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The role of androgens in the control of spermatogenesis: lessons from transgenic models involving a Sertoli cell-selective knockout of the androgen receptor

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Abstract

Androgens play a key role in the control of spermatogenesis, but the cellular and molecular mechanisms involved remain poorly understood. The absence of androgen receptor (AR) expression in germ cells points to somatic cells as the most likely direct targets for androgen action. Sertoli cells are the primary candidates given their intimate morphological and functional interactions with developing germ cells and the fact that they express the AR in a cyclical fashion depending on the stages of spermatogenesis and their cognate androgen responsiveness. Isolated and cultured Sertoli cells have proven of limited help in the study of the effects and mechanisms of androgen action since they lose the expression and/or androgen responsiveness of many potentially relevant target genes. Novel transgenic models in which the AR is selectively ablated in Sertoli cells, such as the SCARKO mice, have made it possible to study the effects of the AR in Sertoli cells, while preserving their natural interactions with surrounding germ cells and somatic cells. These studies unambiguously identify the Sertoli cell as the major target for androgen action in the control of spermatogenesis. Moreover, they show that the effects of androgens on these cells are mainly or exclusively mediated by the classical AR. Selective ablation of this receptor during embryonic life results postpubertally in a block in germ cell meiosis, confirming that progression through meiosis is an early and sensitive target for androgen action. SCARKO mice and related models open a novel avenue to identify molecular mediators of androgen action involved in the control of germ cell development.

Keywords: testis, androgen action, testosterone, meiosis, Cre/loxP.

Introduction

Androgens have a wide variety of effects on both reproductive and non-reproductive target tissues (Mooradian *et al.*, 1987). One of their most intriguing and vital actions, however, concerns the control of spermatogenesis (Verhoeven, 1992a). Spermatogenesis is an extremely complex process that depends on a variety of complementary control mechanisms comprising a genetic program built into the germ cells (Franca *et al.*, 1998a; Brinster, 2002), a local network of bilateral communications between germ cells and surrounding somatic cells (Skinner, 1991; Verhoeven, 1992b; Jegou, 1993; Mruk and Cheng, 2004), and hormonal regulation. At the endocrine level, the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) act as the master switches that turn spermatogenesis on or off (Weinbauer and Nieschlag, 1993; Sharpe, 1994; McLachlan *et al.*, 2002). Follicle stimulating hormone acts directly on the Sertoli cells and supports several aspects of germ cell development (McLachlan *et al.*, 2002; Holdcraft and Braun, 2004b). One of its most important effects, however, is the control of the number of Sertoli cells (Orth, 1984) which, in turn, is a major determinant of spermatogenic capacity (Russell and Peterson, 1984). Luteinizing hormone acts on the Leydig cells in the interstitial compartment of the testis and stimulates the production of androgens. In this way it assures both the physiological levels of circulating testosterone required to support accessory sex tissues and male sexual behavior and the very high intratesticular levels of testosterone that are apparently required for normal spermatogenesis (Weinbauer and Nieschlag, 1993; Sharpe, 1994; McLachlan *et al.*, 2002).

There is a vast amount of experimental evidence indicating that, under natural conditions, both FSH and androgens are required for optimal spermatogenesis (Weinbauer and Nieschlag, 1993; Sharpe 1994; McLachlan *et al.*, 2002; Sharpe, 2006). Interestingly, however, under some circumstances androgens may be sufficient to permit at least qualitatively normal spermatogenesis. High doses of testosterone can initiate, maintain, or restore spermatogenesis in a number of rat, mouse, and primate models where FSH is absent or low. Such models include: intact animals treated with high doses of androgens, hypophysectomized animals, animals treated with gonadotropin-releasing hormone (GnRH) agonists or antagonists, mice that are hypogonadal due to a large deletion in the GnRH gene (*hpg* mice; Sharpe, 1994; Singh *et al.* 1995). Along the same lines, fertility is maintained in transgenic mouse models with a knockout of the FSH receptor gene (Dierich *et al.*, 1998; Abel *et*

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al. 2000) or the FSHβ gene (Kumar *et al.*, 1997) and in human males with an inactivating mutation of the FSH receptor (Tapanainen *et al.*, 1997).

Despite the overwhelming evidence for a key role of androgens in the control of spermatogenesis, our knowledge of the cellular and molecular mechanisms underlying their activity in the testis remains very incomplete.

Mechanisms of androgen action and spermatogenesis

Androgens are steroid hormones and act via a specific receptor, the androgen receptor (AR), a member of the nuclear receptor family. The AR is a 110-kDa protein encoded by a single-copy gene located on the X-chromosome (Quigley *et al.*, 1995; Chang and McDonnell, 2005; Gao *et al.*, 2005). A detailed discussion of the mechanism of androgen action is beyond the scope of the present review, but the most important elements are summarized in Fig. 1. The vast majority of the known effects of androgens are mediated by direct or indirect effects of the activated AR on gene expression. Activation of this receptor occurs via binding of testosterone, the main circulating androgen, or via binding of 5α-dihydrotestosterone, an active metabolite produced in many target tissues under the control of two distinct 5α-reductases (Russell and Wilson, 1994; Wilson, 1996). The activated AR binds in the cell nucleus to specific recognition sequences (androgen response elements, ARE) located in the vicinity of androgen-responsive genes and modulates gene expression in part via interactions with other regulatory factors and in part via the induction of specific changes in the conformation of the chromatin (Verrijdt *et al.*, 2003; Chang and McDonnell, 2005). Some of the effects of androgens may be mediated via another active metabolite, 17β-estradiol, produced by the aromatization of testosterone (Wilson, 1975). Estradiol acts via its own cognate receptors: the estrogen receptors ERα and ERβ (Nilsson and Gustafsson, 2002).

