REPRODUCTION
REVIEW

FSH and testosterone signaling in Sertoli cells

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Abstract

Testosterone and follicle-stimulating hormone (FSH) are required to obtain full reproductive potential. In the testis, somatic Sertoli cells transduce signals from testosterone and FSH into the production of factors that are required by germ cells as they mature into spermatozoa. Recent advances in identifying new signaling pathways that are regulated by FSH and testosterone have allowed for refinement in the understanding of the independent, overlapping and synergistic actions of these hormones. In this review, we discuss the signaling pathways that are regulated by FSH and testosterone as well as the resulting metabolic and gene expression changes that occur as related to Sertoli cell proliferation, differentiation and the support of spermatogenesis.

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Introduction

Male fertility and the process of spermatogenesis are dependent upon the somatic Sertoli cells to produce factors that are required by developing germ cells (reviewed in Sharpe 1994, Griswold 1998). The number of Sertoli cells determines testicular size, germ cell numbers per testis and spermatozoa output (Orth *et al.* 1988). Furthermore, Sertoli cells provide a specialized, protected environment within the seminiferous tubules of the testis for germ cell development. Adjacent Sertoli cells form tight junctions with each other such that nothing larger than 1000 daltons can pass from the outside to the inside of the tubule. This characteristic of Sertoli cells creates what is known as the blood-testis barrier. At the beginning of meiosis, germ cells located outside of the barrier pass through the tight junctions. Once beyond the blood-testis barrier, germ cells are dependent on Sertoli cells to supply nutrients and growth factors (reviewed in Mruk & Cheng 2004). Sertoli cells provide factors required to fuel germ cell metabolism (lactate, transferrin, androgen binding protein), growth regulatory factors (stem cell factor, transforming growth factors alpha and beta (TGF-α and TGF-β), insulin-like growth factor-I (IGF-I), fibroblast growth factor (FGF) and epidermal growth factor (EGF) and hormones that regulate the development of the male reproductive structures or feedback to regulate the hormonal signals affecting Sertoli cells (mullerian-inhibiting substance (MIS), and inhibin) (reviewed in Skinner 2005).

The process of spermatogenesis is regulated by a complex interplay of endocrine and paracrine signals. The master control hormone is gonadotropin releasing hormone (GnRH), a decapeptide produced by specialized neurons in the hypothalamus. Pulsatile GnRH production signals gonadotroph cells in the anterior pituitary to produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that then act on the testis to regulate spermatogenic potential. LH binds to receptors on the surface of Leydig cells in the testis and stimulates the production of testosterone, a steroid hormone that diffuses into the seminiferous tubules. Within the seminiferous tubules only Sertoli cells possess receptors for testosterone and FSH and thus these cells are the major targets of the ultimate hormonal signals that regulate spermatogenesis. In this review, we discuss the molecular mechanisms by which the FSH and testosterone signals that are required to support spermatogenesis are transduced and integrated in Sertoli cells.

FSH signaling mechanisms

FSH binding to the FSH receptor

FSH is a member of the glycoprotein hormone family that includes LH, human chorionic gonadotropin and thyroid-stimulating hormone. These hormones are disulfide-rich heterodimers that share a common α subunit but have unique β subunits that impart hormone specificity. FSH transmits its signals via the 75 kDa FSH receptor (675 amino acids). The FSH receptor is a G protein-coupled receptor that spans the membrane seven times with seven conserved alpha helixes (reviewed in Simoni *et al.* 1997,

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Heckert & Griswold 2002). The gene encoding the FSH receptor consists of 10 exons. The first 9 exons encode the extracellular domain and the last exon encodes the membrane-spanning region. The crystal structure of FSH bound to the hormone-binding domain of the FSH receptor has been solved to reveal that FSH and its receptor interact in a manner that resembles a handclasp. Ten parallel β-strands of the receptor and additional loops just C-terminal to the β stands surround and contact FSH. The receptor wraps around the middle section of the hormone, interacting with C-terminal segments and other loops of both the FSH-α and FSH-β subunits. When FSH interacts with the receptor, the hormone undergoes a series of conformational adjustments and adopts a rigid structure that appears to be required for signaling. As a result of hormone binding to the ectodomain of the receptor, structural changes occur in the seven membrane-spanning domain that illicit guanine nucleotide exchange in associated G_s proteins. There is also evidence that ligand binding causes dimerization of plasma membrane FSH receptors through contacts that are limited to the cytoplasmic domains and that receptor dimerization contributes to signaling (Dias 2005, Fan & Hendrickson 2005).

Sertoli cell ontogeny and FSH signaling

During the last four days of gestation (fetal days 17.5–21.5), plasma FSH levels in rats increase nearly 2-fold to approximately 250 ng/ml at birth and then reach almost 400 ng/ml by 5 days of life. FSH levels in serum transiently peak at about 800 ng/ml by day 35 before reaching steady-state levels of 400 ng/ml in 50 day-old rats (Chowdhury & Steinberger 1976, Ketelslegers *et al.* 1978). Low levels of FSH receptor activity in rat gonads can be detected after 14.5 days of gestation but FSH binding activity increases about four-fold over fetal days 19.5 to 21.5 (Warren *et al.* 1984). This increase in FSH receptors is concurrent with increased Sertoli cell proliferation. It is not clear whether the proliferation is dependent upon increased FSH receptor levels or whether the increased number of Sertoli cells accounts for the elevated FSH

