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Mechanisms of Hormonal Regulation of Sertoli Cell Development and Proliferation: A Key Process for Spermatogenesis

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Abstract: In adulthood, the main function of the testes is the production of male gametes. In this process, Sertoli cells are essential for sustained spermatogenesis, providing the developing germ cells with the physical and nutritional support required. The total number of Sertoli cells in adulthood determines the daily gamete production, since Sertoli cells can support only a limited number of developing germ cells. Considering that Sertoli cell proliferation only occurs during the immature period, proper development and proliferation of the Sertoli cells during the proliferative phase are crucial to male reproductive health in adulthood. The proliferation process of the Sertoli cells is finely regulated by an assortment of hormonal and paracrine/autocrine factors, which regulate the rate and extent of proliferation. In the present review, we discuss the most important hormonal and paracrine factors involved in the regulation of Sertoli cell proliferation, as well as the signaling mechanisms by which they exert their effects.

Keywords: FSH, IGF-1, insulin, reproduction, testis.

1. INTRODUCTION

The testes have basically two main functions: to produce testosterone (steroidogenesis) and to support germ cell development (spermatogenesis) [1]. The testicular somatic cells present within the seminiferous tubules, called Sertoli cells (SCs), are the major structural components of the testes [2]. The most recognized function of SCs is to provide physical and nutritional support to developing germ cells [3]. This dependence of germ cells on SCs support is due to the existence of a blood-tissue barrier that physically divides the seminiferous epithelium into basal and adluminal compartments [4]. Tight junctions, ectoplasmic specialization, gap junctions, and desmosomes constitute this barrier, which is called the blood-testis barrier (BTB), and is formed by adjacent SCs [5]. Thus, the meiotic and post-meiotic germ cells become isolated in the immunologically privileged luminal compartment [6]. As a result, blood-to-germ cell communication is under the tight control of SCs, which control the movement of substances between these two compartments, as well as receiving, integrating and retransmitting hormonal signals that are essential for spermatogenesis [7]. Since haploid germ cells are isolated within the seminiferous tubules, well-functioning adult SCs provide differentiation factors and energy sources to them [1]. Thus, the formation and proper functioning of the SCs and the BTB are crucial for developing germ cells, as this creates an appropriate microenvironment, which enables germ cell development into fully functional spermatozoa [4]. In addition, developing germ cells are unable to metabolize and use glucose as an energy source [8], whereas other glucose metabolites cannot cross the BTB [9]. Lactate is the main energy source of germ cells, and its production and secretion by SCs determine the function and survival of pachytene spermatocytes. Thus, SCs have the task of supplying germ cells with glucose metabolites, such as lactate, to sustain the spermatogenic process [8]. SCs can produce lactate from the metabolism of various substrates, but preferentially glucose [10]. Besides glucose metabolites, such as lactate and pyruvate, SCs also ensure the nutritional support of germ cells by secreting nutrients or metabolic intermediates, such as amino acids, carbohydrates, lipids, vitamins, and metal ions [11]. These cells are responsible not only for the production of energy sources for germ cells, but also produce and secrete many other proteins (such as androgen binding protein and transferrin), growth factors (such as insulin-like growth factor 1 and TNF-α), and inflammatory cytokines (interleukins) [12-15]. Altogether, these compounds ensure the correct development of germ cells. In addition, during the proliferative phase, SCs need substrates such as carbon, nitrogen, and free energy to support the synthesis of new proteins, lipids, and nucleic acids [16].

Despite the irrefutable role of SCs in maintaining spermatogenesis, the adult testes are only capable of producing spermatozoa (in number and quality) if the proliferation and maturation processes of SCs occur properly during the immature period [3]. As reviewed by Sharpe *et al.* [3], the proliferative stage of SCs varies depending on the species. It appears that SCs proliferate during two periods of life, in fetal or neonatal life and in the pre-pubertal period in all species. However, because the neonatal period overlaps with the

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pre-pubertal period in species such as the rat, it is therefore difficult to discern the two periods of SCs proliferation. Consequently, it is assumed that rat SCs proliferate until 15 days of age [3]. The failure in both functioning and development/proliferation of SCs is involved in pathological processes that lead to a reduction in semen quality [1]. This is due to several structural and functional features that change during development of the testes, enabling SCs to support spermatogenesis.

Mature and immature SCs differ extensively from each other in morphological and biochemical aspects. In immature testes, the SCs are proliferating and growing. They lay on the basement membrane and their cytoplasm has projections that fill the center of the seminiferous cords [17]. The seminiferous cords contain only peritubular and germ stem cells in addition to SCs, presenting a solid aspect with the absence of a lumen [18]. On the other hand, after puberty SCs become elongated and BTB begins to be established [1, 3]. Around the onset of puberty, the SCs switch from an immature and proliferative state to a mature and non-proliferative state. Additionally, radical changes in their morphology and functions occur. The nucleus enlarges and the nucleolus becomes more prominent. The tight junctions begin to be formed and create an adluminal compartment, allowing the formation of a fluid-filled lumen [3]. Functionally, SCs enhance their lactate production capacity [19] and lose their proliferative ability [3]. Maturation and proliferation of SCs depend on a tight hormonal and paracrine/autocrine regulation. A broad range of hormones [1, 20], cytokines [15] and growth factors [1, 21] have been described to be related to the proliferation and differentiation of SCs.

Hormonal control of testicular development and spermatogenesis is primarily accomplished by the hypothalamus-pituitary-gonad (HPG) axis as reviewed by Alves and collaborators [20]. This axis functionally connects the brain with the testis and its malfunction leads to infertility [20, 22, 23] (Fig. **1**). The gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), produced and secreted by adenohypophysis, are recognized as the central regulators of testicular functions. FSH mainly regulates SCs proliferation, whereas LH is primarily involved in regulating testosterone production. However, the pituitary regulation exerted by the gonadotropins is not independent. Local mechanisms of regulation within the testis relay this endocrine control. There is a paracrine interaction between peritubular myoid cells, germ cells and SCs, as well as between Leydig cells and the seminiferous tubules and, more specifically SCs, which modulates this pituitary control [3, 24]. A complex assortment of peptides and hormones has been shown to be involved in the regulation of SCs proliferation [1, 21]; hence, it is very difficult to determine the real role of each regulatory protein *in vivo*. Furthermore, the majority of these studies were performed using *in vitro* models; thus, additional evidence for the *in vivo* implications of regulatory proteins that have already been described is still needed. Further studies are required for a full understanding of the hormonal regulation of SCs proliferation. However, it is well known that an appropriate hormonal regulation of such process during the pre-pubertal phase is crucial for normal reproductive function in adulthood [3, 21], since gametes production is directly related to the SC number [3, 25]. Thus, this review is focused on hormonal regulation of the proliferative phase of SCs, with special focus on FSH, insulin and IGF-1 action, since this testicular stage of development is critical for normal reproductive health in adulthood.