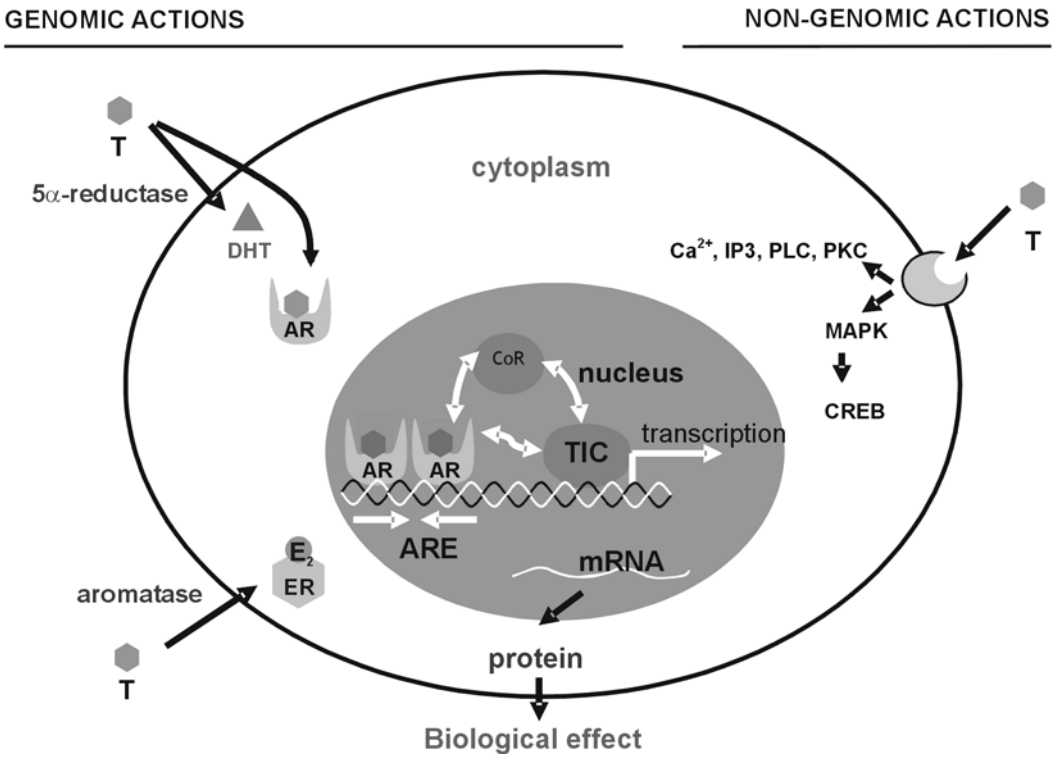

Figure 1. Mechanisms of androgen action. Testosterone (T) enters the target cell by passive diffusion and binds to the androgen receptor (AR) either directly or after conversion into 5α-dihydrotestosterone (DHT) by one of the 5α-reductases. The activated AR complex binds as a dimer to androgen response elements (ARE) in the regulatory regions of androgen target genes and recruits coregulators (CoR) responsible for structural changes in the chromatin, interactions with the transcription initiation complex (TIC), and modulation of gene expression. In a comparable fashion, 17β-estradiol produced by aromatization of testosterone may modulate the expression of estrogen target genes via cognate estrogen receptors (ER). Nongenomic effects of androgens are mediated by alternative signaling pathways involving calcium (Ca^{++}), inositol triphosphate (IP3), phospholipase C (PLC), protein kinase C (PKC), cyclic AMP (cAMP), MAP-kinases (MAPK), and cAMP response element binding protein (CREB). Both the classical AR and as yet unidentified membrane receptors may be involved.

During the last decade, increasing evidence has been presented for the existence of non-genomic responses to androgens (Heinlein and Chang, 2002). In fact, some responses to androgens occur within seconds or minutes and do not seem to involve protein or RNA synthesis. These responses are apparently mediated by alternative signaling pathways involving changes in intracellular calcium, activation of protein kinases, phopholipase C, diacylglycerol or 3',5'- cyclic adenosine monophosphate (cAMP). Such effects have also been described in isolated Sertoli cells, but their *in vivo* significance remains enigmatic (Gorczynska and Handelsman, 1995; Walker, 2003; Fix *et al.*, 2004).

When one tries to apply this knowledge of androgen action to the specific problem of the control of spermatogenesis, it becomes evident that some of the most fundamental questions are still awaiting an answer. The relative contribution of various potential androgen target cells (De Gendt *et al.*, 2004) to the control of spermatogenesis remains a matter of investigation. It also remains an enigma why optimal spermatogenesis apparently requires testicular concentrations of androgens that are far higher than those needed to saturate the AR (Sharpe, 1994). The contribution of active metabolites such as 5α-dihydrotestosterone and 17β-estradiol to the control of spermatogenesis needs further research (O'Donnell *et al.*, 2001; Saunders *et al.*, 2001; McLachlan *et al.*, 2002), and the crucial target genes mediating the control of spermatogenesis by androgens remain unidentified (MacLean and Wilkinson, 2005). This review will focus primarily on what we have learned from a number of recent genetic models on the target cells involved in the control of germ cell development by androgens and on the molecular pathways of androgen action.

