Review

Non-classical actions of testosterone and spermatogenesis

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Testosterone is essential to maintain spermatogenesis and male fertility. In the absence of testosterone stimulation, spermatogenesis does not proceed beyond the meiosis stage. After withdrawal of testosterone, germ cells that have progressed beyond meiosis detach from supporting Sertoli cells and die, whereas mature sperm cannot be released from Sertoli cells resulting in infertility. The classical mechanism of testosterone action in which testosterone activates gene transcription by causing the androgen receptor to translocate to and bind specific DNA regulatory elements does not appear to fully explain testosterone regulation of spermatogenesis. This review discusses two non-classical testosterone signalling pathways in Sertoli cells and their potential effects on spermatogenesis. Specifically, testosterone-mediated activation of phospholipase C and calcium influx into Sertoli cells is described. Also, testosterone activation of Src, EGF receptor and ERK kinases as well as the activation of the CREB transcription factor and CREB-mediated transcription is reviewed. Regulation of germ cell adhesion to Sertoli cells and release of mature sperm from Sertoli cells by kinases regulated by the non-classical testosterone pathway is discussed. The evidence accumulated suggests that classical and non-classical testosterone signalling contribute to the maintenance of spermatogenesis and male fertility.

Keywords: signalling pathways; testis; Sertoli; non-genomic; fertility

1. SPERMATOGENESIS

Spermatogenesis is a multi-step process of germ cell expansion and development which occurs within the seminiferous tubules of the testis that determines male fertility. Spermatogenesis comprises three phases: stem cell renewal and germ cell proliferation, meiosis and differentiation, and spermiogenesis (figure 1*a*). Stem cells located along the basement membrane of the seminiferous tubules divide, resulting in another stem cell and a committed cell called a spermatogonium. The spermatogonia undergo a species-specific number of mitotic divisions, with the final division resulting in differentiated type B spermatogonia. The type B spermatogonia then divide to form preleptotene spermatocytes that detach from the basement membrane as they undergo meiosis to form round spermatids. After undergoing extensive differentiation (spermiogenesis) the differentiated elongated spermatids (now spermatozoa) are released into the tubule lumen (spermiation; reviewed in Sharpe 1994).

Within any one region of the rat seminiferous tubule, the initial spermatogonial mitotic divisions occur once every 12.9 days. The divisions occur progressively earlier or later at positions upstream and

downstream, resulting in a wave of germ cell development down the seminiferous tubule. As a result, there are regular, morphologically defined associations of germ cells that develop and are replenished together in any one region of the tubule. 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). The stages occur in repeating cycles with four and one half cycles of 12.9 days being required to produce mature sperm in the rat (Oakberg 1956; Clermont & Trott 1969).

Developing germ cells are surrounded by somatic Sertoli cells that extend from the basement membrane of the seminiferous tubule to the open lumen. Adjacent Sertoli cells form tight junctions with each other such that nothing larger than 1000 Da can pass from the outside to the inside of the tubule. Together, the tight junctions between all of the Sertoli cells within the tubule form the blood–testis barrier (BTB). At the beginning of meiosis preleptotene spermatocytes ‘pass through’ the BTB. Once the germ cells move beyond the BTB and the BTB reforms behind them, the germ cells no longer have access to serum factors and they become totally dependent upon Sertoli cells to supply nutrients and growth factors (figure 1*b*).

Sertoli cells support of germ cell development is dependent on a complex interplay of endocrine and paracrine inputs. Follicle-stimulating hormone (FSH) and testosterone are the two major endocrine

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Figure 1. (a) The process of spermatogenesis. A type spermatogonia undergo mitotic divisions to become B spermatogonia which then divide and differentiate into preleptotene spermatocytes (PL) that then become leptotene (L), zygotene (Z) and then early mid and late pachytene spermatocytes (EP, MP, LP). After the first meiotic division, secondary spermatocytes are formed (II) that then divide again resulting in haploid round spermatids. The spermatids elongate, shed much of their cytoplasm as residual bodies (RB) and differentiate until mature spermatozoa are formed. (b) The anatomy of the testis. The outline of three seminiferous tubules is shown surrounding the interstitial area containing blood vessels, lymphatic tissue and colonies of Leydig cells. The seminiferous tubules are surrounded by basement membrane and myoid peritubular cells. Spermatogonia are located on the basement membrane. Spermatocytes and spermatids and mature spermatozoa are located apical to the tight junctions between Sertoli cells that make up the blood testis barrier. Sertoli cells extend from the basement membrane to the lumen of the tubule.

signals that act in the testis to regulate spermatogenesis efficiency. FSH acts by binding to the FSH receptor (FSHR), a G-coupled transmembrane receptor on the surface of Sertoli cells that is capable of activating numerous signalling pathways (reviewed in Walker & Cheng 2005). FSH acts synergistically with testosterone to increase spermatogenesis efficiency and fertility (Sharpe 1994; McLachlan *et al.* 1996). Testosterone is essential to maintain spermatogenesis at numerous levels. In the absence of testosterone or the androgen receptor (AR), formation of the BTB is compromised, germ cells are unable to progress beyond meiosis, immature germ cells are prematurely displaced from Sertoli cells and mature sperm cannot be released from Sertoli cells. The disruption of any of these testosterone-dependent steps results in the failure of spermatogenesis and infertility.

