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PHYSIOLOGY OF EPIDIDYMIS

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By

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Lecture

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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يُقْضَىٰ إِلَيْكَ وَحْيُهُ ۖ وَقُل رَّبِّ زِدْنِي عِلْمًا ﴿١١٤﴾

صدق الله العلي العظيم

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Certificate of supervisor

I certify that Laith M. Jabar has completed the fulfillment of her graduation project for the year 2015/2016 under my construction.

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Dedication

To my parents

And to all my members of my family

To my Friends

I dedicate this work

Laith

Acknowledgments

Firstly I would like to thank the Kind Merciful Allah for helping me in completing this work.

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Laith

Summary

Summary

The epididymis performs an important role in the maturation of spermatozoa including their acquisition of progressive motility and fertilizing ability. However, the molecular mechanisms that govern these maturational events are still poorly defined. This review focuses on recent progress in our understanding of epididymal function including its development, role of the luminal microenvironment in sperm maturation, regulation and novel mechanisms the epididymis utilizes to carry out some of its functions.

The epididymis is an amazingly complex tubule consisting of individual microenvironments that serve to mature spermatozoa functionally. Understanding the intricacies of epididymal epithelial cell function and its regulation, the ever-changing luminal microenvironment, and the processes that affect sperm function are essential for the development of new therapies for infertility as well as for contraceptive development. In a broader sense, studying the epididymis has the potential to be of profound significance for understanding basic biological mechanisms including mechanisms of transcriptional control, secretion and turnover of proteins in the extracellular space including mechanisms of quality control for misfolded proteins, and cell–cell communication and cell signaling.

Introduction

Spermatozoa leaving the testis and entering the long convoluted tubule known as the epididymis are non-functional gametes. It is only during transit through the epididymis that spermatozoa undergo maturation and acquire progressive motility and the ability to fertilize ova. Because spermatozoa are, for the most part, synthetically inactive, maturation involves the interaction of spermatozoa with proteins that are synthesized and secreted in a region-dependent manner from the epididymal epithelium. Despite considerable effort, the molecular and biochemical events that are integral for epididymal sperm maturation are unknown. The importance of understanding epididymal function and sperm maturation is emphasized by the fact that up to 40% of infertile men exhibit idiopathic infertility that may reflect sperm maturational disorders. Unfortunately, owing to the lack of alternative therapies, these patients and their partners require assisted reproductive techniques (ART) such as intracytoplasmic sperm injection (ICSI), which utilizes spermatozoa independent of maturational status, to achieve a pregnancy. Although effective, because natural selection processesthat prevent suboptimal spermatozoa from fertilizing ova are bypassed, there can be increased risks of genetic abnormalities being transmitted to the offspring (Cox et al., 2002 & Fedder et al., 2007;). If the mechanisms of sperm maturation were established, it is possible that sperm could be matured in vitro providing an alternative therapy to current reproductive technologies.

The significance of the lack of understanding regarding the functional role of the epididymis in sperm maturation is also underscored by the lack of contraceptives for men. Although much work has been put into developing hormonal methods that interfere with sperm production in the testis, these

approaches have been hampered by cumbersome regimes, extended times before efficacy is achieved, and possible side effects of the administered hormones. Interest has shifted to include identifying epididymal molecules that could serve as targets for non- steroidal-based male contraceptives with the idea that sperm production would occur normally but the spermatozoa would be non-functional (Lei *et.al.*2003).

The purpose of this review is to provide a general background of the epididymis followed by a brief overview of recent progress in the field including advances in our understanding of the epididymal epithelium and its regulation, composition and function of the luminal fluid, as well as changes occurring in spermatozoa during epididymal transit. Because of the limited availability of epididymal tissue from healthy men of reproductive age, the lack of appropriate in vitro models, and the constraint to manipulate the human epididymis experimentally, the majority of these studies have been carried out in rodent models. However, as discussed below, genomic and proteomic analyses of the human epididymis have revealed valuable new information which lends support to the view that, although there may be species differences with regard to where in the epididymis spermatozoa acquire their functions, the human epididymis does serve a role in the functional maturation of spermatozoa.

Literatures review

The Epididymis

The epididymis is the first excretory organ of the male genital system. The epididymis is attached to the testicle and consists of the ductuli efferentes and the ductus epididymidis, surrounded by the testicular albuginea. The ductus epididymidis is very tortuous and extremely long in all species. On the lateral aspect of the testicle, between it and the epididymis, a large space is outlined under the name of testicular bursa(Noakes, et al. 2010). The epididymis has a head, a body, and a tail. The head contains the ductuli efferentes of the testicle, and the origin of the ductus epididymidis. The body encloses the coiled ductus epididymidis. Both the ductuli efferentes and ductus epididymidis are located within the lobules of the epididymis. The ductus epididymidis continues inside of the tail of the epididymis with the ductus deferens. The tail of the epididymis is anchored to the tail of the testicle by the proper ligament of the testicle.

Function of epididymis

1- Transport : As a duct leading from the testes,the epididymis serves to transport spermatozoa.In sexually active males the time involved in transprot is 9 to 1 days in boars,13 to 15 days in rams,and 9 to 11 days in bulls . Several factor contribute to movement of spermatozoa thorugh the epididymis.One factor is pressure from the production of new spermatozoa.As spermatozoa are produced in the seminiferous tubules,they are forced out through the rete testis and vasa efferentia into the epididymis.In a sexually inactive male,they are eventually forced through the epididymis.such movement of spermatozoa is aided by external perssure created by the massaging effect on the testes and epididymis thet occurs during normal exercise.The lining of the epididymis contains some ciliated epithelial cells,but the role of these cilia in facilitating

movement of spermatozoa is aided by ejaculation.During ejaculation,peristaltic contractions involving the smooth muscle layer of the epididymis and a slight negative pressure (sucking action) created by peristaltic contractions of the vas deferens and urethra actively move spermatozoa from the epididymis into the vas deferens and urethra (Hess, et al. 2004).

2- Concentration : A second function of the epididumis is concentration of spermatozoa.spermatozoa entering the epidiaymis from the testis of the bull,ram,and the epididymis,they concentrate to about 4x109(4 billion)spermatozoa per ml. Concentration occurs as the fluids, which suspend spermatozoa in the testes are absorbed by the epithelial cells of the epididymis.Absorption of these fluids principally in the caput and proximal end of the corpus (Noakes, et al. 2010).

