Human Reproduction Update, Vol.22, No.5 pp. 574–587, 2016
Advanced Access publication on June 15, 2016 doi:10.1093/humupd/dmw015

human reproduction update

The human epididymis: its function in sperm maturation

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Submitted on September 29, 2015; resubmitted on April 13, 2016; accepted on April 25, 2016

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BACKGROUND: Spermatozoa acquire their fertilizing ability and forward motility properties during epididymal transit. Our knowledge of gamete physiology is based on studies conducted in laboratory and domestic species; our knowledge of these processes in humans is limited. Medical indications for assisted reproductive technologies (ART) have progressed to include male infertility. Surgical procedures allow collection of spermatozoa from all along the human excurrent ducts, and the former have been used with some success in reproductive medicine. This has raised questions over the role of the epididymis in human sperm physiology.

OBJECTIVE AND RATIONALE: To reanalyze what we now know about epididymal physiology in humans and to assess the relevance of laboratory animal models for understanding human physiology and the pathophysiology of the epididymis.

SEARCH METHODS: A systematic bibliographic search of PubMed for articles published in English before May 2015 was carried out using the search terms 'epididymis' and 'sperm maturation'. Literature on the consequences of vasectomy on the epididymis was also searched.

OUTCOMES: Whereas the proximal epididymis is almost exclusively occupied by efferent ducts, the sperm reservoir capacity is poorly developed in humans. At the molecular level, the human transcriptome and proteome show some segment specificity; conflicting results persist with regard to secretome variation along the tubule. The number of genes regulated along the excurrent ducts in men is lower when compared to rodent species, but remains significant. It is challenging to reconcile biochemical and physiological studies with clinical data obtained from men undergoing reanastomosis of the vas deferens at different points along the excurrent duct. We propose that vasectomy/vasovasostomy is a model to understand the consequences of obstruction on epididymis function in humans.

WIDER IMPLICATIONS: Despite the scarcity of biological material available, the interspecies variability of the male reproductive tract urges us to use modern molecular and cellular biology tools to better understand human epididymis physiology in order to apply ART in a more responsible manner.

Key words: epididymis / sperm maturation / excurrent ducts / male fertility / male infertility / vasectomy / assisted reproductive technologies

Introduction

As for other vertebrate species practicing copulation to achieve internal fertilization, the human male reproductive tract is characterized by a well-differentiated excurrent duct (Bedford, 1979; Jones, 1998a,b). It includes on each side, first the vasa efferentia – originating from the epigenital mesonephric tubules – connected to the rete testis, then the following structures originating from the mesonephric duct (Hess *et al.*, 2001; Cooper *et al.*, 2003): the epididymal tubule, the vas deferens, the ampulla, the seminal vesicle and the ejaculatory duct (Shaw and Renfree, 2014).

The epididymis in particular has been of interest since the pioneering work performed using rabbits in the 1960s by Marie-Claire Orgebin-Crist (Orgebin-Crist, 1967) and Michael Bedford (Bedford, 1967). These authors showed that spermatozoa acquire their fertilizing ability and forward motility properties during epididymal transit. Collectively, these modifications undergone by the transiting male gamete are referred to as sperm maturation (Cooper and Yeung, 2006). Other functions are attributed to the epididymis such as sperm concentration and transport, immunoprotection of the male gamete and its role as a sperm reservoir (Cornwall, 2009; Belleannee *et al.*, 2012).

Anecdotal knowledge of the biological processes leading to fertilization in humans culminated in 1978 with the birth of Louise Brown: the first 'test tube baby' (Steptoe and Edwards, 1978). This technology led to millions of births; this major breakthrough in reproductive biomedicine was recognized by the nomination of Robert Edwards as the 2010 Nobel Prize Laureate (Kirby, 2010). Medical indications for assisted reproductive technologies (ART) (Cooper, 1990, 1993) progressed to include male infertility (French and Sabanegh, 2009). Surgical procedures allowed collection of spermatozoa from all along the human excurrent ducts, and the former were used with some success in reproductive medicine (Schoysman *et al.*, 2001; Shin and Turek, 2013). This has raised questions over the role of the epididymis in human sperm physiology (Schoysman and Bedford, 1986; Cooper, 1990, 1993; Bedford, 1994; Turner, 1995). As a consequence of the development of transgenesis and DNA sequencing technologies, the mouse rapidly became the 'universal' animal model for biomedical research. Our knowledge is based on a wide variety of mammals, and research is now conducted almost exclusively in murine species. Our understanding of the epididymal functions in humans is mainly based on clinical observations of pathological conditions, such as agenesis or obstruction, of the excurrent duct (Schoysman, 1994).

This review is intended to reanalyze what we know about epididymal physiology in humans and to assess the relevance of laboratory animal models for understanding human physiology and the pathophysiology of the epididymis. For general reviews on the epididymis, readers can refer to additional publications (Cooper and Yeung, 2006; Cornwall, 2009; Belleannee *et al.*, 2012).

Methods

A systematic bibliographic search of the MEDLINE database for peer-reviewed original articles was carried out using PubMed. Searches were performed using 'epididymis' and 'sperm maturation' as principal keywords. Literature on the consequences of vasectomy on the epididymis was also searched. Articles published in English before May 2015 were considered. Additional papers cited by primary references were included. A comparative approach was used to understand particularities of the human epididymis with regard to what is known in laboratory animal models. Some transcriptomic data were reanalyzed to allow comparison between species and to better illustrate the consequences of vasectomy on the human epididymal transcriptome previously published by our laboratory.

The vasa efferentia and the proximal epididymis

In contrast to other mammalian species, the vasa efferentia in humans are not straight small tubules connecting the rete testis to the epididymal tubule. Some efferent ducts are coiled, some are branched, while others are endless and do not fuse with the epididymal tubule (Saitoh *et al.*, 1990; Yeung *et al.*, 1991). The histology of the caput epididymis in humans suggests that the proximal portion of the caput epididymis is mainly formed by coiled vasa efferentia. No initial segments can be distinguished by macroscopic examination of the caput epididymis in normal men (Jonte and Holstein, 1987; Turner, 2008). Furthermore, with the exception of some portions of the efferent ducts, no small diameter tubule with the typical cuboidal epithelium that characterizes the initial segment in laboratory animals can be clearly distinguished in the most proximal portion of the human caput epididymis (Jonte and Holstein, 1987; Saitoh *et al.*, 1990).

