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Germ cells, gonads and sex reversal in marsupials

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ABSTRACT The formation of the testis or ovary is a critical step in development. Alterations in gonadal development during fetal or postnatal life can lead to intersexuality or infertility. Several model systems have been particularly useful in studying gonadal differentiation, the eutherian mammal and amphibia, fish, and birds. However, marsupials provide a unique opportunity to investigate gonadal development and the interactions of genes and hormones in gonadal differentiation and germ cell development in all mammals. On the one hand the genetic mechanisms appear to be identical to those in eutherian mammals, including the testis-determining SRY gene. On the other hand, marsupials retain in part the plasticity of the amphibian gonad to hormonal manipulation. It is possible to induce female to male and also male to female gonadal sex reversal in marsupials by hormonal manipulation, and oestradiol can induce male germ cells to enter meiosis at the time the oogonia do. In addition, in marsupials the development of the scrotum and mammary glands are independent of testicular androgens and instead are controlled by a gene or genes on the X-chromosome. Thus marsupials provide a number of opportunities for manipulating the sexual differentiation of the gonads that are not possible in eutherian mammals and so provide a unique perspective for understanding the common mechanisms controlling sexual development.

KEY WORDS: *wallaby, sex differentiation, virilization, pouch, scrotum.*

Introduction

Anne McLaren's name is synonymous with studies of the enigmatic germ cell and her contributions to the field of sexual differentiation are legendary. Although almost all her research has been on the mouse, Anne has always encouraged our research on Australian mammals, and has visited us several times and joined in our marsupial studies: she has become an honorary wallabologist. The work reported here has benefited over the years by vigorous discussions with Anne in locations as diverse as Kangaroo Island, South Australia, Hawaii and the Scottish highlands.

Germ cells and soma

Germ cells are the only potentially immortal cells in the body. The germ line produces the gametes which carry genetic information from one generation to the next, supported and nurtured by the somatic cells (McLaren, 1981; 1990; 1998). Germ cells have a unique developmental pathway in that they arise in the embryonic epiblast and later migrate into the developing gonad along a route specified by a suite of cytokines, growth factors and cell adhesion molecules. Descendants of individual epiblast cells may give rise to different tissues depending on the various signals or local tissue influences they experience during gastrulation. Thus, some cells will form somatic tissues, whilst their nearest neighbours may form the progenitors of the gametes, the primordial germ cells. Primordial germ cells become oogonia in females, and spermatogonia in males. PGCs increase in number during their migration from their extra-embryonic locations to the gonadal ridge. Female germ cells enter meiosis before birth to become oocytes, and most mammals have their full complement of female germ cells by the time of birth, with the number of oocytes progressively decreasing throughout adult life.

Perhaps the most surprising event of early mammalian development is that the cells that will form these germ cells are excluded from the embryo very early in development. Why this occurs is uncertain, but one hypothesis is that it protects them from the DNA methylation by which most of the other cells of the embryo are genomically imprinted.

Almost all our knowledge of germ cell biology in mammals has been based on studies of the mouse. The mouse however, has a complicated egg cylinder, so that following germ cell migration is more difficult than in species with an embryo that develops a more planar orientation. Chickens have been used extensively because their development occurs in this way. However, there is one group of mammals that have an embryo that develops on the surface of

Abbreviations used in this paper: FISH, fluorescent *in situ* hybridization.

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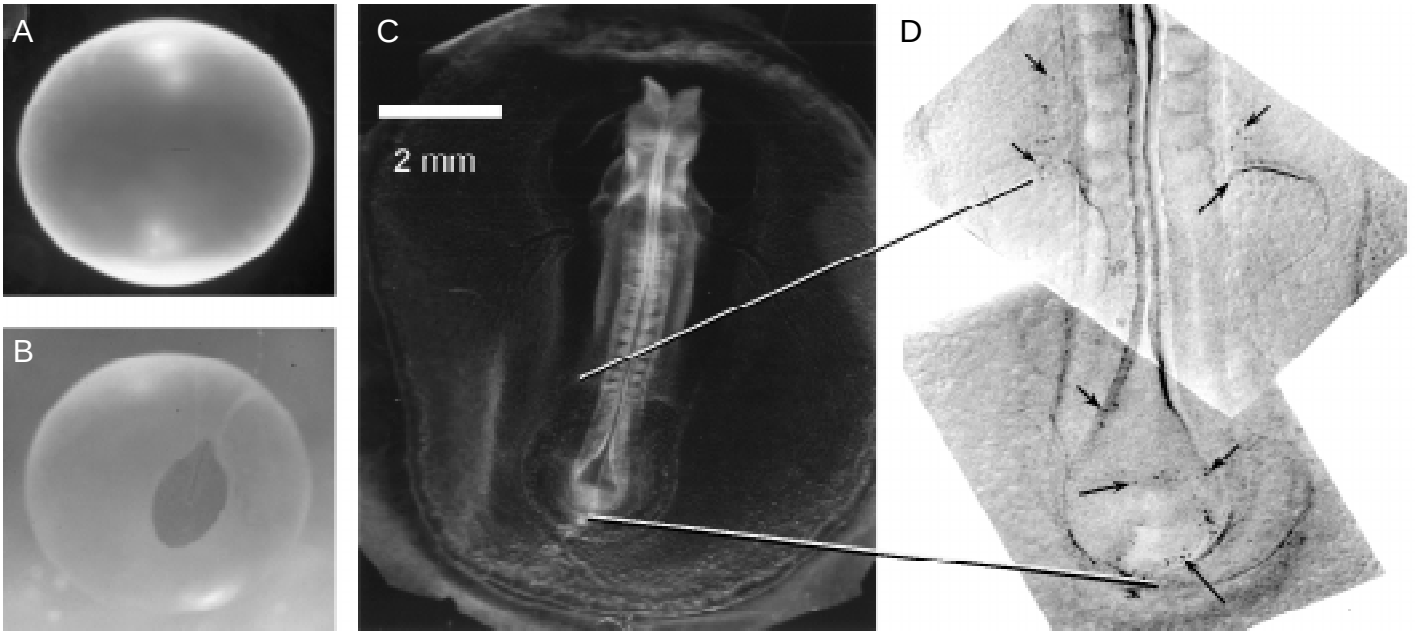

Fig. 1. **Alkaline phosphatase staining of developing vesicles of the tammar wallaby. (A)** *Day 13 of gestation,* **(B)** *day 14 of gestation, and* **(C)** *day 17 of gestation. There is no embryonic disc at day 13, and no alkaline phosphatase staining. At day 14* **(B)** *the entire mesodermal plate is positive, but by day 17 about 80 cells only grouped either side of the neural tube and at the posterior stain positively.* **(C)** *retaken from Ullmann et al., 1997.*

the trophoblast in a planar orientation, namely marsupials. Marsupials were recognised for their potential in developmental biology and in particular for studies of sexual differentiation by C.R.Moore and R.K.Burns early in the 20th century but hardly studied in depth until more recent times (reviewed in Tyndale-Biscoe and Renfree 1987; Wilson *et al.,* 1995). We have used the tammar, *Macropus eugenii*, a small member of the kangaroo family that breeds readily in captivity, as our primary experimental model. In the tammar, the formation of the primitive streak, the development of the node, notochord, definitive endoderm, somites and closure of the neural tube all occur on the surface of the developing embryonic vesicle. These events span d13 and d19 of gestation, a much longer time period than the mouse. The allantois arises later still, at about d20-21. This makes the tammar an ideal model to explore the key signals and tissues that may be involved in germ cell allocation.