3- Storage : A third functin of the epididymis is storage of spermatozoa. Most are stored in the cauda of the epididymis where concentrated spermatozoa are packed into the wide lumen. The epididymis of a mature bull may contain 50 to 74 billion sperm. Capacities of other species are not reported. conditions are optimum in the cauda for preserving the viability of spermatozoa for an extended period.The low pH,high viscosity, high carbon dioside concentration, high potassium-to-sodium ratio,the influence of testosterone, and probably other factors combine to contribute to a lower metabolic rate and extended life.These conditions have not been duplicated outside the epididymis. If the epididymis is ligated to prevent entry of new spermatozoa and removal of old, spermatozoa have remained have alive and fertile for about 60 days.On the other hand,after a long period of sexual rest, the first few ejaculates may contain a high percentage of non fertile spermatozoa(Noakes, et al. 2010).

4- Maturation : A fourth function of the epididymis is that of maturation of spermatozoa. When recently formed spermatozoa enter the caput from the vasa

efferentia they the ability for neither motility nor fertility. As they pass-through the epididymis they sain the ability to be both motile and fertile.If the cauda is ligated at each end,those spermatozoa closet to the corpus increased in fertility for up to 25 days.During the same period,those closest to the vasa deferens exhibited reduced fetilizing ability.Therefore,it appears that spermatozoa gain ability to be fertile in the cauda and then start to age and deteriorate if they are not removed (Hayakawa, et al. 2010).

While in the epididymis,spermatozoa lose the cytoplasmic droplet which forms on the neck of each spermatozoa during spermatogenesis .The physiological significance of the cytoplasmic droplet is not known,but it has been used as an indicator of sufficient maturation of spermatozoa in the epididymis.If a high percentage of spermatozoa in fershly ejaculated semen has cytoplasmic droplets,they are considered immature and have low fertilizing capacity.

Results

Results

The epididymis, located between the efferent ducts and the vas deferens, is a male accessory organ characterized by a single coiled tubule duct with an estimated length of 5–7 m in men (Sullivan, 2004; O'Hara et al., 2011). The anatomic segments of the epididymis include the initial segment, the caput, the corpus and the cauda. Each region consists of a lumen and a polarized epithelium composed mostly of principal and basal cells (Lasserre et al., 2001; Dacheux et al., 2005). Although these four anatomical regions of the epididymis are easily identified in most adult male mammals (Yanagimachi et al., 1985; Smithwick & Young, 2001), histological and ultrastructural segmentation of this organ varies among the different phylogenies of mammals. The rat epididymis is most commonly adopted as an experimental model of study (Figure 1). Several descriptive anatomical and histological studies of the epididymis appeared at the beginning of the twentieth century. The authors hypothesized that the epididymal secretions played a role in the maintenance of sperm vitality, sperm motility (Benoit, 1926) and the capacity to become fertile (Young, 1929a, 1929b, 1931). Relatively little research was done on the excurrent duct system during the ensuing three decades. However, in 1967, Marie-Claire Orgebin-Crist demonstrated that the key event in sperm maturation was not the passage of time but the exposure of the sperm to the luminal environment of the epididymis (Bedford, 1967; Orgebin-Crist, 1967).

The epididymal duct is now recognized as a channel that transports, concentrates and stores the spermatozoa. It is also known that the spermatozoa leaving the testis are immovable, immature and unable to fertilize an oocyte (Yanagimachi, 1994; Flesch & Gadella, 2000), and that under androgen control, the epididymal epithelium secretes proteins within the intraluminal compartment that create a very complex environment surrounding the spermatozoa (Hermo et al., 1994, 2004; Sullivan, 2004). This luminal

compartment stores the spermatozoa until ejaculation and specifically prepares the sperm for fertilization by providing the essentials in terms of temperature, oxygen tension, pH and an available energy substrate (Dacheux et al., 2005). The epididymal duct produces the morphological

Epididymal development

On the basis of histological and ultrastructural differences, the epididymis can be grossly divided into three regions including the caput (head), corpus (body) and cauda (tail) epididymidis. The most proximal epididymal region, in some species such as the mouse, is also known as the initial segment (Fig. 1). Each epididymal region carries out distinctive functions with the caput and corpus carrying out early and late sperm maturational events, respectively, while the cauda region primarily serves as a storage site for functionally mature spermatozoa.

The epididymis is derived from the Wolffian duct and at birth consists mainly of mesenchymal tissue. The epididymis undergoes considerable remodeling including duct elongation and convolution so that by puberty the epididymis has acquired its fully differentiated state consisting of a highly tortuous tubule lined by epithelial cells (Rodriguez et al., 2002). The development of a fully differentiated epithelium is dependent not only on androgens but also requires the influence of luminal factors from the testis (Rodriguez et al., 2002). Considering that the adult epididymis exhibits region-specific characteristics, it is not surprising that homeobox genes, such as Hox genes that control segmental patterning during development, are expressed during epididymal development and participate in the appearance of segment-specific differences (Rao and Wilkinson, 2002). Although studies have established circulating androgens and luminal factors as playing a necessary role in the development of the epididymis, less is known of other factors that

mediate the series of morphogenic events that result in the formation of the adult epididymis (Lei et al., 2003; Zhang et al., 2004).

