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Genes in genital malformations and male reproductive health

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Abstract

Genital malformations constitute the most common birth defects encountered in man and domestic animals. They occur more frequently in genetic males since the participation of many genes is required for sex differentiation to proceed in the male direction. The emerging insight, through the identification of genes involved in the sex differentiation cascade, is that over 85 percent of sex anomalies in human and domestic animal populations are not attributable to chromosome aberrations or to mutations in a known gene. Since a majority of severely malformed individuals are incapable of reproduction, the high rates of these defects have to be the results either of new mutations or of collaboration of environmental factors with genes. Increase in the prevalence of specific malformations in domestic animals often indicates increased concentration of liability genes brought together in the conceptus by inbreeding. However, in human populations where inbreeding is not the norm, such increases may reflect environment-induced new mutations or interaction of environmental agents with hormone sensitive genes. This review summarizes the information currently available on the genetics of major events in male sex differentiation and briefly discusses the collaborative role environment may play in disrupting different components of this process.

Keywords: sex anomalies; genital malformations; sex reversal; testicular cancer; gynecomastia.

Introduction

The increasing trend in the incidence of genital malformations and the decline in human male reproductive health have attracted much concern and controversy in recent years (Foster, 1998; Safe, 2000; Jouannet, et al., 2001; McLahlan, 2001; Damgaard et al., 2002; Degen and Bolt, 2002; Mendes, 2002). Since the essential event in establishing male sex phenotype is the formation of the functional testis, malformations of the internal and external genitalia detected at birth and the compromised reproductive health encountered in adult males, have been thought to be manifestations of a primary assault on the fetal testis inflicted by endocrine disrupting chemicals in the environment (Skakkebaek *et al*., 2001, Damgaard, *et al*., 2002). Domestic animals follow the genetic and ontogenetic pattern of human sex differentiation and share the anthropogenic environment with man. It is, therefore, expected that they may also display biomarkers of disrupted sex differentiation and developmental feminization (Sweeney, 2002). However, the type and frequency of malformations detected in man and domestic animals and among different species of domestic animals, differ mainly due to differences in their genetic background and the embryological clock that shifts the developmental targets of environmental factors (McLahlan, 2001).

Malformation of the male reproductive system, ranging from mild to moderate hypospadias, unilateral or bilateral cryptorchidism, persistence of Müllerian duct derivatives and poorly developed testes, are frequently seen in goats (Basrur and Mckinnon, 1986; Basrur and Yadav, 1990; Basrur, 1993), pigs (Pailhoux *et al*, 2001a, b; c), horses (Basrur *et al*., 1970; McIlwraith *et al.,* 1976), dogs (Meyers-Wallen, 1993) and cats (Millis, *et al*., 1992), even though the genetic and environmental components eventuating these defects in individual species are not well delineated as yet. Breed predisposition for specific birth defects and recurrence in families often point to their genetic etiology. However, recent studies involving experimental exposure of pregnant sows and ewes to endocrine disrupting compounds have shown that antiandrogens and environmental estrogen mimics could cause poor testicular development and / or cryptorchidism in their male offspring and that some of these may have long range effects on subsequent gen-

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erations (McMahon et al., 1995; Bogh et al., 2001b; Sweeney et al., 2000; Sweeney, 2002). It would appear therefore, that some of the endocrine disrupting chemicals in the environment could compound the effects of mutant genes whose functional counterparts are required for normal sex differentiation. This paper briefly reviews what is currently known on the genes involved in normal and anomalous sex differentiation in man and domestic animals in an attempt to show the collaborative role environmental agents may play with these genes to derail sex differentiation and spermatogenesis in man and domestic animals.

Anomalous sex phenotypes

Sex anomalies expressed as malformations of the reproductive organs, constitute the most frequently encountered birth defects in man and domestic animals (Basrur, 1993; Vaiman and Pailhoux, 2000; Vilain, 2002). Although genital malformations and total sex reversal similar to those seen in man occur in domestic animals, the predominant type of sex-related anomalies in domestic animals tend to vary depending upon the species and breed and the selection practice adopted by breeders. As a result, the common type of sex-related abnormalities seen in food animals including cattle, goats, sheep and pigs is male-pseudohermaphroditism through masculinization of the gonads of genetic females although partial feminization and retention of the female reproductive tracts in males also occur in certain breeds and lines of these species (Basrur, 1993; Basrur and Yusoff, 1997; Vaiman and Pailhoux, 2000; Pailhoux et al., 2001c). Male-pseudohermaphroditism is also the most common type of sex anomaly in dogs whereas in cats, the most frequently reported sex anomalies tend to be the Klinefelter-like syndrome (probably due to the ease of detection based on the association of this syndrome with the sex linked orange coat colour gene in this species) and, testicular feminization similar to that seen in man (Meyers-Wallen, 1993; Vella *et al*., 1999). While the familial trend of these sex-related problems in man and domestic animals points to their genetic or chromosomal etiology, it is also unmistakable that total or partial sex reversal is more common in the male (XY) than that in the female (XX), and that the proneness to genital malformations approximates a ratio of 2 : 1 in favour of males in general (Simpson, 1979; Kent and Wachtel, 1987; Vaiman and Pailhoux, 2000; Vilain, 2002). The higher susceptibility of males to sex-related anomalies is easy to reconcile in light of the genetic and developmental hurdles a mammalian conceptus has to pass through to attain its male specific phenotype.

Genes and sex differentiation

Gonadogenesis

The basic tenets of sex determination in mammals explicate that the sex of a conceptus is determined by the male that generates two types of gametes with regard to their sex chromosome constitution. Accordingly, the sex of the conceptus is determined at the time of fertilization, based on whether the male gamete carries an X chromosome or a Y chromosome (Ohno, 1967). The female gamete (ovum) that receives an X chromosome from the male gamete (sperm) at the time of fertilization is destined to develop ovaries and the female phenotype while the ovum that receives a Y chromosome is destined to develop testes and the male phenotype. This long- held view of sex chromosome dependent-sex determination was embellished when it was demonstrated that it is the fetal testis that superimposes the male pattern of growth and the male phenotype on the developing XY fetus (Jost, 1970; 1973). The sole supremacy of the Y chromosome in male sex differentiation was strengthened substantially when a gene from the "sex determination region of the Y" chromosome, referred to as *SRY*, was found to be essential for the induction of testis and subsequent testis -related male phenotype in all mammals (Sinclair *et al*., 1990). However, soon it became evident that individuals lacking a Y chromosome occasionally develop testes and show other evidences of masculinization even in the absence of *SRY*, while others that carry an intact *SRY* sequence often fail to develop the testis and display varying degrees of feminization (McElreavey *et al*., 1993). The genetic and molecular investigations from various laboratories have now demonstrated that gonadal sex differentiation requires genes carried on the sex chromosomes as well as on the autosomes, that the autosomal genes involved in male and female sex differentiation are present in both sexes, and more importantly, that the genes promoting female sex differentiation need to be neutralized (suppressed) before a male conceptus can steer gonadal differentiation in the testis-path way (McElreavey *et al*., 1993; Pailhoux *et al*., 2001c).

Although sex differentiation is often thought to be synonymous with testis differentiation, it is now realized that various important morphogenetic events need to take place even before gonadal differentiation is initiated. These events include the formation of the urogenital ridge as a thickening covered by the coelomic epithelium on the ventro-medial surface of each mesonephros, migration of the primordial germ cells of epiblast origin from the wall of the yolk sac towards the base of the allantois and on to the gonadal ridge, and the arrival of mesenchymal and mesodermal cells from the mesonephric blastema at the gonadal-ridge to home with the germ cells (Byskov and Hoyer, 1988). These migratory events followed by repeated

cell divisions leading to the formation of the bipotential gonad, are similar in the early conceptus regardless of composed of primordial germ cells, mesenchymal and mesodermal cells with the potential to produce protein hormones and steroid hormones, the Wolffian ducts of mesonephric origin and the para-mesonephric Müllerian ducts which begin as invaginations of the coelomic epithelium into the mesenchyme, lateral to the mesonephric duct, and identical external genitalia (Byskov and Hoyer, 1988).

All of the genes participating in early gonadogenesis are not identified as yet. However, several genes encoding transcription factors are expressed in the gonadal ridge, of which two are well studied in man and some of the domestic animals (Payen *et al*., 1995; Pailhoux *et al*., 2001a; 2002; Vilain, 2002). These include the gene coding for the steroidogenic factor (*SF1*) which is a member of the orphan nuclear hormone receptor family, first identified as a common regulator of cytochrome P450 steroid hydroxylases in the gonads and adrenal glands (Luo *et al*., 1994; Achermann *et al.*, 1999). Another gene expressed at the early stage of gonadogenesis is *WT1*, coding for one of the DNA (and RNA)-binding proteins originally isolated from a congenital neoplasm (Wilms' tumour) associated with multiple anomalies including poor adrenal and gonadal function. *WT1* protein which acts as a post transcriptional regulator is first expressed in the mesonephros, and later in the genital ridge. It is also expressed in the bipotential gonad and the fetal testis during differentiation, and in the adult gonads of both sexes (Lahbib-Mansais *et al.*, 1997; Vilain, 2002).

