and paracrine factors, a wide variety of mechanisms can be analyzed in the epididymis. New genome-wide techniques such as ChIP-seq has provided a powerful means for characterizing various aspects of regulation of gene expression and the functional consequences of it. Interestingly, scientific advances during recent years have revealed how intimately different regulatory networks are connected to each other. Steroid hormones regulate miRNA expression, which in turn can regulate steroid hormone signaling either by regulating the levels of steroid receptors or their coregulators or downstream target genes. Interestingly, miRNAs have been suggested to regulate estrogen receptor 1 (*Esr1*) levels in response to AR activation (Delic *et al.* 2010), implying a function

in maintaining proper balance in androgen/estrogen action in cells. Moreover, epigenetic factors modify androgen receptor activity. DNA methylation affects the accessibility of chromosomal sites for AR binding, whereas a group of AR coregulators possessing histone-modifying activity cause changes in chromatin that affect gene activation/silencing. In addition, histone-modifying enzymes can acetylate/deacetylate AR itself consequently activating/repressing AR transcriptional activity (Matsumoto *et al.* 2013). The complexity of regulatory networks merits additional studies, and future research will undoubtedly shed light into the regulation of segment-specific gene expression in the epididymis and thus post-testicular sperm maturation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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