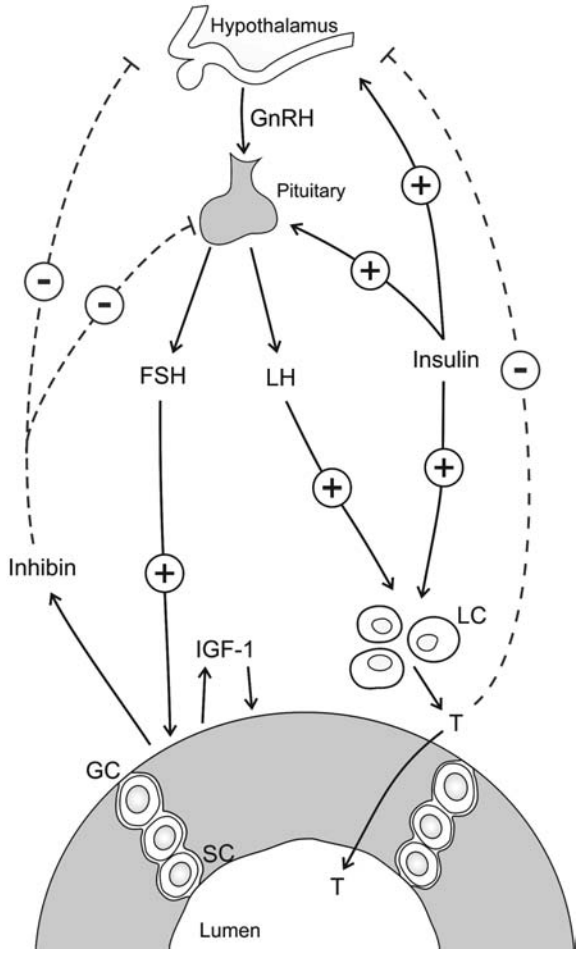

Fig. (1). Simplified representation of the hypothalamus–pituitary–testis axis control of SC proliferation. The hypothalamic GnRH modulates the biosynthesis and secretion of the pituitary hormones, LH and FSH. LH induces testosterone production by Leydig cells, which is involved in the end of SC proliferation period and provides a negative feedback reducing GnRH and LH production. FSH acts on SCs inducing the production and secretion of inhibin and IGF-I, which provide a negative feedback in FSH releasing and a positive feedback in SC proliferation, respectively. Circulating insulin also modulates the HPG axis stimulating testosterone secretion through the action in hypothalamus and pituitary, as well as directly in Leydig cells. Black lines with circled plus – positive feedback; Dashed lines with circled minus – negative feedback; GnRH – gonadotropin-releasing hormone; FSH – follicle-stimulating hormone; LH – luteinizing hormone; T–testosterone; LC – Leydig cell; SC – Sertoli cell; GC – Germ cell.

2. FOLLICLE STIMULATING HORMONE

FSH is critical for male fertility, influencing both SC proliferation in perinatal life and the synthesis of SC-derived products, which are essential for germ cell survival and the proper functioning of the developing and adult testis [26]. Although recent studies have demonstrated a broad range of

hormonal factors involved in SC proliferation and maturation, FSH is still considered the most important regulator of these processes [1]. FSH belongs to the pituitary glycoprotein hormone family, which also comprises LH, chorionic gonadotropin (hCG) and thyroid-stimulating hormone (TSH). These hormones share a common α subunit which associates to a specific β-subunit to form a functional heterodimer [27]. FSH binds to and activates a plasma membrane receptor belonging to the G protein-coupled receptor (GPCR) superfamily. The FSH receptor (FSHR) displays a high degree of tissue specificity, being expressed only in granulosa cells in females and SCs in males, as reviewed by Simoni *et al*. [28]. Classically known as a Gαs protein-coupled receptor, the ability of FSHR to associate with other types of G proteins, such as Gαi, has recently been demonstrated [29, 30]. As FSHR has the ability to associate with different G proteins and stimulate diverse signaling pathways, FSH is able to modulate a vast array of SCs functions, thus regulating testis development and spermatogenesis [31].

In fact, FSH is the main factor responsible for SCs mitogens and several works have shown that FSH stimulates the expression of markers of SCs proliferation exclusively in immature SCs, such as cyclin A2, and D1, c-Myc, and the proliferating cell nuclear antigen (PNCA) [26, 32, 33]. The exact mechanism whereby FSH stimulates SCs proliferation is not well understood, as well as the mechanisms involved in SCs differentiation. Despite the fact that FSH levels and FSHR expression are stable during puberty [28], the signaling pathways involved in SCs response to FSH in the proliferation and differentiation stages are different. At the time of SC transition from the proliferative to differentiated state, there is a change in the signaling pathways triggered by FSH action [34]. Some of these pathways, such as calcium uptake and FSH-mediated ERK activation, occur exclusively in immature SCs during the proliferative stage [29, 32]. This opposite action of FSH on immature and mature SCs seems to be related to the changes in cAMP kinetics. The cAMP basal level is weak in cultured SCs from neonatal rats, which is explained by the low basal activity of phosphodiesterase (cAMP- degrading enzyme) on SCs from 5-day-old rats. In SCs from 19-day-old rats, the basal level of cAMP is high and the phosphodiesterase activity in mature SCs is 4-fold increased [29, 35]. Besides that FSH stimulates the cAMP-independent amino acid transport by A system only in immature rats (from 10 to 20 day-old-rats) [36].

The FSH binding to the FSHR stimulates the activation of the heterodimeric Gα protein, which is followed by dissociation into two molecules, the Gα-subunit and the Gβ/γ heterodimer [37]. This dissociation may result in the activation of adenylate cyclase (AC)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), mitogen-activated protein kinase (MAPK), or phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) signaling pathways, with consequent biological effects, such as changes in SC membrane potential and calcium influx [29, 30, 33]. The Gα-subunit is known to activate the AC, with the consequent formation of cAMP and phosphorylation of PKA [37]. PKA is capable of activating structural proteins, enzymes, and transcription factors, triggering diverse biological effects [38].

