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Function and therapeutic potential of GPCRs in epididymis

Daolai Zhang[1], Yanfei Wang[2], Hui Lin[3], Yujing Sun[3], Mingwei Wang[3], Yingli Jia[4], Xiao Yu[5], Hui Jiang[6], Wenming Xu[7], Zhigang Xu[2], and Jinpeng Sun[8]

[1]Binzhou Medical University - Yantai Campus
[2]Shandong University School of Life Science
[3]Shandong University School of Medicine
[4]Peking University School of Basic Medical Sciences
[5]Shandong University
[6]Peking University Third Hospital
[7]Sichuan University West China Second University Hospital
[8]shandong University

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Abstract

Infertility rates for both females and males have increased continuously in recent years. Currently, effective treatments for male infertility with defined mechanisms or targets are still lacking. G protein-coupled receptors (GPCRs) are the largest class of drug targets, but their functions and the implications on therapeutic development for male infertility largely remain elusive. Nevertheless, recent studies have shown that several members of the GPCR superfamily play crucial roles in the maintenance of ion-water homeostasis of the epididymis, development of the efferent ductules, formation of the blood-epididymal barrier, and maturation of sperm. Knowledge of the functions, genetic variations, and working mechanisms of such GPCRs, along with the drugs and ligands relevant to their specific functions, provide future directions and elicit great arsenal for potential therapy development for treating male infertility.

Abstract

Infertility rates for both females and males have increased continuously in recent years. Currently, effective treatments for male infertility with defined mechanisms or targets are still lacking. G protein-coupled receptors (GPCRs) are the largest class of drug targets, but their functions and the implications on therapeutic development for male infertility largely remain elusive. Nevertheless, recent studies have shown that several members of the GPCR superfamily play crucial roles in the maintenance of ion-water homeostasis of the epididymis, development of the efferent ductules, formation of the blood-epididymal barrier, and maturation of sperm. Knowledge of the functions, genetic variations, and working mechanisms of such GPCRs, along with the drugs and ligands relevant to their specific functions, provide future directions and elicit great arsenal for potential therapy development for treating male infertility.

Keywords : G protein-coupled receptor (GPCR); epididymis; male infertility; ADGRG2; AGTR2; LGR4

Abbreviations: GPCR, G protein-coupled receptor; ADGRG2, adhesion G protein-coupled receptor G2; AGTR2, angiotensin II receptor type 2; LGR4, leucine-rich repeat containing G protein-coupled receptor 4; GPR64, G protein-coupled receptor 64; HE6, human epididymal gene product 6; CFTR, cystic fibrosis transmembrane conductance regulator; CBAVD, congenital bilateral absence of the vas deferens; RAS, renin-angiotensin system; ANGI, angiotensin I; ANGII, angiotensin II; tACE, angiotensin-converting enzyme specific to the testes; AGTR1, angiotensin II receptor type 1; NO, nitric oxide; IPF, idiopathic pulmonary

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fibrosis; GPR48, G protein-coupled receptor 48; ERα, estrogen receptor α· AR, androgen receptor; NHE3, Na^+/H^+ hydrogen exchanger 3; Aqp9, aquaporin 9; BMs, basement membranes; TNF, tumor necrosis factor; GSK3-β, glycogen synthase kinase 3 beta; GPER, G protein-coupled estrogen receptor 1; GPR30, G protein-coupled receptor 30; PAMs, positive allosteric modulators.

Introduction

The infertility rate of humans has continuously increased in recent years and has become a significant social burden (Krausz *et al.* , 2018; Winters *et al.* , 2014). Currently, infertility ranks as the third most common public health concern below cancer and cardiovascular disease. Issues in males and females contribute equally to the increasing infertility rate and nearly 7% of the male population has fertility problems (Krausz *et al.* , 2018; Winters *et al.* , 2014). However, few effective treatments are available for male infertility with defined mechanisms. It is now well accepted that defects in sperm production, decrease of sperm motility, and inability of sperm to interact with the oocyte all contribute to male infertility (Aitken, 2006; Elzanaty *et al.* , 2002).

After spermatogenesis in the testis, the spermatozoa are morphologically complete but immotile and unable to fertilize an oocyte. They must travel through the efferent ductules and the epididymis to acquire the ability to move, capacitate, migrate through the female tract and finally fertilize an oocyte. The efferent ductules are small, coiled tubules that convey sperm from the testis to the epididymis. In mammals, efferent ductules begin with several discrete wide-lumen ducts that eventually merge into highly convoluted tubules with a narrow lumen (Hess, 2015; Joseph *et al.* , 2011). The efferent ductule epithelium contains ciliated cells with long motile cilia and non-ciliated cells with microvillus brush borders (Hess, 2015; Joseph *et al.* , 2011) (Figure 1). It is now commonly accepted that the major function of the efferent ductules is reabsorption of luminal fluid, which increases the concentration of sperm before they enter the epididymis (Clulow *et al.* , 1998; Hess, 2000; Hess *et al.* , 2000).

The mammalian epididymis is an exceedingly long, convoluted ductal system connecting the efferent ductules with the vas deferens. Functionally, the epididymis creates an ideal environment to promote the functional transformation of spermatozoa and their later storage before ejaculation. The epididymis is segmented into four functionally distinct segments: the initial segment (not existing in human epididymis), the caput, the corpus, and the cauda (Abou-Haila *et al.* , 1984; Zhou *et al.* , 2018) (Figure 1). The initial segment, together with the upstream efferent ductules, is responsible for the resorption of the testicular fluid that enters the duct, resulting in a pronounced concentration of the luminal spermatozoa (Abe *et al.* , 1984). The caput epididymis is highly active in protein synthesis and hormone secretion and plays important roles in sperm maturation. The sperm passing through this region begin to obtain the ability to swim in a progressive manner and to recognize an oocyte (Aitken *et al.* , 2007; Chevrier *et al.* , 1992). The functional maturation of the sperm continues in the corpus epididymis and reaches full activity in the distal caudal segment. The caudal segment contains a relatively large lumen, and its surrounding epithelial cells have strong absorptive activity (Hermo *et al.* , 1988). There are four main cell types in the epithelium of the epididymal lumen, namely, narrow cells, clear cells, principal cells, and basal cells. Each cell type has different functions involved in the establishment and regulation of a unique luminal environment (Cornwall, 2009; Shum *et al.* , 2009).

