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Minireview

INSL3 as a Biomarker of Leydig Cell Functionality[1]

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ABSTRACT

Insulin-like factor 3 (INSL3) is a small peptide hormone made and secreted uniquely by mature Leydig cells in the testes of all mammals. Importantly, this expression and secretion appears to be constitutive and therefore reflects the differentiation status and number of the Leydig cells present, differing thereby from testosterone, which is acutely and homeostatically regulated by the hormones of the hypothalamic-pituitary-gonadal axis. As a consequence, the measurement of INSL3 either as mRNA in the testis or as secreted peptide circulating in the blood provides an excellent assessment of Leydig cell differentiation, for example, during fetal development, puberty, or aging or following exposure to endocrine-disrupting agents. Whereas INSL3 is proving increasingly useful as a biomarker for testis status, less is known about its functions, particularly in the adult male. Current evidence points to autocrine, paracrine, and endocrine roles, acting through the G-protein-coupled receptor called RXFP2, although more research is required to characterize these functions in detail.

HPG axis, hypogonadism, INSL3, Leydig cell, puberty, testosterone

INTRODUCTION

Insulin-like factor 3 (INSL3; formerly relaxin-like factor) is a secreted peptide hormone that, in analogy to the structurally related hormones relaxin or insulin, is presumed also to conform to a heterodimeric A-B structure (Fig. 1) of approximately 6 kDa once released from the producing cells [1, 2]. Doubt is attached to this assumption because, first, both the precursor pro-form (pro-INSL3) of approximately 14–18 kDa, which includes a connecting C-peptide domain, as well as the mature A-B heterodimer, have been shown to be equally bioactive [3] and second, at least in the pig, the longer pro-INSL3 has been recently identified as a major circulating form of the hormone [3], although also the mature shorter A-B peptide is present in blood [4]. It appears that whereas the B-domain comprises much of the receptor-binding site, it is essential for activity that this is constrained within a specific conformation by the cosynthesized A-domain [5, 6]. The C-domain is part of the initial gene translation product and does not appear to interfere with receptor binding or activation. Inspection of Figure 1C shows that the C-domain does not in fact occlude the region of the molecule assumed to interact with its receptor via the amino acid side chains shown in red.

In all male mammals, INSL3 is a major secreted product of the interstitial Leydig cells of the mature testes (Fig. 2F). In the adult rat, depending on strain and age, INSL3 concentration in peripheral blood is 1–10 ng/ml, whereas in the interstitial lymph surrounding the Leydig cells it is recorded as being ~400 ng/ml [7]. INSL3 can also cross the blood-testis barrier to be present in luminal fluid from seminiferous tubules, rete testis, or epididymis at a concentration of 10–20 ng/ml [7]. The evidently very high rate of synthesis and secretion is reflected by quite intense immunohistochemical staining of the Leydig cell cytoplasm in all species investigated. In some species (mice, bovine), there is clear punctate accumulation of staining in subcellular organelles, probably corresponding to the Golgi [8] (Fig. 2F). From its gene sequence, with a characteristic signal peptide, its secretory behavior, and the presence of furin-like cleavage signals separating the peptide domains, INSL3 appears to be made in the classical secretory pathway. However, typical dense-core secretory granules have been only rarely observed in Leydig cells [9], suggesting that INSL3 might in fact be using a constitutive unregulated pathway for secretion.

DIVERSE LEYDIG CELL POPULATIONS

In most species, there are two discrete populations of Leydig cells. One population arises during fetal development shortly after the expression of the SRY gene and the early differentiation of the fetal testis from the gonadal ridge. These cells produce INSL3, as well as androgens, essential for the correct development of the male reproductive system [10]. Recent studies show that the fetal Leydig cells mostly make androstenedione, which is converted by fetal Sertoli cells into testosterone [11, 12]. Whereas testosterone appears to be needed for the adequate development of Wolffian duct derivatives and the external genitalia, INSL3 is essential for the initial transabdominal phase of testicular descent, whereby

