Testicular dysgenesis syndrome: from human disorders to mechanistic studies in an animal model

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Disorders of male reproductive development are extremely common in humans. They may manifest either at birth (cryptorchidism, hypospadias) or in young adulthood (low sperm counts, testicular germ cell cancer). There is reasonable evidence that the incidence of most of these disorders has been increasing in recent decades and it has been hypothesised that they form a testicular dysgenesis syndrome (TDS), with a common origin in fetal life. Each of these disorders is a significant risk factor for each of the others and they all share common, pregnancy-related risk factors. It is hypothesised that the disorders arise as a result of maldevelopment of the testis which leads to malfunction of the developing Sertoli and Leydig cells in the fetal testis with the disorders resulting downstream from this cellular malfunction. We have used an animal model in which to explore this hypothesis which involves exposure of pregnant rats to high levels of dibutyl phthalate (DBP) during the last week of gestation. This treatment results in the male offspring in a high incidence of cryptorchidism, hypospadias and impaired spermatogenesis/ infertility in adulthood; it does not induce testicular germ cell cancer or its precursor, CIS cells, but it does induce significant changes to germ cell development in fetal life that has significant postnatal consequences, and these may have some similarities to the origins of CIS cells in the human.

DBP exposure *in utero* leads to profound impairment of fetal Leydig cell function which is manifest as suppression of testicular levels of testosterone and reduced expression of insulin-like factor 3 (Insl3), changes which probably account for the high incidence of cryptorchidism and the cases of hypospadias. There is also a significant reduction in Sertoli cell number by the end of gestation which may also result from the suppression of testosterone levels. However, perhaps the most unique and intriguing effect is that the DBP exposure leads to abnormal aggregation/migration of fetal Leydig cells towards the centre of the testis and this migration appears to interfere with the final phases of seminiferous cord formation and appropriate testicular cell segregation in the fetal testis. This then leads postnatally to the appearance of focal dysgenetic areas that contain malformed seminiferous cords and intratubular Leydig cells. Wherever the intratubular Leydig cells occur, no germ cells survive and this may partly explain the common occurrence of Sertoli cell-only tubules within the adult testis of rats exposed *in* utero to DBP. DBP exposure in fetal life also results in a delay in the normal phases of germ cell development; this is first manifest by delayed entry into quiescence coincident with prolongation of expression of the pluripotency factor OCT4. There are also effects on germ cell proliferation and apoptosis, resulting in a significant reduction in germ cell number at birth. Postnatally, when exposure to DBP has ceased, the affected germ cells show delayed activation of proliferation leading to a major decrease in germ cell number in early puberty. However, by adulthood in normally descended testes, normal germ cell numbers have been restored in DBP-exposed animals though they exhibit a very high rate of infertility. This might indicate that the germ cells have some sort of induced defect.

In addition to the description above, there are numerous other changes that occur in the developing testis and in the expression of specific genes and their encoded proteins and these probably explain some of the changes that are found (in particular in Leydig cell function). Overall, these findings in DBP-exposed rats provide strong support for the TDS hypothesis in humans and suggest that this animal model can be used to help identify the sequence of mechanistic changes that can occur during fetal life in the developing testis which then lead to increased risk of developing the downstream TDS disorders.

Gene expression and the development of male gametes

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The mechanisms involved in self-assembly of complex intracellular structures are poorly understood. An excellent example of this process is formation of the fibrous sheath (FS) during development of the sperm flagellum. It is a unique cytoskeletal structure underlying the plasma membrane, surrounding the outer dense fibers, and extending from the midpiece/principal piece junction anteriorly to the distal end of the principal piece posteriorly. The FS consists of two longitudinal columns connected by circumferential ribs. It assembles from distal to proximal, beginning during the early cap phase and continuing until near the end of spermiogenesis. Our strategy for understanding this process is to identify the individual protein components of the FS, determine when they are synthesized and incorporated into the fibrous sheath, and determine with which proteins they interact. Twenty-six proteins that are integral to or associated with the FS have been identified by us and other investigators. The genes for most of these proteins are expressed only during the postmeiotic phase of spermatogenesis. The majority of these proteins have a structural role, but some have both structural and functional roles, and others have only functional roles. The structural proteins include some that have no other known family members in the genome and some that are germ cell-specific members of diverse gene families. The genes for a few of the FS proteins have been disrupted by gene targeting and found to be essential for sperm to be fully motile, resulting in male infertility. Interactions between only a few of the FS proteins have been defined, but they appear to constitute the scaffold for most of the other FS proteins. However, we are still at an early stage of understanding how the FS self assembles.

Blood-testis barriers re-revisited: a homage to Prof. B. Setchell

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Commencing at the turn of the century, structural and functional data demonstrating the existence of permeability barriers between the blood and the inner part of the seminiferous tubules have been accumulating, giving rise to the concept of the blood-testis barrier (BTB). Structurally, tight junctions between Sertoli cells divide the tubule epithelium into 2 compartments: a basal compartment containing spermatogonia and primary spermatocytes in the early stages of meiotic prophase, and a luminal compartment containing the more advanced spermatocytes and spermatids. These junctions are involved in the polarisation of the Sertoli cells, form the "Sertoli-cell barrier" a key element of the BTB and are responsible for the generation of a luminal physico-cheminal environment different from that of the systemic circulation. In addition to the Sertoli-cell barrier, the concept of the BTB also encompasses the peritubular myoid cell layer in rodents and, more generally, the endothelial lining of the blood and lymphatic vessels in mammals, as stated in a classical review by Ploën and Setchell on this topic (*Blood- testis barriers revisited. A homage to Lennart Nicander. Int. J. Androl. 1992; 15:1-4).*

Although, it has been decades since the first experiments designed to study functional and structural aspects of the BTB were conducted, the deciphering of molecular support and mechanisms underlying the function of this barrier has progressed quite slowly. In contrast, over the same period of time, very significant progress has been made in understanding the molecular support of the blood-brain barrier (BBB). Central to this context is the discovery of the Multidrug resistance (MDR) genes encoding the P-glycoprotein (P-gp) in the endothelium of the brain capillaries.

In our presentation we will re-revisit the concept of BTB by showing how the BTB protects developing germ cells from the harmful effects of cytotoxic compounds (the MDR genes and related ATP-binding cassette transporter family of proteins), but also how the concept of BTB applies to protection against the deleterious influences of infectious agents, and how enzymes in the barrier can provide a catabolic barrier that prevent circulating retinoic acid and retinoic acid synthesized by Leydig cells to enter the seminiferous epithelium.