From studies in rodent species, we learned that water reabsorption by the efferent ducts is as efficient as in the kidney (Hess, 2002). The expression of the Na^+/H^+ ATPase involved in water reabsorption is estrogen-dependent and is high in the vasa efferentia; its expression decreases along the epididymis. In fact, the estrogen concentration is reported to be very high in the proximal epididymis (Hess, 2000; Hess, *et al.*, 2011). The role of estrogens in efferent duct function is well illustrated by dominant estrogen receptor knockout mice. In this model, the testis volume is significantly increased due to water accumulation associated with the inability of the vasa efferentia to reabsorb intraluminal water (Hess *et al.*, 1997). Even though estrogen receptor beta has been immunodetected in human efferent ducts (Saunders *et al.*, 2001), there is no evidence supporting water reabsorption by the vasa efferentia forming the proximal epididymis. Moreover, a clear cutoff for enlarged testicular volume associated with a pathological condition, or macroorchidism, has not been reported. Pathologically enlarged testis is difficult to define due to age, ethnic group and anthropomorphic parameters (Lotti and Maggi, 2015). The effects of epididymis agenesis, estrogen receptor mutation or aromatase inhibitor treatments on human testicular volume have not been reported in the literature, even though cytochrome P450 aromatase is highly expressed in the human vasa efferentia and in the proximal segment of the epididymis (Hinton *et al.*, 1998; Carpino *et al.*, 2004). Some ion transport-associated proteins, possibly involved in water transport, have been immunodetected along the efferent ducts and the proximal human epididymis. Cellular expression and tissue distribution of these proteins possibly involved in water movement show discrepancies between human and animal models suggesting a functional particularity in the human male reproductive tract (Kujala *et al.*, 2007). The role of the human epididymis in water reabsorption remains to be clarified.

In laboratory animals, the initial segment is known to be the more active epididymal segment and to play a major role in sperm

maturation (Cornwall *et al.*, 2002). Expression of the proto-oncogene c-ros is associated with initial segment differentiation in the mouse as it is exclusively expressed in this segment of the differentiated epididymis. Loss of c-ros expression in c-ros knockout mice is associated with the absence of a differentiated initial segment resulting in male infertility (Yeung *et al.*, 1998; Krapf *et al.*, 2012). Whereas normal spermatogenesis and sperm transport occur in these genetically modified animals, their spermatozoa have an osmoregulation deficiency resulting in the inability of the male gamete to efficiently reach the upper part of the female reproductive tract. This experimental evidence supports the involvement of the initial segment in sperm maturation (Cooper *et al.*, 2003). As no initial segment can be histologically distinguished in the human epididymis, we used *in situ* hybridization to study c-ros expression along the human epididymis. It appears that c-ros mRNA expression is not restricted to a defined segment; the transcript is detected with similar intensity along the length of the human epididymis. Both the c-ros transcript and protein are strongly expressed by principal cells and to a lesser extent by basal cells (Legare and Sullivan, 2004).

The efferent ductule morphology, the absence of a differentiated initial segment and unrestricted proto-oncogene c-ros expression along the epididymis suggest that, in humans, the proximal portion of the epididymis differs in structure and function when compared with the classical laboratory animal model.

The anatomy of the epididymis

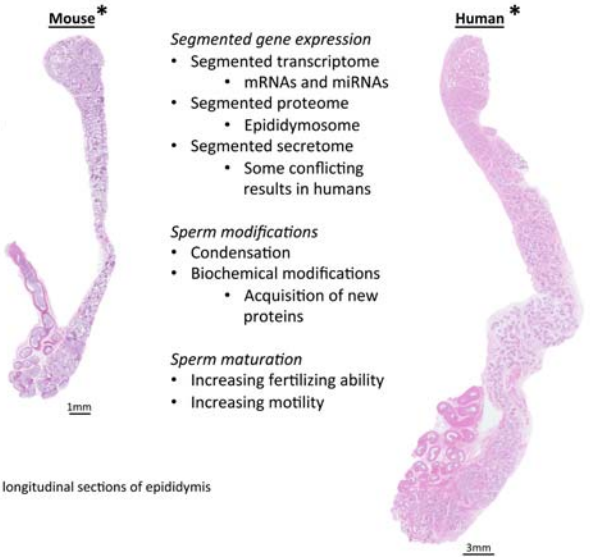
The epididymis is usually divided into three segments; the bulbous proximal caput, the elongated corpus and the swollen distal cauda (Hirsh, 1995; Turner, 2008). Even though this anatomical description can be applied to all mammalian species, the epididymis harbors species-specific morphological characteristics. A particularity of the human epididymis is the poorly differentiated segments: the caput does not show the bulbous appearance that characterizes the proximal excurrent duct in other species. The same observation can be applied to the cauda, which is not characterized by the hallmark swollen tubules of the distal segment in the majority of species. Furthermore, the cauda is poorly distinguishable from the vas deferens at the anatomical level in human and has a limited sperm reservoir capacity; a function associated with the distal portion of the epididymis (see below).

The epididymis in mouse and rat has been subdivided into 10 and 19 intraregional segments, respectively, based on the presence of connective tissue septa (Turner *et al.*, 2007a,b). The latter divide the epididymis into sub-compartments that are impermeable, as shown by the inability of a high-molecular weight dye injected in the intertubular space of a given segment to diffuse into the adjacent space. The septa are readily visualized under a dissecting microscope and are useful for dissecting individual epididymal segments in a reproducible manner. For example, mouse and rat epididymides have been dissected according to the localization of these septa in order to generate transcriptome profiles along the epididymis (Johnston *et al.*, 2005). However, some poorly developed and incomplete septa have been observed along the epididymis in humans. These structures show limited reproducibility from one tissue sample to another and do not exhibit clearly defined epididymal segments as described in rodents by Turner *et al.* (2003). In laboratory animals, partitioning of epididymal interstitium by connective tissue septa may limit the effects of paracrine factors to a specific epididymal segment. To date, no experimental evidence supports this hypothesis. Such segments bordered by connective tissue septum have been described in dogs, marmosets and small primates (Kirchhoff, 2002a,b), Poorly developed connective tissue septa can be observed on longitudinal histological section of the human epididymis (Table I). These are

Table I Comparisons between laboratory rodent and human epididymis.

Particularities	Common features	Particularities

Well-differentiated initial segment
• Responsive to testicular lumicrine factors
• Restricted c-ros expression
 	
Septa defining epididymal compartments
• Hypothesized role in segment differentiation
 	
Progressive increase in intraluminal diameter
 	
Well-developed distal cauda epididymidis
• Sperm reservoir capacity
	Segmented gene expression
	• Segmented transcriptome
	  • mRNAs and miRNAs
	• Segmented proteome
	  • Epididymosome
	• Segmented secretome
	  • Some conflicting results in humans
	
	Sperm modifications
	• Condensation
	• Biochemical modifications
	  • Acquisition of new proteins
	
	Sperm maturation
	• Increasing fertilizing ability
	• Increasing motility
		Undifferentiated initial segment
		• Formed by efferent ducts
		
		Poorly-developed and incomplete septa
		
		Relatively small intraluminal diameter
		
		Poorly developed distal cauda Epididymidis
		• Limited sperm reservoir capacity
		• Well-developed smooth muscle layer surrounding epididymal tubule
		
		Clinical observations
		• In contradiction with physiological observations
		• Epididymal malleability (vasectomy)



*: Haematoxylin-eosin stained longitudinal sections of epididymis from Sullivan's laboratory

