Contractility of the epididymal duct: function, regulation and potential drug effects

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Abstract

During their transit through the epididymis, spermatozoa mature and acquire motility and fertilizing capacity. The smooth muscle cells (SMCs) of the epididymal duct are thought to be responsible for the adequate transport of spermatozoa. Thus, precise regulation of SMC function also represents a prerequisite for sperm maturation thereby contributing to male fertility. In this review, we would like to highlight various aspects of epididymal SMC function and discuss several angles with respect to regulation of contraction and relaxation. Different to the vas deferens, where disturbed SMC pathways resulting in male infertility could be defined, comparable information is missing in the epididymis. We therefore include some vas deferens data which could also be useful for a better understanding of epididymal SMC function. Furthermore, we would like to draw attention to drugs used in clinical practice and their potential (side) effects on contractions in the epididymis.

Reproduction (2018) **156** R125–R141

Introduction

In man, the epididymis contains a caput, corpus and cauda region (Fig. 1A) and is further subdivided into segments (Holstein 1969) by connective tissue septa (Turner *et al.* 2003, Holstein 2008, Domeniconi *et al.* 2016). In different species, the number of segments varies (Turner *et al.* 2003, 2007, Hinton *et al.* 2011). The main structural component of the organ is a single epididymal duct that is upstream (in the caput) connected to the rete testis by 8–12 efferent ducts and downstream (in the cauda) to the vas deferens (Fig. 1A).

Every second, the epididymis receives roughly 1000–1500 spermatozoa that have been released by the testis (Amann & Howards 1980). They need to transit up to 6 m in human, 3 m in rat and 1 m in mice through the highly convoluted (Hinton *et al.* 2011; Fig. 1A and B) epididymal duct in a suitable manner to acquire motility and fertilizing ability (Robaire & Hinton 2015). Total transit time is around 10 days, but differences between species exist (Robaire & Hinton 2015). Proper sperm maturation and transport by the coordinated action of the epididymal SMCs are thus intricately interwoven and require delicate regulation. Beside a brief overview on their structure, the function, regulation and potential clinical relevance of epididymal SMCs is at the focus of this review.

Mechanisms of transport of spermatozoa within the epididymis

In general, transport of spermatozoa within the epididymis might be affected (i) by smooth muscle cell (SMC) contractions (see ‘Muscle contraction’ section), (ii) the luminal fluid (see ‘Luminal fluid and sperm transport’ section) and (iii) presumably specialized ciliated cells (see ‘Transport effects by kinocilia’ section).

Luminal fluid and sperm transport

In the very proximal part of the epididymis, transport of spermatozoa is influenced by a constant fluid production originating from the testis. Nearly all fluids are reabsorbed in the efferent ducts and the initial segment. In fact, in tammar and in rat around 90% of the fluid (of the testis) entering the efferent ducts is reabsorbed (Turner 1984, Jones & Clulow 1987, Clulow *et al.* 1994). Hormonal aspects of fluid resorption are mentioned below (see ‘Estrogens’ in ‘Regulation by hormones’ section).

Transport effects by kinocilia

The efferent ducts consist of an epithelial layer surrounded by SMCs (Kierszenbaum & Tres 2016). Beside cells involved in resorption and secretion, the epithelial layer also comprises ciliated cells (Kierszenbaum & Tres 2016).

Figure 1 The smooth muscle cell layer of the epididymal duct. (A) The diagram illustrates the human epididymal duct (red) with its neighboring structures and the increasing thickness of the SMC layer (cross sections, red) along the duct (Baumgarten *et al.* 1971). (B) The three dimensional aspect of the proximal part of the rat epididymis is visualized after clearing of the tissue by CLARITY. The convoluted epididymal duct is illustrated by staining of the smooth muscle cell layer (using SMA antibodies, red). In addition, the architecture of interstitial blood vessels and the connective tissue septum (arrow) between two segments is visible (collagen 1 staining, green).

In contrast to organs such as the oviduct or the airways where the role of kinocilia for transport function is established (Klein *et al.* 2009, Noreikat *et al.* 2012), the role of ciliated cells in the epididymis is less clear and has not yet been addressed experimentally to our knowledge. Ciliated cells of the efferent ducts were recently suggested to contribute to the transport of spermatozoa in the epididymis (Robaire & Hinton 2015).

Similar to M2 and M3 (and M1) muscarinic acetylcholine receptors (mAChR) shown to be involved in cilia-driven transport in the airways (Klein *et al.* 2009), cholinergic contribution to the transport of spermatozoa is also thinkable, since M2, M3 (and M1) mAChRs were also described in ciliated cells of the efferent ducts (Siu *et al.* 2006). In addition, the presence of NOS in ciliated cells suggests that NO also could have an impact on sperm transport (Davidoff & Middendorff 2000).

Muscle contraction

The relevance of SMC contractility for the transport of spermatozoa seems to be common sense. To our knowledge, however, actual sperm transport as a result of SMC contractility has only occasionally been reported. This chapter intends to give an overview on structure and function of the SMC layer.

Structure and contraction of the smooth muscle layer of the epididymal duct

All efferent ducts become convergent in one single epididymal duct consisting of an epithelial layer and the surrounding SMC layer (Holstein 1969, Kierszenbaum & Tres 2016). The epithelium is classified as a pseudostratified epithelium with (immotile) stereocilia pointing to the lumen (Kierszenbaum & Tres 2016). The SMC layer (Fig. 1A and B) consists of thin SMCs that are predominantly arranged in a circular manner. In the human epididymis, bundles of collagen fibers are found among SMCs and true fibroblasts were also described (Holstein 1969, Baumgarten *et al.* 1971). The thickness of the SMC layer increases along the epididymal duct from caput to cauda (Holstein 1969, Baumgarten *et al.* 1971 and Fig. 1A). In the corpus, outer bundles of longitudinally and obliquely orientated SMCs join the circularly arranged inner smooth muscle layer (Holstein 1969, Baumgarten *et al.* 1971). Toward the cauda, additional large SMCs appear in the outer parts of the duct (Holstein 1969) resulting in a complete further SMC layer (Baumgarten *et al.* 1971) with a predominantly longitudinal orientation of the cells. These large SMCs are more differentiated than the smaller, thin SMCs (Holstein 2008). These thin SMCs are also described as myofibroblasts by some authors (Mewe *et al.* 2006b) and thought to be spontaneously contracting (Holstein 2008). Interestingly, in the quiescent vas deferens only large SMCs were found (Holstein 1969). This arrangement with large SMCs and lacking spontaneous contractility might be important in the context of ejaculation.

