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Recent Advances in Research on the Hormone INSL3 in Male Goats

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Abstract

Insulin-like factor 3 (INSL3), previously called relaxin-like factor (RLF), is essential for testis descent during fetal development and has been implicated in the testicular and sperm functions in adult males. However, similar functions in ruminants remain largely unknown. This chapter will cover recent advancement in our understanding of INSL3 in goats. First, testicular Leydig cells were the sole source of INSL3, with INSL3 expression increasing during development. Second, INSL3 was constitutively secreted as a B–C–A single-chain structure with full biological activity. Third, secreted INSL3 was transported into the seminiferous compartments, where its receptor RXFP2 was expressed on germ cells, thus suggesting that the intratesticular INSL3 hormone-receptor system operates in germ cells. Fourth, functional RXFP2 enabling INSL3 to bind was also identified in the spermatozoa and suggested the existence of the extratesticular INSL3 hormone-receptor system in the spermatozoa. Interestingly, percentages of INSL3-binding spermatozoa were significantly reduced in the semen of subfertile bulls compared to that of fertile bulls, suggesting the potential of this system to diagnose fertility in breeding sires. These fascinating findings will give a new perspective in physiological and/or therapeutic actions of INSL3 on male reproductive processes in domestic ruminants, including goats.

Keywords: insulin-like factor 3 (INSL3), RXFP2, expression, structure, function, testis, spermatozoa, goat

1. Introduction

Fertilizable sperm production is regulated by the organized actions of germ cell types and stage-specific gene products and is supported by the complex interplay of many different molecules, including endocrine and paracrine signaling. Of these, insulin-like factor 3

(INSL3), previously called relaxin-like factor (RLF), is a novel member of the insulin/relaxin gene family, and is produced by both fetal and adult testes [1]. INSL3 is essential for fetal testis, and has been implicated in the testicular and sperm functions in adult males [2]; however, similar functions in ruminants remain largely unknown. Exploring the function of INSL3 in goats is especially intriguing, because goats are useful pilot animal for studying reproductive physiology in ruminants because of their fecundity and precocious sexual development [3] and other advantageous traits, such as small body size, calm behavior, and ease of handling. Here, we provide an overview of the recent progress in research on INSL3 in male goats.

2. Insulin-like factor 3 (INSL3)

The relaxin family peptides consist of seven distinct gene products, which include relaxin (RLN), H1-RLN, RLN-3, INSL3, INSL4, INSL5, and INSL6 (**Table 1**), of which INSL3, like RLN, has been extensively reviewed elsewhere [4] and is expressed almost exclusively both in male and female gonads. INSL3 binds with high affinity to and activates its specific receptor, relaxin family peptide receptor 2 (RXFP2), originally called leucine-rich G protein-coupled receptor 8 (LGR8) [5, 6]. INSL3 plays an essential role in testis descent during fetal development by acting on the gubernaculum via RXFP2, as revealed by mouse models targeting genes encoding either Insl3 or Rxfp2 [7]. After birth, INSL3 possibly plays a role for maintaining spermatogenesis by functioning as a germ cell survival/anti-apoptotic factor and as a germ cell proliferation factor in some species [8–10].

2.1. A short summary of research methods

Before moving on to the main subject, we will briefly introduce a short summary of materials and methods that we actually used in this chapter. First, peptide antibody for INSL3 was generated and subsequently used for immunological approaches such as western blotting

Relaxin family peptides		Receptors
Name	Nomenclature	
Relaxin	RLN	RXFP1
H1-relaxin	H1-RLN	RXFP1
Relaxin 3	RLN3	RXFP3
Insulin-like peptide 3	INSL3	RXFP2
Insulin-like peptide 4	INSL4	?
Insulin-like peptide 5	INSL5	RXFP4
Insulin-like peptide 6	INSL6	?

The data were modified from Ivell et al. [4].

Table 1. The relaxin family peptides and their receptors.

and immunolocalization. Then, INSL3 from goat testes was purified using a series of chromatography steps and the native conformation was examined by tandem MS (MS/MS) analysis. Biological activity of purified INSL3 was examined by cell-based assay based on the cAMP in INSL3 receptor RXFP2-expressing HEK-293 cells. Additionally, the secretory pathway of INSL3 was examined by immunoelectron microscopy. Next, we identified cell types expressing its receptor RXFP2 using both laser capture microdissection and immunostaining with RXFP2-specific antibody and by characterizing its developmental expression pattern and specificity of INSL3 binding. Moreover, we examined the functional RXFP2 protein in the spermatozoa using *in situ* INSL3 ligand binding assay.

2.2. Testicular Leydig cells are the sole source of INSL3 in male goats, with INSL3 expression increasing during sexual development

2.2.1. Generation of an anti-INSL3 peptide antibody

As in other species, *INSL3* transcripts are detected only in the testis in the mature male goat [11]. However, no studies have yet been carried out to identify whether *INSL3* mRNA is translated into INSL3 protein in the goat testis. There are so many commercially available antibodies for INSL3, but none of these antibodies cross-react with goats (http://www.antibodies-online.com). Thus, the first step toward elucidating the possible physiological role of INSL3 in goats is to produce its specific antibody. Based on the cDNA sequence [12], we generated an anti-INSL3 peptide antibody (RLF-A-Ab808) in New Zealand White rabbits against the synthetic peptides of 15 amino-acid residues of the A domain, which shared 100% amino acid homology with boar, bovine, sheep, and goat *INSL3* cDNAs [11]. This peptide antibody was shown by western blotting to recognize the ~12-kDa proINSL3 in the testicular extracts and the ~16-Da recombinant INSL3, which was expressed in *Escherichia coli* by using a construct that expresses the His-tagged proINSL3 sequence containing the B−C−A domain inserted into the pCold-I vector (**Figure 1A**). Furthermore, Ouchterlony gel diffusion test found that one precipitin band between the antibody and testicular extracts of goats and boars or recombinant INSL3 was formed, joined at their ends and fused (**Figure 1B**), indicating that antigens from boars and goats are immunologically identical and are perfectly cross-reactive with antibody. Additionally, the antibody did not cross-react with RLN, which is closely related to INSL3.

