Physiology and evolution of the INSL3/RXFP2 hormone/receptor system in higher vertebrates.

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Highlights

- INSL3/RXFP2 evolution promoted internal fertilisation and viviparity in mammals.
- The INSL3 system is linked to male phenotype and horn growth.
- INSL3 is an ideal monitor of fetal, pubertal and seasonal development.

Abstract

Although the insulin-like peptide hormone INSL3 and its cognate receptor RXFP2 (relaxin-family peptide receptor 2) have existed throughout chordate evolution, their physiological diversification appears to be linked closely with mammalian emergence and radiation. In contrast, they have been lost in birds and reptiles. Both hormone and receptor are expressed from autosomal genes which have maintained their synteny across vertebrate evolution. Whereas the INSL3 gene comprises only two exons closely linked to the JAK3 gene, RXFP2 is normally encoded by 18 exons. Both genes, however, are subject to alternative splicing to yield a variety of possibly inactive or antagonistic molecules. In mammals, the INSL3-RXFP2 dyad has maintained a probably primitive association with gametogenesis, seen also in fish, whereby INSL3 promotes the survival, growth and differentiation of male germ cells in the testis and follicle development in the ovary. In addition, however, the INSL3/RXFP2 system has adopted a typical 'neohormone' profile, essential for the promotion of internal fertilisation and viviparity; fetal INSL3 is essential for the first phase of testicular descent into a scrotum, and also appears to be associated with male phenotype, in particular horn and skeletal growth. Circulating INSL3 is produced exclusively by the mature testicular Leydig cells in male mammals and acts as a potent biomarker for testis development during fetal and pubertal development as well as in ageing. As such it can be used also to monitor seasonally breeding animals as well as to investigate environmental or lifestyle conditions affecting development. Nevertheless, most information about INSL3 and RXFP2 comes from a very limited selection of species; it will be especially useful to gain further information from a more diverse range of animals, especially those whose evolution has led them to express unusual reproductive phenotypes.

Keywords

INSL3, RXFP2, puberty, neohormone, testis descent, horn growth, fetal development, spermatogenesis, antral follicle growth

1. Introduction

Since the discovery of Insulin-like peptide 3 (INSL3; previously called relaxin-like factor, RLF, or Leydig insulin-like, Ley-IL) in 1993 (Adham et al., 1993) and its unique receptor, relaxin family peptide receptor 2 (RXFP2; previously called LGR8) in 2002 (Hsu et al., 2002; Kumagei et al., 2002), research on this hormone/receptor system has grown substantially. Initially envisaged as a relatively recent twig on the evolutionary tree, we now know that the origin of this system is in fact quite ancient and has been subject to very diverse evolutionary pressures, particularly in higher vertebrates. Fortunately, we now have very broad-ranging genomic data from many diverse species, which provide an important map upon which we can trace the path taken by this system in its evolution to present day species (Yegorov et al., 2014). But this map is theoretical and, in many cases, still hypothetical, since actual gene and protein expression, and certainly physiology, for many of the mapped species is mostly unknown, and what information we have is often extrapolated by comparison to a few well studied species. It is important to remember that, just because INSL3 and RXFP2 form an exclusive cognate hormone/receptor couple in humans and rodents, this does not mean that these two molecules have always co-evolved, and that they will always form the same exclusive cognate system in all species. Equally, we know very little indeed about transcriptional and translational regulation of these two genes, even in humans and rodents, and we have almost no information about putative gene promoters and their regulation for any other species, let alone whether these are physiologically functional in a similar way. The comparative endocrinology of the INSL3/RXFP2 system is still highly fragmented, with quite discrete foci around genomics, genetics and breeding, reproductive physiology in males and females, as well as isolated studies in other organs, mostly in humans or rodents. The present review has been undertaken in an attempt to integrate our understanding of this hormone/receptor system in an evolutionary and physiological

context. We have restricted this analysis to higher vertebrates where we have most physiologically relevant information and hence a degree of confidence about certain generalizations.

2. The genomic atlas.

Twenty years ago sequences of INSL3 homologues were only known for mammals, with similar sequences in lower vertebrates being allocated to paralogues of the human RLN3 gene. As a consequence INSL3 was thought of as a new mammalian gene (Wilkinson et al., 2005). Now, with full genomic data and corresponding synteny analysis for several hundred vertebrate and pre-vertebrate species available, we can be confident that INSL3 and its putative receptor RXFP2 were extant in the earliest vertebrates, such as cartilaginous fish, with possible ancestral genes of these even in agnathans, cephalochordates (e.g. *Amphioxus*), and echinoderms (Yegorov et al., 2014). INSL3 and RXFP2 presumably acquired their discrete identities, separate from the structurally related dyad of the peptide hormone relaxin (RLN) and its cognate receptor RXFP1, upon the first and second whole genome duplications (WGD1 and WGD2) which occurred about 540 million years ago at the beginning of the vertebrate radiation (Yegorov et al., 2014). In fish, there then followed a third genome duplication (WGD3) during teleost evolution, besides several local gene duplications and deletions in different lineages. This has been excellently documented by Yegerov and colleagues (Yegorov et al., 2014) and will not be discussed further here. Importantly, the ancestor of all tetrapods, exemplified by the coelacanth, indicated a single INSL3/RXFP2 dyad, though with an additional RXFP2-like gene evident in early vertebrates but not found in mammals (Yegorov et al., 2014).

Amphibia appear to have retained all three of these genes, whereas about 300 million years ago the ancestor of all birds and reptiles lost both INSL3 and RXFP2, though appear to have retained the

RXFP2-like gene (Yegorov et al., 2014), suggesting that this gene is not functionally correlated to the expression of INSL3. Whereas monotremes, like the platypus, retained both INSL3 and RXFP2, these lost the RXFP2-like gene; marsupials on the other hand appear to have retained all three genes (Park et al., 2008; Yegorov et al., 2014). Finally, the RXFP2-like gene was deleted completely about 120-150 million years ago in the ancestor to all modern (eutherian) mammals, implying that all groups of placental mammals initially expressed a functional INSL3/RXFP2 dyad. What then happened and why is unclear, but detailed genomic analysis shows that, among the ancestral parents of the modern Afrotheria, both INSL3 and RXFP2 seem to have lost functionality and progressively introduced deletions and mutations into either or both genes (tenrec, golden mole, elephant shrew, manatee) (Sharma et al., 2018). In contrast, within the same clade, aardvarks, elephants and hyraxes appear to have retained functional sequences for INSL3 and RXFP2 (Sharma et al., 2018. However, it is important to note that these studies only assessed the protein-coding regions of both genes, and neither regulatory nor non-coding regions could be investigated, largely because very little is known about these even for humans and rodents. When genes lose functionality, this may first become manifest by mutations in non-coding regulatory sequences, and only subsequently by mutations in protein-coding domains (Ivell et al., 2000).

