Biology of insulin-like factor 3 in human reproduction

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BACKGROUND: Insulin-like factor 3 (INSL3) is a neohormone that has evolved to address specific mammalian traits, in particular, the first phase of testicular descent towards the scrotum during mid-gestation.

METHODS: A thorough literature search was made in PubMed using the terms INSL3, as well as the older synonyms RLF and Ley-IL.

RESULTS: INSL3 is a major secretory product of the testicular Leydig cells in the fetus and in adult men, and in rodent models, reduction in fetal INSL3 expression is an early marker of the testicular dysgenesis syndrome. In women, it is produced in lower amounts by ovarian theca and luteal cells, and circulating levels are increased in women with polycystic ovarian syndrome. During pregnancy, there is evidence for an interaction regulating the feto-placental unit. The presence of INSL3 in amniocentesis samples taken at 12–14 weeks gestation is absolutely specific for male gender, and levels are predictive of subsequent pre-eclampsia and/or birthweight. INSL3 is also involved in adult traits, such as spermatogenesis and bone metabolism. In adult men, INSL3 is constitutively expressed and secreted into the bloodstream at a constant level, reflecting the number and/or functional capacity of the Leydig cells. In complete contrast, testosterone is highly variable within individuals, is acutely responsive to fluctuations in the hypothalamic–pituitary–gonadal axis and appears to have marginal diagnostic value. INSL3 declines consistently with age in adult men.

CONCLUSIONS: INSL3 promises to become an important new diagnostic tool to characterize those men with late-onset hypogonadism and to add clinical diagnostic value at amniocentesis.

Key words: cryptorchidism / hypogonadism / Leydig cells / feto-placental unit / relaxin-like

Introduction

Insulin-like factor 3 (INSL3) was first described in the early to mid-1990s as the product of a frequent testis-specific gene transcript under the alternative names relaxin-like factor (RLF) or Leydig insulin-like peptide (Ley-IL) (Adham et al., 1993; Pusch et al., 1996). Gene transcripts encoding the novel peptide had been discovered independently from the testes of pigs (Adham et al., 1993) and rodents (Pusch et al., 1996) employing differential cloning techniques. Later, the equivalent sequences were obtained by homologous screening of genomic or cDNA libraries and by RT–PCR from humans (Burghardt et al., 1994; Ivell et al., 1997) and a variety of other species (e.g. Bathgate
et al., 1996; Spiess et al., 1999; Zarreh-Hoshyari-Khah et al., 1999; Klonisch et al., 2001a, b). The sequences obtained revealed a primary amino acid structure with close homology to the relaxin–insulin family of peptide hormones and, consequently, sparked considerable interest in this new testis-specific gene. Because of the difficulty in obtaining or synthesizing the presumed biologically active peptide, experimentation was initially largely restricted to expression studies. The next major milestones were the successful generation of knockout mice in 1999 (Nef and Parada, 1999; Zimmermann et al., 1999) and, subsequently, the identification of the receptor for INSL3 in 2002 (Kumagai et al., 2002). The chemical synthesis of pure peptide agonists and antagonists in the ensuing years then allowed the development of both receptor-binding assays and sensitive immunoassays, besides providing bioactive peptides for functional studies (e.g. Bullesbach et al., 2008; Del Borgo et al., 2006) and a further advance in our understanding of this important new hormone.

Initially, making use of antibodies generated by immunizing rats and rabbits with denatured recombinant protein made in Escherichia coli from full-length cDNA expression vectors (including A-, B- and C-domains), immunohistochemical analysis confirmed high levels of INSL3 expression in the testicular Leydig cells from rat (Fig. 1; Spiess et al., 1999; Sadeghian et al., 2005), mouse (Pusch et al., 1996), hamster (Ivell et al., 2003a), human (Fig. 1; Ivell et al., 1997) and marmoset monkey (Zarreh-Hoshyari-Khah et al., 1999), and also in the equivalent steroidogenic cells of the ovary [mouse: corpora lutea (Pusch et al., 1996); bovine: follicular theca cells and corpora lutea (Bathgate et al., 1996; Irving-Rogers et al., 2002); human: follicular theca cells (Bamberger et al., 1999)], but in no other gonadal cell types. The immunohistochemical results were fully in accord with results from mRNA in situ hybridization, confirming particularly the Leydig cells and ovarian theca cells as the principal, if not exclusive, sites of INSL3 synthesis, and thus potentially at least showing that these steroidogenic cells were also able to make another important hormone-like entity besides steroid hormones. Antibodies raised using short peptide fragments were of mixed success, though succeeded in identifying the sites of INSL3 expression also in the dog testis (Klonisch et al., 2001a), horse testis (Klonisch et al., 2003), human placenta (Hombach-Klonisch et al., 2001), thyroid, mammary and prostate glands (Klonisch et al., 2001a,b; Hombach-Klonisch et al., 2000a, 2003), bovine testis and corpus luteum (Irving-Rogers et al., 2002) and deer testis (Hombach-Klonisch et al., 2000b, 2004). Because all of these antibodies specifically recognized either denatured protein or peptides that were not in a native conformation, none of them proved suitable for the development of specific immunoassays for the measurement of INSL3 in blood and body fluids (but see later). It needs to be emphasized that without measurement of the functional, secreted protein in extracellular fluids, such as blood, the true physiological relevance of a hormone such as INSL3 can only be inferred very indirectly.

**Methods**

To generate this review, a thorough literature search was made in PubMed using the terms INSL3, as well as the older synonyms RLF and Ley-IL. This search covered the complete period from the initial discovery of the INSL3 gene in 1993 to the present day. Literature was filtered to include all articles with physiological relevance to either male or female reproduction, besides those of a more general introductory nature. There is an extensive literature on the molecular genetics of INSL3 and RXFP2 mutations and polymorphisms, which has been recently reviewed in detail elsewhere (Ferlin et al., 2006a, 2008b; Foresta et al., 2008), and is only summarized here.