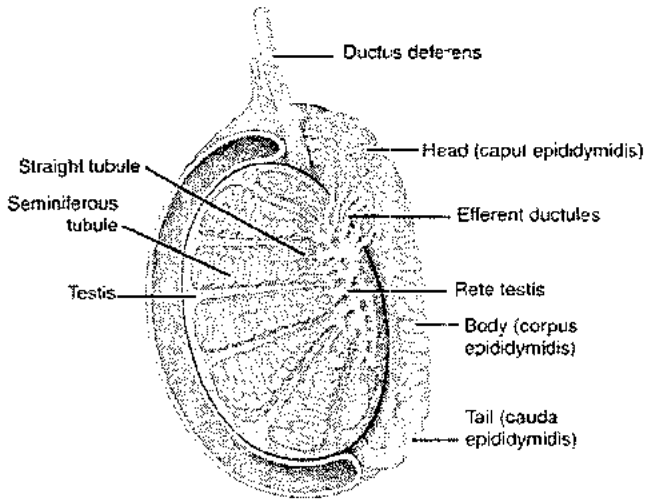

Fig.1 : testis and epididymis

Epithelial–mesenchymal interactions

Several studies have implicated epithelial-mesenchymal interactions as integral for epididymal morphogenesis. Indeed, early studies showed when proximal regions of Wolffian duct epithelium were cultured on seminal vesicle mesenchyme, the epithelium differentiated into seminal vesicle epithelium (Higgins et al., 1989). Bone morphogenetic proteins (BMP), members of the transforming growth factor b (TGFb) superfamily, and their receptors may be involved in this interaction since disruption of the Bmp4, Bmp7 and Bm8a and Bmp8b genes results in epididymal degeneration that is region-specific (Zhao et al., 1998, 2001; Chen et al., 1999; Hu et al., 2004). C-ros, (ROS1), a member of the tyrosine kinase receptor family, may also play an essential role in epididymal development, since mice lacking the rosI gene lack the initial segment and are sterile (Sonnenberg-Riethmacher et al., 1996). Because during kidney development, ROS1 is thought to regulate the extracellular matrix, a storage site for growth factors (Liu et al., 1996), a similar mechanism of action may also occur in the epididymis. Mice with mutations in the SH2 domain protein tyrosine phosphatase (SHP-1) gene ['motheaten' (me), 'viable

motheaten' (mev)] exhibit an aberrant proximal epididymal region similar to that in the c-ros knockout (Keilhack et al., 2001). Since SHP-1 and c-ROS are coexpressed in the epididymis and interact in vitro, SHP-1 was proposed to function as a regulator of c-ros signaling (Keilhack et al., 2001). LGR4, a leucine-rich repeat domain containing G protein-coupled receptor (GPCR) 4 also appears critical for epididymal development since in the LGR4 (Lgr4) knockout mouse the epididymal tubule, especially in the caput, fails to elongate and convolute and the resulting duct is surrounded by a thick condensation of mesenchymal cells (Mendive et al., 2006). The abnormal arrangement of the epithelium and mesenchyme in the LGR4 knockout suggested that altered interactions between these two compartments may cause the phenotype (Mendive et al., 2006). The LGR4 hypomorphic mutant mouse (Lgr4Gt) also exhibits altered post-natal development of the epididymis that lacks the initial segment. Examination of the epididymal ultrastructure demonstrated disruption of the extracellular matrix with an increase in laminin (Hoshii et al., 2007). Thus Lgr4, as postulated for c-ros, may regulate epididymal morphogenesis via maintenance of the extracellular matrix.

Most recently, inhibin beta A, a mesenchyme-derived paracrine factor, was shown to control the coiling of the epithelium in the anterior Wolffian duct, the Anlage of the adult epididymis, thus providing evidence in vivo that interactions between the epithelial and mesenchyme compartments are essential for proper development of the epididymis (Tomaszewski et al., 2007). These studies also demonstrated that the regulation of epididymal coiling was not directly controlled by androgens since the Inhba knockout embryos exhibited normal androgenic parameters.

Epididymal cell structure and function

The adult epididymis consists of a pseudostratified epithelium of several cell types including principal, basal, clear, narrow, apical and halo cells (Fig. 2).

The primary cell type throughout the tubule is the principal cell which constitutes 80% of the epithelium and is, by far, the most studied since it is responsible for the bulk of the proteins that are secreted into the lumen. Less is known regarding the functionof the remaining cell types; however, narrow, apical and clear cells contain the vacuolar Hþ-ATPase and secrete protons into the lumen and thus participate in its acidification (Pietrement et al., 2006; Kujala et al., 2007), while clear cells are also endocytic cells and may be responsible for clearance of proteins from the epididymal lumen. Basal cells do not access the luminal compartment and are in close association with the overlying principal cells, as indicated by the presence of basal cell cytoplasmic extensions between principal cells, and thus may regulate its functions (Veri et al., 1993; Seiler et al., 1999). Halo cells appear to be the primary immune cell in the epididymis, while apical cells may also endocytose luminal components. It is likely that the individual cell types within the epithelium may perform separate as well as integrated functions within the epididymis. In support of this view, recent studies demonstrated that basal cells regulate principal cell electrolyte transport by releasing paracrine factors, specifically by the release of prostaglandin PGE2 (Cheung et al., 2005). Thus, cell–cell interactions within the epithelium can directly affect the luminal environment and ultimately sperm maturation.

The principal cells also form tight junctions with one another and as such form the blood–epididymis barrier. This barrier creates an immunoprotective site within the epididymal lumen that is necessary for sperm maturation. Several androgen-dependent transmembrane proteins including occludin and claudins contribute to the formation of these tight junctions (Cyr et al., 2007). Gap junctions formed by a family of integral proteins known as connexins, are also present between adjacent principal cells both at their apical and lateral

surfaces. These structures, which consist of aligned intercellular pores, allow the transport of molecules ,1 kDa.

Region-specific microenvironments

Within the principal cells, gene expression and subsequently protein synthesis and secretion are tightly regulated and region-specific such that neighboring cells may express very different subsets of genes and gene products. This region-dependent expression contributes to the distinctive luminal protein profile within each epididymal region which is thought to be integral for sperm maturation. While previously it was thought that varying patterns of gene expression along the tubule was loosely associated with different epididymal regions, Turner et al. (2003 demonstrated that the presence of connective tissue septa further subdivides the caput, corpus and cauda epididymidis into discrete intra-regional segments and that region-specific gene expression may in fact be highly ordered and compartmentalized within these precise segments. By using size exclusion dyes and radiolabeled molecules, these authors further demonstrated that the connective tissue septa may also act as barriers restricting the movement of molecules from the interstitial space of one segment to the next. This would allow segment-specific paracrine signaling to occur between stromal and epithelial cells that could regulate the tightly controlled segment-specific expression of genes. Supporting this view, microperifusion studies have demonstrated that the effects of perifused growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF2) and vascular endothelial growth factor (VEGFA) on epithelial cell mitogen-activated protein kinase (MAPK) signaling was restricted to the perifused region only and not neighboring epididymal