Whilst a great deal is known about mammalian germ cell morphology, the migratory pathway, and the ultimate fate of the germ cells as eggs and sperm, much less is known about the enigmatic origins of these all-important cells. Experimental analysis of the early mouse embryo has begun to provide some clues about the control of germ cell allocation.

In the mouse, the primitive streak forms at the onset of gastrulation at 6.5 dpc by an epithelial mesenchymal transition of the epiblast adjacent to the extra-embryonic ectoderm of the egg cylinder (reviewed in Tam & Behringer, 1997). By 7.2 dpc the germ cells are lineage restricted (reviewed in McLaren, 2000). This remarkably brief window of time during which the signals responsible for the establishment of the germ line are expressed was defined by three elegant experiments (Lawson & Hage, 1994; Tam & Zhou, 1996; Lawson *et al.,* 1999). A founding population of approximately 45 cells was identified as lineage-restricted primordial germ cells (Lawson & Hage 1994) when the primordial germ cell precursors moved out of the epiblast and into the posterior primitive streak and extraembryonic mesoderm by around 7.2 dpc (Lawson & Pederson, 1992; Lawson & Hage 1994). Further confirmation of this came from the *in vitro* transplantation experiments of Tam & Zhou (1996) in which genetically marked (LacZ) distal epiblast cells from pre- and early primitive streak stage embryos (6.0 dpc - 6.5 dpc) were transplanted to different locations in the mouse egg cylinder. The differentiation of the cell types depended on their location after 6.5dpc not on their original location in the embryo (Tam & Zhou, 1996). Thus, mouse primordial germ cells (PGCs) are probably allocated early in gastrulation in a group of greater than 40 cells already segregated in the extraembryonic mesoderm. Candidate genes controlling the lineage restrictions must therefore be expressed in the mouse embryo before and during gastrulation. What is the evidence for these genes?

PGCs express the gene *OCT4*, a POU family member that is characteristic of totipotent cells (Schöler *et al.,* 1989). *Oct4* may maintain the potency of germline cells by preventing all other differentiation pathways (Pesce and Scholer, 2000). *Oct4* may also regulate the molecular differentiation of cells in the germline lineage, and might be one of the molecular triggers necessary for the onset of meiosis (Pesce *et al.,* 1998). Apart from OCT4, one factor that may induce germ cell precursors to form PGCs is bone morphogenetic protein 4 (BMP4), a member of the TGFβ superfamily of intercellular signaling proteins (Hogan, 1996). BMPs have various biological effects on different cell types, including stimulation of alkaline phosphatase activity, and have many important roles in morphogenesis of the embryo (Lyons *et al.,* 1991). Recently, BMP4 has been implicated in the formation of primordial germ cell precursors (Lawson *et al.,* 1999). *Bmp4* is expressed early in the extraembryonic ectoderm and later in the extraembryonic mesoderm (Hogan, 1996). It is not expressed in PGCs, but it appears that initiation of the germ cell lineage in the mouse depends on a secreted signal from the previously segregated

extraembryonic trophectoderm lineage. These studies are consistent with the results from the elegant experiments of Tam and Zhou (1996) described above. Thus, it appears that it is the position of the cells, not their origin, that determines their destiny, and that bone morphogenetic protein 4 (Bmp4) is necessary but not sufficient for germ cell differentiation (McLaren, 1999, 2000). Other signals must participate in the process because not all the cells in the region become germ cells. As yet there is no information on *BMP4* and little on *OCT4* in marsupials, but we have recently begun studies on these genes and await the results with interest. In another marsupial, the brushtail possum, OCT4 is present in oocytes in developing follicles (Frankenberg *et al.,* 2001).

Although PGCs are usually morphologically distinctive, there are few biochemical markers which identify PGCs. Germ cells are alkaline phosphatase positive (Byskov and Hoyer, 1994) They cross react with several antigens, such as stage specific embryonic antigen (SSEA-1) which has been especially useful in immunocytochemistry and confocal microscopy of germ cells (Gomperts *et al.,* 1994), tracing the migratory path into the gut epithelium and dorsal mesentery. Another antigen, GCNA-1 is specific for PGCs and in the mouse is expressed in PGCs from their arrival in the genital ridge (Enders & May, 1994). In the tammar, PGC migration occurs along the dorsal mesentery, but the germ cells never enter the hind gut as in the mouse (Ullmann *et al.,* 1997; Jiang *et al.,* 1997). In contrast, in the chicken, germ cell migration to the gonads occurs via the blood stream (Ginsburg & Eyal-Giladi 1987).

Putative PGCs can be identified morphologically as an inner cluster posterior to the primitive streak where the base of the allantois will form (Ginsburg *et al.,* 1990, Anderson *et al.,* 2000). Mouse and human PGCs are alkaline phosphatase positive as are a number of other embryonic tissues. PGCs remain AP positive during their proliferation and migration to the genital ridge (Buehr 1997; Anderson *et al.,* 2000). Similarly in the tammar, primordial germ cells are alkaline phosphatase positive (Ullmann *et al.,* 1997) and in the 15-20 somite stage embryos, about 80 PGCs can be identified in the extraembryonic mesoderm, in the endoderm of the yolk sac and in the embryonic endoderm (Fig. 1) (Ullmann *et al.,* 1997). At the earliest stages examined, when the embryonic disc is just beginning to form, the whole of the presumptive embryonic tissue is alkaline phosphatase positive (Fig. 1). By the time the tammar PGCs have reached the gonad via a migratory pathway that is similar, but not identical, to that of the mouse, they have increased in number, but unlike in eutherian mammals, they continue to divide after birth. Mitotic division does not begin to be replaced by meiotic division until day 25 post partum and peak numbers are reached at about 50 days post partum when all the germ cells are meiotic (Alcorn, 1973; Alcorn & Robinson, 1983). Tammar PGCs also cross react with stage specific antigen SSEA-1 (S.Ullmann, A.McLaren, G.Wijyanti, G Shaw and M B Renfree unpublished observations). However, in our hands we have so far been unable to obtain a strong positive immunocytochemical stain with germ cell nuclear antigen GCNA (G.Wijyanti unpublished observations)

