REVIEW

Diverse functions of insulin-like 3 peptide

Maria Esteban-Lopez¹ and Alexander I Agoulnik¹,²

¹Department of Human and Molecular Genetics, Herbert Wertheim College of Medicine, Miami, Florida, USA
²Biomolecular Science Institute, Florida International University, Miami, Florida, USA

Correspondence should be addressed to A I Agoulnik: aagoulni@fiu.edu

Abstract

Insulin-like 3 peptide (INSL3) is a member of the insulin-like peptide superfamily and is the only known physiological ligand of relaxin family peptide receptor 2 (RXFP2), a G protein-coupled receptor (GPCR). In mammals, INSL3 is primarily produced both in testicular Leydig cells and in ovarian theca cells, but circulating levels of the hormone are much higher in males than in females. The INSL3/RXFP2 system has an essential role in the development of the gubernaculum for the initial transabdominal descent of the testis and in maintaining proper reproductive health in men. Although its function in female physiology has been less well-characterized, it was reported that INSL3 deletion affects antral follicle development during the follicular phase of the menstrual cycle and uterus function. Since the discovery of its role in the reproductive system, the study of INSL3/RXFP2 has expanded to others organs, such as skeletal muscle, bone, kidney, thyroid, brain, and eye. This review aims to summarize the various advances in understanding the physiological function of this ligand–receptor pair since its first discovery and elucidate its future therapeutic potential in the management of various diseases.

Introduction

RXFP2 (previously known as GREAT or LGR8) is a GPCR from the relaxin family peptide receptors, which also contains three other members: RXFP1, RXFP3, and RXFP4 (Halls et al. 2007b). Relaxin is the cognate ligand for RXFP1 and is the most studied ligand–receptor pair of the family, being well-known for its vasodilator and antifibrotic properties. The neuropeptide Relaxin-3, the cognate ligand for RXFP3, has a role in stress and feeding responses, while INSL5 is the cognate ligand for RXFP4 and is involved in gut contractility. RXFP1 and RXFP2 are structurally highly similar and share 60% amino acid sequence identity (Halls et al. 2007b). The RXFP2 cognate ligand is INSL3, which is produced as a preprohormone which, after removal of the signal peptide and cleavage of the C-peptide, gives rise to the mature active hormone consisting of A and B chains linked by two disulfide bonds and an additional disulfide bond within the A chain. INSL3 activation of the RXFP2 receptor causes an increase in cAMP production (Kumagai et al. 2002, Rosengren et al. 2006). Relaxin peptides from some species, such as porcine relaxin (Lin et al. 2004) and human H2 relaxin (Halls et al. 2005), are capable of activating this receptor as shown in cAMP assays in vitro, but only at concentrations far above physiological levels. Interestingly, mouse and rat relaxin do not activate RXFP2 (Halls et al. 2005, Bathgate et al. 2006).

The effect of the INSL3/RXFP2 system on male reproductive tract development was first discovered two decades ago in gene-deficient male mice with cryptorchidism or undescended testes (Nef & Parada 1999, Zimmermann et al. 1999, Overbeek et al. 2001). In humans, this genital malformation has an overall
incidence of around 1–3% in newborn boys and is more common in those who are born prematurely (Kurz 2016). Spontaneous resolution of this abnormality by 1 year of age was reported in less than 10% to more than 50% of affected boys in different studies (Berkowitz et al. 1993, Wenzler et al. 2004). For others, the most common treatment is an orchiopexy, a surgical procedure where the undescended testes are brought into the scrotum. If left untreated, cryptorchidism can lead to infertility and testicular cancer (Ferguson & Agoulnik 2013, Kurz 2016). In females, INSL3 is involved in ovarian follicle maturation and could play a role in the pathology of polycystic ovary syndrome (PCOS) (Pelusi et al. 2013). In addition, a recent line of investigation has elucidated the anabolic functions of the INSL3/RXFP2 system in osteoblasts, skeletal muscles, and other organs, highlighting the role of this ligand–receptor pair outside reproductive physiology (De Toni et al. 2019). The unique RXFP2 structural features, cell surface expression, and confined expression pattern make it a potentially desirable pharmacological target for various diseases, including osteoporosis and hypogonadism, although more work has to be done to further characterize INSL3/RXFP2 signaling and functions.