Androgen target cells in the control of spermatogenesis

One of the primary questions that remains is to define the target cell(s) via which androgens affect germ cell development. It is extremely unlikely that spermatogenesis is controlled by cell-autonomous activation of the AR in germ cells. In fact, although fetal germ cells may express the AR and may be a target for androgens that physiologically inhibit their proliferation (Merlet *et al.*, 2007), most studies on postnatal testes have failed to demonstrate an AR in germ cells (Grootegoed *et al.*, 1977; Bremner *et al.*, 1994). Moreover, male mice chimaeric for androgen-resistant (TfmX/Y) and normal (X/Y) genotype are able to produce offspring from the TfmX/Y component in which the AR is absent (Lyon *et al.*, 1975). Similarly, transplantation of germ cells from TfmX/Y mice into seminiferous tubules of azoospermic mice expressing a functional AR results in complete and qualitatively normal donor-derived spermatogenesis (Johnston *et al.*, 2001). The only possibility that cannot be excluded at the present time is that androgens might affect germ cell development by a mechanism that bypasses the classical AR.

If androgens do not act directly on the germ cells, their actions have to be mediated by somatic cells. In this case, Sertoli cells are primary candidates given their intimate anatomical and functional interactions with developing germ cells. Sertoli cells express the AR (Sharpe, 1994; 2006) and even deploy cyclic changes in AR expression that parallel the androgen responsiveness of the successive stages of the spermatogenic cycle (Parvinen, 1982; Bremner *et al.*, 1994). Unfortunately, most attempts to study androgen effects in isolated cultured Sertoli cells have yielded rather disappointing results. Only a few genes have been identified that are still regulated by androgens under culture conditions, and in general the amplitude of the observed effects is limited (Sharpe, 1994; MacLean and Wilkinson, 2005; Sharpe, 2006). Moreover, some of the effects may be related at least in part to contaminating peritubular cells or to androgen-induced protein stabilization. An example of the former possibility are the stimulatory effects on Sertoli cell transferrin secretion (Perez-Infante *et al.*, 1986; Huggenvik *et al.*, 1987) and the inhibitory effects on FSH-induced aromatase activity (Verhoeven and Cailleau, 1988b). Androgen-induced stabilization may contribute to the effects observed on androgen-binding protein (ABP) production (Louis and Fritz, 1977; Cailleau *et al.*, 1984; Perez-Infante *et al.*, 1986; Verhoeven and Cailleau, 1988a). Nonetheless, it is obvious that the expression of important androgen-responsive target genes is lost under culture conditions. For example, the expression of Reproductive homeobox 5 (Rhox5 also known as Pem), the only androgen-responsive gene known at the present time to be expressed in Sertoli cells *in vivo* and to undergo a 50-fold androgen-induced upregulation during development, is completely lost after 24 h of culture (Sutton *et al.*, 1998). Moreover, although some authors have been able to demonstrate activation of androgen-responsive reporter constructs in isolated Sertoli cells (Ku *et al.*, 1994), others have failed to do so in the absence of cotransfected AR, suggesting that androgen responsiveness is at least partially lost under culture conditions as a consequence of decreasing AR expression (Denolet *et al.*, 2006b). Studies with transformed and immortalized Sertoli cell lines have been equally disappointing (Roberts, 2005). The TM4 cell line, which is widely used, was originally reported to be AR positive but has proven of little help in the identification of androgen regulated genes (Nakhla *et al.*, 1984). Similarly, in MSC-1 cells Rhox gene induction could only be demonstrated after cotransfection with an AR expression construct (MacLean *et al.*, 2005). A SK11 cell line has recently been shown to be AR positive and capable of driving an ARE-reporter gene (Sneddon *et al.*, 2005). This line may hold some promise and merits further investigation. The main conclusion from all these observations, however, is that cultured Sertoli cells and Sertoli cell lines have severe limitations as a paradigm to study

androgen action and that their androgen responsiveness may be strongly dependent on their *in vivo* environment, including the contacts with surrounding cells.

The peritubular myoid cells surrounding the seminiferous tubules are a third potential target for some of the effects of androgens on spermatogenesis. These cells express the AR already during fetal life (from Day 17 post-coitum in rats and even from Day 15.5 post-coitum in mice; Majdic *et al.,* 1995; Merlet *et al.,* 2007) whereas in Sertoli cells, AR expression is only observed from Day 5 after birth on (Bremner *et al.,* 1994). Androgens are required for the normal differentiation of peritubular myoid cells, but they may not be needed for the maintenance of the differentiated state (Schlatt *et al.,* 1993). Direct interactions between germ cells and peritubular cells are limited to the basal compartment of the testis. However, peritubular cells may indirectly affect spermatogenesis by their complex effects on Sertoli cell development. Peritubular cells and Sertoli cells collaborate in the production and deposition of extracellular matrix and in the formation of a basal lamina surrounding the tubules (Dym, 1994). This basal lamina in turn affects morphology and function of both cell types. Moreover, peritubular cells and Sertoli cells participate in a complex network of paracrine interactions (Skinner, 1991; Verhoeven *et al.,* 2000). Androgen-regulated peritubular factors that modulate Sertoli cell function (referred to as P-Mod-S) may play a role in these interactions but their exact contribution as well as the identity of the factors involved, remain elusive (Skinner and Fritz, 1985; Skinner *et al.,* 1988; Swinnen *et al.,* 1990; Verhoeven *et al.,* 2000).