**Molecular structure of the INSL3 gene and protein**

In all species so far investigated, INSL3 is encoded by a very short gene of \(<1\) kb length containing a single intron (Fig. 2). Interestingly, the gene is located close to or within another gene, namely that for Janus Kinase 3 (JAK3; Koskimies et al., 1997; Spiess et al., 1999). In mice, the INSL3 gene commences within the last intron of JAK3 and includes coding sequence shared with the 3’ UTR (mRNA untranslated region) of that gene (Koskimies et al., 1997). Because of this overlapping of mRNA sequence, it is very important that for mRNA hybridization studies (in situ hybridization, northern blotting, etc.), at least in mice, only sequences that are outside of this overlap region are used, otherwise one cannot distinguish INSL3 expression from that for JAK3.

The promoter region of the INSL3 gene appears also to be very short. A region of ca. 100–200 bp (Fig. 2) appears to be sufficient for high levels of expression in mouse Leydig tumour cell-lines (Zimmermann et al., 1998; Koskimies et al., 2002; Sadeghian et al., 2005; Robert et al., 2006), and only a slightly longer sequence (ca. 700 bp) used in transgenic mouse constructs appears to target transgene expression very specifically to the Leydig cells (Shirnesan et al., 2008). This does not necessarily imply that other sequences are not important, but strongly suggests that most, if not all, regulation is limited to a relatively small piece of genomic DNA.

The INSL3 gene encodes a single open reading frame of ca. 180 amino acids, which conforms closely with the structure of the precursor proteins encoding insulin, relaxin and the insulin-like growth factors (IGFs), with a signal peptide immediately preceding a B-domain region, then a C (connecting) domain and finally an A-domain region. It is generally assumed that like relaxin and insulin, but unlike the IGFs, INSL3 is produced mostly as an A-B heterodimer linked by cystine bonds, with post-translational excision of the C-domain. It is important to note that this is only an assumption. To date, the only piece of evidence in favour of this representing the circulating structure

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**Figure 1** INSL3 immunoreactivity in the Leydig cells of healthy adult human (A) and rat (B) testes. Seminiferous tubule diameters for both rat and human tests are approximately 200 μm.
of the hormone is the purification of some A-B heterodimer from the extracts of bull testes (Bullesbach and Schwabe, 2002). It is corroborated by the fact that chemically synthesized A-B heterodimeric INSL3, representing the sequences from mouse, rat, human or sheep, is fully bioactive. However, the uncleaved precursor pro-form (including the C-domain) may also be equally bioactive; certainly this is true for the structurally very closely related relaxin–relaxin receptor system (Zarreh-Hoshyari-Khah et al., 2001). To date, all western blots of testes and other tissues appear only to identify the 14–18 kDa pro-form (e.g. Hombach-Klonisch et al., 2000a). There are several possible reasons for this: the small A-B heterodimer (ca. 6 kDa) may not separate well or remain on western blots; furthermore, there is circumstantial evidence that much of the INSL3 visualized immunohistochemically in Leydig cells is stored in Leydig cells as a Golgi aggregate of the pro-form (e.g. Balvers et al., 1998). Further work is needed here to clarify these issues.

**Regulation of the INSL3 gene**

There have been several independent studies on the regulation of the INSL3 gene and the structure and functionality of the INSL3 gene promoter (Koskimies et al., 1997, 2002; Zimmermann et al., 1998; Sadeghian et al., 2005; Robert et al., 2006). The short INSL3 gene promoter region has up to three independent sites for the binding and interaction of the transcription factor SF-1 (Fig. 2; steroidogenic factor-1; Sadeghian et al., 2005). SF-1 is a very important transcription factor in steroidogenic cells, being essential for the expression of numerous Leydig cell-specific genes involved in steroidogenesis, such as StAR and P450scc. Although binding to each site may differ in intensity between species (cf. rat versus mouse; Sadeghian et al., 2005), it appears that the most proximal of these is the site that is critical for expression of the gene in transfection studies (Fig. 2). It is important to note that an SF-1 site is generally also a site for the binding of other closely related transcription factors, such as Nur77 (NR4a1; Robert et al., 2006), which is also to be found in Leydig cells. Again there are some unanswered questions here. For example, although the transcription factors named above are all to be found also in adrenal steroidogenic cells, there is no evidence for the expression of INSL3 in the adrenal gland at any stage of development, except where there is evidence for the presence there of Leydig-like tumour cells (Lefebvre et al., 2003).

With one exception, all studies looking at the possible regulation of the INSL3 gene by various effectors, either assessing endogenous
expression of INSL3 mRNA in rodent Leydig tumour cells or primary Leydig cell cultures (Balvers et al., 1998; Sadeghian et al., 2005), in bovine ovarian thecal cell cultures (Bathgate et al., 1999) or alternatively assessing reporter expression levels in homologous or heterologous transfection systems (Sadeghian et al., 2005; Lague and Tremblay, 2008), indicate unregulated and constitutive expression of the gene. This includes treatments with a range of established effectors and secretagogues [estrogens, androgens, LH or hCG, db-CAMP, tumour necrosis factor-α (TNF-α), IGF1, atrio-natriuretic peptide, phorbol myristate ester and their combinations]. Only where there are induced changes in the differentiation status (e.g. in bovine ovarian theca cells from antral follicles subjected to long-term hCG stimulation; Bathgate et al., 1999), do we see a change in the specific expression of the INSL3 gene. The exception to these results is a single study by Lague and Tremblay (2008) where they were able to show that both testosterone and the endocrine disrupting chemical (EDC) monoethyl hexyl phthalate at high concentration were able to modulate INSL3 gene expression to a modest degree in both MA-10 Leydig tumour cells and primary immature (Day 29) rat Leydig cells (Lague and Tremblay, 2008). Testosterone stimulated INSL3 mRNA expression, whereas the phthalate was able to suppress this stimulatory effect. We have recently tried to repeat these studies in both MA-10 cells and in adult (Day 90) rat Leydig cells, without success either at the level of mRNA expression or peptide secretion (R. Anand-Ivell and R. Ivell, unpublished). Thus at the present time, and in an acute (<24 h) context, all results appear to be pointing to a constitutive and unregulated expression of INSL3 mRNA mediated via a permissive control of the proximal promoter region of the gene, in particular gonadal steroidogenic cells (Leydig and ovarian theca cells) that have achieved a certain differentiation status.