2.2.2. Source and expression dynamics of INSL3

As revealed by *in situ* hybridization, *INSL3* mRNA is expressed in the Leydig cells that are well known as steroid hormone-producing cells (**Figure 2**), which is consistent with previous study [12]. However, it is unclear whether *INSL3* mRNA was translated into the protein therein. Using the INSL3-specific antibody, INSL3 protein was identified immunohistochemically in the Leydig cells (**Figure 2**), with INSL3 expression increasing during sexual development [11]. Therefore, it is reasonable to draw the conclusion that testicular Leydig cells are the sole source of *INSL3* mRNA and protein in male goats, which is consistent with the findings that INSL3 is expressed by Leydig cells of both fetal and adult testes in a number of species [1].
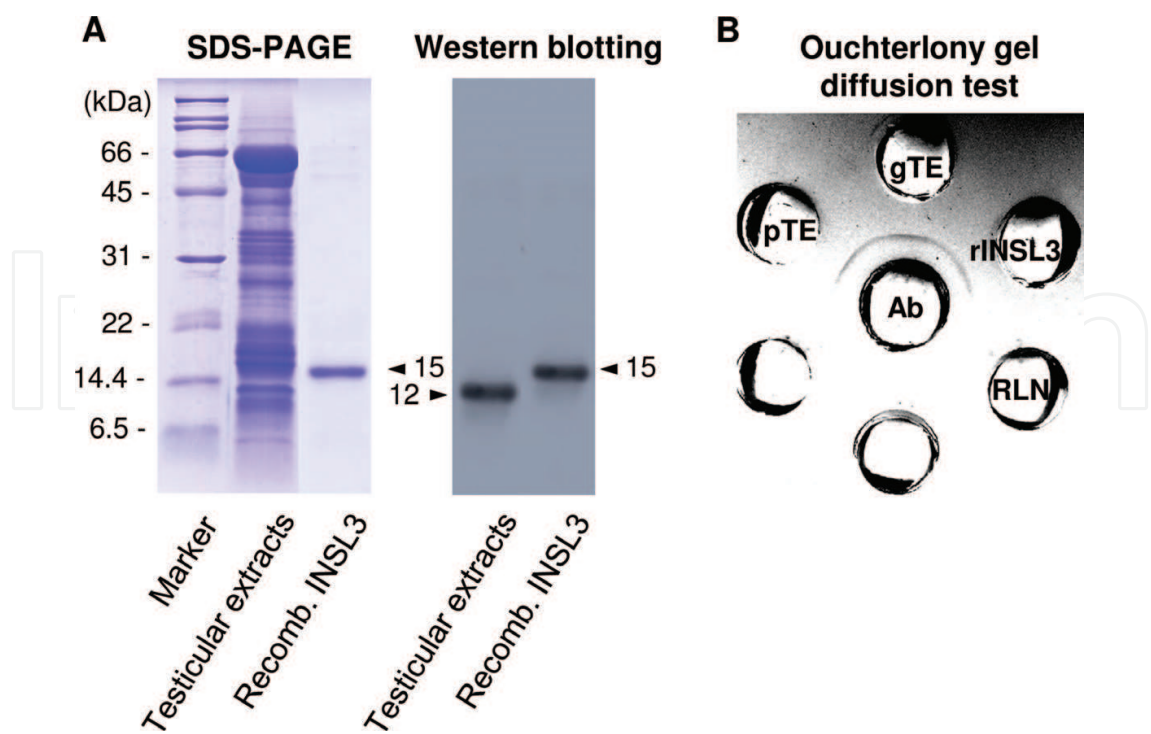
Figure 1. Specificity of anti-INSL3 peptide antibody. (A) SDS-PAGE and western blot analysis of goat testicular extract and recombinant (recomb.) INSL3. (B) Ouchterlony gel diffusion test showing precipitin reactions between the antibody and testicular extracts or recombinant INSL3 (rINSL3). pTE, pig testicular extracts; gTE, goat testicular extracts; RLN, porcine relaxin. SDS-PAGE and western blotting data are unpublished, while Ouchterlony data are derived from Siqin et al. [11].
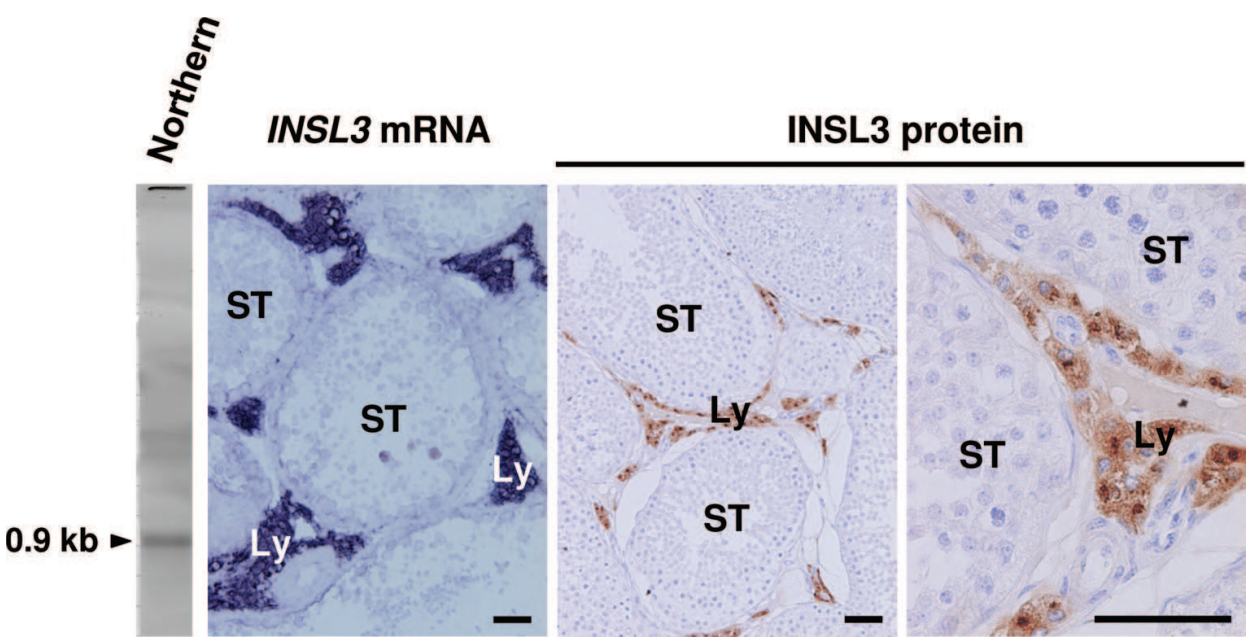

Figure 2. INSL3 expression in the goat testis. *INSL3* mRNA and protein were expressed in the Leydig cells. Northern, northern blotting showing 0.9 kb *INSL3* mRNA in the testis; Ly, Leydig cells; ST, seminiferous tubules. Bars = 50 μm. *INSL3* mRNA data are unpublished, while the protein data are based on Siqin et al. [11].

In common with circulating INSL3, *INSL3* mRNA and protein expression in the testis of rodents and pigs have been reported to be upregulated coincidently with pubertal development [13–15]. In goats, the dynamics of INSL3 production in the testis are fundamentally consistent with those of other species. INSL3 protein was detected as a specific band of ~12-kDa on western blotting of the goat testis (**Figure 3A**), and its signal was high at 1 month after birth, decreased at prepubertal stage (3 months), and then increased markedly at puberty (4 months), remaining at that level through adulthood (**Figure 3B**) [11]. The expression level of *INSL3* gene followed a similar pattern to that of the INSL3 protein [16]. The relatively high expression of INSL3 at 1 month of age might be attributed to the presence of residual fetal
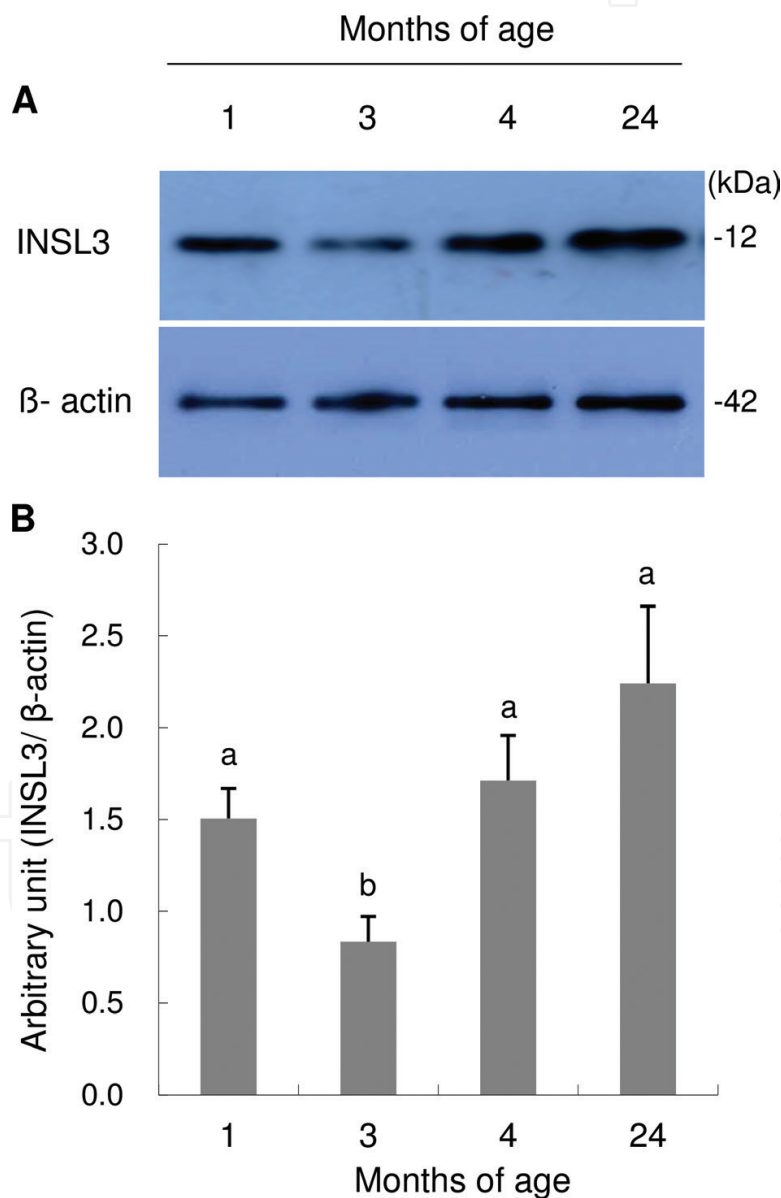

Figure 3. Expression profile of INSL3 protein in the goat testis during development. (A) A representative western blot analysis. (B) Relative level of INSL3 expression. Data were derived from Siqin et al. [11].