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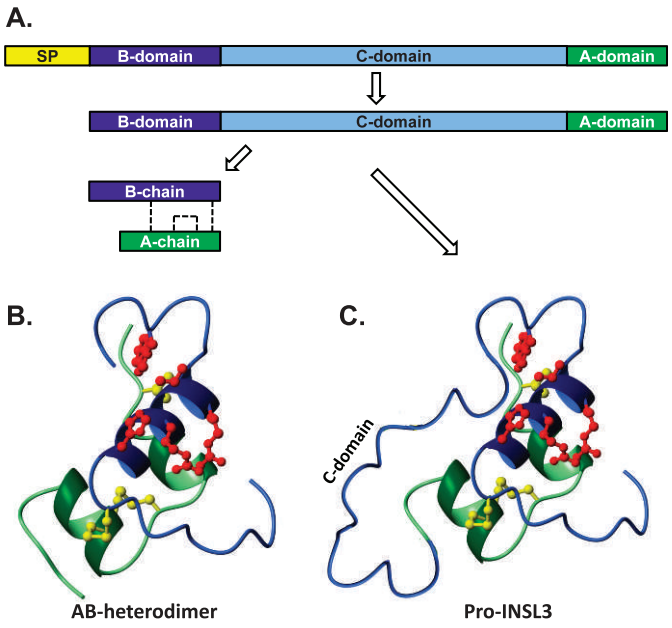

FIG. 1. **A**) Diagrammatic scheme to show expression of the INSL3 gene, first as a transitory prepro-form, including signal peptide (SP), which is removed cotranslationally to generate pro-INSL3. This may then be processed further to result in the A-B heterodimer. Predicted tertiary structure of heterodimeric INSL3 (**B**) and of the INSL3 precursor (pro-INSL3; **C**) based on the known conformation of the two-chain insulin-like heterodimer [67]. The cysteine bridges holding the A- and B-domains in their correct conformation are shown in yellow, and those amino acid side groups involved in receptor interaction are shown in red.

it acts on the specific receptors, called RXFP2, in the gubernacular ligaments linking the testes to the inguinal region of the abdominal cavity. Under INSL3 influence, the gubernacular bulbs thicken, effectively retaining the testes in the lower abdomen while the rest of the body and its organs grow away dorsally [13]. This first phase of testicular descent occurs relatively early in gestation in some species (e.g., ruminants, humans) but is quite late in others, such as rodents, where it is completed only at or after birth [14]. Correspondingly, we find that INSL3 is secreted maximally already in the first half of gestation in humans [15], whereas in rodents maximal levels are observed only at term of pregnancy (Anand-Ivell et al., unpublished). Little is known about the regulation of the fetal Leydig cell population. Whereas in humans at least some aspects of fetal Leydig cell regulation are governed by luteinizing hormone (LH), in mice the differentiation of fetal Leydig cells is independent of LH and rather appears to make use of adrenocorticotropic hormone (ACTH) [16]. We can use INSL3 to monitor this process, for example, in the LH receptor knockout mouse [17; Ivell et al., unpublished] (Fig. 2), where there is no difference in INSL3 expression or Leydig cell density at birth in the LH receptor knockout compared to the wild type. This is quite different for the adult-type Leydig cell population, which also in mice is absolutely dependent on LH secretion (Fig. 2).

In rodents, the fetal Leydig cell population appears to involute mostly during the postnatal decline in gonadotropin secretion, though fetal Leydig cells may still be evident on appropriate immunohistochemical staining, for example, for 17bHSD-10 [18]. With the beginning of puberty, at around Postnatal Days (PND) 10–13 in the rat, new Leydig cells appear to differentiate and proliferate from resident peritubular stem Leydig cells (SLC) to become progenitor Leydig cells