INSL3 receptors and signalling

Although insulin and the IGFs all signal via cell surface receptors with single transmembrane domains and inherent tyrosine kinase activity, both INSL3 and the closely related peptide hormone relaxin make use of an evolutionarily quite different class of receptor. A natural mutant with a cryptorchid phenotype identical to that of the INSL3 use of an evolutionarily quite different class of receptor. A natural mutant with a cryptorchid phenotype identical to that of the INSL3 knockout mouse gave the first clue (Overbeek et al., 2001). The gene responsible turned out to be a G-protein-coupled receptor (GPCR) with seven transmembrane domains and a very large N-terminal ectodomain comprising low-density lipoprotein (LDL)-receptor-like motif, 10 leucine-rich repeat motifs and a cysteine-rich hinge region. This gene first called 'Great' (Overbeek et al., 2001), and subsequently LGR8 (Kumagai et al., 2002), is now formally recognized as RXFP2 (relaxin family peptide receptor 2; Bathgate et al., 2006). The closely related receptor RXFP1 (previously LGR7) is the receptor for relaxin.

In HEK-293T cells, which have been permanently transfected with a DNA construct expressing the RXFP2 gene, INSL3 appears to stimulate the receptor to elicit a Gs-dependent activation of adenylyl cyclase, thereby specifically increasing the concentration of intracellular cAMP (Kumagai et al., 2002; Heng et al., 2008). Other reports suggest that RXFP2 may also couple to inhibitory G-proteins (Kawamura et al., 2004), and thus might be linked to other intracellular signalling systems. At present, it is difficult to elaborate the signalling pathways activated by INSL3 in cell systems that naturally express the RXFP2 receptor, as opposed to those, such as the transfected HEK-293T cells mentioned above, where the RXFP2 gene is overexpressed and where other cofactors required for appropriate receptor signalling may be absent. There appear to be very few accessible cell systems available that naturally express the RXFP2 receptor. Studies using the closely related relaxin receptor, RXFP1, show how important this is; for this system (but not for transfected cells), the naturally expressed receptors absolutely require additional cytoplasmic components for signalling, with the unusual need of a tyrosine kinase activity in order to have optimal cAMP expression (Bartsch et al., 2001; Anand-Ivell et al., 2007).

It is important to recognize that although transfected cell systems are very convenient models with which essential receptor pharmacology for RXFP1 and RXFP2 is studied, cells and tissues which naturally express the receptors may be different. First, we know that the numbers of receptors expressed on the surface of cells that naturally respond to relaxin or INSL3 are generally low, in the order of a few hundred molecules per cell. Secondly, transfection studies are suggesting that a large proportion of the expressed receptors may not be at the cell surface, but distributed on other intracellular membranes, such as those of the endoplasmic reticulum and Golgi apparatus (Kern and Bryant-Greenwood, 2008). Immunohistochemistry for specific RXFP2, as well as RXFP1 epitopes, also suggests a diffuse cytoplasmic distribution for the receptors, rather than a specific localization at the plasma membrane (Ivell et al., 2003b; Anand-Ivell et al., 2006a).

Recent research into the structural biochemistry of the ligand–RXFP2 interaction indicates first that only intact and naturally conformed heterodimeric INSL3 is able to activate the receptor (Bathgate et al., 2006). Certain conformationally constrained B-chain peptides are able to act as antagonists (e.g. Del Borgo et al., 2006; Shabanpoor et al., 2008), further emphasizing the importance of the B-chain for receptor interaction. Particularly, tryptophan B27, valine B19 and arginine B16 are known to be crucial for receptor binding and activation (Bathgate et al., 2006; Bullesbach and Schwabe, 2006). Latest research also suggests that INSL3 is probably interacting with the receptor at two independent locations, with both interactions being essential for appropriate signal transduction, as also is the involvement of the N-terminal LDL motif on the receptor, which however is not part of the peptide binding site (Bathgate et al., 2006; Scott et al., 2006; Kern et al., 2007). New work using overexpressing transfected cell systems is also strongly suggesting that, at least in these albeit somewhat artificial systems, both RXFP2 and RXFP1 can form homodimers and heterodimers with each other in the cell membrane (Kern and Bryant-Greenwood, 2008; Svendsen et al., 2008). This is important in the context of understanding the importance of possible splice variants of the receptors (see below).

Both RXFP1 and RXFP2 (Fig. 3) genes each comprise 18 exons, with many small exons encoding the extracellular hormone-binding domain with the 10 leucine-rich repeats (Bathgate et al., 2006). It is now well known that many similar genes encoding GPCRs are subject to alternative splicing, whereby small exons may be excised or even additional intron sequence included in the final mature mRNA. This has also been shown for RXFP1 and RXFP2 (Muda et al., 2005; Heng et al., 2008). Such splice variants can often lead to frame-shifted coding regions, or the introduction of premature stop codons in the amino acid sequence of the receptor, mostly
resulting in non-functional receptors (e.g. Muda et al., 2005; Kern et al., 2007). Since we now know that RXFP1 and RXFP2 receptors can dimerize, even small relative amounts of an expressed alternatively spliced variant could have significant consequences for signalling even via receptor molecules of normal, full-length appearance (Kern et al., 2007, 2008; Svendsen et al., 2008). Consequently, in any studies of RXFP1 or RXFP2 expression, it is very important that the presence or absence of splice variants is exhaustively explored. We have assembled a panel of oligonucleotide primers for RT–PCR (Fig. 3), which effectively examines the entire coding region for the transcripts of both receptors (Anand-Ivell et al., 2006a; Heng et al., 2008), thereby allowing a comprehensive description of receptor mRNA expression and whether this can be interpreted as potentially functional or not.