Data on contractions of the epididymal duct, which are difficult to obtain from human material, are available from several species. In most parts, the epididymal duct is characterized by spontaneous rhythmic contractions as shown by *in vivo* (Muratori & Contro 1951, Knight 1974, Hib 1976) and *in vitro* (Hib & Caldeyro-Barcia

1974, Markkula-Viitanen *et al.* 1979, Talo *et al.* 1979) experiments. From caput to cauda, contractile frequency decreases while amplitude increases (Risley & Skrepetos 1964, Talo *et al.* 1979, Mewe *et al.* 2006b). In the distal part of the cauda, which stores spermatozoa until ejaculation, only sporadic contractions (Risley & Skrepetos 1964, Talo *et al.* 1979, Hib 1988) with areas devoid of any spontaneous contractility (Mewe *et al.* 2006b) were described. Contractions can spread in both directions and occur against a rising gradient in hydrostatic pressure from caput to cauda (Johnson & Howards 1976, Pholpramool & Triphrom 1984).

In addition, peristaltic movements of the duct have been observed in all parts of the epididymis ((Risley & Skrepetos 1964, Hib & Caldeyro-Barcia 1974, Talo *et al.* 1979, Studdard *et al.* 2002, Mietens *et al.* 2014) our unpublished results). When injecting oil droplets into the caput of the epididymal duct, droplets disperse along the duct. Slow and fast transport in both directions and pendular movements of luminal content were described (Jaakkola & Talo 1982, 1983).

Interestingly, spontaneous contractions of the duct do not only occur in the adult epididymis, but have already been found before mature spermatozoa occur, i.e. also in the prenatal and postnatal epididymis (van de Velde & Risley 1963). This is of interest since polysialylated neural cell adhesion molecule (NCAM) was detectable among SMCs of the epididymal duct before puberty (Simon *et al.* 2015). Polysialylated NCAM is known to prevent the adhesion of cells and thus could theoretically also disturb well-regulated contractions of a SMC layer. Nestin, often preceding expression of polysialylated NCAM during development, does however not mark SMCs of the postnatal epididymal duct (Reckmann *et al.* 2018), but instead marks the vascular SMCs at this time period (Reckmann *et al.* 2018) as shown before in testis (Davidoff *et al.* 2004) and lung (Saboor *et al.* 2016).

Regulation of contractility

In the cauda region, responsible for storage and emission of sperm, SMC activation by adrenergic nerve fibers is

well described (Baumgarten *et al.* 1971). Knowledge about the regulation of smooth muscle function (responsible for sperm transport) in the other parts of the epididymis is however sparse, but various mechanisms may be of importance. Below, we present contributing factors like neuronal input, hormonal, epithelial and sperm influences.

Neuronal input

Early investigations on tissue from stillborn children showed that caput and corpus epididymis are supplied by few nerve fibers whereas the cauda is more densely innervated (Mitchell 1935). These differences of the innervation pattern were also revealed in adult human tissue (Baumgarten *et al.* 1968) and other species (El-Badawi & Schenk 1967, Kaleczyc *et al.* 1993). This finding is in agreement with the special function of the cauda to instantaneously contribute to emission.

Nerve fibers of the epididymis include sympathetic, parasympathetic, peptidergic (e.g. neuropeptide Y (NPY), vasoactive intestine polypeptide (VIP), substance P, GCRP, L-enkephalin, somatostatin), purinergic or nitrergic neurotransmitter fibers (Kunts & Morris 1946, Risley & Skrepetos 1964, Baumgarten *et al.* 1968, Nouhouayi & Negulesco 1985, Ventura & Pennefather 1991, Dun *et al.* 1996). Some important aspects are discussed below.

Adrenergic input

The male reproductive tract receives sympathetic input via adrenergic innervation. In human and rat epididymis, alpha1-adrenergic receptors, known to bind noradrenaline and adrenaline (Graham *et al.* 1996) were described with a predominance of alpha1A-adrenoreceptors (Queiroz *et al.* 2002) and localized to blood vessels, epithelial cells and SMCs (Queiroz *et al.* 2008). Increasing noradrenaline concentrations from testis and proximal epididymis toward the vas deferens (Baumgarten *et al.* 1971) and a higher density of alpha1A-adrenoreceptors were found in the SMCs closer to the cauda epididymidis (Queiroz *et al.* 2002), which

Figure 2 Visualization of noradrenaline effects on spontaneous contractility. (A) Snapshot of a rat epididymal duct section (caput) from a time-lapse imaging movie (Video 1). The time stack was virtually dissected through a plane indicated by the orange bar. (B) Each contraction elicits a small movement of the epididymal duct with changes of its diameter resulting in a series of spikes (marked by vertical arrows). The green arrows show the contractile activity without treatment, while the purple arrows represent the noradrenaline effect. (Figure and Video: courtesy by Christina Seidel.)

emphasizes the relevance of $\alpha 1$ -mediated signaling during emission (Kaplan 2009). In addition to its proposed main function in the cauda epididymidis during emission of sperm, noradrenaline was shown to enhance SMC contractions (Fig. 2 and Video 1) also in other regions of the epididymis (Mewe *et al.* 2006a, 2007, Miettens *et al.* 2014). Organ bath studies over the length of the bovine duct (Fig. 3), however, showed the maximum contractile response to noradrenaline in the mid-part of the cauda (Mewe *et al.* 2007). Functional studies using $\alpha 2$ agonists and blockers showed an influence also of $\alpha 2$ -adrenergic receptors on contraction, especially in proximal parts of the duct (Chaturapanich *et al.* 2002, Mewe *et al.* 2007). Here $\alpha 2$ -mediated effects were suggested to be more important than $\alpha 1$ -mediated ones (Mewe *et al.* 2007). So far, the exact contributions of the different α -adrenoreceptor subtypes to contractile epididymal function have only begun to be explored and require further investigation to identify potential alterations in pathologic conditions.

Video 1

Noradrenaline enhances SMC contractions. The video (<http://movie-usa.glencoesoftware.com/video/10.1530/REP-17-0754/video-1>) from the online version of the article is available at <https://doi.org/10.1530/REP-17-0754>.