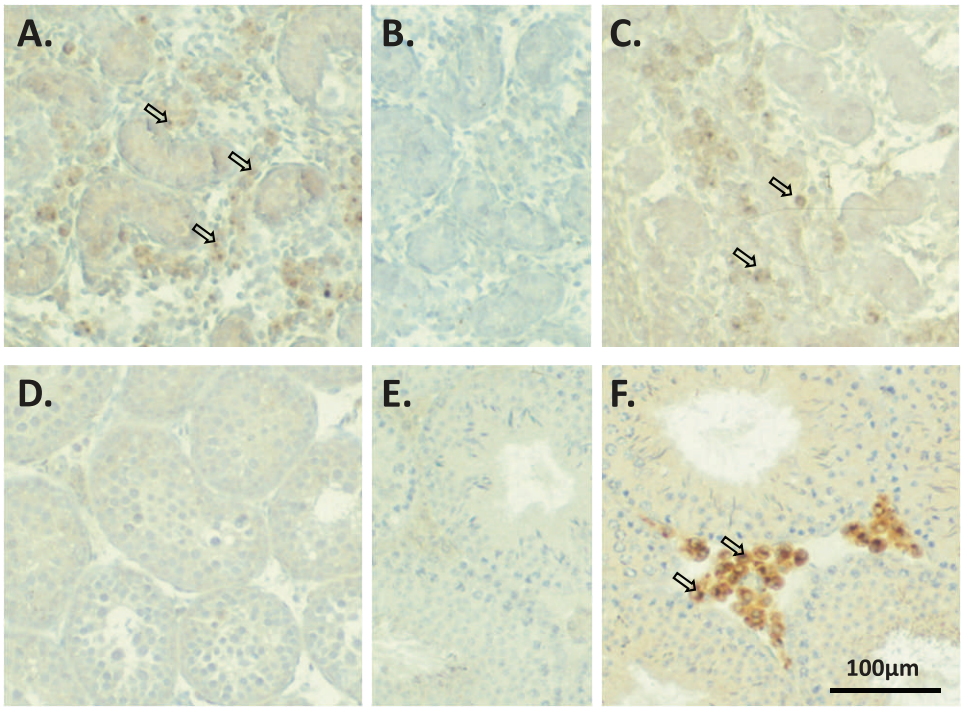

FIG. 2. Immunohistochemical identification of INSL3 epitopes in the testes of LH receptor knockout mice (**A**, **B**, **D**) or their wild-type siblings (**C**, **E**, **F**). Testes were collected and processed either on Postnatal Day 1 to show fetal Leydig cells (**A**–**C**) or at 2 m (**D**–**F**) to illustrate the postpubertal testis. Immunohistochemistry was as in Balvers et al. [8], using as primary antibody the rat polyclonal #63 at a dilution of 1:1000. Mature Leydig cells are indicated by arrows; note the punctate appearance of the staining, suggesting retention of epitopes in the Golgi system. Negative controls (**B** and **E**) were identical to the sections shown in **A** and **F**, respectively, except that the primary antibody was replaced by an equivalent concentration of preimmune serum. Bar in **F** = 100 µm.

(PLC), eventually outnumbering any of the persisting fetal Leydig cells. Following a proliferation phase, these new adult-type Leydig cells undergo further differentiation, first at around PND 28 in the rat, to immature Leydig cells (ILC), then later to mature adult-type Leydig cells, a process that appears to be completed by about 7–8 wk. This process can be monitored by INSL3 expression, which has its first appearance in rats relatively late at about Day 30.

In some species (e.g., humans), there appears to be an intermediate population of Leydig cells manifest in the immediate postnatal phase of infancy. These cells give rise to the peak in testosterone production at 3–4 mo, referred to as the ''minipuberty'' [19]. The origin of these cells is obscure, although there is some evidence to suggest that they may actually represent an independent generation of Leydig cells from resident stem cells [20, 21]. Whether these cells involute again only to redifferentiate during puberty is not known. It appears that the ''minipuberty'' is accompanied also by a burst in INSL3 secretion into the blood [22]. Part of the confusion in interpretation here is due to the fact that Leydig cells are traditionally defined by their mature phenotype; if cells of that description are not identifiable, they are considered to have disappeared or died when in fact they may have only dedifferentiated, for example, in the absence of gonadotropin stimulation. Under normal circumstances, there are very few observations of actual Leydig cell death or apoptosis in the older testis. In rodents, our observation [23; Anand-Ivell and Ivell, unpublished] is that the older Leydig cells (>12 mo mice; >22 mo rats) appear smaller in diameter and show reduced INSL3 expression and secretion, whereas the number of these cells in the interstitium is similar to that in younger animals. In the following sections, unless otherwise noted, we refer to adult fully mature Leydig cells.