Interactions and influence of serotonin, corticotropin releasing hormone (CRH) and melatonin on Leydig cell function in the golden hamster

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It has become increasingly evident that disturbances of the hypothalamic-pituitary-testicular axis account for only a small percentage of male infertility cases (Bartlett *et al.*, 1989). A number of factors have been associated to testicular paracrine regulation but their physiological significance must be examined. In the last decades, the major topics on testicular research have been focused on regulation of steroidogenesis. Particularly, the *fine tunning* exerted by local substances at the cellular level has been taking into account.

In the last years, it has been described the presence and influence of neurotransmitters (N-T) on the gonadal activity at the different stages of sexual development. In addition to the well established actions of different N-T and neuropeptides in the vertebrate central and peripheral nervous systems (Gershon *et al.*, 1977), recent data indicate that significant levels of these compounds are also found in many non neural tissues including sexual organs (Verbeuren, 1989; Zhu *et al*., 1995).

In this context, serotonin (5-HT) has been described in the gonads and accessory reproductive organs of several species (Sowerbutts *et al.*, 1986). Our laboratory initially demonstrated the presence of 5-HT and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA) in rat testes, the influence of 5-HT on rat testicular steroidogenesis, and the existence of 5-HT specific binding sites (Campos *et al.*, 1988). 5-HT was found in rat testicular capsule, interstitial cells as well as in the interstitial fluid, but not in the tubular compartment (Campos *et al*., 1990).

The golden (Syrian) hamster is a seasonal breeder and consequently the pituitary-testicular axis undergoes annual cyclic variations (Desjardins *et al*., 1971; Bartke, 1985). Thus, the golden hamster represents a versatile experimental model where changes in the photoperiod can greatly alter male gonadal function. It is well known that when golden hamsters are exposed to short-day photoperiods (SP less than 12.5 h light/day) undergo a morphological and physiological testicular regression followed by a spontaneous recrudescence phase. The SP-induced gonadal regression is accompanied by a marked decrease in serum levels of FSH, LH and prolactin (Bex *et al*., 1978; Frungieri *et al.*, 1996) as well as a decrease of serum and intratesticular androgen concentrations (Chandrashekar *et al.*, 1989; Frungieri *et al.*, 1996). We have demonstrated that levels of 5-HT and 5-HIAA in hamster testicular parenchyma and capsule are significantly elevated at ages of 36 and 60-90 days, but decreased markedly during their exposure to SP. Thus, testicular 5-HT concentration increases at prepubertal and adult ages when high circulating levels of 3α-androstane,17β-diol (3α-Diol) and testosterone are detected (Frungieri *et al*., 1999). In addition, we have demonstrated that *in vitro* basal and hCG-stimulated testosterone production is significantly inhibited in presence of physiological concentrations of 5-HT via its binding to 5-HT2 and 5-HT1A receptors. This mechanism also involves induction of CRH secretion by 5-HT. In fact, CRH via CRH-R1 receptors has inhibitory actions on testicular steroidogenesis since incubation of hamster Leydig cells with the α-helical CRH antagonist partially or totally reverted the modulatory action of CRH, 5-HT and 5-HT1A / 5-HT2 agonists on the production of cAMP and testosterone (Frungieri *et al.*, 2002). Therefore, the previous results in rat testes (Tinajero *et al.*, 1992) and our data in hamsters, suggest that 5-HT acts as a regulator of the steroidogenesis during sexual development and the photoperiodic induced regression / recrudescence transition of the golden hamster.

It is known that mast cells contain 5-HT in their secretory granules. Frungieri *et al.* (1999) have shown that, in the golden hamster testes, mast cells are localized mainly in the capsule near to the blood vessels. Furthermore, the number of testicular mast cells increases in an age-dependent manner concomitantly with sexual maturation processes. On the other hand, the exposure to SP significantly decreased testicular mast cells number in the capsule. In addition, we also detected immunoreactivity of 5-HT and its key biosynthetic enzyme, tryptophan hydroxylase, in hamster Leydig cells supporting a role of this amine in the regulation of testicular steroidogenesis. Nerve endings in the testicular capsule and the spermatic arteries that penetrate into testicular parenchyma have also been postulated as testicular sources of 5-HT (Dufau *et al.*, 1993; Setchell *et al.*, 1994).

The pineal hormone melatonin (Mel) mediates the influence of photoperiod on the reproduction of many mammalian species.

Whether the day-light signal is interpreted as anti- or pro-gonadotropic will depend on a) the species, b) the duration of night Mel peak, c) the magnitude of the night Mel peak, and / or d) the window of sensitivity

to Mel (Arendt, 1988). Since the Syrian hamster is a seasonal breeder, Mel plays a key role in the regulation of the reproductive function (Bartke, 1985) through the hypothalamic-pituitary axis. The pineal Mel is released into the circulation almost entirely at night and reaches peripheral tissues including the testes. Moreover, there are evidences for the local synthesis of Mel in testes (Tijmes et al., 1996). We have investigated the role of Mel on testicular steroidogenesis in hamsters. Those studies showed an inhibitory effect of Mel on camp and androgen production via its binding to Mel1a receptors and its interactions with the local CRH system (Frungieri *et al.*, 2005). Previously, Mel binding sites have been found in rat and avian testes (Valenti *et al*., 2001).

Furthermore, the effect of Mel on testicular testosterone synthesis involves down-regulation of StAR and steroidogenic enzymes expression (P450scc, 3β-HSD, 17β-HSD) (Frungieri *et al.*, 2005).

N-T receptors are often co-localized on neuron membranes with other receptors, an activation of one receptor can either amplify or antagonize the response involving a co-localized receptor (Wang *et al*., 1999). In this context, the behavioural and biochemical effects of central 5-HT2A receptor activation are modulated by other 5-HT receptor subtypes (5-HT1A) as well as by stimulation of receptors of other N-T and hormones such as norepinephrine (β-adrenergic) and melatonin (Eison *et al.,* 1995).