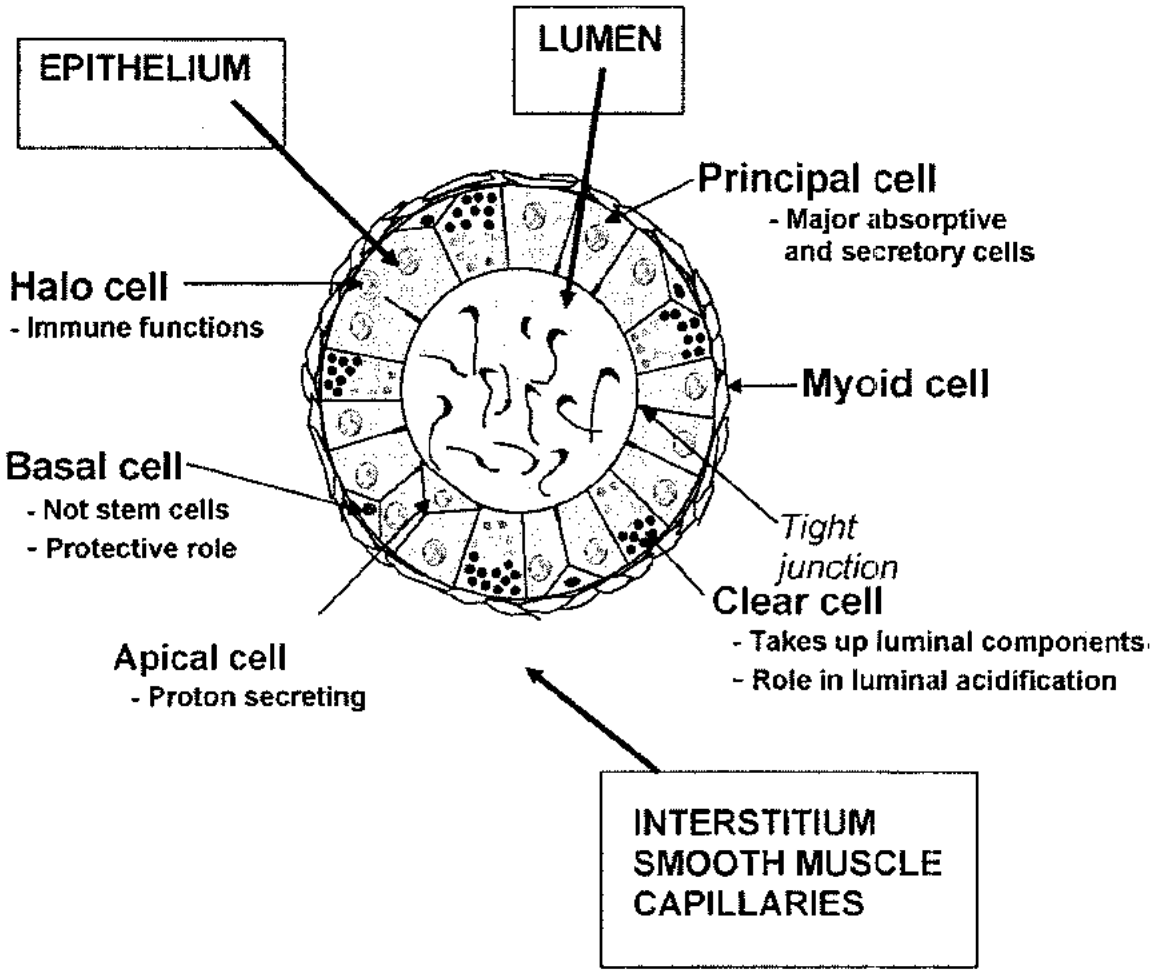

Figure 2 Schematic diagram of the cellular organization in a representative cross-section of the rat epididymis. Modified and reprinted with permission of the author (Robaire et al., 2003), McGill University and publisher, The Van Doren Co, Charlottesville, VA.

segments, presumably reflecting a functional barrier created by the connective tissue septa. However, when growth factors were simultaneously perifused with collagenase, thatdegrades components of the connective tissue septa, MAPK signaling was activated in both the perifused and adjacent epididymal segments (Turner et al., 2007). Thus, the epididymal tubule is a highly ordered and segmented organ with each segment representing a unique physiological compartment. Each compartment possesses distinctive gene expression profiles within the epithelium that dictate segment-specific secretion of proteins into the luminal fluid directly or indirectly affecting sperm maturation. Segment-

specific expression of genes encoding signaling molecules, regulatory proteins, transporters and receptors also contribute to the formation of special microenvironments by allowing the epithelium to respond uniquely to different stimuli such as hormones and other regulatory factors. Identifying and determining the function of segment-specific proteins is of paramount importance for understanding epididymal sperm maturation. For this reason a number of gene profiling studies have been carried out in attempts to identify genes exhibiting regionalized expression in the epididymis (Jervis and Robaire, 2001; Penttinen et al., 2003; Hsia and Cornwall, 2004; Johnston et al., 2005; Oh et al., 2006; Zhang et al., 2006; Jelinsky et al., 2007; Thimon et al., 2007; Li et al., 2008;) as well as proteomic studies to identify proteins (Chaurand et al., 2003; Dacheux et al., 2006; Yuan et al., 2006). These studies have yielded large datasets including novel sequences as well as genes with known identities but previously not known to be expressed by the epididymis. Because of the vast amount of data associated with these studies and the availability of the data to the public, specific genes will not be discussed here, other than to mention that gene sequences included those as potential proteases and protease inhibitors, defensins, transporters, transcription factors, as well as genes associated with metabolism, cell signaling and part of the antioxidant system.