Testosterone is produced by Leydig cells present in the interstitial space of the testis between the seminiferous tubules and then diffuses into the tubules. Because testosterone is produced locally by the Leydig cells, the levels of testosterone in men are 25 to 125-fold higher in the testis (340–2000 nM) as compared to the serum (8.7–35 nM). Testosterone levels are similarly elevated in the rat testis. Numerous assays of rat testicular fluid detected testosterone at levels ranging from 200 to 300 nM (Comhaire & Vermeulen 1976; Turner *et al.* 1984; Awoniyi *et al.* 1989; Maddocks *et al.* 1993; Jarow *et al.* 2001). Two-thirds of the intratesticular testosterone is bioavailable in that it is found free or linked to albumin. The remainder of testicular testosterone is more tightly bound to steroid hormone binding protein or androgen binding protein and is not readily available (Hammond *et al.* 1977; Jarow *et al.* 2005). Thus far, the physiological necessity for high levels of testosterone in the testis is not well understood. It is known that spermatogenesis does not proceed in the absence of relatively high levels of testosterone (more than 70 nM in the rodent; Zirkin *et al.* 1989). Also, the levels of testosterone required to maintain spermatogenesis are much greater than the 1–10 nM that is required for regulation of gene expression via AR binding to gene promoters (Tsai & O'Malley 1994), suggesting that alternative mechanisms of testosterone action are possible.

2. REGULATION OF SPERMATOGENESIS BY TESTOSTERONE

The reduction of testicular testosterone levels after hypophysectomy, immunoneutralization of LH, administration of antiandrogens or destruction of Leydig cells results in the detachment of developing spermatids (step 8–19 spermatids) from the Sertoli cell and the halting of spermatogenesis during the process of meiosis (reviewed in Sharpe 1994). Previous studies of mouse models lacking AR expression in all tissues (ARKO mice), or in which specific testicular cell types lack AR, have confirmed that testosterone is required to maintain spermatogenesis. In the absence of AR, spermatogenesis is halted during meiosis such that germ cells developing past the spermatocyte stage are rare (Yeh *et al.* 2002). In mice in which AR

is knocked out selectively in Sertoli cells (SCARKO mice), spermatogenesis rarely progresses beyond the diplotene spermatocyte stage (De Gendt *et al.* 2004; Tsai *et al.* 2006). Furthermore, studies of SCARKO mice have revealed that AR is required to maintain the integrity of junctional complexes making up the BTB (Meng *et al.* 2005; Wang *et al.* 2006a). Replacement of AR in Sertoli cells with a hypomorph gene having partial AR activity revealed that the progression of round spermatids to elongating spermatids is sensitive to the loss of AR in Sertoli cells and that release of mature spermatids from the seminiferous epithelium requires AR (Holdcraft & Braun 2004).

(a) Classical testosterone actions in Sertoli cells

In the testis, AR is expressed in peritubular myoid cells that surround the seminiferous tubules and in Leydig cells between the seminiferous tubules. Within the seminiferous tubules, only Sertoli cells have receptors for testosterone. Germ cells do not express AR (Sar *et al.* 1990). Thus, Sertoli cells are the major transducer of testosterone signals that are required to support germ cell survival and development. The classical mechanism of testosterone action begins with the diffusion of testosterone through the plasma membrane, and binding to AR that is sequestered in the cytoplasm by heat shock proteins (figure 2). When bound by ligand, AR undergoes a change in structural conformation allowing it to be released from the heat shock protein complex. AR then translocates to the nucleus where it binds to specific DNA sequences called androgen response elements (AREs), resulting in the recruitment of co-activators, and the regulation of gene expression. This classical pathway of testosterone action requires at least 30–45 min to alter gene expression and hours to produce nascent proteins (Shang *et al.* 2002).

Numerous genes and proteins are upregulated in response to testosterone but with the exception of the *Rhox5* (Pcm) homeobox gene, few are known to be induced in Sertoli cells by androgens through AR binding to gene promoter elements (Lindsey & Wilkinson 1996). Recently, microarray assays have identified additional testosterone and AR-regulated genes expressed in the testis by comparing normal mice with mice that have testosterone signalling disrupted. In 8-day-old mice in which testicular testosterone levels are reduced by testosterone propionate treatment for 4, 8 or 16 h, about 220 testis genes were found to be regulated at least twofold at each time point, with 67, 55 and 50 per cent of the genes being downregulated by testosterone, respectively. In 10-day-old SCARKO mice, 40 testis genes were regulated at least twofold differently from wild-type mice, but 28 genes were upregulated and 12 were downregulated by testosterone (Denolet *et al.* 2006). Studies comparing adult wild-type mice with AR hypomorph mice and AR hypomorphs having AR expression further ablated in Sertoli cells identified 46 and 57 testis-expressed genes, respectively, that are regulated at least twofold differently from wild-type mice. Interestingly, in these mouse models about twice as many testis genes were downregulated in the presence of

Figure 2. The classical testosterone signalling pathway. Testosterone diffuses through the plasma membrane and binds with the AR. The AR undergoes an alteration in conformation allowing it to be released from heat shock proteins in the cytoplasm. AR then is able to translocate to the nucleus where it binds to specific DNA sequences called androgen response elements (AREs). AR binding to an ARE allows the recruitment of co-activator and co-repressor proteins that alter the expression of genes to alter cellular function.