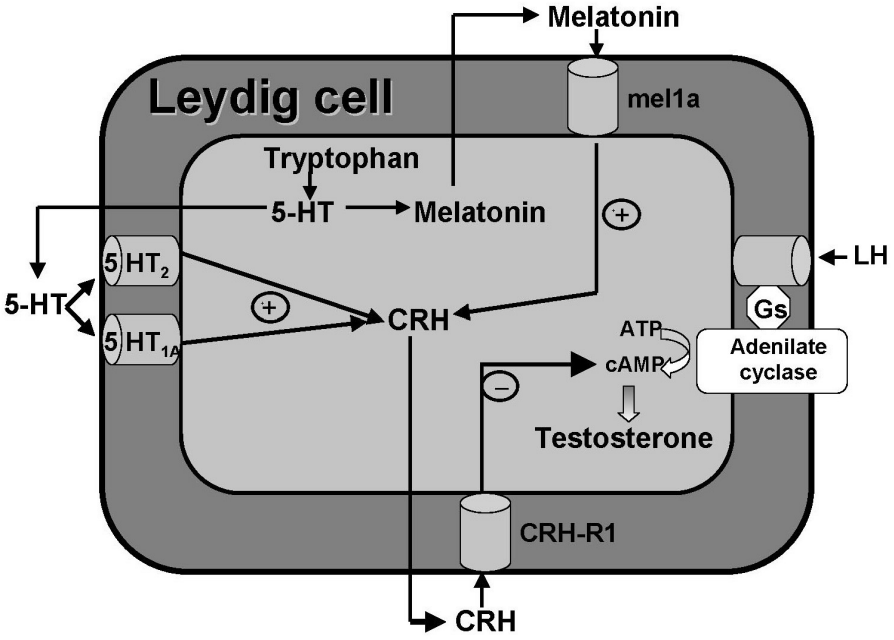

Figura 1: Schematic representation of testicular cross-interactions between serotoninergic, melatonergic and corticotropin-releasing system (CRH) in the golden hamster

Our studies have demonstrated for the first time a similar cross talk between N-T, hormones and factors at the testicular level. In this context, we have found that the serotoninergic and melatonergic inhibition of cAMP and testosterone production is exerted though specific receptors (5-HT2 and Mel1a, respectively) localized on Leydig cells by cross-interaction with the testicular CRH /CRH-R1 system (see details in Fig. 1) (Frungieri *et al.*, 2002; Frungieri *et al.*, 2005). Nevertheless, more studies are required before the biological relevance of our results and, consequently the role of local action of 5-HT, melatonin and CRH in the hamster testis can be placed in its proper perspective. We have also identified the expression of all components of those systems in human testes (Frungieri *et al.*, 2005) but whether their role in the seasonal breeder golden hamster can be extended to non seasonal reproductive mammalian species including man remains to be clarified.

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Androgens and spermatogenesis: lessons from a Sertoli cell-selective androgen receptor knockout

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Testosterone and FSH are the main hormones controlling germ cell development and a vast amount of experimental evidence indicates that quantitatively normal spermatogenesis requires both hormones. Under a number of conditions, however (hypophysectomized rodents, mice with hypogonadotropic hypogonadism due to a large deletion of the LHRH gene (*hpg* mice), man and mice with a mutated and inactivated FSH receptor, mice with an inactivation of the FSHβ gene…) androgens are able to initiate/maintain spermatogenesis and fertility in the virtual absence of FSH. Although the stages at which androgens affect spermatogenesis have been well delineated, the molecular and cellular mechanisms by which they exert their effects on germ cell development remain poorly understood. Spermatogenesis is a prototypical example of a process in which the effects of androgens require complex interactions between cells. Germ cells do not express the androgen receptor (AR) and cell autonomous action of the AR in germ cells is not required for normal germ cell development. Accordingly androgen action is most likely mediated by somatic cells such as Sertoli cells (SC) and peritubular myoid cells but their relative contribution remains a topic of investigation. Moreover, it is obvious that germ cells have major effects on the characteristics of these somatic cells including their androgen responsiveness.

To better delineate the role of the SC in the control of spermatogenesis by androgens we recently developed mice with a selective knockout of the AR in SC (SCARKO) (De Gendt *et al*., 2004). The mice were produced by Cre/*loxP* technology. Transgenic mice carrying an AR with a floxed exon 2 (produced in our laboratory) were crossed with mice expressing the Cre-recombinase selectively in SC under control of the anti-Müllerian hormone gene promoter (kindly provided by F. Guillou, Tours, France). In contrast with mice with a ubiquitous knockout of the AR (ARKO), SCARKO males displayed normal male development and had normally descended testes allowing exploration of the effects of AR inactivation without the confounding influence of cryptorchidism. The selective and complete absence of the AR in SC was confirmed by immunohistochemistry and by PCR measurements showing a complete absence of expression of the homeobox gene *Rhox5,* a marker of androgen action in SC. The testes of SCARKO mice were reduced in size (30% of control) and showed a block in meiosis with very low numbers of round spermatids (3% of control) and absence of elongated spermatids. SC number, however, and number of spermatogonia were essentially normal (Tan *et al*., 2005). Testosterone levels as well as weight of male accessory sex glands were undistinguishable from controls. Nonetheless, for reasons that need further investigation Leydig cell number was reduced (by some 40%) whereas Leydig cell size displayed a (compensatory) increase, with increased numbers of mitochondria and lipid droplets and increased expression of several steroidogenic genes (De Gendt *et al*., 2005). The SCARKO model showed for the first time unambiguously that SC act as the primary target for androgen action in the control of spermatogenesis and that the effects of androgens on spermatogenesis are largely mediated by the classical AR. Moreover it revealed that androgens are essential for the progression of developing germ cells through meiosis.

After the identification of the SC as the key target for androgen action in the testis the main challenge remains to unravel the molecular pathway(s) by which androgens affect germ cell development. Isolated and cultured SC respond poorly to androgens and rapidly lose the expression of many potentially relevant genes. The SCARKO model may provide a unique alternative to study androgen action in SC embedded in their natural microenvironment. The feasibility of this approach has been illustrated by microarray analysis of gene expression in testes from 10-day-old SCARKO and control mice (Denolet *et al*., 2006). At this age androgen action is already evident in SC but testicular cell composition is still comparable in KO and control testes. Statistical analysis identified 692 genes that are differentially expressed and accordingly that depend directly or indirectly on an active AR in SC. For 28 of these genes expression was at least 2 times lower and for 12 at least 2 times 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 regulated by

androgens. For a subset of genes androgen regulation was confirmed by quantitative PCR. Cluster analysis on microarray data from testes from SCARKO and control animals between the ages of 8 and 20 days allowed identification of several genes with an expression pattern strongly resembling that of *Rhox5*. Genes and functions overrepresented in the subset of 692 differentially expressed genes were identified using Onto-Express software. An intriguing observation is that several of the identified genes may be related to tubular restructuring and cell junction dynamics, suggesting that these processes may be important targets for androgen action in prepubertal mice.