Gonadal differentiation

Although the germ cells appear to have their own developmental pathway, they need the supporting cell lineages to develop normally. Thus formation of the testis or ovary is a critical step in sexual differentiation. In all mammals development of the embryo depends on complex site- and stage-specific regulation of gene expression that results in the amazingly complex processes of morphogenesis. In the eutherian mammal many of the genes involved have been identified (Swain & Lovell-Badge, 1999). Steroidogenic factor-1 (*SF1*) and Wilm's tumor-1 (*WT1*) are transcription factors that are essential for genital ridge formation (Kreidberg *et al.,* 1993; Luo *et al.,* 1994). Once the genital ridge is formed, the high mobility group (HMG) transcription factors *SRY* and apparently *SOX9* initiate testis formation in males (Koopman *et al.,* 1991; Foster *et al.,* 1994; Wagner *et al.,* 1994). Testis development can be antagonised by the nuclear hormone receptor DAX1 (Swain *et al.,* 1998), although gonadal differentiation is not affected in *DAX1* knockout mice. Other proteins that influence sexual differentiation and gametogenesis include Mullerian inhibiting substance (MIS), WNT4, Desert hedgehog (DHH) (Behringer *et al.,* 1994; Bitgood *et al.,* 1996; Arango *et al.,* 1999; Vainio *et al.,* 1999) and growth & differentiation factor-9 (GDF9) (Dong *et al.,* 1996). Despite these various advances, however, at present relatively little is known about how these genes interact and about the identity of the intercellular messenger systems that connect them.

In eutherian mammals, all of these events occur in utero, but in marsupials much of gonadal differentiation occurs post-natally, so the young are readily accessible in the pouch for experimental

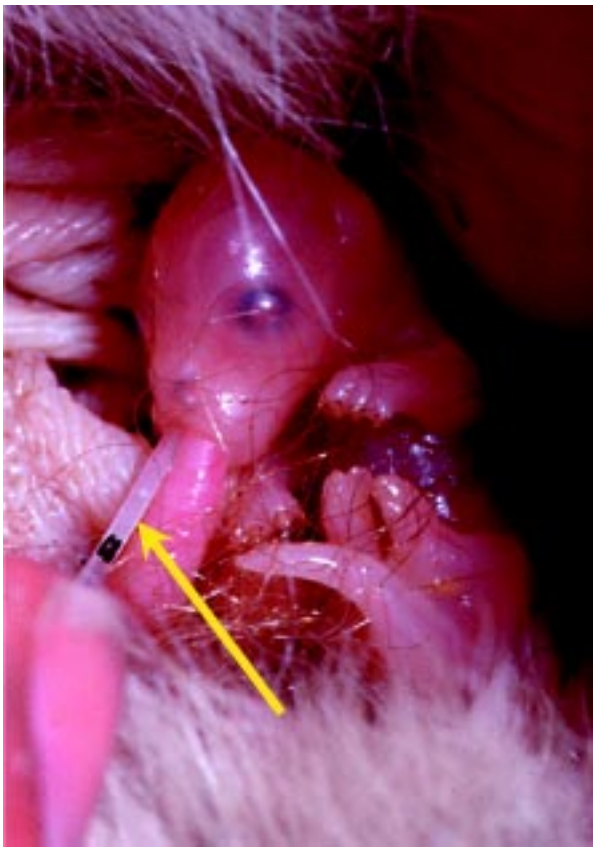

Fig. 2. **Pouch young of a tammar 5 days after birth, showing the oral administration of a hormone**. *Hormone is delivered via a polythene tube inserted beside the teat (yellow arrow) The pouch young drinks the hormone in with each suck of milk. The black mark on the tube allows an accurate volume to be administered.*

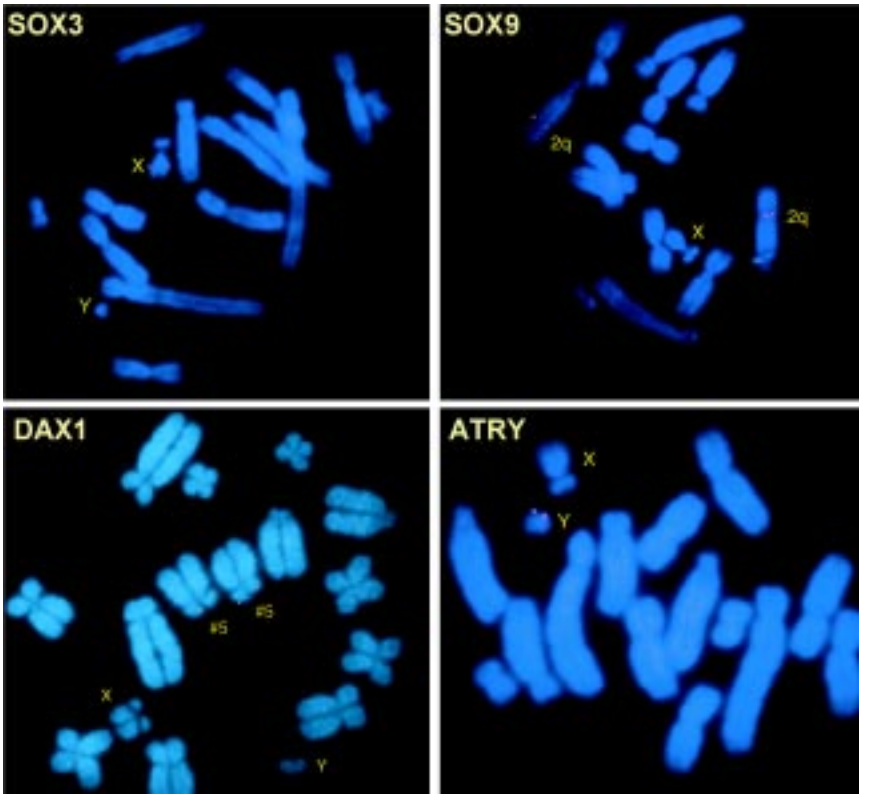

Fig. 3. **Fluorescence *in situ* hybridisation localisation (FISH) of genes on tammar chromosomes.** *SOX3 (on the X chromosome) SOX9 (on 2q), DAX1 (on 5) and ATRY (on the Y chromosome). Photographs courtesy of Dr A.Pask.*

studies of the hormonal control of sexual differentiation (Fig. 2). We have now cloned a number of the genes involved in sexual determination and differentiation in marsupials, and assessed their expression patterns. Our detailed analysis of the sexual differentiation of the tammar has emphasised that the time course of these events is prolonged compared to the mouse (Harry *et al.,* 1995; Renfree *et al.,* 1996; Pask and Renfree, 2001).