3. Genes and gene expression.

The preceding section describes the evolution of genomic sequences but cannot say anything about whether the genes are expressed as mRNA or protein, nor whether whatever is expressed is conventionally functional. There are several ways this might be achieved. Park et al (2008) synthesized the platypus INSL3 homologue based on the DNA sequence and showed that it indeed could activate the human RXFP2 receptor. Alternatively, one might analyse transcript data using RT-PCR, EST libraries, or global mRNA-Seq for different tissues. This can provide structural and/or

quantitative expression data. Finally, specific antibodies can be raised against predicted unique peptide sequences and applied in western blots or immunohistochemistry, with the important caveat that antibody specificity must be very rigorously validated. Such approaches were all used in the early exploration of INSL3 and RXFP2 in a small selection of modern mammals (e.g. Ivell et al., 1997; Balvers et al., 1998; Zarreh-Hoshyari-Khah et al., 1999; Ivell et al., 2003; Sagata et al., 2015).

INSL3 is encoded by a relatively small gene, comprising just two exons (Fig 1). For the rat, mouse, bovine and human genes there is also some information on the functionality of the upstream promoter region (Koskimies et al., 1997; Dai et al., 2017a; Sadeghian et al., 2005; Tremblay et al., 2009). This region appears to be restricted to less than 1000 nucleotides since the INSL3 gene is located very close to the 3' end of the JAK3 gene in humans, rodents and ruminants (Koskimies et al., 1997; Safford et al., 1997; Spiess et al., 199), and it is assumed that the latter sequences are unlikely to interfere with INSL3 regulation. In the species studied, within this upstream region there are three separate sites for interaction with the transcription factor SF-1, or related factors, any or all of which appear to be used at least *in vitro* to induce up-regulation of the gene (Koskimies et al., 1997; Dai et al., 2017a; Sadeghian et al., 2005; Tremblay et al., 2009). Since the synteny in relation to JAK3 and this region appears to be maintained in all the mammalian genomes studied, it seems likely that the regulation of the INSL3 gene promoter is similar in most species. SF-1 responsive elements, while binding the transcription SF-1 (NR5A1) or its paralogue Nur77 (NR4A1) to elicit an activation of the gene, may also mediate activation by steroid receptors in a non-classical manner (Tremblay et al., 2009; Ivell et al., 2014; Dai et al., 2017a). This may explain why in some studies evidence suggests that INSL3 expression can be modulated to a small degree by steroid hormones (Tremblay et al., 2009; Dai et al., 2017a). However, most studies indicate that INSL3 expression is both cell-specific and constitutive within that cell type (Balvers et al., 1998; Sadeghian et al., 2005).

Several studies have indicated that both in cell culture and *in vivo* the INSL3 gene product may be secreted as either the B-A-C pro-form precursor which includes the connecting C-domain or as the processed A-B heterodimer (Fig 1), where the C-peptide has been cleaved, presumably by PC1/2-type proteolytic enzymes (Zarreh-Hoshyari-Khah et al., 1999; Büllesbach & Schwabe, 2002; Minagawa et al, 2012; Siqin et al., 2013). Whether or not this cleavage occurs *in vivo* may depend on species, cell type, and/or level of expression. For example, in the male pig evidence suggests that most circulating immunoreactive INSL3 occurs as the B-A-C pro-form (Minagawa et al., 2012), whereas in the human, the cleaved A-B heterodimer appears to be the prevailing form (Albrethsen et al., 2020a).

Several studies, however, report that in addition to the conventional INSL3-encoding gene transcript, alternatively spliced transcripts can be expressed (Zarreh-Hoshyari-Khah et al., 1999; Sadeghian et al., 2005; Yang et al., 2020). Mostly, these represent peptides comprising an additional C-terminal sequence added to the B-domain, followed by a stop codon, and thus missing any of the downstream peptide sequence normally representing the C-terminus of the C-domain and the A-domain (Fig 1). While in themselves lacking the structure to activate the RXFP2 receptor, a recent study suggests that they may in fact act antagonistically (Yang et al., 2020), similar to the B-chain dimer peptides that had been artificially created (Del Borgo et al., 2006; Shabanpoor et al., 2011). Such alternative splice products have been identified for rat, human and marmoset monkey (Zarreh-Hoshyari-Khah et al., 1999; Sadeghian et al., 2005; Yang et al., 2020); whether they are generally expressed in other mammalian species or are expressed as peptide and are functional *in vivo* is not known, although immunohistochemistry using a specific antibody in the marmoset monkey suggests that such products are probably very rare (Zarreh-Hoshyari-Khah et al., 1999).

RXFP2 is a G-protein-coupled receptor (GPCR) with a very large extracellular domain, which when expressed in cells by transfection, signals via adenylate cyclase and the generation of cAMP

(Kumagai et al., 2002; Heng et al., 2008). Its natural ligand INSL3, at least for those species where it has been studied, interacts first via the large LRR (leucine-rich repeat)-repeat region within the extracellular domain of the receptor (Fig 2), this complex then appears to bend downwards towards the cell membrane where then the N-terminal LDLa-domain of the receptor, as well as the A-domain of INSL3, can interact with the extracellular loops of the receptor transmembrane region (Halls et al., 2015). Only then can activation and generation of cAMP occur. The LRR-region comprises 10 small LRRs, each of which is encoded by a separate exon all of which are in-frame with each other. Altogether, the RXFP2 gene in all species for which genome sequences are available (Yegorov et al., 2014), comprises 18 exons (Fig 2), and appears to be highly conserved across all vertebrates. However, in the bovine, human, and rat, numerous alternatively spliced variants have been reported (Muda et al., 2005; Anand-Ivell et al., 2006; Heng et al., 2008; Dai et al., 2017b). Mostly these involve the splicing out of one or more of the LRR-encoding small exons, which nevertheless retain the reading frame, though sometimes also novel exons can be introduced (Fig 2). It is unclear at the present time whether these alternatively spliced variants are physiologically relevant. Certainly any loss of specific INSL3 binding, or altered receptor conformation is likely to affect INSL3 signalling, though it has been suggested that some functionality may be retained due to receptor dimerization and what is referred to as complementation, whereby a partially defective GPCR may be complemented and repaired by dimerising with a functional partner (Svendson et al., 2008; Kern & Bryant-Greenwood, 2009; Rivero-Müller et al., 2010). As for the ligand INSL3, so also for RXFP2, we have no information at all about the extent and functionality of any splice variants *in vivo* for any species (though see below).