In the gonads of the male (XY) fetus, the primordial germ cells continue to proliferate and form progressively elongating cords of germ cells and mesonephric (supporting) cells encased by peri-tubular mesenchymal and myoid cells. The mesonephric cells within the cords differentiate into pre- Sertoli cells, while those outside the elongating sex cords (in the interstitium) differentiate into Leydig cells. Several genes have been identified to be active and/or to be inactivated, during the differentiation of the fetal gonad into the testis. Of these, the already mentioned Y-chromosome-specific *SRY* which encodes a nuclear factor encompassing a conserved high mobility group (HMG) protein is thought to be the key factor in initiating the cascade of gene regulations that eventuate testicular induction in mammals (Sinclair *et al*., 1990; Pailhoux *et al*., 2001b). This protein product of *SRY*, made up of a DNA binding and DNA bending domain, is detected in the gonadal ridge of mammals just prior to the hormonal phase of testicular differentiation. In the human conceptus, *SRY* protein is localized in the pre-Sertoli cells as early as at 7 weeks of gestation (Harley ,2002). Expression of *SRY* is essential for the repression of genes which normally interfere with testicular differentiation and for the activation of genes which are required for testis differentiation. One of the autosomal genes activated by *SRY* is *SOX9,* which codes for an *SRY*-related high mobility box protein family of transcription factors (Kent *et al.*, 1996; Pailhoux *et al.*, 2001c). *SOX9* protein which is highly conserved in all mammals, birds and reptiles (Kent *et al*., 1996; Morais da Silva *et al.*, 1996) with a DNA binding domain and a transactivating domain, is first localized in the cytoplasm of "pre-Sertoli cells" of the bipotential gonad (Harley, 2002). However, coincident with *SRY* expression in the male fetal gonads, *SOX9* is transported to the nucleus of the pre-Sertoli cells and through its interaction with *SF1*, activates the anti-Müllerian hormone (*AMH*) gene in fetal Sertoli cells (Vaiman and Pailhoux, 2000; Harley, 2002). Anti Müllerian hormone, a glycoprotein dimer belonging to the transforming growth factor. family, interacts with a signalling receptor and a ligand binding receptor in the target cells and causes the regression of the paired Müllerian ducts of the fetus (Picard *et al*, 1989; Scherer, 2002). According to Scherer (2002), the up regulation of *AMH* gene by various transcription factors including *SOX9, SF1* and *WT1* constitutes the major step in male sex differentiation.

Another gene involved in the testicular differentiation pathway is referred to as *DMRT1*, coding for a putative transcription factor homologous to the double sex (*dsx*) gene of *Drosophila* and to one of the male abnormal (*mab) genes (mab –3 gene)* of the nematode *Caenorhabditis elegans* (Zarkower, 2001). *DMRT1*, located on human chromosome 9p, is expressed exclusively in the Sertoli cells and germ cells of fetal testis (Zarkower, 2001). It codes for a testis-specific protein carrying a zinc finger DNA binding motive, involved in the differentiation of the testis in man and a variety of vertebrates and invertebrates (Ottolenghi *et al.*, 2000; Zarkower, 2001). Recently, another testis specific gene has been identified to play a role in fetal testis differentiation (Olesen *et al*., 2001). This X-linked gene referred to as fetal and adult testis expressed (*FATE*) transcript, has been noted to be co-expressed with *SRY* in fetal testis (at 7 weeks of gestation in humans), is thought to be essential for the differentiation of male germ cells in the fetus and adults (Olesen, *et al*., 2001).

While all these genes need to be expressed for testis differentiation to proceed, two genes expressed during the earlier stages of gonadogenesis have to be suppressed in order to accomplish this process. One of these is *DAX1* (a gene located in the dosage-sensitive sex reversal region of human X chromosome short arm) that codes for an unusual member of the nuclear hormone receptor super family proteins. *DAX1* plays the

role of a repressor of transcription activators, through the transcription-silencing domain of its protein product which is conserved in all mammalian species tested (Bardoni *et al*., 1994; Nachtigal *et al.*, 1998; Pailhoux *et al.*, 2001a; Vilain, 2002). *DAX1* which inhibits the *SF1*-mediated steroidogenesis in male fetal gonads, is thought to be an "anti-testis" gene (Swain and Lowell-Badge, 1999). Expression of this gene is turned off by *SRY* in the male gonad, even though one dose of *DAX1* product is expected to be present in normal XY individuals (Goodfellow and Camerino, 1999). It is believed that *DAX1* in two doses (in XX individuals and in males carrying a duplication of this region on their X chromosome), blocks the up-regulation of *AMH* expression (Zanaria et *a*l, 1994; 1995; Nachtigal *et al.,* 1998; Zhang *et al.*, 2000; Vilain, 2002). Another gene that needs to be repressed before testis differentiation can be accomplished is *WNT4* which belongs to the group of intercellular growth and differentiation factors involved in the reproductive system development. WNT4 protein, first isolated form *Drosophila*, is one of the wingless-type integration site family of proteins that serve as locally acting cell signals (Vainio *et al.*, 1999; Heikkila, *et al.*, 2001). *WNT4* gene is first expressed in the mesonephros and in the coelomic epithelium of the bipotential gonad of XX and XY fetuses up to the time of bifurcation of the male and female gonadal differentiation pathways. At the fork in this process, *WNT4* is down regulated coinciding with the activation of the steroidogenic enzyme 3. -hydroxysteroid dehydrogenase (*3. -HSD*) gene, in the fetal testis (Heikkila *et al.*, 2001; Jordan *et al*., 2001).

In contrast to the situation in the male fetus, only a few genes involved in the morphogenesis of fetal ovary have been identified to date. Since gonadogenesis in the female fetus takes place in the female hormonal milieu provided by the mother, it is still not well understood what genes other than those expressed in the bipotential gonad (*SF1*, *WT1*, *DAX1* and *WNT4*) are essential for fetal ovarian differentiation. However, the expression of *WT1* and *SF1*, initiated during the urogenital ridge formation and continued during the growth of the bipotential gonad, is gradually reduced in the female fetal gonad coinciding with the elevated expression of *DAX1* gene (Scherer, 2002). Also, *SOX9* is not detected beyond the very early stage in the differentiating fetal ovaries (Harley, 2002). On the other hand, unlike in the fetal testis, *WNT4* is continuously expressed in the developing fetal ovary (Heikkila *et al*., 2001). Since *SRY* is not present in normal females, its absence allows *DAX1* gene to be expressed, which, in turn, inhibits the testis promoting genes (Pailhoux *et al*., 2001c; Vilain, 2002). Continued expression of *DAX1* and *WNT4* is thought to be essential for the survival of female germ cells and for the differentiation of the fetal ovary (Scherer, 2002). Furthermore, the expression of the cytochrome P450 aromatase gene (*CYP19*) required for the conversion of androgens to estrogen, is detected in the somatic cells of the fetal ovary much earlier in gestation than that in fetal testis (Pailhoux *et al*, 2001a; 2002) indicating that estrogen is synthesized by the pre-follicular cells of the fetal gonad long before morphological evidence of ovarian differentiation is detected. Estrogen synthesis continues in fetal ovaries during the oogonial phase and during the formation of the ovigerous cords and it is turned off just prior to the initiation of meiosis in female germ cells (Dominguez *et al*, 1988; 1990).

Differentiation of Intern*al* and Extern*al* Genit*al*ia

Further differentiation of the male reproductive system is dependent on the functional integrity of the fetal testis (Jost, 1973). Main aspects of this process concern the development of organ components for transport, maturation, storage and delivery of the male gametes (to be generated in the adult testis) out of the paired embryonic excretory ducts of the mesonephros (Wolffian ducts), the urogenital sinus and the genital tubercle. Morphogenetic events leading to the development of these structures are initiated after gonadal differentiation, unlike the inhibitory effect unleashed on the Müllerian ducts adjacent to the testis, just after the formation of the seminiferous tubules (Pailhoux *et al*., 2001a, b). The cranial segment of the mesonephric ductules connected to the straight tubules of the rete testes, become differentiated into the convoluted efferent ductules which join at their posterior end to form the epididymis while the central segment of the Wolffian duct differentiates into the vas deferens, and the lower portion, through budding of its wall, forms the seminal vesicle (Byskov and Hoyer, 1988). The closure of the urogenital sinus, elaboration of the accessary sex gland, and the differentiation of the genital tubercle and the genital swelling respectively into the penis and the paired protrusion at the ventral abdominal wall to form the scrotum, are all accomplished through the steroid hormones secreted during the hormonal phase of testicular development (Jost, 1972; Byskov and Hoyer, 1988). Thus, testosterone secreted by the Leydig cells through the activation of 3β- hydroxy steroid dehydrogenase gene, is required for the male duct system differentiation while a more potent androgen produced by the conversion of testosterone by one of the 5α reductase enzymes within the target cells is required for the differentiation of the external genitalia. This process involving the urogenital sinus closure, the elaboration of the urethral and prostate glands, the elongation of the genital tubercle and fusion of the urethral folds over the urethral groove (in a posterior to anterior direction) to form the penis and penile urethra, and the movement and relocation of the genital swellings posterior to the

genital tubercle prior to fusion, to form the scrotum, are all influenced by the androgen dihydrotestosterone (Byskov and Hoyer, 1988).