In immature rats, FSH promotes a biphasic effect on the membrane potential of SC, which is characterized by a short membrane hyperpolarization (30 seconds) followed by a prolonged depolarization (6 minutes) [31, 39]. The AC activator forskolin also produces a rapid hyperpolarization of the SC membrane which is blocked by tobultamide and glibenclamide (K_{ATP} blockers), indicating the activation of Gαs by FSH [31, 40]. ATP reduction, resulting from the formation of cAMP, causes ATP-sensitive potassium channels (K^+_{ATP}) to close, and leads to membrane hyperpolarization [31, 41]. After the hyperpolarizing effect, the stimulation of SCs with FSH promotes a rapid (60 seconds) and sustained (10 minutes) depolarization and increase of intracellular calcium levels. The depolarizing effect is a result of calcium influx through L-type voltage-dependent calcium channels (VDCC) and is inhibited by verapamil, an L-type VDCC blocker [30, 40]. In addition, perfusion of the seminiferous tubules with the pertussis toxin (PTX), which inhibits Gαi-Gβ/γ dissociation, prevents the depolarization phase, suggesting that depolarization is mediated by Gαi activation [31]. Experiments performed by Gorczynska and co-workers [42] revealed the involvement of both Gαs and Gαi in FSH-stimulated calcium increase in immature SCs. Additionally, GPCRs could bind to a more than one type of G protein. This dual mechanism explains the biphasic electrophysiological response of SCs from immature rats to FSH action described above [31]. The implication of both Gαs and Gαi heterotrimeric complexes is also observed in FSH-mediated extracellular-signal-regulated kinases (ERK) phosphorylation in immature SCs [29]. ERK1/2 is a part of the MAPK family, the activation of which promotes a series of signal transduction pathways that regulate cellular processes, such as cell proliferation and differentiation [43]. Crépieux and colleagues showed that this ERK phosphorylation is cAMP/PKA-dependent and is stage-specific, as it is observed in SC primary culture from 5- and 12-day-old but not 19-day-old rats.

Several works have shown that AC/cAMP/PKA pathway is involved in calcium uptake [30, 42]. Gorczynska *et al*. [42] suggested that in response to FSH, cAMP/PKA mediates protein phosphorylation to activate calcium channels or their regulators. The exact mechanism through cAMP/PKA stimulated by FSH mediates VDCC modulation is not clear. Some studies performed in muscle tissues and chromaffin cells have indicated that PKA phosphorylates the α1-subunit of the VDCC resulting in calcium current potentiation [44, 45]. However, to date, there have been no studies showing this mechanism in SCs. Nevertheless, SC incubation with AC and PKA inhibitors (MDL, $(Bu)_2$cAMP, and staurosporine) only partially prevents FSH-stimulated calcium uptake, suggesting the involvement of other pathways in the calcium influx [42].

Experiments using EGTA (a calcium chelator) and verapamil revealed that the main source of calcium is the extracellular compartment by influx through type VDCC; however, voltage-independent calcium channels and intracellular sources are also found to be associated [42, 46]. Calcium is an important intracellular signaling messenger, which is involved in several cellular processes, including cellular proliferation. The FSH-induced calcium uptake observed exclusively in immature rat SCs strongly suggests its involvement in SC proliferation [32, 47]. Nonetheless, besides the effect

of AC/cAMP/PKA on regulating calcium influx, it also occurs through an AC/cAMP-independent pathway [30, 31, 42]. After Gαi activation, FSH-mediated the dissociation of the Gαi β/γ heterodimer, resulting in calcium influx through L-type VDCC and [^{14}C]-MeAIB transport [31, 36, 40]. The FSH-mediated depolarization of the SC membrane is prolonged and potentiated by [^{14}C]-MeAIB [40]. FSH acts on [^{14}C]-MeAIB influx, increasing the activity (V_{max}) of System A of amino acid transport, which is Na^+- and energy-dependent [48, 49]. The System A mediates the symport of neutral amino acids with small side chains (alanine, serine, and glutamine) and with sodium [50]. The non-metabolizable alanine analogue MeAIB is also a substrate for this system [51]. The FSH-mediated increase in System A activity provides a source of nitrogen from neutral amino acids required for protein and nucleotide biosynthesis that is essential for cell growth and metabolism [16, 51]. Alanine can be converted to pyruvate and used as an energy substrate by SC [10]. Glutamine is the main amino acid in the body and has an important role in the proliferation of several tissues [52, 53]. Nevertheless, the FSH actions on calcium uptake and [^{14}C]-MeAIB transport are blocked by PTX, verapamil and wortmannin (a PI3K blocker) [31]. PI3K is a member of the cytosolic lipid kinase family [54], which accumulates at the plasma membrane when it is stimulated [55]. The PI3K family is divided into classes I, II, and III [56]. Class I comprises of a p110 catalytic subunit and a regulatory adapter subunit; Class I converts extracellular signals into the intracellular environment [56]. This PI3K class is subdivided into class IA (isoforms a, b, and g), activated by tyrosine kinase receptors or non-receptor tyrosine kinases, and class IB (PI3Kγ) which is exclusively activated by GPCRs and mostly regulated by the Giβ/γ subunit [57]. Considering that the effects of FSH are blocked by PTX and wortmannin, its action seems to involve the PI3Kγ isoform [31]. Once activated, PI3Kγ converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidyl-inositol-3,4,5-trisphosphate (PIP3) [56]. PIP3 binds to pleckstrin-homology (PH) domains of signaling proteins and enhances phosphorylation [54]. The activation of these proteins initiates a complex downstream process of events that controls protein synthesis, actin polymerization, cell survival, and cell cycle entry [56]. The best-characterized downstream target of PI3K, PKB, is a key regulator of cell growth, proliferation and metabolism [58]. Acting through PKB, FSH increases the glucose uptake [59], calcium and MeAIB [33]. These effects are blocked by verapamil, indicating L-type-VDCC involvement [31]. Experiments performed by Viard [60] showed that PKB stimulates the trafficking of calcium channels to the plasma membrane, possibly by phosphorylation of the B regulatory subunit (CavB2) of VDCC. Considering that AC/PKA inhibitors only partially decrease FSH-induced calcium influx, whereas wortmannin completely blocks this FSH effect; there is a clear evidence of cross-talk between the AC/cAMP/PKA and PI3K/PKB signaling pathways activated by FSH, modulating VDCC and calcium influx in immature SCs.