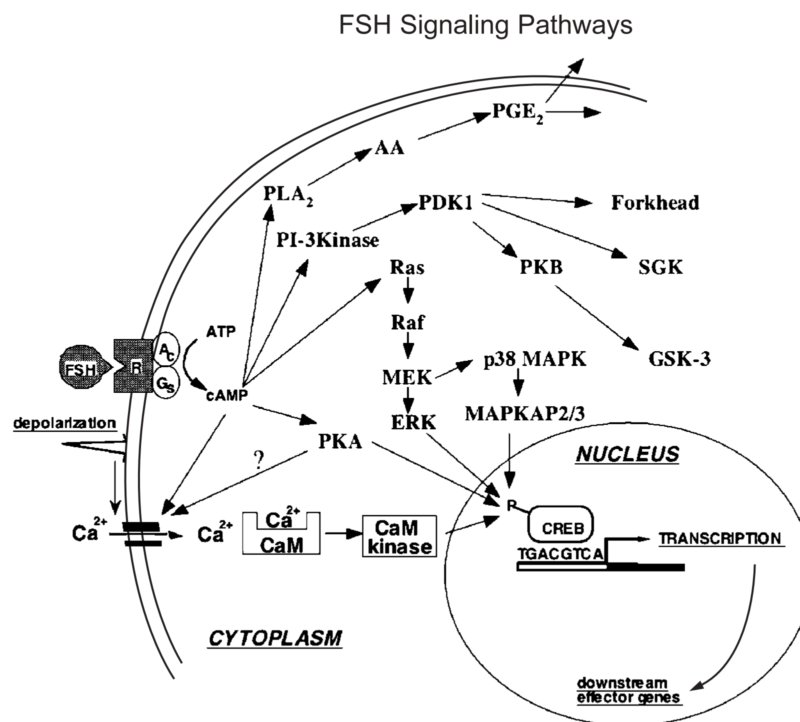

Figure 1 Signaling pathways activated by FSH are displayed. Initially FSH binding to the FSH receptor causes receptor coupled G proteins to activate adenylate cyclase (AC) and increase intracellular cAMP levels. Multiple factors can be activated by cAMP in Sertoli cells including PKA that can phosphorylate a number of proteins in the cell and also regulate the expression and activity of numerous transcription factors including CREB. FSH also causes Ca^{2+} influx into Sertoli cells that is mediated by cAMP and perhaps PKA modification of surface Ca^{2+} channels. Depolarization of the cell is also involved in Ca^{2+} influx. Elevated Ca^{2+} levels can activate calmodulin and CaM kinases that have multiple potential downstream effects including the phosphorylation of CREB. During puberty, FSH activates the MAP kinase cascade and ERK kinase in Sertoli cells most likely via cAMP interactions with guanine nucleotide exchange factors (GEFs) and activation of Ras-like G proteins. ERK is capable of activating transcription factors including SRF, c-jun and CREB. In granulosa cells, FSH also activates the p38 MAP kinase. FSH and cAMP also likely act through GEFs to activate PI3-K and then phosphoinositide dependant protein kinase (PDK1) and PKB in Sertoli cells. Studies of granulosa cells identified Forkhead transcription factor (Forkhead), SGK (glucocorticoid-induced kinase) and GSK-3 (glycogen synthase kinase-3) as additional downstream targets of the PI3-K pathway. FSH also mediates the induction of PLA_2 and the subsequent release of arachadonic acid (AA) and the activation of eicosanoids such as PGE_2 that may act as intracellular or extracellular signaling agents.

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binding activity that is detected. Ketelslegers and colleagues assayed FSH binding activity in testis extracts after birth and determined that by 2 days of life FSH receptors are approximately 40 fmol/testis (Ketelslegers *et al.* 1978). FSH receptor numbers per testis then continually increase up to a plateau of 1000 fmol/testis 60 days after birth. Studies by Bortolussi *et al.* resulted in similar trends but receptor concentrations were found to be 3 to 10-fold lower (Bortolussi *et al.* 1990). Both groups observed that after birth the concentration of receptors in the testis increases about 3-fold, peaking at 15 days after birth after which receptor levels fall in the adult to levels approximately that of the 2 day-old Sertoli cell. These changes in concentration reflect the increase in FSH receptors due to Sertoli cell proliferation 2 to 15 days after birth and the subsequent dilution of Sertoli cells due to the expansion of germ cells (Orth 1984, Meachem *et al.* 1996). The number of FSH receptors per Sertoli cell actually remain relatively constant from 2 to 21 days after birth (1500–1900 receptors/cell) but increase by another 2-fold each by 40 days and again by 60 days after birth (Bortolussi *et al.* 1990).

Cyclical regulation of the FSH receptor in the adult

In the adult rat seminiferous tubules, spermatogenesis occurs in a cyclical fashion such that, within any one region of the tubule, spermatogonial stem cells divide approximately every 12 days. As a result, there are regular, defined associations of germ cells that develop and are replenished together in any one region. Each step of the development of the associated germ cells can be divided into stages that have defined physiological characteristics and cell associations (cell association stages I–XIV in rats). One cyclical characteristic that has been characterized is FSH-mediated production of cAMP in Sertoli cells. In the adult rat and human, serum FSH levels remain relatively constant. However, the expression of FSH receptors on Sertoli cells varies more than three-fold in a cyclical and stage-specific manner such that receptor levels are highest in stages XIII–II and minimal during stages VII–VIII (Heckert & Griswold 1991). The levels of FSH-induced cAMP production in Sertoli cells closely follows the levels of FSH receptor with highest levels of cAMP observed in stages XIV–VI (Kangasniemi *et al.* 1990).

FSH and intracellular cAMP are important regulators of FSH receptor levels in cultured Sertoli cells and *in vivo*. FSH stimulation results in a transient down-regulation of FSH receptor expression 4 to 8 h after addition of the hormone. By 16 h, receptor mRNAs return to control values (Themmen *et al.* 1991, Maguire *et al.* 1997). The levels of FSH receptor are determined primarily by the activity of the FSH receptor gene promoter region. A major FSH-regulated element within the rat FSH receptor promoter is a regulatory motif matching the defined core sequence CANNTG called an E-box that is located 23 bp upstream of the transcription start site. This E-box was shown to bind the helix-loop-helix transcription factors USF-1 and USF-2 (upstream stimulatory factor 1 and 2); activators of genes that support differentiation (Heckert *et al.* 1998). Interestingly, FSH and cAMP were recently shown to induce the expression of the Id2 gene, a member of the inhibitor of differentiation family of gene regulators that repress E-box mediated transcription (Scobey *et al.* 2004). Overexpression of another Id protein (Id1) was shown to decrease FSH receptor promoter activity by up to 50% (Goetz *et al.* 1996). It remains to be determined whether FSH-mediated induction of Id proteins is responsible for the cyclical down-regulation of the FSH receptor gene.