In general, an appropriate microenvironment established by the efferent ductules and epididymis is required for sperm to undergo maturation and acquire progressive motility and the ability to fertilize oocyte during their transit. To date, the exact molecular mechanism involved in maintaining the effective microenvironment in the efferent ductules and epididymis remains elusive, which creates significant obstacles to developing effective treatments for male infertility. Therefore, there is an urgent need to understand the regulatory mechanisms in the efferent ductules and epididymis involved in both physiological and pathological processes, and this knowledge will provide potential drug targets for developing effective therapies.

G protein-coupled receptors (GPCRs), also called seven-transmembrane receptors, are a group of important drug targets, accounting for approximately one-third of all clinically marketed drugs (Hauser *et al.* , 2018; Santos *et al.* , 2017). Although the roles of GPCRs in cardiovascular disease, neuronal disease, diabetes

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and many other diseases have been extensively investigated(Desimine *et al.* , 2018; Dong *et al.* , 2017; Hauser *et al.* , 2017; Kim *et al.* , 2020; Lammermann *et al.* , 2019; Li *et al.* , 2018; Liu *et al.* , 2017; Srivastava *et al.* , 2015), there is a significant knowledge paucity in regard to the functions of GPCRs in the efferent ductules and epididymis. GPCRs were well known for carrying out their selective functions through coupling to different G protein subtypes or arrestins(Manglik*et al.* , 2020; Staus *et al.* , 2020; Wingler *et al.* , 2020). In general, the binding of ligands (such as hormones, neurotransmitters or sensory stimuli) induces conformational changes in the transmembrane and intracellular domains of the receptor, thereby allowing interactions with heterotrimeric G proteins or arrestins. For G protein signaling, activated GPCRs act as guanine nucleotide exchange factors (GEFs) for the α subunits of heterotrimeric G proteins, catalysing the release of GDP and the binding of GTP for G protein activation. Different G protein couples to downstream effectors. For example, the Gs couples to adenyl cyclase whereas the Gq connects to the phospholipase C(Flock *et al.* , 2017; Flock *et al.* , 2015; Furness *et al.* , 2016; Isogai *et al.* , 2016; Ritter *et al.* , 2009; Sounier *et al.* , 2015; Venkatakrishnan *et al.* , 2016). The activated GPCRs are also phosphorylated by a group of GPCR kinases (GRKs)(Homan *et al.* , 2014; Komolov *et al.* , 2017; Reiter *et al.* , 2006), leading to the recruitment of a different type of arrestins. The interaction of GPCRs with arrestins turns on a second wave of signalling(Desimine *et al.* , 2018; Dong *et al.* , 2017; Kumari *et al.* , 2016; Lefkowitz *et al.* , 2005; Liu *et al.* , 2017; Reiter *et al.* , 2006; Shukla *et al.* , 2014; Wang *et al.* , 2018; Yang *et al.* , 2018; Yang *et al.* , 2015). Even a single type of GPCR can initiate a broad range of physiological processes through arrestin engagement by scaffolding different downstream effectors(Hara *et al.* , 2011; Liu *et al.* , 2017; Luttrell *et al.* , 1999; Miller *et al.* , 2000; Peterson *et al.* , 2017; Srivastava *et al.* , 2015; Tobin *et al.* , 2008; Xiao *et al.* , 2007; Yang *et al.* , 2018; Yang *et al.* , 2015). However, the exact roles of the G protein subtype or arrestins downstream epididymis GPCRs remain cloudy.

At present, there are no U.S. Food and Drug Administration (FDA)-approved drugs targeting GPCRs in the efferent ductules or epididymis for the treatment of male infertility. In contrast, there are more than 470 GPCR-targeted drugs for therapies treating other diseases in clinical markets (Hauser *et al.* , 2018). Nevertheless, recent research has elucidated the expression patterns and functions of several important GPCRs in the efferent ductules and epididymis, such as adhesion G protein-coupled receptor G2 (ADGRG2), angiotensin II receptor type 2 (AGTR2), and leucine-rich repeat containing G protein-coupled receptor 4 (LGR4), and has successfully developed the corresponding ligands to regulate their functions, illuminating the possibility of therapeutic developments regarding male infertility (Figure 1). Here, we review the existing progress of GPCRs in epididymis and efferent ductules, and suggest potential therapeutics directions by targeting these GPCRs for male infertility.

Function of ADGRG2 in fluid reabsorption and epididymis development

Few GPCRs have tissue-specific distributions in male reproductive systems. ADGRG2, also called G protein-coupled receptor 64 (GPR64) or human epididymal gene product 6 (HE6), has attracted substantial attention for its specific expression and essential function in male reproductive systems. It is specifically expressed in the efferent ductules and the proximal epididymis, with much lower expression levels in other tissues (Table 1) (Kirchhoff *et al.* , 2008; Obermann *et al.* , 2003). Further studies confirmed the functional importance of ADGRG2 in male fertility. The human and mouse *ADGRG2/Adgrg2* gene is localized on chromosome X. *Adgrg2* $^{-/Y}$ mice exhibit reduced sperm numbers, decreased sperm motility and increased number of spermatozoa with deficient heads or angulated flagella (Davies *et al.* , 2004). Moreover, dysfunction in the fluid resorption of the efferent ductules is observed, which might eventually lead to the above-mentioned phenotypes in *Adgrg2* $^{-/Y}$mice (Table 1) (Gottwald *et al.* , 2006; Zhang *et al.* , 2018).

ADGRG2 belongs to the adhesion GPCR subfamily, and all members of this family share a very large N-terminal domain(Fredriksson *et al.* , 2003; Hamann *et al.* , 2015; Hu *et al.* , 2014; Kishore *et al.* , 2017; Liebscher *et al.* , 2013; Paavola *et al.* , 2012; Paavola *et al.* , 2011; Sun *et al.* , 2013; Wang *et al.* , 2014). Many members of this family have been shown to function through G protein coupling (Folts *et al.* , 2019; Purcell *et al.* , 2018). Without known endogenous ligands, these adhesion GPCRs display significant constitutive activity once their N-terminal region is removed by autocleavage (Demberg *et al.* , 2015; Hamann