The gene coding for the enzyme 5α reductase - type 2, is expressed during the penile urethral differentiation (Kim *et al*.,2002). The androgen-mediated differentiation of the duct system and external genitalia also depends on the functional integrity of the androgen receptor gene which, in humans, is located on the X chromosome (Meyer *et al*., 1975; Ohno, 1979; Gottlieb *et al*, 1999). Androgen receptor (AR) protein is a ligand dependent transcription factor belonging to the steroid nuclear receptor super family (McPhaul *et al*., 1991; Gottlieb *et al*., 1999). This protein which has a C-terminal binding domain and an N-terminal modulatory domain, regulates the transcription of specific genes, by binding androgen-AR complexes to regulatory DNA sequences, close to the target genes. In a male conceptus in humans, the epithelial cells of the skin, urethral plate in the glans, and the stroma and epithelium of the tubular urethra of the penile shaft are all strongly AR positive as early as 12 weeks of gestation and in the fetal penis between 16 and 20 weeks of gestation (Kim *et al.,* 2002). Androgen receptors increase progressively in the epithelium in this region, specifically on the ventral aspect of the urethra, while 5α reductase - type 2 expression is localized in the ventral seam stroma of the remodelling urethra in male fetus at 16 to 20 weeks of gestation (Kim *et al.,* 2002). Presence of these receptors underlines the androgen-dependence of penile differentiation and point to the vulnerability of this process to disruptive events leading to hypospadias (Kim *et al.,* 2002). Furthermore, studies on mice indicate that the development of male external genitalia requires one of the fibroblast growth factors (Fgf 10) since mice experimentally rendered deficient for this signalling protein develop abnormal glans penis as in human hypospadias (Haraguguchi *et al*., 2000).

Descent of the Testis

In a majority of mammals, the fetal testis migrates from the posterior pole of the kidney and pass through the abdominal wall to eventually reach the scrotum (Byskov and Hoyer, 1988; Hutson *et al*., 1997). The testis is already lodged in the scrotum by the time of birth in cattle, sheep and goats; however, in dogs, cats, pigs and horses, descent is completed after birth (Gier and Marion, 1970). Testicular descent occurs in several stages beginning with the nephric displacement initiated by the time fetal gonad has differentiated into the testis and is situated on the dorsal wall of the abdominal cavity, anchored to the ventral surface of the corresponding kidney by the cranial suspensory ligament (CSL). The second stage characterized by the growth and trans-abdominal movement of the testis, is aided by various factors including the degeneration of the peritoneal fold that anchors the testis to the abdominal wall, rapid growth of the abdominal pelvic region, regression of the Müllerian ducts elicited by AMH, and the differentiation of the duct system induced by the steroid hormones secreted by the interstitial cells (Byskov and Hoyer, 1988; George and Wison, 1988). The third stage (the inguinal passage) is facilitated by the movement of the gubernaculum which is a mesenchymal cord wrapped by peritoneum extending from the caudal end of the nephrogenic cord along the lateral abdominal wall to the developing scrotum. Increasing abdominal pressure leads to the herniation of the posterior peritoneal membrane to create an outpocketing referred to as the processus vaginalis (vaginal process) which extends when abdominal pressure increases, to form the inguinal canal along the course of the inguinal portion of the gubernaculum. Dilatation of the inguinal ring, combined with the regression of the gubernacular ligaments and the increasing intra-abdominal pressure forces the flaccid fetal testis into the inguinal ring. The outgrowth of the gubernaculum through the inguinal canal guides the movement of the testis while the degeneration of the gubernaculum and vaginal ring contraction during the perinatal period cause the testis to descent into the scrotum (Leipold *et al*., 1986; Vanderbroeck and Maghninrogister, 1995). Even though the earlier stages of gubernacular growth are not androgen dependent in some species, the final stage of testicular descent, accomplished by gubernacular regression, is androgen-dependent (Levy and Husmann, 1995). More recently, a precholesterol sterol of testicular origin, referred to as meiosis activating sterol (T- MAS) has also been noted to play a part in testicular descent (Bogh *et al*., 2001a).

Genes involved in the different phases of testicular descent, are all not identified as yet. However, various genes and different environmental factors are known to influence this process. Recent studies have shown that a mutation in the gene coding for the Leydig cell genereted insulin-like hormone (Insl 3), could interrupt testicular descent (Nef and Parada, 1999). Insulin like hormone 3, expressed in the developing testis is thought to act in an androgen-dependent manner to induce growth and differentiation of the gubenaculum, and to mediate the intra-abdominal testis descent while its absence leads to total (abdominal) retention of the testis (Nef and Parada, 1999; Zimmermann *et al*., 1999; McLahlan, 2001). Another gene envisaged to be involved in testicular descent is one of the homeobox (*HOX*) containing genes which play key roles in the morphogenesis of segmental structures along the primary body axis (Maconochie *et al*., 1996; Ma *et al*., 1998; Emmen *et al*., 2000). Analysis of human cases suggests that one of the *HOX* genes

(*HOXA 10*) may play a part in the development and differentiation of the urogenital mesenchyme (Konlon *et al*., 1999) and that a *HOX* mutation may be involved in some cases of cryptorchidism and associated defects (Cortes *et al*., 1998; 2001; Podlassek *et al*. 1999). In addition, the expression of the spermatid-specific heat shock protein (HSP70), conserved in all scrotal mammals, and active during spermiogenesis in the adult testis, is postulated to be involved in the testicular growth and descent pathway (Tsunekawa *et al*., 1999). It is also believed that gubernacular regression, at least in the rodents, may indirectly be influenced in an androgen-dependent manner by the neurotransmitter (a calcitonin gene-related peptide) generated by the sensory branch of the genito-femoral nerve, through its action on the muscular component of the gubernaculum (Hrabovsky *et al*., 2000).

In contrast to these interrelated series of events in the male (induction of testis → duct system differentiation → urogenital differentiation → testicular descent), the female fetal reproductive system differentiation appears to be much less complicated. In the female fetus, the Wolffian ducts fail to differentiate (in the absence of testosterone) and persist only in a remnant form, while the differentiation of the Mullerian ducts takes place in a cranio-caudal direction (in the presence of maternal hormones) to give rise to the female internal genitalia. The cranial portion of the Mullerian duct (derived from the coelomic epithelium) gives rise to the oviducts, while the mid portion gives rise to the uterine horns which fuse at their posterior aspect to form the body of the uterus, and the caudal portion gives rise to the utero vaginal plate with the participation of both Mullerian and Wolffian components to form the cervix and the anterior vagina (Byskov and Hoyer, 1988). The urogenital tubercle of the female fetus undergoes only limited growth and remains mainly exposed on the surface as a cleft into which the vagina and urethra open, unlike the urogenital tubercle of the male fetus which is enclosed by fusion at the pelvic portion and undergoes considerable growth (George and Wilson, 1988). Another major difference between the male and the female conceptus at this stage is that the differentiation of internal genitalia and the uro-genital system in the latter, does not require the fetal ovary or the fetal pituitary gland since decapitation or castration of the fetus has no effect on the development of female genitalia (Byskov and Hoyer, 1988). More recently, it has been demonstrated that the WNT signalling proteins are involved in the differentiation of female genitalia. In situations where *WNT4* gene is altered or absent, the epithelial cells of the fetal Müllerian ducts are not responsive to maternal estrogen and the differentiation of the female internal genitalia does not proceed (Vainio *et al*., 1999). Deficiency for other members of these signaling proteins (*Wnt*-5a or *Wnt*-7a) also leads to malformations of the internal and external genitalia in the mouse. For example, *Wnt-5a* is required for the differentiation of the genital tubercle in the female mouse while *Wnt-7a* is required for the differentiation of the Müllerian ducts into oviducts, showing that the female reproductive system differentiation is not entirely accomplished in a default pathway (Heikkila *et al* 2001; Parr and McMahon, 1998).