Mechanisms of INSL3 binding and activation of RXFP2

INS3 treatment of HEK293T cells transfected with RXFP2 causes activation of the classical adenyl cyclase (AC) pathway via activation of $G_{s}$, resulting in increased cAMP production. At its N-terminus, RXFP2 contains a large extracellular domain with a low-density lipoprotein domain (LDLa) joined with a linker to a ten leucine-rich repeat domain (LRR), followed by a seven transmembrane helical domain (TM) (Fig. 1). Within the GPCR family, the LDLa module is only present in RXFP1 and RXFP2 (Halls et al. 2007b). Mutagenesis studies have shown the importance of the module for receptor expression in the cell surface and cell signaling.
have been designed through systematic efforts to induce α-helicity in the isolated INSL3 B-chain by constraining the B-chain with a short region of A-chain or dimerizing the B-chain (Shabanpoor et al. 2010).

**RXFP2 cellular signaling**

Signal transduction has been primarily studied in HEK293T cells transfected with RXFP2. Stimulation of RXFP2 in these cells results in its coupling to Gαs, which leads to cAMP production and further activates CRE-dependent gene transcription (Halls et al. 2007a). On the other hand, the coupling of Gαi3 also occurs, which is involved in negative modulation of cAMP accumulation in HEK293T cells (Halls et al. 2006). Unlike in relaxin/RXFP1 signaling, there is no evidence of RXFP2 signaling in HEK293T cells through the Gαi3 isof orm, which inhibits cAMP production (Halls et al. 2009) (Fig. 1).

Additional studies performed on cells that endogenously express RXFP2 have provided insight into cell-dependent responses to INSL3 treatment. Rat gubernacular and mouse Leydig cells showed increased production of cAMP upon stimulation with INSL3 (Kumagai et al. 2002, Pathirana et al. 2012), while the opposite effect was reported in rat female and male primary germ cells (Kawamura et al. 2004). Stimulation with INSL3 resulted in an inhibition of intraoocyte cAMP levels and forskolin-induced cAMP in seminiferous tubular cells. Studies using myotubes derived from C2C12 cells have shown increased phosphorylation of AKT, mTOR, and S6 after INSL3 treatment, identifying a possible downstream cAMP pathway activated by INSL3/RXFP2 (Joglekar et al. 2018). In the human osteoblast cell line MG-63, INSL3 stimulation also increased intracellular cAMP production (Joglekar et al. 2008). Further investigations of INSL3/RXFP2 signaling in primary human osteoblasts have shown increased MEK and ERK1/2 phosphorylation via the AC/cAMP/PKA pathway (Joglekar et al. 2011). Surprisingly, a classical regulatory mechanism of GPCR signaling, beta-arrestin-induced internalization and desensitization, does not appear to be a major factor in RXFP2 regulation. RXFP2 has a sustained cAMP response when stimulated with INSL3, which may be due to reduced recruitment of beta-arrestin to the cell surface and attenuated internalization of the receptor (Callander et al. 2009).

To date, only a few studies have been performed to analyze whole transcriptome changes in response to INSL3. To identify downstream pathways activated by INSL3/RXFP2 signaling, rat gubernacular bulb cells
isolated from embryos on day 17 were treated with INSL3 and analyzed using Affymetrix microarrays (Johnson et al. 2010). Male gubernacular bulb cells responded to INSL3 with changes in expression of genes involved in cAMP, BMP, WNT/β-catenin, pluripotency and several other signaling pathways, including genes playing a role in osteoblasts, osteoclasts, and chondrocyte participation in rheumatoid arthritis. As expected, gene ontology (GO) analysis revealed changes in the expression of genes involved in GPCR signaling, extracellular, and plasma membrane functions. In addition, several GO related to neurogenesis were altered by INSL3 (Johnson et al. 2010).

More studies need to be conducted to fully understand the signaling mechanisms of INSL3/RXFP2 in various cells and their biological significance. This is also important for efficient targeting of this receptor in future therapeutic applications, providing for the specificity of response while avoiding potential harmful off-target effects.