Sexual differentiation in marsupial mammals has many similarities with that of eutherian mammals (Renfree, 1994; Shaw *et al.,* 1990). Marsupials have an XX-XY sex determining mechanism, and have a homologue of the testis-determining gene *SRY* on their Y chromosome which is first detected in the gonad before differentiation of the supporting cell lineage into Sertoli cells (Harry *et al.,* 1995). However, like the human but unlike the mouse, SRY is widely expressed in male tissues until at least 40 days after birth expressed in a range of non-gonadal tissues in male pouch young and adults. Although it occurs at the right time and in the right tissue, there is as yet no direct evidence that *SRY* is testis-determining in marsupials, but it is presumed to induce differentiation of the wallaby testis at about the time of birth.

In the tammar *SF1* transcripts are present in adrenal, kidney and gonads of both males and females from earliest age examined but until day 8 after birth the signal in males appears to be considerably stronger (DJ Whitworth, AJ Pask, G Shaw, RR Behringer and MB Renfree, unpublished observations). *WT1* is also expressed strongly in the tammar kidney throughout development (J. Paplinska, A Pask and MB Renfree unpublished observations)

One *SRY*-related gene, SOX3 is expressed in the developing mouse gonad at the same time as *SRY* (Collignon *et al.,* 1996; Foster and Graves *et al.,* 1994). Based on this coincident expression Graves (1998) proposed that *SOX3* and *SRY* might control the activation of another *SOX* gene, *SOX9*, and therefore testis development. However although *SOX3* is located on the X-chromosome in marsupials (Foster and Graves, 1994; Pask *et al.,* 2000b) (Fig. 3), it is not expressed in the developing gonad of male or female tammar wallabies (Pask *et al.,* 2000b). Thus this gene is not necessary for sexual differentiation of the gonad in this species, and so may not be needed in eutherian mammals either.

SOX9 appears to be the key downstream regulatory gene needed to induce testicular differentiation in all eutherian mammals so far studied, as well as in reptiles and chickens (Kent *et al.,* 1996; Western *et al.,* 1999; Smith *et al.* 1999) through its interactions with several other genes, in particular *DAX1* and *WNT4.* The timing and cell specific expression of SOX9 suggests that it may be directly regulated by *SRY* (Wagner *et al.,* 1994; Kent *et al.,* 1996, Swain and Lovell-Badge, 1999). *SOX9* expression in the male gonad increases at the onset of testicular differentiation but it is abolished in the ovary (Morais da Silva *et al.,* 1996). Transgenic introduction of the key sex determining gene *SRY* has only been tested in the mouse, and here, while it produced a testis in a female, the resultant sex-reversed animal was infertile (Koopman *et al.,* 1991), suggesting that *SRY* does not cause sexual

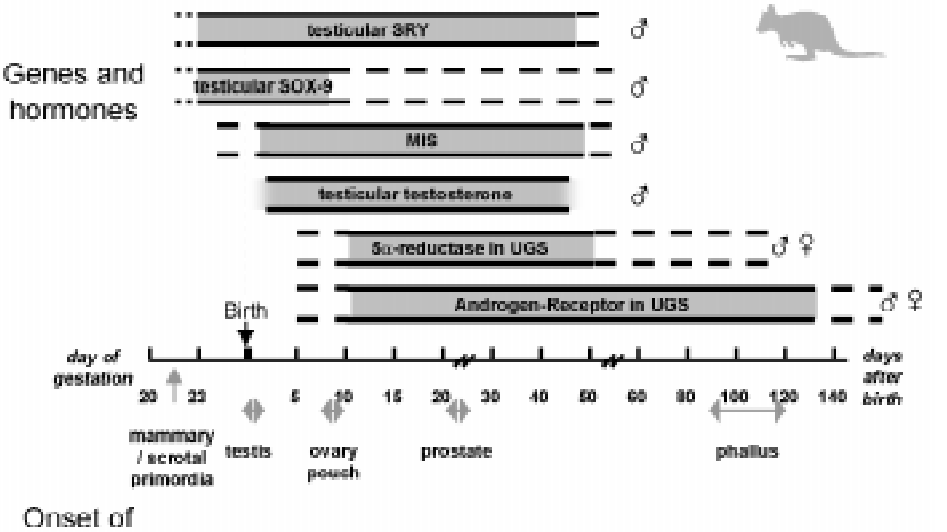

Fig. 4. Diagrammatic summary of the time during development that the various genes, hormones and sexual dimorphisms are present in the tammar wallaby pre- and post-partum. *UGS, urogenital sinus.*

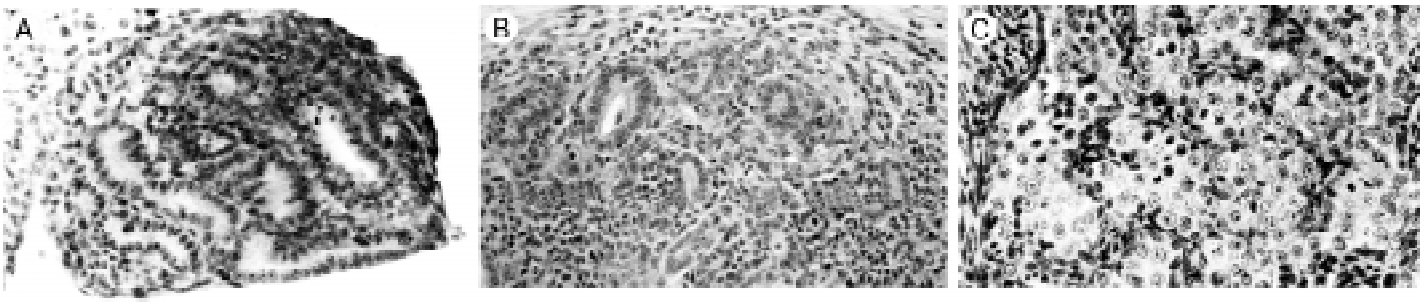

Fig. 5. **Sex reversal of tammar ovaries**. *Seminiferous cord–like structures formed in tammar ovaries after culture for 4 days in the presence of rhMIS* **(A)**, *or transplanted to the flanks of male neonates for 25 days* **(B)**, *compared to an ovary transplanted to a female pouch young that has a normal ovarian appearance* **(C).** *(Data from Whitworth et al., 1996).*

differentiation of germ cells. *SOX9*, which is essential for testis development in man and mouse (Sinclair *et al.,* 1990) and for normal skeletal development (Kanai and Koopman, 1999), is expressed in both tammar ovaries and testes from late gestation until adulthood (Harry *et al.,* 2001) consistent with its importance in the vertebrate sex determining pathway. We have also recently found that *SOX9* is expressed in the scrotal and mammary primordia at days 24, 25 and 26 of the 26.5 day gestation (Harry *et al.,* 2001). Expression was undetectable by day 1 post-partum in the scrotum and by the day of birth in mammary gland, but we do not yet understand the significance of this observation.