It is concluded that SCARKO mice represent a powerful tool for the further analysis of androgen action in the testis

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Estrogens and the male reproductive tract

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It is an honor to participate in this 1st International Symposium on Animal Biology of Reproduction, which is given in honor of Dr. Brian P. Setchell, a true renaissance scientist whose publications have influenced every reproductive physiology graduate over the past 30 years. Historically, it has been known from the 1930's that developmental exposure to high dosages of estrogens could induce malformations in the male reproductive tract and that in some species high concentrations of estrogen are excreted in the urine [23, 29]. By the 1970's, estrogen synthesis in the testis had been well established and estrogen receptor (ER)-like proteins were found in the epididymis. Several species exhibited remarkably high levels of estrogens in either the rete testis and testicular lymphatic fluids or semen [10, 11, 22, 23, 25]. In 1982, Dr. Setchell published concentrations of oestrone sulphate in spermatic venous blood and lymph ranging from 400 to 1500 ng/ml in the adult stallion [37]. Yet, as late as the early 1990's, a function for estrogen in the male reproductive tract remained elusive. However, as for many other fields of study, the arrival of new technology through targeted gene disruption, improved immunohistochemical antibodies and more selective chemical ER modulators, permitted a burst of new discoveries that have now established specific physiological functions for estrogen in the adult testis and male reproductive tract. Some major discoveries include the following:

a) Aromatase and estrogen synthesis was shown in spermatids and spermatozoa, as well as Leydig cells, which provided a source of estrogen in rete testis fluid [1-11, 16, 19-25, 27, 38];
b) Immunohistochemical staining for ER demonstrated an abundance of ERα, particularly in the efferent ductule epithelium of every species examined [22, 23, 25];
c) ERβ was localized in all tissues of the male reproductive tract, but a distinct function has not been found [18, 22, 23, 26], although others have shown that estradiol treatment of the hypogonadal (*hpg*) mouse results in qualitative spermatogenesis, suggesting an effect on ERβ in Sertoli cells [13];
d) ERα knockout mouse (ERKO) and antiestrogen-treated rodents were infertile, with subsequent back-pressure atrophy of the testis [12, 17, 22, 23, 25, 28, 30, 33];
e) ERα disruption induced complete inhibition of fluid reabsorption by efferent ductule epithelium, the mechanism involving decreases in NHE3 and aquaporins and loss of epithelial microvilli [17, 22, 23, 25, 30, 33, 36];
f) The aromatase knockout mouse (ArKO) exhibited disruption of spermatogenesis but only with aging, but also enhanced with soy-free diet [29, 35];
g) ER antagonist ICI 182,780 blocked estradiol's stimulation of qualitative spermatogenesis in the hpg mouse, which raises the possibility for an ERβ function in the testis [13].

Although many discoveries have been made, there appears to be a lull in the further advancement of our understanding of estrogen function in the adult male since the early 2000's. This could be in part due to the resurgence of studies in environmental toxicology, with new concerns over endocrine disruptors and environmental estrogens, which greatly improved our understanding of male reproductive tract sensitivity toward estrogen and estrogen-like compounds. It may also reflect a continuation of the underlying assumption by some reproductive endocrinologists that "The presence of ERs in the immature and adult male reproductive tract may be residual from the stem cells of which the tract is derived and may be essential for the development of the male reproductive tract"[15]. From a clinical standpoint, some do not consider the epididymis to be so very important, as the assisted reproductive technologies (ART) have allowed medicine to fertilize eggs *in vitro* with testicular and caput sperm, thus bypassing epididymal function.

The future of this interesting field of research is difficult to predict, but hopefully some of the following areas of study will lead to a better understand of the mechanisms of estrogen action in the male:

a) Localization of ERα and β and aromatase in the male reproductive tract of new species. ERα in particular is found in different regions in different species [23, 25]. Recently, aromatase was found expressed in Leydig and Sertoli cells of the turtle testis and ERα was shown in the testis and epididymis [14].
b) Regulation of ER expression in the male reproductive tract. Our recent study suggests that in the male tract, ERα and β have constitutive expressions and may function in the absence of

endogenous estradiol. However, exogenous estradiol downregulates ERα [31, 32].

c) Recognition of nuclear steroid receptor coactivators and corepressors within the male reproductive tract. Our recent studies have localized several cofactor proteins that are uniquely positioned within the male tract and may be responsible for species differences in response to antiestrogens (unpublished data). These data help to explain tissue and chemical-specific responses to estrogens and antiestrogens.

d) Other steroid metabolites. DHT and estradiol are considered to be the major metabolites of testosterone, but other compounds, such as 5α-androstane-3β-17β-diol (3β-diol), a metabolite of DHT, may have greater influence on male reproduction than previously considered. 3β-diol does not bind androgen receptor, but rather ER and thus may alter the balance of steroid function in the male reproductive system during variations in testosterone and estrogen levels [34].

In conclusion, it has been shown that estrogen's abundance in the male reproductive system has classical receptor mediated targets of action, but ERα and β show species and tissue-specific expressions. It is now well-established that ERα activation is essential for male fertility but a specific function for ERβ remains to be clearly demonstrated. Although many questions remain and conflicting data are noted in the literature, current studies are focused on novel aspects of estrogen receptor activity and regulation in the male reproductive tract.

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From brain to gonad: new neuropeptides emerge

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Introduction

Reproduction in vertebrates depends on release of several different neuropeptides from the brain. The classical neuropeptide is gonadotropin-releasing hormone (GnRH), which binds to its receptor on pituitary cells, gonadotropes, thereby activating the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). In turn, these hormones activate gametogenesis and steroidogenesis. In addition, GnRH is expressed in the gonads as a local factor.