LACK OF ACUTE REGULATION

Leydig cells are experimentally convenient cells that have been well characterized for their regulated secretion of testosterone (or progesterone) under the acute influence of LH, or equivalent cAMP-generating agents. Pulses of testosterone are released and/or their enzymatic synthesis is up-regulated within minutes of LH stimulation, hence exhibiting in vivo the well-known correspondence between testosterone entering the circulation and LH pulsatility [24]. There appears to be no such relationship in regard to INSL3 secretion and synthesis. It seems that INSL3 is released from Leydig cells in an unregulated fashion more or less as soon as it has been made. As a consequence, there is a very good quantitative association between INSL3 peptide measured in blood or culture media or visualized by immunohistochemistry and the INSL3 mRNA content of the Leydig cells [25–27].

CHRONIC REGULATION OF LEYDIG CELL FUNCTION

It is important to emphasize that the above regulation is referring to short-term *acute* effects in the order of minutes or hours only and must be clearly separated from *chronic* effects occurring over several days or longer periods. Leydig cells are highly adaptive cells whose differentiation status chronically reflects the status of the hypothalamic-pituitary-gonadal (HPG) axis and, in particular, LH. Thus, Leydig cells differentiate during puberty largely under the influence of increased amplitude and frequency of LH pulsatility [28]. This differentiation is evident as massively increased specific gene transcription on a per cell basis, by an increase in cytoplasmic and nuclear size, by a concomitant increase in smooth endoplasmic reticulum for steroidogenesis, and by the adoption of a specific, mature adult-type Leydig cell morphology [29, 30]. Chronic down-regulation of the HPG axis, for example, by chronic application of a gonadotropin-releasing hormone (GnRH) antagonist [31], or steroidal male contraception [32], or in seasonal breeders [33], causes Leydig cells to involute, becoming effectively less differentiated. INSL3 expression mirrors these changes. Thus, whereas Bay et al. [34] show no effect of human chorionic gonadotropin (hCG) on peripheral INSL3 concentration in hypogonadal patients after 72 and 96 h of treatment, like Foresta et al. [35], they observed a large effect after 1 or more months of treatment, by which time the immature Leydig cells of the hypogonadal men have differentiated under hCG influence.

INSL3 VERSUS TESTOSTERONE

For various reasons, testosterone is a complex parameter in adult males of any species. Besides the issues surrounding its bioavailability, leading to discussion as to whether total testosterone, bioavailable testosterone, or so-called free testosterone are the more meaningful parameters [36], the steroid is generated and metabolized in a highly dynamic manner, leading to high within-individual and diurnal fluctuations, especially in mice [37]. Moreover, its synthesis is regulated acutely (minutes and hours) by the hormones of the HPG axis, primarily LH, acting on the steroidogenic enzymes and steroid transport proteins, such as the steroidogenic acute regulatory protein. This is a consequence of activation of cAMP- and protein kinase A-dependent signaling pathways in Leydig cells via the mediation of the LH receptor [38, 39]. This in turn can be acutely desensitized by higher concentrations of LH [40]. Most significant, the steroid produced by the Leydig cells forms part of a negative feedback loop regulating the production (pulse amplitude and frequency) of LH by the pituitary. Together these mechanisms ensure an acutely variable though, within a normal biological range, consistent level of testosterone in the blood. In addition, in circumstances where circulating testosterone is insufficient to trigger the negative feedback loop controlling the HPG axis, testosterone is also chronically influenced by the long-term differentiation status of the Leydig cells. Thus, the measurement of testosterone reflects effects at the hypothalamic, pituitary, and testicular levels; it responds to both acute and chronic physiology; and it can furthermore be influenced in its biological relevance by the concomitant variations in binding proteins and its metabolism in target organs.