In the tammar, another gene, *ATRX*, known to induce sex-reversal in humans is expressed in the developing adrenal, mesonephros, and in the adult epididymis, prostate, spleen, testis, brain, kidney and lung, whereas a new gene, *ATRY* is expressed only in the developing testis and adult testis (Pask *et al.,* 2000a) (Fig. 3).

Another gene, desert-hedgehog (*DH*H) is a member of a family of genes that encodes secreted proteins that control many aspects of growth and patterning during animal development. Although *DHH* expression in pre-Sertoli cells is one of the earliest indications of male specific gene expression in the gonad, it has not yet been well studied. In fetal male mice *Dhh* is expressed in Sertoli cell precursors shortly after the activation of Sry and expression continues into the adult (Bitgood *et al.,* 1996). *Dhh* is not expressed in the ovary. Male mice with a *Dhh* null mutation are viable but infertile. There is a complete absence of mature sperm due to defects in early and late spermatogenesis. *Dhh* may play distinct roles in the regulation of mitosis and meiosis in the male germ line, although it is not an essential germ-cell mitogen (Bitgood *et al.,* 1996). Despite its obvious interest, there is little other information available on *DHH* function in sexual differentiation, other than the recent identification of hedgehog interacting protein, HIP, an alternative signaling protein (Chuang & McMahon, 1999). We have recently established that *DHH* is expressed in the marsupial testis during the period of sexual differentiation in a manner similar to that seen in eutherian mammals (C-A Mao, JL Harry, D Whitworth, RR Behringer, G Shaw, A Pask and MB Renfree unpublished observations). *DHH* transcripts are expressed in both male and female tammar gonads from at least two days before birth to four days after birth assessed by RT-PCR, but with Northern analyses *DHH* is only present in measurable quantities in testes.

The female pathway of development was thought to be the result of the absence of the testis-determining factor (*SRY*) and consequent absence of MIS and testosterone. Over-expression of *DAX1* interferes with *SOX9* activation by *SRY* causing male to female sex reversal (Swain *et al.,* 1998). However it is now known that it does not have a critical role in female development since the *DAX1* knockout does not interfere with normal female development (Yu *et al.,* 1998). However, in males *DAX1* is essential for the maintenance of spermatogenesis (Yu *et al.,* 1998). *DAX1* cannot function in a dose-dependent manner in marsupials, as we now know that *DAX1* is autosomal (Fig. 3) and is located on tammar chromosome 5 (Pask *et al.,* 1997).

Recent evidence has implicated another gene, *WNT4*, as critical for female development (Vainio *et al.,* 1999). *WNT4* is a signaling molecule that is essential in both sexes for the formation of the Müllerian duct, but in the ovary it suppresses the development of Leydig cells. The *WNT4* gene has a unique role in sexual differentiation, in that it is the first gene identified to have a direct role in the differentiation of the ovary and female urogenital system. WNT4 was first identified through mutations affecting nephrogenesis of the murine kidney (Stark *et al.,* 1994). It is expressed in the mouse embryonic kidney (the mesonephros) and the developing gonads of both XX and XY fetuses and also the Müllerian ducts of female embryos.

After sexual differentiation expression of *WNT4* is restricted to the developing ovary and down regulated in the testis. Male mice homozygous for a *WNT4* null allele develop phenotypically normal testis but die shortly after birth due to kidney failure. *WNT4* mutant females are masculinised: the Müllerian ducts never form, testosterone biosynthesis is activated and the Wolffian ducts develop. Later the female germ cells are lost and seminiferous-like cords form (Vainio *et al.,* 1999). Testosterone production from the ovary is due to the development of male gonad Leydig cells, within the ovary. The absence of Müllerian ducts in homozygous null females is not due to regression of the developing ducts, but due to a failure for them to develop at all. *WNT4* is therefore essential for the development of the Müllerian ducts and the differentiation of a viable ovary by suppressing the development of Leydig cells and maintaining the female germ line. This is a major new finding which alters our previous perception of the female differentiation pathway as being the passive or default state. We are currently cloning *WNT4* in the tammar to examine its expression profile during ovarian development, and also during gonadal sex reversal.

Development of scrotum and pouch

In male tammars (O *et al.,* 1988), marsupial antechinuses (Bolton 1983), American opossums (Renfree *et al.,* 1990) and