AR as were upregulated (Eacker *et al.* 2007). Although the various models of disrupted testosterone signalling provide varying results and identify different testosterone-regulated genes, an unexpected common theme is relatively high percentage of genes that are down-regulated by testosterone. This finding suggests that testosterone does not act solely via AR binding to ARE motifs to upregulate gene expression.

At least one conserved ARE was identified within 6 kb of the transcription start site in the 65 per cent of genes found to be regulated by AR in AR hypomorph mice. One of the AR-regulated genes, Kallikrein 27, a protease expressed in Leydig cells, was found to be regulated by AR through an ARE in the kallikrein 27 gene promoter (Eacker *et al.* 2007). However, at present there are no reports of newly identified testosterone-regulated genes in Sertoli cells being assessed to determine whether they are regulated via AREs.

Many AREs can bind and respond to glucocorticoid or progesterone receptors (non-specific AREs). Other AREs are capable of interacting only with AR (specific AREs). A mouse model in which the second zinc finger of AR is replaced by that of GR has been used to assess ARE-regulated gene expression. These SPARKI (specificity-affecting AR knock in) mice express an AR that is only capable of recognizing non-specific AREs. Analysis of the SPARKI mice revealed that the expression levels of the *Rhox5*, *Tsx* and *Drd4* genes were severely reduced. In contrast, the expression of *Eppin*, *PCI* and *Tubb3* mRNAs were

not greatly altered in the SPARKI mice (Schauwaers *et al.* 2007). These results suggest that some Sertoli cell genes are directly regulated through AR-specific AREs, whereas other genes are regulated via AR-non-specific AREs or by another mechanism.

(b) *Non-classical testosterone pathways in Sertoli cells*

A series of findings argue that testosterone may act via mechanisms other than the classical pathway to support spermatogenesis. These findings include: (i) testosterone is present in the testis at levels much greater than that needed to regulate transcription via AREs, (ii) relatively few genes in Sertoli cells are regulated by testosterone and fewer yet are known to be regulated through AREs and, (iii) a large percentage of testosterone-regulated genes are inhibited in the presence of AR. Studies in Sertoli cells and in other cell types have confirmed that testosterone can act through non-classical mechanisms to rapidly activate kinase signalling pathways and eventually alter the expression of genes that do not have known AREs or are not dependent on AR-promoter interactions (reviewed in Walker 2003; Rahman & Christian 2007). Thus far, testosterone has been found to act via two non-classical pathways in Sertoli cells. Testosterone stimulation can depolarize the Sertoli cell and cause calcium influx ($[Ca^{2+}]_i$) into Sertoli cells. Testosterone can also activate a series of kinases resulting in activation of the MAP kinase cascade

Figure 3. Non-classical testosterone signalling pathways. Left side: testosterone interacts with the classical AR which then is able to recruit and activate Src, which causes the activation of the EGF receptor via an intracellular pathway. The EGF receptor then activates the MAP kinase cascade most probably through Ras, resulting in the sequential activation of RAF and MEK and then ERK that activates p90-kinase, which is known to phosphorylate CREB on serine 133. As a result, CREB-regulated genes such as lactate dehydrogenase A (LDH-A) and early growth response 1 (EGR1) and CREB can be induced by testosterone. Right side: Testosterone interacts with a receptor in the plasma membrane that has characteristics of a G_q coupled G-protein coupled receptor (GPCR). Phospholipase C (PLC) is activated to cleave PIP_2 into IP_3 and DAG. Lower concentrations of PIP_2 inhibit K^+ATP channels causing membrane depolarization and Ca^{2+} entry via L-type Ca^{2+} channels.

and phosphorylation of the CREB transcription factor. At present, it is not yet known to what extent the two pathways are interlinked.

(i) Testosterone-mediated Ca^{2+} influx

The influx of $[Ca^{2+}]_i$ into freshly isolated Sertoli cells is observed within 20–40 s in response to testosterone (Gorczyńska & Handelsman 1995). Furthermore, in studies of primary Sertoli cells cultured for 1–4 days, testosterone or the synthetic androgen agonist, R1881 (methyltrienolone), at levels of 1 pM to 1 nM, induced transient increases (2–3 min) in intracellular Ca^{2+} ($[Ca^{2+}]_i$) within 20 s of addition, whereas higher concentrations of testosterone or R1881 (more than 10 nM) caused rapid and sustained increases (more than 15 min) in $[Ca^{2+}]_i$ (Lyng *et al.* 2000). Testosterone-mediated increases in $[Ca^{2+}]_i$ are abolished by EGTA and L-type Ca^{2+} channel current inhibitors, indicating that Ca^{2+} levels increase due to influx of extracellular Ca^{2+} via L-type Ca^{2+} channels (Gorczyńska & Handelsman 1995; Lyng *et al.* 2000).