**INSL3 physiology in the fetus and cryptorchidism**

The principal phenotype of mice where the INSL3 gene has been ablated (Nef and Parada, 1999; Zimmermann et al., 1999) or where the RXFP2 gene has been ablated or mutated (Bogatcheva et al., 2003) is primary cryptorchidism. Specifically, the gubernacular ligament linking the embryonic testis to the inguinal body wall fails to develop and thicken, and thus fails to retain the testes in the inguinal region while the rest of the body grows and extends dorsally. This is referred to as the first or transabdominal phase of testicular descent, and is followed by the migration of the testes through the inguinal canal into the scrotum, which appears to be largely an androgen-dependent process (Ivell and Hartung, 2003). The rodent gubernaculum has been shown to express the RXFP2 gene at a high level (Kubota et al., 2002; Anand-Ivell et al., 2006a, b; Feng et al., 2007), and primary cultures of gubernaculums respond to INSL3 with increased DNA synthesis (Kubota et al., 2002). The INSL3 responsible for gubernacular thickening is produced by the fetal population of Leydig cells within the embryonic testis. This is a discrete population of Leydig cells from those of the adult testes, though they both may share a common pool of stem cells. The fetal Leydig cells are derived from relatively undifferentiated mesenchymal cells in the dorsal wall of the coelom and appear to originate from a common pool that also gives rise to the steroidogenic cells of the adrenal gland, though this tissue appears not to produce INSL3. mRNA in situ hybridization and immunohistochemistry (McKinnell et al., 2005) suggest that in the mouse and rat, the INSL3 gene is expressed very soon after gonadal gender is determined (e.g. around embryonic Day 12 in the mouse).

In the human, we have been able to measure INSL3 in amniotic fluid (Fig. 4A) obtained from women undergoing routine amniocentesis (Anand-Ivell et al., 2008). INSL3 is only detectable in fluid collected from women carrying a male fetus, but not a female fetus, further verifying for the first time that INSL3 is uniquely a product of the testis, and that the fetal adrenal, which is also well developed in females, is not contributing. Matching its role in the first phase of testicular descent, INSL3 is maximal in amniotic fluid at weeks 12–14 of gestation and declines thereafter to undetectable levels by the end of pregnancy (Fig. 4A). In this study, interesting significant associations were also shown between second trimester INSL3 levels and later pre-eclampsia (Fig. 4B), or with adjusted birthweight (Anand-Ivell et al., 2008). However, sample size did not allow an assessment of genital defects or cryptorchidism. Amniotic fluid levels were presumed to reflect the circulating levels of INSL3 in fetal blood. The first measurements of INSL3 in fetal blood have been made via cord-blood sampling at birth (Bay et al., 2007), i.e. at a time when the local synthesis in the testis is probably already very low and only a remnant of what it would have been earlier in pregnancy. Nevertheless, it has been possible to show that there is a significant difference in the levels of INSL3 in the cord blood of young boys suffering cryptorchidism compared with normal controls (Bay et al., 2007).

Taken together, these results strongly support the view that also in humans INSL3 is probably responsible for at least part of the transabdominal phase of testicular descent. There have been numerous attempts to identify mutations or polymorphisms in either the INSL3 gene or that of its receptor, RXFP2, associated with cryptorchidism (e.g. Ferlin et al., 2006a, 2008b; El Houate et al., 2007; Yamazawa et al., 2007). The failure of the testes to descend into the scrotum is a very common congenital ailment, and depending on geography can be observed in as high as 8% of all newborn boys, though most estimates suggest a frequency of 2–4% (Cortes et al., 2008; Foresta et al., 2008; Virtanen and Toppari, 2008). If corrected early enough by appropriate minor surgery, there are probably no long-lasting effects, though a delay in repositioning the testes is associated with loss of fertility and an increased incidence of testicular cancer (Kaleva and Toppari, 2005;
Pettersson et al., 2007). It is difficult at this stage to be certain that fertility loss and testicular cancer are solely consequences of having cryptorchidism and associated testicular heating. There is also a strong likelihood that all three symptoms, including cryptorchidism, are themselves the consequences of an earlier developmental disturbance, referred to as the testicular dysgenesis syndrome (TDS; Skakkebaek et al., 2001; Sharpe and Skakkebaek, 2008).

Although several polymorphisms have been identified in both the INSL3 and RXFP2 genes, with the exception of the rare T222P mutation in the RXFP2 gene (Bogatcheva et al., 2007), none have been found to causatively associate with a cryptorchid phenotype. The T222P mutation creates a markedly altered receptor that appears unable to respond to INSL3 stimulation in vitro (Bogatcheva et al., 2007). However, recent studies suggest that being a heterozygous carrier of the T222P mutation does not increase the susceptibility to cryptorchidism (El Houate et al., 2008; Nuti et al., 2008). Nevertheless, in several studies of relatively large cohorts of cryptorchid subjects, there does appear to be significantly more INSL3 or RXFP2 polymorphisms cumulatively associated with cryptorchid than with normal subjects, suggesting a pleiotropic contribution by these genes (Ferlin et al., 2006a).

The TDS postulates that early in the embryonic development of the testis, shortly after male sex determination has occurred, the molecular events that lead to the accurate differentiation of Sertoli cells, Leydig cells and germ cells are exposed to external disrupting influences, which can have subtle effects on quantitative aspects of the mature phenotype of all three cell types (Skakkebaek et al., 2001; Sharpe and Skakkebaek, 2008). The effects are manifest in the neonate by reduced androgenization (cryptorchidism, hypospadias, micropenis and shorter anogenital distance) and in the adult by lower sperm counts, increased incidence of testicular cancer and lower circulating testosterone. The cause for the original developmental disturbance has been traced to the influence on the pregnant mother of so-called environmental EDCs acting on the early developing testis at a time when, particularly, this organ is undergoing major differentiation activity and, evidently, the protective capacity of mother and placenta is not high. Most of the symptoms of TDS can be modelled in rats by exposing pregnant dams to moderate-to-high amounts of any of a range of chemical substances, which include natural and synthetic estrogens, fire retardants, plasticizers (e.g. phthalates), some fungicides and pesticides, and many common components of personal care products. Research is showing first that there is a sensitive time window for exposure, approximately between embryonic Days 12 and 17 in the rat. Secondly, some of the earliest detectable events involve altered gene expression.
specifically of Leydig cell products (including INSL3), though since all three major testicular cell types (Leydig cells, Sertoli cells and germ cells) are mutually interacting at this time, it is difficult to determine whether only one cell type is the primary target of EDC action.