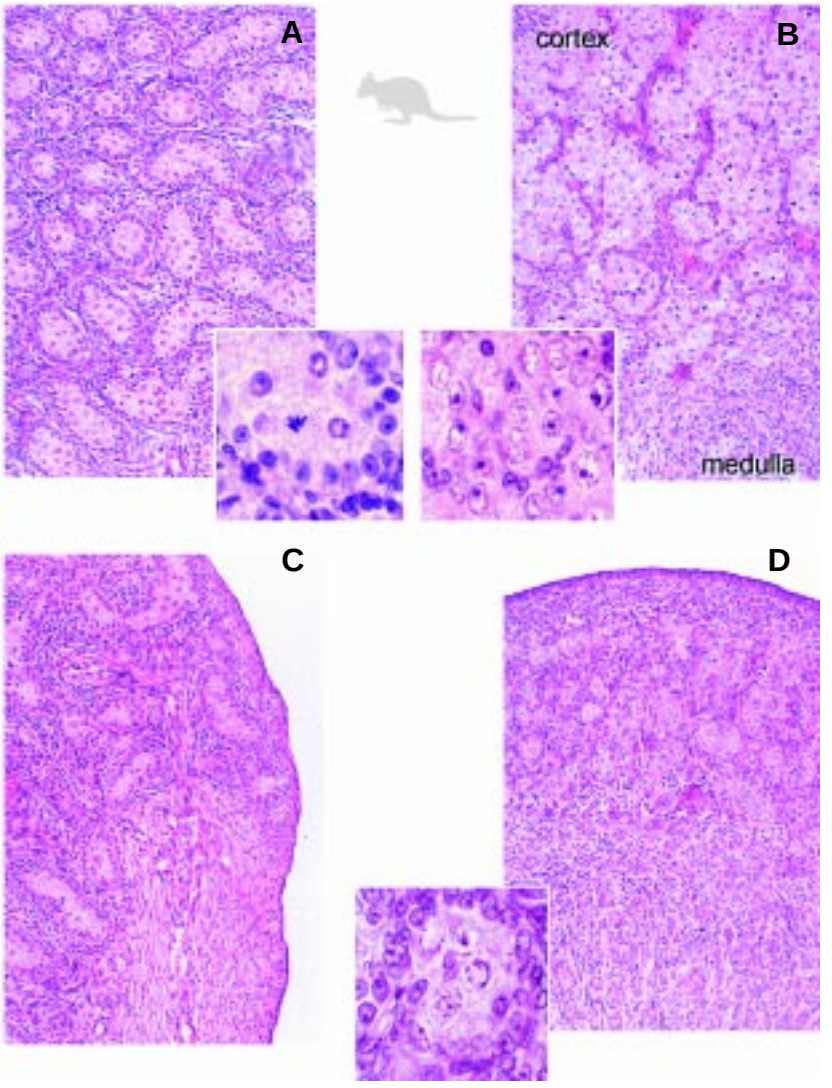

Fig. 6. **Sex reversal of tammar testes collected at day 50 post partum after oestradiol treatment of pouch young for 25 days**. **(A)** *Normal day 50 testis and insert showing mitotic germ cells.* **(B)** *Normal day 50 ovary, with meiotic germ cells.* **(C)** *Testis day 50 after oestrogen treatment, with disrupted cords and poorly retained seminferous tubules.* **(D)** *Sex reversed testis day 50 after oestradiol treatment, showing an ovarian like medulla, cortex, and insert with germ cells in various stages of meiosis. (Data from Coveney et al., 2001).*

brushtailed possums (Ullmann 1993) scrotal bulges are visible on or before the day of birth. In the tammar, scrotal bulges only develop in genetic males, and primordia of the mammary glands only develop in genetic females (Fig. 4). Both structures differentiate several days before birth and well before the gonadal primordia differentiate into a testis or an ovary (Alcorn, 1975; O, *et al.,* 1988; Renfree *et al.,* 1995; Wilson *et al.,* 1995). Interestingly there is a dichotomy between male Australian and American marsupials in that mammary primordia are never present in the former, whereas a reduced number occur in fetuses and pouch young of the latter (Renfree *et al.,* 1990; Robinson *et al.,* 1991). Treatment of wallaby with androgens or oestrogens has no effects on the presence of the mammary, pouch or scrotal primordia or their development (Shaw *et al.,* 1988). Thus, formation of the mammary gland, scrotum and gubernaculum precede gonadal differentiation and so cannot be hormonally determined. This necessitated revision the paradigm that mammalian sexual differentiation is totally dependent on testicular hormones (Renfree & Short, 1988; Renfree *et al.,* 1995).

The decision about whether a scrotum or mammary glands will form in marsupials is controlled by an X-linked genetic mechanism and is independent of gonadal sex (O *et al.,* 1988, Renfree & Short 1988; Sharman *et al.,* 1990; Cooper 1993; Shaw *et al.,* 1997). Marsupial "Klinefelter" (XXY) male intersexes have no scrotum, but have mammary glands and a pouch, despite testes, a normal male internal reproductive anatomy and a phallus. "Turner" XO female intersexes have ovaries but no mammary glands or pouch, and have an empty scrotum. Thus, the number of X chromosomes determines the presence of a pouch or scrotum. Some intersexual marsupials have bilaterally asymmetrical reproductive systems with a hemi-pouch and a hemi-scrotum suggesting that the pouch and scrotum are developmental alternatives regulated locally by morphogens controlled by an X chromosome switch (Cooper, 1993). This switch may result from a dosage effect mechanism, where one X chromosome is activated and the other partly reactivated (Cooper *et al.,* 1977; Renfree & Short, 1988; Sharman *et al.,* 1990). Alternatively, a hypothesis implicating parental imprinting has been proposed whereby the development of a scrotum is dependent on the presence of a maternal X and the pouch/mammary anlagen upon the presence of a dominant paternal X chromosome (Cooper, 1993). Identification of the gene system controlling this aspect of marsupial sexual differentiation may provide clues to the mechanisms regulating sexual dimorphisms preceding gonadal differentiation in eutherians. If there is an effect of the morphogenetic field of the scrotum on the presence of mammary primordia, as suggested by Renfree *et al.,* (1990) and Robinson *et al.,* (1991), it is difficult to imagine how these intersexes develop. We are currently testing these hypotheses by transplanting scrotal tissue into females to see if the transplanted scrotal tissue produces morphogens that suppress mammary or pouch development.

The scrotum of eutherians was thought to be homologous with that of marsupials, but it now seems more probable that the scrotum has evolved many times in mammals, and we know that not all mammals develop one (Renfree, 1992). In eutherian mammals the scrotum lies caudal to the penis and the scrotum develops from the labio-scrotal folds under the influence of androgens during development. In contrast, in male marsupials the scrotum arises from the abdominal wall cranial to the phallus, and it is androgen independent. The descent of the testes appears to be controlled by similar mechanisms, although the route of descent is less convoluted in marsupials than in eutherians (Hutson *et al., 1988).*

The pouch, which gives the marsupials their family name, is clearly an adaptation that has evolved many times within the group. It is a highly variable organ, from a deep structure to a shallow one, with a forward or backward opening, and with a variable number of

mammary glands and teats (Tyndale-Biscoe and Renfree, 1987, Renfree, 1992; 1993). The earliest marsupials are generally thought to have been pouchless, as are many extant marsupials (Renfree, 1993). The association of mammary development and pouch formation in the majority of marsupial species that have pouches suggests mammary development may drive pouch formation.

Gonadal sex reversal

Gonadal sex reversal in mammals can occur through exposure to hormones such as oestradiol or MIS or by inappropriate expression of some genes. We have demonstrated sex reversal, both male to female and female to male, in marsupials where sexual differentiation occurs in the young in the pouch (Whitworth *et al.,* 1996; Coveney *et al.,* 1998; 2001).

In eutherian mammals, over-expressing the dosage-sensitive sex-reversing gene on the X-chromosome, *DAX1*, causes male-to-female sex reversal by antagonising the action of SRY (Swain *et al.,* 1998). However, in mice *DAX1* knockout does not prevent testicular development although spermatogenesis fails (Yu *et al.,* 1998). *WNT4* appears to be a key gene for the maintenance of female germ cells (Vainio *et al.,* 1999). In both eutherians and marsupials the germ cells also play a key role: loss of female germ cells can cause formation of seminiferous- like cords in a developing ovary, but loss of male germ cells has no effect on testicular formation (Burgoyne, 1988; McLaren, 1991; Whitworth *et al.,* 1996; Whitworth, 1998). The chromosomal sex of the germ cells does not determine whether they enter meiosis or mitotic arrest; this depends on the environment in which they find themselves (McLaren, 1995).