Wassermann and colleagues demonstrated that testosterone even at lower than physiological concentrations (0.1–10 nM) induces depolarization of the Sertoli cell membrane within 30 s and for up to 5 min. The K^+ATP channel agonist diazoxide nullified

the depolarizing effect of testosterone and the K^+ATP channel inhibitor glibenclamide mimicked testosterone actions, indicating that testosterone affects K^+ATP channel activity (Von Ledeber *et al.* 2002). Follow-up studies showed that the phospholipase C inhibitor U73 122 and the G-protein inhibitor pertussis toxin block testosterone-mediated increases in membrane potential and Ca^{2+} (Loss *et al.* 2004). These findings suggest a model in which testosterone stimulation causes the activation of an unidentified G_q type G protein coupled receptor and the activation of phospholipase C that then hydrolyses PIP_2 in the plasma membrane to produce IP_3 and diacylglycerol (DAG). The decrease in the levels of PIP_2 , an inhibitor of ATP-mediated activation of K^+ATP channels, promotes the closing of the channels, causing an increase in membrane resistance and depolarization of the cell. As a result, voltage dependent L-type Ca^{2+} channels open and allow the influx of Ca^{2+} (Von Ledeber *et al.* 2002; figure 3, right). Similar pathways of testosterone-mediated Ca^{2+} have been reported for osteoblasts, macrophages, prostate cells and myotubules (Lieberherr & Grosse 1994; Benten *et al.* 1999; Estrada *et al.* 2003; Sun *et al.* 2006). Interestingly, oestradiol has been found to work through an as yet unidentified membrane associated receptor that has the characteristics of a G_q receptor. Oestradiol also elicits the activation of phospholipase C (PLC) and

elevated $[Ca^{2+}]_i$ (Kelly & Ronnekleiv 2008). Therefore, oestradiol and testosterone may act via the same or similar membrane associated receptors to elicit Ca^{2+} influx. In Sertoli cells *in vivo*, testosterone-mediated regulation of PLC, PIP2, IP3, DAG and Ca^{2+} levels may have important effects on gene transcription, the secretion of products required by germ cells and the stability of the cytoskeleton including restructuring of adherens junctions between Sertoli cells and germ cells as well as enabling the mobility and development of germ cells (Loss *et al.* 2004).

(ii) *Testosterone-mediated activation of Src, EGFR, the map kinase cascade and CREB*

Stimulation of primary cultured Sertoli cells isolated from rat testes with levels of testosterone similar to or lower than that found in the testis (10–250 nM) resulted in the phosphorylation of the CREB transcription factor on Ser 133 and ERK1/2 MAP kinases at Thr202/Tyr204 and Thr185/Tyr187 within 15 min. Phosphorylation at these positions induces CREB-mediated transcription and ERK kinase activity. This initial rapid activation suggested that testosterone activates ERK and CREB via a non-classical mechanism. RNA and protein synthesis inhibitors did not affect testosterone-mediated CREB phosphorylation, further supporting a non-classical mechanism of action. Neither oestradiol nor the progestin agonist R5020 were capable of inducing CREB phosphorylation in Sertoli cells, confirming that testosterone is the major steroid mediator for Sertoli cells. The MAP kinase pathway inhibitor PD98059 blocked CREB phosphorylation, indicating that ERK activation was upstream of CREB activation. The induced CREB and ERK phosphorylation was found to be rapid and sustained (1 min to more than 12 h; Fix *et al.* 2004). The sustained activation of CREB is important for the regulation of CREB-mediated transcription because CREB must be activated for at least 20 min to induce changes in gene transcription (Shaywitz & Greengard 1999). The activation of CREB in Sertoli cells is particularly significant because activated CREB has been shown to be essential for maintaining spermatogenesis. Specifically, infection of rat Sertoli cells *in vivo* with an adenovirus expressing a CREB mutant that cannot be phosphorylated on Ser 133 resulted in the apoptosis of spermatocytes and at least a 75 per cent reduction in the number of haploid spermatids (Scobey *et al.* 2001).

Earlier studies of testosterone-mediated influx of Ca^{2+} had raised the possibility that novel receptors for testosterone were present in the plasma membrane or on the surface of Sertoli cells. Support for this idea included the findings that testosterone–BSA conjugates, which cannot pass through the plasma membrane, increased Ca^{2+} influx into Sertoli cells in a manner similar to stimulation with free testosterone (Lyng *et al.* 2000). The studies by Wasserman's group showing that testosterone causes Ca^{2+} influx via phospholipase C, and the closing of K_{ATP}^+ channels also implicated a G protein coupled receptor for testosterone (Von Ledeberg *et al.* 2002; Loss *et al.* 2004). Although these findings are consistent with the

presence of testosterone receptors on or in the Sertoli plasma membrane, thus far a specific membrane receptor has not been identified in Sertoli cells.

Whereas it is possible that a novel membrane receptor could be responsible for transducing testosterone signals into activation of factors required for Ca^{2+} influx, the classic AR was found to be required for testosterone-mediated activation of ERK and CREB. Specifically, RNA interference studies in which AR expression was knocked down showed that testosterone could no longer induce CREB phosphorylation. Also, testosterone could not increase CREB phosphorylation in Sertoli cells derived from AR-defective tfm rats except after over-expression of wild-type AR (Fix *et al.* 2004).

Localization studies determined that most of Sertoli cell AR is present in the cytoplasm and nucleus. However, confocal microscopy studies and western analysis of membrane extracts identified a population of AR that was located close to or associated with the membrane. Furthermore, immunofluorescence studies as well as western immunoblot analysis of Sertoli cell membrane extracts showed that AR levels at the membrane transiently increased for 5–15 min after testosterone stimulation before returning to basal levels. Additional studies were performed using total internal reflection fluorescence microscopy in which antigens can only be detected within 70–100 nm of the plasma membrane. These studies identified punctuate AR immunoreactivity that by definition must be near the plasma membrane, although there is no evidence at this time that AR is present in the membrane (Cheng *et al.* 2007).