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et al. , 2015; Hu *et al.* , 2014; Kishore *et al.* , 2016; Purcell *et al.* , 2018; Sun *et al.* , 2013; Wang *et al.* , 2014; Zhang *et al.* , 2018). The transmembrane and cytoplasmic regions remained after cleavage are usually referred to as the β subunit. Our data showed that in cells overexpressing either full-length ADGRG2 or the ADGRG2-β subunit, significant constitutive Gs or Gq coupling activity was observed, which was confirmed by several parallel studies assessing artificial ligands or specific cellular contexts (Demberg*et al.* , 2015; Hamann *et al.* , 2015). These studies suggested that ADGRG2-mediated Gs or Gq signaling may play important roles in the regulation of fluid resorption in the efferent ductules and epididymis (Figure 1). However, the exact functions of G protein subtypes in maintaining the microenvironment of the efferent ductules or epididymis are still unknown, and the downstream effectors involved in controlling the luminal ion/water homeostasis balance in these tissues also remain elusive. Interestingly, immunostaining assays revealed specific expression of ADGRG2 on the apical membrane only in non-ciliated cells (in the efferent ductules) and principal cells (in the epididymis), not in ciliated cells (Kirchhoff *et al.* , 2008). The non-ciliated cells in efferent ductules are frequently referred as principal cells in the epididymis (Burkett *et al.* , 1987). Cellular expression specificity of ADGRG2 suggests a cell type-specific function of ADGRG2 in the regulation of ion/water homeostasis in the efferent ductules and epididymis. The specific expression pattern of ADGRG2 allowed us to develop a non-ciliated cell-specific labeling technique by exploiting the promoter of the*ADGRG2* gene. Using this newly developed method, we successfully isolated non-ciliated cells and showed that a diminished constitutive chloride current was the cause of the imbalanced pH state in the efferent ductules and dysfunction in fluid resorption in*Adgrg2* $^{-/Y}$ mice (Zhang *et al.* , 2018).

Further analysis combining $Gq^{-/+}$ and*Adgrg2* $^{-/Y}$ mouse models, pharmacological intervention and cell labeling techniques demonstrated that ADGRG2 regulated Cl^- and pH homeostasis through Gq-dependent coupling between the receptor and the anion channel CFTR (cystic fibrosis transmembrane conductance regulator) (Figure 1)(Zhang *et al.* , 2018). CFTR and ADGRG2 colocalized at the apical membrane of non-ciliated cells, accompanied by selective high expression of Gq in the same cells. Through coupling to Gq, ADGRG2 maintains the basic CFTR outward-rectifying current, which is required for fluid resorption and sperm maturation (Figure 1) (Zhang*et al.* , 2018). In addition to G protein signaling downstream of GPCRs, arrestins (members of a family related scaffold proteins) are known not only to mediate endocytosis of these receptors but also to perform many G protein-independent or G protein-cooperative functions (Dong *et al.* , 2017; Liu *et al.* , 2017; Smith *et al.* , 2018; Yang *et al.* , 2018; Yang *et al.* , 2017b).

Importantly, whereas disruption of β-arrestin-2 has no significant effects on the fluid resorption function, β-arrestin-1 deficiency impaired pH and Cl^- homeostasis in the efferent ductules and initial segment of the epididymis (Zhang *et al.* , 2018). Further investigation confirmed the coexistence of ADGRG2, CFTR, β-arrestin-1 and Gq in the same protein complex (Figure 1), while β-arrestin-1 deficiency abolished the colocalization of ADGRG2 and CFTR on the apical membrane. These data suggested that the ADGRG2/β-arrestin-1/Gq/CFTR supercomplex localizes at the apical membrane of non-ciliated cells and functions as a regional signaling hub, controlling fluid reabsorption and maintaining pH and Cl^- homeostasis in the efferent ductules and initial segment of the epididymis (Figure 1) (Zhang *et al.* , 2018). The ADGRG2/CFTR interaction in the epididymis represents yet another example of the functional divergence between the two β-arrestin isoforms, already established in several other tissues/organs(Lymperopoulos, 2018; Lymperopoulos *et al.* , 2019; Srivastava *et al.* , 2015). For example, in the heart,β-arrestin-1 and -2 initially thought of as functionally interchangeable, actually exert diametrically opposite effects in the mammalian myocardium.β-arrestin-1 exerts overall detrimental effects on the heart, in contrast, β-arrestin-2 is overall beneficial for the myocardium(Lymperopoulos *et al.* , 2019).

Consistent with our findings that inhibition of ADGRG2 or Gq activity caused fluid resorption dysfunction, recent clinical studies have revealed that multiple *ADGRG2* mutations are associated with male infertility. For example, p.Glu516Ter, p.Leu668ArgfsTer21, p.Arg814Ter, or p.Lys818Ter results in the absence or truncation of the seven-transmembrane domain, which might abolish receptor coupling to downstream Gq and Gs proteins and eventually lead to male infertility (Figure 2A, Table 2) (Khan *et al.* , 2018; Patat *et al.* , 2016; Yuan *et al.* , 2019). The p.Cys570Tyr missense mutation is located close to the GPS region of ADGRG2, which may affect its autoinhibitory mechanism mediated by the N-terminal subunit (Yang

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et al. , 2017a). In contrast, the p.Cys949AlafsTer81 frame shift mutation, the missense p.Lys990Glu and p.Arg1008Gln mutations produce a protein with an intact seven-transmembrane domain, but all of these mutations cause changes in the C-terminal region of ADGRG2, which may be involved in arrestin recruitment and the corresponding signaling (Figure 2A, Table 2) (Patat *et al.* , 2016; Yang *et al.* , 2017a; Yuan *et al.* , 2019). Therefore, different *ADGRG2* mutations may cause the same male infertility phenotype through distinct cellular signaling mechanisms.

Notably, the mutations of ADGRG2 in human mentioned above are clinically associated with congenital bilateral absence of the vas deferens (CBAVD). In general, CBAVD involves a complete or partial absence of the Wolffian duct derivatives. In most cases of CBAVD, it is generally presumed that the genital tract abnormality is developed by a progressive atrophy related to abnormal electrolyte ion balance and dysfunction of fluid homeostasis in the male excurrent ducts rather than agenesis. This model is supported by the link between CBAVD and mutations of the gene encoding the CFTR chloride channel (Patat *et al.* , 2016). In our recent report, we have demonstrated a functional coupling between the ADGRG2 and the CFTR serves as the key event in maintenance of the Cl^- and pH homeostasis in efferent ductules and epididymis,of which a persistent dysfunction may finally cause progressive atrophy of the efferent/epididymis ductules (Zhang *et al.* , 2018). Thus, the impairment of the ADGRG2/CFTR coupling may directly relate to the CBAVD in the male infertility patients.