In marked contrast to the above, INSL3 is constitutively expressed by the Leydig cells, is not acutely regulated by the hormones of the HPG axis, and in healthy individuals shows minimal diurnal or within-individual fluctuation, even over several weeks or months [2]. Thus, unlike testosterone, INSL3 provides a less ambiguous measure of Leydig cell functionality (i.e., a combination of Leydig cell differentiation status and the absolute numbers of Leydig cells in the testes). The best supporting evidence for this was provided by the FAMAS (Florey Adelaide Male Aging Study) study of nearly 1200 Australian men [41]. First, it could be shown that among men who have had one testis removed (uniorchid) and presumably about half the number of Leydig cells compared to normal intact men (with both testes), INSL3 levels were significantly lower than in the intact control group, unlike testosterone levels that did not significantly differ, largely due to the acute compensation by the HPG axis [41]. Second, in the same group of uniorchid men, there was a significant *negative* correlation between circulating LH and INSL3, reflecting that under conditions where testosterone production is likely to be rate

limiting, testes with lower Leydig cell functionality will have low INSL3 but induce high LH as part of the HPG homeostatic mechanism to elevate testosterone. This contrasts with what is observed in intact men, where INSL3 correlates *positively* with LH [34, 41]. Finally, this study revealed that there is a steady 12% per decade reduction in circulating INSL3 between the ages of 40 and 80, whereas for testosterone, the equivalent reduction is only 6% per decade, again due to compensatory control by LH, which significantly increases over this period [41].

INSL3 DURING PUBERTY

In rodents, shortly after birth there are still some persistent fetal Leydig cells that appear to be expressing INSL3, though these cells are not regulated by LH (Fig. 2). As the testes start to grow, the fetal Leydig cells either involute or are simply outnumbered by the proliferation of the new adult-type Leydig cell pool from small peritubular mesenchymal stem cells (SLC). In the rat or mouse, INSL3 is considered a relatively late marker of Leydig cell differentiation, mRNA and protein being first apparent at about PND 30 (rat) or PND 20 (mouse), that is, in the so-called ILC [8, 27, 42]. Both mRNA and protein (determined immunohistochemically) then increase to reach a maximum at about PND 40–45 (rat) or PND 35 (mouse). When we look at the concentration of INSL3 circulating in the blood, this initially parallels the mRNA levels within the testes [7, 27], suggesting again that it is more or less constitutively secreted as soon as it is synthesized. Then, at least in the rat, circulating INSL3 concentration rises dramatically to reach a maximum at around PND 42 but thereafter declines to attain a reduced adult steady state that then persists [7, 27]. This is not the case for testosterone measured in the same samples, which reaches its maximum also at or shortly after PND 42 but then remains relatively constant through early adulthood. The ''overshoot effect'' seen for INSL3 is what one would expect if, during puberty, LH pulses increase in amplitude and frequency until Leydig cells have attained an advanced differentiation status but then become reduced in intensity once that status is reached and testosterone synthesis can be maintained by the acute homeostatic actions of the HPG axis. This implies that after puberty, Leydig cells may attenuate somewhat their differentiation status and activity, a change that is directly reflected by the circulating INSL3 concentration. This picture is inevitably oversimplified since it takes no account of other steroids generated by Leydig cells, for example, the large amounts of androsterone, 3alpha-DIOL, or DHT, produced by PLC and ILC [43] or the relative expression levels of the key enzymes involved. We only know that by about 8–10 wk, Leydig cells have stabilized in their phenotype, consistent with a matured HPG axis [44].

The close relationship between INSL3 secretion and Leydig cell functionality and the relatively small within-individual variance in circulating INSL3 values have made this an ideal parameter with which to assess factors such as maternal endocrine disruptors on Leydig cell differentiation. Recently, we have been able to show that maternal exposure to diethylstilbestrol or to dibutylphthalate significantly alters the adult-type Leydig cell differentiation trajectory, either substance causing an advance in puberty, largely by modulating the trade-off between Leydig cell proliferation and differentiation [27]. This study during normal rat puberty yielded very similar results to a previous study using the ethane dimethylsulfonate rat model of adult-type Leydig cell differentiation [26] where circulating INSL3 showed that the trajectory of new Leydig cell differentiation in this model is kinetically very comparable to that during puberty [27].

In human puberty, the situation appears to be less clear in that, while circulating INSL3 concentrations increase as expected, by Tanner stage 5 they appear still not to have attained by some large margin the concentrations observed in young adulthood [45, 46]. This would imply that there is still substantial development of the Leydig cells in young men subsequent to reaching Tanner stage 5 at around 15–17 yr of age.