Novel GnRH peptides

The first GnRH peptide (GnRH-I), isolated from pig brain, was ten amino acids in length. An identical form was isolated from other mammals, frogs and sturgeon. Eventually, distinct GnRH forms were isolated from salmon, chicken and other vertebrates. To date, 14 distinct GnRH peptides are identified by, protein, cDNA or gene structure. Common to GnRH peptides is length, presence of signature amino acid sequences at the N- and C-termini and conserved post-translational modifications. A striking discovery was that most vertebrates from fish to human each have two or three forms of GnRH in the brain. Humans share their GnRH-II form with bony fish, amphibians, reptiles, birds and some mammals; although rodents, sheep, cow and chimpanzee have lost the gene or a functional form of GnRH-II.

New GnRH receptors-new targets

The GnRH receptor is a G protein-coupled receptor, originally isolated from mouse (GnRH-RI). Binding of GnRH-I to this receptor triggers an intracellular signaling pathway, the inositol triphosphate path. A second type of GnRH receptor (GnRH-RII) was discovered later; activation is strongest with the ligand GnRH-II. Not understood is why most mammals including rhesus and green monkey have a functional type II receptor but human, chimpanzee, rodents, cow and sheep do not. In contrast, some fish have multiple forms of the GnRH receptor; zebrafish, for example, express four GnRH receptors. Among these receptors, there is some selectivity for their ligands and some distinct localization. Hence, the target tissue for GnRH can vary from known reproductive tissues (brain, pituitary, gonad) to tissues not thought to be directly related to reproduction (e.g., eye, skin, gill).

Kisspeptin, upstream of GnRH

A recently discovered neuropeptide, kisspeptin (metastin), is present in the brain and acts on GnRH neurons to control GnRH release. The kisspeptin receptor, also a seven-transmembrane receptor coupled to a G protein, is located on GnRH neurons. Thus, to date, kisspeptin is the most upstream regulator of reproduction if viewed from the brain to gonad.

Direct action of GnRH in gonad

GnRH is proposed to act also as a local factor in the gonads. From fish to humans, GnRH cDNAs have been isolated from gonads but the level of expressed protein is very low. The origin of a direct action of GnRH on the gonad is well rooted in evolution. Ancestral to vertebrates, the protochordates lack a pituitary but have GnRH receptors; they respond to injections of protochordate GnRHs with the rapid release of gametes.

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Constancy of the germinal epithelium in vertebrates

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Germ cells originate from a specialized epithelium, the germinal epithelium. Both male and female germinal epithelia are composed of somatic cells and germ cells. In males, the somatic cells are the familiar Sertoli cells which form the borders of spermatocysts in some amphibians, teleosts, and lower vertebrate taxa. They surround individual developing sperm in higher taxa. In females, the germinal epithelium is composed of epithelial cells, oogonia, and oocytes in early development. In fishes with cystovarian ovaries these cells line the ovarian lumen and in amphibians, they cover the ovarian surface. The epithelial cells become prefollicle cells when associated with oogonia that have entered meiosis and become oocytes. At the completion of folliculogenesis, they are the familiar follicle cells that surround the oocyte in a follicle.

During the annual reproductive cycle of fish, changes in the testicular germinal epithelium have been used to determine reproductive classes. This is possible because regressed, early maturation, mid maturation, late maturation, and regression classes can be recognized in histological preparations based upon stages of germ cells present and whether continuous and/or discontinuous germinal epithelia are present. The criteria for basing annual reproductive classes upon the morphology of the germinal epithelium began with common snook but have not yet been applied to other fish on a consistent basis or applied to other vertebrates. However, they can be used to determine the reproductive classes of perciform fish such as seatrout (*Cynoscion* nebulous, Sciaenidae), mullet (*Mugil cephalus*, *M. curema*, *M. gyrans*, Mugilidae), southern puffer (*Sphoeriodes nephelus*, Tetraodontidae), black seabass (*Centropristis striata*, Serranidae), and *Micropterus salmoides*, Centrarchidae) and may be used to determine reproductive classes of all perciform fish. Because the testes in the lower vertebrates (such as hagfish, lampreys, and elasmobranches) are polyspermatocystic, other criteria for assignment of reproductive classes must be developed. Our experience shows that the germinal epithelia in higher vertebrates (reptiles, birds, and mammals) cannot be classified as continuous or discontinuous.

In females, the germinal epithelia are always discontinuous. Individual or small groups of germ cells in cell nests are scattered among the epithelial cells. It is characteristic for oogonia to produce meiotic oocytes in the female germinal epithelium before puberty; conversely, spermatogonia produce meiotic germ cells (spermatocytes) during and after puberty. Our work and that in the literature indicates that the female germinal epithelium and the process of folliculogenesis are consistent between vertebrate taxa, i. e., ovarian follicles in vertebrates are homologous structures. They should all be defined identically.

The glycolytic engine fuels sperm motility

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There are a surprising number of glycolytic enzyme variants in mammalian sperm, including several with restricted expression in the male germline. New features of this central metabolic pathway continue to be uncovered. We recently identified two novel aldolase A variants in mouse sperm that are encoded by intronless retrogenes and a third splice variant with a distinctive N-terminus. Multiple glycolytic enzymes are localized in the principal piece, which is the longest segment of the sperm flagellum. At least four of these enzymes are anchored to the fibrous sheath, along with a number of key components of signal transduction pathways. This compartmentalization and enzyme diversity suggests that energy production in mammalian sperm may be regulated by novel mechanisms.

Our gene targeting studies of two isozymes expressed only during spermatogenesis indicate that glycolysis is essential for maintaining sperm motility and male fertility in the mouse. Males lacking glyceraldehyde 3-phosphate dehydrogenase-S (GAPDHS) are infertile and produced sperm that do not exhibit progressive motility. Mating behavior, testis histology, testis weights, seminal vesicle weights and general characteristics such as body weight are normal in these mice. Although indistinguishable from wild-type sperm at the light microscopic level, sperm lacking GAPDHS have subtle ultrastructural defects in the fibrous sheath, with wider spacing between some of the circumferential ribs. Moreover, sperm ATP levels are only 10% of wild-type levels immediately after isolation from the cauda epididymis. These levels decline further within 30-60 min, while ATP levels of wild-type sperm are maintained for at least 4 h when incubated under identical conditions. Oxygen consumption is not altered, indicating that mitochondria are functional in sperm lacking GAPDHS but cannot provide sufficient ATP to support motility and fertilization. We also produced mice lacking phosphoglycerate kinase 2 (PGK2). This isozyme catalyzes the reaction immediately after GAPDHS in the glycolytic pathway in sperm. We expected the reproductive phenotype of *Pgk2*$^{-/-}$ mice to be identical to *Gapdhs*$^{-/-}$ males, without defects in the fibrous sheath since PGK2 is not tightly bound to this cytoskeletal structure. As expected, sperm motility and male fertility are severely impaired in *Pgk2*$^{-/-}$ mice and fibrous sheath ultrastructure is indistinguishable from wild-type sperm. However, the *Pgk2*$^{-/-}$ males sire occasional small litters. In further comparisons, we found that sperm motility and ATP levels are consistently higher in sperm from *Pgk2*$^{-/-}$ males during the first 30 min after isolation from the cauda epididymis. These studies suggest that glycolysis may be regulated by distinct mechanisms in male gametes and confirm the importance of this metabolic pathway for sperm energy production and function.