FSH signal transduction pathways

FSH binding to its receptor is known to activate at least 5 signaling pathways in Sertoli cells (Fig. 1). These pathways are discussed below.

cAMP-PKA pathway

In the first FSH-regulated pathway to be identified, the binding of FSH to its receptor catalyzes the exchange of GDP for GTP that leads to the disassociation of G_α and $G_{\beta\gamma}$. The GTP bound form of the G_α protein stimulates adenylate cyclase resulting in the production of cAMP (Zhang *et al.* 1991). Increased intracellular cAMP concentrations release the catalytic subunit of protein kinase A (PKA) from repressor subunits allowing phosphorylation of numerous cellular proteins. One target for the increase in cAMP and PKA is a class of transcription factors that bind to cAMP response elements (CREs). Specifically, the CRE binding protein (CREB) transcription factor is rapidly activated after being phosphorylated on serine 133 by PKA in response to FSH stimulation (Walker *et al.* 1995). The importance of CREB phosphorylation for fertility was identified using *in vivo* studies in which a mutant CREB isoform that could not be phosphorylated was over expressed exclusively in Sertoli cells in rat testes. Testes receiving the mutant CREB displayed disrupted spermatogenesis in greater than 40% of the seminiferous tubules due to the apoptosis of spermatocytes and subsequent loss of more than 75% of spermatids (Scobey *et al.* 2001).

Once phosphorylated, CREB is able to activate transcription from the promoters of numerous genes. Two examples include the CREB gene promoter and an internal promoter within the highly homologous CREM gene that results in production of the inducible cAMP early repressor (ICER) repressor of CRE-mediated gene transcription (Molina *et al.* 1993, Walker *et al.* 1995). After being induced by CREB, ICER represses transcription from its own promoter and the CREB promoter (Molina *et al.* 1993, Walker *et al.* 1998). Transient down-regulation of CREB due to the expression of ICER prior to the auto-inhibition of ICER may explain the oscillating cAMP-mediated

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CREB mRNA expression that was observed in Sertoli cells during the spermatogenic cycle (Walker & Habener 1996). FSH has also been implicated in the expression of various isoforms of CREM that are expressed in spermatoctyes and spermatid germ cells and are required for survival (Foulkes *et al.* 1993, Nantel *et al.* 1996). However, this regulation of germ cell CREM by FSH must be indirectly mediated through as yet unidentified Sertoli cell factors.

MAP kinase pathway

The MAP kinase cascade and ERK kinases are activated by FSH in cultured rat Sertoli cells following the dual coupling of the FSH receptor to G_s and G_i heterotrimeric proteins. The activation of ERK kinase is dependent on PKA and Src kinases although the exact signaling pathway has not been characterized. FSH activates ERK kinase in Sertoli cells cultured from 5 and 11 day-old rats but not 19 day-old rats (Crepieux *et al.* 2001). Thus, this FSH-regulated pathway may not play a large role in maintaining spermatogenesis or other processes in adult rats. However, it is significant that the timing of FSH-mediated activation of the MAP kinase cascade is limited to the period of Sertoli cell proliferation that occurs in the first 15 days after birth because FSH stimulates Sertoli cell proliferation via this pathway. The FSH and ERK-dependent induction of cyclin D1 and E2F, two promoters of entry into the cell-cycle, also suggests that mitogenic effects of FSH are at least partly mediated by the MAP kinase cascade during puberty (Crepieux *et al.* 2001).

In contrast to cultured cells, Sertoli cells from 5 day-old rats placed into suspension had the opposite response to FSH. Under these conditions the gonadotropin repressed ERK activity via a cAMP dependent but PKA independent mechanism that required the activation of a tyrosine phosphatase (Crepieux *et al.* 2002). One clue toward understanding this mechanism for FSH-mediated inhibition of ERK activity in Sertoli cells has been provided by studies of granulosa cells that identified a 100 kDa tyrosine phosphatase that binds to and maintains lower basal levels of ERK kinase activity. In granulosa cells, ERK kinase activity is only activated after FSH-mediated phosphorylation of the phosphatase releases it from ERK (Cottom *et al.* 2003). It is possible that a similar tyrosine phosphatase may be utilized in Sertoli cells to limit the activation of ERK and downstream factors. Studies of granulosa cells also identified another MAP kinase, p38 kinase, as being induced by FSH, thus raising the possibility of p38 kinase regulation by FSH in Sertoli cells (Maizels *et al.* 1998).

Calcium pathway

FSH (10–1000 ng/ml) causes an increase in intracellular Ca^{2+} within seconds of stimulation (Grasso & Reichert 1989, Gorczynska & Handelsman 1991, Sharma *et al.* 1994, Lalevee *et al.* 1997). In freshly isolated Sertoli cells in suspension, FSH causes an influx of Ca^{2+} through the plasma membrane via both voltage gated and voltage independent calcium channels (Gorczynska & Handelsman 1991). Sertoli cells in culture for two days respond to FSH with Ca^{2+} release from intracellular stores that then cause Ca^{2+} influx due to calcium release-activated current (Lalevee *et al.* 1997). In both cases it is thought that FSH-mediated elevation of intracellular Ca^{2+} is predominately due to increases in cAMP, and that PKA may contribute to alter channel activity (Gorczynska *et al.* 1994, Sharma *et al.* 1994, Lalevee *et al.* 1997). One result of increasing intracellular Ca^{2+} is the activation of calmodulin and CaM kinases that may affect cytoskeletal structure of Sertoli cells and phosphorylation of transcription factors including CREB (Spruill *et al.* 1983, Franchi & Camatini 1985, Wu *et al.* 2001). In addition, Ca^{2+} likely plays an important role in Sertoli–Sertoli junction dynamics, although most studies showing the importance of Ca^{2+} in tight junction dynamics have been performed in other cell types (Franchi & Camatini 1985, Mruk & Cheng 2004).