It's worth noting that the infertile patients are usually identified at their adult age, whereas the animal model normally has a shorter life span. This could explain the ADGRG2 knockout mice did not develop the CBVAD in their life time. For an ADGRG2-targeted therapy for treating male infertility, a systematic screening for male sterility gene, and the identification of the genetic mutations in ADGRG2 or CFTR, as well as genetic or pharmacological intervening in the early stage of a male patient carrying the mutations could be considered.

Currently, the endogenous ligands for ADGRG2 are still unknown. However, the ADGRG2 β-subunit itself shows significant constitutive G protein activity and is able to activate the CFTR current in transfected HEK293 cells (Zhang *et al.* , 2018). Therefore, further investigation is needed to determine whether constitutive ADGRG2 activity is sufficient to maintain the microenvironment of the epididymis and efferent ductules or whether an endogenous ADGRG2 ligand is required in this process. It is worth noting that a 15-amino acid peptide derived from the N-terminus of the ADGRG2 β-subunit was shown to activate ADGRG2 with low affinity (Table 3) (Demberg *et al.* , 2015). Further modification of ADGRG2 ligands derived from this peptide might increase the activity of certain ADGRG2 mutants and exhibit therapeutic potential. Alternatively, we have also shown that activation of angiotensin II receptor type 2 (AGTR2) in the efferent ductules is able to rescue fluid resorption dysfunction in isolated efferent ductules derived from *Adgrg2*$^{-/Y}$ mice (Zhang *et al.* , 2018). Thus, further investigation is warranted to determine whether specific therapeutic methods such as treatment with a selective agonist need to be developed for different ADGRG2 mutants or whether a general rescue approach such as AGTR2 activation is sufficient to treat patients carrying *ADGRG2* mutations.

Endogenous angiotensin system and AGTR2 in epididymis

The epididymal lumen and efferent ductules contain a complete local renin-angiotensin system (RAS) including renin, angiotensin I (ANGI) and angiotensin II (ANGII) in the seminal fluid, the angiotensin-converting enzyme specific to the testes (tACE), and angiotensin II receptor type 1 (AGTR1) and angiotensin II receptor type 2 (AGTR2) in the basal cells of the epididymis (Leung *et al.* , 2003; Saez *et al.* , 2004; Speth *et al.* , 1999; Wong *et al.* , 1990; Zhao *et al.* , 1996). Importantly, ANGII in the epididymal lumen is mainly produced through the cleavage of ANGI by angiotensin I-converting enzyme (ACE) (Langford *et al.* , 1993; Sibony *et al.* , 1994). Deficiency in tACE leads to male infertility through impairing the function but not the production of sperm, implying that the RAS plays an important role in sperm maturation (Esther *et al.* , 1996; Hagaman *et al.* , 1998; Krege *et al.* , 1995).

AGTR1 and AGTR2 have been found in a radio-ligand binding assay to be expressed in the epididymal

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lumen. In particular, AGTR2 was specifically detected in basal cells and found to be required for the proton-secretion function of the epididymal lumen (Figure 1 and 2B, Table 1) (Shum *et al.* , 2008). Unexpectedly, AGTR2 was absent in clear cells, which regulated proton secretion. Further studies showed that AGTR2 activated the nitric oxide (NO)-cGMP pathway in response to ANGII stimulation in basal cells (Figure 1). NO produced by basal cells quickly diffuses to clear cells, activating soluble guanylate cyclase. Then, the elevation of the cGMP concentration mediated by guanylate cyclase triggers the apical accumulation of V-ATPase in the microvilli, ultimately leading to increased proton secretion (Figure 1) (Shum *et al.* , 2008). This model is consistent with the essential role of ANGII production and the requirement for tACE in the maintenance of the proper luminal ion/water environment and sperm maturation. Thus, a delicate signaling network between basal cells and adjacent clear cells modulated by the receptor AGTR2 may contribute to the finely tuned microenvironment of the luminal space of the epididymis.

Interestingly, male infertility may result from dysfunction in the proton balance in the efferent ductules without significant impairment of AGTR2 function, suggesting that an AGTR2-targeted treatment may have therapeutic potential. In our recent study, although administration of 1 μM ANGII had no significant effect, applying 100 nM ANGII restored pH homeostasis and fluid reabsorption in efferent ductules derived from*Adgrg2* $^{-/Y}$ mice. This rescue effect was blocked specifically by PD123319, an AGTR2 antagonist, but not by an ANGII antagonist (Zhang *et al.* , 2018). Therefore, the specific agonists of AGTR2 could be considered as therapeutic drugs to treat male infertility associated with a significant impairment in the pH balance in the efferent ductules or epididymis.

For AGTR2, both peptide-based agonists and small chemical compound agonists have been developed, which have therapeutic potential to treat several human diseases (Table 3) (Bennion *et al.* , 2018; Hallberg *et al.* , 2018). Sarile and saralasin are two peptide AGTR2 agonists that have been approved by the FDA to treat hypertension and used in the clinic for a short period (Table 3) (Guimond *et al.* , 2014; Hallberg *et al.* , 2018). These peptides inactivate AGTR1 but activate AGTR2. Currently, it remains unknown whether the blockade of AGTR1 activity is dispensable for the normal function of the efferent ductules or epididymis. Therefore, the application of these two peptides for the treatment of sperm obstruction in male infertility requires further evaluation. Recently, β-Pro^{7}AngIII was reported to show high selectivity for the activation of AGTR2 but no significant effect on AGTR1 (Hallberg *et al.* , 2018), providing an alternative choice for peptide-based AGTR2 activation therapy in male infertility. Small-molecule compounds have also been developed to target AGTR2 activation for clinical treatment. For example, MP-157 was used as an AGTR2 agonist for cardiovascular disease treatment in a phase I clinical trial, whereas C21/M24 was examined in a phase II exploration of idiopathic pulmonary fibrosis (IPF) (Table 3) (Hallberg *et al.* , 2018). Testing these small-molecule compounds or their derivatives will be of great interest for developing treatment for male infertility related to impaired pH homeostasis in the efferent ductules or epididymis.

LGR4, an essential GPCR for epididymal development

LGR4, also called G protein-coupled receptor 48 (GPR48), is a member of the LGR subgroup of the rhodopsin-like GPCR superfamily, which derives its name from a large extracellular domain consisting of multiple leucine-rich repeats (Figure 2C). LGR4 is widely expressed in multiple human and mouse tissues, with the highest expression levels in the epidermis and hair follicles of the skin, pancreatic islet cells, and epithelial cells in the male and female reproductive organs (Van Schoore *et al.* , 2005; Yi *et al.* , 2013).