A novel experimental paradigm to study androgen action in Sertoli cells in vivo: Sertoli cell-selective knockout of the androgen receptor

To further delineate the contribution of Sertoli cells to the control of spermatogenesis and to overcome the problems of loss of differentiated function and androgen responsiveness in cultured cells, several groups have recently developed transgenic models in which the AR gene is selectively ablated in Sertoli cells (Chang *et al.,* 2004; De Gendt *et al.,* 2004; Holdcraft and Braun, 2004a). In these models, Sertoli cells preserve their natural environment, including their contacts with surrounding cells. All of these groups have employed Cre/*loxP* technology (Fig. 2). This technology makes use of the Cre (Cyclization recombination) recombinase of the bacteriophage P1, which mediates an efficient site-specific recombination between 34 bp recognition sequences known as *loxP* sites. To produce ubiquitous or cell-selective knockouts by this approach, two transgenic strains need to be developed. In one strain, a functionally critical region of the targeted gene is "floxed" by the introduction of a *loxP* site both at its 5'-upstream and at its 3'-downstream end. If these *loxP* sites are oriented in the same direction, Cre recombinase action will result in the excision of the floxed region. To this end, mice of the floxed transgenic strain need to be crossed with another transgenic strain that expresses the Cre recombinase either ubiquitously or in a cell-selective way. In the former case, Cre-mediated excision of the floxed region results in a ubiquitous knockout. In the latter case, a cell-selective gene inactivation is the expected end-result. Three models with a Sertoli cell-selective inactivation of the AR have been described, and their main characteristics are summarized in Table 1. In two of the models (Chang *et al.,* 2004; De Gendt *et al.,* 2004), mice with a floxed exon 2, were crossed with mice expressing the Cre recombinase under the control of the Anti-Müllerian Hormone (AMH) promoter. The AMH-Cre mice were derived from the same source (Lecureuil *et al.,* 2002). The third model uses mice in which *loxP* sites, oriented in an opposite direction, are inserted upstream and downstream of exon 1, allowing Cre-mediated inversion of exon 1. These mice also carry a neomycine selection cassette in the first intron of the floxed AR allele, resulting in a hypomorphic phenotype (Holdcraft and Braun, 2004a). The mice with the floxed exon 1 are crossed with mice expressing an AMH-Cre construct developed in the laboratory of the same authors.

Table 1. Comparison of Sertoli cell-selective knockout models.

Model	$Ar^{flox(ex1\text{-}neo)/Y}$; AMH-Cre	$S\text{-}AR^{-/y}$	SCARKO
Mutated AR allele	inversion of exon 1+neo*	deletion of exon 2	deletion of exon 2
Development of male reproductive tract	normal	normal	normal
Testicular descent	normal	normal	normal
Testis weight**	60%	29%	28%
Seminal vesicle weight**	81%	ND	108%
Spermatogenic defect	transition RS to ES	block in meiosis	block in meiosis
Serum testosterone**	4210%	~33%	100%
Serum LH**	2214%	~450%	94%
Serum FSH**	259%	~115%	134%
Rhox5 (Pem) expression**	20%	ND	< 1%

* A neo cassette is retained in intron 1; ** Values expressed as % of normal male control.
ND: not determined; RS: round spermatids; ES: elongated spermatids.