**INSL3 in male reproductive physiology**

In mammalian male reproductive organs, INSL3 is produced mainly in the testicular Leydig cells (Ivell et al. 1997, Balvers et al. 1998, Zarreh-Hoshyari-Khah et al. 1999, Pitia et al. 2017) and RXFP2 is expressed in testis in Leydig cells as well as in germ cells, especially at the postmeiotic stage (Feng et al. 2007, Huang et al. 2012, Pitia et al. 2017). INSL3 is considered an indicator of normal Leydig cell function and overall reproductive health in men (Ivell et al. 2013). Human male fetuses produce INSL3 during gestation, measured at approximately 0.12 ng/mL in amniotic fluid (Bay et al. 2008). This level of circulating INSL3 is maintained from birth until the age of 3 months, at which point it begins to decrease (Bay et al. 2007). The INSL3 level rises again through puberty and is sustained between 0.5 and 1 ng/mL in adult men (Bay et al. 2005). During puberty, luteinizing hormone (LH) drives Leydig cell maturation, which coincides with a spike in INSL3 production that positively correlates with an increase of sex hormones such as follicle stimulating hormone (FSH), LH, and testosterone (Ferlin et al. 2006). Low INSL3 serum levels are characteristic of orchidectomized and infertile men, individuals with Klinefelter’s syndrome, hypogonadotropic hypogonadism, and cryptorchidism (Bay et al. 2005, Ferlin & Foresta 2005). Males with anorchism have no detectable levels of INSL3 (Bay et al. 2005). To further investigate the correlation between INSL3 and LH, subjects with unilateral orchietomy were treated with a single dose of human chorionic gonadotropin (hCG), resulting in elevated testosterone levels around 3 days after treatment but no impact on INSL3 levels (Bay et al. 2005). However, when hypogonadotropic hypogonadism subjects were treated with repeated doses of hCG over several months, INSL3 levels increased (Bay et al. 2005). In agreement with these findings, normal human male subjects who were gonadotropin deprived had decreased INSL3 levels. Such patients had spontaneous partial recovery of INSL3 to 38.9% of baseline as opposed to an almost complete recovery of testosterone at 80.2% (Bay et al. 2006). It was also shown that direct hCG stimulation of Leydig cells in vitro does not increase INSL3 expression (Sadeghian et al. 2005). These findings suggest that long-term LH stimulation of Leydig cells is required for INSL3 expression but testosterone production is not coregulated with INSL3 in mature Leydig cells.

Testicular descent during embryogenesis consists of a transabdominal phase followed by an inguinoscrotal phase. The INSL3/RXFP2 system has a determining role in the development of the gubernaculum ligament and testicular descent during the transabdominal phase (Nef & Parada 1999, Zimmermann et al. 1999, Overbeek et al. 2001, Huang et al. 2012). The inguinoscrotal phase of testes descent is known to be androgen mediated, but studies have also shown the possible synergistic involvement of INSL3/RXFP2 during this phase (Yuan et al. 2010). Genetic ablation of Insl3 in mice has resulted in various degrees of intraabdominal cryptorchidism, the most common congenital birth defect in newborn boys. Insl3−/− adult mice showed decreased testes size, lesions in the seminiferous tubules, absence of spermatid and mature sperm, and gubernaculum underdevelopment (Nef & Parada 1999, Zimmermann et al. 1999). Interestingly, Insl3−/− males had normal copulatory behavior, normal seminal vesicles and prostate weights, which suggests that the mutants had normal androgen production and that this form of cryptorchidism is androgen independent (Nef & Parada 1999, Zimmermann et al. 1999). On the other hand, transgenic female mice overexpressing Insl3 develop bilateral inguinal hernias due to the descent of the ovaries into the processus vaginalis via outgrowth of the gubernaculum (Adham et al. 2002).

Rxfp2 involvement in cryptorchidism was discovered in mutant mice with a 550 kb deletion containing the gene in chromosome 5 (Overbeek et al. 2001). Breeding of these mutant mice, known as crsp, resulted in bilateral intraabdominal cryptorchidism in male homozygotes. All crsp/crsp adult males had small testes size and epididymis, absence of spermatogenesis, vacuolization of Sertoli cells, lesions of the seminiferous tubules and were
infertile (Overbeek et al. 2001). Recently, more sophisticated genetic tools were used to generate transgenic mouse lines to further prove the involvement of Insl3 and Rxfp2 in testicular descent. Rxfp2 with LacZ reporter allowed for the detection of Rxfp2 expression in gubernaculum, Leydig cells, and postmeiotic spermatogenic cells in testis (Huang et al. 2012). Cryptorchidism was also observed when Rxfp2 was deleted specifically in gubernacular embryonic mesenchymal tissue using a Cre/loxP approach, but the abnormal phenotype was not found after deletion of Rxfp2 in gubernacular striated or smooth muscle cells (Huang et al. 2012). This finding further demonstrates the importance of Rxfp2 for testicular transabdominal descent via regulation of the outgrowth of the gubernaculum in mice.