Disrupted male sex differentiation

Sex Reversal

As the foregoing sections show, a large number of genes participate in the masculinization of the male fetus, and consequently, absence or alteration of any one of these genes has the potential to disrupt male sex differentiation. Various cases of sex reversal reported earlier in human XY individuals have since then been attributed to mutations of genes in the testis differentiation pathway (Simpson, 1979; 1987). For example, a mutation involving a base pair substitution in the DNA binding domain of *SF1*, even in heterozygous state, causes feminization of XY individuals due to haplo-insufficiency for *SF1* transcript (Ozisik *et al*., 2002; Achermann *et al*., 1999). Similarly, absence of functional *WT1* which normally codes for various transcription factors, causes failure of gonad and kidney development in homozygotes and XY sex reversal in heterozygotes (Villain, 2002). Mutations in the open reading frame of *SRY,* with or without any influence on the DNA binding and bending property of its high mobility group (HMG) domain, account for a substantial proportion of XY gonadal dysgenesis (Scherer, 2002). Also, duplication or mutation in *SOX9* gene causes XY sex reversal and skeletal defects in humans (Morais da Silva *et al*., 1996; Huang *et al*., 1999). Deletion of a small segment in one member of chromosome pair 9 in humans, rendering a hemizygous status for the *DMRT1* locus, causes failure of testicular development and feminization of a variety of non-gonadal tissues in man (Ottolenghi *et al*, 2000; Vilain, 2002; Zarkower, 2001). Failure to down regulate *DAX1* or *WNT4* (due to duplication of the Xp segment carrying *DAX1* gene or a mutation in *WNT4* locus) also causes XY sex reversal in humans (Zanaria *et al*., 1994; 1995; Zhang *et al*., 2000; Jordan *et al*., 2001).

Another factor that affects males more profoundly than the females, is the variety of missense and nonsense mutations in exons 4 to 8 of the X-linked androgen receptor gene (Meyer *et al*., 1975; Gottlieb *et al*., 1999; Zenteno *et al*., 2002). While severe mutations in this X-linked intracellular androgen receptor gene cause complete androgen insensitivity, less severe mutations cause compromised masculinization often

with impaired fertility since both testosterone and 5-dihydrotestosterone (DHT) require functional AR for their action (McPhaul *et al*, 1991). In the absence of a normal AR gene, the primary male sexual development before birth and the development of secondary sex characteristic after birth (at puberty in humans) are all affected. A considerable proportion of XY males displaying a spectrum of female characteristics are androgen insensitive since the rates of *de novo* mutations of AR gene, leading to complete androgen insensitivity (in cases of nonsense mutations) or partial insensitivity (in cases of missense mutations) are relatively high (Gottlieb *et al* 1999; Chavez *et al*., 2001). Females carrying AR mutations are not affected to the same degree since androgens, although essential, are only required around puberty and for sexual function in adult state. This, coupled with the inactivation of one of the two X chromosomes, leads to the diminished expression of X-linked genes in female mammals and allows these mutations to be transmitted through the female line. In milder androgen insensitivities the phenotypic impact of the mutant gene on the male may be impaired spermatogenesis with reduced sperm production or with low, but sufficient sperm count to be fertile (Gottleib *et al*, 1999).

Sex reversal of (XY) males among domestic animals is not uncommon although the rigorous selection practice generally tends to keep the prevalence low, at least in food animals. For example, cases of male sex reversal in cattle have been identified in various countries in the past (Basrur *et al*., 2001b). While some of these were isolated cases, others clearly displayed recurrence in families and led to the elimination of the suspected perpetrators from breeding program (Henricson and Akesson, 1967; Chapman *et al*., 1978; Gustavsson *et al*.,1981; Sharma *et al*, 1980; Hare *et al*., 1994). Similar cases have been reported in other domestic animals including goats, sheep and dogs although the incidence of male sex reversal in domestic animals is generally lower than that for female sex reversal (Phailhoux *et al*., 2001c). An exception to this pattern is seen in horses in which sex reversal of the genetic (XY) male is relatively common. XY mares generally carry small abdominal gonads (in the ovarian position) and possess a small uterus (Kent *et al*., 1986; Kent-First, 2000). A survey revealed that this condition occurs in different breeds of horses including Thoroughbred, Quarter horse, and Arabians and different breeds of ponies (Kent *et al*, 1986). The affected horses appear phenotypically female and often the only clues to their status as sex reversed "mares" are their total lack of interest in males and absence of other signs of estrus during the breeding season, their XY karyotype, low plasma testosterone concentration and indications of gonadal dysgenesis (Kent and Wachtel, 1987). Breeding histories revealed that "sex reversal" was transmitted by carrier females in 5 out of 6 pedigrees examined (Kent and Wachtel, 1987), and that in one interesting pedigree, by a "stallion" that had sired over 500 foals including fertile XX and XY foals, feminized (XY) mares and female foals carrying a deletion on the long arm of one of the X chromosomes (Kent and Wachtel, 1987). Subsequent studies on these and other unrelated "mares" showed that a majority of these are SRY negative and display gonadal dysgenesis (Pailhoux *et al*., 1995; Kent-First, 2000; Vaiman and Pailhoux , 2000).

Genital Malformations

As stated before, sex reversal may be manifested in a variety of phenotypes ranging from mild forms of genital malformations to ambiguous external genitalia with total sterility, depending up on the specific stage at which sex differentiation was disrupted. These biomarkers of disrupted sex differentiation which also occur in otherwise "normal" males, include hypospadias and cryptorchidism, which are the most frequently detected genital malformations in humans and domestic animals (Husmann, 1998; 2000; Hussain *et al*., 2002). In domestic animals, hypospadias is often seen in association with intersexuality in goats, pigs, dogs and sheep (Hamori, 1983; Basrur and Yadav, 1990; Basrur, 1993). In ovine and caprine hypospadias, the corpus cavernosum urethrae is absent and the urethral orifice is located as a long slit beneath the anus or on the ventral side of the penis (Dennis, 1979; Dennis and Leipold, 1979; Basrur and Yusoff, 1997). In cattle and sheep, this urethral closure defect (resulting in clefts of varying length) may be accompanied by the bifurcation of scrotum (Hamori, 1983). In dogs displaying isolated hypospadias the urinary meatus may be located in the glans, the penile shaft, the periscrotal junction or the perineum (McEntee, 1990).

Hypospadias characterized by abnormal development of the glans and ventral displacement of the urethra, with or without midline closure defect, has long been associated with hyper estrogenism (exposure of the fetus to estrogen during the formation of external genitalia) in domestic animals (McEntee, 1990). In humans, mutations in 5α- reductase, type- 2 enzyme have been implicated in mild to severe forms of familial hypospadias (Stock *et al* 1995). Other factors associated with hypospadias are poor intrauterine growth, low birth weight, low levels of testosterone in maternal circulation due to the effect of antiandrogens on the placental-fetal unit, multiple urogenital malformations and the rank of birth of the afflicted infant, with the first child being more prone to this malformation (Key *et al*., 1996; Hussain *et al*, 2002). Even though considerable geographical differences exist in the incidence and severity of this malformation in human popula-

tions, an increase in the incidence of hypospadias is noted in the United States, Australia, and various European countries since 1970 (Paulozzi *et al*., 1997; Dolk, 1998; Weidner *et al*., 1998; Pierik, *et al*., 2002). A more recent study from North America indicates a 10-fold increase in the incidence during a 13- year period from 1987 to 2000 (Hussain *et al*., 2002). Exposure to endocrine disrupting chemicals (from hazardous waste landfill sites in the vicinity or dietary exposure to phyto-estrogens during pregnancy) has also been noted to be associated with the increased incidence of these malformations (Weidner *et al*, 1998). Since the differentiation of the genital tubercle into male external genitalia requires the signaling factor protein, *Fgf* 10 (Haraguchi *et al*., 2000; McLahlan, 2001), it is possible that estrogen mimics or anti-androgenic compounds in the environment contribute to this malformation by disrupting this signal during the formation of the penile uretha in the developing fetus.

Cryptorchidism

Abnormal testicular descent leading to the retention of one or both testes outside the scrotum (cryptorchidism), occurs as an isolated defect or in association with hypospadias and other urogenital anomalies in man and domestic animals (Vandenbroeck and Maghuinrogister, 1995; Hutson *et al*., 1997; Husmann, 1998; Cortes, *et al*., 2001). Cryptorchidism is relatively rare in cattle, but it is frequently seen in sheep and goats (Ladds, 1993). An overall prevalence of 3.3 percent has been reported among the slaughtered goats surveyed in South India (Mathew and Raja, 1978) and a 2 percent prevalence in the West African dwarf breed of goats, with abdominal retention of the right testis occurring more frequently (Ezeasor, 1985). Dennis and Leipold (1979) noted that 1.5 percent of new born lambs in their survey in West Australia displayed reproductive system malformations and that 34 percent of these were cryptorchids with or without hypospadias. Cryptorchidism, as an isolated defect, accounts for approximately 13 percent of the male dogs presented at the clinics (Meyer-W*a*llen, 1993), with the small breeds (Chihuahua, Yorkshire Terrier and Miniature Dachshunds) showing a greater representation among them (Hayes and Wilson, 1986). In a survey of 1345 cats admitted to the clinics for neutering, cryptorchidism was seen more frequently in the Persian breed (29 percent) and among the white coat colour cats in other breeds, with an over all prevalence of 1.7 percent in that study (Millis *et al*., 1992). Two of the domestic animal species in which the prevalence rate for cryptorchidism reaches strikingly high proportions are the pig and the horse. The prevalence rate may reach as high as 20 percent in some lines of pigs (Rothschild *et al*., 1988; Lingaas and Ronningen, 1991; Pailhoux *et al*., 2001c). Although cryptorchidism in the horse has previously been shown to have a 3 to 4 percent overall prevalence rate (Cox *et al*., 1979), in some breeds of horses including American Quarter horses and ponies, it far exceeds this estimation (Hayes, 1986; Cox, 1993). Among the Newfoundland ponies, 25 percent of the males are afflicted with this developmental anomaly (Rowan Lalonde, Agriculture Canada: Personal Communication). Since the genital tract development and the pattern of testicular descent and tunic relationships in the pig and the horse are similar to that in man, interruption of testicular descent leading to varying degrees of cryptorchidism in these two species have received much attention in the past (Gier and Marion, 1970; Smith, 1975; McMahon *et al*., 1995; Husmann, 1998; Muller and Parks, 1999).