4. The neohormone hypothesis.

The radiation and success of modern mammals was largely due to two major changes to reproductive physiology which effectively made progeny more independent of environmental change and adversity. The first of these was lactation, providing the newborn young with a constant and reliable source of nutrition in the first days and weeks after birth. This appears to have occurred in the common ancestor to monotremes, marsupials and eutherians. The second innovation was the development of internal fertilisation and the retention of the embryo inside the mother with appropriate nutrition for the growing embryo provided by the formation of a placental interface between mother and offspring. This began in marsupials but was developed much further in eutherian mammals. Whilst the anatomical and physiological features of internal fertilisation and placentation are well known, these must be accompanied by the co-evolution of appropriate control systems manifest as specific and novel hormone-receptor relationships. This has led to the coining of the term 'neohormones' to describe those novel hormone systems whose development was essential to regulate this new mammalian physiology (Ivell & Bathgate, 2006; Anand-Ivell et al., 2013). The molecules themselves are likely to have been pre-existing but in different roles and were co-opted and adapted specifically to regulate the special physiology needed to make retention of the embryo in the mother for as long as possible a success. Table 1 summarizes the diverse physiological and behavioural requirements to be regulated by neohormone systems, with examples of some of the hormones involved. Prominent amongst these are the INSL3/RXFP2 system, as well as the closely related relaxin (RLN)/RXFP1 system. Note, also, the involvement of the oxytocin (OXT)/OXTR system in many of these novel aspects. It may be significant too that paralogues of RLN also evolved by gene duplication along with mammalian emergence, including INSL4, INSL6, and RLN1 in humans, possibly responding to the same positive selection pressure.

5. Testicular descent and the evolution of the scrotum.

For internal fertilization to occur, one essential prerequisite is for the timed introduction of fertile sperm into the female oviduct at a time when the unfertilized egg is released from the ovary. To achieve this so that sperm are in the optimally primed state to achieve fertilization, it was essential to both store the sperm in the male system and to create a trigger which effectively initiated a series of biochemical activation events in the sperm upon their introduction into the female tract at the appropriate time. In most mammals this seems to have involved the evolution of exteriorised testes and the development of a scrotum (Ivell, 2007; Kleisner et al., 2010). The principal selection pressure appears to be a requirement for sperm storage at a temperature which is lower than abdominal temperature but nonetheless controlled. Although spermatogenesis occurs within the testes, the sperm are continually released and stored outside the testes in the cauda epididymis. The epididymis is a Wolffian derivative which sequentially secretes a series of highly specific products into its lumen which interact and modify the transiting sperm before these are stored in the final caudal sector, which is located at the lowest and coolest region of the scrotum at approximately 5^{o}C below abdominal temperature. Here the sperm can be stored for up to several weeks prior to ejaculation. Upon ejaculation, the sperm are ejected from the cauda epididymis, mixed quickly with further secretions from the prostate gland, seminal vesicles and other glands, and injected into the female tract (either into the vagina in most mammals, or directly into the uterus, for example, in pigs), from where they are rapidly transported to the site of fertilization in the oviducts. The rapid changes in temperature, nutrients, electrolytes, pH, and hormonal milieu which occur upon ejaculation act as the trigger to initiate sperm capacitation and their fertilizing ability.

How the scrotum evolved is unclear. But it involved the retention after sex determination of the testes in the inguinal region through the development of the gubernacular ligament linking the basal pole of the testes to the inguinal skin, later to become the scrotum, while the kidneys and other abdominal organs move away through body growth in an antero-dorsal direction. In most mammals this first phase of testicular descent occurs in the fetus during the second and third trimesters of

pregnancy. This does not occur in females who retain the ovaries in a peri-renal position. There then follows a second phase of testicular descent whereby the testes pass through the inguinal canal and abdominal wall and enter the scrotum which develops as a vascularized skin sac on the outside of the body.

Hormonally, the first phase of testicular descent is induced by the expression and secretion of large amounts of INSL3 by the Leydig cells of the fetal testes (Anand-Ivell & Ivell, 2014; Hutson et al., 2015). In humans this begins at around weeks 10-12 of pregnancy, when high concentrations of INSL3 can also be detected in amniotic fluid from male fetuses only. In rodents, this first phase occurs relatively later, towards the last days of pregnancy and may continue into early postnatal life (Anand-Ivell & Ivell, 2014). This fetal INSL3 interacts with RXFP2 receptors which are induced in the gubernacular ligaments, probably involving androgen-dependent up-regulation of the RXFP2 gene (Yuan et al., 2010). In male mice, inactivation of either the INSL3 gene or that of its receptor RXFP2, leads to cryptorchidism with a failure of the first phase of testicular descent (Nef & Parada, 1999; Zimmermann et al., 1999; Bogatcheva et al., 2003). Interestingly, in female mice over-expression of INSL3 in the fetus leads to the formation of lower abdominal hernia (Feng et al., 2006), suggesting a way that the externalization of the fetal testes may have evolved via controlled induction of a hernia accompanying the retention of the testes in the inguinal region.

Whilst the absence of testis descent into a scrotum is obviously the pre-mammalian situation, it is hard to identify any present-day mammals representing this primitive condition and it seems likely that both testis descent and testis retention may have occurred several times independently during mammalian evolution. Although marsupials exhibit testis descent and have a scrotum, the positioning of the scrotum anterior to the penis implies that this occurred separately from the event in placental mammals, where the scrotum is invariably posterior to the penis (Kleisner et al., 2010). The retention of the testes in the abdominal cavity (testicondy) appears to be common amongst the

Afrotheria (elephants, hyraxes, etc.), though whether this is a primitive trait is less clear. Importantly, both the genes for INSL3 and RXFP2 indicate variable levels of mutation and deletion within this clade (Sharma et al., 2018), with the signature species, the elephant, showing no such changes in spite of being primarily testicond. Although it does not exclude mutations within control regions of the genes, this does suggest that the loss of these genes within the clade is more likely to be a secondary consequence of testis retention or that, in some species more than others, the INSL3/RXFP2 dyad is required for other important physiological functions besides testis descent. Unfortunately, there is still no information on the expression of either INSL3 or its receptor RXFP2 at the mRNA or protein level for any of these species, nor about the cells or tissues where they might be expressed and hence the physiologies with which they may be involved.