Phosphatidylinositol 3-kinase (PI3-K) pathway

FSH was first found in granulosa cells to activate phosphatidylinositol 3-kinase (PI3-K) that generates specific inositol phospholipids that are bound by and activate protein kinase B encoded by the akt gene (PKB/akt) (Gonzalez-Robayna *et al.* 2000). More recently, FSH was also found to activate PKB in a PI3-K-dependent manner in Sertoli cells (Meroni *et al.* 2002). The most favored mechanism for PI3-K activation is via FSH-mediated increases in cAMP levels. Using inhibitors of PI3-K, it was shown that FSH acting through PI3-K contributes to metabolic processes required to support germ cells. Specifically, PI3-K inhibitors reduced the ability of FSH to induce the activity of lactate dehydrogenase (LDH) required to produce lactate for germ cells and γ-glutamyl transpeptidase (γ-GTP) a transporter of amino acids across the plasma membrane. Also dependent on PI3-K is the uptake of glucose that is converted to lactate for the germ cell energy needs and transferrin secretion that is vital for maintenance of spermatogenesis (Meroni *et al.* 2002). The seven membrane-spanning domain of the FSH receptor has been found to interact with the protein APPL (adapter protein containing PH domain, PTB domain and leucine zipper motif) that in turn interacts with the p110α catalytic subunit of PI3-K and with inactive PKB/akt (Nechamen *et al.* 2004). This juxtapositioning of signaling factors likely potentiates FSH activation of PI3-K mediated events.

Phospholipase A_2 (PLA_2) pathway

FSH through the activation of phospholipase A_2 (PLA_2) leads to the release of the arachadonic acid second messenger and its subsequent metabolism to prostoglandin E_2

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and other eicosanoids that function as intracellular and extracellular signals. As a result, adenylate cyclase activity and androgen aromatization are stimulated in Sertoli cells and germ cells may be affected via their G-protein coupled eicosanoid receptors (Jannini *et al.* 1994). Further studies will be required to better characterize the downstream factors that are affected by FSH activation of the PLA_2 pathway.

Because FSH can activate at least 5 signaling pathways in Sertoli cells it might be expected that some crosstalk occurs to organize the final desired cellular consequences. Although potential crosstalk has not been extensively studied, it has been proposed that oligomerization of FSH receptors may provide a mechanism bringing together the components of multiple signaling pathways (PKA, PI3-K, PKB/akt and PLA_2) to refine and control intracellular signaling (Nechamen *et al.* 2004). It should be noted that much of the valuable progress made toward characterizing FSH signaling has been performed with immature Sertoli cells. Further work will be necessary using fully differentiated Sertoli cells to understand the activation of these pathways and their roles in supporting spermatogenesis in adults.

Desensitization of the FSH receptor

Prolonged exposure of target cells to FSH results in a decreased response with time (desensitization). For example, a two-hour pretreatment with FSH reduces a subsequent FSH-mediated induction of cAMP production by 70% or more (Verhoeven *et al.* 1980, Troispoux *et al.* 1999). Desensitization can initiate within minutes of FSH binding to the FSH receptor. In this process, the receptor is phosphorylated on the first and third intracellular loops by G protein-coupled receptor kinases that allows arrestin proteins to bind to the intracellular domain of the receptor, thereby uncoupling the receptors from G proteins and preventing further signal transduction. Arrestins also facilitate internalization of receptors by interacting with clathrin and concentrating receptors in clathrin coated pits (Nakamura *et al.* 1998*a*, Nakamura *et al.* 1998*b*, Troispoux *et al.* 1999, Marion *et al.* 2002). With longer term exposure to FSH, Sertoli cells respond by up-regulating phosphodiesterase activity to decrease cAMP levels (Conti *et al.* 1983) and by down-regulating FSH receptor expression (Themmen *et al.* 1991). When viewed together, the FSH-mediated activation of phosphodiesterase, the desensitization of the FSH receptor and down-regulation of the receptor as well as the stage-specific control over FSH receptor levels suggests that limiting the timing and duration of FSH signaling is important for Sertoli cell function. In support of this idea, a mouse transgenic model of constitutive FSH action displays elevated testosterone levels and is infertile (Kumar *et al.* 1999).

Regulation of gene expression by FSH

The multi-pronged signaling actions of FSH gene expression provide for extensive alterations in the expression of genes in Sertoli cells due to the activation of a number of transcription factors. In addition to the CRE binding factors mentioned previously, FSH stimulation results in translocation of NF-κB to the nucleus due to the release of NF-κB from its cytoplasmic anchoring partner IκB, most likely due to PKA phosphoylating IκB and marking it for degradation (Shirakawa & Mizel 1989, Ghosh & Baltimore 1990, Delfino & Walker 1998). FSH rapidly and transiently induces AP1 activity by stimulating the transcription of the AP1 components c-fos and jun-B while inhibiting c-jun (Hamil *et al.* 1994). The androgen receptor (AR) is also induced by FSH, thus FSH regulates the androgen responsiveness of Sertoli cells (Verhoven & Cailleau 1988, Blok *et al.* 1989, Sanborn *et al.* 1991, Blok *et al.* 1992). In granulosa cells, FSH induces LRH-1 (the orphan nuclear receptor liver homologue 1) (Saxena *et al.* 2004), and HIF-1 (hypoxia inducible factor 1) via the PI3-K pathway (Alam *et al.* 2004). The potential significance of FSH regulation of LRH-1 and HIF-1 has not yet been investigated in Sertoli cells.