In addition, PI3K/PKB/Akt activates the mammalian target of rapamycin (mTOR), a serine/ threonine kinase, which plays an important role in the control of protein synthesis [61]. After being activated, mTOR interacts with many proteins (mTOR, mLST8/GBL, Raptor, PRAS40, and DEPTOR) to form the mTOR complex 1 (mTORC1) [33]. Activation of mTORC1 results in phosphorylation of several downstream targets such as p70S6K, which are involved in protein synthesis [33]. The activation of PI3K/PKB/mTORC1 by FSH is blocked by wortmannin and rapamycin (mTOR inhibitor) [33, 62]. However, the PKA blocker H89 did not alter the phosphorylation of PKB, indicating that this pathway is PKA-independent [59]. The key role of FSH on SC proliferation could be observed in the recent studies performed with animal models lacking FSH (FSHBKO) and the FSH receptor (FSHRKO). Adult FSHRKO mice are fertile, besides the low SC number and germ cells [63-65]. Another study with FSHBKO and FSHRKO mice showed reduction in numbers of spermatogonia, spermatocytes and spermatids, as reviewed in [66]. Also, both *in vivo* and *in vitro* analyses revealed that FSH requires the insulin/IGF signaling pathway to mediate its proliferative effects on immature SCs. Thus, it becomes clear that FSH is not the only factor responsible for SC proliferation. Otherwise, the local production of paracrine factors, such as insulin and IGFs, is also crucial, and the insulin signaling family is the major intra-testicular signal that is responsible for regulating SC number, testis size and sperm output in mammals [21].

3. INSULIN RECEPTOR SIGNALING FAMILY

The insulin receptor tyrosine kinase family comprises of insulin receptor (IR), IGF-1 receptor (IGF-1R) and insulin-related receptor (*Irr*), and is present in virtually all cell types [67]. This family of receptors is indispensable for pre- and postnatal growth and development in both rodents and humans [68]. Pups lacking IR die from ketoacidosis within 4 days of birth [68], whereas those who do not express IGF-1R die at birth from respiratory failure and show several abnormalities, including reduced size [68, 69]. The absence of both IR and IGF-1R affects mice development even more severely [70]. On the other hand, Irr-deficient mice are viable and no physiological function has been attributed to this receptor [67]. The involvement of the insulin receptor signaling family in the male reproductive function has been studied since the 1970s, with studies focused on insulin and IGF-1 action in all of the different cell types of the testes [71-73]. In the last few decades, plenty of data were published providing new insights concerning the importance of these hormones in male reproductive physiology [21, 23, 33, 67, 74-76]. Also, a recent study from Pitetti and collaborators [21] demonstrated *in vivo* that IR and IGF-1R act in a synergistic manner to regulate SC number and testis size. Thereby, both receptors and their associated downstream signaling molecules are required for the appearance of male gonads, and thus for male sexual differentiation [21, 67, 76].

IR and IGF-1R are membrane-bound glycoproteins. They display a similar overall structural organization, which consists of two α- and two β-subunits linked by disulfide bonds (Fig. **2**). The intracellular β-subunit is a ligand-stimulated tyrosine kinase domain, while the extracellular α-subunits configure the ligand binding sites. Both receptors display a similar structural organization, with overall similarity at the amino acid sequence level of approximately 50% [77]. In addition to that, their receptors, insulin and IGF-1 are struc-

turally very similar, and thus both hormones are capable of binding and eliciting effects through all members of the insulin receptor signaling family [77]. To further complicate matters, the signaling pathways activated by insulin and IGF-1 are largely overlapping [78], inducing many of the same signaling pathways. The activation of both receptors leads to the phosphorylation of common substrates that activate a network of downstream effectors, including the PI3K and ERK pathways (Fig. **2**). Both signaling pathways are associated with proliferation, differentiation and cell survival [21, 79]. Thereby, this capability of binding to all receptors of the family makes the investigation of the action of insulin and IGF-1 difficult when both receptors are expressed in the same cell [78]. Despite these similarities, IR and IGF-1R are not functionally redundant molecules and one cannot compensate for the absence of the other. The functional difference between these receptors becomes clear in the distinct phenotype of IR- and IGF-1R-deficient mice [21, 78]. Therefore, both hormones play an essential role in regulating SC proliferation, and are crucial to male reproductive function [21]. Although several factors are related to the proliferation and differentiation of SCs, insulin and IGF-1 signaling are intimately involved in such processes and, among FSH, seem to be the most important signals in regulating the final number of SCs during the pre-pubertal period [21].

3.1. Insulin

Insulin has long been recognized as the main regulator of energy homeostasis, but this hormone also modulates proliferation and differentiation in virtually all cell types. Unfortunately, the relevance of insulin for the development of the male reproductive system has been underestimated and the data accumulated until now are poor. On the other hand, increasing evidences have recently demonstrated the fundamental role of this hormone in male reproduction [21, 23, 67, 75, 80]. The effects of insulin are mediated by the insulin receptor (IR), which is widely expressed in testicular tissue [22]. After insulin binding and the consequent induction of IR phosphorylation, the classical signaling mechanisms activated are mediated by adaptor proteins, such as insulin receptor substrate-1 (IRS-1). The adaptor proteins subsequently recruit diverse effector proteins containing Src homology 2 (SH2) domains, such as PI3K. In turn, these effector proteins activate different signaling pathways that are involved in modulating metabolism, proliferation and differentiation [81, 82]. Although the molecular mechanisms have not been completely described yet, it is clear that insulin affects reproductive function at multiple levels. A number of studies have demonstrated the involvement of insulin in testicular development [21, 67], in modulating testicular cells function [74, 75, 83, 84], or even influencing HPG axis functioning [22, 23].