Cholinergic input

In addition to its potential effect in ciliated cells (see 'Luminal fluid and sperm transport' section), cholinergic signaling also mediates smooth muscle contractions *in vitro* and *in vivo* (Laitinen & Talo 1981, Pholpramool & Triphrom 1984, Siu *et al.* 2006). M3 mAChRs were found in the smooth muscle layer of the rat epididymal duct, mainly in the cauda (Siu *et al.* 2006).

Despite of many experimental studies, the functional relevance of the cholinergic input on contractions of the epididymal duct is unclear. Still, it is assumed that adrenergic innervation is more potent.

Nerve injury and experimental denervation

A neuronal input certainly contributes to the coordinated function of the epididymal smooth muscle, but in men suffering from spinal cord injury, impaired fertility in the majority of patients seems to be rather associated with ejaculatory and erectile dysfunction as well as secretory dysfunction of male accessory sex glands, while there is no evidence for specific deficits of sperm maturation and transport along the length of the epididymis (Brackett *et al.* 2010, Brackett 2012, Ibrahim *et al.* 2016). Data from a sympathectomy rat model also suggest intact epididymal function and sperm isolated from the

Figure 3 Stimulating effects of oxytocin and noradrenaline on spontaneous contractility as well as relaxing effects of cGMP on the bovine epididymal duct and proximal vas deferens. Colored lines illustrate maximal and minimal response to oxytocin (blue) and noradrenaline (black) (in percent of contraction) along the different epididymal regions. Data were normalized to the respective maximum effects in the mid-dist. cauda. The red line represents relaxing effects of the membrane-permeable cGMP analog 8-Br-cGMP on spontaneous contractility of the epididymal duct and has its greatest impact in the corpus and proximal cauda. Oxytocin and noradrenaline have clearly distinct contractile effects. While oxytocin has its peak effect in the mid-distal cauda, noradrenaline has a relatively weaker effect in this region and elicits its maximum response in the distal cauda. We suggest a function of noradrenaline in ejaculation while oxytocin ensures antegrade flow of sperm.

epididymal cauda showed normal fertilizing capacity (Kempinas *et al.* 1998). However, it remains to be elucidated in how far potential dysfunction of the distal cauda is also involved in the described disturbances of emission/ejaculation in case of nerve injury and experimental denervation.

Regulation by hormones

Androgens epididymal function is well known to be androgen dependent. Testosterone produced by testicular Leydig cells is reduced in the epididymis by 5- α reductase to dihydrotestosterone (Robaire & Hinton 2015). Temporary deletion of the Leydig cells by ethane dimethane sulfonate revealed reduced volume of the epididymis and epididymal duct (Yang *et al.* 2006) potentially suggesting impaired sperm transport.

Presence of classical androgen receptors (ARs) in the epididymis has been intensively investigated in many species (Robaire & Hinton 2015). Beside epithelial cells, AR was repeatedly described in SMCs of the epididymal duct (Zhou *et al.* 2002, Trybek *et al.* 2005) indicating transcription-based testosterone effects in these cells. This might explain long-term effects of androgen (or androgen withdrawal) on contractility (Hib & Ponzio 1977, Din-Udom *et al.* 1985) most likely due to changes of cellular signaling or structure. Differences between efferent duct ligation (no lumicrine (Hinton *et al.* 2000)

effects) and bilateral castration (neither lumicrine nor systemic effects) are of interest (Din-Udom *et al.* 1985).

As far as we know, the phenotype of various mouse models with deletion of the AR in SMCs has not yet been investigated in the epididymis different to the testis (Welsh *et al.* 2009) and prostate (Welsh *et al.* 2011). Corresponding epididymis data would improve our knowledge of direct androgen effects in epididymal SMCs. Deletion of AR in principal cells of the proximal epididymis resulted in obstructive azoospermia (Krutskikh *et al.* 2011). However, a possible contribution of SMCs in this process is unclear.

Castration (although not proving direct effects on SMCs) was shown to result in spontaneous contractions in the normally quiescent vas deferens (MacDonald & McGrath 1980, Johns *et al.* 1983, Burnstock & Verkhatsky 2010). Data clearly showing comparable results in the distal epididymis, likewise quiescent, seem to be missing. However, increased contraction amplitudes in caput and corpus as well as increased basal intraluminal pressure in corpus and cauda were described after castration (Din-Udom *et al.* 1985) suggesting that testosterone has relaxing effects on epididymal smooth muscle.

There are only few studies describing rapid effects of androgen on epididymal contractility. In guinea pig, testosterone was shown to stimulate spontaneous contractions of isolated duct segments within 60 min (da Silva e Souza *et al.* 1974). Most likely, such effects are not mediated by classical ARs. Instead, steroid actions via G protein-coupled plasma membrane receptors, also suggested for the epididymis (Robaire & Hamzeh 2011) or cation channels as described for progesterone in spermatozoa (Lishko *et al.* 2011, Strücker *et al.* 2011) are thinkable.

Estrogens In addition to a testicular origin (Hess *et al.* 2011), estrogen might also be produced locally in the epididymis, since aromatase was shown to be expressed in various epididymal structures (Hess *et al.* 2011, Oliveira *et al.* 2012).

Classical estrogen receptors (ESRs) were described in the epididymis of different species and primarily found in the epithelium (Hess *et al.* 2011, Rago *et al.* 2018), but also in SMCs (Oliveira *et al.* 2012, Rago *et al.* 2018). Regional differences of ESR1 expression are suggested to induce varying estrogen effects along the epididymis (Hess *et al.* 2011). Apparently, estrogen influences a calcium-sensitizing RhoA/Rho kinase pathway in SMCs of the epididymis, possibly resulting in an increase of contractility (Fibbi *et al.* 2009). ESR1-KO mice showed disturbed fluid resorption in the efferent ducts resulting in male infertility (Hess *et al.* 1997). In contrast, aromatase-knockout mice did not show such defects of the efferent ducts (Toda *et al.* 2008). Blocking the activity of aromatase resulted in changes of endothelin-

1-dependent contractions mediated by oxytocin (Filippi *et al.* 2005).

Different to androgens, studies directly describing rapid effects of estrogen on contractility of the epididymal duct are missing. G protein-coupled ESRs as binding sites in a non-classical signaling pathway of estrogen were described in epithelial cells of the rat corpus and cauda and suggested to play a role in sperm maturation (Martinez-Traverso & Pearl 2015). Recent findings in the human proximal epididymis, however, revealed G protein-coupled ESRs not only in epithelial cells, but also in the smooth muscle layer (Rago *et al.* 2018) pointing to the existence of rapid effects on SMC contractility also by estrogen.