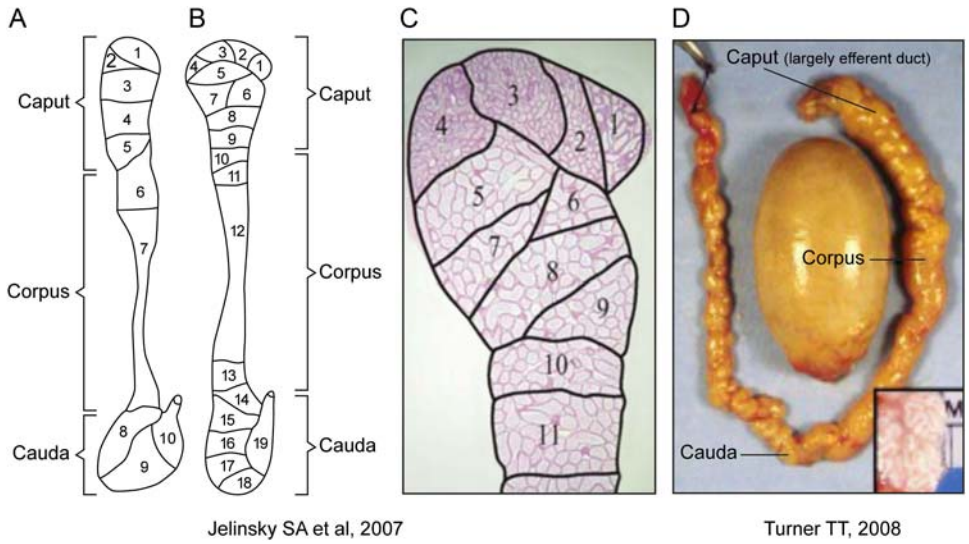


Figure 1 Anatomy of the epididymis. Schematic representation of the mouse (**A**) and rat (**B**) epididymis illustrating the partitioning of epididymal interstitium by connective tissue septae. (**C**) Micrograph of longitudinal histological section of the proximal region of the rat epididymis. The connective tissue septae are drawn according to (B). (**D**) Photograph of the human epididymis dissected from the testis. Inset: higher magnification illustrating the organization of the epididymal tubule. (A), (B) and (C) Reprinted with permission from Jelinsky *et al.* (2007) and (D) Reprinted with permission from Turner (2008).

incomplete and do not define compartments, as those characterizing rodent species do (Fig. 1).

The histology of the epididymis in all species studied reveals a thicker epididymal epithelium in the proximal epididymis; the thickness progressively decreases along the tubule (Moore and Pryor, 1981; Hermo *et al.*, 1994; Hermo and Robaire, 2002). As a result of water reabsorption, the sperm concentration increases along the epididymis together with the lumen diameter (Moore, 1996). The human epididymis exhibits the same trend. In general, the intraluminal diameter of the epididymal tubule is relatively small all along the organ when compared to animal models. This may be related to the relatively rapid sperm transit along the human epididymis. As described below, obstruction of the excurrent duct as a result of vasectomy has a marked effect on epididymal histology. Although progressive histological modifications are observed along the epididymis, the anatomical descriptions suggest that segmentation of the excurrent duct is not well organized in humans in comparison to other investigated mammalian species.

The epididymal proteome, secretome and transcriptome

The spermatozoon is a highly polarized cell that has eliminated the majority of cytoplasm and organelles during the spermiogenesis process. This cell is known to be transcriptionally and translationally silent. Intact ejaculated spermatozoa, as well as isolated subcellular compartments such as the head, tail, nuclei and membranes have been subjected to proteomic analysis. More than 5000 proteins have been identified in the human sperm lysate proteome (Wang *et al.*, 2013). Sperm proteomes of globozoospermic (Liao *et al.*, 2009) and asthenozoospermic (Siva *et al.*, 2010; Parte *et al.*, 2012) semen samples, as well as of spermatozoa that failed to fertilize (Xu *et al.*, 2012) have been compared with normozoospermic sperm cells in order to understand the pathophysiology of male infertility. Proteomic technologies have also been applied to understand biochemical modifications underlying sperm capacitation (Ficarro *et al.*, 2003; Secciani *et al.*, 2009) and epididymal sperm maturation. Proteomes of seminal plasma obtained from men presenting with different pathologies affecting reproductive function have also been investigated to identify markers of reproductive tract constituents (Batruch *et al.*, 2012; Rolland *et al.*, 2013; Drabovich *et al.*, 2014). In this context, proteomes of prostate (Garbis *et al.*, 2011, O'Hurley *et al.*, 2015), epididymis (Li *et al.*, 2011; Liu *et al.*, 2014), prostatic (Drake *et al.*, 2010) and epididymal fluids (Dacheux *et al.*, 2006, 2009), and those of extracellular vesicles, prostasomes (Utleg *et al.*, 2003) and epididymosomes (Girouard *et al.*, 2011) have been published.

Like the testis, the intraluminal compartment of the epididymis is immunoprotected (Mital *et al.*, 2011; Dube and Cyr, 2012; Guiton *et al.*, 2013). In the 1980s, substantial research efforts were devoted to the identification of epididymal-specific proteins playing a role in sperm maturation in order to develop an immunocontraceptive method for men, pests and domestic species (Khole, 2003; Aitken *et al.*, 2004; Hinton and Cooper, 2010). Exhaustive analysis by 1D and 2D-gel electrophoresis was used to characterize the proteome of intraluminal fluids collected from all along the epididymis in different livestock and laboratory animal species (Dacheux *et al.*, 2009). Metabolic labeling using ^{35}S-methionine was coupled to electrophoretic analyses to distinguish the *in situ*-secreted proteins from those originating from upper epididymal segments. This type of labelling also allowed identification of proteins of epididymal origin, which were

added to the sperm cells during the maturation process. Coupled to modern liquid chromatography–mass spectrometry/mass spectrometry technologies, these experimental approaches enabled the identification of many of these proteins, and their functions in sperm/epididymis physiology have been hypothesized. From all of these studies, it can be concluded that the protein composition of the intraluminal compartment varies along the epididymis and that each segment has its own proteome and secretome signatures. Hundreds of proteins have been identified in epididymal fluid, and some species differences are evident. In all eutherian species, <20 proteins represent 80–90% of the total epididymal intraluminal protein content including clusterin, prostaglandin D synthase, glutathione S-transferase, lipocalin 5 and Niemann–Pick Type C2 disease protein. According to Dacheux *et al.*, albumin represents almost 50% of the total protein content of human epididymal fluid, with clusterin and Niemann–Pick Type C2 disease protein comprising the other two major protein constituents (Dacheux *et al.*, 2006, 2009).

A catalog of human epididymal secreted proteins has been generated using 2D-gel electrophoresis followed by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (Stoffel *et al.*, 1991; Li *et al.*, 2010a,b). Of the 408 proteins present in the epididymal intraluminal compartment, 207 were detected on spermatozoa and may thus be involved in sperm maturation and functions (Li *et al.*, 2010a,b). Some could be of testicular origin (Li *et al.*, 2011) or secreted by both the epididymis and the prostate (Li *et al.*, 2011; Liu and Liu, 2015). These studies are in constrast to the description by Dacheux *et al.* of the relatively similar secretome found along the length of the epididymis. The discrepancy between these studies could be explained by variation in epididymal intraluminal fluid collection techniques and by screening criteria used for identification of proteome constituents (Dacheux *et al.*, 2009).

Whether the secretome of the epididymal fluid shows variations along the human epididymis as observed in animal studies remains an open question. Water reabsorption as well as protein reabsorption play an important role in proteome modification along the epididymis. It remains to be determined just how selective these biophysical events are in the human epididymis, if they occur.