Increasing observations have demonstrated that pathological conditions which compromise insulin production/action, such as Diabetes Mellitus (DM), are usually accompanied by altered testosterone levels [22, 23, 85]. These findings revealed the importance of insulin in regulating testosterone secretion, in both men and in animal models. Indeed, insulin seems to influence testosterone secretion and production either as a direct effect on Leydig cell steroidogenesis [84, 86] or through modulation of the HPG axis [22, 23]. In the central nervous system (CNS), IR is widely distributed, including the hypothalamus and pituitary [87]. Insulin is also described to be expressed in the CNS in higher levels than that observed in plasma [88], although the relative importance of insulin in these regions remains to be fully determined [89]. The insulin interaction with hypothalamic neurons modulates the secretion of gonadotropins,

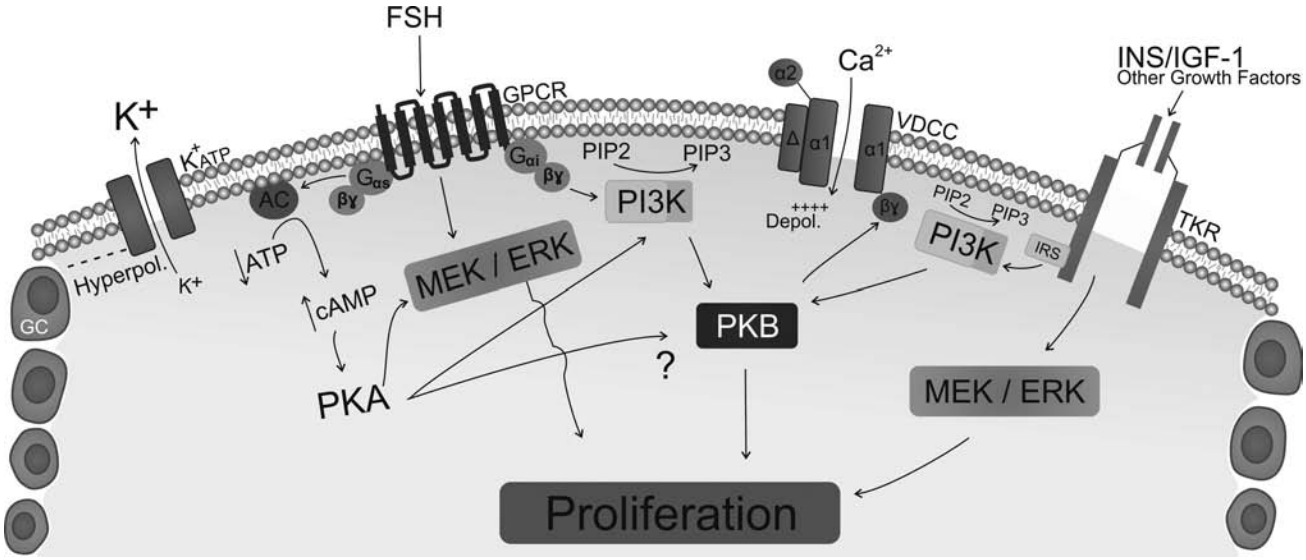

Fig. (2). Schematic representation of hormonal regulation of SCs proliferation. The main hormones described activate mainly the PI3K/PKB and MEK/ERK signaling pathways to induce SC proliferation. K^{+}_{ATP} – ATP-sensitive potassium channel; AC – adenylate cyclase; ATP – adenosine triphosphate; cAMP – cyclic adenosine monophosphate; PKA –phosphokinase A; FSH – follicle-stimulating hormone; GPCR – G protein-coupled receptor; Gαs – G protein isoform s; Gαi – G protein isoform i; MEK/ERK - mitogen extracellular kinase /extracellular signal-regulated kinase pathway; PI3K – phosphatidyl inositol 3-kinase; PKB – protein kinase B; IRS – insulin receptor substrate; VDCC – voltage-dependent calcium channel; TKR – tyrosine kinase receptor; PIP2 and PIP3 – phosphatidyl inositol biphosphate and phosphatidyl inositol triphosphate; SC – Sertoli cell; GC – germ cell.

altering serum hormone levels that are essential for spermatogenesis [90]. In both men and rodents, insulin clearly affects LH plasma levels, and consequently testosterone concentrations, by the action on the CNS neurons, modulating HPG axis activity [23, 90]. The exact mechanism by which insulin regulates LH secretion in the CNS is not yet well established. However, alterations in LH levels are a common complication of DM and culminate in reduced testosterone concentrations, which impairs male fertility, reducing spermatogenesis and libido [23]. In the Akita mouse model, which is a model of type 1 diabetes resulting from a mutation in the ins2 gene, the LH and testosterone levels were restored after exogenous insulin treatment [23]. In addition, Ballester and colleagues [22] found that diabetic rats present reduced FSH levels, thereby indicating a role for insulin in regulating FSH secretion. Thus, insulin might regulate SC proliferation not only by a direct effect in SC, but also through modulating FSH action. However, further investigations are necessary to corroborate these hypotheses.

Insulin also alters testosterone concentration by a direct action in Leydig cells [84]. This direct effect modulates steroidogenesis through regulation of aromatase activity and expression, as well as by modulating LH receptors in these cells [73, 84, 86]. Although the effects of testosterone on postnatal SC proliferation are controversial, it has an important influence on prenatal testicular development [91, 92]. As insulin is also indispensable to prenatal SC proliferation [67], the interrelationship of these hormones may play an important role in prenatal testicular development, as well as in SC proliferation.

For the cells to be able to proliferate, they need to adapt their metabolism to support the conversion of available nutrients; however, the way in which cell metabolism is regulated to balance the production of ATP remains unclear [93]. It is known that regulation of SC metabolism is also important for spermatogenesis [10]. Since a vast array of hormones regulates SC metabolism, it is probable that this hormonal modulation is also involved in SC proliferation and development. Insulin directly affects SC metabolism, stimulating glucose transport [80], lactate production [83] and amino acid accumulation [80], altering the expression pattern of glucose transporters, and modifying important glycolytic enzymes activity and expression [75]. Also, SCs cultured in the absence of insulin present reduced acetate production and altered caspase-dependent apoptotic signaling [94, 95]. Insulin also induces a calcium-dependent membrane depolarization in immature SCs, which is, curiously, mainly induced through IGF-1R activation [74]. Some of these effects were demonstrated in both mature [19, 75] and immature [19, 80] SCs, and may be crucial for SC development. As the germ cells depend on SC metabolism, these alterations seem to be associated with impaired spermatogenesis, and perhaps SC proliferation, but the molecular mechanisms remain unknown. Furthermore, several studies show that metabolic diseases such as DM, which are associated with insulin deregulation, are related to male infertility [22, 96-98]. Since insulin has an important role in the regulation of SC metabolism, this deregulation may be a possible mechanism by which insulin deregulation may be involved in the development of infertility.