Regulation of epididymal cell function

Control by luminal fluid

The epididymis is highly dependent on androgens, primarily dihydrotestosterone, for function and will be reduced to 25% of its normal weight following castration. Restoration of circulating testosterone reverses the cellular changes in the caput, corpus and cauda epididymidis but not in the initial segment (Ezer and Robaire, 2002). Early studies suggested that the initial segment was controlled by additional regulatory factors since ligation of the

efferent ducts, which connect the testis to the epididymis and are the passageway for spermatozoa and luminal components, resulted in a profound regression of the initial segment region (Fawcett and Hoffer, 1979). Because efferent duct ligation does not affect circulating androgen levels, these studies suggested that the maintenance of initial segment morphology required components in the luminal fluid, i.e. lumicrine regulation, including spermatozoa (Scheer and Robaire, 1980; Garrett et al., 1990; Hinton et al., 1998; Turner and Riley, 1999). Gene expression studies together with expression profiling revealed a subset of initial segment-expressed genes, including those encoding secretory proteins that are down-regulated following efferent duct ligation such Cst8 (CRES), Ggt_pr4 (GGT), Gpx5 (GPX), Lcn8 (MEP17), Etv4 (PEA3) and others suggesting that luminal factors are not only needed for the maintenance of initial segment morphology but for function as well (Scheer and Robaire, 1980; Garrett et al., 1990; Cornwall et al., 1992; Hinton et al., 1998; Hsia and Cornwall, 2003; Avram et al., 2004; Sipila et al., 2006). Because the mRNAs for several of these genes were profoundly decreased within hours after efferent duct ligation, argues for a direct effect of the loss of testicular factors on gene expression rather than indirect effects due to regression of the epithelium (Hinton et al., 1998) (Cornwall, unpublished observations).

Control by luminal proteins

While it is not known if one or many testicular factors are required to maintain initial segment function, studies by Lan et al. (1998) suggested that bFGF may be one such factor. Administration in vitro of FGF2 but not EGF to efferent duct ligated rats restored Ggt_pr4 mRNA, protein and activity in the initial segment to control levels. Furthermore, these investigators proposed that FGF may elicit its effects on Ggt_pr4 gene expression via activation of the ras-

raf-MAPK pathway and downstream activation of the ETV4 transcription factor (Hinton et al., 1998; Lan et al., 1998). Most recently, studies by these investigators suggested, not surprisingly, that not all testis-regulated genes respond in the same way to changes in ETV4 transcriptional activity.

The administration of a ETV5-dominant negative plasmid by in vivo electroporation to the rat initial segment, resulted in the downregulation of ETV5, ETV4 and ETV1 mRNAs in the initial segment as well as putative target genes Ggt_pr4, Srd5a1 (steroid 5 alpha reductase) and Gpx5 (Yang et al., 2006). However, although the testisregulated genes Cst8 and Lcn8 contain ETS binding sites within their promoters, they did not respond to the dominant negative, suggesting that there either may be several testicular factors each differentially regulating specific subsets of genes or that one or a few testicular factors may mediate different downstream effects via the activation of multiple signaling pathways and subsequent effector molecules (Yang et al., 2006). In support of alternative signaling pathways that mediate initial segment function, the mRNAs of Cst8 and the related cystatin E2 as well as Lcn8 and Lcn9, all of which depend on luminal factors for expression, are profoundly down-regulated in mice lacking the HE6/Gpr64 gene, a member of the LNB-7TM subfamily of GPCR expressed in the epithelium of both the efferent ducts and initial segment (Davies et al., 2007). Because preliminary studies suggested HE6/Gpr64 interacted with a profilin-like molecule, known regulators of the cytoskeleton, HE6/Gpr64 may regulate the microenvironment of the initial segment by its association with adaptor/scaffolding proteins ultimately affecting signal transduction pathways and downstream target genes (Kirchhoff et al., 2006, 2008). Indeed, mice lacking the HE6/Gpr64 gene are infertile owing to dysregulation of fluid resorption in the efferent ducts (Davies et al., 2004).

Control by lipids

Other molecules that appear critical for the maintenance of the epididymal epithelium and subsequently sperm maturation includeoxysterols, derivatives of cholesterol. Mice lacking the nuclear oxysterol receptor (LXR) a and b isoforms exhibited a disruption of the caput epididymidis characterized by a localized disruption of the epithelium, especially in the proximal caput regions, including the accumulation of lipids, and an accumulation of amorphous material in the epididymal lumen (Frenoux et al., 2004; Saez et al., 2007). Spermatozoa within the lumen were structurally abnormal with detached heads and tail angulation. Curiously, the effects were not observed until rather abruptly around 6 months of age suggesting that compensatory mechanisms may for a time prevent the expression of the phenotype due to the loss of LXR. The LXRs bind DNA as obligate heterodimers with retinoid X receptors and control the elimination of cholesterol by regulating genes involved in its catabolism, transport and uptake. Thus in addition to proteinaceous factors, lipids also play an integral role in the regulation of epididymal function.

Control by spermatozoa

Other studies suggest that in addition to factors in the fluid, spermcells themselves may regulate the epididymal epithelium (Garrett et al., 1990). Ejaculated bovine spermatozoa washed free of seminal plasma and incubated with primary cultures of caput, corpus and cauda epididymidal cells affect the epithelial secretory profile in an epididymal region and temperature-dependent manner (Reyes-Moreno et al., 2008). Although further studies are needed to determine whether similar results are obtained with testicular or epididymal spermatozoa, these studies provide tantalizing evidence that cell–cell communication between spermatozoa and the epithelium may direct epididymal function.

Signaling molecules and transcription factors

Several molecules that play roles in the developing embryo are also expressed in the adult epididymis and may regulate epididymal functions. These include the Rhox (reproductive homeobox X-linked) and ladybird-like homeobox genes (Lbx) and sonic hedgehog (Shh). Homeobox genes encode transcription factors that typically regulate developmental events including limb development and organogenesis but can also be expressed in adult tissues. The Rhox genes are a cluster of over 30 genes that are expressed in a cell type-specific manner in reproductive tissues including the epididymis (Maclean et al., 2005). Rhox 5, in particular, may function in sperm maturation since spermatozoa from mice lacking Rhox5 exhibited reduced fertility in part because of impaired sperm motility. Because the individual Rhox family members exhibit different region-specific expression patterns in the epididymis, as well as diverse amino acid motifs that interact with the DNA, it suggests that they regulate a broad range of downstream target genes and biological functions. The homeobox gene Lbx2, typically known to be expressed in the nervous system, is expressed in a region-dependent manner in the epididymis and thus may also contribute to the development of the epididymis as well as regulation of adult epididymal function (Moisan et al., 2008). Hedgehog proteins are extracellular signaling molecules that play roles in the regulation of patterning processes during embryonic development. Shh is also expressed in the adult epididymis (Turner et al., 2006). Inhibition of the Shh pathway by the administration of cyclopamine reduced the ability of mouse cauda epididymidal spermatozoa to initiate motility following dilution suggesting that Shh was important for sperm motility maturation (Turner et al., 2006).