Oxytocin and vasopressin Oxytocin receptors (OTRs) are membrane receptors which transduce rapid effects (Leng & Sabatier 2017). OTRs were found in the SMC layer of the epididymal duct (Whittington *et al.* 2001, Mewe *et al.* 2007). In response to oxytocin epididymal contractions increased *in vitro* (Hib 1974, Studdard *et al.* 2002, Filippi *et al.* 2005) and *in vivo* (Melin 1970). Unexpectedly, oxytocin has opposite effects in the different regions of the organ and showed relaxing effects in corpus and proximal cauda (Mewe *et al.* 2007). Relaxing effects were suggested to be epithelium-dependent, since epithelium-denuded corpus segments showed contractile effects (Mewe *et al.* 2007). Maximum contractile response to oxytocin was found in the mid-cauda comparable to noradrenaline (Fig. 3). This may be useful for a selective emptying of the cauda during emission. Reduced contractile effects of adrenaline and oxytocin in the distal cauda might ensure an orthograde sperm transport (Mewe *et al.* 2007).

Vasopressin, a peptide hormone and potent vasoconstrictor, also stimulated epididymal duct SMCs and enhanced contractility of caput and cauda epididymidis (Jaakkola & Talo 1981, Studdard *et al.* 2002).

Oxytocin and vasopressin are nonapeptides that differ only in two amino acids. Especially in high concentrations vasopressin can also bind to the OTR and vice versa (Song & Albers 2017). Therefore, it is necessary to always consider vasopressin when observing oxytocin effects. Using the selective OTR agonist xOT, Mewe and colleagues could mimic contractile oxytocin effects in the bovine duct, pointing to OTRs as main mediators of the observed oxytocin effects (Mewe *et al.* 2007).

Relevance of the epithelium for contractility

Mewe *et al.* (2006a) removed the epithelium of proximal parts of the bovine duct and observed a quiescent duct without contractions. After addition of endothelin-1 or noradrenaline contractions could be induced. However, only prostaglandin (PG) F2a (see below, 'Prostaglandins')-induced phasic activity similar to the

profile of spontaneous contractions in the intact duct (Mewe *et al.* 2006b).

While experimental data support a role for various epithelial factors (see below) in regulating epididymal contractile function their overall significance *in vivo* is less clear and warrants further exploration. Endothelin-1/Endothelin-converting enzyme 1 and the peptide endothelin-1 were detected in the epididymis (Peri *et al.* 1997). Reappearing contractions induced by endothelin-1 after removal of the epithelium (Mewe *et al.* 2006b), see above) points to an epithelial source of this peptide. Its receptors ET_A and ET_B , localized to the SMC layer, were shown to influence these contractions (Peri *et al.* 1997). Spontaneous contractions of the epididymal duct were discussed to be mediated by a paracrine loop between endothelin-1 and oxytocin ((Filippi *et al.* 2005) see 'Regulation by hormones' section).

eNOS Mewe and colleagues described the expression of endothelial nitric oxide (NO) synthase (eNOS), one of the three NO synthase (NOS) isoforms (Fig. 4) (Knowles & Moncada 1994), see 'NO/cGMP' section), in the bovine epididymis as a modulator for spontaneous phasic contractions (Mewe *et al.* 2006a). eNOS was not only detected in endothelial cells, but also in SMCs and epithelial cells of the epididymal duct (Mewe *et al.* 2006a). As shown for the vasculature (Busse & Fleming

1998), a mechanism of stretch-induced eNOS activation and subsequent relaxation is also thinkable for the epididymal duct. The maximum relaxing potency of NO (cGMP) pathways (see 'NO/cGMP' section) was found in the corpus region of the epididymal duct (Fig. 3) different to oxytocin and noradrenaline effects (Mewe *et al.* 2007). Stretch would be induced by the local luminal sperm content resulting in circumscribed SMC relaxation of the duct at the same position to improve sperm maturation.

Serotonin Beside its role as a monoamine neurotransmitter, serotonin was found in the caput epididymidis and could be detected in epithelial cells (in addition to mast cells and neuroendocrine cells) (Jimenez-Trejo *et al.* 2007). Immunoreactivity was also observed for serotonin transporter and receptors (Leung *et al.* 1999, Jimenez-Trejo *et al.* 2007). While serotonin responses of SMC contractions in the prostate (Killam *et al.* 1995) and vas deferens (Hay & Wadsworth 1982) were reported, the role of (epithelial) serotonin in epididymal contractions remains unclear.

Angiotensin Binding sites for angiotensin II were shown from caput to cauda epididymidis and localized to the epididymal duct by receptor autoradiography (Grove & Speth 1989). Angiotensin II affected expulsions of spermatozoa in the cauda (Grove & Speth 1989) and

Figure 4 cGMP pathways induce smooth muscle relaxation. Relaxation of smooth muscle cells is predominantly mediated by cGMP signaling and components of this pathway are found within the male reproductive tract. The messenger molecule cGMP is generated by the soluble guanylyl cyclase sGC and different membrane-bound guanylyl cyclases (e.g. GC-A, GC-B). Nitric oxide (NO), generated by NO synthases (nNOS, eNOS, iNOS), activates sGC. Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) activate GC-A. C-type natriuretic peptide (CNP) is the ligand of GC-B. Activity of GC-A and GC-B is modulated by at least three different mechanisms: 1. Degradation of the natriuretic peptides by the neutral endopeptidase (NEP) at the cell surface. 2. Metabolic clearance of the natriuretic peptides by internalization after binding of peptides to the natriuretic peptide clearance receptor (NPRC). 3. Desensitization of GC-A and GC-B by intracellular de-phosphorylation of the receptors. In addition to GC-A and GC-B there are further membrane-binding GCs (not shown). The key enzyme of cGMP-mediated SMC relaxation is cGMP-dependent protein kinase I (PKG I). The duration of a given cGMP signal depends on the activity of different phosphodiesterases (PDEs) which hydrolyze cGMP (and/or cAMP).

induced contractions in the vas deferens mediated by angiotensin receptor 1 (Sum & Cheung 1995, Ban *et al.* 2002). Data on contractile effects in the proximal regions of the epididymal duct are less defined. Angiotensin-converting enzyme (ACE), responsible for cleaving active angiotensin II from the prehormone angiotensin I, was found in epididymal epithelial cells and in high concentration in seminal plasma (Kryukova *et al.* 2015). Mice lacking (somatic) ACE or angiotensinogen showed normal fertility (Hagaman *et al.* 1998).