Probably, the clearest evidence of insulin-mediated SC proliferation was provided by Pitetti and collaborators in 2013. Using knockout mouse models, these authors demonstrated *in vivo* that insulin receptor is crucial in regulating immature SC proliferation, maturation and ultimately the total pool of adult SCs. The molecular mechanisms behind insulin-mediated SC proliferation are not fully understood, but may be explained by the signaling pathways which are activated by this hormone. The binding of insulin to its cognate receptor can stimulate an assortment of different signaling pathways, which makes it difficult to identify how insulin regulates SC proliferation [99, 100]. The most commonly observed signaling pathways stimulated by this hormone in different cell types are the PI3K/PKB and MEK/ERK pathways. The MEK/ERK pathway is a well-known signaling pathway activated by insulin and growth factors in several cell types [101], including SCs [102]. Growth factors stimulate this signaling pathway, inducing cell proliferation through the GTPase Ras. Active Ras binds to and activates Raf-1, which in turn, phosphorylates and activates MEK, which then phosphorylates and activates ERK [79]. Other growth factors, such as transforming growth factor-α [102], have been demonstrated to induce SC proliferation through ERK activation. However, it is not yet known whether insulin is capable of inducing SC proliferation through this signaling pathway. On the other hand, the PI3K/PKB signaling pathway was previously shown to be activated in SC by FSH and insulin and regulates the proliferation and metabolism of these cells, respectively [33, 80]. We recently demonstrated that PI3K/PKB signaling pathway is involved in insulin-induced glucose, amino acid and calcium uptake in immature rat SCs [80]. Similarly to MEK/ERK signaling, PI3K/PKB is also usually associated with cell proliferation [82]. The involvement of PI3K/PKB signaling in FSH-induced SC proliferation has already been demonstrated [33]. Considering that FSH-induced amino acid transport occurs only in immature SCs and is associated with PI3K signaling, it is possible that the regulation of SC metabolism by insulin is an important step in SC proliferation. However, the exact role of this signaling pathway in insulin- and IGF-1-regulated SC proliferation remains to be elucidated. Notwithstanding, the main docking proteins phosphorylated by the insulin signaling family are the insulin receptor substrate (IRS) proteins, which are involved in PI3K/PKB activation [103]. The four IRS proteins identified to date (IRS1-4) show structural homology, but studies with knockout models indicate that they serve as complementary signaling molecules rather than redundant. In rodent models, the lack of IRS1 causes severe growth retardation and the absence of IRS2 is responsible for diabetes development. IRS3 and IRS4 deletion causes minimal alterations in metabolism, endocrine system and growth [76]. IRS1 and 2 are expressed in human testis [104], and Griffeth and collaborators [76] demonstrated that the deletion of IRS2, but not IRS1, causes a 45% reduction in testis size as a consequence of a decrease in the cell population within the seminiferous tubules, including SCs, spermatogonia and spermatocytes. These evidences strongly suggest that insulin-induced SC proliferation is mainly due to the activation of IRS2/PI3K/PKB signaling, which is known to regulate proliferation. However, the relative role of Raf-1/MEK/ERK pathway in this process cannot be excluded. Finally, other growth factors that induce IRS activation, such

as IGF-1, might regulate SC proliferation through this same signaling pathway.

3.2. Insulin-like Growth Factor 1

Insulin-like growth factor 1 (IGF-1) is a small polypeptide with structural homology to pro-insulin and can be produced by several tissues. This growth factor may act as an autocrine/paracrine signal [105] or as a classical hormone, since it circulates in the plasma associated with cognate binding proteins [69]. The IGF-1 action is mediated by signaling pathways initiated in response to autophosphorylation of IGF-1R on specific tyrosine residues with the consequent recruitment of several adapter proteins [106]. IGF-1R, a transmembrane tyrosine kinase, is widely expressed in the testes, including in SCs [71, 107, 108]. Generically, activation of the receptor following binding of the secreted IGF-1 elicits assorted cellular responses including proliferation, and the protection of cells from programmed cell death [109]. Similar to insulin and its receptor, the activation of IGF-1R leads to the stimulation of diverse signaling pathways that have been implicated in the effects of growth factors on cell survival and proliferation, such as PI3K/PKB and MEK/ERK pathways [110]. In addition to the expression of IGF-1R, SCs also express and secrete IGF-1 and IGF-binding proteins (IGFBPs), predominantly IGFBP3, which inhibits the effects of exogenous IGF-1 on SCs [111]. Thus, SCs possess all of he necessary components for generating, receiving and modulating IGF-1 action [105], which is also known as the IGF-1 system. Furthermore, in SCs, the IGF-1 system can be modulated by FSH, which stimulates IGF-1 production whilst inhibiting the secretion of IGFBP3 by immature SCs *in vitro* [111, 112]. The indispensable role of IGF-1 and its receptor in male reproduction has long been demonstrated [113], and several studies have shown that IGF-1 regulates SC metabolism [74, 83, 114], proliferation [21, 115] and differentiation [113].

IGF-1 exerts important effects on SC metabolism, similar to those observed for insulin, such as stimulating lactate production [83], glucose transport [74, 83] and transferrin production [116]. Indeed, distinguishing the effects of IGF-1 and insulin in cells that express both IR and IGF-1R is very difficult, since both hormones can bind to both receptors and activate many of the same downstream signaling molecules [78]. Some effects that can be observed in the action of these hormones are also very similar. However, recent studies have provided new insights into the effects and specificity of insulin and IGF-1 [78, 117, 118]. Versteyhe and collaborators [118] demonstrated that IGF-1 and insulin, even acting upon the same receptor, specifically regulate different groups of transcripts from the other ligand in the culture of fibroblasts. However, the authors could not draw a definitive conclusion concerning whether one ligand creates a more metabolic or mitogenic response in comparison to the other. On the other hand, previous experiments with IR- and IGF-1R-deficient mice demonstrated that IR is more important in fuel metabolism, whereas IGF-1R is more associated with growth regulation [68, 78]. Despite the effects on SC metabolism, IGF-1 seems to be the most significant regulator of SC proliferation [21]. According to Pitetti and collaborators [21] SCs lacking IR are less affected then those lacking IGF-1R. Even the effect of FSH on SC proliferation seems to be, at least in part, dependent on IGF-1 signaling [21].