Leydig cells, which often persist in the testis at early postnatal stages [17] and express high levels of *INSL3* mRNA and protein similar to those of adult Leydig cells, as observed in rats [18].

As in the case of other species, INSL3 expression in goats appears to correspond fundamentally to the developmental status of Leydig cells. With the establishment of a functional hypothalamic-pituitary-gonadal (HPG) axis during puberty, goat Leydig cells appear to be most responsive to LH at puberty as characterized by a dramatic increase in Leydig cell number and cell diameters [16]. In addition, in other species, INSL3 expression in Leydig cells has been reported to be induced under the long-term effects of LH/hCG and is not acutely regulated by LH/hCG and other hormones influencing Leydig cell differentiation, such as insulin-like growth factor1 (IGF1) [19, 20].

2.3. Goat INSL3 is constitutively secreted from testicular Leydig cells as a B–C–A single-chain structure with full biological activity

2.3.1. Structure of INSL3

Unlike other peptides from the relaxin/insulin family, very little is known about the native conformation of INSL3. INSL3 is predicted from the cDNA sequence to be biosynthesized as a precursor protein (pro-INSL3) containing A- and B-domains connected by a C-domain and is assumed to undergo proteolytic processing to remove the C-domain peptide. The precursor then matures to an A–B heterodimer linked by two disulfide bonds to form an active hormone, as do the other members of this family of peptides (**Figure 4**) [1]. This prediction has been supported by the findings that a synthetic INSL3 peptide, which consists of an A–B heterodimer with site-specific sequential disulfide bonds in some species, such as humans [21] and rats [22], stimulates cAMP production by binding to its receptor, RXFP2 [5]. Furthermore, this is corroborated by a report that native INSL3 exists as an A–B heterodimer when isolated from bovine testis [23].

However, recent study concerning native INSL3 purified from boar testes demonstrated for the first time that the native INSL3 exists as a monomer comprising three domains, B–C–A, with site-specific disulfide bonds and full biological activity [24]. Therefore, there appears to be not only a bovine form of native INSL3 that exists as an A–B heterodimer [23], but also a porcine form that exists as a B–C–A single chain [24]. In fact, the molecular mass of INSL3 detected by western blot analysis in tissue extracts from several species, including goats [11], corresponds to that of pro- INSL3 (B–C–A single-chain form) as deduced from their cDNA sequences. Hence, it is worth proving whether native INSL3 of other species, including goats, exists as a B–C–A single chain that corresponds to a porcine model of native INSL3 but not the bovine model.

When purified the protein using a series of chromatography steps and examined the native conformation by MS/MS (tandem MS) analysis, the native goat INSL3 is isolated as a 12-kDa protein with a B–C–A single-chain structure, in which the C-domain does not undergo proteolytic processing (**Figure 5A**) [25]. This is consistent with the structural features of porcine native INSL3 comprising a B–C–A single-chain form [24] but quite different from those of native bovine INSL3 [23] in that C-domain does not undergo processing. Clarification of

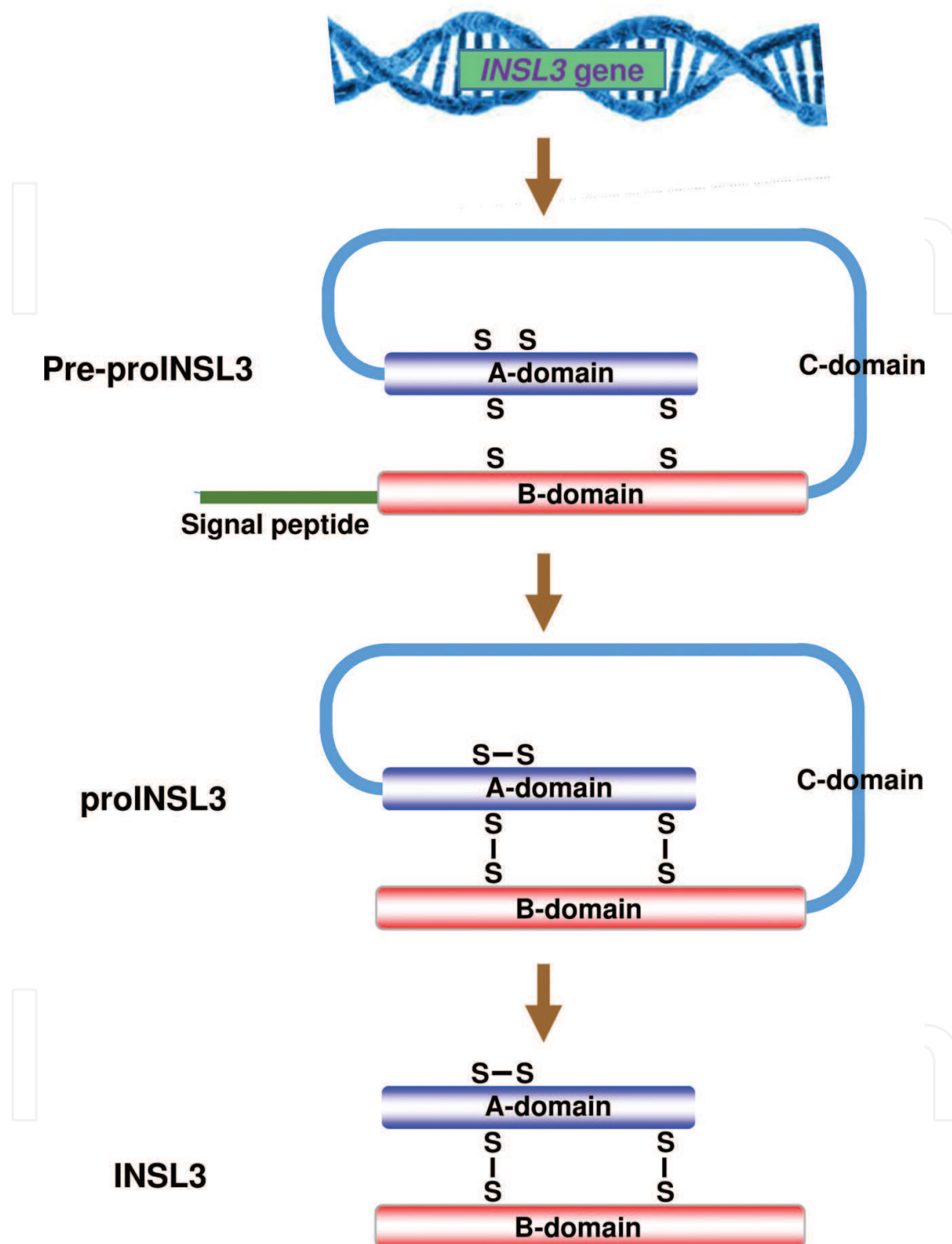**Figure 4.** Predicted biosynthetic process of INSL3. INSL3 is predicted from the cDNA sequence to be biosynthesized as a precursor protein (prepro-INSL3) containing: (1) a signal peptide that presumably permits the nascent protein access to the rough endoplasmic reticulum (RER) before being excised; and (2) A- and B-domains connected by a C-domain. After cleavage of the signal peptide in the lumen of the RER, pro-INSL3 is assumed to undergo proteolytic processing to remove the C-domain peptide and then to mature to an A–B heterodimer linked by two disulfide bonds to form an active hormone like the other members of this group of peptides. Predicted biosynthetic process of INSL3 is based on Ivell and Bathgate [1].
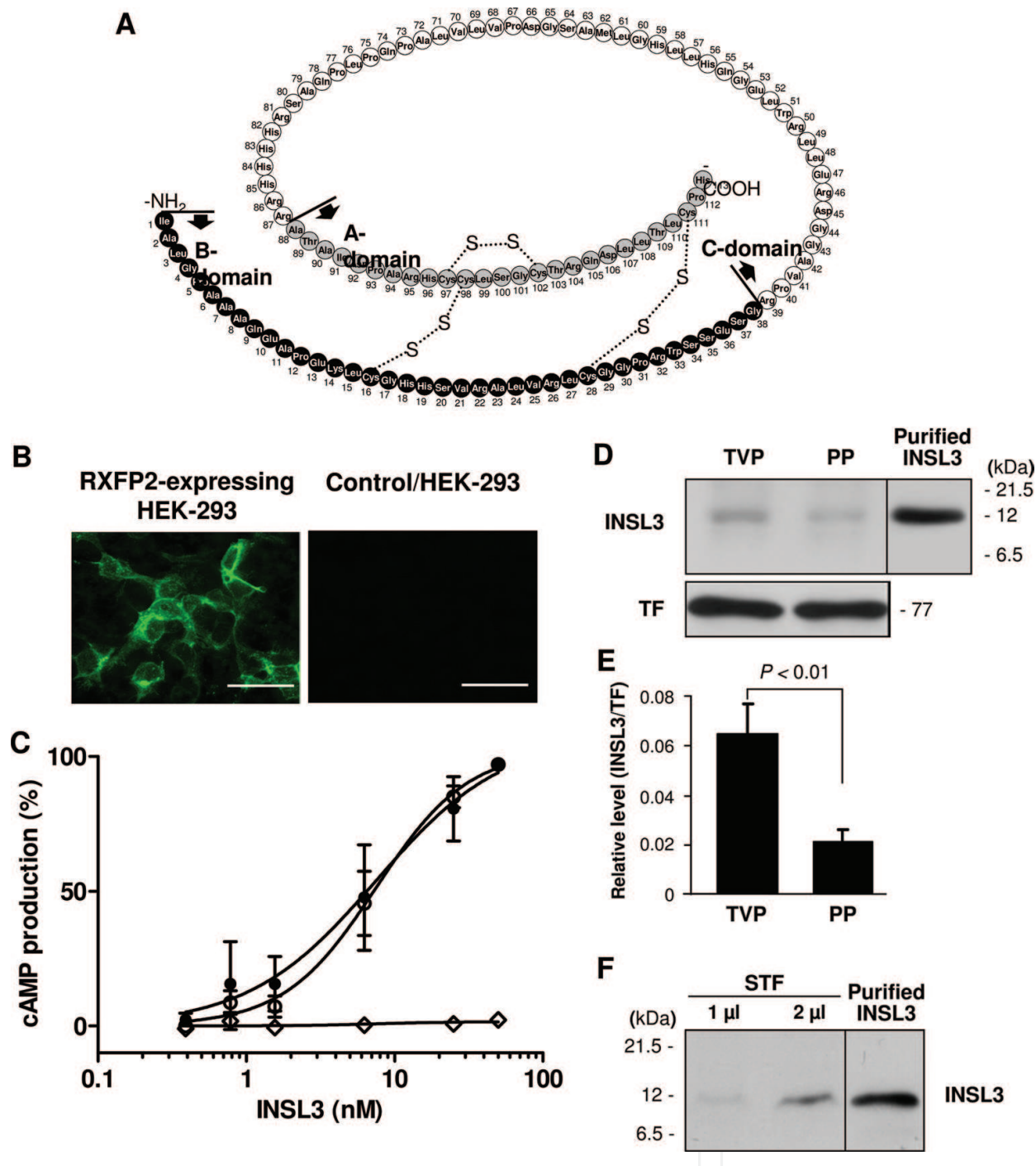
Figure 5. Structure, biological activity, and secretion of native goat INSL3. (A) Primary structure of goat INSL3 determined by MS/MS analysis. (B) RXFP2-expressing or control HEK-293 cells transfected with the expression construct or empty vector for detecting the biological activity of INSL3. (C) Stimulation of cAMP production by INSL3 in RXFP2-expressing HEK-293 cells. Purified goat INSL3 (●) stimulated dose-dependent cAMP production with EC_{50} values comparable with those of the synthetic A–B heterodimeric human INSL3 (○), indicating the retention of full bioactivity. In contrast, purified INSL3 did not stimulate cAMP production in the control/HEK-293 cells (◇). (D) A representative western blot analysis showing secretion of INSL3 into testicular venous plasma (TVP) and peripheral plasma (PP). (E) The relative level of each message normalized to that of TF (transferrin). INSL3 level was three-fold higher in TVP than that in PP. (F) A representative western blot analysis showing secretion of INSL3 into seminiferous tubular fluid (STF). Data are derived from Siqin et al. [25].