Prostaglandins PGs are synthesized from arachidonic acid by cyclooxygenase (COX)-1 or -2 in almost every nucleated cell. In the rat epididymis, for example, basal cells of the epithelium were described to contain COX-1 and to secrete PGE2 and PGD2. COX-2 was found in principal cells (Wong *et al.* 1999). PGs are synthesized as needed and, with a short half-life, suitable as regional messengers of limited duration. Whereas COX-2 was found in the proximal parts of the rat epididymis (but not in the cauda) (Stanfield & Khan 2003), Lazarus and colleagues (Lazarus *et al.* 2002) localized the enzyme in the mouse to the distal cauda. COX-1 was described in all parts of the mouse epididymis and increased from caput to cauda (Lazarus *et al.* 2002). PGD2, PGE1, PGE2 and PGF2alpha were found to be active in the epididymis (Bartke & Koerner 1974, da Silva e Souza *et al.* 1975, Hib & Oscar 1978, Cosentino *et al.* 1984, Sorrentino *et al.* 1998). At least in part, their local concentration was influenced by androgens (Bartke & Koerner 1974). Beside direct effects on contractility, PGs were also suggested to modify the action of neurotransmitters and hormones such as noradrenaline and acetylcholine (da Silva e Souza *et al.* 1975, Hib & Oscar 1978).

Interestingly, the function of the single PGs might differ. Cosentino *et al.* (1984) reported increasing contractions after addition of PGF2alpha to the isolated rat caput epididymidis, whereas PGE2 had an opposite effect (Cosentino *et al.* 1984). In the isolated cauda epididymidis of the guinea pig, PGE1, PGE2 and PGF2alpha showed stimulating effects on contractility (da Silva e Souza *et al.* 1975). Increasing contractility was also observed by Hib and Oscar (1978) who infused PGE2 and PGF2alpha systemically (Hib & Oscar 1978).

Influence of sperm/luminal factors in contractility of the epididymal duct

While the influence of epithelial cells on smooth muscle contractions is conceivable by their direct neighborhood, it is noteworthy that luminal factors or sperm can also modify contractile activity as shown by Mewe *et al.* who demonstrated spontaneous burst-like phasic contractions instead of regular phasic contractions after rinsing the lumen of sperm and other luminal factors (Mewe *et al.* 2006b).

Influence of elements of the SMC layer on contractions of the epididymal duct

Interstitial cells of Cajal Interstitial cells (ICCs) of Cajal (1911) function as pacemakers for SMCs and are found throughout the gastrointestinal tract. Their occurrence has also been suggested in the genitourinary tract (Lang *et al.* 1998). In the bovine epididymal duct, 'atypical muscle cells' with cytoplasmic extensions may represent epididymal ICCs (Mewe *et al.* 2006b). The Ca²⁺-activated chloride channel TMEM16A is known to be involved in spontaneous SMC contractility and was found in ICCs (but not in typical SMCs) of the gastrointestinal tract. Thus, TMEM16A-positive epididymal cells could indicate ICCs (Huang *et al.* 2009). Most recently, TMEM16 was suggested to be also functionally active in epididymal epithelial cells (Gao *et al.* 2016). In these cells, TMEM16 is coupled to the Ca²⁺ channel TRPV6. Interestingly, pore mutation or knockout of TRPV6 (Weissgerber *et al.* 2011, 2012) leads to severe defects of Ca²⁺ absorption in the epididymal duct resulting in male infertility.

Relaxation of SMCs regulated by cGMP signaling

NO/cGMP

Relaxation of SMCs is predominantly mediated by cyclic guanosine monophosphate (cGMP) signaling and components of this pathway are found within the male reproductive tract. The messenger molecule cGMP is generated by either soluble (cytosolic) guanylyl cyclase (sGC) or membrane-bound particulate guanylyl cyclases (pGC, e.g. GC-A, GC-B, Fig. 4) (Kuhn 2016). In the epididymis, sGC is – apart from certain epithelial cells (Shum *et al.* 2008) – mainly expressed in SMCs of the epididymal duct (Mewe *et al.* 2006a, Mietens *et al.* 2012) and mediates its relaxing effects when stimulated by NO (Mewe *et al.* 2006a). NO effects were prevented by prior application of the sGC blocker ODQ (Mewe *et al.* 2006a) showing that the action of the NO donor is dependent on cGMP synthesis. NO is generated by the constitutive NOS isoforms neuronal (nNOS) and eNOS (see 'Relevance of the epithelium for contractility' section) as well as inducible NOS (iNOS) (Fig. 4) (Knowles & Moncada 1994). All isoforms were found within the epididymal duct (epithelium (see 'Relevance of the epithelium for contractility' section), SMC layer) or its direct neighborhood (nerve fibers (see 'Neuronal input' section), blood vessels) (Burnett *et al.* 1995, Dun *et al.* 1996, Zini *et al.* 1996, Wiszniewska *et al.* 1997, Mewe *et al.* 2006a), allowing NO to reach its receptor sGC in the SMC layer of the duct. NOS activity was described in the epididymis of different species. NOS activity was found in all three regions of the rat epididymis (Burnett *et al.* 1995) and is particularly high in the cauda consistent with a rich supply of nNOS-immunoreactive fibers (Dun *et al.*

1996). Inhibition of NOS increased spontaneous contractility of the epididymal duct (Mewe *et al.* 2006a) and points to a basal activity of NO-generating enzymes. After castration of rats, NOS activity was significantly reduced in all regions of the epididymidis, indicating an involvement of androgen in epididymal NOS activity (Chamness *et al.* 1995). Especially the NOS/NO/cGMP pathway of SMC relaxation might provide a crucial mechanism to ensure epididymal sperm maturation (see 'Relevance of the epithelium for contractility' section (eNOS) and Fig. 4).

Natriuretic peptides/cGMP

C-type natriuretic peptide (CNP), the ligand for the pGC GC-B (Fig. 4), exerts local activities (Kuhn 2016) and is abundant in rat and bovine seminal plasma (Chrisman *et al.* 1993, Hosang & Scheit 1994). Epididymal epithelial and SMCs produce CNP (Nielsen *et al.* 2008, Thong *et al.* 2014) and GC-B was described in turtle (Kim *et al.* 2000), bovine (Mewe *et al.* 2006a), human, mouse (Thong *et al.* 2014) and rat (Müller *et al.* 2011) epididymis. In agreement with relaxing CNP effects on isolated parts of the bovine epididymal duct (Mewe *et al.* 2006a), GC-B was shown to be expressed in the wall of the epididymal duct including the SMC layer, both at the mRNA level by laser capture microscopy (LCM) and RT-PCR (Thong *et al.* 2014) as well as at the protein level (by receptor autoradiography (Mewe *et al.* 2006a) and immunofluorescence (Thong *et al.* 2014)).