A scrotum is also absent in Cetacea and in other aquatic mammals. Testicondy in these species appears to be secondary, with the testes retained within the abdominal cavity (whales) or subcutaneously (seals) to provide both streamlining and to avoid excessive cooling. Interestingly in some of these species there is an additional testicular blood plexus which still allows cooler cutaneous blood to reduce gonadal temperature below core body levels (Rommel et al., 1992, 1995). For all of these species, there appear to be no evident mutations within the coding regions of either INSL3 or RXFP2 (Yegorov et al., 2014), though also here we have no precise mRNA or protein expression data for any of these species.

6. INSL3, horns and the skeletal axis.

In the mature male mammal, where it has been measured, INSL3 circulates as a hormone in relatively high concentration (1-10 ng/ml or greater; Table 2). This is different from female mammals (see below) where concentrations in peripheral blood are either undetectable or generally $\leq$0.1

ng/ml (Table 2). It is logical therefore to find a relationship between INSL3 and skeletal physiology, particularly in species where there is a male gender bias in size or strength. In particular, INSL3 has been shown to modulate osteoblast and osteoclast physiology (Ferlin et al., 2017), and mice and/or humans with defective INSL3 or RXFP2 genes indicate a significant osteopenia or osteoporosis (Ferlin et al., 2008). This association with INSL3 or its receptor RXFP2 is most marked in the context of horn growth in ruminants. RXFP2 has been identified in several genomic studies in ruminants as a significantly associated gene locus linked to the selection of horn-related traits during domestication, including polling, whereby a normally horned breed lacks horns (Johnston et al., 2011; Gautier & Naves, 2011; Allais-Bonnet et al., 2013; Wiedemar et al., 2014; Aldersey et al., 2020). It was further shown that fetal horn buds in cattle express RXFP2 (Allais-Bonnet et al., 2013; Wiedemar et al., 2014). The development of horns amongst wild ungulate species is clearly linked to male reproductive behaviour and breeding privilege in the context of internal fertilisation and viviparity, thus suggesting that this role of the INSL3/RXFP2 system is fully in accord with the neohormone hypothesis (Table 1). There is still very little research being carried out on such aspects, particularly as regards expression studies and physiology. More relevant information on different species would be very welcome.

7. INSL3 as a biomarker of Leydig cell functional capacity.

The principal site of INSL3 synthesis in the male mammals which have been studied are the mature steroidogenic Leydig cells of the testes. Only the well differentiated Leydig cells of the fetal testes or those which develop during puberty in the adolescent animal express INSL3 mRNA and peptide. As discussed above, the expression of the transcription factor SF-1, or the related factor Nur 77, appears to be sufficient for high expression of the INSL3 gene, at least *in vitro* (Koskimies et al., 1997; Robert et al., 2006). These factors are expressed in several cell types, though especially the

steroidogenic Leydig cells, and probably account for the almost constitutive expression of INSL3 by these cells. Although adrenocortical cells also express SF-1, these cells do not normally express INSL3 (Lefebvre et al., 2003), implying that other cell-specific factors may be involved. Unlike testosterone and the steroidogenic pathway, there is only little if any evidence for a diurnal rhythm in secretion, at least in human (Chong et al., 2015; Albrethsen et al., 2020b). Although acutely quasi-constitutive, INSL3 expression does depend on the differentiation state of the Leydig cells. Thus, immature Leydig cells as in pre-puberty or early adolescence, as well as those dedifferentiated by GnRH treatment or steroidal contraceptives indicate very reduced or negligible INSL3 expression (Ivell & Anand-Ivell, 2009). Furthermore, as Leydig cells age, they also indicate a reduced differentiation state, which is manifest as a reduced expression and secretion of INSL3 (Anand-Ivell et al., 2006b). Taken together, these observations show that measuring the circulating concentration of INSL3 in peripheral blood is a very good parameter by which to assess the overall number and differentiation state of the Leydig cells (Leydig cell functional capacity) and hence their ability to make testicular steroids such as testosterone, without the technical problems associated with the large daily fluctuations found with that hormone (Ivell et al., 2014).

Amongst the fields where INSL3 measurement has proved useful is in the assessment of male pubertal development, for example in cattle (Kawate et al., 2011; Anand-Ivell et al., 2019) or pigs (Minagawa et al., 2014;), and the factors which may influence this. Also, INSL3 could prove helpful in studying the suppression and recovery of testicular function associated with seasonal breeding (Hombach-Klonisch et al., 2004; Ivell et al., 2013) or possibly social dominance. For example, in non-breeding Djungarian hamsters under a short-day lighting regime, INSL3 expression is almost undetectable, unlike when shifted to long days (Ivell et al., 2013). Similarly, deer show a markedly increased expression of INSL3 in the testes upon entering the breeding season (Hombach-Klonisch et al., 2004).

The differences in INSL3 expression between different species and different breeds within species are still largely unexplored. We have developed several different well-validated assays to measure INSL3 using species-specific antisera (Ivell & Anand-Ivell, 2009; Vernunft et al., 2016; Anand-Ivell et al., 2009, 2011; Minagawa et al., 2014). Table 2 summarizes some of the observations made with these and similar homologous and heterologous assays. Firstly, because of the divergence of the INSL3 peptide sequence amongst different mammal species, while there is some cross-reaction of antibodies between some species, for others the antibodies do not cross-react. It is therefore very important that all such applications are appropriately validated. Secondly, there is substantial variation in the circulating INSL3 concentration between species, and between different breeds even within a species (Table 2). For example, whereas male ruminants indicate circulating INSL3 concentrations in the adult of up to 10 ng/ml or more, for dogs the equivalent concentration is only approximately 0.2-0.4 ng/ml. Human males, for which most data are available suggest a normal adult range of between 0.5 and 2.5 ng/ml (Bay et al., 2005; Anand-Ivell et al., 2006; Albrethsen et al., 2020b), declining at approximately 15% per decade with age (Anand-Ivell et al., 2006).