The forkhead transcription factors carry out multiple roles in the epididymis. Several studies suggest that Foxa family members such as Foxa2 [Fox(forkhead box) subclass A], play a role in steroid hormone-responsive gene promoters including that for lipocalin 5 (Lcn5) (Yu et al., 2006). The epididymal expression of Foxi1 is also necessary for normal sperm function since spermatozoa from mice lacking Foxi1 showed a high incidence of tail angulation and a decreased ability to migrate through the female tract resulting in decreased fertility (Blomqvist et al., 2006). Foxi1 in the epididymal narrow and clear cells regulates the expression of the B1-subunit of the vacuolar Hþ-ATPase proton pump as well as carbonic anhydrase II and the chloride/bicarbonate transporter pendrin. Because acidification of the epididymal lumen requires the function of the vacuolar Hþ- ATPase proton pump and is necessary for sperm maturation (Yeung et al., 2004; Pastor-Soler et al., 2005), the fertility defect in mice lacking Foxi1 may reflect impaired epididymal sperm maturation as a result of increased luminal pH.

Epididymal luminal environment

The majority of studies in the epididymis have focused on identifying specific epididymal secretory proteins and their functional roles in sperm maturation ultimately to identify novel targets for male contraception or provide new treatments for male infertility. However, equally important as these individual proteins, is understanding the complex epididymal luminal milieu as a whole, since perturbations in the microenvironment that surrounds the sperm cell could affect maturation. Indeed, alterations in the luminal pH affect sperm maturation (Yeung et al., 2004; Pastor-Soler et al., 2005). The epididymal lumen is also rich in inorganic ions and small organic molecules which create an environment that is hyperosmotic relative to serum (Turner, 2002). During epididymal transit, spermatozoa may acquire the capacity to

regulate their cell volume, possibly by the uptake of luminal components that function as intracellular osmolyte reserves, so that upon exposure to the iso-osmotic secretions of the female tract osmotic shock does not occur (Cooper and Yeung, 2003; Cooper, 2007). Osmolites may also function within the epididymal lumen to affect protein folding or interactions.

The epididymal lumen contains perhaps the most complex fluid found in any exocrine gland resulting from the continuous changes in composition as well as the presence of components in unusually high concentrations for reasons not yet known, or those not present in other body fluids (Dacheux and Dacheux, 2002). The caput epididymidis is the most metabolically active region secreting 70–80% of the total overall protein secretion in the epididymal lumen. Moreover, by the time spermatozoa migrate into this region, 99% of the fluid accompanying them has been resorbed, resulting not only in a profound concentration of spermatozoa but luminal components as well (Clulow et al., 1998). While such an environment may be integral for sperm maturation, an environment low in water content creates a situation of macromolecular crowding which places unique stresses on luminal proteins that can alter their behavior and lead to protein misfolding and aggregation (Minton, 2005).

Other stressors such as inappropriate ionic strength, oxidative stress pH and temperature extremes are also known to promote the unfolding of fully folded native proteins and the formation of misfolded, aggregated proteins which can be cytotoxic. Considering that this same epididymal environment must protect spermatozoa and allow maturation, it is likely that mechanisms are in place to prevent or remove aggregated proteins. Several recently published reports provide evidence that the epididymal fluid does not just consist of a large pool of soluble proteins in their native conformations, but rather also contains proteinaceous aggregate structures of varying molecular mass. In particular, the

amyloidogenic prion protein is present in the epididymal lumen both in insoluble exosome-like membranous vesicles (epididymosomes) (Ecroyd et al., 2004), and in a soluble highmolecular mass lipophilic complex in association with hydrophobic proteins (Ecroyd et al., 2005). The chaperone clusterin, which is known to interact with hydrophobic proteins to maintain their solubility, is also detected in the soluble prion protein complex. This suggests that these structures may be a means to maintain proteins in their soluble state either to prevent aggregation and precipitation and enable clearance or to allow hydrophobic proteins to be transferred between cells such as the epididymal epithelium and spermatozoa. It must also be considered that high molecular mass proteinaceous structures in the epididymal lumen carry out biological functions.

Other evidence for the presence of protein aggregates in the epididymal lumen comes from studies examining molecular chaperones in the reproductive tract. Both heat-shock protein 1 (HSPD1, HSP60) and tumor rejection antigen 1 (TRA1, a member of the heat-shock protein 90 family) colocalize to large electron-dense bodies in the epididymal lumen. These structures seem not to be membrane-bound and are larger than epididymosomes suggesting unique structures (Asquith et al., 2005). Because proposed functions of TRA1 include the folding of denatured proteins as well as multimer assembly (Nigam et al., 1994), its function in epididymal lumen may be as a means of extracellular quality control, specifically to refold proteins from non-native (denatured or misfolded and potentially aggregation prone) to native conformations or alternatively to facilitate clearance from the epididymal lumen.

Our studies of the cystatin CRES in the epididymal luminal fluid demonstrated that it was present not only in monomeric forms but in soluble SDS-sensitive and SDS-resistant forms as well as insoluble forms as defined by

its precipitation following high-speed centrifugation (von Horsten et al., 2007). Within the epididymis CRES is synthesized and secreted by the proximal caput epididymidal epithelium, accumulates in the lumen of the midcaput, but abruptly disappears from the distal caput epididymidal lumen (Cornwall and Hann, 1995). Although the mechanism(s) for the sudden disappearance of CRES is not known, its self-aggregation to high-molecular mass forms may contribute to the inability to detect the monomeric forms of CRES in distal epididymal regions. As found for cystatin C, which is a proven amyloidogenic protein, studies of CRES demonstrated that it also forms amyloid in vitro, raising the possibility of amyloid formation within the epididymal lumen (von Horsten et al., 2007).

Because amyloid structures can be cytotoxic and associated with disease, it is likely that the epididymis has mechanisms to guard against the cytotoxicities of protein aggregates. While intracellular mechanisms to control aggregated proteins are well-characterized, to date little is known in any organ system mechanisms that control proteins that may aggregate once they are secreted into the extracellular space. Our studies of CRES demonstrated that it is a substrate for transglutaminase cross-linking and that following exposure to transglutaminase, CRES aggregation was not of the amyloid-type (von Horsten et al., 2007). Thus, transglutaminase cross-linking may be one mechanism the epididymis utilizes to regulate the formation of potentially cytotoxic aggregate structures.