A series of independent studies have identified FSH-inducible genes in Sertoli cells that have direct effects in supporting spermatogenesis. These genes include the FSH receptor (Maguire *et al.* 1997), double sex-and-mab 3 related transcription factor (Dmrt), which has been implicated in sex determination and testis differentiation (Chen & Heckert 2001), transferrin, which is required to transport iron to germ cells (Suire *et al.* 1995, Chaudhary & Skinner 1999), androgen binding protein which may play a role in regulating androgen activity in the testis (Morris *et al.* 1988) as well as vascular endothelial growth factor (VEGF) (McLean *et al.* 2002) that may act on receptors present on germ cells from the spermatogonia to round spermatid stages of development (Nalbandian *et al.* 2003). FSH also up-regulates GDNF (glial cell line derived neurotrophic factor), a member of the TGF-β superfamily that regulates the proliferation of germinal stem cells (Tadokoro *et al.* 2002). Additional genes known to be regulated by FSH-inducible cAMP and CREB include lactate dehydrogenase (LDH-A), that controls the synthesis of the major fuel source for germ cells (Short *et al.* 1994), stem cell factor (kit ligand) that potentiates the survival and expansion of spermatogonia (Taylor *et al.* 1996), as well as aromatase (Schteingart *et al.* 1995), plasminogen activator (Nargolwalla *et al.* 1990) and insulin like growth factor (IGF-I) (Suwanichkul *et al.* 1993). Recent microarray analyses suggest that at least 300 genes in Sertoli cells are up or down-regulated by FSH (McLean *et al.* 2002, Sadate-Ngatchou *et al.* 2004*a*). Remarkably, the microarray studies identified few new FSH-regulated genes that have known or potential direct influences on germ cell survival or

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development. However, the relative lack of information provided related to spermatogenesis may be due to the less differentiated status of the Sertoli cells used for the studies that were isolated from immature 20 day-old rats or adult hpg mice that both have few germ cells beyond the spermatocyte stage. Further gene expression studies employing mature Sertoli cells will be useful to better understand the results of FSH signaling.

Testosterone signaling

Testosterone levels and AR expression during Sertoli cell development

In contrast to FSH, it is well established that androgen is absolutely essential for the maintenance of spermatogenesis (reviewed in Sharpe 1994, McLachlan *et al.* 2002). Although dihydrotestosterone (DHT) is crucial for the development of the male reproductive tract, testosterone is the androgen in the testis that regulates spermatogenesis. In fetal male rats, serum testosterone levels are elevated beginning 3 to 4 days prior to birth and remain high (0.5 ng/ml) until 8 days after birth. Testosterone concentrations progressively decrease to about 0.2 ng/ml during post-natal days 8 to 24. From days 30 to 55 testosterone levels rise to stable adult levels (1–2 ng/ml) (Ketelslegers *et al.* 1978). Adult concentrations of intratesticular testosterone in the rat are approximately 50 to 100-fold higher than that found in serum. Although the physiological advantages of elevated testosterone levels in the testis are not understood, the higher testicular levels of testosterone are important because full spermatogenic capacity requires 70 ng/ml and spermatogenesis is dramatically compromised at testosterone concentrations below 20 ng/ml (Zirkin *et al.* 1989).

In the testis, only Leydig cells, peritubular cells and Sertoli cells express AR. No AR is expressed in germ cells of the mature testis (Lyon *et al.* 1975). In rat Sertoli cells, the levels of AR are low or below detection until 5 to 10 days after birth but then increase up to 35 or 60 days of age (Buzek & Sanborn 1988, Bremner *et al.* 1994, Zhou *et al.* 1996). Adult AR levels increase and decrease in a cyclical fashion. Specifically, analyses of *in situ* hybridization and immunocytochemistry results revealed that AR levels progressively increase during cell association stages II through VII of the spermatogenic cycle and then decline sharply

Table 1 Androgen-regulated genes. Three classes of androgen-responsive genes are listed: Androgen-regulated genes in which there is no proof of AR-DNA interactions, Androgen-regulated genes with known AR-DNA interactions and Androgen-regulated genes with known AR-DNA interactions that are expressed in Sertoli cells. The transcriptional response to androgen, activation (A) or repression (R) is noted.

Gene	Antrogen response	Reference
Antrogen-regulated genes		
HMAK	A	Xia *et al.* 2002
SPAK	A	Qi *et al.* 2001
FSH-β	A	Spady *et al.* 2004
Fibroblast growth factor 2[1]	A	Rosini *et al.* 2002
CDK2, CDK4	A	Lu *et al.* 1997
p16	R	Lu *et al.* 1997
p27	A	Chen *et al.* 1996
AlbZIP	A	Qi *et al.* 2002
NKX3.1	A	He *et al.* 1997
PART-1	A	Lin *et al.* 2000
Prostase	A	Nelson *et al.* 1999
Prostein	A	Xu *et al.* 2001
Fatty acid synthase	A	Swinnen *et al.* 1997
c-myc	A	Lim *et al.* 1994
Antrogen-regulated genes with known AR-DNA interactions		
Prostate specific antigen (PSA)	A	Luke & Coffey 1994, Sun *et al.* 1997
Kallikrein 2 (KLK2)	A	Sun *et al.* 1997, Mitchell *et al.* 2000
Probasin	A	Rennie *et al.* 1993, Claessens *et al.* 2001, Zhang *et al.* 2004
Tyrosine aminotransferase	A	Denison *et al.* 1989
p21	A	Lu *et al.* 1999
Neutral endopeptidase 24.11 (NEP)	A	Shen *et al.* 2000
Sex-limited protein (Slp)	A	Verrijdt *et al.* 2000
Ventral prostate C3[2]	A	Tan *et al.* 1992, Claessens *et al.* 1993
Androgen receptor[3]	A	Grad *et al.* 1999
Glycoprotein hormone α sununit[4]	R	Jorgensen & Nilson 2001
Androgen-regulated genes with known AR-DNA interactions on Sertoli cells		
Pem	A	Lindsey & Wilkinson 1996

[1] FGF2 is activated by AR but no binding data is available.
[2] AR binds to the first intron of C3.
[3] AR binds to AREs in exons of the AR gene.
[4] AR down-regulates not by binding DNA but by interacting with c-jun and AP2 to inhibit their binding to DNA.