INSL3 AND AGING

The FAMAS cross-sectional study showed that INSL3 declines gradually with age (12% per decade) from early adulthood on [41], presumably reflecting the functional capacity of the Leydig cells. This decline also corresponded to the lesser decline in testosterone as well as the change in Leydig cell functional capacity as measured by the testosterone/LH ratio [41]. Significantly, more detailed analysis of this cross-sectional study showed that INSL3 was a robust parameter showing an inverse relationship to smoking (unlike testosterone) and was otherwise a useful predictor of metabolic syndrome [47]. Histological examination of aging human testes [48] suggests that there is both a loss of Leydig cells with age and a reduction in differentiation status (cells are smaller and less morphologically differentiated), presumably concomitant with loss of LH sensitivity [49]. This is comparable to what has been observed in old rats [7, 23], where INSL3 expression also appears to be down-regulated with age. The FAMAS study showed that even among young healthy men there is a broad range of INSL3 expression (0.5–1.5 ng/ml); this range is maintained as men age [41]. What will be particularly interesting is to identify other features that are associated with the progressively increasing proportion of aging men with low ($<$0.5 ng/ml) circulating INSL3. In this context, it is interesting to note that INSL3 has recently been shown to be associated with bone metabolism and that people with an inactivating mutation in their INSL3 receptor, RXFP2, have significant osteopenia [50], as do INSL3 knockout mice [50]. Taken together, such results suggest that INSL3 could become a very useful parameter in the differential diagnosis of conditions such as late-onset hypogonadism.

SEASONAL BREEDING AND INSL3

Seasonal breeding mammals offer an excellent model with which to study in a controllable fashion the differentiation events otherwise associated only with puberty. In male mammals such as deer or hamsters, changing day length is accompanied by a long-term annual cycle of attenuation of the HPG axis, followed by a renewed puberty-like reactivation of the hypothalamic and pituitary hormones [51]. This is accompanied by behavioral and physical changes to accommodate and synchronize seasonally restricted breeding activity. During the attenuation phase, LH and follicle-stimulating hormone production is minimal, and consequently both spermatogenesis and steroidogenesis and hence also testicular size are all drastically reduced. Histologically, during this attenuation phase, there appears to be an involution of the Leydig cells, which become small and indistinct. In deer, measurement of INSL3 at the mRNA level and at the immunohistochemical level shows that this does not appear to be due to a loss or apoptosis of Leydig cells [33]; rather, these seem to dedifferentiate into small peritubular fibroblast-like cells, not very different from prepubertal Leydig stem cells (SLC). Presumably, these cells will redifferentiate again once