INSL3/RXFP2 signaling induces WNT and BMP developmental pathways and drives morphogenetic changes in gubernaculum (Johnson et al. 2010). It was shown that Rxfp2−/− male mice embryos experienced a dramatic decrease in expression of developmental signaling molecules β-catenin, NOTCH1, and WNT1 in the gubernaculum (Kaftanovskaya et al. 2011). The disruption of normal RXFP2 expression resulted in the failure of the testes to descend away from the caudal pole of the kidney at day E16.5, as well as an underdeveloped gubernaculum (Kaftanovskaya et al. 2011). To further understand the pathways leading to the failure of gubernacular development, β-catenin or Notch1 was ablated in the gubernacular ligament. In these males, the gubernacular ligament lacked the muscle layers, which was consistent with previous observations in Rxfp2-deficient mice (Kaftanovskaya et al. 2011).

Recently, a mammalian phylogenetic study in Afrotherians revealed that four testicond species from this lineage had a loss of functional RXFP2 and INSL3, which correlates with a lack of gubernaculum and testes descent, demonstrating the highly evolutionarily conserved function of this ligand–receptor pair (Sharma et al. 2018).

Orchidopexy in Insl3−/− and crsp/crsp male mice recovered spermatogenesis at least partially, suggesting that the increased testicular temperature as a result of cryptorchidism may be a contributing factor for the defects observed in mouse male germ cell differentiation (Zimmermann et al. 1999, Overbeek et al. 2001). Additionally, conditional deletion of Rxfp2 in germ cells beginning from premeiotic stages did not affect spermatogenesis, fertility, or germ cell survival in adult male mice (Huang et al. 2012).

Although studies in mice showed that deletion of Insl3 or Rxfp2 does not appear to have a direct impact on spermatogenesis or germ cell survival, other studies have drawn links between INSL3 levels and improved survivability of germ cells. Studies in gonadotropin compromised male rats showed the ability of INSL3 to suppress germ cells apoptosis (Kawamura et al. 2004). In a human contraceptive study, subjects who were given a combination of testosterone and progesterone as a contraceptive treatment for 24 weeks but did not display azoospermia had higher serum INSL3 levels compared to those who were azoospermic (Amory et al. 2007). This suggests that INSL3 may prevent apoptosis in male germ cells as non-azoospermic subjects also have higher sperm concentration, which positively correlated with INSL3 serum levels (Amory et al. 2007). Treatment of boar with neutralizing antibody against INSL3 also showed similar results, in which lower levels of available INSL3 reduced anti-apoptotic XIAP and BCL2 levels and sperm concentration (Minagawa et al. 2018). Taken together, these studies demonstrate that INSL3 may play a role in regulating apoptosis and turnover of male germ cells, especially when spermatogenesis is under stress conditions.

Clinical case studies in cryptorchid patients have identified dozens of mutations in INSL3 and RXFP2 genes (Gorlov et al. 2002, Bogatcheva et al. 2003, 2007, El Houate et al. 2007, Ayers et al. 2019) (Fig. 2). Functional analysis of these INSL3 mutants in HEK293T cells transfected with the RXFP2 receptor showed a decreased cAMP response for V18M, P49S, R73X and R102C mutants, suggesting potential clinical relevance (Bogatcheva et al. 2003, El Houate et al. 2007). A recent genetic analysis in patients with testicular torsion has also described mutations in the INSL3 gene, including a T60A polymorphism in the C-peptide region (Capra et al. 2018), a common polymorphism that was previously identified with the same frequency in cryptorchid and control patients (Lim et al. 2001). A missense mutation variant T222P in the LRR of RXFP2 impairs receptor expression on the cell surface and has been strongly associated with cryptorchidism (Gorlov et al. 2002, Bogatcheva et al. 2007). However, a more recent screen of cryptorchidic subjects in Spanish and Italian populations revealed that carrying this mutation may lead to increased risk of cryptorchidism only in the Italian population (Ares et al. 2011). Another missense mutation, G499E, was identified in the third TM domain of RXFP2 by conducting whole-exome sequencing of four brothers who presented with bilateral cryptorchidism (Ayers et al. 2019). A mutagenesis study further validated that G499E is a loss-of-function mutation. The G499E RXFP2 receptor showed no ligand...
binding or activation when stimulated with INSL3, and the mutant receptor expression on the cell surface was about 12% of the WT RXFP2 (Ayers et al. 2019). Significantly, in all studies, only heterozygous mutant carriers were found. Thus, while mutation analysis in cryptorchid patients suggests a possible contribution of INSL3 and RXFP2 in this abnormality in men, further studies are necessary to prove a direct cause-effect relationship.