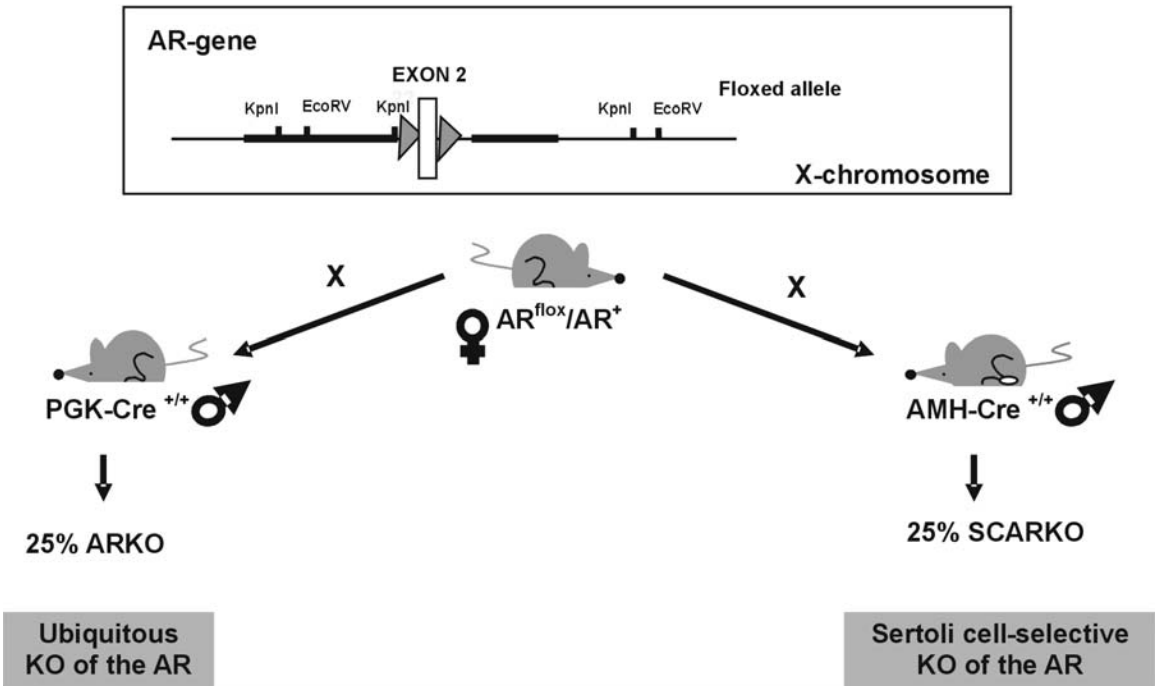

Figure 2. Generation of mice with a ubiquitous knockout of the androgen receptor (ARKO) or a Sertoli cell-selective knockout of the androgen receptor (SCARKO) using Cre/loxP-technology. Top panel: structure of the AR allele with a floxed exon 2, residing on the X-chromosome. The grey arrowheads indicate the location of the LoxP sites. Lower panel: crossing strategy to obtain ARKO and SCARKO mice. ARKO mice are produced by crossing females heterozygous for the floxed AR allele with male mice expressing the Cre recombinase ubiquitously under control of the phosphoglycerate kinase-1 promoter (Lallemand *et al.*, 1998). SCARKO mice are produced by crossing the same females with males expressing the Cre recombinase selectively in Sertoli cells under the control of the anti-Müllerian hormone promoter (AMH-Cre).

The two models with Sertoli cell-selective excision of exon 2 yield mice (referred to as S-AR$^{-/y}$ and SCARKO "Sertoli cell-selective AR knockout", respectively) with a similar phenotype: the animals are phenotypically male and show normal development of external and internal male sex organs. Body weight is identical to that of wild-type males (Fig. 3). The testes are normally descended but are reduced to less than 30% of the normal size. In both models, spermatogenesis is arrested at meiosis with a severe reduction of secondary spermatocytes and round spermatids and the absence of elongated spermatids. A noticeable difference is that, in the S-AR$^{-/y}$ mice, testosterone levels are markedly reduced along with an increase in LH and an increase in AMH mRNA expression (Chang *et al.*, 2004). In the SCARKO mice, on the contrary, testosterone and LH levels as well as AMH expression are normal (De Gendt *et al.*, 2004; Tan *et al.*, 2005). The reason for these differences remains unclear. Both strain differences and technical factors related to the measurement of the mentioned parameters could play a role.

The model with an inversion of exon 1 results in mice with a different phenotype (Holdcraft and Braun, 2004a). As mentioned above, male mice with a floxed exon 1 and a neomycine selection cassette in intron 1 (Ar$^{flox(ex1\text{-}neo)/Y}$) already display a hypomorphic phenotype. Their AR protein levels are markedly decreased, which results in a reduction in testis weight (reduced to 81% of the Ar$^{+/Y}$ control) and seminal vesicle weight (reduced to 80% of the control) and in a marked decrease of the number of sperm in the epididymis (4% of control values). The circulating levels of testosterone and LH are increased 40-fold and 24-fold, respectively. Interestingly, the reduction in AR activity apparently mainly affects the late stages of spermatogenesis near the time of spermiation. When Ar$^{flox(ex1\text{-}neo)/Y}$ mice are crossed with AMH-Cre mice, the Cre recombinase causes an inversion of exon 1 and accordingly a further inactivation of the AR selectively in Sertoli cells (Ar$^{flox(ex1\text{-}neo)/Y}$; AMH-Cre). This is reflected in a further decrease in testis weight (60% of the Ar$^{+/Y}$ control) and in a further decrease in the number of epididymal sperm (0.9% of control). The serum levels of testosterone and LH and the weight of the seminal vesicles remain comparable to the values observed in the Ar$^{flox(ex1\text{-}neo)/Y}$. The defect in spermatogenesis in these Ar$^{flox(ex1\text{-}neo)/Y}$; AMH-Cre animals is apparently mainly located at the level of the transition from round to elongated spermatids. Both the finding of a spermatogenic block during spermiogenesis

rather than during meiosis and the finding of a more limited reduction in testis weight and testicular Rhox5 (Pem) expression suggest that the Sertoli cell-selective AR ablation in the $Ar^{flox(ex1\text{-}neo)/Y}$/AMH-Cre model is less complete than in the two models with excision of exon 2. The fact that Cre-mediated inversion is in principle reversible might explain at least part of this discrepancy. The dramatic increase in testosterone and LH in the $Ar^{flox(ex1\text{-}neo)/Y}$/AMH-Cre model might further enhance the observed phenotypic differences.