Until recently, evidence did not support a role for the ubiquitin/ proteosome pathway in extracellular control in any organ system with the exception of studies in the epididymis showing that ubiquitin was associated with structures in the luminal fluid (Fraile et al., 1996; Sutovsky et al., 2001). Most recently, a biologically active proteosome was identified in the human alveolar space in

the lung (Sixt et al., 2007), while further studies in the epididymis demonstrated that components of the ubiquitin pathway including ubiquitin activating enzyme E1, ubiquitin carrier enzyme E2 and ubiquitin C-terminal hydrolase PGP9.5/UCHL1 are present and active within the epididymal luminal fluid (Baska et al., 2008). Thus in the epididymis, pathways that normally function within the cell may also occur in the extracellular environment and as a result provide the mechanisms to appropriately maintain the luminal environment ultimately protecting the maturing spermatozoa. Indeed, spermatozoa from mice lacking the ubiquitin ligase Herc4 exhibit angulated tails, reduced motility and reduced fertility supporting a role for the ubiquitin pathway in sperm maturation (Rodriguez and Stewart, 2007).

Epididymosomes

The epididymis utilizes common as well as unique mechanisms to deliver secretory proteins to the sperm surface. Most secreted proteins possess the typical signal sequences indicating trafficking through the Golgi and subsequent packaging and release from secretory granules (merocrine secretion). However, several studies have shown that epididymal spermatozoa also acquire proteins that lack signal sequences suggesting an unusual secretion pathway in the epithelium. Differential extraction of spermatozoa indicates some proteins act like integral membrane proteins and, in fact, are thought to be anchored to the sperm plasma membrane by a glycosylphosphatidylinositol anchor (Kirchhoff and Hale, 1996; Cooper, 1998).

In the epididymal lumen, several of these proteins are associated with membranous vesicles known as epididymosomes. While previously thought to be an artefact of fixation, these small membrane-bound vesicles originate from the epididymal epithelial cells in a process known as apocrine secretion. This type of secretion involves the formation of apical blebs containing various

sized vesicles from the epithelial cells and once the blebs have detached they are thought to fragment and release the small vesicles (Aumuller et al., 1999) (Fig. 3). Although similar type vesicles have been known for some time to be secreted by the prostate (prostasomes) and present in the semen where they have proposed roles as protection for sperm against complement, enhancement of motility and stabilization of the sperm membrane, Yanagimachi et al. (1985) was the first to describe such vesicles in the epididymal lumen and showed interactions of these vesicles with spermatozoa.

Analysis of proteins associated with the epididymosomes reveals protein profiles quite different from that of proteins in the lumen. Proteins associated with epididymosomes include P26h, believed to be involved in zona pellucida binding, HE5, macrophage migration inhibitory factor, ubiquitin and glutathione peroxidase, all of which have been shown to be transferred to spermatozoa in the epididymis (Kirchhoff and Hale, 1996; Sutovsky et al., 2001; Frenette et al., 2003; Saez et al., 2003). However, not all proteins associated with epididymosomes are transferred to spermatozoa suggesting that only some proteins have the ability to be transferred or that complete fusion and transfer of vesicles to spermatozoa does not occur (Sullivan et al., 2005). Studies examining epididymosomes in the cauda fluid from the ovine epididymis identified different subsets of proteins present in these vesicles including dipeptidyl peptidase V, neprilysin, mannosidase and actin, but observed no interactions of such vesicles with spermatozoa also suggesting that transfer of proteins may be by a subtle exchange rather than complete fusion(Gatti et al., 2005). It is also possible that epididymosomes are heterogeneous with different protein compositions depending on downstream functions.

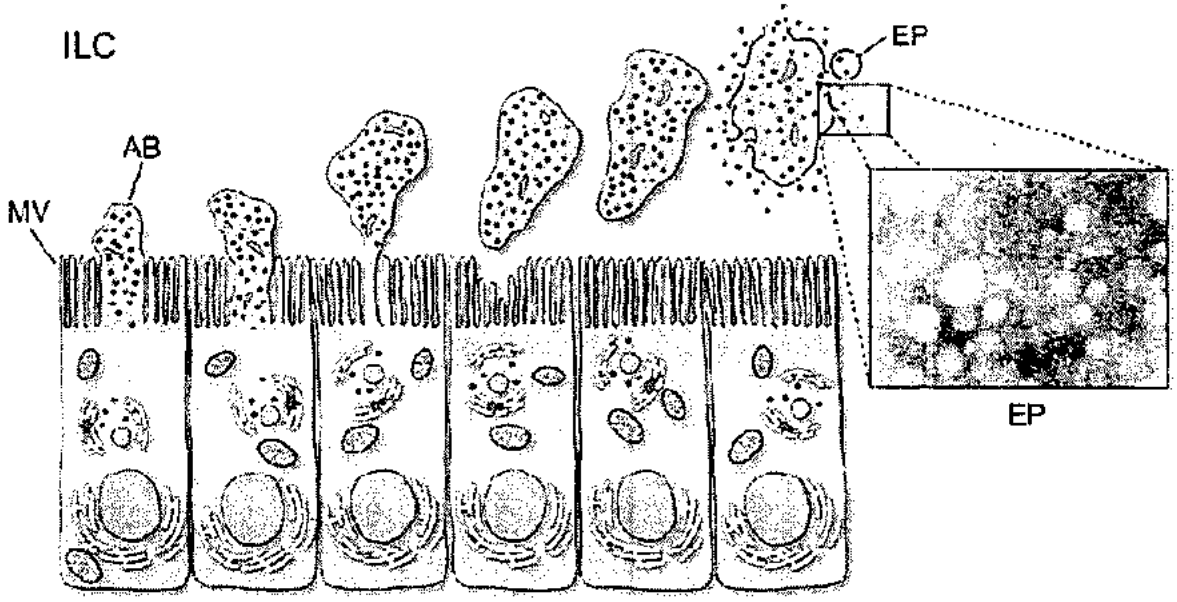

Figure 3 Schematic diagram of apocrine secretion in principal cells of the epididymis. Inset, electron micrograph of epididymosomes. AB, apical bleb; EP, epididymosome; ILC, intraluminal compartment; MV, microvilli. Reprinted with permission of the author, University of Laval and the publisher Blackwell publishing (Sullivan et al., 2007).