native goat INSL3 and its functional characterization would be a major step toward developing a specific immunoassay system for measuring INSL3 in blood and body fluids, whereby the yet unidentified endocrine, paracrine, and/or autocrine aspects of INSL3 can be explored, thereby giving insight into the potential role of INSL3 in goat testicular function.

2.3.2. Biological activity and secretion of INSL3

INSL3 mediates its effect by stimulating cAMP production through its specific cell-surface receptor, RXFP2 [5]. A reliable bioassay system based on the cAMP production in HEK-293 cells transfected with either mouse or human RXFP2 has been employed to examine the biological activity of INSL3. Treatment of RXFP2-expressing HEK-293 cells with the goat INSL3 resulted in a dose-dependent increase in intracellular cAMP production with an EC_{50} values (approximately 7 nM) comparable with those of the synthetic A–B heterodimeric human INSL3 peptide (**Figure 4**), indicating the retention of full bioactivity (**Figure 5B** and **C**).

Furthermore, western blot analysis of testicular venous and peripheral plasma and also of seminiferous tubular fluid demonstrated that the INSL3 secreted was present as the 12 kDa of molecular nature in blood and body fluid, revealing that most secreted INSL3 is not processed (**Figure 5D–F**) [25]. These results establish that goat INSL3 is secreted from testicular Leydig cells into the blood and body fluids within the lumen of the seminiferous tubules as a biologically active 12-kDa B–C–A single-chain peptide. Therefore, the native goat INSL3 differ from insulin, in which the prohormone undergoes proteolytic processing to form the active hormone, but is quite similar to insulin-like growth factors (IGFs) [26], as well as native porcine INSL3 [24], in that the proforms are not processed into two-chain peptides and exert full bioactivity.

2.3.3. INSL3 regulation by constitutive secretory pathway

It is important to note what secretory pathways are responsible for regulating INSL3 production and why INSL3 is secreted as a single-chain peptide. It is now accepted that endocrine cells producing peptide hormone possess regulated or constitutive secretory pathways, and that most secretory proteins that are synthesized as prohormones on the RER are transported to the cis-Golgi network and then through the trans-Golgi network (TGN) [27]. In the regulated secretory pathway, prohormones are sorted into secretory granules for processing and storage before their release (**Figure 6**). In contrast, the constitutive secretory pathway is thought to be the default pathway by which prohormones exit the TGN and are rapidly released without storage [27, 28]. Moreover, the major proteolytic enzymes mediating protein processing are prohormone convertase 1/3 (PC1/3) in the secretory granules in the regulated pathway and furin in the TGN in the constitutive pathway [27, 29]. For example, relaxin, which belongs to the same family as INSL3, is stored in secretory granules [30–32], and they undergo processing by PC1/3 [33].

Immunoelectron microscopy demonstrated that INSL3 localizes to the TGN within the goat Leydig cells, where secretory storage granules were undetectable, suggesting that goat INSL3
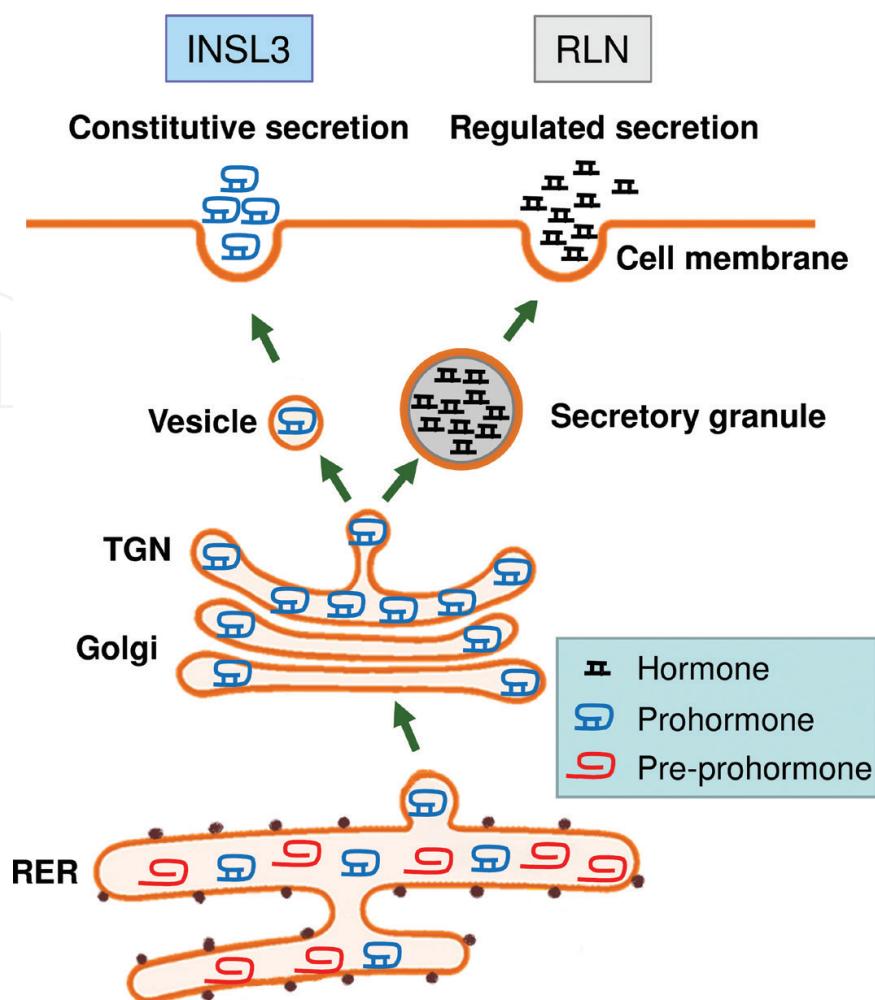
Figure 6. Regulation of INSL3 by constitutive secretory pathway. INSL3 and relaxin (RLN) are synthesized as preprohormone in rough endoplasmic reticulum (RER), converted to prohormone, and later transported to trans-Golgi Network (TGN). Pro-INSL3 is rapidly released by constitutive secretion without storage. In contrast, pro-RLN is packed into secretory granules, where pro-RLN is converted to RLN and condensation occurs before release. Based on our data [25, 30–32].