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP), which act as hormones in the regulation of blood pressure and fluid volume homeostasis, bind to the pGC GC-A (Fig. 4). GC-A was also shown to be expressed in the bovine and rat epididymis (Mewe *et al.* 2006a, Müller *et al.* 2011) and could be localized to ductal SMCs (Mietens *et al.* 2014). In agreement, ANP- and CNP-induced relaxation of the bovine epididymal duct could be prevented by the GC-A/GC-B blocker HS142-1 (Mewe *et al.* 2006a). Different to bovine tissue, only ANP, but not CNP, resulted in significant relaxing effects in the rat epididymal duct (Mietens *et al.* 2014), suggesting further, yet undefined functions of CNP/GC-B signaling in the muscle layer of the duct.

Activity of the natriuretic peptide receptors GC-A and GC-B is modulated by at least three different mechanisms (Fig. 4). Availability of the ligands is controlled by the plasma membrane protein neutral endopeptidase (NEP, also known as metallo-endopeptidase EC 3.4.24.11; enkephalinase, neprilysin, CD10, CALLA) (Terawaki *et al.* 2007) which cleaves and inactivates a variety of biologically active peptides including natriuretic peptides (Corti *et al.* 2001). By this, NEP regulates the local concentrations of natriuretic peptides at the cell surface (Pankow *et al.* 2009, Potter 2011). In the epididymis, cleavage of CNP and reduction of CNP-induced cGMP production by NEP was described

(Thong *et al.* 2014). Another mechanism to control responses to natriuretic peptides is binding to a further natriuretic peptide receptor, the clearance receptor (NPRC), devoid of GC activity, which binds all natriuretic peptides with equal affinity and provides metabolic clearance of the circulating natriuretic peptides by internalization (Potter 2011). Further, activity of GC-A and GC-B is known to be downregulated by intracellular de-phosphorylation of the receptors ('desensitization') (Potter & Garbers 1992, Müller *et al.* 2006).

In addition to GC-A and GC-B, there are further membrane-binding GCs. So far, only GC-C, known to be involved in intestinal ion transport and epithelial turnover was also described in epithelial cells of the epididymis (Jaleel *et al.* 2002). It is not known, however, whether GC-C influences local smooth muscle contractility.

cGMP-dependent protein kinases

The key enzyme of cGMP-mediated SMC relaxation is cGMP-dependent protein kinase I (PKGI, Fig. 4) (Hofmann & Wegener 2013) that was detected in all parts of bovine and rat epididymal duct using immunoblotting (Mewe *et al.* 2006a, Müller *et al.* 2011) and crosslinking experiments with fluorescein-coupled cGMP (Mewe *et al.* 2006a,b). Interestingly, in the aging epididymis, a significant decrease of PKGI expression was found (Müller *et al.* 2011). Immunohistochemical analyses revealed the kinase in the muscle wall of the epididymal duct and also in some epithelial cells (Mewe *et al.* 2006a).

The inhibitory effect of the membrane-permeable cGMP analog 8-Br-cGMP on contractions of the epididymal duct was clearly reduced by the PKG inhibitor Rp-8-Br-cGMPS as well as by iberiotoxin, thapsigargin and indomethacin, pointing to PKGI as a main target for cGMP and to large-conductance Ca^{2+} -activated K^{+} channels, the sarcoplasmic endoplasmic reticulum Ca^{2+} -ATPase and COX-1 as possible targets of PKG (Mewe *et al.* 2006a).

Phosphodiesterases

The duration of a given cGMP signal depends on the activity of phosphodiesterases (PDEs, Fig. 4). They hydrolyze the intracellular second messenger and thereby control cGMP levels (Bender & Beavo 2006). Among the 11 families of PDEs described so far, PDEs differ in their substrate specificity and are able to hydrolyze cGMP (PDE5, PDE6, PDE9), cAMP (PDE4, PDE7, PDE8) or both cyclic nucleotides (PDE1, PDE2, PDE3, PDE10, PDE11), thus mediating a possible cross-talk between these two second messenger systems (Bender & Beavo 2006).

PDE5 was exclusively localized to the SMC layer of the human and rat epididymal duct, both at the mRNA level by LCM in combination with RT-PCR and at the protein

level by immunoblotting and immunohistochemistry (Müller *et al.* 2011, Mietens *et al.* 2012). In contrast, there was no evidence for PDE5 expression in the epididymal epithelium (Mietens *et al.* 2012). The PDE5 inhibitor sildenafil interfered with epididymal cGMP degradation *in vitro* (Mietens *et al.* 2012), and with contractility of the rat epididymal duct *ex vivo*, as shown by contraction force recordings (Mietens *et al.* 2012) and time-lapse imaging (Mietens *et al.* 2014).

The non-specific PDE inhibitor isobutyl methyl xanthine (IBMX), which inhibits most of the PDEs described so far (Lugnier 2006), blocked most of the cGMP-degrading activity of epididymal protein fractions. In the epididymal duct IBMX resulted in a more pronounced reduction of contractions compared to PDE5 inhibitors, suggesting expression of further yet undefined PDEs that might affect contractility in the epididymal duct. In agreement, transcripts of PDE1, PDE2 and PDE3 were detected in the smooth muscle layer of the rat epididymis (our unpublished results). The absence of PDE3A and PDE3B in the corresponding human probes (Fig. 5) points to species-specific differences in the epididymal expression of cGMP pathway components. The existence of other PDEs than PDE5 is also implied by the finding that relatively small amounts of the cGMP-hydrolyzing PDE5 are expressed in rat epididymis compared to large amounts of the cGMP-generating sGC, GC-A and GC-B (Müller *et al.* 2011).