LRG4 has been shown to play an important role in postnatal epididymal development in mice. In *Lgr4* knockout mice, the epididymal tubule, especially the caput region, fails to elongate and convolute, and the resulting duct is surrounded by a thick condensation of mesenchymal cells. This abnormal cellular organization suggests that LGR4 is important for epithelial-mesenchymal interactions (Table 1) (Mendive *et al.* , 2006). Furthermore, the expression levels of estrogen receptor α (ERα) and androgen receptor (AR) are dramatically reduced in the epididymis of male *Lgr4* knockout mice, which in turn leads to decreased expression of Na^+-K^+-ATPase, Na^+/H^+hydrogen exchanger 3 (NHE3), and aquaporin 9 (Aqp9) (Li *et al.* , 2010). LRG4 upregulates ERα expression via the cAMP/PKA signaling pathway (Figure 1). Downstream of the LRG4-cAMP-PKA pathway, CREB binds to a Cre motif in the ERα promotor and activates its

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expression (Li *et al.* , 2010).

The pivotal role of LGR4 in the epididymis is further supported by a*Lgr4* hypomorphic mutant mouse line ($Lgr4^{Gt}$) that was developed through gene-trap insertional mutagenesis. Short and dilated epididymal tubules are detected in homozygous $Lgr4^{Gt/Gt}$ mice, which have only one-tenth the normal *Lgr4* expression level. Moreover, multilamination and distortion of the basement membranes (BMs) is observed in the caput region, and the initial segment is completely lost (Hoshii *et al.* , 2007). *Lgr4* knockout or hypomorphic mice also show deficits in the testes and efferent ductules (Qian *et al.* , 2013), which together with the epididymal defects eventually lead to male infertility in mice.

Overexpressed LGR4 has been found to activate heterotrimeric Gs proteins to elevate intracellular cAMP levels (Gao*et al.* , 2006). Moreover, R-spondins and norrin were identified as LGR4 ligands that could bind LGR4 and stimulate the Wnt signaling pathway (Table 3) (Carmon *et al.* , 2011; de Lau *et al.* , 2011; Deng *et al.* , 2013; Glinka *et al.* , 2011). Recently, tumor necrosis factor (TNF) superfamily member 11 (TNFSF11, also known as RANKL) was identified as a novel LGR4 ligand (Table 3) (Luo *et al.* , 2016). TNFRSF11A (also called RANK) was considered to be the sole receptor for TNFSF11 until LGR4 was found to compete with RANK and suppress canonical RANK signaling. TNFSF11 binds to LGR4 and subsequently activates the Gq and glycogen synthase kinase 3 beta (GSK3-β) signaling pathway (Luo *et al.* , 2016). At present, synthesized agonists or antagonists of LGR4 have not been reported.

Complex functions ofG protein-coupled estrogen receptor 1 (GPER) in the epididymis

GPER, also known as G protein-coupled receptor 30 (GPR30), was first identified as a receptor that demonstrated MAP kinase (Erk1/2) activation by binding to estrogen (Prossnitz *et al.* , 2007). Compounds such as the GPER antagonist fulvestrant (ICI 182780) and GPER agonist G-1 can also modulate GPER to induce rapid nongenomic cellular responses (Bologa *et al.* , 2006; Lucas *et al.* , 2010; Revankar *et al.* , 2005). Unlike the other members of the GPCR family that mainly reside on the plasma membrane, GPER is broadly localized on the endoplasmic reticulum and nuclear envelope as well as the plasma membrane (Figure 1) (Funakoshi *et al.* , 2006; Prossnitz *et al.* , 2007; Thomas *et al.* , 2005).

GPER has been detected in many male reproductive structures, such as the testes (Cassault-Meyer *et al.* , 2014; Gautier *et al.* , 2016; Lucas *et al.* , 2010), spermatozoa (Arkoun *et al.* , 2014; Cassault-Meyer *et al.* , 2014; Gautier *et al.* , 2016), and prostate (Rago *et al.* , 2016). It has also been found in the efferent ductules and epididymis (Cao *et al.* , 2017; Hess *et al.* , 2011; Katleba *et al.* , 2015; Krejcirova *et al.* , 2018; Lu *et al.* , 2016; Malivindi *et al.* , 2018; Martinez-Traverso *et al.* , 2015; Menad *et al.* , 2017; Pereira *et al.* , 2014; Rago *et al.* , 2018), indicating that GPER may play important roles in sperm maturation, protection and storage (Table 1). For instance, in the corpus epididymis of postnatal pigs, GPER participates in sperm maturation by affecting the formation of the blood-epididymal barrier (Katleba*et al.* , 2015). In the caudal epididymal epithelium in immature rats, GPER induces a pathway involved in cAMP-CFTR-chloride secretion to regulate osmotic pressure in response to a perfusion solution and thus affects sperm motility (Figure 1) (Cao*et al.* , 2017).

In addition, the relative abundance of GPER in the efferent ductules and each part of the epididymis, the cellular localization of GPER, and the molecular weight of the protein differ depending on the species, developmental stage, and physiological cycle studied (Krege *et al.* , 1995; Krejcirova *et al.* , 2018; Lu *et al.* , 2016; Pereira *et al.* , 2014). Therefore, the role of GPER in the efferent ductules and epididymis appears to be complex. The first GPER-specific agonist, G-1, has been identified through virtual and biomolecular screening (Table 3) (Bologa *et al.* , 2006). Based on the synthesis of the G-1 analog as well as additional screening, two GPER-specific antagonists, G15 and G36, were also identified, both of which inhibit estrogen- and G-1-stimulated cell proliferation *in vivo* (Table 3) (Dennis *et al.* , 2009; Dennis *et al.* , 2011). Recently, a series of indole-thiazole derivatives were identified as new GPER agonists (O'Dea *et al.* , 2018). These newly identified agonists and antagonists provide very useful tools for further evaluation of the therapeutic potential of GPER in treating male infertility, given the potential complex function of GPER in male systems. Overall, the evaluation of GPER as a drug target in male infertility requires further investigation, and the

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new compounds identified for specific regulation of GPER activity will certainly accelerate this assessment.