During this critical time window in pregnancy, the Leydig cells are producing two major hormonal products: besides testosterone (which may become aromatized to estrogen in target tissues), INSL3 appears to be produced in very large amounts by the fetal Leydig cells. The fact that it is easily detectable in human amniotic fluid at around 14 weeks of gestation (Anand-Ivell et al., 2008; Bay et al., 2008), where it is already considerably diluted from what must be in fetal blood is suggesting that this is a very significant and obviously gender-specific hormone for the fetus at that time. Significantly, comparative data for testosterone and INSL3 in human amniotic fluid show that girl fetuses also produce significant amounts of testosterone (approximately half the male levels), presumably of adrenal origin, whereas they generate no detectable INSL3 (Anand-Ivell et al., 2008). It is well known that in terms of feto-placental physiology there are several gender-specific effects that can influence both birthweight and later health outcomes (e.g. cardiovascular disease, obesity and insulin-sensitivity). Our study on INSL3 in human amniotic fluid highlighted two significant associations. First, INSL3 in mid-pregnancy (15–17 weeks) was significantly predictive of later (third trimester) pre-eclampsia (Fig. 4B), and secondly, INSL3 levels correlated with subsequent birthweight corrected for gestational age (Anand-Ivell et al., 2008). Both observations strongly suggest that INSL3 may be influencing the feto-placental unit in a gender-specific fashion. It should be noted that the presence of INSL3 and its receptor in the human placenta has been reported (Hombach-Klonisch et al., 2001), and supports this viewpoint. Furthermore, this could provide an explanation for the subtle affects of EDCs on other aspects of physiology and disease, by influencing—via reduced fetal INSL3—the role of placental function on later health outcomes.

**INSL3 physiology in the adult male**

INSL3 is a major circulating hormone in the adult male. The sole contributors to circulating levels are the ~250 million Leydig cells of the adult human testis. This has been confirmed by mRNA hybridization and/or by immunohistochemistry (Fig. 1) for a wide range of species (rat, mouse, pig, human, marmoset monkey, lemur, deer, camel, cat, dog, bovine, sheep and goat). These adult-type Leydig cells differentiate during puberty under the influence of LH from a population of stem cells, and are quite discrete from the fetal Leydig cell population referred to above. The latter appear to involute during infancy and remain insignificant for the remainder of life (Ivell et al., 2003a). From studies in rats, the Leydig stem cells from which the adult population derives are small spindle-shaped, relatively undifferentiated mesenchymal cells which are attached to the outer surface of the seminiferous tubules. With the establishment of a functional hypothalamic–pituitary–gonadal (HPG) axis during puberty, LH induces initial proliferation and then post-mitotic differentiation of the Leydig cells. It is important to note that the final number of Leydig cells achieved during puberty remains largely unchanged throughout life with subsequently no detectable cell division or cell death. This makes the adult-type Leydig cells some of the oldest cells of the body.

Circulating INSL3 concentrations in humans (Foresta et al., 2004; Bay et al., 2005; Anand-Ivell et al., 2006b) and rats (R. Anand-Ivell and R. Ivell, unpublished) reflect the number and differentiation status of the Leydig cells. During infancy, INSL3 is at or below the level of detection of the currently available assays (see below). There is a steady increase during puberty to reach relatively stable adult levels by the age of about 18 years in the human (Ferlin et al., 2006b). In fact, the initial pubertal increase in circulating INSL3 is one of the earliest serum indicators of initiating puberty in boys (Wikstrom et al., 2006). The average young (35–40 years) human male has ~1.3 ± 0.5 ng/ml INSL3 in his circulation and this value declines steadily throughout life (Fig. 5) to reach an average level of about 0.8 ± 0.4 ng/ml in old age (75–80 years; Anand-Ivell et al., 2006b). Confirming the testicular origin of circulating INSL3, men whose testes have been surgically removed as part of cancer treatment have almost undetectable circulating INSL3 (Anand-Ivell et al., 2006b).

One of the more interesting features of INSL3 expression is that it appears to be completely independent of acute regulation by hormonal factors, and is thus independent of the homeostatic feedback regulation by the HPG axis. It is important to differentiate this acute situation from longer term regulation that involves differentiation or dedifferentiation processes. For example, for Leydig cells in culture, none of the effectors known to stimulate steroidogenesis in an acute context (e.g. LH or hCG, db-cAMP, atrial natriuretic peptide, TNF-α, phorbol myristate acetate, testosterone and estradiol) have any effect on INSL3 gene or peptide expression (Balvers et al., 1998; Sadeghian et al., 2005). hCG application to men whose HPG axis had been suppressed by GnRH treatment is without effect on circulating INSL3 levels, unlike on androgen levels, over a short period of a few days (Bay et al., 2005).

In contrast, any circumstance that affects the long-term differentiation status of Leydig cells is indeed reflected by the amounts of INSL3 produced. Thus, seasonally breeding mammals show a regular cycle of high and low INSL3 expression depending upon whether their testes are in a phase of development or involution.

**Figure 5** INSL3 concentration in the blood of 1164 men sampled as part of the Florey Adelaide Male Ageing Study (Anand-Ivell et al., 2006b). Republished with permission from Wiley-Blackwell.
respectively (Hombach-Klonisch et al. 2000b, 2004; Ivell et al., 2003a). A suppressive contraceptive regime of GnRH agonist or antagonist plus a steroid hormone (testosterone or progesterone) to suppress the functioning of the HPG axis leads to a chronic dedifferentiation of the Leydig cells and concomitant loss of INSL3 expression, with restoration of the HPG axis leading to a full recovery of the normal circulating INSL3 levels (Amory et al., 2007; Bay et al., 2006; R. Ivell and R. Anand-Ivell, unpublished data). Similarly, men treated with androgens as part of a hormone replacement therapy also show reduced INSL3 levels because of the resultant chronic suppression of the HPG axis (Anand-Ivell et al., 2006b). Immunohistochemically, Leydig tumour cells that have re-entered the cell cycle, and consequently lost much of their post-mitotic differentiation status, also indicate a markedly reduced INSL3 expression (Klonisch et al., 1999).