**INSL3 in female reproductive physiology**

In females, INSL3 is produced primarily in the follicular theca interna cells of the ovary, where RXFP2 expression is also found (Bamberger et al. 1999, Zarreh-Hoshyari-Khah et al. 1999, Kawamura et al. 2004, Dai et al. 2017b). Lower levels of INSL3 and RXFP2 expression have also been detected in the corpus luteum and uterus (Balvers et al. 1998, Bamberger et al. 1999, Zarreh-Hoshyari-Khah et al. 1999, Li et al. 2011, Dai et al. 2017b). INSL3 in healthy women is first detectable during the late stages of puberty (Hagen et al. 2015), and serum levels remain around 79 pg/mL, which is much lower than in men (Anand-Ivell et al. 2013). INSL3 levels in women fluctuate in a phasic manner throughout the menstrual cycle and become undetectable after menopause (Anand-Ivell et al. 2006). The secretion of INSL3 is at its lowest levels during menses and spikes to significantly higher levels during the follicular phase of the menstrual cycle, concordant with the recruitment of growing antral follicles (Anand-Ivell et al. 2013). The highest levels of INSL3 positively correlated with pre-ovulatory ovarian hormones, such as anti-Müllerian hormone (AMH) and inhibin B (Anand-Ivell et al. 2013). The LH spike during ovulation negatively correlates with INSL3 and pre-ovulatory hormones and this is a causal relationship that was also described in studies on bovine theca cells (Anand-Ivell et al. 2013, Dai et al. 2017a). In primary bovine theca interna cells, LH at low doses stimulates production of INSL3, but higher LH levels comparable to those measured during ovulation had an inhibitory effect on INSL3 production (Dai et al. 2017a). Through further testing, it was revealed that INSL3 production is stimulated by LH and estradiol, acting through the protein kinase A pathway (Dai et al. 2017a). A feedback loop between INSL3 and steroidogenesis in theca cells was established when siRNA knockdown of RXFP2 sharply reduced CYP17A1 expression and androstenedione secretion (GliSter et al. 2013). Further, the inhibition of CYP17A1 reduced androgen secretion and INSL3 and RXFP2 expression. Additionally, treatment of theca cells with BMP6 dramatically downregulated expression of INSL3 along with CYP17A1 and several other key steroidogenesis genes. Taken together, these studies showed that estrogen-induced INSL3 production is a key mediator during follicular phase steroidogenesis and that LH that peaks at ovulation negatively regulates INSL3.

Abnormal levels of INSL3 in women have been shown to correlate with polycystic ovary syndrome (PCOS) (Pelusi et al. 2013, Shaikh et al. 2016, Seyam & Hefzy 2018). Patients with PCOS were categorized by...
their menstrual cycles into amenorrheic, eumenorrheic, or oligomenorrheic (Pelusi et al. 2013). Amenorrheic and oligomenorrheic groups had significantly higher levels of INSL3 and AMH compared to control women, which suggests a potential role in the follicle arrest and anovulation typically seen in this disease (Pelusi et al. 2013). Women with abnormal anatomical characteristics such as ectopic ovaries were also identified from a sample of PCOS women with positively correlated INSL3 and androgen levels (Seyam & Hefzy 2018). A recent study suggested that the common polymorphism T60A of the INSL3 gene could increase the risk of developing PCOS (Shaikh et al. 2016). Although this finding would need to be confirmed, it provides an argument toward a relationship between INSL3 and development of PCOS.