8. INSL3 and spermatogenesis.

RXFP2 expression using antibodies and/or mRNA has been identified in rodents, pigs, goats, bulls and humans within the seminiferous compartment of the testes, particularly on pre- and post-meiotic germ cells, and to a lesser extent on spermatogonia, spermatids and spermatozoa (Kawamura et al., 2004; Anand-Ivell et al., 2006a; Filonzi et al., 2007; Siqin et al., 2010; Kohsaka, et al., 2013; Pitia et al., 2015, 2017). There is no expression on Sertoli cells. Since it has also been shown for pigs and rats that INSL3 from the Leydig cells of the interstitial compartment appears able to cross the blood-testis barrier and enter the seminiferous compartment in considerable quantities (Anand-Ivell et al., 2009; Minagawa et al., 2014), it is logical to assume that there may be a role for

INSL3 in relation to spermatogenesis or sperm function. Such a role has also recently been supported by studies on the ancestral INSL3 in zebrafish (Assis et al., 2016). However, in mammals, results are less clear cut. For pigs, both passive and active immunisation against INSL3 to reduce the levels of the testicular hormone leads to a negative impact on spermatogenesis, with significantly increased germ cell apoptosis (Sagata et al., 2015; Minagawa et al., 2018). In rats, intratesticular injection of an INSL3 receptor antagonist leads to a reduction of testis size probably due to marked germ cell loss (Del Borgo et al., 2006); and in humans, it was shown that men subjected to a prolonged steroidal contraceptive regimen indicated most impact of this on sperm production in those men whose residual circulating INSL3 was also lowest (Amory et al., 2007). In contrast, for mice whose RXFP2 gene expression in male germ cells was genetically prohibited, there appeared to be no effect of this on spermatogenesis (Huang et al., 2012). Such experiments need to be carefully distinguished from those where loss of INSL3 or RXFP2 function occurs genetically or *in utero*, and there is a consequent failure of testis descent, which itself causes multiple problems with spermatogenesis.

There is some evidence to suggest that RXFP2 may also be expressed in or on free spermatozoa (Feugang et al., 2011, 2015; Pitia et al., 2017; Shokri et al., 2020), or on cells of the epididymal epithelium (Anand-Ivell et al., 2006b; Filonzi et al., 2007), from where it might be transferred to transiting or stored sperm by transcytosis of epithelial vesicles. Effects of INSL3 on ejaculated sperm function have also been shown for the human, but only at very high, supraphysiological INSL3 concentrations (Shokri et al., 2020), so that a natural role *in vivo* must be doubted. Our own studies using human sperm indicated no effect at physiological concentrations (unpublished). Nevertheless, there does appear to be a correlation between RXFP2 expression on spermatozoa and sire fertility in bulls (Pitia et al., 2017). Regarding this point, we recently explored the effect of INSL3 on sperm fertility in pigs, and found that INSL3 might contribute not to sperm capacitation, but to sperm-

oocyte interaction by stimulating the expression of adhesion molecules (Minagawa et al., unpublished).

For all species, where the normal, as opposed to mutant, RXFP2 receptor has been cloned, expressed and pharmacologically tested (mouse, rat, bovine, human; Dai et al., 2017b; Halls et al., 2017), the EC50 values are all in the peri-nanomolar range, with significant activation still possible at picomolar concentrations of INSL3. Such concentrations are well within the normal circulating concentration in adult male mammals (Table 2) and likely to be physiologically significant also for female mammals, at least locally within tissues, if not systemically. An *in vitro* requirement for much higher INSL3 concentration in order to see a physiological response is therefore likely to be artefactual or through a less specific mechanism.

9. INSL3 and female reproductive physiology.

In the female mammal, the ontogenetically equivalent cells to the Leydig cells are the steroidogenic theca interna cells of the mature ovarian follicle. These are the vascularized layer of cells encompassing the antral follicles following their selection under the influence of the gonadotropins LH and FSH to become the growing follicles of the estrous (menstrual) cycle, one or more of which will release a fertile oocyte after the LH surge. In most species, only the theca cells of healthy growing antral follicles express INSL3, and only within the follicular phase of the estrous cycle (Ivell & Anand-Ivell, 2018). This expression appears to be quite consistent amongst those mammalian species assessed, including monkey, human, mouse, and cow (Balvers et al., 1998; Irving-Rodgers et al., 2002; Hanna et al., 2011; Anand-Ivell et al., 2013; Satchell et al, 2013). In the rat, pre-antral follicles also express INSL3 (Xue et al., 2014). No other organ or cell type in the non-pregnant female appears to secrete INSL3 at a level able to influence the peripheral concentration. Consequently, it

was shown for cows and women that measurement in the circulation of INSL3 deriving from such follicles reflects their growth (Satchell et al., 2013; Anand-Ivell et al., 2013); less healthy or atretic follicles immunohistochemically fail to show any INSL3 (Irving-Rodgers et al., 2002). Although corpora lutea which result from follicles following ovulation also appear to express INSL3 mRNA (Balvers et al., 1998; Bathgate et al., 1999; Hanna et al., 2011), it is still unclear whether these tissues are able to secrete INSL3 peptide into the bloodstream.

As in the male gonad, also within the ovary, the INSL3 receptor RXFP2 is found both on the INSL3-producing steroidogenic theca cells, as well as on oocytes (Kawamura et al., 2004; Xue et al., 2014; Dai et al., 2017b). Recently, we have shown for the bovine that the local theca cell INSL3-RXFP2 system is essential for the adequate production of the androstenedione precursor required by the inner granulosa cells for the synthesis of estrogens (Dai et al., 2017a; Ivell & Anand-Ivell, 2018). The role of RXFP2 on the oocyte, shown for the cow, as well as in rodents and monkeys (Kawamura et al., 2004; Xue et al., 2014; Dai et al., 2017b), is still unclear. One early study in rats suggests a role in the Ca^{2+}-signalling mechanisms surrounding oocyte maturation and ovulation (Kawamura et al., 2004). Inactivation of the INSL3 gene in mice leads to reduced ovulations and smaller litter size (Nef & Parada, 1999; Spanel-Borowski et al., 2001), emphasizing the importance of this system in follicle development and physiology.

When a pregnant female mammal is carrying a male fetus, then a further source of INSL3 is introduced. Namely, male but not female fetuses express large amounts of INSL3 from the fetal testes, whose purpose is to promote the first transabdominal phase of testicular descent (see above). This fetal INSL3 can not only be detected in fetal blood and in amniotic or allantoic fluids (pig, rat, human; Anand-Ivell et al., 2008; Anand-Ivell & Ivell, 2014; Vernunft et al., 2016), but is also measurable in extra-fetal fluids, such as maternal blood (bovine; Anand-Ivell et al., 2011), and can even transfer from male to female fetuses within the uterus (pigs; Vernunft et al., 2016). It is still

unclear what role this fetal INSL3 may have outside the fetus, but it evidently represents a fetal sex-specific hormone capable of communicating with the placenta and maternal tissues, as well as with female fetuses in multiparous species, and may underlie some of the biased physiology specifically associated with male fetal gender. That female fetuses in multiparous species mostly show no evidence of gonadal dislocation is of course due to the lack of androgen-dependent expression of the RXFP2 receptor in the ligaments attaching the ovary to the body wall. However, very few mammals have been examined in this context, and a broader comparison of species could prove interesting.