Possibly the most convincing data for this distinction between chronic and acute effects of the HPG axis on INSL3 production were provided by a study of unilaterally orchidectomized men (Anand-Ivell et al., 2006b). These men will have only half the normal number of Leydig cells. Consequently, the HPG axis is up-regulated with increased LH production in order to maintain normal levels of testosterone, which are not significantly different from intact controls (Anand-Ivell et al., 2006b). Circulating INSL3 levels, however, are significantly reduced in these semi-orchiectomized subjects, being intermediate between intact controls and the concentration observed in a bilaterally orchidectomized man (Anand-Ivell et al., 2006b). Significantly, when LH values are plotted against INSL3 values for the unilaterally orchidectomized subjects (Fig. 6), there is an inverse correlation, implying that the poorer the functioning of the remaining Leydig cells in the one intact testis, the greater the amount of LH is needed to produce a normal concentration of testosterone (Fig. 6). However, these poor quality Leydig cells produce a lower amount of INSL3, because this production is independent of the acute regulation by LH (Anand-Ivell et al., 2006b).

In terms of physiological function in the adult male, there is accumulating evidence to suggest a role in maintaining spermatogenesis, though there may also be other functions in male reproduction. Analyses of where the RXFP2 receptors are expressed in adult male rats and mice show that within the testis there are receptors on both the Leydig cells themselves, as well as on both pre- and post-meiotic stages of differentiating germ cells, with most being on post-meiotic spermatids (Anand-Ivell et al., 2006a, Feng et al., 2007; Filonzi et al., 2007). This was confirmed by immunohistochemistry for the human testis (Anand-Ivell et al., 2006a). There are no receptors on Sertoli cells or any other cellular component of the testes. We have so far not been able to determine any effect of INSL3 on adult-type rat and mouse primary Leydig cells, nor on the mouse Leydig cell tumour cell-line MA-10 (R. Anand-Ivell and R. Ivell, unpublished), though it has been reported to stimulate the mouse TM3 embryonic Leydig cell-line (Feng et al., 2007). However, the high local concentration of INSL3 in the testicular interstitial fluid that bathes the Leydig cells in vivo is such that any Leydig cell receptors are likely to be permanently down-regulated or desensitized.

Three studies support a role for INSL3 on post-meiotic male germ cells. First, treatment of immature rats with a GnRH agonist leads to considerable germ cell apoptosis. This apoptosis can be largely inhibited by co-treatment of the animals with INSL3 (Kawamura et al., 2004). Secondly, application of an INSL3 antagonist by direct injection into the rat testis leads to a significant reduction in testis weight, equivalent to that seen under a GnRH agonist-induced suppression of spermatogenesis (Del Borgo et al., 2006). Thirdly, in an analysis of men undergoing suppression of spermatogenesis using a contraceptive regime of GnRH agonist plus testosterone or equivalent to suppress the HPG axis, subjects could be subdivided into good responders (few residual sperm) and poor responders (many residual sperm). The latter group had significantly higher levels of circulating INSL3 (Amory et al., 2007), implying a possible protective effect vis-à-vis germ cell apoptosis. Finally, as will be discussed later, in the ovary, there is also evidence that INSL3 has an anti-apoptotic role with regard to follicle (oocyte) selection (Spanel-Borowski et al., 2001; Irving-Rodgers et al., 2002). There is also RXFP2 gene expression in other parts of the male reproductive system, including the epididymis and vas deferens (Feng et al., 2007; Filonzi et al., 2007), though as yet there is no information as to any function for INSL3 in these tissues.

One of the most exciting new pieces of information to have appeared recently is the report that INSL3 has a role to play in bone metabolism (Ferlin et al., 2008a). Men carrying the T222P mutation of the RXFP2 gene had significantly osteopaenia, mice with the RXFP2 gene ablated exhibited marked osteoporosis and cultured primary human or mouse osteoblast cells responded significantly to INSL3 stimulation by increased cAMP production and cell proliferation (Ferlin et al., 2008a). Thus, it appears that in addition to the role of steroid hormones, the gender-specific expression of INSL3 might also be a contributing factor to the reduced incidence of osteoporosis in men.

Two other tissues also appear to engage INSL3 in their metabolism. These are the prostate gland and the thyroid gland. In the former, cells from benign prostate hyperplasia as well as from some but not all prostatic carcinomas expressed both INSL3 and RXFP2 mRNA, and some

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**Figure 6** Peripheral INSL3 concentration in blood from 10 unilaterally orchidectomized men collected as part of the Florey Adelaide Male Ageing Study. There is a highly significant (P < 0.01) negative correlation with peripheral LH (Anand-Ivell et al., 2006b). Republished with permission from Wiley-Blackwell.
cells appeared to respond to the hormone by altering their motility (Klonisch et al., 2005). For thyroid medullary carcinoma, INSL3 expression was detected in some cancer cell-lines and tissues though not in normal human tissue; RXFP2 gene expression was detected throughout, suggesting that also the thyroid gland may be a responsive target tissue for INSL3 (Hombach-Klonisch et al., 2003). It should be noted, however, that making use of the very extensive data set (ca. 1200 men) of the large South Australian FAMAS cohort, there was no evidence for any specific association between circulating INSL3 levels and incidence of prostate disease, prostate specific antigen or any thyroid-related parameters, nor it should be noted at this stage with bone density (Anand-Ivell et al., 2006b).

**INSL3 physiology in the adult female**

Although INSL3 is primarily a male hormone, it is nevertheless detectable in the peripheral blood of adult women, with levels close to or just above the level of assay detection (Bay et al., 2005; Gambineri et al., 2007). The ovary is effectively quiescent in the female fetus, and hence unlike in the male, not only is INSL3 not measurable (Anand-Ivell et al., 2008), but also there appears to be little relevance for this hormone there. In women, INSL3 is expressed in the ovary, in those cells that represent the ovarian equivalent to the Leydig cells, namely the follicular theca cells and their later derivatives as cells of the corpus luteum (Bamberger et al., 1999). Interestingly, whereas in women expression levels in the ovary appear to be relatively low, reflecting local but probably not any systemic functions for the hormone, in the cow and sheep ovarian expression of INSL3 in the same cells is very high (Bathgate et al., 1996, 1999; Irving-Rodgers et al., 2002). INSL3 has also been detected in the mouse ovary (Balvers et al., 1998) and in the ovary of the marmoset monkey (Zarreh-Hoshyan-Khah et al., 1999).