The localization of a population of AR near the plasma membrane has important ramifications for rapid testosterone signalling because signalling pathway factors such as G-protein coupled receptors and kinases are often found in clusters at the plasma membrane. Src kinase is one signalling factor that is found tethered to the plasma membrane (Resh 1996). Co-immunoprecipitation studies determined that Src and AR interactions increased for at least 15 min after stimulation of Sertoli cells with testosterone. Analysis of Src activation as measured by phosphorylation of Tyr 418 revealed that testosterone activated Src kinase in a rapid (within 1 min) and sustained manner (at least 2 h; Cheng *et al.* 2007). Studies by Migliaccio *et al.* (2000) determined that androgen stimulation triggers direct association of the proline rich region of AR and the SH3 domain of Src. Pre-incubation of Sertoli cells with the Src kinase inhibitor PP2 or over-expression of a dominant negative Src kinase blocked ERK kinase phosphorylation, indicating that Src acted upstream of ERK. Additional studies demonstrated that testosterone-mediated activation of Src caused the phosphorylation and stimulation of the EGF receptor (EGFR) via an intracellular pathway and that the EGFR acted downstream of Src to activate the MAP kinase pathway including ERK (Cheng *et al.* 2007).

Downstream of the EGFR, the non-classical signalling pathway is completed with the activation of the MAP kinase cascade, including the sequential activation of Raf, MEK and ERK kinases. ERK then

can stimulate p90^{Rsk} kinase, which is known to phosphorylate CREB on Ser 133 (Xing *et al.* 1996). When phosphorylated, CREB becomes an activated transcription factor and mediates transcription of CREB-responsive genes. Testosterone stimulation of Sertoli cells was found to induce at least three CREB-regulated genes (*LDH-A*, *EGR1* and *CREB*) that do not contain known AREs (Fix *et al.* 2004). It remains to be determined whether testosterone activates other transcription factors and other genetic programs. However, testosterone activates numerous kinases in Sertoli cells for an extended period; thus it would be expected that many classes of transcription factors would be affected. Because testosterone stimulation acts at the genomic level to activate CREB-mediated transcription, the term non-genomic is not the most accurate descriptor of testosterone action and is the reason that this testosterone pathway is described as non-classical. Alternatively, the FASEB rapid steroid signalling working group has adapted the terms membrane-initiated steroid signalling and nuclear-initiated steroid signalling (NISS) to describe the known mechanisms of signalling by steroid hormones.

(iii) FSH effects on non-classical testosterone signalling

FSH acts synergistically with testosterone to increase spermatogenesis efficiency and fertility in the male (Sharpe 1994; McLachlan *et al.* 1996). However, FSH has been shown to inhibit phosphorylation of ERK, a target of the non-classical signalling pathway, in mature Sertoli cells (Crepieux *et al.* 2001). Western analysis of Sertoli cells co-treated with testosterone and FSH demonstrated that FSH blocked testosterone-mediated phosphorylation of ERK. Treatment with forskolin, a potent inducer of cAMP production, further inhibited ERK phosphorylation. Addition of the phosphodiesterase inhibitor IBMX amplified the effects of FSH and forskolin. In contrast, pretreatment with the PKA inhibitor H89 restored testosterone-mediated ERK phosphorylation (W. H. Walker 2009, unpublished observations). Together, these studies indicate that FSH acts via cAMP and PKA to block phosphorylation of ERK. FSH treatment did not result in the down-stream inhibition of CREB because FSH is known to strongly induce CREB phosphorylation via increasing cAMP levels and activating PKA to phosphorylate CREB on Ser 133 (Walker *et al.* 1995). In agreement with the known properties of FSH, stimulation of Sertoli cells with FSH + testosterone increased CREB phosphorylation above that of testosterone alone and co-stimulation with FSH + T + H89 reduced CREB phosphorylation to levels achieved with testosterone alone (W. H. Walker 2009, unpublished observations). Together, these results indicated that FSH acts through cAMP and PKA to inhibit ERK while using the same effectors to activate CREB. Furthermore, FSH inhibition of the non-classical testosterone pathway is only relevant for processes reliant on factors that act upstream of CREB. This result is also consistent with the finding that CREB phosphorylation must be maintained in Sertoli cells to support the survival of spermatocytes (Scobey *et al.* 2001).

Studies performed to identify the site at which FSH blocks the non-classical signalling pathway determined that FSH acted upstream of MEK kinase and downstream of Src and EGFR. Further studies determined that testosterone-inducible Raf kinase activity was reduced by FSH and that Raf-1 kinase phosphorylation was reduced in the presence of FSH (W. H. Walker 2009, unpublished observations; figure 4, region 1). These data indicate that FSH increases cAMP levels, resulting in the activation of PKA that then blocks Ras activation of Raf-1 or Raf-1 activity directly. FSH inhibition of non-classical testosterone signalling at Raf-1 is supported by previous work demonstrating that there are at least four pathways by which cAMP and PKA are able to downregulate Raf (Wu *et al.* 1993; Mischak *et al.* 1996; Schmitt & Stork 2001; Dhillon *et al.* 2002; Enserink *et al.* 2002; Chong & Guan 2003; Dumaz & Marais 2003; Wang *et al.* 2006b).