Two adenosine receptors with opposite functions in the epididymis

Adenosine receptors consist of four members, namely, A_1, A_{2A}, A_{2B}, and A_3. Adenosine receptors are activated by adenosine and transmit signals through classic G protein-cAMP or β-arrestin pathways (Table 1) (Geldenhuys *et al.* , 2017). Most adenosine receptors have been suggested to be present in the epididymis (Table 1) (Haynes *et al.* , 1998b; Minelli *et al.* , 1995).

The A_1 and A_2 adenosine receptors have been shown to regulate the contractility of the vas deferens and epididymis (Table 1) (Brownhill *et al.* , 1996; Haynes *et al.* , 1998a; Haynes *et al.* , 1998b). Interestingly, it seems that the A_1 and A_2 receptors have opposite effects on the contractility of the epididymis: the A_1 receptor enhances the contractility, whereas the A_2 receptor inhibits the contractility (Haynes *et al.* , 1998b). This phenomenon might be explained by the difference in their G protein-coupling selectivity (van Galen*et al.* , 1992). In the epididymis, A_2 adenosine receptors increase intracellular cAMP levels (Haynes *et al.* , 1998b), consistent with the generally accepted view that A_2 adenosine receptors are coupled to Gs-protein and activate adenylyl cyclase to increase intracellular cAMP levels (Figure 1) (Fredholm *et al.* , 1994). Further investigation showed that the A_{2A} receptor mediates potassium channel activation through protein kinases A and G in rat epididymal smooth muscle (Haynes, 2000). This result is consistent with the finding that A_2receptor activation stimulated cAMP-dependent protein kinase A, which in turn modulated potassium channel activity in arterial or skeletal muscles (Barrett-Jolley *et al.* , 1996; Kleppisch *et al.* , 1995). In contrast, the A_1 adenosine receptor is likely coupled to effectors through Gi/o proteins, although confirmative evidence is still lacking (Haynes *et al.* , 1998b).

Adenosine (and its precursor ATP) has been used for several decades to treat cardiac arrhythmias through activating A_1adenosine receptors (Szentmiklosi*et al.* , 2015). Adenosine is also the gold-standard agent to create maximum coronary hyperemia through activating A_{2A}adenosine receptors (McGeoch *et al.* , 2008). However, given that adenosine can activate various adenosine receptors, it inevitably produces some undesirable adverse effects. To avoid nonspecific global adverse reactions, selective agonists of A_1, A_{2A}, and A_3 adenosine receptors have been developed, some of which are currently undergoing clinical trials (Jacobson *et al.* , 2019). For example, the A_1 adenosine receptor partial agonist trabodenoson (INO-8875) was tested for the treatment of glaucoma and ocular hypertension, but it failed in a phase 3 trial because its primary endpoint was not achieved (Table 3) (Jacobson *et al.* , 2019). The moderately selective A_{2A} adenosine receptor agonist regadenoson was first approved as a pharmacological stress agent in 2008 and is currently being tested in various clinical trials for cardiovascular treatment and diagnosis (Table 3) (Jacobson *et al.* , 2019). The moderately selective A_3 adenosine receptor agonist IB-MECA (CF101, piclodenoson) is being tested in a phase 3 clinical trial for the treatment of autoimmune anti-inflammatory diseases (Table 3) (Jacobson *et al.* , 2019).

An important limitation of adenosine receptor agonists is agonist-induced desensitization (Mundell*et al.* , 2011). The application of either partial agonists or positive allosteric modulators (PAMs) may circumvent desensitization and improve therapies. Currently, only adenosine and regadenoson are approved for human use (Jackson *et al.* , 2018). However, many adenosine receptor agonists and PAMs (such as the A_1 adenosine receptor PAM benzoylthiophenes) are being tested in humans, and it is of great interest to test the effects of these compounds on the regulation of epididymis functions and the treatment of male infertility.

Future questions and perspectives

Numerous GPCRs are expressed in the efferent ductules and epididymis, which consist of various cell types. Thus, the following questions arise. (1) Which GPCRs are expressed in a particular cell type? (2) How do these GPCRs contribute to the development and normal physiological functions of the epididymis and efferent ductules? (3) Can any of these GPCRs functionally compensate for each other? (4) If so, is it possible to activate an alternative GPCR in the epididymis or efferent ductules to rescue the dysfunction of a particular GPCR, such as in cases of infertility caused by ADGRG2 mutations? (5) Is there crosstalk between different GPCRs or between GPCRs and other membrane proteins in specific cell types? (6) Are endogenous ligands

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of the GPCRs in epididymis and efferent ductules constantly produced in the local environment to actively regulate specific physiological processes of epididymis development and sperm maturation? (7) Do second messengers downstream of GPCRs, such as cAMP and calcium, have distinct functions in different types of cells in the epididymis and efferent ductules, and how are they regulated by different GPCRs? (8) Are location bias (signaling compartments) and effector bias important for the regulation of different GPCRs expressed in the epididymis and efferent ductules? (9) What are the endogenous ligands for ADGRG2, AGTR2, GPER and LGR4 in the local male fertility system? (10) Do FDA-approved drugs targeted to GPCRs with known functions in the epididymis, such as AGTR2 and adenosine receptors, have beneficial effects on male fertility? (11) Are there regional drug delivery systems that can target specific GPCRs in the epididymis to decrease the side effects of GPCR ligands? To answer these questions, a systematically investigation of the GPCR expression in epididymis and efferent ductules by transcriptional analysis and the single cell sequencing; utilization of the conditional knock mice driven by the specific epididymis or efferent ductile marker Cre; combined with the molecular and cellular approaches to delineate the mechanism underlying the specific GPCR functions in male infertility and the usage of the biochemical approach and the proteomics and metabolomics to identify the endogenous ligands for specific GPCR such as the ADGRG2, will lay an important foundation for evaluation of these GPCRs as potential therapeutic targets for male infertility treatment. Moreover, usage of the specific known chemical ligands for these GPCRs, united by the selective drug delivery methods and assessment of the effects of these ligands in male infertility mice models will provide further information for drug development toward these GPCRs.

Conclusions

(1) Male infertility rates have continuously increased in recent years, and few effective treatments with known targets and defined mechanisms exist. Recently, the identification of mutations in specific GPCR superfamily members related to male infertility and the increased understanding of the detailed molecular mechanisms involving these GPCRs in the regulation of sperm maturation and homeostasis of the microenvironments of the epididymis and efferent ductules have provided new clues on the potential development of therapies to treat male infertility, given that these receptors account for almost 1/3 of current clinical drug targets.