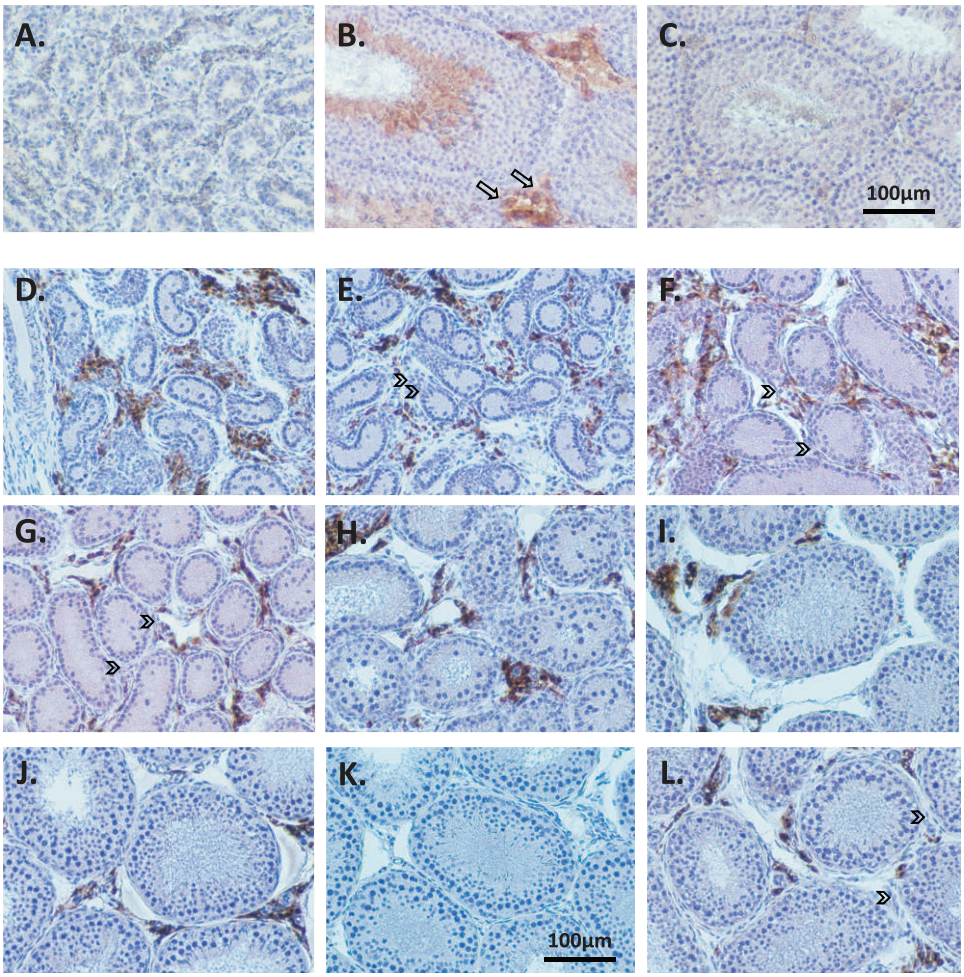

FIG. 3. Application of INSL3 immunohistochemistry to the testes of seasonally breeding mammals. **A**) Adult hamster (transferred to short days, nonbreeding). **B**) Adult hamster (long day, breeding); note that besides the Leydig cells (arrows), also the elongating spermatids stain positively for INSL3 epitopes in this species. **C**) As in **B** but using pre-immune serum. **D**–**L**) Sheep testes from birth through puberty to adulthood. **D**, day 1; **E**, 2 wk; **F**, 4 wk; **G**, 6 wk; **H**, 4 mo; **I**, 5 mo; **J**, 6 mo (July, breeding seasons); **K**, as **J**, but using preimmune serum as negative control; **L**, 12 mo (January, nonbreeding season). Note particularly in **E**–**G** and **L** the many unstained Leydig cell precursors in the interstitium (arrowheads). Immunostaining in the hamster employed the rat polyclonal antiserum #63 [8], while for the sheep testes, the rabbit polyclonal #33 [68] was used. The protocol was as in Balvers et al. [8]. Bars in **C** and **K** = 100 µm.

the hormones of the HPG axis are reactivated at the beginning of the next breeding season. Similar observations have been made in the laboratory hamster subjected to alternating short- or long-day lighting regimes (Ivell et al., unpublished; Fig. 3, A–C). Here it is interesting to note that in this species there is also specific INSL3 immunostaining within the seminiferous compartment in elongating spermatids (Fig. 3B). Whether this is because of local synthesis uniquely in spermatids in this species or, less likely, whether this represents an accumulation of INSL3 into the seminiferous compartment sequestered by RXFP2 on the germ cells, possibly at a time before the Sertoli cell tight junctions of the blood-testis barrier are established [52], is not known. In the sheep, where testicular seasonality though not seasonal breeding is less pronounced, there appears to be less morphological involution of the Leydig cells (Ivell et al., unpublished; Fig. 3). In this figure, this can be seen both at the beginning of puberty (cf. Fig. 3, E and F), where there is a clear population of unstained Leydig cell precursors (arrowheads) in addition to residual fetal Leydig cells, as well as in the transition from long day (July; Fig. 3J) to short day (January; Fig. 3L), where both unstained and INSL3-stained interstitial cells are clearly evident. Given the new molecular tools available with which to assess INSL3 expression, it will be interesting to take a more detailed look at this unusual cycle of Leydig cell differentiation, involution, and redifferentiation and its obvious relevance in the context of revitalizing Leydig cell functional capacity in the aging male.