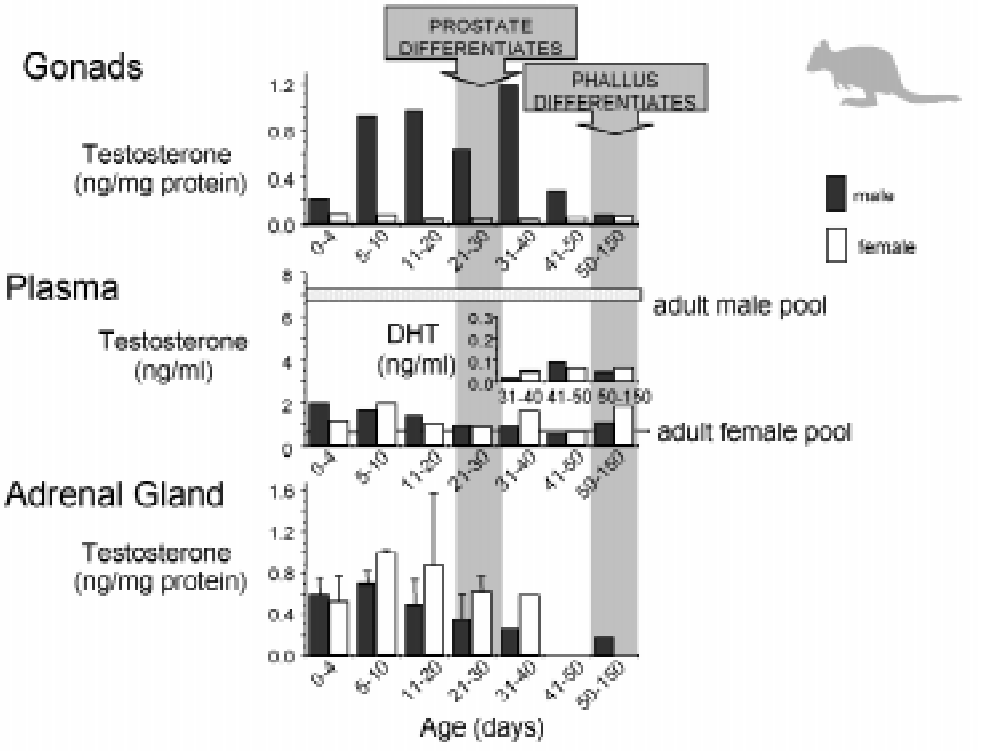

Fig. 7. **Summary of the concentrations of testosterone, dihydrotestosterone and timing of prostatic and phallic development in the male and female tammar pouch young and compared to adult male and female pools**. *Top panel, concentrations in gonads; middle panel concentrations in plasma and lower panel, concentrations in adrenals. Black bars: male; white bars: female. Data from Renfree et al., 1992 and Wilson et al., 1999.*

As the Sertoli cells differentiate they interact with the other cell types, inducing Leydig cell differentiation, germ cell proliferation and seminiferous tubule formation. Leydig cells produce testosterone which stimulates the development of the Wolffian ducts into the vasa deferentia, epididymides and seminal vesicles. The Sertoli cells produce the protein hormone Müllerian inhibiting substance (MIS) that causes regression of the Müllerian ducts which would otherwise form the oviducts, uterus and upper vagina. *GATA4*, a transcription factor that has a sexually dimorphic expression pattern (Viger *et al.,* 1998) may enhance MIS gene transcription through a direct interaction with the nuclear receptor SF1 (Tremblay & Viger, 1999). *GATA4* and *MIS* have similar expression patterns. In eutherian mammals female germ cells enter meiosis before birth to become oocytes, but male germ cells go through a period of mitotic arrest, and do not enter meiotic stages until puberty (Byskov & Høyer, 1994). Marsupial germ cells follow a similar pattern, except that these stages occur after birth. Female germ cells enter meiotic stages between day 25 and 50 post partum, and male germ cells go into mitotic arrest progressively from day 25 after birth (Alcorn, 1975; D. O'Brien, G. Shaw, A. McLaren and M.B.Renfree, unpublished results). In males, germ cells apparently play no role in the early differentiation of the testis, since seminiferous cords, Sertoli cells and Leydig cells can all develop in the absence of germ cells. However, loss of germ cells from ovaries can lead to the formation of seminiferous-like tubules in marsupials (Whitworth *et al.,* 1996), as in eutherians (reviewed in Burgoyne, 1988; McLaren, 1991; Whitworth, 1998), suggesting that in females an interaction between the germ cells and the supporting cell lineage inhibits Sertoli cell formation in ovaries.

Sertoli and granulosa cells are thought to be derived from a common progenitor supporting cell line, and both express MIS in the adult. Exposure of developing females to MIS can lead indirectly to a transformation of granulosa cells into Sertoli cells and the formation of seminiferous tubules. Such sex-reversed gonads may go on to produce testosterone and MIS as occurs in freemartin heifers (Short *et al.,* 1969). In transgenic female mice that chronically overexpress human MIS, germ cells are lost within two weeks and the ovaries develop seminiferous tubule-like structures (Behringer *et al.,* 1990). Maximal reduction of germ cells occurs between 16 dpc and birth when most transgenic oocytes are in leptotene of meiotic prophase, compared to normal oocytes that have reached pachytene (Lyet *et al.,* 1995). In male MIS transgenics with the highest levels of MIS expression there is feminization of external genitalia, impairment of Wolffian ducts and undescended testes (Behringer *et al.,* 1990). Serum testosterone decreases in adult transgenic males, suggesting that high concentrations of MIS

can interfere with Leydig cell development of function (Lyet *et al.,* 1995). Male MIS deficient mice and mice with MIS receptor mutations have functional testes, but they also develop female internal genitalia so sperm cannot leave the testis (Behringer *et al.,* 1994; Mishina *et al.,* 1996). Both *MIS* and *MIS-R* mutant males also have Leydig cell hyperplasia, suggesting that MIS normally inhibits Leydig cell proliferation. In female transgenic mice chronically expressing MIS, but deficient for the MIS-R, folliculogenesis and germ cell development are normal and females are fertile (Mishina *et al.,* 1999). Clearly MIS has a large number of effects, not only on the Müllerian ducts, but also on the Leydig cells in males and on the germ cells of females. In the tammar MIS transcripts and MIS protein first appear in developing testis at very onset of cord formation. There is weak transcription in gonads before the day of birth but this we assume to be below functional level. MIS protein is not expressed in Sertoli cells before they align into seminiferous cords.

We can induce gonadal sex reversal in female neonatal marsupials by treating with MIS. (Fig. 5) We have confirmed that the female sex reversal is due to germ cell loss (Whitworth *et al.,* 1996). Neonatal ovaries transplanted to male hosts or treated with MIS *in vitro* develop seminiferous-like tubules (Fig. 5) (Whitworth *et al.,* 1996). This sex reversal was invariably associated with loss of premeiotic female germ cells, suggesting that interactions between female germ cells and somatic cells are needed to prevent testis-like differentiation in the developing gonad. This suggests that the indifferent gonad of the female will form a testis in the absence of germ cells. MIS hastens germ cell loss in female gonads, but MIS is not an essential factor for the induction of gonadal sex reversal. Thus, the toxic effect of MIS on the female germ cells is likely to be due to dysregulation of signalling through the MIS-R on the granulosa cells.