is not produced by the regulated secretory pathway but by the constitutive secretory pathway in Leydig cells (**Figure 6**) [25]. This is consistent with the findings that *INSL3* mRNA is constitutively expressed in rodent Leydig cells [13, 14, 20]. If this is the case, furin, rather than PC1/3, would mediate processing of INSL3 in goat testes. However, goat INSL3 did not actually have an RXK/RR sequence for cleavage catalyzed by furin [29]. Therefore, it is reasonable to conclude that goat testicular INSL3 is regulated by the constitutive secretory pathway and that the A–B heterodimeric INSL3 is not produced due to a lack of the RXK/RR motif at the furin cleavage site required for processing pro-INSL3.

2.4. INSL3-receptor RXFP2 network functions in testicular germ cells

2.4.1. An RXFP2-specific antibody that is suitable for detecting RXFP2

To investigate what effects INSL3 actually exerts in testis, the identification of the possible site(s) of action of INSL3 within the testis is necessary. Identifying the cell type(s) that

expresses the receptor RXFP2 and specific INSL3 binding in target cells would be a major step toward understanding the as-yet-unknown function of INSL3 in goats. Previous study determined the partial cDNA sequence of goat RXFP2 and demonstrated the high level of expression of this gene in mature testes, suggesting that INSL3 is involved in testicular function [34]. However, the specific cell type(s) that expresses RXFP2 in the testis have remained unidentified because of the absence of reliable anti-RXFP2 antibodies.

Recently, we successfully identified a highly specific anti-RXFP2 antibody that was suitable for detecting RXFP2 at protein level by analyzing the characteristics of commercially available RXFP2 antibodies that were directed against different parts of human RXFP2 [9, 35]. Identified RXFP2-specific antibody (GTX108235; GeneTex), which was directed against the human RXFP2 endodomain (C-terminal intracellular domain), recognized the cell-surface antigen by double immunofluorescence in HEK-293 cells transiently transfected with a FLAG-tagged mouse *Rxfp2* cDNA construct (HEK-293 expressing FLAG-RXFP2): RXFP2 signal overlapped with FLAG-RXFP2, which was recognized by the FLAG antibody, when the fluorescence images were merged (**Figure 7**). The characterization of the antibody has been described elsewhere [9, 16]. Notably, the antibody can react with not only functional 85-kDa receptor form that enables INSL3 to bind, but also smaller sized degradation forms, precursor forms, or splice variants of RXFP2, as verified by western blotting and far-western blotting of cell lysates from HEK-293 expressing FLAG-RXFP2 [9].

2.4.2. Germ cell types expressing RXFP2 mRNA and protein in the mature testis

Cell types expressing *RXFP2* mRNA and protein in the goat testis were identified by RT-PCR of cells captured using laser capture microdissection (LCM) and immunohistochemistry using the characterized RXFP2-specific antibody [16]. *RXFP2* transcripts were mainly expressed in spermatocytes and round spermatids, and in the Leydig cells (**Figure 8A**), whereas RXFP2 protein was detected specifically in the same cell types in which *RXFP2* mRNA was expressed
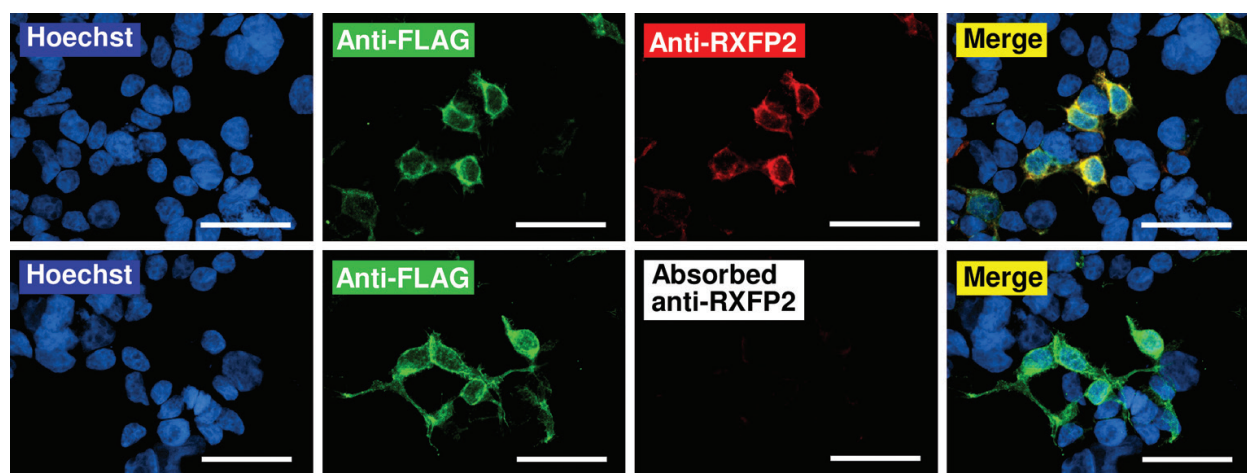