Although FSH levels in serum constantly increase and decrease with a periodicity of 1–3 h, the levels of FSH present in the testis during the 12.9 day, 14 stage (I–XIV) cycle of the seminiferous epithelium in the rat are relatively constant. Thus, it might be expected that FSH stimulation would continuously block ERK activation. However, FSH-initiated signalling in Sertoli cells varies greatly during the cycle of the seminiferous epithelium because expression of the FSH receptor on the surface of Sertoli cells is cyclical (reviewed in Walker & Cheng 2005). FSH receptor expression peaks during stages III–IV and is lowest during stages VII–X of the cycle of the seminiferous epithelium (Kangasniemi *et al.* 1990). Testosterone levels in serum also rise and fall in a pattern similar to FSH, but testosterone levels in the testis remain uniformly high. In contrast, AR levels in Sertoli cells are extremely low and testosterone-dependent actions are minimal during much of the 12.9 day cycle except when AR levels peak during stages VII–VIII (Bremner *et al.* 1994). Therefore, during stages VII–VIII when FSHR levels and FSH signalling efficiency is lowest and AR levels are highest, a transient spike of testosterone-mediated phosphorylation of ERK is possible. In agreement with this hypothesis, phosphorylated ERK levels in Sertoli cells *in vivo* are only elevated in stage VIII tubules adjacent to the heads of sperm in the process of being released and in the adluminal regions adjacent to the developing germ cells. The increased staining for phosphorylated ERK is absent by stage X (Chapin *et al.* 2001). Because FSH and testosterone act synergistically, it is possible that FSH-mediated inhibition of ERK activity in Sertoli cells during much of the cycle of the seminiferous epithelium and transient increases in ERK activation during stages VI–VIII may be required to support germ cell processes at specific stages of development.

3. REGULATION OF SPERMATOGENESIS BY NON-CLASSICAL TESTOSTERONE SIGNALLING

Combining the observations from *in vivo* studies in which either testosterone levels are reduced, AR is knocked out or a less functional hypomorph AR allele is expressed in the testis, it has been learned

Figure 4. Regulation of spermatogenesis processes by non-classical testosterone signalling. The non-classical signalling pathway resulting in Src, ERK and CREB activation is shown for a stage VII–VIII seminiferous tubule cross section. (1) FSH-mediated increases in cAMP and PKA activity result in blocking of the non-classical testosterone signalling pathway at the Raf kinase step so that p-ERK levels would be restricted except for stages VII–VIII when FSHR levels are lowest and AR expression is highest in the Sertoli cell. (2) ERK activation contributes to testosterone-mediated Sertoli–germ cell adhesion and ERK activation may be particularly important during stages VII–VIII to remodel the connections between Sertoli cells and spermatids as they begin to elongate. (3) Testosterone entering the Sertoli cell from the interstitial space or the tubule lumen acts through Src kinase to promote the release of mature sperm from the Sertoli ES, most probably by Src phosphorylation of adaptor proteins (filled round circles) that anchor the cell–cell adhesion proteins (represented by lines extending from Sertoli cell to mature sperm) to actin filaments.

that testosterone and AR actions in Sertoli cells are required for at least three major cell adhesion processes that affect fertility (Chang *et al.* 2004; De Gendt *et al.* 2004; Holdcraft & Braun 2004; Wang *et al.* 2006a). Specifically, testosterone is required for (i) the formation of connections between Sertoli cells that make up the BTB, (ii) maintaining the connections between Sertoli cells and haploid spermatid germ cells and (iii) the release of mature sperm from Sertoli cells. Investigations into the specific roles of classical and non-classical testosterone signalling in regulating processes critical to maintaining

spermatogenesis have only recently been initiated. Nevertheless, initial results suggest that the non-classical pathway contributes to maintaining spermatogenesis by regulating Sertoli–germ cell adhesion.

One recent study has argued that the non-classical pathway is not required for maintaining spermatogenesis. The Handelsman group has studied the effects of an AR mutant lacking exon III encoding 50 amino acids including the second zinc finger (ZF2) DNA-binding domain. Male mice in which the ZF2 domain was removed from AR specifically in Sertoli

cells were found to have smaller testes due to the loss of germ cells and the mice were found to be infertile. These results were provided as evidence that the DNA-binding activity and the classical signalling pathway of AR are required for fertility and that the non-classical pathway is not required (Lim *et al.* 2009). However, it is possible that removal of 50 amino acids from the AR protein may have other effects in addition to abolishing DNA-binding activity. The relatively large deletion (6% of the protein) may alter the protein structure of AR and disrupt androgen binding, AR interactions with Src or other activities required for non-classical signalling. Unfortunately, the non-classical activities of the ZF2 deleted AR have not yet been reported and it remains to be determined whether the ZF2 deleted AR is a specific monitor of the classical pathway.

(a) *Testosterone-mediated maintenance of the blood–testis barrier*

The BTB is a series of tight and adherens junctions between adjacent Sertoli cells that divides the basal and adluminal compartments of the seminiferous epithelium and separates post-meiotic germ cells from the outside environment and the blood supply (Dym & Fawcett 1970). Sertoli cells must supply the germ cells beyond the barrier with required nutrients, minerals and growth factors (reviewed in Mruk & Cheng 2004; Skinner 2005). During stages VII and VIII the BTB is disrupted and reformed as preleptotene spermatocytes pass through the barrier (Dym & Fawcett 1970; Bremner *et al.* 1994). The findings that testosterone up-regulates three tight junction components (Occludin, Claudin 11 and Claudin 3; Meng *et al.* 2005; Wang *et al.* 2006a) is consistent with the requirement for AR to reform tight junctions in stage VIII after pre-meiotic cells pass through the BTB. Presently, it is not known whether classical or non-classical mechanisms are used to regulate the production of tight junction proteins. It remains to be determined whether testosterone-mediated activation of kinases regulates the stability or localization of proteins at the BTB.