Rodent models have also proved useful in establishing the role of the INSL3/RXFP2 system in female reproduction (Nef & Parada 1999, Kawamura et al. 2004, Li et al. 2011). Deletion of the Insl3 gene resulted in abnormal estrous cycle and reduced fertility (Nef & Parada 1999), further demonstrating the involvement of INSL3 in maintaining healthy reproductive functions. Rxfp2 and Caveolin 1 (Cav1) have been associated with epithelial and stromal cell growth and overall homeostasis in the mouse uterus, as the rate of uterine cyst development sharply increased when both Cav1 and Rxfp2 were deleted in females (Li et al. 2011). INSL3 induces oocyte maturation in rats, shown by a dose-dependent increase in germinal vesicle breakdown (GVBD) after INSL3 treatment (Kawamura et al. 2004).

**Role of INSL3 in bone and skeletal muscle physiology**

INSL3/RXFP2 signaling has been shown to play a role in maintaining normal bone characteristics in both mouse and human (Ferlin et al. 2008, 2011). Young men with cryptorchidism carrying the T222P mutation in the RXFP2 have significantly reduced bone density, resulting in osteopenia and osteoporosis (Ferlin et al. 2008). Expression of RXFP2 has been shown in human osteoblasts and osteocytes, as well as in mouse osteoblasts (Ferlin et al. 2008, Di Nisio et al. 2018) Treatment of primary human osteoblasts with INSL3 showed a dose-dependent increase in proliferation (Ferlin et al. 2008). Moreover, femurs from Rxfp2−/− mice had significantly decreased bone mass and trabecular number compared to WT mice. A histomorphometric analysis of the lumbar spine of mutant mice also revealed reduced bone formation rate and mineralization surface compared to WT (Ferlin et al. 2008). Furthermore, INSL3 has been shown to regulate the expression of genes involved in differentiation and maturation of primary human osteoblasts, such as ALP, COL1A1, COL6A1, and Osteonectin (Ferlin et al. 2011). Treatment of primary human osteoblasts with INSL3 improved mineralization of the bone matrix (Ferlin et al. 2011). Decreased INSL3 levels in Klinefelter’s syndrome patients are correlated with increased levels of serum sclerostin, which is involved in bone catabolism by inhibiting osteoblasts differentiation and stimulating osteoclasts activation (Di Nisio et al. 2018). This relationship was further explored in cultured osteocytes, which had reduced expression of sclerostin when treated with INSL3. The negative correlation between INSL3 and sclerostin provides insights into the impact of INSL3 on bone health and its potential therapeutic value (Di Nisio et al. 2018).

RXFP2 was also suggested to have an important role in maintaining the proper function of muscular tissues (Ferlin et al. 2018). INSL3 treatment of myotubes differentiated from C2C12 skeletal muscle cells resulted in increased cell size compared to untreated control. Expression of myosin heavy chain can be induced by INSL3, which leads to increased protein synthesis in these cells (Ferlin et al. 2018). Denervated muscles in Rxfp2−/− mice developed greater muscle loss compared to WT mice. Additionally, the tibialis anterior muscle had significantly decreased beta-oxidative fibers, while the soleus muscle showed a significant decrease in fast and slow fibers in these mice. The soleus muscle absolute force, when normalized to the muscle mass of Rxfp2−/− mice, was also decreased compared to WT mice (Ferlin et al. 2018).

The emerging role of INSL3/RXFP2 in the musculoskeletal system reveals potential new targets for INSL3 and synthetic RXFP2 agonists in the treatment of diseases associated with bone and muscle loss.

**INSL3 role in other organs**

In adult rat forebrain, high Rxfp2 gene expression has been found in the thalamus, frontal and motor cortices. Receptor autoradiography showed INSL3 radioligand binding of RXFP2 in the thalamus and striatum nucleus (Sedaghat et al. 2008), suggesting a potential role of the INSL3/RXFP2 system in motor and sensory brain functions. Additionally, INSL3 and RXFP2 in the eye may play a role in wound healing (Hampel et al. 2013). Protein expression has been found in the human and mouse...
ocular surface and tears. A scratch test assay performed on human conjunctival and corneal epithelial cells showed increased migration and proliferation when treated with INSL3 (Hampel et al. 2013). A mouse corneal ulcer model was used to test the topical application of INSL3, which was determined to be effective in re-epithelialization and healing of corneal wounds (Hampel et al. 2013). INSL3 radioligand binding was also detected in the glomeruli of the renal cortex of post-natal and adult rats (Fu et al. 2006). It has been proposed that INSL3 in the kidney inhibits glomerular cell proliferation, which may be beneficial in targeting glomerular diseases that are associated with uncontrolled mesangial cell proliferation (Fu et al. 2006).