Figure 7. Verification of RXFP2 antibody specificity by double immunofluorescence labeling in HEK-293 cells transiently transfected with a FLAG-tagged mouse *Rxfp2* cDNA construct (HEK-293 cells expressing FLAG-RXFP2). RXFP2 signal overlapped with FLAG-RXFP2, which was recognized by FLAG antibody, when the fluorescence images were merged. In contrast, RXFP2 signal was abolished by preabsorbing the antibody with recombinant porcine RXFP2. Bars = 50 μm. Data are derived from Sagata et al. [9].
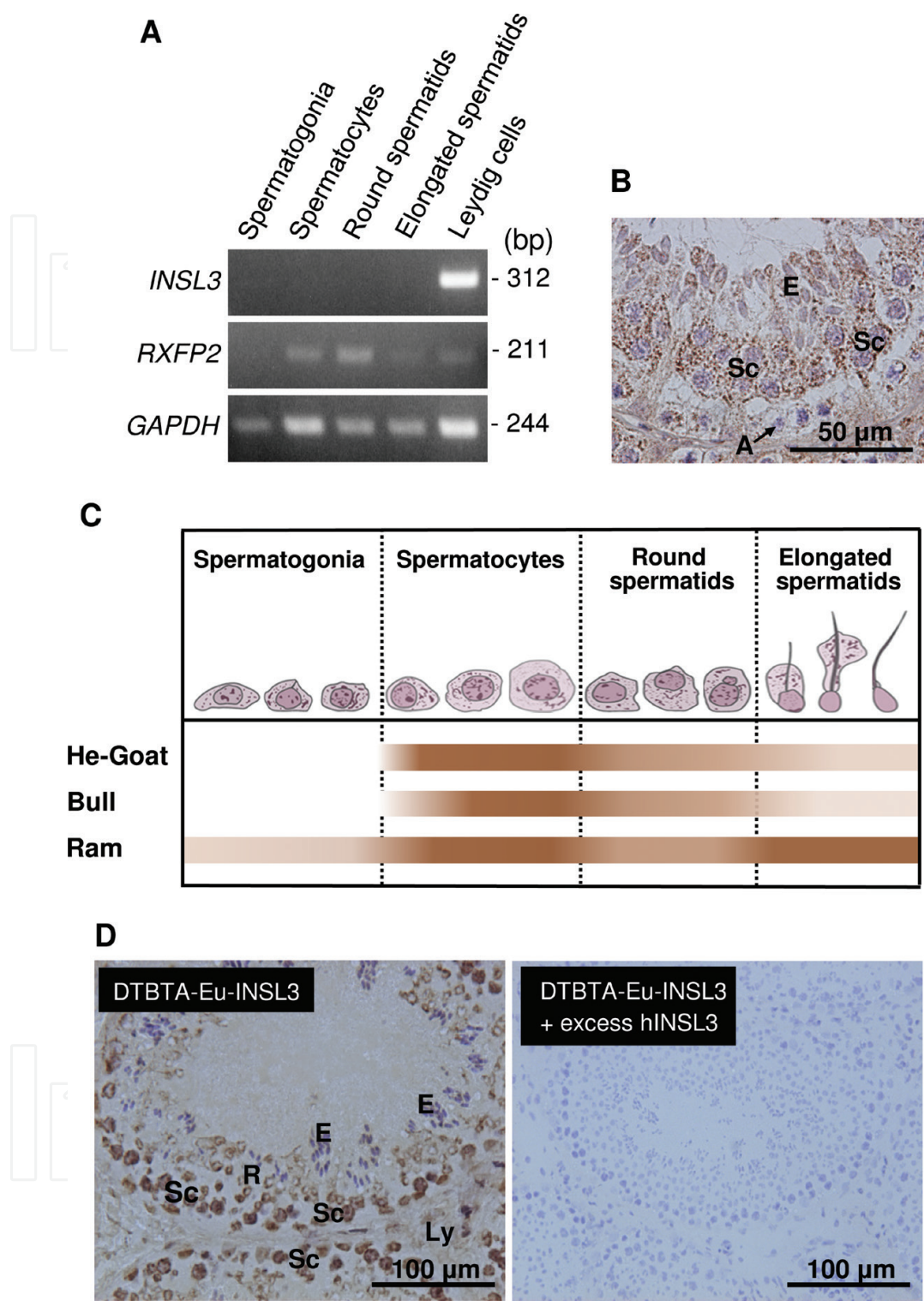
Figure 8. RXFP2 expression and INSL3 binding in germ cell in adult goat testis. (A) Specific cell types expressing *RXFP2* mRNA. Cells were collected by laser capture microdissection (LCM) microscopy and mRNA was detected by RT-PCR. *INSL3* transcripts were present only in Leydig cells, whereas *RXFP2* transcripts were highly expressed in Leydig cells, spermatocytes, and round spermatids and weakly expressed in elongated spermatids but not in spermatogonia. (B) A representative immunostaining of RXFP2 in the testis. (C) Comparison of RXFP2 protein expression during spermatogenic cycle among domestic ruminants. The brown colored bars indicate RXFP2 expression, with a darker shade reflecting a higher level of expression. (D) *In situ* INSL3 binding in germ cells. INSL3 binding was detected in germ cells but not in Leydig cells in sections incubated with DTBTA-Eu-INSL3. The binding was inhibited by excess hINSL3. A, type A spermatogonia; Sc, spermatocytes; R, round spermatids; E, elongated spermatids; Ly, Leydig cells. Data were derived from Pitia et al. [16, 36].

(**Figure 8B** and **C**). Especially, RXFP2 expression in meiotic and postmeiotic germ cells in goats are fundamentally consistent with our studies in boars [9, 35], bulls, and rams [36], as well as other reports in humans [37] and rats [8].

2.4.3. INSL3 ligand binding to germ cells

An *in situ* ligand assay with DTBTA-europium (Eu)-INSL3 was useful for determining whether INSL3 can bind RXFP2 expressed in Leydig and germ cells, since the binding of ligand to specific receptors on target cells initiates downstream physiological effects. DTBTA-Eu, which is a recently developed luminescent lanthanide chelate label, has several advantages such as not interfering with the biological activity of the labeled proteins [15]. In goat testes, INSL3 mainly binds to spermatocytes, with weak binding of round and elongated spermatids, in a hormone-specific and saturable manner (**Figure 8D**) [16]. Unexpectedly, no binding of INSL3 to Leydig cells was detected, despite their expression of *RXFP2* mRNA and protein, indicating that a functional RXFP2 receptor is expressed only in germ cells.

As shown in **Figure 5D–F**, goat INSL3 secreted from testicular Leydig cells was released not only into the blood circulation but also into seminiferous tubules [25]. This finding suggests that INSL3 enters the seminiferous compartment across the blood-testis barrier (BTB), which is created by inter-Sertoli tight junctions and adherens junctions, by mechanisms which are still unclear. Similar findings were also found in boar and rat INSL3 [9, 14]. These results taken together reveal that INSL3 secreted from the Leydig cells is transported into the seminiferous compartments and is capable of encountering germ cells, where its receptor RXFP2 is expressed, thus enabling INSL3 to bind (**Figure 9**). This suggests that INSL3 could act as a paracrine factor in seminiferous germ cells in the goat testis. Unfortunately, it remains unknown what functions INSL3 actually exerts on germ cells in the goat testis. One possibility is that INSL3 plays a role in the maintenance of spermatogenesis by regulating cellular processes such as apoptosis through the receptor RXFP2 on seminiferous germ cells in a paracrine manner. In fact, there is compelling evidence that INSL3 contributes to the maintenance of spermatogenesis by acting as a germ cell survival/antiapoptotic factor in testes of rats [8] and boars [9], and as a germ cell proliferation factor in zebrafish [10].

In contrast, the reason why INSL3 did not bind to Leydig cells is unclear, despite their expression of *RXFP2* mRNA and protein. However, there is the possibility that RXFP2 receptors in Leydig cells might have been saturated with endogenous INSL3 *in vivo*, leading to their long-term desensitization/internalization [38]. Another possibility is the expression of RXFP2 splice variants in Leydig cells, as shown in rodent testes [37]. In fact, RXFP2 variants that lack part of the ectodomain (N-terminal extracellular domain) components that are essential for ligand-receptor interaction often yield nonfunctional products [39, 40].