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during or immediately after stage VII to become barely detectable in stages IX–XIII (Bremner *et al.* 1994, Vornberger *et al.* 1994, Shan *et al.* 1995). The levels of androgen receptor are highest in stage VII and thus this stage is thought to be the most regulated by and sensitive to testosterone (Kerr *et al.* 1993). From various models in which testosterone is withdrawn from rats, it has been confirmed that in the absence of testosterone, progressive germ cell degeneration begins during stage VII of the spermatogenic cycle (reviewed in Sharpe 1994).

Testosterone signaling: the classical mechanism

The classical mechanism by which androgens and other steroid hormones exert their effects is initiated with the diffusion of the hormone into a target cell through the plasma membrane. The hormones then bind with high affinity to specific intracellular receptor proteins that are present in the cytoplasm and/or nucleus. The binding of the steroid to its receptor produces conformational changes that result in the formation of a "transformed" or activated receptor that has high affinity for specific DNA-binding sites (Tsai & O'Malley 1994). Once the steroid-receptor complex is formed, it acts as a ligand-inducible transcription factor that is able to recruit coactivator proteins and stimulate gene transcription (Bagchi *et al.* 1992). The entire process required to initiate gene expression via this classical mechanism takes at least 30–45 min (Shang *et al.* 2000, Shang *et al.* 2002), and the length of time required to produce significant levels of nascent proteins is in the order of hours.

Testosterone regulation of gene expression

Recently, microarray analyses were performed using testis tissue from hpg mice that lack germ cells but contain the somatic cells expressing AR. Although cell-specific information was not produced, 4, 8 and 12 h after injection these studies identified 56, 129 and 48 genes, respectively, that were regulated by testosterone. Interestingly, within these relatively brief incubation periods, more genes were down-regulated than up-regulated by about a 2:1 ratio. In contrast, of the 234 genes regulated by testosterone after 24 h, approximately 2 genes were up-regulated for every down-regulated gene. As was the case for FSH regulated genes, microarray analysis identified few genes that had known or potential direct roles in regulating spermatogenesis (Sadate-Ngatchou *et al.* 2004*b*).

Although many genes can be regulated by androgens, relatively few are known to be regulated by androgen receptor binding to androgen response elements in promoters and fewer yet have been proved to be regulated by this classical mechanism in Sertoli cells (Table 1). In fact, only the gene that encodes the Pem transcription factor is known to be induced by AR-DNA interactions in Sertoli cells (Lindsey & Wilkinson 1996). Thus, based on this limited knowledge of testosterone-regulation of gene expression in Sertoli cells, it is not clear how testosterone acts via gene regulatory mechanisms to support spermatogenesis (see 'The testosterone paradox' below).

Testosterone elevates intracellular Ca^{2+} levels in Sertoli cells

In addition to known classical actions of testosterone in mediating AR-regulated transcription, androgen (but not other steroids) elevates intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in freshly isolated, suspension cultures of Sertoli cells within seconds of stimulation (Gorczynska & Handelsman 1995, Lyng *et al.* 2000). In Sertoli cells, testosterone-mediated elevation of $[Ca^{2+}]_i$ required the influx of extracellular Ca^{2+}, suggesting that calcium channels in the plasma membrane play a role in testosterone-calcium signaling (Lyng *et al.* 2000). Sertoli cells express at least 4 types of voltage sensitive calcium channels (VSCC) in the plasma membrane (L, N, P/Q and T types) (D'Agostino *et al.* 1992, Fragale *et al.* 2000). However, L-type channels have been implicated as the major conveyers of testosterone-induced Ca^{2+} into Sertoli cells. (Lyng *et al.* 2000). Testosterone linked to BSA, which is unable to pass through the plasma membrane, also induced Ca^{2+} influx suggesting that testosterone actions may be mediated directly at the membrane (Gorczynska & Handelsman 1995). Unlike FSH that elevates intracellular Ca^{2+} via a cAMP-dependent mechanism, testosterone does not increase cAMP levels in Sertoli cells (Gorczynska & Handelsman 1995, Fix *et al.* 2004). Even though the signaling pathways resulting in elevated $[Ca^{2+}]_i$ would appear to be different, FSH and testosterone effects on intracellular Ca^{2+} are not additive (Gorczynska & Handelsman 1995). This result suggests that FSH and testosterone may act on similar Ca^{2+} channels.

It has been proposed that Ca^{2+}-dependent actions of androgen in Sertoli cells involve the closing of an ATP-dependent K^+ channel because testosterone-mediated increases in $[Ca^{2+}]_i$ were nullified by the K^+ channel agonist diazoxide (Silva *et al.* 2002). This finding raises the possibility that depolarization of Sertoli cells due to testosterone closing of K^+-ATP channels is the mechanism that causes Ca^{2+} uptake through L-type voltage dependent Ca^{2+} channels. An alternative possibility is that androgens may open K^+ channels in Sertoli cells as testosterone and DHT reportedly contribute to vasodilation by opening a large conductance, calcium- and voltage-activated K^+ channel in coronary myocytes (Deenadayalu *et al.* 2001).