In the horse the testis may be retained anywhere along its developmental pathway, with all parts including the epididymis located within the abdominal cavity (abdominal cryptorchidism) or part of the testis or its appendages located in the inguinal region (inguinal cryptorchdism). The testis may occasionally be noticed to have traversed the inguinal canal to lodge itself either subcutaneously beside the prepuce or in the femoral triangle. Over 60 percent of cases represent abdominal cryptorchidism while the balance may be retained mainly in the inguinal region and rarely at other ectopic sites (Leipold *et al*., 1986). Prevalence of cryptorchidism in the major breeds of horses in North America reveals that the American Quarterhorse and ponies have higher proneness to this malformation than other breeds including Thoroughbred, Standardbred and Arabian breeds (Hayes, 1986; Cox, 1993). The American Quarter horse is over-represented in cases of cryptorchidism recorded in surveys from various locations in North America, including that from Canada (Cox, 1993; Basrur and Cote: unpublished data).

Uunilateral cryptorchidism is more common than bilateral in the horse regardless of the breed and the epididymal tail is often located in the processus vaginalis in the abdominally retained testis, either loosely attached to the body of the epididymis or at a distance from the testis, as in cases refered to as detached epididymis (McEntee, 1990; Cox, 1993). Studies from various centers have shown that abdominal retention of the right testis is often associated with detached epididymis (in 50 percent of the cases) while no such trend is detected for inguinal cryptorchidism (Cox, 1993; Hawkins *et al*., 2002). Leydig cells of the retained testis show no overt regressive changes and in some cases may be hyperplastic and may continue to produce testosterone, albeit at reduced levels relative to that of descended testis (Arighi *et al*., 1986). In unilateral cryptorchids, the scrotal testis undergoes compensatory hypertrophy and generally functions normally, although impaired spermatogenesis and abnormal

sperm morphologies are occasionally detected in unilateral cryptorchid ponies (Basrur *et al*, 1979; Cox, 1993).

The cause of the relatively high rate of cryptorchidism in the horse is not known. Although some of the cases diagnosed as cryptorchid have shown chromosome anomalies and proved to be intersexes, this type represents only a small percentage of equine cryptorchidsm reported (Basrur *et al*., 1970; McIlwraith *et al*., 1976; Kubien *et al*., 1993). The size and histological features of the retained testes indicate that the growth of fetal testis is interrupted around 5 months in gestation (Smith, 1975). In normal situations the fetal horse testis grows to an enormous size (140 percent of the adult size) by 8 months of gestation under the influence of the highly elevated estrogen levels in the mare. When the mares hormone level drops drastically after the 8th month, the testis undergoes a substantial reduction (as much as 40 percent) in size. The high levels of maternal estrogen leading to the growth of the fetal testis during gestation has been proposed as one of the causes of the late descent of testis in the horse and the drop in maternal estrogen and elevated levels of androgen produced by the fetal testis are thought to be the major causes of shrinkage of the gubernaculum and fibrosis and contraction of the vaginal ring during the perinatal period (Leipold *et al*., 1986; Mueller and Parks, 1999). Shrinkage of the gubernaculum, coupled with the increased intra-abdominal pressure, allows testicular passage through the inguinal region to enter the scrotum (between 9 and 11 months of gestation) although testicular descent is generally completed only within two weeks after birth in the horse (Leipold *et al*., 1986). Cox (1993) has hypothesized that the main cause of interrupted testicular descent in the horse may be its excessive growth during the abdominal phase or its failure to "regress" just prior to the inguinal phase. These events in sex differentiation are generally influenced by genetic and environmental factors (McLahlan, 2001).

Cryptorchidism recurs in families and displays great variability in the pattern of occurrence and in the degree of manifestation In older reports from different parts of Europe equine cryptorchidism was variously described as a dominant autosomal trait, a trait caused by two mutant genes (one of which was thought to be located on the sex chromosome), or as a simple autosomal recessive trait (Leipold *et al*., 1986). While it is possible that a genetic heterogeneity exists for cryptorchidism in the horse breeds of different countries, no simple genetic component is recognized to date for this defect in different breeds or within the same breed of horses within the same country nor has any single environmental agent claimed the sole causative role for this birth defect. However, the familial and sporadic nature of this malformation in a variety of horse breeds would indicate that cryptorchidism, like other reproductive system malformations, is not controlled by a single locus and that many genes and many environmental factors may have parts to play in this birth defect.

Among the environmental factors that could influence this malformation is prenatal exposure to estrogen-like compounds in pasture or feed. These estrogen mimics through their action on insulin-like hormone 3 (Insl 3) gene (or other genes) could interfere with gubernacular growth or regression and, in turn, interrupt testicular descent (Zimmermann, etal., 1999; Nef, *et al*., 2000). Studies on sows exposed to an anti-androgen (Flutamide) at different stretches of time after mating, have demonstrated that testicular descent is interrupted in androgen-deprived male piglets and that gubernacular regression is an androgen-dependent process (McMahon *et al*., 1995). These investigators also showed that the transabdominal stage of testicular descent, at least in the pig, is also androgen-dependent and that the anti-androgen induced abdominal retention may be associated with epididymal malformation (McMahon *et al*., 1995). The vulnerability of testicular descent to estrogenic and anti-androgenic influence is further underlined by the observation that estrogen receptor genes are expressed in the mesenchymal and stromal cells of fetal reproductive organs including efferent ductules, epididymis, ductus deferens, seminal vesicles and urogenital sinus (Pelletier and El-Alfi, 2000; Pelletier *et al*., 2000; Nielsen *et al*., 2001). All these observations indicate that the high prevalence rates of this genital defect in lines of pigs and horse breeds, and the increased, but differing, incidences of cryptorchidism among new born babies in some countries, may be attributable to the different genetic loci and different environmental factors contributing to this maldevelopment. Because of the multifactorial nature of this developmental defect any search for a specific mutation in a single gene (or for a single environmental agent) may not prove successful as some of the recent studies on human cryptorchidism have shown (Baker *et al*., 2002). The process of testicular descent which depends on normal *in utero* growth and regression of the testis, followed by spatially and temporally coordinated events in the development of the androgen and estrogen dependent components of the genitalia, could be subverted at any stage by the mutant genes harbored by the fetus at different loci and their impact can be exaggerated by one or more endocrine disrupting agents with the potential to "feminize" the process of male sex differentiation.

Other indicators of reduced reproductive health

Sex reversal and genital malformations similar to those discussed above have been induced in laboratory animals experimentally exposed to endocrine dis-

rupting compounds (EDCs) and the developmental defects encountered in individuals occupationally exposed to these compounds have been regarded as biomarkers of feminization detectable at birth (Garcia, 1998; McLachlan, 2001; Skakkebaeck *et al*., 2001). Other indicators of reproductive problem due to a primary assault on the testis (generally detected later in life) include testicular hypoplasia, poor quality and quantity of spermatozoa in the seminal plasma, testicular cancer and abnormal secondary sexual characteristics including gynecomastia (Damgaard *et al*., 2002).

Testicular hypoplasia which is a gross diagnostic term used for the presence of smaller size testes on one or both sides (unilateral or bilateral), can result from a variety of factors impacting on the number, function and fate of male germ cells in domestic animals (Stafford, 1972; Teplitz *et al*., 1974; Vale-Filho *et al*., 1984; McEntee, 1990; Basrur *et al*., 2001a). Evidence for an increase in testicular hypoplasia in human populations is provided by a substantial (11 percent) reduction in testicular size between the period of 1981 and 1991 and a 27 to 56 percent reduction in spermatogenesis evidenced by poor quality semen (Pajarinen, *et al*., 1997). Although this aspect has been debated to some extent, Damgaard *et al* (2002) have reviewed the data on human populations tested from different parts of the world and have illustrated that impaired spermatogenesis reflected in the quantity and quality of semen, is on the increase in various countries (Safe, 2000; Skakkebaeck, *et al*., 2001; Jounnet, *et al*., 2001).