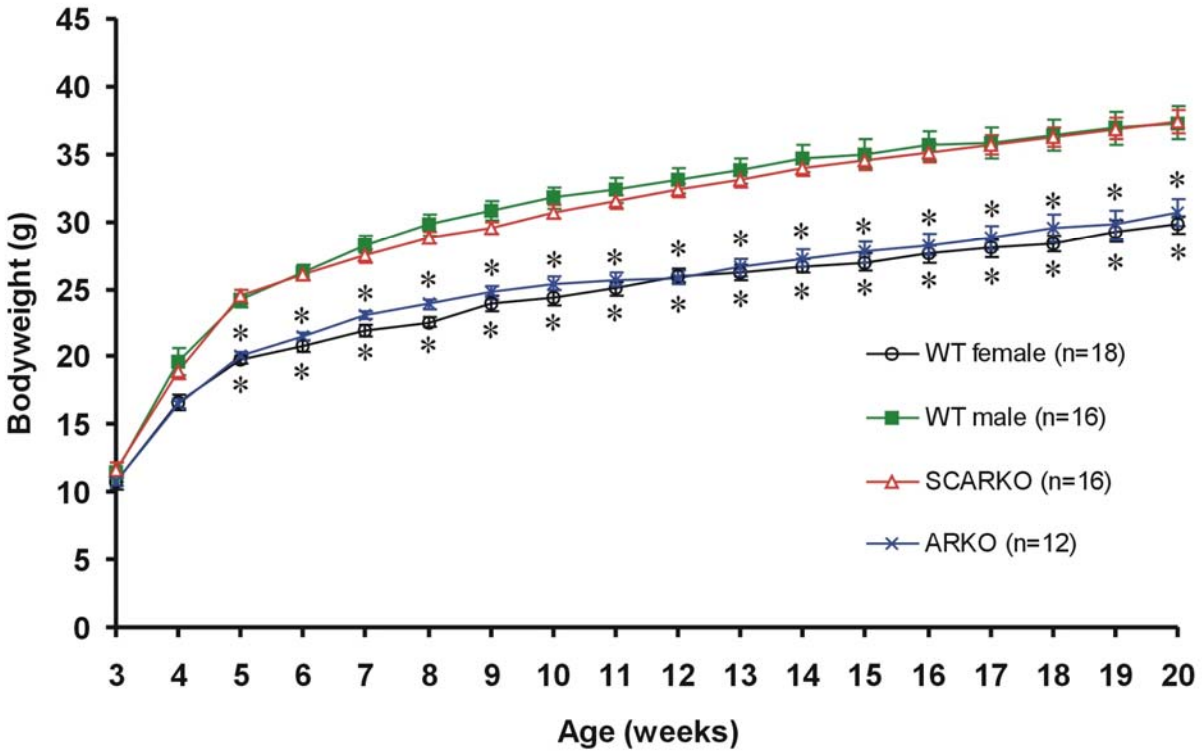

Figure 3. Growth curves of SCARKO, ARKO, and wild-type (WT) male and female mice from week 3 until week 20. Data are expressed as means ± SEM. *Significantly different ($P < 0.05$) from WT males by ANOVA supplemented with a Tukey-Kramer multiple comparison test. n = number of animals.

Consequences of Sertoli cell-selective androgen receptor ablation as studied in the SCARKO model

A detailed analysis of the effects of Sertoli cell-selective ablation of the AR on Sertoli cell, germ cell, and Leydig cell development and function has been made in the SCARKO model developed in our laboratory (De Gendt *et al.*, 2004; 2005; Tan *et al.*, 2005).

Interestingly, the number of Sertoli cells (as estimated from the Sertoli cell nuclear volume per testis) turned out to be comparable in SCARKO and control testes. This is in contrast to animals with a spontaneous mutation resulting in a ubiquitous knockout of the AR (Tfm or testicular feminized mice) or in transgenic mice with a general AR knockout (ARKO) both of which show a progressive reduction in the number of Sertoli cells from Day 2 onwards (Johnston *et al.*, 2004; Tan *et al.*, 2005; Scott *et al.*, 2007). Since cryptorchidism as such does not cause a comparable reduction in Sertoli cell number (Johnston *et al.*, 2004), these data suggest that androgens affect Sertoli cell proliferation at an early stage of development and that these effects are not mediated by the Sertoli cell AR. It is tempting to speculate that peritubular cells might act as the mediators of this effect. Several key genes in Sertoli cell development are apparently also not affected by the ablation of the AR. AMH expression, for instance, decreases postnatally in a comparable fashion in control and SCARKO testes. Time patterns of $p27^{kip1}$, SGP-2 (sulfated glycoprotein 2), and GATA-1 expression also are essentially comparable. Nonetheless, the expression of several genes with representative functions in Sertoli cells (Rhox 5, PDGF-A [platelet-derived growth factor A], claudin-11) is markedly reduced and microarray analysis reflects early repercussions on the expression of many other genes (Tan *et al.*, 2005; Denolet *et al.*, 2006a).

The most marked effects of Sertoli cell-selective AR ablation are noted in germ cell development. Sertoli cell AR-knockout testes show a marked reduction in seminiferous tubular diameter and in tubular lumen volume per testis (De Gendt *et al.*,

2004); the latter probably reflecting a defect in tubular fluid secretion, a known target of androgen action (Sharpe *et al.*, 1994). Stereological measurements of nuclear volumes per testis revealed that the ability of the Sertoli cells to support spermatogonial development is largely preserved but that the number of spermatocytes and round spermatids is reduced down to 64% and 3% respectively of the control level. Elongated spermatids are completely absent. The block in meiosis is confirmed by a reduction in the expression of meiotic markers such as Spo-11 (sporulation protein 11), Hsp70-2 (heat shock protein 70-2), SCP-3 (synaptonemal complex protein 3), and pro-acrosin binding protein down to 20-50% of control values, and an absence of the expression of postmeiotic markers such as transition proteins and protamines (Fig. 4). The block in meiosis is accompanied by a 5-fold increase in the number of apoptotic germ cells (De Gendt *et al.*, 2004; Tan *et al.*, 2005).

Surprisingly, ablation of the AR in Sertoli cells also markedly affects Leydig cell development and function. Despite the fact that androgen and LH levels in SCARKO mice are undistinguishable from those observed in control littermates, the number of Leydig cells in adult SCARKO testes is reduced by ~50% as compared to the control. This effect is apparently largely neutralized by an increase in Leydig cell size accompanied by an increase in mitochondria and lipid droplets and an enhanced expression of several steroidogenic enzymes (De Gendt *et al.*, 2005). The mechanisms responsible for these changes in Leydig cell development remain elusive. The decreased production of PDGF-A, a growth factor known to be involved in Leydig cell development, might be part of the explanation (Gnessi *et al.*, 2000). These effects may be reinforced by an increase in estrogen sulfotransferase activity in SCARKO Leydig cells leading to reduced estrogen action and increased testosterone production.