The availability of human epididymal tissue for research purposes is very limited. Postmortem delays are often incompatible with mRNA extraction. Prostatic cancers are the major indication for castration. These patients are typically advanced in age and have generally received hormonal treatments incompatible with the maintenance of the differentiated status of the adult epididymis. Under these circumstances, tissues obtained from orchiectomy are rare and generally unsuitable for biochemical or molecular biology studies. Furthermore, knowledge of the influence of temperature on testis and epididymis physiology (Bedford, 2015) precludes the use of biological material obtained from orchiectomy performed for cryptorchidism as a means of studying epididymis physiology (unpublished results). Therefore, our knowledge of the human epididymis transcriptome is limited by poor access to normal tissue.

By using subtracted cDNA libraries, Kirchhoff *et al.* were the first to identify genes that are mainly/exclusively expressed by human epididymal tissues (Kirchhoff, 1999, 2002a,b; Robaire and Hinton, 2002). Northern blots and *in situ* hybridization revealed the segment specificity of certain transcripts. This was the first molecular level demonstration of segment-specific gene expression along the human epididymis. The encoded genes were named HE1–12; HE standing for 'Human Epididymis' (Kirchhoff, 1999). Sequence analysis of some of these clones led to the assignment of hypothetical functions: HE1 was hypothesized to be involved in cholesterol transport (Kirchhoff *et al.*, 1996); immunolocalization of HE2 suggested that it may be involved in gamete fusion as well as in bacterial defense activity (Osterhoff *et al.*, 1994); the structure analogies of HE4 with protease inhibitors suggested that this protein could be a decapacitation factor (Clauss *et al.*, 2002); HE5 (CD52) has a glycosylphosphatidylinositol (GPI) anchor and could be involved in sperm binding (Kirchhoff *et al.*, 1996), and others may be involved in innate defense against microbial infection (Kirchhoff, 2002a,b).

Although early studies on human epididymal mRNAs were not very informative with regard to epididymal functions in sperm maturation, they demonstrated that some genes are expressed in a tissue-specific manner by the epididymis, and the expression of some is restricted to defined segments along the human epididymis. Other approaches, such as the production of monoclonal antibody libraries against human ejaculated spermatozoa allowed identification of epididymal-originating proteins with segment-specific expression along the epididymis (Boue *et al.*, 1995; Li *et al.*, 2011). All of these evidences support the concept of human epididymis segmentation. However, discrepancies remain with regard to molecular studies, the poorly differentiated morphology of the epididymis and the relatively homogeneous secretome profiles along the human organ.

Gene chips are powerful tools for characterizing gene expression in a given tissue under different differentiation or pathological situations and are available for an increasing number of species. As stated above, in both mouse and rat, the epididymis is divided by connective tissue septa defining 10 and 19 epididymal segments, respectively. Epididymides from both species were dissected accordingly, and gene expression was analyzed by microarrays and compared between the two species. In rat, out of 16 000 qualifiers expressed in segmental analysis of epididymal gene expression, >3500 were differentially regulated by more than 4-fold between any two segments. In these studies, a total of 492 genes were represented on both rat and mouse microarrays and differentially expressed by more than 4-fold between segments in both species. Thus, it appears that gene expression is highly segmented along the epididymis of these two rodent species (Johnston *et al.*, 2005; Jelinsky *et al.*, 2007). Availability of human epididymal tissues is very limited, and there are no clear anatomical criteria allowing discrimination between the different epididymal segments. Therefore, the human epididymis is arbitrarily dissected into caput, corpus and cauda, as no differentiated initial segment or connective tissue septa can be distinguished. The human epididymal transcriptome has been described by three groups (Zhang *et al.*, 2006; Dube *et al.*, 2007; Thimon *et al.*, 2007). The absence of anatomical criteria to dissect the human epididymis may cause some variability in results as well as the origin of the tissue. The Zhang study was based on one 26-year-old fertile donor (Zhang *et al.*, 2006), the Dube conclusion was based on four patients undergoing orchiectomy for testicular cancer (Dube *et al.*, 2007), whereas data for the study by Thimon were obtained from epididymides from three healthy donors through collaboration with a local organ transplantation program. Despite methodological variations, the three studies concluded that gene expression was indeed segmented along the human epididymis (Fig. 2). The number of differentially expressed

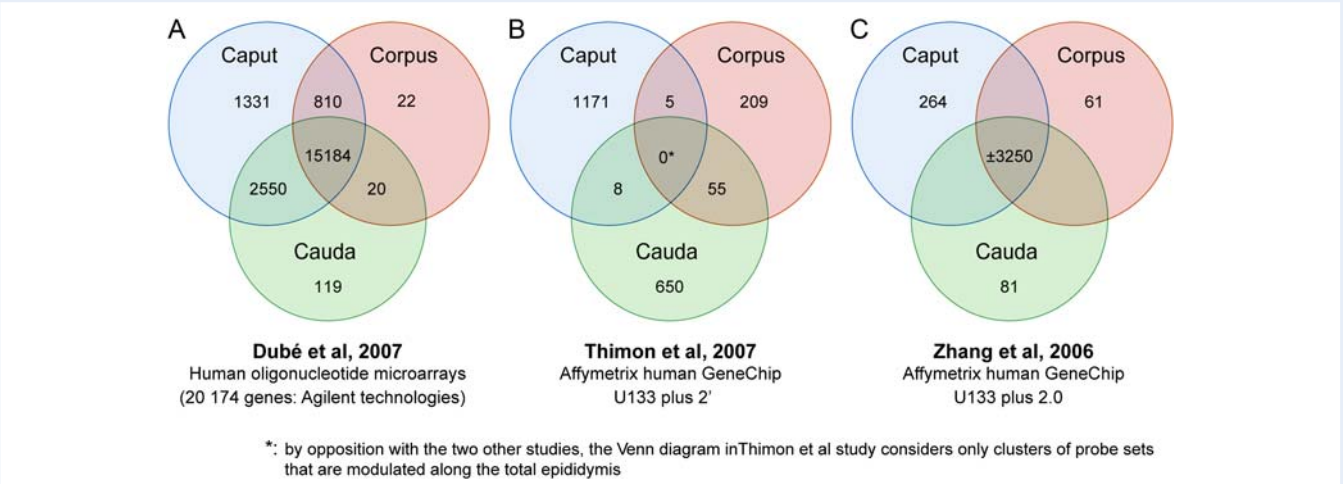


Figure 2 Transcriptome of the human epididymis. Venn diagrams of mRNA distribution in caput, corpus and cauda human epididymis from three independent studies. Venn diagrams were generated from the transcriptomes of mRNAs isolated from epididymal tissues (**A**) from men undergoing orchidectomy for testicular cancer, reproduced with permission from Dube *et al.* (2007), (**B**) from healthy men obtained through collaboration with an organ transplantation program, adapted from Thimon *et al.* (2007), (**C**) from a 26-year-old fertile donor, from Zhang *et al.* (2006) (drawn according to authors' description).

genes showed variability from one study to the other, and the number of segment-specific transcripts expressed along the human epididymis may appear lower when compared with rodent species. This may be due to limitations in our ability to dissect the human epididymis into well-defined segments. It has been shown, however, that gene expression is regulated in a segment-specific manner along the human epididymis (Belleannee *et al.*, 2012).