Impaired Spermatogenesis

Various gene mutations, chromosome defects and environmental factors impact on the process of spermatogenesis (Johnson *et al*., 2000). Among these are mutations in the genes involved in the regulated synthesis and function of DNA methyl transferase during spermatogenesis and a mutation in the gene coding for a 120 kDa testis-specific isoform of the hormone sensitive lipase enzyme (Trasler, 1998; Chung *et al*., 2001). The testis specific lipase is required for the maintenance of seminiferous epithelium and the intercellular bridges of the differentiating post meiotic germ cells (Chung *et al*., 2001). Deficiency created by targeted disruption of the gene for this hormone sensitive lipase has recently been demonstrated to elicit abnormalities in spermiogenesis leading to sterility in the male mouse (Chung *et al*., 2001). In addition, genes located on the Y chromosome and clusters of genes contained in its different segments, are thought to play important roles in spermatogenesis (Tiepolo and Zuffardi, 1976; Chandley and Cooke, 1994; Pryor, *et al*., 1997; Burgoyne, 1998). These include, in addition to the testis-inducing gene (SRY) described in previous sections, the gene for the zinc finger protein on the Y (*ZFY),* a conserved homologue of the X-linked *ZFX* required for the maintenance of germ cells and fertility, one of the 3 adenine nucleotide translocase genes referred to as *ANT3*, the basic protein genes 1 and 2 (*BPY1* and *BPY2*) and one of the 3 genes coding for ornithine decarboxylase antizyme (referred to as antizyme 3: *AZ3*) involved in proteosomal degradation of protein targets (Luoh *et al*., 1997; Burgoyne, 1998; Ivanova *et al*., 2000). Unlike the 2 other antizymes (*AZ1* and *AZ2*), ornithine decarboxylase antizyme 3 is expressed exclusively in the post meiotic stage germ cells of mammalian testis (Ivanova, *et al*., 2000). Specificity of its expression, starting at the earliest step of spermiogenesis and terminating in late spermatid stage, and the important role it plays in reducing the polyamine accumulation in spermatids engaged in nuclear remodelling into sperm head, indicate that *AZ3* mutation may be one of the key factors leading to oligospermia (Ivanova *et al*., 2000).

Although all of the genes involved in spermatogenesis have not been assigned to map positions on the Y chromosome of domestic animals to date, some of these have been mapped and deletion of Y chromosome segments has been shown to be associated with male sterility in some domestic animals species (Basrur *et al*., 2001a; b). In this regard, the human multicopy gene family of "azoospermia factor" (AZF) region, is worth mentioning (Ferlin *et al*., 1999). The azoospermia factor (AZF) region consists of 3 entities referred to as AZFa, AZFb and AZFc (Vogt *et al*., 1992; Ma *et al*., 1993; Chandley and Cooke 1994; Burgoyne, 1998). In situations involving a deletion of AZFa, germ cells are arrested early in spermatogenesis leading to the "Sertoli cell only syndrome" where as in AZFb deletions, germ cells are arrested at the spermatogonial stage leading to azoospermia and in AZFc deletions, meiosis is completed but spermiogenesis is disrupted leading to mis-shapen sperm heads and oligospermia (Vogt *et al*., 1992). The AZFc deletion which is the most prevalent in men with idiopathic azoospermia includes the reiterated gene cluster referred to as "deleted in azoospermia" (*DAZ*) gene, generally associated with severe oligospermia and /or azoospermia (Reijo *et al*., 1996; Pryor, *et al*., 1997; Chang *et al*., 1999). The phenotypes of AZFc deletions vary greatly from mild oligospermia to azoospermia with varying degrees of spermatogenesis arrest (Stuppia *et al*., 1996). Among the other genes known to impinge on spermatogenesis, is the testis specific protein coding gene on the Y (*TSPY*), which occurs in tandem repeat clusters of 20 to 40 copies per human Y chromosome (Vogt, 1996) and as moderately repeated clusters varying from 50 to 200 copies per bovine Y chromosome (Jakubiczka *et al*., 1992; Vogel *et al*., 1996; 1997). The bovine *TSPY*, like its human counterpart, is

expressed exclusively in the premeiotic germ cells of adult bull testis and germ cells of fetal bovine testis (Vogt, 1996).

TSPY containing region, like other regions of the essentially heterochromatic Y chromosome of man and domestic animals, is prone to breakage (Vogt, 1996; Vogel and Motulsky, 1997; Vogel *et al*, 1998). Since the impact of a breakage and / or deletion of a Y chromosome segment is generally confined to the testis, carriers of these defects are likely to survive, albeit with impaired testicular function. However, vulnerability of the Y chromosome to microdeletions on exposure to environmental agents have not been examined to date although compromised spermatogenesis and sperm head malformations have been observed in bulls subject to a Y chromosome break in meiosis, and in laboratory animals exposed to sublethal doses of insecticides and herbicides including carbofuran and glyphosate (Yousef *et al*., 1995; Basrur *et al*., 2001a, b). Other environmental agents tested for specific effects on the spermatogenesis are methoxyclor (MXC) and octylphenol (OP) both of which, if administered during gestation to the mother, or by oral route to the perinatal or juvenile stage male offspring, reduce the number of spermatogonia and Sertoli cells in the testis, decrease testicular size at birth and impair spermatogenesis when they reach adult age (Johnson *et al*., 2000; Sweeney, *et al*., 2000; 2002; Staub *et al*., 2002). A survey of male farm sprayers exposed to the chlorinated phenoxy herbicide, 2,4-D, commonly used as a lawn care herbicide in various parts of the world including North and South America, had revealed a decline in sperm count and an increase in abnormal sperm morphology in exposed indivuduals (Lerda and Rizzi, 1991; Sanborn, *et al*., 2002). Arbuckle and Sever (1998) also had reported an increase in time for women to conceive if their male counterparts were exposed to pesticides (Curtis, *et al*., 1999). Specifically, women whose male partners were involved in mixing and applying glyphosate, carbaryl and a phenoxy herbicide, 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB), within 3 months prior to conception, were at higher risk of spontaneous abortion (Arbuckle *et al*., 2001). The increase in time required for conception and spontaneous abortions in these women could possibly have resulted from sperm damage sustained by their male partners, and the consequent early embryo death and resorption (Curtis *et al*., 1999; Nurminen, 2001). Although exposure to physiological levels of 2,4-D alone is thought to pose no adverse effect to DNA or to human health, the reproductive risk to farm workers indicative of a cumulative effect of the same herbicide or a combined effect with other agents, has not been challenged (Arbuckle *et al*., 2001; Garabrant and Philbert, 2002). In addition, some of the hormonally active and popular herbicides and other estrogen mimics in the environment, have been shown to have the potential for causing adverse effects on the fetus or on the infant exposed to these agents by various mechanisms even in the absence of demonstrated genotoxicity (Foster, 1998; McLahlan, 2001; Hunt *et al*., 2003). For example, bisphenol A (BPA) an estrogen mimic used in the production of polycarbonate plastic lining for cages and cans in daily use have recently been shown to be a potent aneugen, (causing aneuploidy) through its disruptive effect on the meiotic spindle (Hunt *et al*., 2003). Atrazine, an activator of aromatase activity, is known to disrupt male sex differentiation in rodents and amphibians and 2,4-D has been shown to cause spindle abnormalities in dividing cells (tripolar and unipolar spindles, malorientation of mitotic apparatus in relation to cellular axis) and initiation of cell division in differentiated cells (Basrur, *et al*., 1976; Friedmann, 2002; Tavera-Mendoza *et al*., 2002). More importantly, one of these widely used herbicides (2,4-D), was detected in the seminal plasma of 50 percent of farmers working with this agent (Arbuckle, 1999; Arbuckle *et al*., 1999). Furthermore, forest pesticide applicators exposed to 2,4-D and showing high levels of this herbicide in their urine, were noted to show high serum levels of luteinizing hormone (LH) and increased DNA instability relative to that of men minimally exposed to this herbicide (Garry, *et al*., 2001). These observations, taken together with the findings on 2,4-D-elicited sperm damage and evidence of embryo mortality in different studies, suggest that some of these hormonally active agents could cause chromosome loss or deletion of a Y chromosome segment during spermatogenesis, or impose alterations in the pituitary gonadal axis (by their presence in the testis) and lead to male-mediated embryo mortality, fetal loss or malformed fetuses (Lerda and Rizzi, 1991; Arbuckle and Sever, 1998; Savitz *et al*., 1997; Weidner *et al*., 1998; Arbuckle *et al*., 1999; Abell *et al*., 2000; Garry *et al*., 2001).

Testicular Cancer

Surveys conducted from different parts of the world during the past few decades indicate that the incidence of testicular cancer has increased 3 to 4 times in Northern Europe and that this trend is evident in countries where reliable registries are maintained (Moller 1998; 2001; Damgaard, *et al*., 2002). An older survey on 2000 human testicular tumours in Canada and the United States had shown that 3.6 percent of these were associated with undescended testis (Mostofi, 1973). However, more recent reports indicate that the risk of cancer in cryptorchid men vary from 5 percent in a survey from the United States to 10 percent in a similar survey from Spain (Cortes, *et al*., 2001; Germa-Lluch *et al*., 2002). Mostofi (1973) had proposed that the factors contributing to tumour development in the

cryptorchid testis may include the presence of abnormal germ cells, endocrine disturbance and gonadal dysgenesis. This view has been supported by the higher prevalence of gonadoblastomas and dysgerminomas in sex reversed (XY) males with dysgenetic gonads (Simpson , 1979; 1987).