Contractility and infertility

Epididymitis: aspects of contractility

Investigations of pathological processes in the epididymis resulting in male infertility are of high interest. In this context, epididymitis was recently reviewed (Michel *et al.* 2015, Taylor 2015), but data on the smooth muscle layer of the epididymal duct and possible alteration in case of epididymitis are nearly completely missing. Interestingly, a mouse model of ascending infection with uropathogenic *E. coli* (UPEC) showed a decrease of the ductal diameter in upstream segments 3 days after infection (Stammler *et al.* 2015) suggesting infection-induced effects on SMC contractility. After 7 days of infection in the same UPEC mouse model, ductal obstruction in the infected region was described (Michel *et al.* 2016). In human chronic epididymitis, similar obstructive changes of the duct were observed (Stammler *et al.* 2015, Michel *et al.* 2016). These findings indicate that infection and inflammation also target structure and function of epididymal SMCs.

Molecular mechanisms: lessons from the vas deferens

So far, male infertility cannot be related to the absence or mutation of single molecules that affect contractility of the epididymal duct. In the vas deferens, however,

specific molecules impairing contractility were shown to be crucial for male fertility.

Purinergic signaling

Knockout of the purinergic P2X1 receptors, for example, reduced male fertility by 90% (Mulryan *et al.* 2000) and in a double knockout model of the frequently co-expressed P2X1 receptor and alpha1-adrenergic receptor (Ventura & Pennefather 1991), vas deferens contractions were disrupted and resulted in male infertility (White *et al.* 2013). P2X1 receptors are ATP-gated cation channels

Figure 5 Expression and localization of specific PDEs in human epididymis. (A and B) Laser-assisted microdissection combined with (C) RT-PCR analysis of PDE3A (236 bp), PDE3B (242 bp) and PDE5A (245 bp) of isolated epithelial cells (Ep) and SMCs of the human epididymis. SMA marks SMCs (140 bp) and NEP (411 bp) identifies epithelial cells. Lung tissue (Lu) and (amplification of) the housekeeping gene beta-actin (199 bp) served as a positive control. '+', '-' and '0' represent lanes with reverse transcriptase, without reverse transcriptase and water control. PDE3A and PDE3B could not be detected in isolated epithelial cells and SMCs. PDE5A was only detected in SMCs. (Data and figure: courtesy by Dr Caroline Feuerstacke.)

regulating Ca^{2+} influx, which mediates vas deferens contractions upon sympathetic nerve stimulation (Lee *et al.* 2000). Additionally, P2X1 and P2X2 receptors were identified in the epididymal SMC layer (Lee *et al.* 2000). Interestingly, purinergic signaling seems to have differential effects on longitudinal vs circular SMCs in the vas deferens (Amobi *et al.* 2012). It is currently unknown if a similar differential regulation occurs in the cauda epididymidis, which also shows an outer longitudinal SMC layer (Baumgarten *et al.* 1971). It is conceivable that P2X receptors contribute to the regulation of both, epididymal contractility and sperm release from the cauda. Purinergic signaling may be further modulated by NTPDase1, an extracellular ectonucleotidase, which was found in the tunica muscularis of the vas deferens (Kauffenstein *et al.* 2014). The absence of NTPDase1 leads to an enhanced contractile response to extracellular ATP in the vas deferens, desensitization of the P2X1 receptor and reduced receptor protein expression (Kauffenstein *et al.* 2014). Both, NTPDase1 and 2, are also expressed in epididymal SMCs (Martín-Satué *et al.* 2009).

Alpha1-adrenergic signaling

In alpha1-adrenergic receptor-knockout mice (Sanbe *et al.* 2007), reduced contractions of the vas deferens were reported. Deletion of one of the three subtypes (alpha1A) markedly decreased contractions while in triple (1A, 1B, 1D) KO mice, vas deferens contractions were completely eliminated resulting in reduced sperm content in the ejaculate and reduced pregnancy rate (Sanbe *et al.* 2007). Until now it remains unknown whether contractions of the cauda epididymidis are similarly affected in these mouse models and in how far epididymal effects contribute to the described effects on fertility.

NPY

In men and various mammals, high levels of NPY were found in noradrenergic nerves supplying the muscle layer of the vas deferens (Lundberg *et al.* 1983, Jen *et al.* 1997). NPY inhibited the contractile response to electrically evoked field stimulation (Allen *et al.* 1982). In the epididymis, however, NPY had no effect on field stimulation-induced contractions of the isolated duct of the cauda region. It was suggested that NPY has post-junctional actions, which mask its pre-junctional effects (Haynes *et al.* 1997).

VIP

In the vas deferens, vasoactive intestine polypeptide (VIP) inhibited electrically induced contractions. Investigations on the presence of VIP in the epididymis of the guinea pig showed an exclusive localization in the caudal parts and especially in the SMC layer

(Kastin *et al.* 1978, Greenberg *et al.* 1985) suggesting a role of VIP on relaxation to improve sperm storage. However, functional studies in the epididymis are necessary to confirm this hypothesis. Moreover, VIP- but not tyrosine hydroxylase-positive fibers were described to co-express nNOS (Dun *et al.* 1996).

NOS and acetylcholine

Regarding the cholinergic system of the vas deferens, it was found that cholinergic nerve fibers were less abundant than adrenergic ones and preferentially reach the lamina propria (for review, Koslov & Andersson 2013). Around 50% of VACHT-positive fibers also contained NOS (Dixon *et al.* 2000). Unpublished data of our group, investigating the epididymal duct in the rat cauda, suggest that cholinergic effects result in smooth muscle relaxation mediated by NOS.

Potential side effects of medications

Male infertility may be related to disorders of spermatogenesis, post-testicular sperm maturation or sexual function ensuring ejaculation of spermatozoa. The impact of drugs on male fertility has recently been reviewed (Semet *et al.* 2017), but still, data on drug effects or side effects on epididymal contractility is sparse. Medications that target smooth musculature potentially interfere with epididymal contraction and thus could prevent the correct maturation and transport of sperm. A range of drugs in clinical use might affect contractility of the epididymal duct.

Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) interfere with PG synthesis (see 'Influence of sperm/luminal factors in contractility of the epididymal duct' section) and could alter local smooth muscle function. Indomethacin, an example for NSAIDs, was reported to reduce epididymal contractility (da Silva e Souza *et al.* 1975) and to inhibit responses to noradrenaline or acetylcholine (Hib & Oscar 1978). Different NSAIDs are widely used as analgetics and acetyl salicylic acid is additionally used to inhibit platelet aggregation. Possible side effects on male fertility are, however, largely unknown.

PDE inhibitors

Drugs interfering with PDEs (see 'Phosphodiesterases' section) are gaining importance in different clinical fields and potential side effects of PDE inhibitors are conceivable.