INTRATESTICULAR FUNCTION OF INSL3

While the focus here has been primarily on the role of INSL3 as a biomarker of Leydig cell functional capacity, it is also important to understand the possible roles of INSL3 within the testis. The main functions of INSL3 appear to be to induce the first phase of testicular descent during fetal development [53, 54] or to modulate follicle development during the estrous cycle in female mammals [55]. Recently, an additional endocrine role for INSL3 has emerged with the discovery of marked activity in regard to osteoclast function and bone remodeling [50, 56]. Moreover, RXFP2-linked quantitative trait loci appear to be associated with horn growth in sheep [57] and cows [58]. Within the testis, the specific receptors for INSL3, called RXFP2, are expressed on both meiotic and postmeiotic germ cells within the seminiferous tubules as well as in a much lower amount on the Leydig cells themselves, suggesting both paracrine and autocrine roles [59]. Supporting

the former, Kawamura et al. [60] showed that INSL3 protected germ cells against apoptosis on treatment with a GnRH agonist, and Del Borgo et al. [61] showed that intratesticular injection of an INSL3 antagonist led to substantial germ cell loss, together pointing to an antiapoptotic function of INSL3. Moreover, in a clinical study assessing the consequences of a steroidal contraceptive regimen in men, it was shown that specific germ cell loss was more effective in men who had lower circulating INSL3 concentrations [62]. Supporting this concept, we were able to show that INSL3 is indeed able to cross the established blood-testis barrier in adult rats from the interstitium to the seminiferous compartment in sufficient amounts to be able to activate receptors there [7]. Conventional knockout mice for either the INSL3 gene or that of its receptor, RXFP2, have proved difficult to assess because the primary phenotype of such male mice is cryptorchidism, which itself is highly deleterious to spermatogenesis. Attempts to correct this cryptorchidism surgically suggest no major phenotypic difference in the adult testis [53]. Recently, a conditional RXFP2 knockout mouse has been generated using Cre-LoxP technology, wherein the RXFP2 gene has been specifically deleted in late male germ cells only, thus avoiding cryptorchidism and its consequences [63]. Although this mouse has been assessed only after attainment of sexual maturity, initial observations suggest that the RXFP2 expressed in these germ cells, at least in the mouse in early adulthood, is dispensable for male fertility [63]. However, more studies are required to look at effects at younger or older ages or under proapoptotic insult.

RXFP2 is also expressed at a low level on Leydig cells [59], and it has been recently shown that when Leydig cells are cultured at a very reduced cell density, such that endogenously produced INSL3 is also at a low concentration in the medium, added INSL3 is significantly able to induce steroidogenesis [64]. No such effects are evident when Leydig cells are cultured at high concentration [59]. This result is comparable to recent observations using the female equivalent of Leydig cells, the theca interna cells of ovarian follicles. Here added INSL3 is able, via RXFP2, to induce increased androgen production [65]. The RXFP2 receptor appears to act via adenylyl cyclase to raise the intracellular levels of cAMP [66], thus acting in a similar way to LH. Now, in the adult testis, there are already very high concentrations (~400 ng/ml) of INSL3 present in the interstitial compartment [7]. It is therefore very unlikely that endogenous INSL3 will be physiologically relevant for adult Leydig cells in the sexually mature testis, which are likely either to have their RXFP2 receptors desensitized or to be anyway sufficiently activated in terms of cAMP by pituitary LH. The situation may be quite different early in puberty during the first spermatogenic wave, when LH is not yet available in substantial amounts and when initial INSL3 secretion could be quantitatively quite relevant. This could also be true for RXFP2 receptors on germ cells since early in puberty the blood-testis barrier is not yet fully established and even low interstitial concentrations of INSL3 could reach the seminiferous compartment quite easily.

CONCLUSIONS

INSL3 is a major secretory product of phenotypically mature Leydig cells. Moreover, because it appears to be expressed in the constitutive pathway, its secretion into the testicular interstitial space and hence into the bloodstream largely reflects INSL3 gene expression and the differentiation status of the Leydig cells as well as their absolute number. This constitutive nonpulsatile expression gives INSL3 a great advantage over testosterone as a parameter since, unlike the latter, it is not acutely regulated by the hormones of the HPG axis and thus shows relatively low within-individual variation over quite long periods. For these reasons, we have found INSL3 measurement to accurately reflect the dynamics of Leydig cell differentiation and proliferation in situations such as puberty, aging, or endocrine disruption.

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