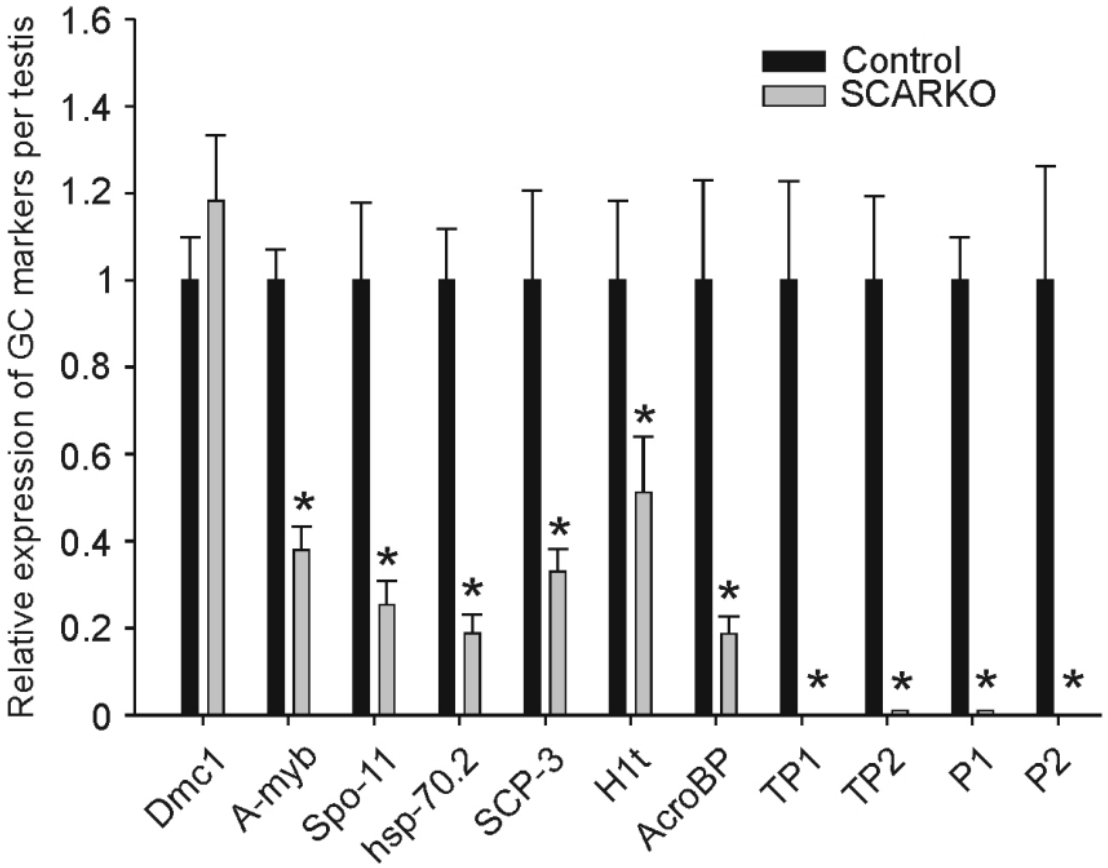

Figure 4. Changes in expression of germ cell meiotic and postmeiotic marker genes in 50-day-old SCARKO mice as compared to control mice. Gene expression is measured by quantitative RT-PCR and results are expressed as a fraction of the control, arbitrarily set equal to 1. Values shown are means ± SEM for 6-8 animals. Data indicated by an asterisk are significantly different ($P < 0.05$) from the respective control as assessed by a two-sample *t*-test. Meiotic marker genes: *Dmc1*, disrupted meiotic cDNA 1; *A-myb*, A-homologue of the transforming gene of avian myeloblastosis virus; *Spo11*, sporulation protein 11; *hsp-70.2*, heat shock protein 70.2; *SCP-3*, synaptonemal complex protein 3; *H1t*, testicular histone 1; *AcroBP*, proacrosin-binding protein. Postmeiotic marker genes: *TP1*, transition protein-1; *TP2*, transition protein-2; *P1*, protamine 1; *P2*, protamine 2.

Lessons from the SCARKO model

The SCARKO model demonstrates for the first time unambiguously that the Sertoli cell is the main mediator of the effects of androgens on germ cell development. In fact, the testicular phenotype of the SCARKO males reflects a defect that is as severe, or even more severe, than that observed in a variety of models in which testosterone levels and action in the testis are severely reduced, such as rats treated with implants releasing low doses of testosterone and estradiol (McLachlan *et al.*, 2002), or mice with a LH receptor knockout (Lei *et al.*, 2001; 2004; Zhang *et al.*, 2001). Furthermore, the model shows that the classical AR acts as the main mediator of these effects and that, at least for those effects of androgens needed to proceed through meiosis, alternative signaling pathways play no role or only a secondary role. Finally, the SCARKO model stresses that progression through meiosis is an important target of androgen action. The model cannot completely exclude that other androgen target cells may contribute to the control of spermatogenesis. In fact, some observations suggest that peritubular myoid cells may also play a role. The finding, for instance, that Sertoli cell number is reduced in mice with a ubiquitous knockout of the AR (Matsumoto *et al.*, 2003) but not in SCARKO mice suggests that androgens affect Sertoli cell proliferation but that this effect does not depend on the Sertoli cell AR (Johnston *et al.*, 2004; Tan *et al.*, 2005; Scott *et al.*, 2007). Similarly, the observation that transferrin mRNA levels are strongly reduced in ARKO mice and much less in SCARKO mice is compatible with the hypothesis that the effects of androgens on transferrin production in Sertoli cells may be mainly mediated by peritubular cells (Tan *et al.*, 2005). Recent experiments showing that animals with a knockout of the AR in peritubular myoid cells display a reduced testis size with oligozoospermia but normal fertility seem to confirm that peritubular cells may also contribute to the effects of androgens on germ cell development (Zhang *et al.*, 2006). Some caution may be needed in the interpretation of these data, however, since the Transgelin-Cre construct used to induce the AR ablation in peritubular cells may also inactivate the AR in vascular smooth muscle cells (Holtwick *et al.*, 2002), and since it has been demonstrated that testosterone may play a role in the control of testicular blood flow and vasomotion (Damber *et al.*, 1992).