Control of gene expression

With the highly segmented transcriptome along the excurrent duct, the epididymis is an ideal organ with which to study gene expression control mechanisms. For obvious reasons, information on these mechanisms is scarce in humans, but an abundance of literature is available for rodent species (Cornwall *et al.*, 2002; Belleannee *et al.*, 2012). Many factors have been shown to be involved in gene expression with steroid hormones being the best-known regulatory factors of epididymal differentiation and function. Testosterone secreted by Leydig cells is found at much higher concentrations in the intraluminal fluid of the proximal epididymis than the levels found in circulation (Viger and Robaire, 1996). Once secreted by Leydig cells, testosterone binds to the transporter androgen binding protein secreted by Sertoli cells. The complex is found in the lumen of seminiferous tubules and transported to the epididymal fluid where it is rapidly endocytosed by principal cells. These cells have high 5α-reductase activity that rapidly metabolizes testosterone to the most active androgen, dihydrotestosterone (Robaire and Hamzeh, 2011; Sipila *et al.*, 2011). Circulating androgens can also be transformed to estradiol by epididymal P450 aromatase (Shayu and Rao, 2006). Both androgens and estrogens are known to regulate epididymal gene expression (Hess *et al.*, 1997; Hess *et al.*, 2001; Henderson *et al.*, 2006). Deprivation of the major source of androgen by castration has a major impact on the epididymis. This is well illustrated in humans by orchiectomy or by administration of GnRH antagonists, both of which impact the epididymis. Testosterone supplementation in castrated animals is not sufficient to completely restore epididymal functions (Avram *et al.*, 2004; Robaire and Hamzeh, 2011). Other non-steroidal factors of testicular origin, named 'lumicrine factors' have been hypothesized to play a role in epididymal gene expression and differentiation (Hinton *et al.*, 1998). Experimental evidence in rodent species clearly demonstrated that lumicrine factors affected expression of specific genes in the epididymis (Sipila *et al.*, 2011). Some growth factors, in particular fibroblast growth factor 2, have been proposed to be lumicrine factor candidates (Kirby *et al.*, 2003). It appears that these effects occur in the initial segment, and that lumicrine factors have a minor impact on the distal part of the epididymis. As the initial segment is undifferentiated or poorly differentiated in the human epididymal organ, the importance of lumicrine factors other than steroids may not be relevant to human epididymal physiology and sperm maturation.

Spermatozoa *per se* have been shown to affect epididymal physiology and protein secretion (Reyes-Moreno *et al.*, 2008). Thus, the male gamete may be a lumicrine factor as it can cross-talk with the epididymal epithelium (Garrett *et al.*, 1990). Spermatozoa added to primary cell cultures of bovine caput, corpus and cauda epididymal epithelial cells modulate protein synthesis and secretion, as well as cell proliferation (Reyes-Moreno *et al.*, 2008). Whether these observations can be extrapolated to humans remains to be shown.

Since the last decade, there has been increasing interest in small non-coding RNAs and their functions in transcription, RNA stability and translation. Micro RNAs (miRNAs) in particular are small, 19–22-nucleotide long RNAs that have post-transcriptional silencing properties on complementary mRNAs. Their presence has been demonstrated in all biological fluids studied, and they are proposed to be involved in many biological signaling pathways and pathologies

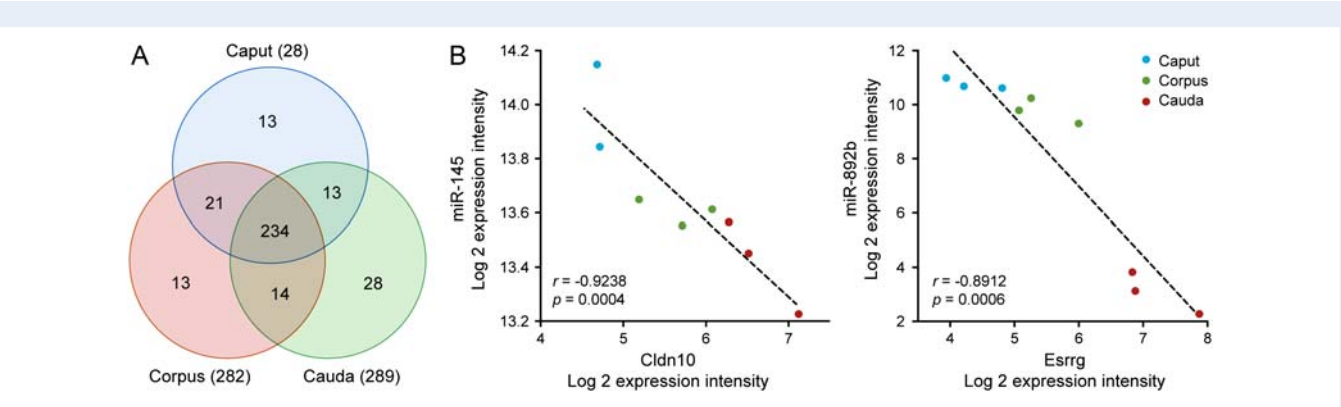


Figure 3 miRNAs in the human epididymis. Venn diagram of miRNA distribution in caput, corpus and cauda human epididymis (**A**). Correlation between the expression of Claudin 10 (Cldn10) and Esrrg with the expression of miR-145 and miR-892b, respectively. Correlation was based on the mRNA and miRNA expression detected in caput, corpus and cauda from three donors. Each dot represents a segment of a given donor (**B**). Reproduced with permission from Belleannée *et al.* (2012).

(Bobrie *et al.*, 2011). miRNAs have been described in human epididymis, and a negative correlation exists between the decreasing number of miRNAs and the mRNA population in the epididymis from childhood through adulthood to elderly men. This suggests a role for miRNAs in androgen-dependent gene expression in the human epididymis (Zhang *et al.*, 2010). Whereas the miR-888 miRNA cluster is highly conserved among primate species and predominantly expressed in the epididymis (Landgraf *et al.*, 2007), it is absent in other mammalian species (Li *et al.*, 2010). This suggests that these miRNAs play a major role in epididymal gene expression, and that these molecular controls have some peculiarity unique to primates. Out of the 847 human miRNAs analyzed by microarrays, 35 were differentially expressed in specific epididymal segments including 5 members of the miR-888 cluster, which showed elevated expression in the corpus/cauda region (Belleannee *et al.*, 2012). *In silico* analyses of both mRNA and miRNA microarrays of human caput, corpus and cauda epididymal tissues strongly supported the role of miRNAs in segmented gene expression along the human epididymis (Fig. 3). These studies are the most convincing arguments in favor of a well-orchestrated control of segmented gene expression along the human epididymis (Belleannee *et al.*, 2012).