The INSL3/RXFP2 system has also been tested in cancer pathology (Klonisch et al. 2005, Hombach-Klonisch et al. 2010). INSL3 in situ hybridization and immunoreactivity was shown in benign prostate hyperplasia and neoplasia. Stimulation of human prostate carcinoma cell line PC-3 with INSL3 resulted in cAMP production and showed a positive correlation with increased cell migration (Klonisch et al. 2005). RXFP2 expression has also been detected in human thyroid carcinoma tissues (Hombach-Klonisch et al. 2010). Expression of INSL3 in xenotransplants with the FTC133 human thyroid carcinoma cell line showed increased tumor growth in nude mice compared to FTC133 cells not transfected with INSL3 (Hombach-Klonisch et al. 2010). Additionally, in vitro INSL3 treatment of these tumor cells led to increased motility, which is indicative of enhanced tumor metastatic capacity. Treatment of HUVECs with INSL3 also showed significant tube formation, similar to the VEGF control, which suggested the promotion of angiogenesis (Hombach-Klonisch et al. 2010). As future studies elucidate the role of the INSL3/RXFP2 in these systems, there may be additional opportunities for therapeutic intervention with RXFP2 agonists or antagonists.

### Conclusion and future directions

The role and involvement of the INSL3/RXFP2 system in health and disease has greatly expanded beyond the initial finding of its role in testicular descent (Table 1). Recent studies suggest the importance of this system for maintaining overall bone and skeletal muscle health, which may hold great potential for treating common age-related disorders associated with osteoporosis or muscle loss. In addition, links to PCOS, corneal healing and several cancers have also been described, further underscoring the importance of INSL3/RXFP2 signaling outside reproductive physiology. Therefore, further efforts should be focused on defining INSL3 functions in human physiology along with designing pharmaceuticals that target this signaling system.

To date, no clinical studies have been conducted targeting INSL3 or RXFP2 or has a systemic analysis of recombinant INSL3 stability in vivo been reported. Studies of the effect of INSL3 injections on testicular functions in rats have shown that INSL3 can pass through the blood-testis barrier (Anand-Ivell et al. 2009) and protect against GnRH antagonist-induced apoptosis in germ cells (Kawamura et al. 2004). Various approaches previously used to improve the stability of relaxin (Muppidi et al. 2019, Nagorniewicz et al. 2019, Sun et al. 2019) can be applied to INSL3 to design biologicals with the full spectrum of INSL3/RXFP2 downstream signaling. Alternatively, small molecule agonists have proven to be an attractive alternative to therapies with peptide ligands due to improved stability and potential

### Table 1 Summary of the known physiological roles of the INSL3/RXFP2 system and related pathologies.

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<th>Organ</th>
<th>Physiology</th>
<th>Pathology</th>
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<td>Testis</td>
<td>- Transabdominal phase of testis descent</td>
<td>- Cryptorchidism</td>
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<td>- Gubernaculum development</td>
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<td>- Germ cell survival</td>
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<td>Bone</td>
<td>- Maintain bone density</td>
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<td></td>
<td>- Osteoblast maturation</td>
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<td>Skeletal muscle</td>
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<td>Eye</td>
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oral bioavailability. A high-throughput screening of small molecules successfully identified the first RXFP1 agonist, compound ML290, which has antifibrotic effects recently demonstrated in a mouse model of liver fibrosis (Kaftanovskaya et al. 2019). Due to the structural similarities of RXFP1 and RXFP2, the same approach that was used for the selection and optimization of ML290 can be applied to identify a small molecule agonist that specifically targets RXFP2. The development of such stable agonists will not only provide novel insights into RXFP2 signaling mechanisms, but potentially also become useful pharmacological agents with various clinical applications.

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

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Diverse functions of insulin-like 3 peptide

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