Toxicity related to PDE4 inhibitors was reported in rat epididymis with dilatation of efferent ducts and the initial segment followed by the formation of sperm granulomata in caput and cauda of the epididymis (Heuser *et al.*

2013). PDE4 inhibitors are currently developed for the treatment of inflammatory diseases like asthma, chronic obstructive pulmonary disease (COPD) (Rabe 2011), rheumatoid arthritis and psoriasis (Pagès *et al.* 2009, Sakkas *et al.* 2017, Torres & Puig 2017). With apremilast approved for the treatment of psoriasis, and roflumilast used in COPD treatment, PDE4 inhibitors are already in clinical use.

PDE5 inhibitors like sildenafil, tadalafil or vardenafil are used in the treatment of erectile dysfunction (Smith-Harrison *et al.* 2016), but also to treat pulmonary hypertension (Ghofrani *et al.* 2006) thereby exposing more and younger patients. Interestingly, long-term treatment with sildenafil did not change spontaneous contractility of the epididymis in a rat model (Mietens *et al.* 2014). Tadalafil, gaining additional importance for the treatment of benign prostatic hyperplasia (BPH), was not tested in that study.

Further PDE inhibitors may be of importance in the future. PDE10 inhibitors are under development to treat psychiatric and neurological disorders (Chappie *et al.* 2009, Abdel-Magid 2013). PDE2 represents an interesting target for psychiatric, cognitive (Abdel-Magid 2017, Zhang *et al.* 2017) and cardiovascular (Bobin *et al.* 2016) diseases and PDE3 inhibitors are used in acute therapy for heart failure (Movsesian 2016). Regarding potential side effects on epididymal contractility, it is of interest that PDE3 seems to be missing especially in the human epididymis (see 'Phosphodiesterases' section and Fig. 5).

Nitrates/NO-releasing drugs

Also of widespread use in the treatment of coronary artery disease are substances like nitrates (e.g. ISDN, ISMN, molsidomine), which release NO (see 'NO/cGMP' section and Fig. 4). We could demonstrate the importance of the NO/cGMP signaling system for the regulation of spontaneous epididymal contractility in rat (Mietens *et al.* 2012, 2014) and bull (Mewe *et al.* 2006a, 2007).

Adrenergic receptor antagonists and agonists

Alpha-adrenergic as well as cholinergic pathways have been shown to be functional in the regulation of epididymal contractility (Chaturapanich *et al.* 2002). Many drugs in clinical use that interfere with these signaling pathways may therefore alter epididymal sperm transport and maturation.

Alpha1-adrenergic receptor antagonists like tamsulosin or silodosin are in clinical use for the management of BPH (Fibbi *et al.* 2009, Gacci *et al.* 2016). While adverse effects on vas deferens contractility with ejaculatory disorders were reported in men (Kobayashi *et al.* 2008), data on epididymal effects are only available from rat (Chaturapanich *et al.* 2002) and bovine (Mewe *et al.* 2006b) models.

Alpha1-adrenergic receptor antagonists like prazosin, doxazosin or terazosin are also used to treat arterial hypertension.

Clonidine, an alpha2-adrenergic receptor agonist (see 'Neuronal input' section), used to lower blood pressure was shown to enhance epididymal contractility and to alter sperm parameters in the rat (da Silva Júnior *et al.* 2014).

In contrast to alpha-adrenergic signaling, beta-adrenergic influence seems not to affect epididymal contractility (Chaturapanich *et al.* 2002), but in a rat model, reversible histologic changes were reported during administration of beta-adrenergic blockers (el-Sayed *et al.* 1998).

Psychotropic drugs

Psychotropic medications like anti-depressants or anti-psychotics interfere with various signaling, thus, unwanted effects on epididymal contractions are likely, but remain largely unknown. An inverse stimulating effect on epididymal contractility with reduced sperm count and quality was reported for drugs like sibutramine, an appetite suppressant (Borges *et al.* 2013) or bupropion used for smoking cessation and as an antidepressant (Cavariani *et al.* 2015). The drugs interfere with the re-uptake of noradrenaline and dopamine thereby modulating these signaling systems with potential side effects on epididymal contractility.

Anti-cholinergic drugs

Cholinergic signaling at the level of the epididymis could be disturbed by drugs that have anticholinergic side effects like anti-depressants. Such drugs are usually given over longer time periods.

Inhaled atropine, an anticholinergic that is used as a bronchodilator, may rather have local effects in the respiratory system. In the rat, effects of anti-muscarinic drugs were associated with reduced fertility, but effects on the epididymis were not clearly discriminated from effects on other organs in the male reproductive tract; however, adverse effects seem to recede with discontinuation of the treatment (Ban *et al.* 2002).

Ca²⁺ antagonists

Ca²⁺ antagonists, such as nifedipin, are established in the treatment of hypertension, for their relaxing effect on vascular SMCs. Side effects on the SMC layer of the epididymal duct are well conceivable, but compared to the vas deferens (Blakeley *et al.* 1981), the epididymis was barely investigated.

ACE inhibitors

The epididymal lumen seems to be protected from ACE inhibitors, established in the treatment of arterial hypertension, by the blood-epididymis barrier

(Wong & Uchendu 1990), but side effects mediated by SMCs are possible (see 'Relevance of the epithelium for contractility' section).

Interference with androgens

Androgen effects on epididymal contractility (see 'Regulation by hormones' section) may be disturbed by the 5- α reductase (see 'Regulation by hormones' section) inhibitor finasteride used in BPH treatment. In a rat model, this drug caused decreased male infertility (Robaire & Henderson 2006).

Conclusion

Currently, our knowledge about the molecular mechanisms orchestrating epididymal contractions and transport processes is still limited as is our insight into potential side effects of pharmacologic treatment. Thus, further studies are needed to better explore and define the role of the epididymis for male infertility.

Declaration of interest

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

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG, Germany), with grant KFO 181 and as part of an International Research Training Group project (GRK 1871/1, P6). Funding was also received from the University Hospital Giessen and Marburg.

Acknowledgements

The authors are most grateful to Christina Seidel who contributed Fig. 2 and Video 1 and to Dr Caroline Feuerstacke who contributed the data presented in Fig. 5. Excellent technical support by Sabine Tasch, Ingrid Schneider-Hüther and Andre Kaschtanow is also greatly appreciated. Moreover, they thank Prof. Hans-Christian Schuppe for fruitful discussions.

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Received 13 December 2017

First decision 11 June 2018

Revised manuscript received 17 May 2018

Accepted 11 June 2018