It is obvious that androgens affect several steps in the complicated cascade of germ cell development. Recent studies have mainly focused on the adhesion of round spermatids to Sertoli cells, the conversion of round spermatids into elongated spermatids, and spermiation as important targets for androgen action (Sharpe, 1994; McLachlan *et al.*, 2002; Beardsley and O'Donnell, 2003; Wong *et al.*, 2005). The SCARKO model, however, stresses that androgen action is already critical to convert spermatocytes into spermatids. The reason that the SCARKO model focuses on meiosis as a target of androgen action is obviously the very early and complete ablation of the AR gene preventing any further germ cell development. In fact, the AMH-Cre construct becomes active and inactivates the AR gene from Day 15 of embryonic development onwards (Lecureuil *et al.*, 2002), whereas under physiological conditions AR expression in Sertoli cells is observed only from Day 5 after birth (Bremner *et al.*, 1994). As a consequence, meiotic progression is completely arrested during initiation of spermatogenesis precluding the study of effects of androgens on later stages of germ cell development.

Effects of androgens on meiosis have been observed in numerous other models such as hypophysectomized rats or rats in which pituitary gonadotropin secretion has been reduced to undetectable levels by administration of estradiol benzoate or clomiphene (Kalra and Prasad, 1967; Steinberger and Duckett, 1967; Steinberger, 1971). In most of these models, spermatogenesis proceeds only to the pachytene spermatocyte level and administration of testosterone or other androgens induces completion of meiosis and formation of spermatids (Kalra and Prasad, 1967; Steinberger and Duckett, 1967; Steinberger, 1971; Chowdhury and Steinberger, 1975; Chemes *et al.*, 1976). Hypophysectomy apparently does not result in a complete abolition of all androgen effects in the testis. In fact, treatment of hypophysectomized animals with the anti-androgen flutamide, causes a major decrease in the production of preleptotene spermatocytes and a significant reduction in cell numbers from preleptotene until late pachytene spermatocytes (Franca *et al.* 1998b). Also, in mice with a knockout of the LH receptor gene, testosterone treatment increases progression through meiosis and restores spermatogenesis (Lei *et al.*, 2004; Pakarainen *et al.*, 2005). In this model too, residual androgen action in the testis is present and can be neutralized by flutamide (Zhang *et al.*, 2003). Mice with congenital gonadotropin deficiency due to a major deletion in the GnRH gene (*hpg* mice) represent another exciting and relevant experimental paradigm. In these animals, postpachytene spermatocytes and spermatids are absent. Qualitatively normal spermatogenesis can be restored by androgen treatment (Singh *et al.*, 1995). Interestingly, dose-response curves suggest that completion of meiosis is the most androgen-sensitive step in germ cell development. In fact, at the lowest dose of testosterone studied (testosterone-filled silastic capsules of 0.06 cm), formation of round spermatids is already 79% maximal whereas elongated spermatids are only 17% maximal. These data suggest that identification of the androgen-regulated genes allowing progression through meiosis may be critical to understand the regulation and molecular basis of androgen-driven induction and maintenance of spermatogenesis (Handelsman *et al.*, 1999).

The SCARKO model offers a unique opportunity to identify these androgen-regulated genes. Differences in gene expression are already present on Day 10, a time at which there is still no evidence for morphological differences of the testes between SCARKO and control animals (Denolet *et al.*, 2006a). In this way, the model allows study of the effects mediated specifically by the AR in Sertoli cells under conditions in which the natural environment of the Sertoli cells, in particular the germ cell complement, is comparable to control (wild-type) animals. In addition, by comparing gene expression in SCARKO and control testes, effects of endogenous androgens rather than effects of non-physiological exogenous androgens are observed. A first series of microarray studies using SCARKO testes has allowed the identification of a set of 692 genes that are differentially expressed on Day 10 and that accordingly depend directly or indirectly on an active AR in Sertoli cells. For 28 of these genes, expression was at least 2-fold lower and for 12 at least 2-fold higher in SCARKO than in control testes. The physiological relevance of the identified genes was supported by the observation that some of them have previously been demonstrated to be essential for male fertility or to be androgen-regulated. For 9 genes selected from this series, androgen regulation could be confirmed in prepubertal mice treated with anti-androgens and in organotypical cultures. For 4 of the autologous rat genes, androgen regulation could even be confirmed in isolated Sertoli cells. The exact role of the corresponding genes is under study, but the available evidence suggests that several of them may be related to tubular restructuring and cell junction dynamics, suggesting that these processes may be important targets for androgen action in prepubertal testes. In this context, it is of interest to note that several other recent studies also point to effects of the Sertoli cell AR in maintaining tubular structure as well as Sertoli cell cytoskeletal organization, polarization, and barrier formation (Meng *et al.*, 2005; Wang *et al.*, 2006; Eacker *et al.*, 2007; O'Shaughnessy *et al.*, 2007).

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