Functions in sperm maturation

Clinical observations

In vertebrate species practicing internal fertilization, spermatozoa leaving the testis are unable to fertilize the egg. They have to transit through a minimal length of the epididymis in order to do so. They then acquire their forward motility and their ability to efficiently encounter the egg and its vestments. This 'sperm maturation' dogma is based on IVF assays or artificial inseminations performed with spermatozoa collected at different sites along the epididymis. From all of these experimental procedures performed in different animal species, it became clear that the male gamete has to transit a certain portion of the epididymis in order to be able to fertilize; the first functional spermatozoa are collected at approximately the mid-corpus region (Cooper, 1986). Few studies reported pregnancy outcomes following cervical insemination with spermatozoa collected along the human epididymis (Schmidt *et al.*, 1976; Bedford, 1980; Schoysman, 1981). A low percentage of pregnancies was obtained with spermatozoa collected from the proximal segment; the success rate increased with the use of spermatozoa collected more distally (Schoysman and Bedford, 1986).

Before the availability of ART to overcome male fertility pathologies, microsurgical procedures were used to solve anatomical male infertility problems. Surgical recanalization and reanastomosis were performed in men presenting with agenesis of the distal part of the excurrent duct, obstruction or for vasectomy reversal (Schoysman, 1994). In the case of vasectomy reversal, anastomosis of the vas deferens is performed at a different level along the epididymis when obstructive granuloma is present as a result of vas deferens occlusion after vasectomy. If successful, as evaluated by the presence of spermatozoa in the ejaculate, these surgical procedures can result in pregnancies. In a series of studies published by Schoysman and Bedford, it appears that the more distally along the epididymis, the epididymo-vasostomy (reanastomosis of the epididymal tubule with the vas deferens) is performed, the higher the chances of natural conception, demonstrating the beneficial effect of epididymal transit on sperm functionality (Schoysman and Bedford, 1986). However, a case reported by Bedford described a natural pregnancy occurring after reanastomosis of the vas deferens up to one of the efferent ductules bypassing the total length of the epididymis (Bedford, 1994). In a series of provocative papers, Silber clearly questions the function of the epididymis in humans (Silber *et al.*, 1977; Silber, 1977, 1980, 1988). He reported pregnancy outcomes for men undergoing microsurgical epididymo-vasostomy for vasectomy reversal. In this published series, the level of reanastomosis of the vas deferens along the epididymal tubule did not impact pregnancy outcome to the extent that was previously reported by Bedford and Schoysman. These studies were particularly controversial in the andrology field during the 1990s (Cooper, 1990, 1993; Bedford, 1994).

In cases of obstructive azoospermia, spermatozoa are collected upstream of the acquired obstruction or the agenesis, along the

length of the epididymis, in order to perform ART. Thousands of take-home babies have been conceived using these male gametes that are usually poorly motile, but viable and morphologically normal (Palermo *et al.*, 2014a,b,c). Intracytoplasmic sperm injection (ICSI) must be performed with these partially mature spermatozoa in order to obtain fertilized eggs, i.e. egg activation and reestablishment of diploidy (Neri *et al.*, 2014). The surgeon performing sperm retrieval for ART procedures endeavors to recover sperm as distally as possible to optimize the outcome of these procedures. To our knowledge, standard IVF is not performed when aspirated spermatozoa are used due to the inability of these male gametes to accomplish all of the steps leading to fertilization (Shin and Turek, 2013). This poses the question of how these successes obtained following surgical reanastomosis of the male tract or sperm aspiration, and ART procedures to overcome pathologies affecting male fertility are informative with regard to epididymal physiology in men.

Physiological evidence

Epididymal sperm maturation involves both modifications to flagellar beating and acquisition of properties necessary to efficiently encounter the egg's vestments, especially the zona pellucida (an acellular glycoprotein coat surrounding the egg). Work on animal models has taught us that these physiological changes involve sperm surface modifications that occur during epididymal transit. These include: an increase in total negative surface charge (Moore and Akhondi, 1996); modifications to lectin-binding properties (Hermo *et al.*, 1992); changes in membrane lipid composition (Jones, 1998a,b; Rejraji *et al.*, 2006); remodeling of raft membrane microdomains (Girouard *et al.*, 2011) and of other plasma membranes structures (Cuasnicu *et al.*, 2002); modifications to surface glycoproteins (Tulsiani, 2006) and surface antigen relocalization (Hunnicutt *et al.*, 1997, Belmonte *et al.*, 2000). All of these changes involve complex interactions between epididymal secretions and male gametes: modifications that are essential for the acquisition of sperm fertilizing ability (Haidl *et al.*, 1994; Turner, 1995; Moore, 1996; Cooper and Yeung, 2006).

Some sperm parameter modifications occurring during epididymal transit have been described in humans (Blaquier *et al.*, 1988; Lasserre *et al.*, 2001; Cooper and Yeung, 2006). Sperm chromatin condensation increases during epididymal transit of the human spermatozoa, in addition to forward motility (Haidl *et al.*, 1994). There is also an increased capacity of zona-free hamster oocyte penetration by transiting spermatozoa (Hinrichsen and Blaquier, 1980; Moore and Akhondi, 1996). Information on biochemical modifications undergone by the male gamete in the human male reproductive tract is scarce. Tezon *et al.* established a human epididymis organ culture system that was responsive to androgens (Tezon and Blaquier, 1981; Tezon *et al.*, 1982). Metabolic labelling revealed that proximal segments are the more active in protein synthesis. Androgens have a stimulatory effect on the synthesis of only a few of these proteins (Tezon *et al.*, 1985a,b). Evidence is presented suggesting the association of androgen-dependent secreted proteins with epididymal spermatozoa; these proteins remain associated with spermatozoa after ejaculation (Tezon *et al.*, 1985b; Ross *et al.*, 1990). The use of interfering antibodies allowed the identification of epididymal-originating proteins associated with ejaculated human spermatozoa; P34H and FLB1 play specific roles in fertilization such as zona pellucida binding (Boue *et al.*, 1994) and oocyte penetration (zona-free hamster egg penetration assay) (Boue *et al.*, 1995), respectively.

The origin and functions of P34H in particular have been well documented. This protein is synthesized and secreted by the human corpus epididymis where it is added to the sperm surface covering the acrosome (Boue *et al.*, 1996). This protein is a dicarbonyl reductase playing multiple functions according to the cell type synthesizing this protein, the sub-compartment localization and the differentiation status (Lee *et al.*, 2013; Ebert *et al.*, 2015). Complementary DNA sequencing revealed the absence of a signal peptide (Legare *et al.*, 1999). As the protein is GPI anchored to the sperm surface, it has been hypothesized that epididymal principal cells use the apocrine pathway to secrete P34H into the intraluminal compartment. Specific anti-P34H antibodies inhibit sperm–zona pellucida binding *in vitro* without affecting other fertilization steps (Boue *et al.*, 1994). Furthermore, the absence of P34H is associated with cases of male infertility (Boue and Sullivan, 1996) (Moskovtsev *et al.*, 2007) and is a predictor of standard IVF failure (Sullivan *et al.*, 2006). Thus, the epididymal protein P34H is added to the sperm surface during its transit along the human epididymis and plays a major role in sperm physiology (Sullivan, 1999; Sullivan, 2004; Desrosiers, *et al.*, 2006). It also supports the concept that epididymal dysfunction can be involved in the pathophysiology of male infertility.