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Seminal plasma proteins: from the cauda epididymis to the site of fertilization

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Seminal plasma is a complex milieu containing basically spermatozoa and fluids of cauda epididymis and accessory sex glands. The fundamental tenet is that several proteins of these fluids interact with sperm and modulate diverse sperm functions in both adult and prepubertal males. In this regard, ongoing studies are dedicated to generate comprehensive protein maps of these fluids; investigate prospective associations between those proteins and fertility; and understand how such proteins interact with sperm and affect the process of fertilization. Major proteins of cauda epididymal fluid (CEF) have been identified as albumin, carboxylesterase-like urinary excretory protein, cholesterol binding protein, glutathione peroxidase, clusterin, prostaglandin-D synthase, gelsolin, N-acetyl-β-glucosaminidase and transferrin, in addition to several other low abundance constituents (Moura *et al*., 2006a). General attributes of many of these proteins suggest they are important for sperm function (Gatti *et al*., 2004). Proteins of accessory sex gland fluid (AGF) include albumin, acidic seminal fluid protein, ADP-ribosyltransferase 5, BSP proteins, cathepsin L, clusterin, ecto 5'-nucleotidase, tissue inhibitor of metalloproteinase 2, nucleobindin, osteopontin, phospholipase A_2 and spermadhesin Z13 (Moura *et al*., 2006b). Contact of epididymal sperm with AGF at ejaculation induces complexes changes in the protein profile of sperm membrane and, in general, knowledge about functions of AGF proteins is certainly more solid than in the case of CEF proteins, as they are certainly involved in capacitation, sperm motility, protection against oxidative mechanisms and acrosome reaction. We have demonstrated that a select group of cauda epididymis and accessory sex gland fluid proteins is related to fertility. Sires with superior fertility expressed more cathepsin D and α fucosidase and less prostaglandin D synthase in the CEF; and more BSP 30 kDa, osteopontin and phospholipase A_2 and less spermadhesin Z13 in the AGF (Moura *et al*., 2006c,d). Enhancing effects of accessory sex gland fluid on oocyte-penetrating capacity of epididymal sperm in vitro related to higher amounts of albumin, BSP 30 kDa, osteopontin, phospholipase A_2 and clusterin and lower of nucleobindin and spermadhesin Z13 in the AGF itself (Moura *et al*., 2006e). It is intriguing that some proteins are linked with aspects of fertility assessed both in vivo and in vitro, and current challenges are focused on understanding their physiological relevance. For instance, BSP proteins represent 86 % of all AGF proteins (Moura *et al*., 2006b) and mediate sperm capacitation and sperm binding to the oviductal epithelium (Manjunath and Thérien, 2002; Gwathmey *et al*., 2006). BSPs bind to midpiece, equatorial and acrosome region of sperm. The presence of nucleobindin in male fluids was first reported by our recent publications (Moura *et al*., 2006b,e) and it seems to bind to sperm. Nucleobindin contains Ca2+ binding motifs (Wendel *et al*., 1995) and, interestingly, was originally found as a structural element of bone extracellular matrix (Peterson *et al*., 2004). These features resemble those of osteopontin (OPN), another bone protein identified in the bull accessory sex gland fluid. The expression of OPN is 4.5 times greater in high than in low fertility sires, making it one of the most significant seminal plasma markers of reproductive performance (Cancel *et al*., 1997; Moura *et al*., 2006d). OPN adheres to post-equatorial segment and acrosome of ejaculated sperm, but only to the former after sperm is in contact with isthmic and ampullary oviductal fluid. Indirect immunocytochemistry also demonstrates OPN binding to the zona pellucida and probably the oolema (unpublished results) and antibodies against OPN reduces sperm-oocyte binding, fertilization and development of bovine embryos (Goncalves *et al*., 2003, 2006). Following this same line of research, enhancing effects of OPN on fertilization of swine oocytes have been demonstrated (Hao *et al*., 2006). Such findings support empirical associations between OPN and fertility, but gaps still exist concerning how OPN connects with sperm and potentially influence gene expression in the embryo. In summary, factors of paternal origin interact with sperm before and after ejaculation, affect sperm physiology in the female reproductive tract and early embryonic development. In addition to experiments conducted with adult bulls, studies using young rams indicate that expression of key seminal plasma proteins is timely orchestrated, since early prepuberty, with developmental states at which sperm acquires motility. The picture emerging is that such proteins probably signify crucial phases of epididymal and accessory sex gland development. Information gained from all these fields will help us to better understand mechanisms that define male fertility and selection of superior sires.

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Androgens and androgen receptor in prostate development and regulation