In fact, IGF-1 and FSH actions on regulating SC function and proliferation are closely related [105, 111, 119]. Several authors have shown that IGF-1 and FSH interact with each other to regulate SC proliferation [21], aromatase regulation, and PKB phosphorylation [105]. Once both hormones are known to activate PI3K/PKB, it could be postulated that interaction between IGF-1 and FSH is due to the modulation of this signaling pathway. Khan and collaborators [105] suggested that the effect of FSH on PKB phosphorylation could be due to a combination of increased secretion of endogenous IGF-1, decreased IGFBP3, and the maintenance of the synergistic action of FSH on IGF-1-dependent PI3K activation. Although Khan and co-workers [105] demonstrated that FSH amplifies IGF-1-stimulated PKB signaling but is not able to induce PKB phosphorylation alone. Jacobus *et al*. [31] demonstrated that the FSH-induced calcium influx in immature SCs is due to the activation of PI3K but independent of IGF-1 action. It is probable that the molecular signaling by which IGF-1 stimulates SC proliferation is similar to that proposed for insulin. In addition, the interaction between IGF-1 and FSH is clear, but the molecular mechanisms must be further investigated in order to provide a full understanding of the hormonal regulation of SC proliferation process. The specific mechanisms by which IGF-1 regulates SC proliferation still require further elucidation, and although this growth factor, as well as insulin, is responsible for stimulating around 60% of SC proliferation [21], other growth factors and cytokines also appear to be involved in this process.

4. OTHER REGULATORY FACTORS

There are strong evidences that FSH, IGF-1 and insulin are the main regulators of SC proliferation. However, the effects of these hormones are not sufficient to explain the entire process. In this context, many other hormones, growth factors and inflammatory cytokines have been demonstrated to be involved in the SC proliferation process.

4.1. Growth Factors

IGF-1 is the most characterized growth factor that regulates SC proliferation. However, a number of other growth factors have been found to stimulate the growth of these cells. Several other growth factors and their receptors are expressed in the testes, including fibroblast growth factor (FGF), IGF-2, epidermal growth factor (EGF), transforming growth factor α (TGF-α), and the transforming growth factor β (TGF-β) superfamily, which includes TGF-β, activins and inhibins, which seem to have a relevant impact on SC proliferation *in vitro* [120-124]. The transforming growth factor β (TGF-β) superfamily is defined as a group of over 40 ligands. The members of this family best studied in mammalian reproduction include: the TGF-βs 1–3, the inhibins and activins, the bone morphogenetic proteins (BMPs), the anti-Müllerian hormone, the growth and differentiation factors (GDFs) and the distantly related glial cell line-derived neurotropic factor (GDNF) [125]. TGF-β itself and its receptors are expressed within the testis [Konrad 2006], but its relative importance on SCs proliferation remains to be fully elucidated. Among the TGF-β superfamily members, activin

A seems to be most important regulator of SC proliferation. According to Boitani and co-workers [126], activin A stimulates SC proliferation *in vitro* in a defined phase of postnatal testis development, when the proliferative activity of these cells is approaching its final stage. Activin A seems to maintain SC mitotic activity by an FSH-dependent manner at this stage, working as a fine modulator of FSH action on SC proliferation [126]. Petersen and colleagues [102] also described TGF-α as a potent stimulator of SC proliferation *in vitro* in a dose-dependent manner. However, there is not enough *in vivo* evidence corroborating the biological relevance of these growth factors as regulators of SC proliferation. It appears that all of these growth factors are able to regulate SC proliferation at some extend, contributing to regulate such process, and their redundant effect ensures that SC proliferation does not depend on a single one of them [1]. The conflicting information regarding whether TNF-α stimulates or not the SC proliferation [102, 127] reinforces the necessity of *in vivo* studies. Several growth factors, especially those of TGF-β superfamily, are likely to be involved in the regulation of SC proliferation, and they must be further investigated.

4.2. Inflammatory Cytokines

Inflammatory cytokines produced by macrophages in response to inflammatory stimuli may also be produced by cultured SCs, and appear to be involved in regulating SC proliferation [1, 15]. The cytokines expressed by SCs include interleukin-1 and 6 (IL-1 and IL-6) and tumor necrosis factor α (TNF-α). *In vitro* studies have demonstrated that all of these inflammatory factors alter SC metabolism, *i.e.*, enhancing transferrin production. IL-1, but not IL-6 and TNF-α, which may also enhance lactate production and secretion [1]. In isolated SCs, both IL-1 and TNF-α stimulate the proliferation of immature cells [1]. However, the physiological relevance of these cytokines in such processes remains obscure due the lack of *in vivo* studies. Moreover, no alterations in testicular phenotype of animal models with disrupted IL or TNF receptors were observed, and no testicular pathology associated with it has been described [1].

4.3. Estrogens

The testes of several mammals express aromatase, and thereby produce significant amounts of estrogen [121], which plays an important role in male reproductive function and fertility (as reviewed in Lazari *et al.* [128]). Estrogens exert biological effects by interacting with the classical nuclear estrogen receptors (ESRs) ESR1 and ESR2 (also known as ERa and ERb) and through the non-classical membrane receptor, a G protein-coupled estrogen receptor 1 (GPER) [129, 130]. It was demonstrated by Royer and collaborators [129] that 17β-estradiol may induce the proliferation of 15-day-old cultured SCs. The same study demonstrated that this effect of E2 might occur due the activation of both ER and GPER, as both induce CREB activation *via* stimulation of the MAPK and PI3K signaling pathways. It is important to note that aromatase expression in testicular cells vary throughout the testis development. The mRNA levels for aromatase are higher in SCs from 20-day-old than from 10- or 30-day-old animals, whereas the aromatase transcripts are undetectable in SCs from adult rats. However, in these animals, aromatase can be found in Leydig cells, pachytene spermatocytes and round spermatids [131]. Thus, SCs are the major source of estrogens in immature animals, while Leydig and germ cells synthesize these hormones in adult animals [132]. Taking into account the different pattern of aromatase expression throughout testis development and the association of E2 with SC proliferation [129], the paracrine effects of estrogens must be considered to the full understanding of physiological importance of E2 in all testicular cells' development.