Testicular neoplasms are relatively rare in domestic animals probably due to the relatively early age at which males among farm animals are castrated for stock-rearing purposes. However, breed predisposition to specific type of testicular tumours, is recognized in domestic animals (McEntee, 1990). In cattle, Sertoli cell tumours (also referred to as tubular adenomas) occur more frequently in some breeds (Ladds and Saunders, 1976). In a survey from Queensland, 6 out of 800 slaughtered Shorthorn bulls were noted to have Sertoli cell tumors (Ladds and Saunders, 1976). Another study from Canada revealed two cases of Sertoli cell tumours in the descended testis of unilaterally cryptorchid sons of a Shorthorn bull (P*a*lmer, *et al*., 1980). Sertoli cell tumours were more prevalent among the testicular tumours detected in new born Holstein bull calves while interstitial cell tumours were more common in Guernsey bulls (McEntee, 1990). Apart from these breed-associated cases, tumours detected in bulls under 5 years of age tended to be of Sertoli cell origin while Seminomas (germ cell tumours) were more frequently seen in older bulls (McEntee, 1990). Testicular tumours are rare in cats, but common in dogs and horses. In one survey, the prevalence of testicular cancer was 13.6 fold higher in cryptorchid dogs compared to normal dogs, approximately a third of these being seminomas (McEntee, 1990). In the horse, seminomas are more frequently detected in the inguinal and scrotal testes while interstitial cell tumours and teratomas are more common in the abdominally retained testis (Leipold *et al*., 1986; McEntee, 1990). McEntee (1990) had suggested that the prevalence of interstitial cell tumours, which tend to be smaller than other types of testicular tumours, is probably much higher in horses than currently assumed since the surgically removed retained testis is not routinely examined for the presence of these small tumours while seminomas seen in older horses are generally recorded.

Mostofi (1973) had reported that testicular tumours constitute the fourth most common cause of death involving 11-13% of all cancer deaths, in the 15 to 34 year age group in North America. More recent surveys from Canada show that testicular cancers approximate 1 to 2 percent of all cancers in Ontario in men of 25 to 34 years of age and that the incidence has increased by 59.4 percent in the 32 years starting from 1964 to 1996, with a steady overall increase of 2 percent per year (Holowaty et al, 1996; Weir et al., 1999). Etiological factors implicated in this increase in testicular cancer include the influence of transplacental access of estrogen or estrogen mimics to the fetus at the critical stage of testicular differentiation. This assumption is based on the occurrence of testicular tumours in men who were exposed to diethyl stilbestrol (DES) at 7 to 10 weeks of their *in utero* life, and on experimental evidence from laboratory animals exposed to estrogen mimicking agents including DES (Conley *et al*., 1983; McLahlan *et al*., 1998; Klotz, 1999). Further support for the deleterious effect of estrogens on the fetal testis comes from observations on dizygotic twins in human populations (Klotz, 1999). It has been reported that the risk for testicular cancer in male twins (and breast cancer in female twins) are higher in dizygotic twin pregnancies where maternal estrogen levels are higher than that in monozygotic twin pregnancies (Swerdlow, *et al*., 1997).

In one of the Canadian survey mentioned above, the incidence of testicular germ cell cancers (seminomas and non-seminomas) was higher in men in the 15 to 29 year age group although non-seminomas tended to decline after 1990 (Weir *et al*, 1999). A similar survey from the United States also revealed a continued increase in the over all rate of seminomas during the period from 1973 to 1998, with the exception that the rate of increase steadily declined for white men while it doubled for black men at corresponding periods (McGlynn *et al*., 2003). Results of these North American surveys underline the differences in proneness to testicular germ cell cancers based on race, age and year of birth of the individuals (Weir *et al*., 1999; McGlynn, 2003). The elevated rates of seminomas and nonseminomas in men in the younger age group (15 to 29 years) compared to that in older men, raises some questions (Klotz, 1999; Weir *et al*., 1999). The answer may be found in the fact that the pubertal hormonal stimulus normally proffered to the young adults during sexual maturation, also provides for extensive germ cell loss and renewal in the testis. It is conceivable that in this hormonal milieu, testicular cells which sustain genetic alterations (by gene mutations) or functional modulation (by environmental agents) could gain a proliferative advantage over normal cells or undergo malignant transformation. Recent studies on human testicular germ cell tumours have shown that one of the tumour suppressor genes, *RASSF1A,* located on the short arm of chromosome 3 (3p21.3), is rendered epigenetically inactive by promoter hyper-methylation (Honorio *et al*., 2003). Interestingly, these investigators noted promoter methylation in more than twice as many nonseminomas as in seminomas (83 Vs 40 percent), indicating that the epigenetic events may have an additive effect on the progression of these germ cell tumours from seminomas to nonseminomas (Honorio *et al*., 2003).

Gynecomastia

One of the overt markers of feminization de

tectable in adult males is gynecomastia, characterized by persistent and often progressive enlargement of the breast. This condition, recognized as glandular proliferation leading to unilateral or bilateral enlargement of mammary glands in man or as galactoceles in young boys, also occur in domestic animals either spontaneously or due to the presence of testicular tumours (Panchdevi and Pandit, 1979; Carlson, 1980; McEntee, 1990; Braunstein, 1993; Al Salem and Nazer, 2002). Spontaneous development and persistence of udder in domestic male goats, referred to as milking buck syndrome (MBS) has been reported from various parts of the world (Rieck *et al*, 1975; Panchadevi and Pandit, 1979; Nair *et al*, 1981; Dafalla *et al*, 1990; Basrur and Yadav, 1990; Basrur, *et al*., 1997). The udder of the lactating bucks, located either in the periscrotal region or behind the penis carries functional lactiferous glands with extensive parenchyma consisting of groups of tubulo-alveolar secretory units separated by interlobular connective tissue septa (Basrur, *et al*., 1997). Hand milking of these bucks yields varying amounts (ranging from 25 to 1500 mL) of milk which is higher in fat and protein contents compared to that of normal does milk. In one survey, approximately 11 percent of the bucks being tested for artificial insemination (AI) service, were noted to be MBS (Hamori, 1983). Age at onset of lactation varied from 7 months to 8 years in the goats studied from Canada while the youngest age at which lactation was noted in Europe was 15 months (Marx *et al* 1975; Basrur *et al*., 1997).

The peak periods at which evidence of human gynecomastia is manifested (neonatal, pubertal and later stages in life) indicate that the stimulus for lactiferous glands may be endocrinological (Braunstein, 1993). Since estrogens stimulate growth of ductal epithelium and androgens inhibit its proliferation, either estrogen stimulation or target organ antagonism by antiandrogens or other factors, are suspected to play a part in this condition. Also other conditions implicated in hormonal imbalance (testicular dysgenesis and testicular cancers of germ cell, Leydig cell or Sertoli cell origin, adrenal neoplasms and paraneoplastic diseases, testicular feminization and androgen insensitivity syndrome) are associated with gynecomastia (McEntee, 1990; Braunstein, 1993). Furthermore, Olsson *et al*. (2002) have noted that men surgically treated for gynecomastia are at increased risk for testicular cancer, indicating that a hormonal imbalance may play a causal role in testicular malignancies and gynecomastia.

Enhanced sensitivity to estrogenic agents, either inherited or acquired during testicular differentiation in the fetus, or in adult life, could lead to protracted neonatal gynecomastia or persistent pubertal gynecomastia (Braunstein, 1993). Among the environmental factors known to induce gynecomastia in humans are diet, accidental estrogen ingestion, occupational exposure to pyrethroid pesticides, estrogenic contraceptives or embalming compounds (Braunstein, 1993). Another cause of human gynecomastia is androgen deficiency triggered by the displacement of androgen from the male breast tissue. The drugs known to interact competitively with androgen receptors and lead to estrogenic effect on the breast tissue in man include sprionolactone, cyproterone acetate, flutamide and cimetidine (Braunstein, 1993). Prolonged treatment with estrogen also causes enlargement of teats with dilated cisterns and ducts and mammary tissue formation and spreading, in bulls and rams (Hamori, 1983). The milking bucks studied from Canada belonged to different herds and were not treated with any hormones even though the possibility of their being exposed to estrogenic agents in pastures cannot be ruled out (Basrur *et al*., 1997).