(b) *Testosterone is required for Sertoli–spermatid adhesion*

Spermatocytes and round spermatids are connected to Sertoli cells via desmosomes that use intermediate filaments as their attachment sites. During stage VIII of the cycle of the seminiferous epithelium when spermatids first begin to elongate, Sertoli–germ cell desmosome anchoring proteins are replaced with new specialized adherens junctions called ectoplasmic specializations (ES). The ES, which has stronger connections than desmosomes, is linked to the actin cytoskeleton through adapter proteins and is maintained until the release of mature sperm. The ES is composed of (i) endoplasmic reticulum located near the periphery of the cell, (ii) the actin cytoskeleton located closer to the plasma membrane, and (iii) the proteins that comprise the adherens junction.

At least three types of protein–protein interactions cooperate at adherens junctions to secure spermatids

to Sertoli cells: (i) Cadherin–cadherin: transmembrane cadherin proteins expressed by elongated spermatids and Sertoli cells interact in the intracellular space to link the two cells. In Sertoli cells, cadherin is bound by β or γ catenin adapter proteins that are in turn linked indirectly to the actin cytoskeleton via α catenin. Phosphorylation of β or γ catenin results in loss of cell adhesion. (ii) Nectin–afadin–ponsin: The extracellular region of the nectin transmembrane protein that is produced by both cells contributes to cell–cell connections. Nectin is then bound by afadin and ponsin in the cytoplasm. The α -catenin protein links afadin to the actin cytoskeleton. (iii) Integrin $\alpha 6 \beta 1$ –laminin $\gamma 3$: Integrin $\alpha 6 \beta 1$ that is expressed by the Sertoli cell and laminin $\gamma 3$ that is produced by the spermatid interact to form a linkage. The transmembrane integrin $\alpha 6 \beta 1$ protein is known to be associated with numerous kinases including Src and ERK. The focal adhesion kinase (FAK) links integrin $\alpha 6 \beta 1$ to paxillin that binds vinculin to anchor the complex to the actin cytoskeleton (reviewed in Lui *et al.* 2003; Mruk & Cheng 2004; Wong & Cheng 2005).

In one study of Sertoli cell-specific AR knockout mice, only one of the eight ES proteins that was assayed (gelsolin) was found to be regulated (repressed) at the RNA level in the absence of AR (Wang *et al.* 2006a), yet these proteins are cyclically induced during stage VIII to form new connections between Sertoli cells and more mature germ cells (elongated spermatids). These data suggest that testosterone-mediated transcriptional regulation through AR is not a major factor in the regulation of the ES, which is consistent with the idea that ES integrity is regulated predominately by non-classical signalling events.

Co-cultures of Sertoli cells with germ cells from adult rats were used to assay the effects of non-classical testosterone signalling on Sertoli–germ cell attachment. Stimulation with testosterone increased germ cell attachment to the Sertoli cells by almost 50 per cent but the addition of testosterone with the Src kinase inhibitor PP2, the ERK kinase inhibitor PD98059 or flutamide reduced germ cell attachment to below basal levels (W. H. Walker 2009, unpublished observations). These results indicate that testosterone increases the efficiency of germ cell attachment to Sertoli cells and that both the Src and ERK kinases that are activated by the non-classical pathway are required to facilitate Sertoli–germ cell attachment. Presently, it is not known whether direct Src actions are required or whether it is the activation of ERK by Src that is required to support Sertoli–germ cell adhesion (figure 4, area 2).

Further studies using Sertoli cells isolated from AR-defective tfm rats were performed in which the Sertoli cells were infected with adenovirus constructs that expressed either wild-type AR, an AR mutant that can only activate the classical pathway, or an AR mutant that can only activate the non-classical pathway. Co-culture of the adenovirus infected Sertoli cells with germ cells revealed that testosterone can only increase the attachment of germ cells in the presence of an AR that is capable of activating the non-classical pathway (W. H. Walker 2009, unpublished observations). These results indicate that the

non-classical pathway of testosterone action contributes to Sertoli–germ cell adhesion and that the DNA-binding activity of AR and the classical pathway is not sufficient to permit testosterone-induced increases in germ cell binding.

(i) *In vivo models for ERK regulation of Sertoli–germ cell adhesion*

The significance of ERK signalling in maintaining ES connections between Sertoli cells and maturing germ cells has been demonstrated by two *in vivo* models that mimic ES disruption during spermatogenesis. The first model is based on the use of subdermal testosterone and oestradiol (TE) implants in adult rats, which lower the intratesticular T level and induce the loss of stage VIII and later spermatids from the epithelium (McLachlan *et al.* 1996; Beardsley & O'Donnell 2003). During this TE-induced loss of germ cells, ERK is significantly activated (Wong *et al.* 2005). This activation is preceded by an initial activation of focal adhesion kinase (FAK) and c-Src. Also N-cadherin and β -catenin associations are broken, accompanied by a surge in β -catenin tyrosine phosphorylation, whereas later reassociation of N-cadherin and β -catenin was associated with a loss of tyrosine phosphorylation (Zhang *et al.* 2005). A second model using Adjudin, a chemical that causes disruptions of the ES, resulted in the activation of ERK at the time when germ cells are depleted from the epithelium (Siu *et al.* 2005; Xia & Cheng 2005). Pretreatment with U0126, an inhibitor that specifically blocks MEK (the kinase upstream of ERK) activity, partially blocked the Adjudin-induced germ cell loss, confirming the need for ERK in the regulation of ES dynamics (Xia & Cheng 2005).