2.5. The potential of INSL3 hormone-receptor system as a novel parameter for predicting fertility in breeding sires

2.5.1. Expression and functionality of RXFP2 in the spermatozoa

INSL3 possibly plays a role in sperm function as suggested by the detection of its receptor *RXFP2* mRNA or protein in spermatozoa of rodents [41] and boars [42]. However, there
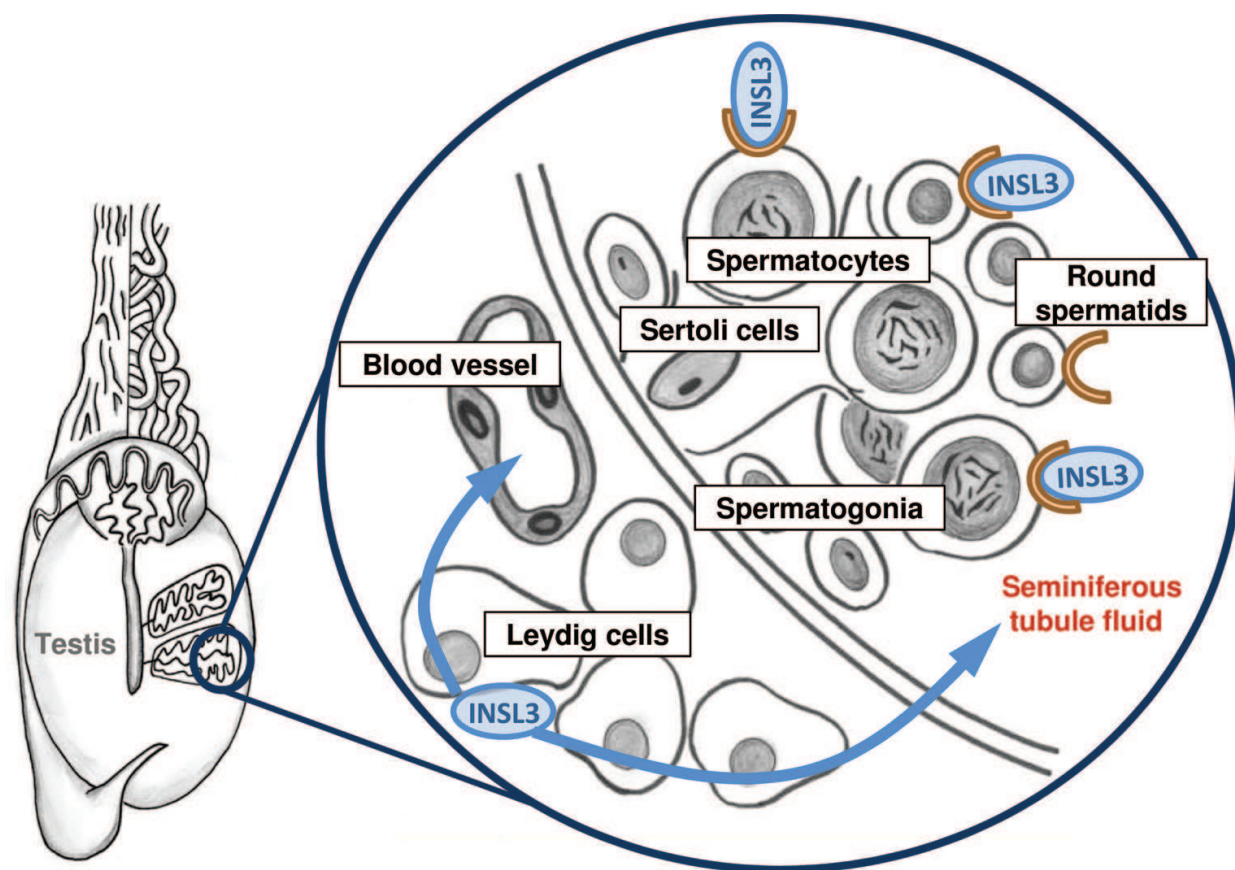
Figure 9. INSL3 secreted from Leydig cells is released not only into the blood circulation, but also into the interstitial and seminiferous compartments, where receptor RXFP2 is expressed mainly in the germ cells, suggesting its involvement as a paracrine factor. Based on our data [16, 25].

was no evidence whether RXFP2 is expressed in spermatozoa of domestic ruminants, including goats. With the help of immunofluorescence using RXFP2-specific antibody, we found that RXFP2 was expressed in the equatorial segment and midpiece of goat spermatozoa (**Figure 10A**). Similar findings are also found in bull and ram spermatozoa [36].

Furthermore, we investigated the functionality of RXFP2 in these spermatozoa using *in situ* ligand binding assay with DTBTA-Eu-INSL3 and clearly showed the presence of functional RXFP2 enabling INSL3 to bind with the equatorial segment of he-goat (**Figure 10B**), bull, and ram spermatozoa, suggesting that a functional INSL3 hormone-receptor system operates in the equatorial segment of ruminant spermatozoa [36]. It has been reported to date that *RXFP2* mRNA is detected in the spermatozoa of rats [41] and boars [42] and its protein is distributed throughout the entire spermatozoon in boars [43]. However, the reason for discrepancy in receptor distribution is unclear, but it might be due to differences in animal species and antibodies used. It is important to note where INSL3 acting on spermatozoa comes from. INSL3 has been reported to be produced in theca cells in the ovary of some species such as rats [8] and cows [44]. In addition, high levels of INSL3 is detected in follicular fluid of rhesus macaques [45]. Therefore, INSL3 in follicular fluid entered into oviduct at the time of ovulation appears to operate in spermatozoa.
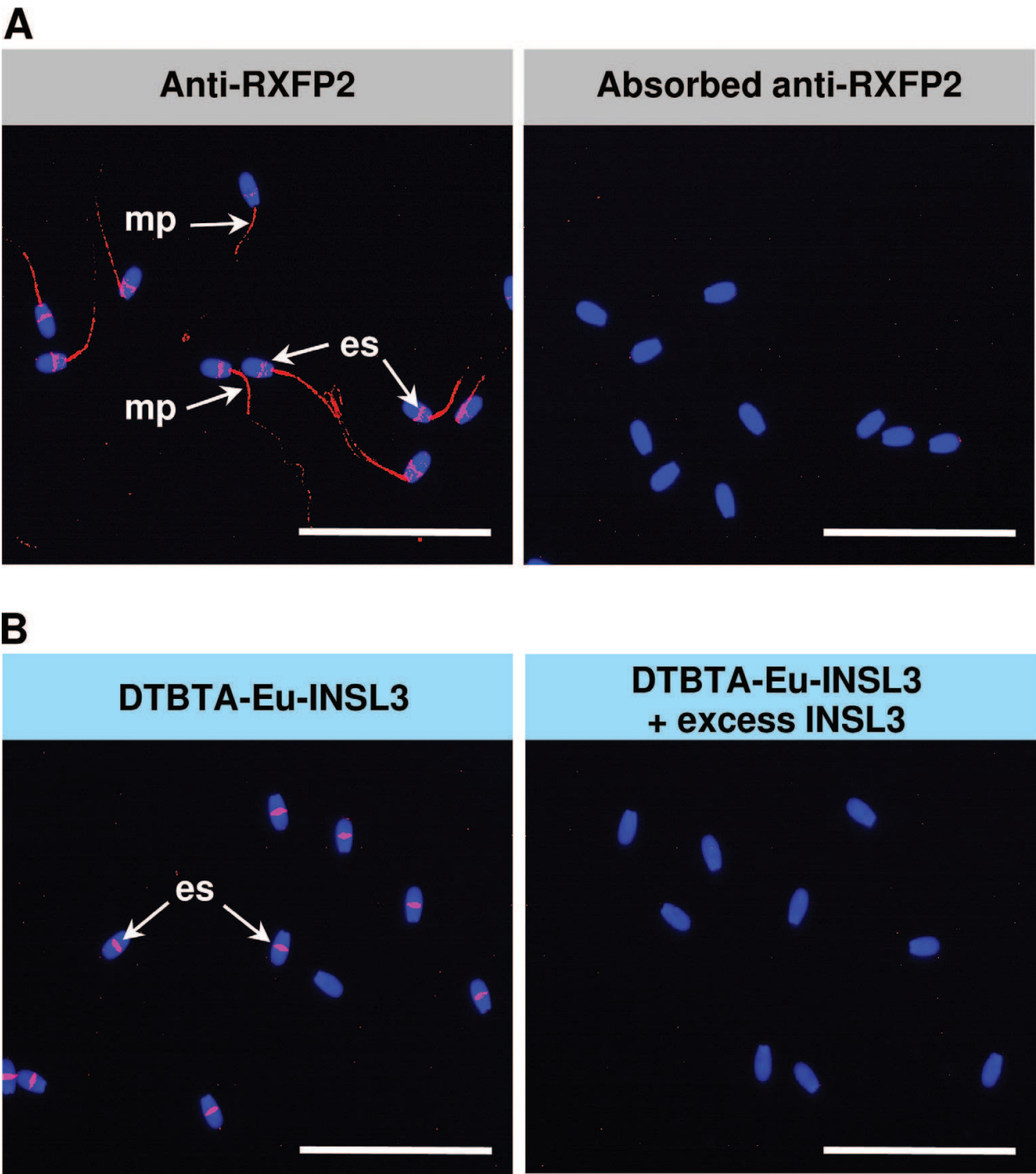
Figure 10. Expression and functionality of RXFP2 in the goat spermatozoa. (A) Immunolocalization. RXFP2 signals were observed in the equatorial segment and midpiece of the spermatozoa, and were blocked using the preabsorbed antibody. (B) *In situ* INSL3 binding. INSL3 binding was exclusively detected in the equatorial segment of goat spermatozoa incubated with DTBTA-Eu-INSL3. The binding was inhibited in the presence of excess hINSL3. es, equatorial segment; mp, midpiece. Bars = 50 μm. Data were derived from Pitia et al. [36].