The testosterone paradox

Testosterone actions present an interesting paradox in that numerous genes and proteins are up-regulated in response to stimulation but few genes have been characterized that are known to be induced with this steroid through the classical mechanism of AR binding to specific promoter elements. At least two observations support the hypothesis

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that testosterone may act through alternative mechanisms to complement classical AR actions in Sertoli cells. First, Sertoli cells require testicular testosterone levels greater than 70 nM to support full spermatogenesis even though testosterone binding to AR and gene expression responses to testosterone are saturated at 1 nM (Rommerts 1988, Zirkin *et al.* 1989, Veldscholte *et al.* 1992, Sharpe 1994). Second, $[Ca^{2+}]_i$ levels are elevated in primary Sertoli cells within seconds of androgen stimulation and thus cannot be dependent on AR-DNA interactions and initiation of gene expression (Steinsapir *et al.* 1991, Gorczynska & Handelsman 1995, Lyng *et al.* 2000). Together, these observations suggest that testosterone acts in Sertoli cells through pathways in addition to classical mechanisms to regulate spermatogenesis.

Testosterone activates MAP kinase and the CREB transcription factor in Sertoli cells

Increasing evidence has been gathered demonstrating that androgen can directly activate cellular signaling pathways by nongenomic pathways that are independent of AR binding to DNA (reviewed in Cato *et al.* 2002, Heinlein & Chang 2002, Silva *et al.* 2002). Particularly relevant is the activation of the MAP kinase pathway by androgens. In other cell types, androgen can induce a number of factors that have been implicated in regulating the MAP kinase cascade including PKA, calmodulin, phospholipase C, protein kinase C and guanine nucleotide exchange factors (GEFs) (Finkbeiner & Greenberg 1996). All these factors have been found to be capable of initiating the MAP kinase cascade by stimulating a Ras or a Ras-like protein to activate a Raf MAP kinase kinase kinase (MAPKKK) (Pearson *et al.* 2001).

Testosterone-mediated activation of MAP kinase was recently characterized in Sertoli cells. Physiological levels of testosterone (10–250nM), but not estradiol or progesterone, rapidly (within 15 min) induced the phosphorylation and activation of the Erk MAPK and CREB in Sertoli cells from 15 day-old rats. CREB phosphorylation increased about 3-fold within 1 to 15 min and 5-fold 1 to 2 hrs after stimulation with the non-hydrolizable androgen agonist R1881. Erk phosphorylation followed similar kinetics and the MAP kinase pathway inhibitor PD98059 blocked testosterone-mediated induction of CREB phosphorylation thus supporting the hypothesis that androgen-induced CREB phosphorylation is mediated via the MAP kinase pathway. Further studies have been performed demonstrating that testosterone activates Src kinase in Sertoli cells (Cheng J and Walker WH, unpublished results). A scaffold protein called modulator of nongenomic actions of the estrogen receptor (MNAR) was shown to facilitate the activation of Src kinases and MAPK by androgen as MNAR also forms a complex with AR and Src (Wong *et al.* 2002, Unni *et al.* 2004). One result of testosterone-mediated stimulation of the MAP kinase cascasde and CREB was the induction of CREB-mediated transcription. Specifically, testosterone was found to induce the expression of three CREB-regulated genes in Sertoli cells (Fix *et al.* 2004).

Although other receptors may be involved, three lines of evidence support the hypothesis that AR mediates testosterone-induced Erk and CREB phosphorylation. First, the AR antagonist flutamide inhibited testosterone-mediated phosphorylation of CREB. Second, CREB was not phosphorylated in Sertoli cells lacking AR activity after RNA interference knockdown of AR expression.

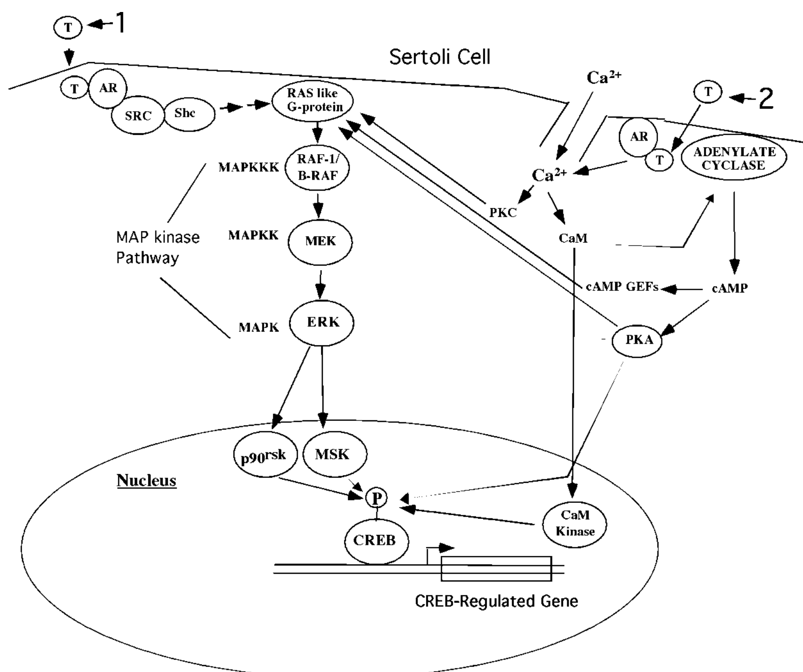

Figure 2 Potential testosterone signaling pathways in Sertoli cells: Two potential pathways are proposed for testosterone-induced CREB phosphorylation. In one pathway (left side, 1), testosterone (T) binding to AR allows AR to bind with and activate Src tyrosine kinase (SRC) resulting in the stimulation Ras and Raf-1 kinase and the activation of the MAP kinase pathway. In the second pathway (right side, 2), testosterone induces Ca^{2+} influx into Sertoli cells that then may cause calmodulin (CaM) to stimulate CaM kinase to translocate to the nucleus and transiently phosphorylate CREB within 1 minute. Ca^{2+} may also stimulate a slower, more persistent pathway in which protein kinase C (PKC), guanine nucleotide exchange factors (GEFs) or PKA stimulate Ras or a Ras like GTP binding protein resulting in the activation of the MAP kinase pathway. Both pathways are capable of inducing CREB phosphorylation and CREB-mediated gene expression.

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Third, testosterone could not increase CREB phosphorylation in Sertoli cells containing the testicular feminization (tfm) AR mutant that displays dramatically decreased androgen binding capacity (Fix *et al.* 2004).