At first glance the *in vivo* models of ES disruption do not appear to agree with the results of co-culture studies in that ERK is activated when the ES is disrupted *in vivo* but activated ERK is required to increase Sertoli–germ cell adhesion in co-culture studies. One hypothesis to unify the disparate results is that ERK kinase activity is required to initiate the process of ES formation and Sertoli–elongating spermatid connections, but extended ERK activation results in disrupted ES formation or does not allow for the adherence process to be completed. This hypothesis is consistent with the idea that FSH acts to limit ERK activity except during stages VII–VIII when the ES is being formed. Another possibility is that Src kinase is inappropriately activated in the *in vivo* models and acts to disrupt the ES as described in §3c below. Clearly, additional studies are required to determine how ERK and/or Src regulate the creation and dismantling of the ES *in vivo*.

(c) *Testosterone-regulated SRC kinase is required to release mature sperm*

In the rat, mature spermatozoa are released from Sertoli cells during stage VIII. In the absence of testosterone, the sperm are not released but are instead phagocytized by the Sertoli cells (Holdcraft & Braun 2004). To determine whether the non-classical pathway may contribute to the testosterone-mediated

disruption of the ES and the release of sperm, sections of seminiferous tubules from rats containing mature sperm just prior to their release were cultured. Treatment of the cultured seminiferous tubule sections with testosterone or testosterone + various signalling pathway inhibitors revealed that co-stimulation with PP2 decreased sperm release by 42 per cent. The inhibition of sperm release by PP2 exceeded that by the AR antagonist flutamide in the absence of testosterone stimulation (30% below control levels; W. H. Walker 2009, unpublished observations). These results suggest that Src kinase, which is induced by the non-classical testosterone signalling pathway, is an important regulator of sperm release and fertility (figure 4, area 3).

The finding that Src activity is required for sperm release is not unexpected because it has been demonstrated that Src phosphorylation is transiently induced immediately prior to the release of sperm during stages VII–VIII of the seminiferous cycle. Src is structurally associated with cell adhesion regulatory proteins at the ES that links Sertoli cells with elongated spermatids and maturing spermatozoa (Lee & Cheng 2005); the levels of phosphorylated Src and Erk increase dramatically and activation of Src is required for the release of sperm (Wang *et al.* 2000; Chapin *et al.* 2001; Zhang *et al.* 2005). Src has been shown to phosphorylate β -catenin and N-cadherin proteins that contribute to the extracellular bridge to the maturing spermatid. As a result of the Src-dependent phosphorylation, N-cadherin diffuses away from β -catenin and the linkage to the actin cytoskeleton is broken so that the mature sperm can be released (Kinch *et al.* 1995; Roura *et al.* 1999; Xia & Cheng 2005). Together, these observations suggest that stage-specific, testosterone-mediated activation of Src located at the ES may be responsible for the disassembly of adherens junctions at the ES and the release of mature sperm.

(i) *Src and Sertoli–germ cell adhesion*

Src knockout mice normally survive only three to five weeks after birth but can survive for 1 year (Soriano *et al.* 1991; Lowell & Soriano 1996). There are no detailed studies of Src effects on spermatogenesis in the knockout mice except for the report that ‘on rare occasions Src knockout mice can reproduce’ (Lowell & Soriano 1996). Studies of the effects on inhibiting Src activity *in vivo* suggest that Src has different effects on Sertoli connections with less mature germ cells versus more mature elongated spermatids. Three days after injection of the Src inhibitor PP1 into the testis or into the jugular vein, spermatocytes and round spermatids were absent, but elongating spermatids remained (Lee & Cheng 2005). Presently, the mechanism for the loss of earlier germ cells after inhibition of Src activity is not known. However, the retention of the elongated spermatids is consistent with the need for Src activity to disrupt the ES and permit the release of sperm from Sertoli cells (Russell & Clermont 1977; Kerr *et al.* 1993; O'Donnell *et al.* 1996).

4. CONCLUSION

Although it has been known for more than 50 years that testosterone is required for maintaining

spermatogenesis and fertility, the mechanisms by which testosterone acts are only now being characterized. The paucity of spermatogenesis-enhancing genes known to be regulated by testosterone via the non-classical pathway and the necessity for high testosterone levels in the seminiferous tubules has been a paradox that can now be explained by the characterization of the non-classical pathway. The evidence available suggests that the classical and non-classical testosterone pathways both contribute to maintaining spermatogenesis and fertility. The new information showing that non-classical testosterone actions increase germ cell attachment to Sertoli cells and permit the release of mature sperm suggests that the non-classical pathway may support these processes and perhaps others that are required for fertility. Further *in vivo* studies will be required to confirm the importance of the non-classical pathway. Transgenic mice in which Sertoli cells lack wild-type AR but express only AR mutants that are capable of activating one of the pathways would provide important information regarding the genes and proteins that are regulated by each pathway as well as the processes that are supported by each pathway. Knowledge of the specific regulatory mechanisms and factors used by testosterone to maintain spermatogenesis will permit the design of new approaches to limit or enhance male fertility.

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