Post-pubertal follicular selection and maturation are processes involving the continual differentiation of the cells that comprise the follicles: oocytes, theca and granulosa cells. It is, therefore, not surprising that INSL3 mRNA levels and peptide are reported to vary markedly depending on the differentiation status of the cells concerned (Bathgate et al., 1999; Irving-Rodgers et al., 2002). Similarly, in cell culture, INSL3 mRNA expression by theca cells can be modulated by those hormonal factors (IGF1 and LH or hCG) that are known reflecting local but probably not any systemic functions for the hormone in these tissues are not clear. We have recently identified the RXFP2 receptor at the mRNA level also in human myometrial cells, but it does not appear to signal here in any conventional fashion (Heng et al., 2008).

**Measurement of INSL3**

In tissue fragments, INSL3 gene expression can be estimated by using INSL3 gene transcripts as a surrogate, measuring the mRNA by quantitative RT–PCR, or by northern blotting. Alternatively, immunohistochemistry can estimate protein expression in cells. None of these are substitutes for measuring the free hormone in the circulation, in other body fluids or in culture media. Four immunoassays have been described in the literature (Bullesbach et al., 1999; Boockfor et al., 2001; Foresta et al., 2004; Bay et al., 2005; Anand-Ivell et al., 2006b), all making use of a purified (extracted or chemically synthesized) A-B heterodimer of INSL3 as immunogen and tracer. Because of the high species-specificity in INSL3 sequences, these assays tend to be restricted to application in one species only. For the human, two assays have been used most commonly. One is a commercial assay (Phoenix Pharmaceuticals, Burlingame, CA, USA) available in both a RIA format and as an ELISA, although published studies to date have only made use of the former (e.g. Foresta et al., 2004). Although results appear to be comparable to those achieved by the other assay (see below), personal correspondence with other groups suggests a higher degree of within- and between-sample variability, which may be due to the assay format rather than to the specificity of the antibodies used.

The second assay was developed by us in Hamburg and, subsequently, used in both Copenhagen (Bay et al., 2005) and with further modifications in Adelaide (Anand-Ivell et al., 2006b). It makes use of a chemically synthesized A-B heterodimer of human INSL3 of very high purity, and polyclonal antibodies raised against this peptide in rats (Bay et al., 2005). It is established on a time-resolved fluorescent immunoassay (TRFIA) platform as a simple sandwich assay: microtitre plates are coated with second anti-rat IgG antibody and incubated with a competing mixture of primary rat anti-human INSL3 antibody, sample and tracer (Europium-labelled INSL3). The advantage of the TRFIA format is that background fluorescence is kept very low, and there is no need for any prior extraction of the peptide. The assay is highly sensitive with a working range currently in our hands of 20 pg/ml to 5 ng/ml using 100 µl samples and both intra- and inter-assay coefficients of variation across the range of <10% (usually much less; Anand-Ivell et al., 2006b, 2008). We have
also tested a wide variety of structurally related substances, showing that the antisera are highly specific for human INSL3. As blank matrix for measurements in serum or plasma, we have found that a pooled sample of post-menopausal female serum is optimal, being completely free of any detectable INSL3. For other body fluids, such as amniotic fluid, we have standardized the assay using the assay buffer as blank matrix (for details, see Anand-Ivell et al., 2008). Extensive control studies show that it works equally well for plasma as for serum samples, and that over short periods INSL3 in solution appears to be relatively stable at room temperature (at least for several hours). Serum samples are stable frozen at −20 or −80°C for long periods, though for transport where dry ice shipments are problematic, we have found that freeze-drying with an efficient lyophilizer able to achieve <100 µBar vacuum and maintain samples frozen throughout the drying process is also reliable. The resultant dried samples can be kept and shipped at room temperature for several days without loss of specific INSL3 immunoreactivity following reconstitution with water. Any kind of excessive heating, either in drying or storage, causes a denaturation of INSL3, with the resultant loss of conformation-dependent immunoreactivity.

We have recently developed (R. Anand-Ivell and R. Ivell, unpublished) a highly specific assay for rodent Insl3, also on a TRFIA platform, with similar detection parameters to the assay for human INSL3 described above. Rats and mice are the most commonly used laboratory models with which to study Insl3 function, both in vivo and in vitro, and we anticipate that this new assay will prove very useful in the evaluation of Insl3 physiology.

**Importance of INSL3 as a novel clinical parameter**

There are three areas of clinical importance where INSL3 promises to become a major new parameter of considerable diagnostic value. The first of these is in the area of andrology and, in particular, in the definition of age-related hypogonadism. There has recently been substantial debate about the role of androgen supplementation in the ageing male. This has been summarized to suggest that there is indeed a case to be made for such treatment in cases of true age-dependent (i.e. late-onset) primary testicular androgen deficiency (Wang et al., 2008; Gooren, 2009). The difficulty is how to define this. It is well established that circulating total testosterone declines with age (e.g. Anand-Ivell et al., 2006b), and in some cases androgen therapy can improve cognitive function, bone mineral density, muscle tone and certain cardiovascular and reproductive parameters (Stanworth and Jones, 2008; Gooren, 2009). However, total testosterone is not a very reliable diagnostic parameter. Many attempts have been made to separate out the biologically relevant component of this, either as ‘free testosterone’, or as receptor-responsive testosterone using a bioassay. In addition, it is well known that most routine assay systems for the measurement of testosterone have high intra- and inter-assay variability, with the result that currently some experts are advocating a physical mass spectrometry-based assay rather than an immunological one, particularly for apparently low testosterone levels as in ageing men (Sikaris et al., 2005; Diver, 2006; Wang et al., 2008). Partly, the problem also arises because of high biological variability, with a single daily measurement often not being representative of an individual’s capacity to generate androgens (Diver, 2006). Repeat measurements within an individual can be extremely disparate. The second main issue is that a low androgen level, particularly in ageing men, can have many reasons (reviewed in Veldhuis et al., 2008): (i) reduced GnRH pulsatility; (ii) hence reduced pituitary LH output; (iii) lower Leydig cell capacity to respond to LH; (iv) reduced Leydig cell capacity to generate steroid hormones; (v) increased or altered androgen metabolism and/or clearance; (vi) disturbed steroid feedback to the brain and pituitary. Androgen supplementation only makes sense where this will not be effectively compensated by suppressing the HPG axis or where the endogenous capacity of the Leydig cells to produce androgens is severely limited. One of the key pieces of knowledge that is required here in order to make a decision is an assessment of the Leydig cells’ capacity to generate testosterone, i.e. whether the decline in testosterone is truly due to a reduced steroidogenic capacity or to some other cause. At present, the only practical parameter that can be applied here is the T/LH ratio (Andersson et al., 2006; De Kretser, 2006) as a measure of the testicular capacity to respond to LH, though this is subject to high within-individual variability.