10. Other roles for the INSL3/RXFP2 system

For a very limited number of species, there is also evidence for local INSL3/RXFP2 systems operating in other tissues and organs. For example, in humans and mice both INSL3 and its receptor are found associated with the corneal epithelium of the eye and linked to the lacrimal apparatus, and *in vitro* INSL3 appears to promote corneal growth and repair (Hampel et al., 2013). Both INSL3 and RXFP2 are also expressed by epithelial cells within human thyroid cancer, though apparently not in healthy tissue, and *in vitro* assays suggest an effect on cancer cell differentiation, growth, and angiogenesis (Hombach-Klonisch et al., 2010).

In the rat, RXFP2 appears to be expressed quite moderately in the kidney, particularly within the mesangial cells of the Bowman's capsules; and *in vitro* INSL3 is able to modulate the growth and differentiation of these cells (Fu et al., 2006). It is to be noted that an altered electrolyte balance is listed as one of the phenotypic traits recorded for RXFP2-deficient mice (*http://www.informatics.jax.org/humandisease.shtml*), suggesting that INSL3 may have a physiological role in regulating renal physiology, at least in rodents.

Finally, it is well established that, again in the rat, there is noted RXFP2 expression particularly within the thalamic region of the forebrain (Sedaghat et al., 2008), with suggested links to sensorimotor function. However, except for the bovine hypothalamus (Bathgate et al., 1996), for no other mammal, including the rat, is there any evidence for a local synthesis of INSL3 within the brain, and whether or not INSL3 can cross the blood-brain barrier from the general circulation is still unclear.

11. Synthesis.

The receptor RXFP2 and its unique cognate ligand INSL3 form a paracrine and endocrine dyad, largely conserved across mammals. Where RXFP2 has been analysed in different mammalian species, there is no evidence for any other ligand being able to interact with the receptor at physiological concentrations. The related hormone relaxin can only activate RXFP2 at greatly supraphysiological concentration, thus any reference of this receptor being an alternative relaxin receptor is a misnomer. The INSL3/RXFP2 dyad or its ancestor is represented in the genomes of some of the earliest vertebrate orders, including teleosts, where it is linked specifically to spermatogenesis with INSL3 expression uniquely in Leydig cells (Assis et al., 2016). Thus, both the expression of INSL3 in mammalian Leydig cells as well as its role in modulating spermatogenesis may be considered primitive. The dyad was lost in all reptiles and birds but maintained in some form or other in all mammalian groups, including monotremes and marsupials, as well as in Amphibea. The successful radiation of mammals was dependent on the evolution of viviparity and internal fertilization, which demanded the co-evolution of novel regulatory systems, referred to as neohormones. The INSL3/RXFP2 dyad was evidently co-opted to promote and support the new physiology, leading across male mammals to its novel roles in testicular descent, and probably bone strength and horn formation. Our knowledge of the role played by INSL3 and RXFP2 in these novel

physiologies is still limited to only a few common species. Whether the important role of INSL3 and RXFP2 in female physiology is primitive or also a reflection of a neohormone role is unclear, largely because there is so little comparative data. Particularly amongst the Afrotheria (elephants, manatees, hyraxes) which exhibit varying degree of testicondy, and indicate in some species deletions and inactivation of the INSL3 and RXFP2 genes, there is a need to gather more detailed expression data at both mRNA and protein levels, besides functional information. Moreover, we need to understand better the reasons for the diversity of expression levels evident in different species of mammals, or in different breeds within a species. Finally, INSL3 is proving to be a valuable biomarker to monitor development in the fetus, during puberty and in ageing, under normal healthy conditions, as well as following environmental insults, or changes in feeding. However, such applications are limited to a few species only; it would be helpful to extend such knowledge to encompass a wider range of species, including those under threat.

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849

850 **Legends to Figures**

851

852 **Figure 1**

853 **Biosynthesis of rat Insl3**. **A**. Location and synteny of the *Insl3* gene on chromosome 16p14,
854 indicating the close juxtaposition to the *Jak3* gene. **B**. Structure of the Insl3 gene, indicating exons 1
855 and 2, as well as additional exons 1a determined by RT-PCR (Sadeghian et al., 2005) and 1b predicted
856 from the genomic sequence. **C.** The normal prepro-Insl3 precursor protein which, upon correct
857 processing and folding as in **D**, can after removal of the signal peptide (SP) and cleavage of the C
858 (connecting) peptide give rise to the secreted functional heterodimer, **E**. In the event that additional
859 exon 1a is used, then the B-domain is extended followed by a Stop codon to yield an abbreviated
860 peptide which may be a putative antagonist. If exon 1b is used, the reading-frame is maintained to
861 yield a putative precursor with a 22 amino acid longer C-domain (Genbank XP_006252882).

862

863 **Figure 2**

864 **Biosynthesis of bovine RXFP2**. **A**. Location and synteny of the *RXFP2* gene on bovine chromosome
865 12. **B**. Composition and alternative splicing of the bovine *RXFP2* gene, normally comprising 18 exons.
866 Alternative splicing can add up to two additional exons (exons 5a and 11a) or may lead to loss of
867 exons 13 and/or 15 in the illustrated examples, based on sequenced RT-PCR products or EST
868 fragments (Dai et al., 2017b). The numbers below the genomic structure indicate the positions of
869 PCR primers used for multiple RT-PCR analysis (Dai et al., 2017b). **C**. RXFP2 precursor protein
870 indicating signal peptide (SP) and the key peptide domains (LDLa, low density lipoprotein a; LRR,
871 leucine-rich repeat; hinge domain; 7TM (7-transmembrane) domain; and C-terminal intracellular
872 signaling domain). **D**. Folded structure of the mature RXFP2 protein, indicating the relative locations
873 of the various peptide domains and their specific interactions (double-headed arrows) with the
874 INSL3 peptide (after Ivell & Anand-Ivell, 2018).

875

876

877 **Legends to Tables**

878

879 **Table 1**

880 **Reproductive physiology and behaviour acquired with mammalian emergence and regulated by**

881 **neohormone systems**.

882 RLN, ovarian relaxin; OXT, oxytocin; INSL3, insulin-like peptide 3; hCG, human chorionic

883 gonadotropin; IFNτ, interferon tau; TGFβ, transforming growth factor beta; PGF2α, prostaglandin

884 F2apha; PRL, prolactin; and their respective receptors. ? , not known.

885

886 **Table 2**

887 **Circulating INSL3 concentration in diverse mammals**.