There is increasing evidence that epididymal principal cells secrete extracellular microvesicles named epididymosomes. Described in different species, epididymosomes are involved in the trafficking of selected subsets of epididymal proteins to spermatozoa (for review: Sullivan, 2013). The proteome of epididymosomes collected during vasectomy procedures is characterized by numerous proteins with potential roles in sperm physiological functions (Thimon *et al.*, 2008a,b). This provides indirect evidence supporting the key role of the epididymis in human reproductive physiology.

There is sufficient physiological and biochemical evidence to support the role of the epididymis in human sperm maturation, i.e. the acquisition of fertilizing ability. The challenge remains as to how this evidence can be reconciled with clinical data obtained from men undergoing reanastomosis of the vas deferens at different points along the excurrent duct.

Vasectomy: a model to understand human sperm maturation

During the early days of epididymal research, two possibilities were considered to explain sperm maturation: the epididymis was actively involved in this process or sperm maturation was a post-testicular time-related event. To distinguish between these two possibilities, rabbit epididymis was ligated upstream of the site of apparition of fertilizing spermatozoa and male gametes were collected at different time points upstream of the ligation site. At the initial time points, the collected spermatozoa were dysfunctional or immature. After a certain period of time, a longer period than the time required to complete the epididymal journey, functional spermatozoa appeared upstream of the ligation. From these experiments, it was concluded that under obstruction, the proximal segment of the epididymis is

able to differentiate or to reprogram itself to mimic the functions of the downstream epididymal segments (Bedford, 1967; Bedford *et al.*, 1973). If this is the case, it would explain the pregnancy outcome in men having undergone reanastomosis of the vas deferens along the epididymis, a surgery performed to overcome excurrent duct ligation, obstruction or agenesis (Legare *et al.*, 2001).

Vasectomy consists of ligation of the scrotal portion of the vas deferens (Art and Nangia, 2009). This contraceptive procedure performed on a large scale is an interesting model with which to understand the consequences of obstruction on epididymal physiology in men (Sullivan *et al.*, 2011). A very limited number of reports describe the consequences of vasectomy on the human epididymis because of the scarcity of specimens. Only a few histological studies have been published, which mainly describe the formation of granuloma and epididymal distension after vasectomy in men (Bedford, 1967; McDonald, 1996). At the morphological and histological levels, responses to vasectomy vary from one species to another, questioning the relevance of animal models for studying post-vasectomy sequelae (Bedford, 1976; McDonald, 2000). The immune response against spermatozoa following vasectomy has been exhaustively studied using different laboratory animals including non-human primates (Alexander, 1973; Clarkson and Alexander, 1980). In the late 1970 s, it was shown that production of anti-sperm antibodies occurring after vasectomy in monkeys could result in circulating immune complexes exacerbating atherosclerosis (Alexander, 1982). The knowledge that vasectomy can result in anti-sperm antibody production in men raised serious concerns regarding the safety of this procedure. Since then, many epidemiological studies have investigated possible correlations between vasectomy and an increase in the incidence of different pathologies including atherosclerosis (Clarkson and Alexander, 1980) and prostate cancer (Khan and Partin, 2004). It is now generally believed that no such correlation exists (Nienhuis *et al.*, 1992; Goldacre *et al.*, 2005; Liu *et al.*, 2015). This emphasizes the fact that results concerning the consequences of vasectomy obtained using animal models, including primates, must be extrapolated to men with caution.

The effects of vasectomy on different epididymal physiological parameters, such as trans-epithelial water reabsorption (Hohlbrugger and Pfaller, 1983), intratubular hydrostatic pressure (Johnson and Howards, 1976) and fluid movement in the epididymis (Turner *et al.*, 1990), the local renin–angiotensin system (Saez, *et al.*, 2004) and blood–epididymal barrier (Turner *et al.*, 1979), have been described using laboratory animals. Whereas gene expression patterns and intraluminal protein compositions along the epididymis have been exhaustively studied during the last two decades, the consequences of vasectomy on these biochemical parameters are poorly documented. Vasectomy selectively affects expression of a cysteine-rich secretory protein in the rat caput epididymidis (Turner *et al.*, 1999) and of an HE2-like mRNA in the corpus segment of the cynomolgus monkey (Doiron *et al.*, 2003). At least in the rat, the consequences of vasectomy on epididymal protein secretion are irreversible following vasovasostomy (Turner *et al.*, 2000). Many studies (Bedford, 1967; Bedford, 1994; McDonald, 1996), including some of our own (Legare *et al.*, 2001, Doiron *et al.*, 2003, Saez *et al.*, 2004), emphasize the fact that vasectomy affects the epididymis, but these results cannot be directly extrapolated to humans.

As previously described, P34H (DCXR) is a sperm protein of epididymal origin involved in fertilization in humans. After vasectomy, the gene encoding P34H is not expressed by the corpus epididymis as in normal men, but is expressed instead in the caput segment of the human epididymis (Legare *et al.*, 2001). Consequently, ejaculated spermatozoa of some vasovasostomized men lack this epididymal protein. In normal men the thickness of the epididymal epithelium varies from one segment to the other; the maximum thickness being found in the distal caput–corpus. After vasectomy, the maximum thickness is reached in the proximal caput epididymidis. In vasectomized men, the thickness of the epithelium in the proximal caput is similar to that which is characteristic of the corpus epididymidis in normal men. In fact, P34H follows the same shift in expression as the shift in maximal epithelium thickness observed in the epididymis of vasectomized men (Legare *et al.*, 2001). These observations on the consequences of vasectomy on human epididymis correlate with the changes in distribution of fertile sperm along the epididymis after ligation. Thus, it appears that in animal models, as in humans, the pattern of differentiation observed along the epididymis is modified after obstruction (ligation or vasectomy). This could be the underlying physiological mechanism explaining the recovery of fertility in men presenting with surgical epididymo-vasostomy.

Transcriptomic studies reveal that vasectomy affects the pattern of gene expression along the length of the human epididymis (Thimon *et al.*, 2008a,b) (Fig. 4). The distribution pattern of miRNAs along the excurrent duct is also affected by vasectomy (Belleannee *et al.*, 2013). Other observations support the hypothesis that in some vasovasostomized men, spermatozoa are unable to undergo some of the necessary steps leading to fertilization as a result of epididymal defects occurring after vasectomy; defects that are irreversible following vasovasostomy (Legare *et al.*, 2001, 2004, 2006, 2010; Boudreau *et al.*, 2009). This may explain the discrepancies between surgical success of vasectomy reversal and pregnancy outcome. If we accept the postulate that vasectomy/vasovasostomy is a model to understand the consequences of obstruction on epididymis function in humans, these studies explain the discrepancy between physiological observations made on normal epididymides and clinical data collected on obstructed epididymides.

Sperm reservoir

In rats, the epididymis reaches 6 m in length and can be as long as 50 m in large domestic animals. In humans, the epididymal tubule measures a modest 5 m. Sperm transit along the human epididymis takes 2–4 days (Bedford, 1994), which is quite rapid when compared with the 10- to 12-day journey in laboratory and domestic animals. As with the other segments, the cauda epididymis is poorly developed in humans (Johnson and Varner, 1988). This correlates with the very limited sperm reservoir capacity in our species. After performing semen analyses on successive ejaculates from healthy young men, Bedford (1990) determined that the reservoir capacity in humans does not exceed the number of male gametes necessary to produce two to three normal semen samples. Again, human epididymal function is far from being as efficient as in other mammalian species (Amann and Howards, 1980).