The functional significance of epididymosomes in sperm maturation remains to be elucidated. It is possible that they evolved to ensure the safe delivery of some proteins to the sperm cell and perhaps to particular sperm domains without possible damage by luminal proteases. Alternatively, given the complexities of sperm maturation and the vast multitude of cellular and extracellular events that the epididymis must carry out for maturation to occur, it is also possible that the epididymis has developed new strategies to deliver cellular proteins and their associated functions to the sperm surface rather than synthesize a secretory protein that carries out the same function as its cellular form. It is also possible that, in addition to the delivery of proteins to the sperm surface, the epididymosomes act as signaling centres or scaffolds within the luminal compartment affecting protein function independently of that associated with spermatozoa. Indeed, the epididymosomes are believed to

contain lipid raft-like structures which in somatic and germ cells are thought to function as sites of signaling complexes (Brown and London, 1998). Finally, it is possible that epididymosomes contribute to the clearance of proteins from the lumen by binding and delivering proteins to cells for endocytosis.

Sperm maturation

As spermatozoa migrate from the proximal to the distal regions of the epididymis, they undergo a series of morphological, biochemical and physiological changes with the end result being spermatozoa that have acquired the function of progressive motility and the ability to fertilize an ovum. Microscopic studies have demonstrated that during epididymal transport, spermatozoa undergo remodeling that includes changes in the dimension and appearance of the acrosome and nucleus, in some species migration of the cytoplasmic droplet along the tail, as well as structural changes in intracellular organelles (Olson et al., 2002).

The sperm plasma membrane, a highly compartmentalized structure, in particular, is modified during transit with changes in overall phospholipids and cholesterol (Jones et al., 2007). Spatially separated lipids and proteins are re-organized during maturation possibly allowing the formation of signaling complexes critical for fertilization. Lipid rafts have been described in mouse, guinea pig, human, and porcine spermatozoa (Travis et al., 2001; Cross, 2004; Shadan et al., 2004) and may function as large multimolecular signaling assemblies that re-organize and aggregate during capacitation inducing signaling cascades. In many species, a reduction in sperm cholesterol is one of the first steps that triggers signal transduction cascades during capacitation including tyrosine phosphorylation of sperm proteins.

Removal of cholesterol from immature testicular spermatozoa does not induce tyrosine phosphorylation suggesting that downstream signaling molecules have not yet assembled. The failure of immature spermatozoa to undergo tyrosine phosphorylation has also been proposed to reflect their inability to generate sufficient ATP required for subsequent phosphorylation events (Aitken et al., 2007).

Mass spectrometry has also been used to compare the protein profiles of immature caput spermatozoa with that of mature cauda spermatozoa. These studies revealed a number of proteins that increased with epididymal maturation including several that arephosphorylated such as glucose-regulating protein, heat-shock

protein 70, actin, b-tubulin, lactic acid dehydrogenase and the mitochondrial proteins aconitase and b subunit F1 ATPase (Aitken et al., 2007). Thus, the ability of specific sperm proteins to be phosphorylated may represent a key maturational step possibly as a result of the development of signaling complexes.

The topography of the sperm surface also changes in a domainspecific manner during epididymal transit. Atomic force microscopy demonstrated that particles ranging in size from 20 to 60 nm were associated with the acrosomal cap, equatorial segment and postacrosomal region, and varied depending on whether the spermatozoa were isolated from the initial segment or caudal regions (Takano and Abe, 2004; Jones et al., 2007). These studies were interpreted as reflecting changes in protein associations with the sperm surface as part of the maturational process. Indeed, the protein composition of the spermatozoon changes as the cells mature with some proteins disappearing, others being modified, while others change their cellular localization. The biochemical alterations in sperm proteins reflect either those proteins that were

synthesized during spermatogenesis or those that were secreted by the epididymal epithelial cells and interact with the maturing sperm. Sperm proteins that were produced during spermatogenesis and are modified such as by deglycosylation or proteolytic processing during epididymal transit include ADAM family members fertilin and cyritestin, CE9, a-mannosidase and many others (Table I). Other sperm proteins including SPAM1 and b-galactosyltransferase exhibit new localization patterns during

Table I Epididymal sperm proteins

Sperm proteins modified or relocalized during epididymal transit	Epididymal proteins that interact with spermatozoa
Spam1[1]	CRISP1[11]
ADAM2[2], ADAM3[3], ADAM15[4], ADAM24[5]	P26h[12]
α-mannosidase[6]	Clusterin[13]
CE9[7]	HE1[14], HE2[15], HE4[16], HE5[17], HE12[18]
β-galactosidase[8]	HEL75[19]
Basigin[9]	SPAG11[20]
α-enolase[10]	Eppin[21]
Grp78/Hsp70[10]	Cystatin 11[22]
Endoplasmin[10]	SED1[23]
Phosphatidylethanolamine binding protein[10]	
Lactate dehydrogenase 3[10]	
Testis lipid-binding protein[10]	
Cytokeratin[10]	
β-subunit F1-ATPase[10]	

[1](Phelps et al., 1990); [2](Lum and Blobel, 1997); [3](Frayne et al., 1998); [4](Pasten-Hidalgo et al., 2008); [5](Zhu et al., 2001); [6](Tulsiani et al., 1995); [7](Nehme et al., 1993); [8](Scully et al., 1987); [9](Saxena et al., 2002); [10](Baker et al., 2005); [11](Cohen et al., 2000); [12](Legare et al., 1999); [13](Sylvester et al., 1991); [14](Kirchhoff et al., 1996); [15](Osterhoff et al., 1994); [16](Kirchhoff et al., 1991); [17](Kirchhoff and Hale, 1996); [18](Saalmann et al., 2001); [19](Lin et al., 2008); [20](Yenugu et al., 2006); [21](Richardson et al., 2001); [22](Hamil et al., 2002); [23](Ensslin and Shur, 2003).

epididymal transit which may in part be triggered by proteolytic processing (Phelps et al., 1990). Epididymal proteins that are known to interact with spermatozoa and thus may be involved in their maturation include the CRISP family proteins, P26h, P34h, SPAG11, Eppin and others (Table I).