Although the physiological significance of RXFP2 in the equatorial segment of ruminant spermatozoa, including goats, is still unknown, it might be related to sperm fusion with the oocyte membrane during fertilization. A recent study on knockout mice has demonstrated

that gamete fusion is mediated by β-catenin through formation of β-catenin/E-cadherin complex, which facilitates adhesion on both sperm and oocyte plasma membranes [46]. Both β-catenin and E-cadherin are expressed on the acrosomal and/or midpiece regions of the spermatozoa in bulls [47], humans [48], and mice [46]. As INSL3 appears to activate the Wnt/β-catenin canonical pathway through RXFP2 in gubernaculum cells [49], it seems reasonable to assume that INSL3 may contribute to gamete fusion by upregulating β-catenin required for formation of β-catenin/E-cadherin complex via RXFP2 in the equatorial segment of ruminant spermatozoa. The lack of INSL3 binding to the midpiece could be attributed to the expression of RXFP2 splice variants therein, although there is currently no evidence for such variants in the spermatozoa of any species. If they are indeed present, RXFP2 splice variants may affect receptor functionality and prevent INSL3 binding in spermatozoa.

2.5.2. Potential to diagnose subfertility in sires

Reproductive efficiency in domestic ruminants is a major factor that affects profitability in meat and dairy industries. In sires, normal sperm production and good semen quality are essential in maximizing reproductive performance. Artificial insemination (AI) with frozen semen has been employed in modern breeding programs of dairy and meat livestock to improve economically important traits. Especially, in cattle, it is prominent. However, even when semen quality parameters such as sperm viability and motility are good, some prized sires are still adversely affected by low fertility (subfertility). Thus, it is important to explore novel molecular markers for an accurate assessment of male fertility from the perspective of sperm production and semen quality.

Although serum INSL3 levels are positively correlated with sperm production [50], sperm number [50], and sperm morphology [51] in humans, we examined the potential to diagnose subfertility from the spermatozoa [36]. Since samples suitable for this analysis were available in bulls, we used bull spermatozoa as an alternative to goat spermatozoa. *In situ* ligand binding assay revealed that the functional receptor RXFP2, enabling INSL3 to bind, was expressed only in the equatorial segment of both fertile and subfertile spermatozoa (**Figure 11A**). However, when sperm population expressing the functional RXFP2 receptor was evaluated, the percentage of INSL3-labeling spermatozoa was significantly reduced ($P < 0.05$) in the semen of subfertile bulls compared to that of fertile bulls (**Figure 11B**). This suggests the possibility to diagnose subfertility in bulls based on sperm population expressing the functional RXFP2 receptor. To date, many proteins such as seminal plasma protein, α-L-fucosidase, cathepsin, clusterin, fertility-associated antigen, or osteopontin might be useful fertility markers in bulls [52]. Additionally, the immunoassayable levels of hormones, such as follicular stimulating hormone, testosterone, and RLN, have also been suggested as useful indices for predicting the fertility potential of sires [53–56]. Thus, the present evaluation, based on the properties of the INSL3 hormone-receptor system in the spermatozoa of ruminant sires, might represent an effective means by which fertilizing potential can be assessed from the standpoint of sperm quality, respectively.
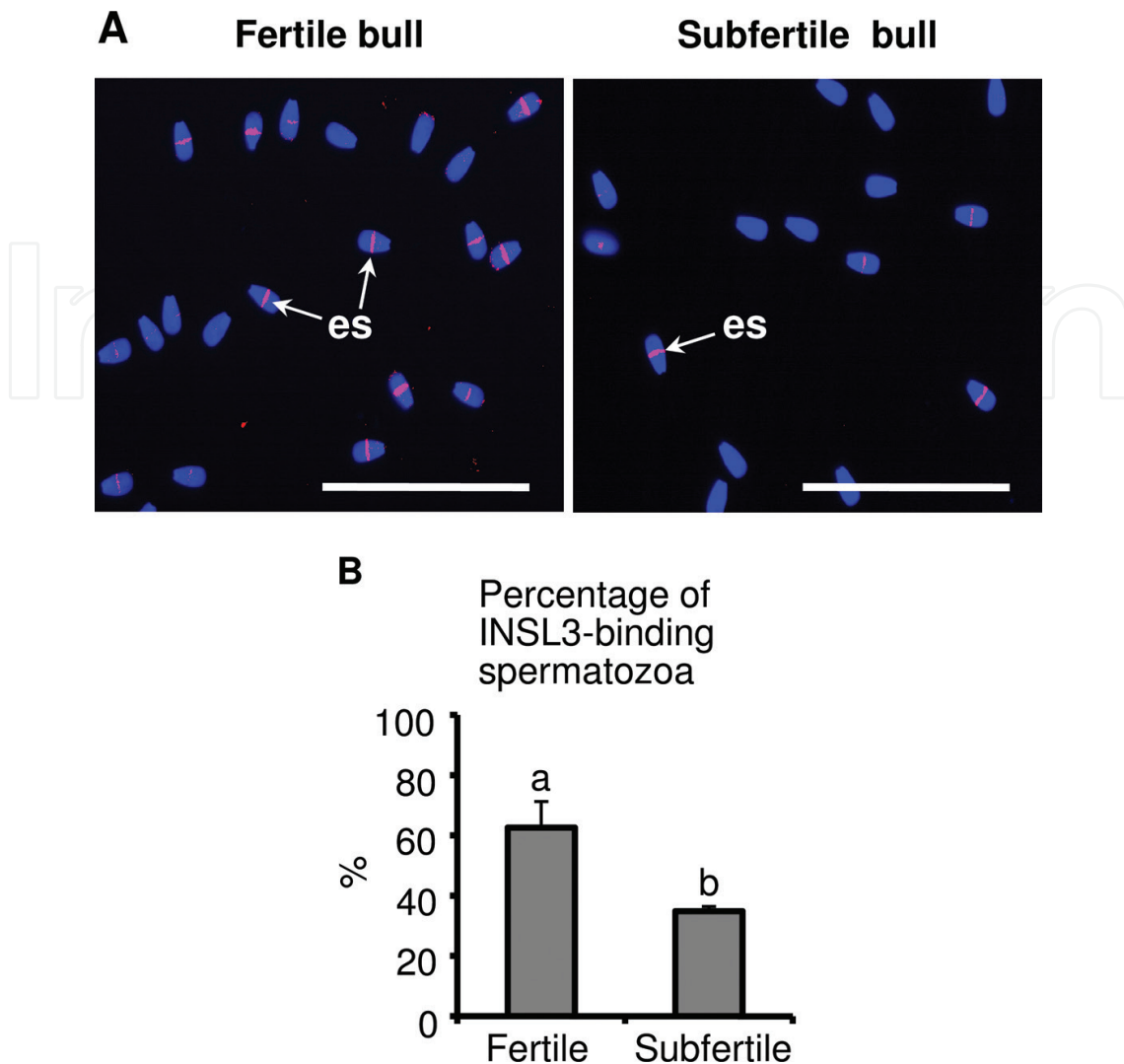
Figure 11. Evaluation of sperm population expressing the functional RXFP2 receptor enabling INSL3 to bind in the semen of fertile and subfertile bulls. (A) Binding of INSL3 in bull spermatozoa *in situ*. INSL3 binding signals were visualized only in the equatorial segment of both fertile and subfertile bull spermatozoa. es, equatorial segment. Bars = 50 μm. (B) Morphometric analysis. Percentages of INSL3-binding spermatozoa were significantly reduced in the semen of subfertile bulls compared to that of fertile bulls. Values are expressed as mean ± SE, and values with different letters are significantly different ($P < 0.05$). Data were derived from Pitia et al. [36].

3. Conclusions

While peptide hormone INSL3 is essential for fetal testis, and possibly acts as an important player in testicular and sperm functions in adult males, there has been very little progress in understanding the functions of INSL3 in male ruminants, including goats. In this review, we have undertaken an understanding of both the structure and function of INSL3 in male goats to fill a gap in our knowledge. From a structural point of view, we provided evidence that goat INSL3 is constitutively secreted from Leydig cells as an unprocessed B–C–A form

retaining the C-domain with full biological activity. The reason why the INSL3 exists as a B–C–A form is unclear, but the C-domain does not appear to interfere with receptor binding and activation. Additionally, clarification of native goat INSL3 will facilitate development of a specific immunoassay system for monitoring INSL3 in blood and body fluids. In contrast, from a functional point of view, we provided the evidence for a functional receptor that binds INSL3 in testicular germ cells and in spermatozoa, implying that the intra- and extratesticular INSL3 hormone-receptor system operate in male goats. We also found the potential of this system as a novel parameter for predicting fertility in breeding sires. However, it remains unknown what functions INSL3 actually exerts on testicular germ cells and on spermatozoa in this species. Finally, the findings outlined here will help in the discovery of new target tissues/organs and receptor-expressing cells, not only in male goats but also in female goats, thereby giving insight into the potential role of INSL3 in those organs.

Acknowledgements

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