888 INSL3 concentration is given as mean + SD, except where indicated. *median and 95% confidence

889 intervals; ** approximate ranges. (yr., years; mo., months).

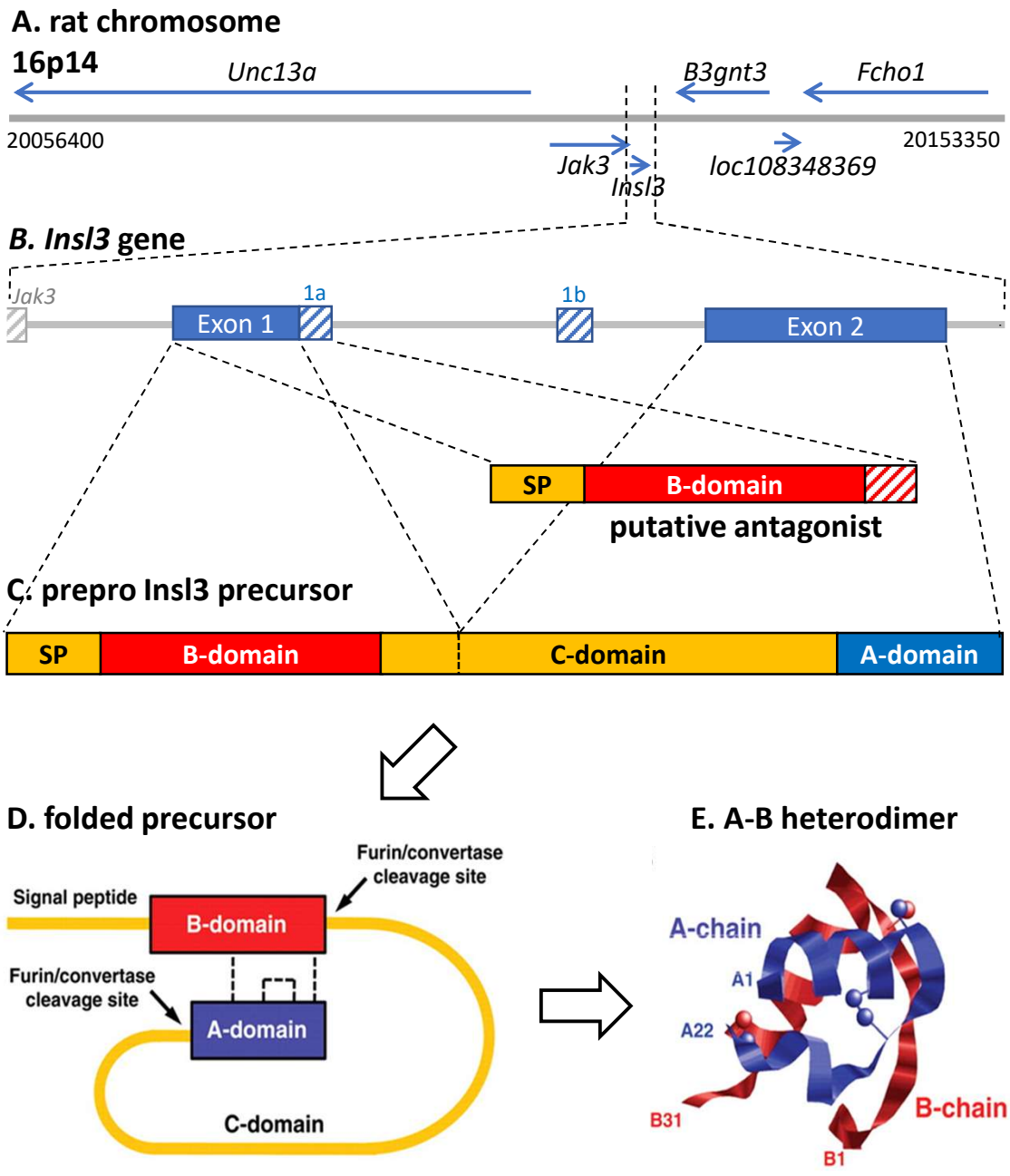
890

891

892

893

Fig 1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Fcho1
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Jak3
Insl3
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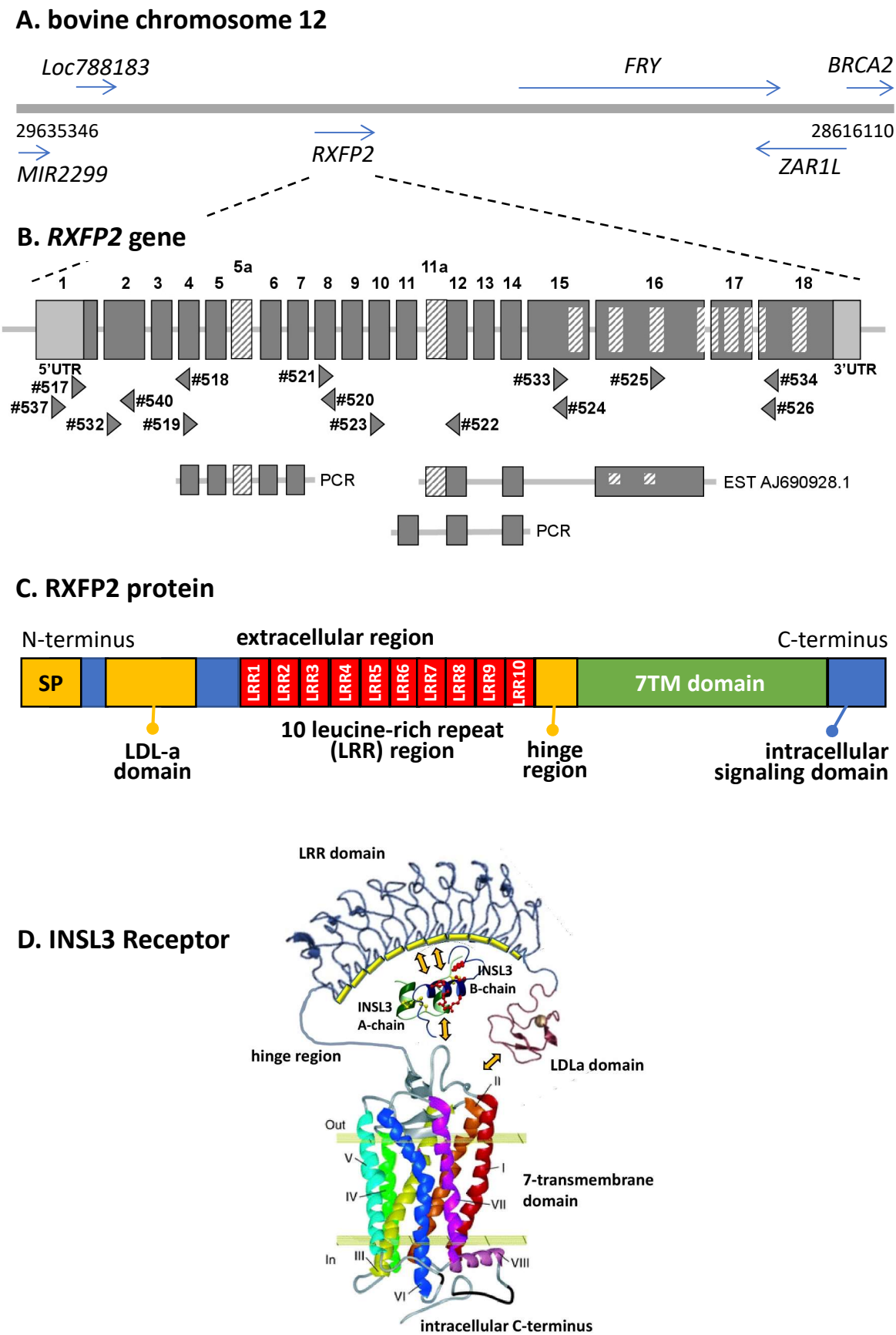
Jak3
1a
1b
Exon 1
Exon 2
SP
B-domain
putative antagonist
C. prepro Insl3 precursor
SP
B-domain
C-domain
A-domain
D. folded precursor
Signal peptide
B-domain
Furin/convertase cleavage site
Furin/convertase cleavage site
A-domain
C-domain
E. A-B heterodimer
A-chain
A1
A22
B31
B-chain
B1