Occurrence of milking buck syndrome indicates that it is a familial trait; however, the genetic component and the environmental factors in caprine gynecomastia still remain to be established (Hamori, 1980; Basrur *et al*., 1997). Chromosome analysis on a polled milking buck of German Fawn breed had shown a deletion of the Y chromosome even though in that case, gynecomastia was only one of the many manifestations of feminization (Rieck *et al*., 1975). None of the milking bucks examined from Canada exhibited a Y chromosome deletion and 8 out of 9 of these were of normal fertility (Basrur *et al*., 1997). In some cases of gynecomastia in humans (associated with adrenal neoplasms) and in domestic animals subjected to prolonged exposure to estrogen, azoospermia is a common occurrence (Limone *et al*., 1989; McEntee, 1990; Leiberman and Zachmann, 1992). The normal fertility, normal male (XY) sex compliments and the lower than normal testosterone levels detected in milking bucks suggest that the caprine condition (MBS) may be similar to the familial gynecomastia reported in humans (Hemsell *et al*., 1977; Berkovitz *et al*., 1985; Basrur *et al*., 1997). Men affected with gynecomastia in these families displayed normal male karyotype and normal testicular function, in spite of the 10 and 50 fold increase in their extra glandular aromatase activity (Hemsell *et al*., 1977; Berkovitz *et al*., 1985). The pattern of inheritance of this condition was thought to follow that of a sex linked (or sex limited-autosomal dominant) trait and the difference in aromatase activities in different families was attributed to varying severity of the mutations (Berkovitz *et al* 1985). More recently, a point mutation (at codon 870) in the sex linked androgen receptor gene was noted to be associated with gynecomastia in otherwise "normal" men and the investigators hypothesized that bilateral gynecomastia may be one of the phenotypes of the milder form (single amino acid substitution type) of mutation in the AR gene (Chavez *et al*., 2001; Zenteno *et al*., 2002).

Determinants of genital malformations

Anomalous sex differentiation in humans, manifested as hypospadias, micropenis, cryptorchidism and \ or total sex reversal, is reported to be as high as one in every hundred newborn babies (Vilain, 2002). The disturbing part of these statistics is that only less than 15 percent of these cases can be attributed to mutations in any of the known genes involved in the sex differentiation pathway or to a chromosome anomaly (Vilain, 2002; Scherer, 2002). This would imply that many more genes involved in this process are yet to be discovered or that the etiologies of these disorders are not exclusively genetics. Regardless of these alternatives, since a majority of the individuals afflicted with these malformations are not capable of reproduction, their relatively high prevalence has to be the results of new mutations or the results of collaboration of environmental factors with hormone sensitive genes in the sex differentiation pathway. Some of the genes expressed (or effectively suppressed) during testis differentiation including *SF1* and *DAX1*, interact with estrogen and estrogen receptors and these and other male sex determinants are also expressed in other endocrine organs of the fetus and adults, indicating their pleiotropic potential in the mutant forms (Zhang *et al*., 2000; Ozisik, *et al*., 2002; Scherer, 2002). Implicit in the perception of a gene-environmental collaboration is the multifactorial etiology of birth defects, which is worth considering in the context of genital malformations and other aspects of feminization of males.

It is evident that male sex differentiation, because of its complex and inter-related component steps, requires not only the participation of a large number of genes at different loci, but also proper timing and levels of expression of these genes. In situations where two or more of these loci are occupied by their mutant counterparts, the developmental process dependent on stage specific products of these genes, will be compromised to varying degrees based on the number of such loci (and their overall concentration) in the conceptus. Smaller number of these mutant genes may not interrupt the birth of an overtly normal individual if their presence can be compensated for (by other genes) and the concentration of altered (mutant) genes is within the load that permits the conceptus to proceed through the normal developmental "threshold" (Nicholas, 1996). However, in some cases the sheer number of these mutant genes may overwhelm the process in such a way that a developmental disruption becomes detectable (Nicholas, 1996). Since an increase in the number of these mutant genes increases the liability of the conceptus to a malformation, these genes are referred to as liability genes and the malformations of this category are referred to as threshold traits. In other words, a malformation of this type reflects that the concentration of liability genes in the conceptus is beyond that which allows its normal development (Nicholas, 1996). The organ affected and the type and severity of the disruption elicited are factors that determine the *in utero* fate of the conceptus. Minor disruptions that do not impede the survival of the conceptus are likely the ones generally encountered in man and domestic animals. Sex reversal and genital malformations fall under this category of polygenic threshold traits that are controlled by many loci and are compatible with fetal survival since normal gonads and normal reproductive system are not vital for the survival of the fetus.

Prevalence of such malformations in any population will generally be low because of the relatively large number (or concentration) of liability genes required to create a specific developmental crisis (Nicholas, 1996). In domestic animals increase in liability genes is brought about by inbreeding, as demonstrated by the increased incidence of familial traits such as sex reversal, cryptorchidism and other sex-related problems in stallions and boars and gynecomastia in goats. However, the concentration requirement (the number of loci need to be occupied by liability genes) may be reduced if the conceptus carrying a few liability genes, is exposed to adverse environmental influence (Nicholas, 1996). How exactly environmental factors augment the disruptive potential of the liability genes is not known. However, the fact that the extensive use of pesticides is prevalent in technologically advanced countries including Canada (Nurmimen, 2001, Damgaard et al., 2002; Weir, 2002), gives some indications on the possible mechanisms of their interaction in light of the observations described below.

Maternal exposure to pesticides is known to be associated with relatively high reproductive risk (Abell, *et al*., 2000; Arbuckle and Sever, 1998). Specifically, Arbuckle and associates have shown that preconception exposure to pesticides containing glyphosate, carbaryl and 2,4-D, increases the rate of spontaneous abortion in women and that the risk doubles with increasing maternal age, showing that exposure to these agents (and maternal age) contribute to the loss of the embryo or fetus (Arbuckle and Sever, 1998; Arbuckle, 1999; Arbuckle etal., 2001). Intersestingly, exposure of older women to more than one type of pesticides (2,4-D and carbaryl, for example) led to a 27-fold increased risk of spontaneous abortion, compared to that in women of their age group exposed to carbaryl alone (Arbuckle *et al*., 2001). Elevated rate of spontaneous abortions in these women would indicate that exposure to these pesticides had caused the break down of one or more major organ system development (through induced gene mutations, chromosome defects or severe hormonal imbalance in the mother) at the level that is incompatible with *in utero* survival of the fetus. However, it is conceivable

that the impact of these or other pesticides on fetal reproductive system (as opposed to other major somatic organ system of the fetus), leading to genital malformations, is not incompatible with *in utero* survival. Studies on women occupationally exposed to pesticides suggest that first trimester exposure poses an elevated risk of birth defects including cryptorchidism in male children and that the risk is higher in agricultural regions of high pesticide use (Garcia-Rodriguez *et al*., 1996; Cheek and McLachlan, 1998; Garcia, 1998; Weidner *et al*., 1998; Garcia *et al*., 1999). The potential of these environmental contaminants to find their way to human or domestic animal fetus through transplacental route is further illustrated by the presence of pesticide metabolites in the amniotic fluids of pregnant women and by the faulty gonadal and reproductive system development noted in domestic animals experimentally exposed to some of these compound (McMahon, *et al*., 1995; Foster, *et al*., 2000; Sweeney *et al*., 2000; 2002).

Maclahlan (2001) has discussed the different ways in which estrogen mimics and antiandrogens which do not cause detectable DNA change, could disrupt the normal developmental mechanisms. These include acute or persistent modulation of expression of hormone responsive genes coding for stage-specific proteins required for a developmental process. Some estrogen mimics have already been demonstrated to simulate the effects of gene mutations. For example, the phenotypes of a HOXA gene mutation and Insl 3 mutation have been simulated by prenatal exposure of mice to estrogens or diethyl stilbestrol (Ma *et al*., 21998; Konlon *et al*., 1999; Nef and Parada,1999; Emmen *et al*., 2000; Nef *et al*., 2000). The phenotypic impact in these cases is thought to be elicited by altering the programmed methylation- demethylation cycle of pertinent genes. Methylation- demethylation pattern are organ- specific and some of the enzymes involved in these processes are sex-specific (Trasler, 1998). It is conceivable that disruptions of these events by hormonally active agents could lead to male-specific anomalies and other manifestations of feminization.

Conclusion

Sex reversal and sex-related anomalies similar to those seen in man occur in domestic animals. Even though the rates of sex anomalies vary greatly among lines, breeds and species of domestic animals, the prevalence rates of some of these is much higher in domestic animals in which high degree of inbreeding occurs by choice or by shortage of males maintained for breeding. Information similar to that on human sex differentiation is available on some domestic animals (Pailhoux, *et al*., 2001c; 2002). Sweeney, *et al*., (2002) have shown the effect of endocrine disruptors on the fate and function of testes in male lambs. Furthermore, observations by Bogh *et al*., (2001b) on the long range effects of estrogenic agents on sows, manifested as hyper-proliferation of genital tract epithelium, precocious onset of puberty in second generation sows and reduced litter size of their offspring, in a multigeneration studies indicate that the genital malformations and reduced reproductive health encountered in domestic animals, may be augmented by the environment they share with human populations.

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