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The prostate gland is an accessory sex gland and as such is largely dependent on androgens for its induction, development and functioning. Perhaps most importantly, prostatic tumor growth is likely dependent on androgens and androgen receptors. In this presentation I will review some aspects of prostatic development which is accordingly dependent on androgens and represent windows for endocrine disruption, leading to underdevelopment or predisposes the organ to prostatites and cancer, adding some new results from our laboratory. The fetal growth phase results from the androgen production by the fetal testis and is susceptible even to the fetal positioning with respect to adjacent male and female fetuses, exposing the organ to up to 30% higher levels of estrogen which, besides to androgens, also affects prostatic physiology. The early postnatal growth seems to result from a testosterone surge taking place immediately after birth. This phase results from intense proliferation and is associated with important morphogenetic events such as branching and ductal canalization of previously solid cords. This phase is particularly sensitive to estrogenic endocrine disruptors, as they might affect prostatic growth in opposite directions depending on estrogenic potency and dosing, leading to pubertal prostate enlargement or growth inhibition due to androgen insensitivity. Before puberty the organ is largely quiescent though responding to somatotrophic stimuli as the organ parallels the body weight gain[1]. This period is largely insensitive to androgen blockade or estrogen exposition, though sensitive to androgens. At puberty the organ respond to the increasing testosterone levels acquiring its final size and function. High dosis testosterone in adult predisposes the organ to carcinogens. Androgen deprivation promotes time dependent regressive changes in the organ, with marked epithelial and stromal remodelling[2,3]. High estrogen exposure in adults has both indirect (via hypothalamus-pituitary-gonad axis) and direct (via estrogen receptors) effects decreasing size and function[4]. I will present three series of results which are directly concerned to the current subject: (1) insulin modulation of androgen receptor expression in smooth muscle cells; (2) the effect of dietary fat acid on prostatic growth and (3) the effect of a hyperandrogenic environment on the growth and displastic growth of the female prostate. Working on isolated prostatic smooth muscle cells one observed that these cells express very low levels of androgen receptors, that estrogen has distinct effect on androgen receptor expression in the presence or absence of testosterone and that insulin modulates the levels of AR protein, thus modulating the ability of the cells to respond to the same testosterone levels. Dietary fat is a prognostic to prostate cancer. It is apparent that saturated fatty acids might predisposes to prostate cancer. We have shown the fatty acid migh have opposite effects on prostatic growth. Using linseed oil (up to 52% polyinsaturated fatty acid) we restricted prostatic growth while feeding rats with pork fat (saturated fatty acid) we promoted prostatic growth, which included epithelial hyperplasia. The effects were associated with variations in the androgen receptor expression and then responsivity to androgens, and also PPARγ. Finally, we have demonstrated that the female Mongolian gerbil possess an underdeveloped prostate[5], which is active under normal hormonal conditions, but remains at a limited size. We have then chalenged the organ with a high dosing of testosterone, simulating the hyperandrogenic condition of certain women[5]. The results demonstrate that the female gerbil prostate express the androgen receptor and respond to testosterone, increasing its size, modifying the proportion of epithelial luminal cells and showing signs of displastic growth. It was then concluded that prostatic overgrowth and displasia should be included in the symptons of hyperandrogenism.

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Epididymis as a model to study innate immunity and the role of antimicrobial proteins in the male reproductive tract

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In the rete testis spermatozoa are incapable of forward motility or oocyte fertilization, capacities they acquire in the epididymis. The epididymis contains a highly specialized coiled tubule wherein the spermatozoa mature as they pass through the caput and corpus to be stored in a decapacitated state in the cauda. In this functional role, the epididymis would be expected to express proteins involved in host defense, including defensins that protect the maturing sperm throughout development and maturation. Defensins are small cationic peptides involved in innate host defense against invading pathogens. β-defensins, the oldest defensin subfamily, display a particular cysteine spacing and pattern of intramolecular bonding and have both antimicrobial and cell signalling functions. They are found primarily in several epithelial tissues in contact with the environment, including the reproductive tract of mammals and invertebrates. Expression of a broad range of β-defensins in the male reproductive tract of different species and on sperm surfaces in the epididymal lumen and in ejaculate have also suggested their possible role beyond innate immunity, in events related to male fertility. In humans, many β-defensin genes are located within a cluster on chromosome 8p23. The *SPAG11* (sperm associated antigen 11) gene [also known as *EP2* in monkey, *HE2* in human, and *Bin-1b* (*Spag11e*) in rats], is contained in this cluster and is unusual among human β-defensins because of its complex genomic structure and mRNA splicing pattern. Different from the classical primate defensin genes, *SPAG11* is a single gene derived from 2 ancestrally independent β-defensin genes joined by read-through transcription governed by promoter choice (promoters A and B) and species-specific exon recruitment mechanisms that result in at least 20 alternatively spliced mRNAs differentially expressed along epididymis and other tissues of the male reproductive tract. In a combination of activities unique to male tract host defense proteins, SPAG11 isoforms and other β-defensins have been shown not only to kill bacteria, but also to interact with spermatozoa affecting motility and zona-pellucida recognition. The fundamental contributions of both activities to animal health and productivity prompt us to investigate the structure and function of the *SPAG11* gene in cattle (*Bos taurus*). The bovine SPAG11 gene maintains features observed in primate including: 1) conserved chromosomal location within a cluster of β-defensin genes on chromosome 27q1.2; 2) conserved fusion gene structure producing at least 6 transcripts initiated at both A (*SPAG11C*, *SPAG11D*, *SPAG11U-W*) and B promoters (*SPAG11E*); 3) species-specific exons giving rise to transcripts not previously found in primates; 4) dominant constitutive expression of transcripts in tissues from the male reproductive tract; 5) developmental regulation of transcript expression in reproductive and non-reproductive tissues from fetal and adult bulls, 6) presence of protein (*SPAG11D and C*) in the epithelium of epididymis and testis including in late stage of spermatids, Sertoli cells and on isolated epididymal spermatozoa; 7) *in vitro* antibacterial activity for recombinant full length SPAG11D isoform and its C-terminal peptide. Thus, the forces that determine SPAG11 structure and function in these mammalian lineages may be related to the evolution of SPAG11 isoforms to perform both immune and reproductive functions. Questions raised by these results are "Do SPAG11 gene products have a role during the response of the male reproductive tract against microbial infection?" and "What are the factors involved in the regulation of SPAG11 gene expression?". We are conducting investigations to address these questions using as experimental model the Wistar rat epididymis challenged in vivo and in vitro with lipopolysaccharide (LPS) from E. coli. These investigations will have important implications for our understanding of innate immunity in the male reproductive tract.