Uniquely amongst mammals, male-to-female gonadal sex reversal can be induced in marsupials by treating neonatal males with oestradiol (Burns, 1961; Fadem and Tesoriero, 1986; Shaw *et al.,* 1988; Coveney *et al.,* 1998; 2001). Depending on the stage that treatment starts, the reversals range from partial suppression of seminiferous tubule development to the development of a morphologically normal ovary with cortex, medulla and apparently healthy germ cells. Oestrogen treatment of neonatal males not only sex-reverses the gonad but also causes abnormalities in Müllerian duct regression and testicular descent (Shaw *et al.,* 1988; Coveney *et al.,* 1998). A feminizing effect of oestrogens on gonadal development is also seen in fish, amphibians, reptiles and birds and may reflect a fundamental process underlying gonadal differentiation. Whilst oestrogens have not been shown to sex reverse gonads of male eutherian fetuses, a recent study has shown that the double knockouts of both oestrogen receptors (α and β) causes ovarian sex-reversal in female mice (Couse *et al.,* 1999). How oestrogens cause gonadal sex reversal in marsupials is unclear, but the Sox9 promoter contains consensus binding sites for *GATA1* (Kanai & Koopman, 1999), a transcription factor expressed in Sertoli cells (Yomigida *et al.,* 1994) whose transactivational activity can be directly modulated by protein-protein interactions with oestrogen-receptor-α (Blobel *et al.,* 1995). Interactions between the oestrogen receptor and genes on the sex-determining pathway may be a fundamental aspect of sexual differentiation since oestrogen plays a key role in sexual differentiation in reptiles (Pieau, 1995; Crews, 1996) and birds (Abinawanto *et al.,* 1996).

Oestrogens also affect germ cell development. Male pouch young treated orally with oestradiol benzoate from the day of birth have significantly fewer germ cells, but in contrast to normal males in which meiosis begins at puberty, the germ cells of treated males become surrounded by follicle-like cells and surprisingly enter meiosis by 50 days post-partum, at the normal time for XX germ cells in ovaries (Fig. 6; Coveney *et al.,* 2001). This therefore appears to be a case of germ cell sex reversal.

Virilization

As in eutherian mammals, the developing marsupial testes, but not the ovaries, produce Müllerian inhibiting substance (MIS) and testosterone (Hutson *et al.,* 1988; George and Wilson, 1985; Renfree *et al.,* 1992, Wilson *et al.,* 1999) although adult ovaries have detectable MIS protein in granulosa cells (DJ. Whitworth, personal communication). Tammar wallaby testes produce MIS for a longer period than in the mouse, from birth to at least day 100, although Müllerian duct regression is essentially complete by day 25 (Hutson *et al.,* 1988; Whitworth *et al.,* 1997).

Testosterone is the principal androgen present in the testis at the time of marsupial sexual differentiation (Renfree *et al.,* 1992; Fadem & Harder, 1992) although some species, such as the brush tailed possum the adult testis may secrete smaller amounts of DHT (Curlewis and Stone, 1985). In the tammar, gonadal testosterone concentrations are low in male and female gonads at birth, but in males they rise between day 2 and day 10 with the formation of seminiferous tubules (Renfree *et al.,* 1992) (Fig. 7). Testicular testosterone concentrations are elevated from soon after birth until about day 40 post partum (p.p.), declining to low levels by day 50 (Renfree *et al.,* 1992) by which time sexual differentiation of the internal genitalia is essentially complete. However, circulating concentrations of testosterone are not sexually dimorphic in plasma (Fig. 7) (Wilson *et al.,* 1999), both male and female pouch young have similar low peripheral plasma testosterone concentrations, presumably of adrenal origin, presenting a dilemma to explain how the urogenital system becomes sexually differentiated.

Testosterone produced by the neonatal testis stimulates the Wolffian duct to form the vas deferens and epididymis. Whilst virilization of the urogenital sinus is also androgen dependent (Shaw *et al.,* 1988, Lucas *et al.,* 1997, Ryhorchuk *et al.,* 1997; Butler *et al.,* 1998), prostatic development does not commence until 3 weeks after the onset of testosterone production (Figs. 4 and 7).

Daily treatment of female neonates with testosterone from birth to day 25 induces the formation of prostatic buds, but since these are similar to those of control males the prolonged treatment does not enhance development (Shaw *et al.,* 1988). Treatment of male pouch young with the androgen receptor blocker flutamide from day 20 to 45 dramatically reduced prostatic development compared to control treated males (Butler *et al.,* 1995). These experiments suggest that androgen is acting to induce prostate development in a narrow window between days 20 and 25 after birth (Fig. 4).

The phallus is also androgen-dependent. The phallus does not become sexually dimorphic until about day 100 p.p. (Butler *et al.,* 1999; Leihy *et al.,* 2000), despite the fall in testicular testosterone by day 50. The dependence of the phallus on the testis for differentiation has been clearly demonstrated. Tyndale-Biscoe &

Hinds (1989) removed testes from day 10 male pouch young and grafted these into similar aged female pouch young. The castrated males did not develop a phallus whereas the female pouch young with testicular grafts developed a penis, a clear demonstration that the testicular hormones are critical for sexual differentiation of the external genitalia.

In eutherian mammals low levels of circulating testosterone are converted in the urogenital sinus and phallus to the more potent androgen 5α-dihydrotestosterone (DHT), and defects in the 5α-reductase gene interfere with normal male sexual differentiation. However, in tammars the delay in virilization of the urogenital sinus and phallus cannot be due to lack of DHT, since the urogenital sinus and phallus both contain 5α-reductase in high concentrations by day 10-11 (Renfree *et al.,* 1992) (Fig. 7). Recently we have identified another androgen, 5α-androst-3α,17β-diol (Adiol) in male tammar pouch young plasma (Shaw *et al.,* 2000) and have shown that administration of this hormone can virilize both the prostate and phallus in developing females (Leihy *et al.,* 2000). Thus, Adiol may be a key hormone regulating virilization, not only in marsupials, but also in all other mammals.

Conclusions

The studies of marsupials have directed attention to the fact that the hormonal control of sexual differentiation overlies a genetic control system which is essentially similar to that of eutherian mammals, but with a few significant differences such as the direct genetic control of the development of the pouch and the scrotum, and the plasticity of gonadal development in response to gonadal hormones. The sex reversal models using neonatal marsupials are a fascinating system that will continue to inform our understanding of the differentiation of germ cells, somatic cells and sex in mammals.

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