In humans, the sperm reservoir capacity of the cauda epididymidis is comparable to that found in experimental abdominal epididymo-cryptorchidism in the rat. This observation makes sense with

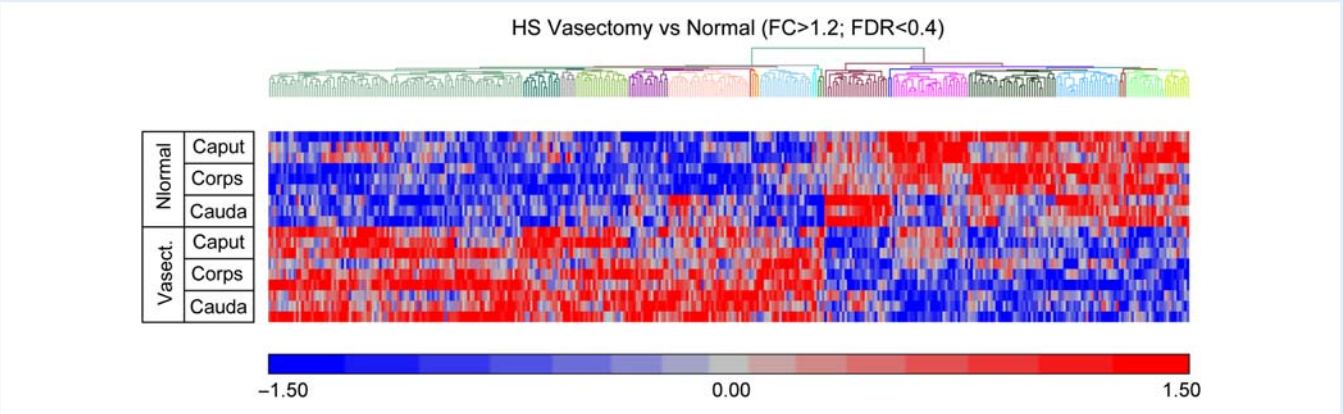


Figure 4 Effect of vasectomy on the human epididymis transcriptome. Heat map of hierarchical clustering of 397 probe sets (corresponding to 332 known mRNAs) of caput, corpus and cauda epididymidis of normal and vasectomized (Vasect.) men. Each cell in the matrix represents the expression level of each mRNA affected by vasectomy. The blue-red scale indicates the intensity level of expression. P-value <0.05 with a correction factor FDR < 0.4. Adapted from Thimon *et al.* (2008a,b). HS: *Homo sapiens*, FC: fold change, FDR: False Discovery Rate. The pseudocolor representation of FC is shown such that red indicates high and blue represents low changes.

knowledge of the effect of temperature increase on epididymal sperm reservoir function (Amann, 1981; Bedford, 1994). Speculation exists over whether the poorly differentiated cauda segment in humans is due to a newly acquired adaptation to lifestyle affecting scrotal temperature (Bedford, 2015) or to a reproductive strategy in our species with little inter-individual sperm competition pressure. Sperm production by the human testis is in fact notoriously very low when compared with other mammalian species (Amann and Howards, 1980; Johnson and Varner, 1988; Amann, 2008). This could help to explain the poor sperm reservoir capacity in our species. The fact remains that the distal portion of the epididymis is poorly developed in men (Johnson, 1982).

Conclusions

In rodents, the proximal epididymis (initial segment and caput) is the most responsive segment to intraluminal factors proposed to control downstream gene expression. Consequently, the caput epididymis is very active in gene expression characterized by a segment-specific transcriptome signature. In these species, septa define different compartments along the epididymis. It has been hypothesized that these structures are also involved in the regulation of gene expression. There is no doubt that the anatomy of the human epididymis is peculiar when considering the absence of an initial segment, the proximal caput formed by efferent ducts and the presence of poorly defined and incomplete septa that do not clearly demark epididymal compartments. These observations raise questions concerning the functional significance of this organ in humans. The rapid sperm transit, the relatively small diameter of the epididymal tubule and the poorly differentiated cauda segment also give the impression that the epididymis may not be as important for sperm maturation in human as shown in laboratory animals. In contrast, transcriptomic and proteomic studies teach us that the pattern of gene expression varies along the human epididymis with the caput segment having the highest transcriptomic activity. Together with the available information concerning epididymal physiology in normal men, these observations support the concept of epididymal sperm maturation in humans.

The epididymis in humans is 'malleable' as shown by the consequences of vasectomy on the epididymal transcriptome, in particular in the proximal segment. Almost all our knowledge of epididymal sperm parameters is based on sperm cells collected along the male tract in cases of epididymis obstruction and agenesis. If spermatozoa present in the epididymis under vasectomy are comparable to those collected in obstructed organs, conclusions on human epididymal function may be distorted. It should be borne in mind that spermatozoa that have undergone only a partial epididymal transit have limited fertilizing ability, and that ART, such as ICSI, have to be applied to generate zygotes with these male gametes.

The rapid evolution of reproductive technologies applied to overcome male infertility has jeopardized efforts to understand epididymal physiology during the previous two decades. The limited number of studies exploring epididymal physiology in humans conducted during the 1980s and 1990s clearly support the concept that the epididymal secretome is essential for the acquisition of fertilizing ability by the male gamete. The role of the human epididymis in sperm function has been questioned. Even though all the information regarding functions of the excurrent duct obtained with animal models cannot be extrapolated to humans, it remains true that the epididymis is essential for sperm function in our species. A summary of comparisons between human and rodent epididymides is presented in Table I.

Recent studies performed with laboratory and domestic animals have shed light on the mechanisms of epididymis–sperm interaction and how epididymal gene expression is regulated. The role of extracellular microvesicles (epididymosomes) in transferring proteins to the maturing spermatozoa and in trafficking miRNAs along the male tract is of particular interest. Epididymosomes are also secreted by the epididymal epithelium in humans, and their functions in sperm maturation cannot be excluded. The possibility that epididymosomes transport small RNAs that modulate sperm functions is particularly intriguing.

There is increasing evidence that parental lifestyle and the environment influence phenotypes of the next generation (Rando and Simmons, 2015). It has recently been demonstrated in rodents that small RNAs derived from transfer RNA (tRNA) degradation are acquired by spermatozoa during epididymal transit; these small tRNAs mediate epigenome modifications of the male gamete (Sharma *et al.*, 2015). If such epigenetic modifications to sperm exist in humans, then ART using sperm collected at different sites along the male tract should be seriously re-evaluated. Obviously, there are major aspects of epididymal physiology, which remain to be explored.

Despite the scarcity of biological material available, the interspecies variability of the male reproductive tract urges us to use modern molecular and cellular biology tools to better understand human epididymis physiology in order to apply ART in a more responsible manner.

Acknowledgements

Dr Muriel Kelly is acknowledged for text editing. Mrs Christine Légaré and France Couture from Graphic core facility of the CRCHUQ are acknowledged for figure designs.

Authors' roles

Both authors were equally involved in conception and writing of this review.

Funding

Canadian Institutes for Health Research to RS laboratory.

Conflict of interest

The authors have no conflict of interest to declare.

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