Fig 2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FRY
BRCA2
29635346
28616110
RXFP2
MIR2299
ZAR1L
B. RXFP2 gene
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5'UTR
3'UTR
#517
#537
#532
#540
#519
#518
#521
#520
#523
#522
#533
#524
#525
#534
#526
PCR
EST AJ690928.1
PCR
C. RXFP2 protein
N-terminus
extracellular region
C-terminus
SP
LRR1
LRR2
LRR3
LRR4
LRR5
LRR6
LRR7
LRR8
LRR9
LRR10
7TM domain
LDL-a domain
10 leucine-rich repeat (LRR) region
hinge region
intracellular signaling domain
D. INSL3 Receptor
LRR domain
INSL3 B-chain
INSL3 A-chain
hinge region
LDLa domain
Out
In
I
II
III
IV
V
VI
VII
VIII
7-transmembrane domain
intracellular C-terminus

Table 1

Reproductive physiology and behaviour acquired with mammalian emergence and regulated by neohormone systems

Biological feature	**Neohormone candidates**	**Receptors**
Viviparity and uterine accommodation of the embryo (placentation)	RLN	RXFP1
Internal fertilization and its coordination	?	?
Appropriate male and female sexual behaviour	OXT, INSL3	OXTR, RXFP2
Maternal recognition of pregnancy	hCG, IFNτ	LHCGR, IFNR1
Adjustment of cardiovascular function, electrolyte and fluid balance in pregnancy	RLN	RXFP1
Adjustment of maternal immune tolerance to accommodate genetically different sperm and embryo	TGFβ, RLN	TGFBR, RXFP1
Thermoregulation and a constant core temperature	?	?
Regulation of the birth process, postnatal uterine involution and regeneration	OXT, RLN, PGF2α	OXTR, RXFP1, PGFR
Perinatal analgesia	?	?
Breast development and lactation	PRL, RLN, OXT	PRLR, RXFP1, OXTR
Appropriate maternal behaviour	OXT	OXTR
Scrotal testes, testicular descent, and reduced scrotal temperature	INSL3	RXFP2
Post-testicular sperm maturation, storage and capacitation as an adaptation to internal fertilization	?	?
Post-reproductive survival to provide extended care for offspring	?	?

Table 2

Circulating INSL3 concentration in diverse mammals

Species	Breed/ Condition	INSL3 (ng/ml)	References
Human	young men (35-44 yr.) young men (19-40 yr.)	1.29 ± 0.47 1.3 (0.9-2.7)*	Anand-Ivell et al., 2006b Albrethsen et al., 2020a
	old men (75-80 yr.)	0.79 ± 0.39	Anand-Ivell et al., 2006b
	adult women (30-38 yr.)	0.08 ± 0.01	Anand-Ivell et al., 2013
Macaque	adult male	0.44 ± 0.13	Hanna et al., 2010
	adult female	0.12 ± 0.01	Hanna et al., 2010
Rat	*Sprague-Dawley*		
	adult male (3 mo.)	2.81 ± 0.27	Anand-Ivell et al., 2009
	old male (24 mo.)	0.94 ± 0.03	Anand-Ivell et al., 2009
	adult female (3 mo.)	0.08 ± 0.03	Anand-Ivell et al., 2009
	Wistar		
	adult male (3 mo.)	1.51 ± 0.09	Anand-Ivell et al., 2009
	old male (24 mo.)	0.76 ± 0.21	Anand-Ivell et al., 2009
Mouse	*CBA* male (2 mo.)	0.78 ± 0.03	Anand-Ivell et al., 2009
	CBA female (2 mo.)	0.05 ± 0.01	Anand-Ivell et al., 2009
Pig	*Duroc* adult male	12.7 ± 1.86	Minagawa et al., 2014
	Landrace adult female	0.18 ± 0.02	Vernunft et al., 2016
Bovine	*Japanese Black* adult male (18-24 mo.)	7-8, 5-7 **	Hannan et al., 2015 Weerakoon et al., 2018
	Holstein-Friesen adult male (8-12 mo.)	5.1 ± 1.8	Anand-Ivell et al., 2019
	Holstein-Friesen adult female	0.18 ± 0.01	Anand-Ivell et al., 2011
Sheep	*Merino* adult female	0.10 ± 0.01	Anand-Ivell et al., 2011
Goat	*Shiba* adult male	25 ± 7	Hannan et al., 2017
Horse	Adult intact male	19.9 ± 17.7	Tsogtgerel et al., 2020
Dog	diverse breeds adult male	0.49 ± 0.36 0.29 ± 0.09	Pathirana et al., 2012 Anand-Ivell (unpublished)