4.4. Thyroid Hormones

It is known that thyroid hormones regulate diverse effects in SCs, such as lactate production, GLUT1 mRNA levels, aromatase activity, SC proliferation and others [133-136]. The high quantities of thyroid hormone receptors present in neonatal SCs indicate that immature SCs may be an important thyroid hormone target [137]. Indeed, the involvement of thyroid hormones in the establishment of the adult SC population have been extensively studied, and the evidences indicate that thyroid hormones can affect SC proliferation both directly and indirectly [138]. Indirectly, triiodothyronine (T3) has been demonstrated to stimulate inhibin production by SCs, which in turn might inhibit FSH production leading to a reduction in SC proliferation [133]. On the other hand, van Haaster and collaborators [134] demonstrated that 6-n-propylthiouracil (PTU)-induced hypothyroidism in rats, retards SC differentiation and prolongs SC proliferation period. Thus, hypothyroidism leads to an increase in the SC number and testis size [134]. However, subsequent studies demonstrated that treatment with T3 directly affects SC proliferation, reducing the SC proliferative activity, as well as the SC proliferation period and the final number of SCs [92, 133, 135]. T3 also stimulates the *in vitro* maturation of SC, thereby indicating that T3 terminates SC proliferation and stimulates the functional maturation of these cells [138]. Finally, Palmero and colleagues [135] demonstrated that T3-dependent effects in cultured SCs are age-dependent, with no significant effects observed after finishing the proliferative period of these cells. Thus, the involvement of the thyroid hormones in SC proliferation process is clear but also an intricate process that requires better elucidation. However, it is clear that the levels of thyroid hormones early in life are important for the terminal differentiation of SCs and, therefore, for determining adult testis size.

4.5. Androgens

During the fetal life, Leydig cells produce high amounts of androgens, especially testosterone. The intratesticular testosterone concentration is high at the end of fetal life, and decreases after birth remaining low until puberty, when it increases again [139, 140]. Androgens are responsible for the development of the male reproductive tract, the formation and maintenance of the BTB and spermatogenesis. Although testosterone is capable of acting through both classical and non-classical mechanisms, most of testosterone effects are associated to the classical mechanism. In the classical mechanism, the androgen diffuses through the plasma membrane and binds to the intracellular androgen receptor (iAR) localized in the cytoplasm or nucleus. Then the hormone-receptor complex formed is translocated to the nucleus where it binds to androgen response elements (AREs), result-

ing in the recruitment of co-activators, and in the regulation of gene expression [141, 142]. The iAR is present in Leydig cells and peritubular cells since fetal life. A slight iAR expression takes place in SCs after postnatal day 5 and becomes progressively stronger during pre-pubertal development. Until recently, it was believed that androgens have little or any effect on SCs proliferation due to the absence of iAR in the beginning of the proliferative phase. However, over the last few decades several studies have been evaluating the involvement of androgen on SCs proliferation.

Orth and co-workers [143] revealed that *in vivo* testosterone treatment decrease [H^3] thymidine incorporation by SC in hemicastrated and intact animals, suggesting an inhibitory effect of testosterone on SCs proliferation. In their work, Buzzard and colleagues [92] showed that testosterone treatment of cultured SCs not only causes a progressive suppression of SCs proliferation but also increases the expression of the cell cycle inhibitors p27Kip1 and p21Cip1 and induces the expression of Gata-1, a marker of SCs differentiation. Thereby contributing to SC maturation process. Subsequent studies have been carried with genetic loss-of-function and gain-of-function animal models. However, the results regarding the SC proliferation in these genetic models are controversial. Works developed using *Tfm* mutant mice [144], which lack functional androgen receptor, and AR Knockout mice (ARKO) [63, 91] show a decrease in SCs number. However, in both approaches it is impossible to establish whether the observed effects result from the lack of androgen action specifically on SCs or on other testicular cells, such as peritubular cells. Nevertheless, the works performed with SC-selective AR knockout mice (SCARKO) did not show any reduction in SCs number [63, 64, 91] or alteration in the maturation markers of SCs [91]. These results suggest an indirect effect of androgens on SCs proliferation. It is known that the several factors secreted by peritubular cells (such as Activin A) acts in a paracrine manner influencing SCs physiology [145-147]. On the other hand, experiments with TgSCAR, a gain-of-function transgenic (Tg) mouse model, show a reduced SC proliferation. Recently, Hazra and collaborators [148] developed the TgSCAR model, which provides premature expression of AR on SCs (SCAR). TgSCAR animals showed strong immunodetection of AR in SC from pnd 5 comparing with the sparse and very weak SC staining observed in WT littermates. The total SC number was significantly reduced in TgSCAR resulting in smaller testis. TgSCAR testis also present premature SC maturation, which is demonstrated by an accelerated formation of the seminiferous lumen and early onset of meiotic phase. In a subsequent study of the same group the TgSCAR mouse model also show an accelerated LC development and a decreased LC number, which are tightly linked to the total SC population [149]. The results from this gain-of-function model suggest that the iAR expression ontogeny on SC is carefully orchestrated in order to protect SC from early maturation.

Besides the androgen classical action it is necessary to take in account the involvement of the non-classical effects of androgens in the regulation of SCs proliferation. The non-classical action of androgens promotes the activation of intracellular signaling pathways, and alter cellular process within seconds to minutes [150]. The androgen non-classical pathway causes the influx of calcium through activation of an unidentified membrane receptor. This membrane receptor activates phospholipase C (PLC) which causes hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP_2) [151]. The lack of PIP_2 reduces negative charges near membranes and produces closing of K^+_{ATP} channels and opening of the voltage-gated calcium channels (VDCC) resulting in calcium influx [151, 152]. The calcium influx stimulated by testosterone may be involved in several physiological processes such as cytoskeleton rearrangement, secretion, gene transcription and even cell proliferation [28].

5. FINAL CONSIDERATIONS

The hormonal regulation of SC proliferation is an intricate process, which involves a vast array of hormones, growth factors and cytokines that modulate the SC division and development in a hormonal and paracrine/autocrine manner. Recent studies performed *in vivo* have made clear that FSH, IGF-1 and insulin are the most important regulators of SC proliferation. However, these hormones alone do not explain the whole process, and others hormones, growth factors, and cytokines have also been demonstrated to be involved in this process, but to a lesser extent. These factors seem to act as potential paracrine and/or autocrine mediators, produced locally within the testis and finely regulating SC proliferation. There are enough evidences to corroborate that several factors, such as activin A and other TGF-β family members, TGF-α, TNF-α, and IL-1, have a contribution to define the final number of SCs. However, little is known about their relative importance in this process in *in vivo* approaches, such as in specific knockout for receptors in SC. Finally, further studies addressing the *in vivo* effects of these regulatory factors are essential for the complete understanding of the SC proliferation process. Also, the precise mechanism of action of these hormones, such as intracellular signaling pathways and their interactions, could give us insights to a better comprehension of hormonal regulation of SC proliferation, as well as possible therapeutic targets for infertility.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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