(2) In addition to ADGRG2 and AGTR2, GPCR superfamily members such as LGR4, GPER, and adenosine receptors are known to play important roles in the regulation of postnatal epididymal development, the formation of the blood-epididymal barrier, the maintenance of osmotic pressure in a perfusion solution and the contractility of the epididymis (Table 1). The repertoire of the physiological roles of these GPCRs and other uncharacterized GPCRs, as well as further detailed studies of these receptor connecting to male infertility development, provide entirely novel therapeutic opportunities for the treatment of male infertility.

(3) Currently, various small-molecule compounds, peptide ligands and endogenous ligands have been found or developed to target AGTR2, LGR4, GPER and adenosine receptors (Table 3). It is worth noting that several such compounds or ligands have been approved by the FDA for the treatment of diseases other than male infertility. Therefore, there is great interest in testing these ligands and compounds in male infertility animal models to examine their therapeutic potential. It is also worth noting that endogenous or high-affinity ligands involved in the regulation of ADGRG2 have not been identified. Such tools are greatly needed to understand the function of ADGRG2 in male fertility and evaluate the potential role of ADGRG2 as a therapeutic target in male infertility.

(4) Only a small number of the signaling pathways downstream of GPCRs have been characterized in detail in the efferent ductules and epididymis, and these pathways have shown unique signaling properties, although they sometimes share signal-transducing effectors (Figure 1). For example, both ADGRG2 and GPER have been shown to couple to Gs in the epididymis; however, they exhibit distinct subcellular microdomain biases in their signaling. ADGRG2 forms a signal transduction complex with β-arrestin-1, Gq and CFTR on the apical membrane, whereas GPER forms a complex with Gs at the endoplasmic reticulum, nuclear envelope and plasma membrane (Figure 1). Therefore, even when sharing effectors, the location bias of each GPCR may determine its detailed specific functions in the epididymis and efferent ductules. This possibility raises the question of whether activation of an alternative GPCR in the epididymis or efferent ductules will be

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able to rescue the dysfunction of a particular GPCR, such as in cases of infertility caused by the ADGRG2 mutations.

(5) Collectively, the complex signaling of GPCR members in the epididymis and the specific physiological roles of these GPCRs that contribute to male fertility are worthy of further detailed investigation. In addition, the prospect of using their ligands highlights new opportunities for potential therapies development for male infertility.

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Figure legends

Figure 1. Schematic showing GPCR signaling and functions in the epididymis and efferent ductules.

Above: The efferent ductules are a series of tubules that connect the rete testis to the epididymis. The epithelia of the efferent ductules are mainly composed of two cell types, ciliated cells and non-ciliated cells. The epididymis is composed of one highly convoluted tubule. The epididymis is segmented morphologically and functionally into following distinct regions: the initial segment (not existing in human epididymis), the caput, the corpus, and the cauda. Each part consists of several cell types, including principal cells, narrow cells, clear cells, and basal cells. Inset: G protein-coupled estrogen receptor 1 (GPER) activates cAMP-CFTR-chloride transportation to maintain the osmotic pressure of the perfusion solution. ADGRG2 is located exclusively on the apical membrane in non-ciliated cells. ADGRG2/β-arrestin-1/Gq/CFTR forms a supercomplex that maintains pH and chloride anion homeostasis. AGTR2 is specifically detected in basal cells and is essential for the proton-secretion function of the epididymal lumen through activation of the nitric oxide (NO)-cGMP pathway. Different members of the adenosine receptor family have opposite effects on the contractility of the epididymis. LGR4 activates Gs to increase intracellular cAMP levels, which promote ERα expression.

Figure 2. GPCR mutations associated with disease.

Schematic representation of the structures of ADGRG2 (A), AGTR2 (B), and LGR4 (C). The approximate positions of different mutations are indicated. Abbreviations: PLL domain, pentraxin/laminin/neurexin/sex-hormone-binding-globulin-like domain; GPS, G protein-coupled receptor proteolytic site; LRR, leucine-rich repeats.

Tables

Table 1. GPCRs with known functions in epididymis or efferent ductules

Receptor name	Family (GRAFS) name	Expression	F
ADGRG2(GPR64)	Adhesion	efferent ductules; proximal epididymis (non-ciliated cells; principal cells)	f
AGTR2	Rhodopsin	basal cells	p
LGR4 (GPR48)	Rhodopsin	epithelial cells in the reproductive organs	e

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Receptor name	Family (GRAFS) name	Expression	F
GPER (GPR30)	Rhodopsin	testis; spermatozoa; prostate; efferent ductules; epididymis	f
Adenosine receptor	Rhodopsin	epididymis	c

Table 2. Disease-related SNP analysis in GPCRs

GPCR	dbSNP rs# cluster id	dbSNP allele change	Protein residue change	Amino acid pos	Ac
ADGRG2(GPR64) Xp22.13	rs879255540	->T	Glu [E]>*	516	Co
		G>A	Cys [C]>Tyr [Y]	570	Co
	rs879255539	CTGTG>AGA	Leu [L]>Arg [R]	668	Co
		C>T	p.Arg [R]>*	814	Ot
		A>T	p.Lys [K]>*	818	Co
	rs879255538	T>-	Cys [C]>Ala [A]	949	Co
		A>G	p.Lys [K]>Glu [E]	990	Co
		G>A	p.Arg [R]>Gln [Q]	1008	Co
AGTR2 Xq23	rs121917810	G>T	Gly [G]>Val [V]	21	X-
		T>-	Phe [F]>Leu [L]	134	no
	rs5191	G>A	Arg [R] >Lys [K]	248	no
LGR4(GPR48) 11p14.1	rs587777005	C>T	Arg [R]>*	126	Lo

Table 3. Potential therapeutic ligands targeting to GPCRs in epididymis

Receptor	Ligand	Structure (or Sequence)	Mode of action	Highest status	References
ADGRG2	Tethered peptide agonist	TSFGILLDLSRTSLP	Agonist		Demberg et al., 2015
AGTR2	Angiotensin II (ANG II)	Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8	Agonist	Clinic	Guimond et al., 2014; Hallberg et al., 2018
	Saralasin	[Sar^1,Val^5,Ala^8]Ang II	Agonist	Clinic	Guimond et al., 2014; Hallberg et al., 2018
	Sarile	[Sar^1,Ile^8]Ang II	Agonist	Clinic	Guimond et al., 2014; Hallberg et al., 2018
	MP-157	No structural formula is disclosed	Agonist	Phase I	Hallberg et al., 2018
	C21/M24		Agonist	Phase II	Hallberg et al., 2018
	C38/M132		Antagonist		Hallberg et al., 2018