The measurement of INSL3 protein offers a much more reliable and consistent parameter to assess the functional status (i.e. differentiation status and/or number) of Leydig cells. First, its expression is acutely independent of the HPG axis. It appears to be constitutively expressed as a function only of the differentiation status and/or number of Leydig cells. For example, in both rats and humans, we have observed that less INSL3 is produced by ageing Leydig cells (Paust et al., 2002; Anand-Ivell et al., 2006b), and as mentioned above, is significantly reduced in men with only one testis, i.e. it appears not to be compensated by up-regulation of the HPG axis (Anand-Ivell et al., 2006b). There is an excellent correlation between circulating levels of INSL3 and the T/LH ratio (Anand-Ivell et al., 2006b). In repeat sampling of individual men, we have shown that there is only ca. 21.1 ± 4.5% or 0.18 ± 0.05 ng/ml (mean ± SEM; n = 9) within-individual variation between INSL3 values in blood samples taken 2 and 4 weeks apart from the same subjects over a broad range of values (0.4–1.6 ng/ml) (R. Anand-Ivell and R. Ivell, unpublished). This compares to the broad range of between-individual variation in intact men (ca. 0.3–3.5 ng/ml; Anand-Ivell et al., 2006b). Finally, in a comprehensive analysis of multiple data sets derived from the FAMAS male ageing study, INSL3 was shown to be the one parameter that best correlated with age, to a greater degree than measurement of testosterone, though it correlated strongly with both total and bioavailable testosterone (Atlantis et al., 2008). All observations so far suggest that INSL3 could be a very important indicator of the true testicular capacity to generate androgens, and thus might be a good discriminator of those subjects who might benefit best from androgen therapy.

The second field where measurement of INSL3 would be of clinical value is in the assessment of amniotic fluid obtained at routine amniocentesis. First, it is an absolute discriminator of fetal gender in samples taken at 12–14 weeks. In addition, we have shown that there are significant differences in INSL3 levels in amniotic fluid where there are subsequent high-risk pregnancy outcomes (fetal birthweight and pre-eclampsia) for male babies (Anand-Ivell et al., 2008). The initial study involved only a limited number (250) of male samples, and a larger, more extensive sample set would be required to evaluate this more thoroughly.
Finally, circulating INSL3 concentrations are significantly elevated in women with PCOS (Gamberini et al., 2007; R. Ivell and R. Anand-Ivell, unpublished). The small sample numbers so far assessed do not suggest that INSL3 could be used alone as an important diagnostic parameter. However, as one of several parameters, it has the potential to provide important supporting information to other diagnostic parameters.

Neohormones and the evolution of INSL3

Both INSL3 and the closely related ovarian hormone relaxin (H2 relaxin) evolved relatively recently with the emergence of mammals from lower vertebrates. They appear to have evolved by repeated duplication from a common ancestral gene, that for relaxin-3 (Wilkinson et al., 2005), specifically to address the new demands of mammalian physiology (viviparity, lactation, scrotal testes and post-reproductive survival). Such newly evolved hormones linked to specifically mammalian traits have been called ‘neohormones’, and are potentially of great interest as drug targets, because they refer to supplementary rather than ancient and essential functions (Ivell and Bathgate, 2006; Ivell et al., 2007). Although INSL3 appears to be more a hormone of male reproductive physiology, including testicular descent, ovarian relaxin has a broad range of functions both in relation to specifically female reproductive functions (breast development, cervical ripening, uterus remodelling, embryo implantation and gestational adjustment of osmotic balance) and more general ones (as an anti-fibrotic agent, vasodilation, neoangiogenesis and wound healing) which can be considered as part of a post-reproductive phenotype.

The ancestral hormone from which both INSL3 and ovarian relaxin have evolved, relaxin-3, appears to function in mammals mostly as a neurohormone in the brain (Liu et al., 2003; Ma et al., 2007). There appears to be only negligible, if any, expression of relaxin-3 in gonadal tissues. This is of considerable interest, since in non-mammalian vertebrates there are molecules, which we now recognize as relaxin-3 orthologues, that are highly expressed in both ovary and testis. In the frog and the chicken, as well as in some fish species, this ‘relaxin’ is very highly expressed in the interstitial (Leydig) cells of the testis or in the ovary (Steinetz et al., 1998), i.e. those cell types that express INSL3 and/or relaxin in mammals. It is, therefore, tempting to speculate that some of the mammalian functions attributed to INSL3 might include historical remnants of the ancestral hormone of lower vertebrates. In addition, the evolution of the neohormones, INSL3 and ovarian relaxin involved, however, another event, namely the acquisition of a new class of receptors. Although INSL3 and relaxin make use of two members of the leucine-rich repeat containing (LGR) class of GPCR receptors, relaxin-3 acts through a member of a quite different class of GPCR receptor, namely GPCR135 (Liu et al., 2003; Wilkinson and Bathgate, 2007). Neither ovarian relaxin nor INSL3 can act at this receptor. Thus, while INSL3 is indeed expressed with similar tissue-specificity to the ancestral hormone of lower vertebrates, mammalian evolution brought with it the evolution of a completely new ligand-receptor system, implying that also the physiology being regulated by this system is likely to be new too.

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