Two models were devised to explain how testosterone may activate MAP kinase, CREB phosphorylation and CREB-mediated transcription in Sertoli cells (Fig. 2). In the first pathway, testosterone binds to AR causing the recruitment and activation of Src, thereby initiating a series of events leading to the activation of the MAP kinase cascade by the Ras G-protein. In the second pathway, testosterone binding to AR causes an increase in $[Ca^{2+}]_i$ that activates numerous potential intermediates that are capable of stimulating Ras or a Ras like G-protein. Results from studies of nonclassical estrogen actions raise the possibility that additional factors may be involved in transmission of androgen signals. Specifically, estrogen-estrogen receptor interactions trigger G protein dependent activation of Src that then stimulates matrix metalloproteases to release heparin bound epidermal growth factor (HB-EGF) from the surface of the cell. The HB-EGF then binds to the EGF receptor resulting in the activation of the MAP kinase cascade (Razandi *et al.* 2003).

Regardless of the exact pathway for the nonclassical regulation by testosterone, this newly discovered process is particularly relevant for providing an answer to the paradox of how testosterone can support spermatogenesis yet regulate few genes via AR-promoter interactions. The androgen-induced increases in Src kinase and the MAP kinase cascade that have been characterized thus far have the potential to regulate the expression of many more genes than is possible by direct AR-promoter interactions. Thus, it is likely that nonclassical actions of testosterone in Sertoli cells will be found to be a necessary compliment to the classical actions that are required to maintain spermatogenesis. Determining the factors that are required for testosterone induction of MAP kinase and CREB activity in Sertoli cells will be essential to better understand testosterone regulation of spermatogenesis and androgen regulation of other target tissues.

Integration of the FSH and testosterone signaling pathways

FSH and testosterone both initiate signaling pathways that contribute to the support of spermatogenesis and the hormones share some common characteristics. For example, FSH or testosterone can independently support Sertoli cell proliferation although FSH is apparently more effective (Griswold *et al.* 1977, Meachem *et al.* 1996, Haywood *et al.* 2003, Allan *et al.* 2004, Johnston *et al.* 2004). Stimulation of Sertoli cell proliferation may be due to the shared ability of FSH and testosterone to activate the MAP kinase pathway in pubertal Sertoli cells. FSH and testosterone induce phosphorylation of ERK kinase to similar levels but FSH is about 2-fold more effective in phosphorylating CREB (Fix *et al.* 2004), suggesting that other factors in addition to the MAP kinase pathway may be responsible for more efficient FSH-mediated Sertoli cell proliferation. Because phoshorylated CREB in Sertoli cells was shown to be essential for spermatocyte survival (Scobey *et al.* 2001), the less efficient phosphorylation of CREB in the presence of testosterone alone may be one contributing factor responsible for the reduced fertility observed with androgens in the absence of FSH. For full fertility, it may also be necessary for CREB to be phosphorylated during specific stages of the spermatogenetic cycle, a condition that may be better controlled with the cyclical up-regulation of the FSH receptor and cAMP production (Kangasniemi *et al.* 1990). FSH was only found to activate MAP kinase in pubertal rat Sertoli cells 5 and 11 days after birth but not in 19 day-old rats. Thus far, testosterone activation of the MAP kinase pathway has only been tested in Sertoli cells isolated from 15 day-old rats. It remains to be determined whether testosterone can act via MAP kinase to support CREB activation and spermatogenesis in adult rats.

Another characteristic that is shared by FSH and testosterone is the elevation of $[Ca^{2+}]_i$ levels in Sertoli cells, although the mechanisms by which elevated $[Ca^{2+}]_i$ might support spermatogenesis are not yet known. Together, the shared signals that FSH and testosterone transmit likely explain the redundant functions that these hormones display in supporting spermatogenesis.

There are also differences in the actions of FSH and testosterone. These differences may account for the fact that testosterone but not FSH is capable of maintaining spermatogenesis independently. One difference is that testosterone does not up-regulate cAMP production in Sertoli cells. These findings suggest that either elevated cAMP levels are not required for spermatogenesis or that redundant mechanisms are present. Other possible differences between FSH and testosterone include the regulation of the PI3-K and PLA_2 pathways. FSH has been shown to stimulate the PI3-K and PLA_2 signaling pathways but thus far there is no evidence that testosterone affects these signaling mechanisms in Sertoli cells. However, other steroid hormones have been shown to activate the PI3-K and PLA_2 pathways in other systems by non-classical mechanisms (Bagowski *et al.* 2001, Honda *et al.* 2001, Fiorini *et al.* 2003). In the future, it is possible that testosterone may be found to also regulate the PI3-K and PLA_2 pathways. A final difference between FSH and testosterone is the up and down regulation of gene expression via AR-DNA interactions that can only be accomplished after testosterone stimulation. Perhaps future studies will identify genes uniquely regulated by testosterone-induced AR-DNA interactions that are required to maintain spermatogenesis.

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Conclusions and future perspectives

In recent years, the characterization of the signaling pathways regulated by FSH and testosterone has been an important step toward the understanding of how these hormones support spermatogenesis. For example, with the finding that FSH and testosterone are both capable of activating the MAP kinase pathway and Ca^{2+} influx, it now seems reasonable that some of the actions of FSH and testosterone overlap. Nevertheless, many of the mechanisms by which these hormones support spermatogenesis and the required factors that they regulate remain to be fully characterized, especially for testosterone. It is likely that further study of the nonclassical (nongenomic) testosterone signaling will identify additional testosterone-regulated genes and factors that are required to support spermatogenesis. Also, microarray or similar gene expression studies employing mature Sertoli cells in the context of a normal testis with germ cells present will be important for identifying the FSH and testosterone regulated factors that are required for spermatogenesis. With this information the characterization of the mechanisms by which FSH and testosterone act hopefully will be facilitated.

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