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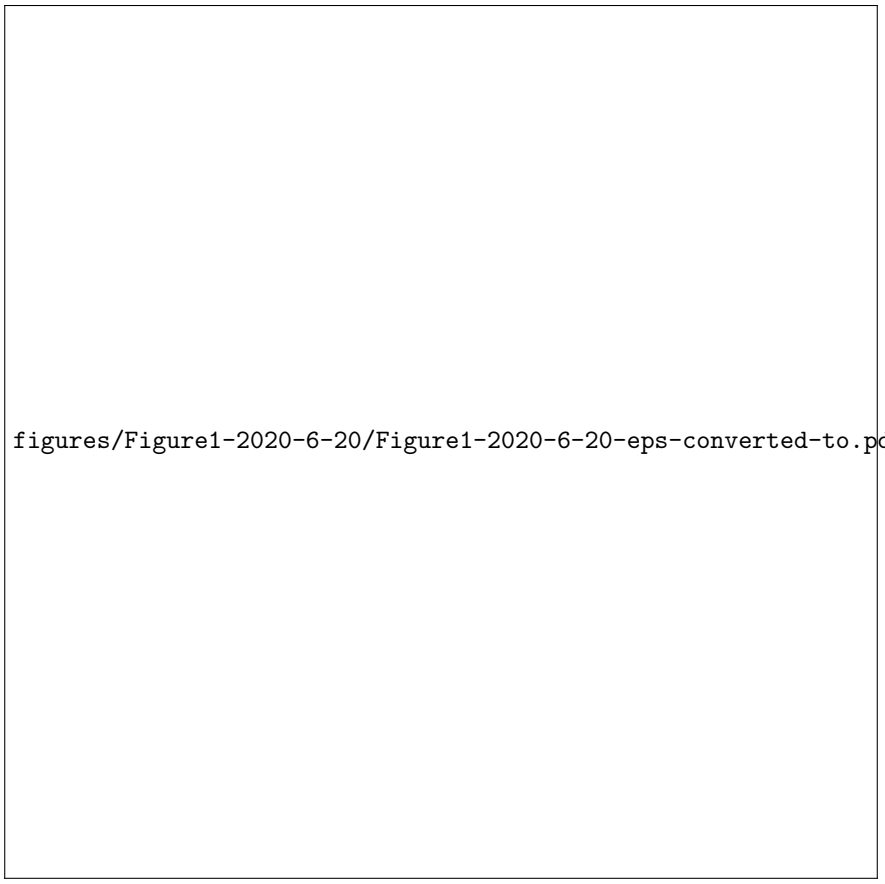
Receptor	Ligand	Structure (or Sequence)	Mode of action	Highest status	References
LGR4	R-spondins	R-spondin1-4(RSPO1-4)	Agonist		Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011
	Norrin	MRKHVLAASFSMASLLVTMGDTDSKTDSSFIMDSDPRRQMGHHYVDSISHPLYE	Agonist		Ohlmann et al., 2013
	TNFSF11(RANKL)	Tumor necrosis factor (TNF) superfamily member 11	Agonist		Luo et al., 2016
GPER	G-1		Agonist		Bologa et al., 2006
	G15		Antagonist		Dennis et al., 2009; Dennis et al., 2011
	G36		Antagonist		Dennis et al., 2011
A_1AR	Trabodenoson (INO-8875)		partial agonist	Phase III	Jacobson et al., 2019
$A_{2A}AR$	Regadenoson		agonist		Jacobson et al., 2019
A_3AR	IB-MECA		agonist	Phase III	Jacobson et al., 2019



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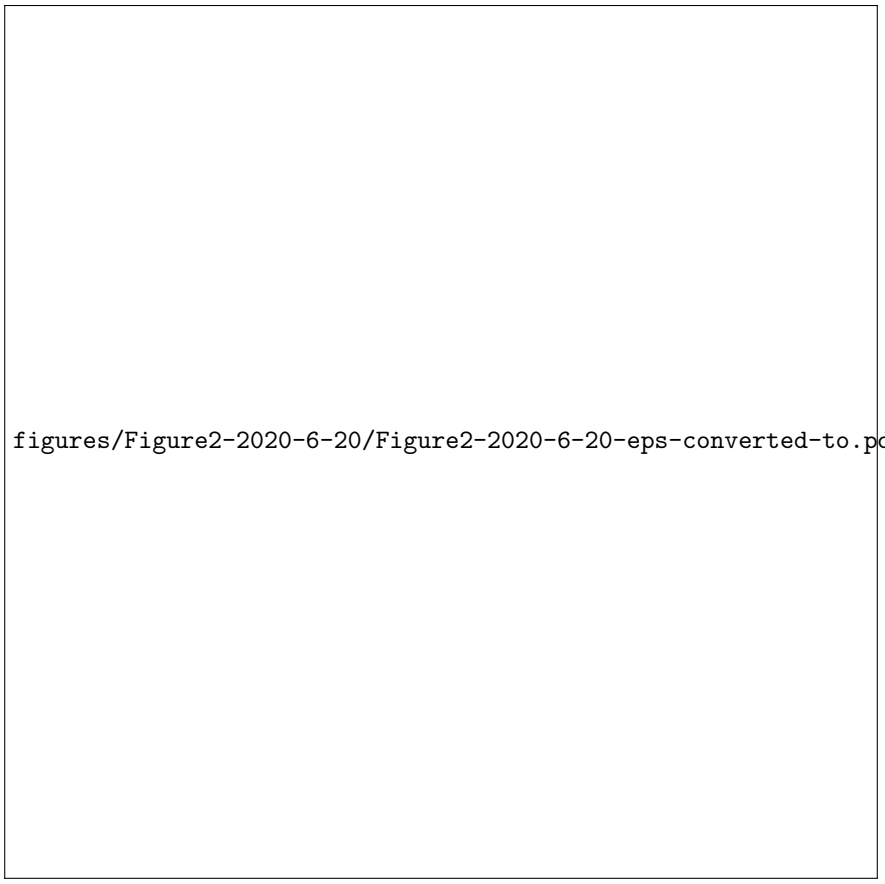

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