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The epididymis as a target organ for toxic substances

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The epididymis is the organ in which sperm mature by developing the capacity for progressive motion and fertilizing ability. The process of maturation is orchestrated via complex interactions between the epididymal epithelium, luminal fluid, and sperm. Nowadays there is heightened awareness about the possibility that sperm number and quality are declining in the human population as a consequence of exposure to environmental pollutants. Thus far, all attention has been focused on the testis as the "target" organ, but it should take in consideration that if a toxicant accelerates sperm transit time through the epididymis, less sperm will be available for ejaculation. Moreover, under such conditions, sperm would have less opportunity to undergo the maturational process. While many toxicants have been shown to produce alterations in epididymal sperm, of the hundreds of known or suspected male reproductive toxicants tested to date, relatively few have been linked with epididymal toxicity due to the fact that it is inherently difficult to establish that a toxicant exerts its direct action on the epididymis. One way to control experimentally for any testicular factor contributing to an observed toxicity in the epididymis is to evaluate sperm fertilizing ability in the proximal cauda epididymidis shortly following the onset of toxicant exposure, using *in utero* insemination[1]. In the rat, sperm are evaluated 4 days after exposure as this period of time is required for the sperm to travel from the caput to the proximal cauda. Using this protocol, we showed that gossypol, a potential male contraceptive, produces direct effects on the epididymis[2]. Among the first compounds linked with epididymal toxicity are α-chlorohydrin, methyl chloride and cyclophosphamide. The antifertility effects of these compounds were seen within few days of dosing. While most studies demonstrate toxic effects on the epididymis following postpubertal exposure, an increasing number of studies are providing data to support the notion that the developing epididymis is extremely sensitive to toxic insult. Recently, we showed that the epididymis concentrates fenvalerate, a pyrethroid insecticide, both after *in utero* and adult exposure. In this talk we will focus on results of our laboratories, showing the epididymis as a target organ for toxicants. The use of SP22[3], a sperm membrane protein, that is a potential biomarker of fertility, will also be discussed.

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Development of Transplanted Spermatogonia and Sertoli Cells in Irradiated Testes

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Testicular cell transplantation has been used to investigate the biology of spermatogonial stem cells and restore fertility in mice whose own germ cells were genetically defective or damaged by cytotoxic treatment. The preparation of the recipient testes is an important step for efficient transplantation and, although busulfan has been widely used in rodents, it would not be practical in larger species. Localized irradiation is an excellent alternative. The efficiencies of transplantation and development of donor spermatogonial stem cells into colonies of differentiated spermatogenic cells were similar when testicular germ cells from prepubertal mice or rats were transplanted into the irradiated mouse testes or into testes of busulfan-treated mice.

But not all species are like the mouse. In the rat, and particularly in certain inbred strains, spermatogonial stem cells that survive irradiation or chemotherapy treatment, proliferate but undergo apoptosis as they try to differentiate. However, it was not known whether the radiation damage to the rat testes that resulted in blocking spermatogonial differentiation was due to damage to the somatic cells or the spermatogonia. Transplantation of stem spermatogonia from irradiated adult rats into the testes of irradiated nude mice, which do not show the differentiation block of their own spermatogonia, permitted differentiation of the rat spermatogonia into spermatozoa. Conversely transplantation of spermatogonial stem cells from untreated prepubertal rats into irradiated rat testes showed that the donor spermatogonia were only able to colonize along the basement membrane of the seminiferous tubules but could not differentiate. Thus the radiation-induced block to differentiation in rat testes is due to injury to the somatic compartment, not the spermatogonia. We were able to modulate the effects of that injury to the somatic compartment by suppression of testosterone, which allowed the differentiation of the transplanted stem spermatogonia from donor rats, as well as the endogenous surviving stem cells.

In addition to colonization by spermatogonial stem cells, transplantation of tubule cells from prepubertal rats into the seminiferous tubules of irradiated rats also resulted in colonization by donor Sertoli cells. The Sertoli cells produced at least three different types of colonies: (1) spherical structures in the lumen surrounding a core of unidentified cells, (2) filling tubules that were divided into minitubules by fibroblastic or peritubular-like cells, and (3) a single epithelial cell layer along the basement membrane. When the tubule cells were transplanted into the interstitial space, the donor Sertoli cells from the immature rats formed irregular, but otherwise normal-appearing, tubular structures. Surprisingly, both intratubular colonization by Sertoli cells and interstitial development of donor-derived tubules stimulated endogenous surviving spermatogonia in adjacent tubules to differentiate.

These results have implications for the proposals of autologous transplantation of cryopreserved spermatogonia, harvested from prepubertal testes prior to radiation or chemotherapy, back into the testicular tubules after puberty. The success of such a procedure might be limited by somatic damage and may require hormonal treatments or transplantation of somatic elements to restore the ability of the tissue to support spermatogenesis.

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Reproductive functions in long-lived and short-lived mice: trade-offs with aging?

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Relationships between reproduction and longevity are complex and poorly understood. Mutations that extend life often produce reproductive deficits or sterility. Calorie restriction (CR), the only intervention that effectively delays aging in a wide variety of species has generally negative effects on reproduction. In laboratory stocks of mice and rats, severe CR leads to sterility or markedly reduced fertility, although reproductive aging was delayed in some studies of CR animals. In women, increased longevity has been linked both to reduced number of offspring and to maintenance of fertility into advanced age. In fruit flies, selection for reproduction late in life produced lines of long-lived animals.

Laboratory mice with reduced somatotropic signaling due to growth hormone (GH) deficiency or resistance live much longer than normal (wild-type) animals and exhibit numerous symptoms of delayed aging as well as various reproductive deficits. In GH receptor knock-out (GHRKO) mice, puberty is delayed in both sexes, litter size is reduced and incidence of infertility is increased. Female Ames and Snell dwarf mice are infertile but this reproductive defect has been linked to deficiency of prolactin rather than GH and thus may be unrelated to the mechanism(s) of increased longevity. Normal fertility was reported in female mice with partial resistance to insulin-like growth factor 1 (IGF-1), an important mediator of GH actions, while IGF-1 deficiency or complete IGF-1 resistance prevent normal reproductive development.

Transgenic mice overexpressing GH have drastically shortened lifespan and various symptoms of accelerated aging. In these animals, sexual maturation is advanced and litter size is increased. However, GH transgenic mice exhibit also various reproductive deficits including failure to become pregnant from post-partum estrus, increased intervals between litters, luteal failure and dramatically reduced reproductive lifespan.

The complex and seemingly inconsistent relationship between GH signaling, reproduction and aging fits the concept of antagonistic pleiotropy. This concept was proposed to explain how physiological characteristics and genetic traits that promote aging and shortened lifespan may have escaped elimination by natural selection. Actions of GH promote early reproductive development and contribute to reproductive potential of young adults. The ability of GH signaling to accelerate aging and shorten life becomes evident mainly during the late reproductive and post-reproductive period when the force of natural selection is rapidly declining. This interpretation may be particularly pertinent to small rodents which in their natural habitat are subject to heavy predation and early mortality, conditions that favor selection for rapid reproductive development and maximal reproductive effort early in life, while the potential for longevity may be of little if any evolutionary advantage.

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