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Sexual differentiation of the urogenital system of the fetal and neonatal tammar wallaby, Macropus eugenii

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Abstract In male tammar wallabies, the scrotum is the first organ to become sexually differentiated, 4–5 days before birth (day 22 of gestation). This is followed by enlargement of the gubernaculum and processus vaginalis one day before birth. However, the indifferent gonad does not show any signs of testicular cord formation or androgen production until later, at around the time of birth; this is more pronounced at 2 days post-partum (p.p.), when the testis takes on a characteristic rounded appearance. Primordial germ cells proliferate throughout the testis at this time, although the testis does not become significantly heavier than the ovary until around 80 days p.p.. In females, the appearance of the mammary glands is the first sign of sexual differentiation 4–5 days before birth. The indifferent gonad first shows signs of developing an ovarian cortex and medulla 7 days after birth. The migrating germ cells are confined to the cortex, and first start to enter meiosis about 25 days after birth. The Wolffian (mesonephric) ducts are patent to the urogenital sinus in fetuses at day 21 of gestation. In the female they have started to regress by 10 days p.p. and only rudiments remain by day 25 p.p.. The Müllerian (paramesonephric) ducts develop adjacent to the cranial pole of the mesonephros at about day 25 of gestation and grow caudally to meet the urogenital sinus between days 2 and 7 p.p.. The Müllerian duct of the female develops a prominent ostium abdominale by day 9 p.p., but this structure has completely regressed in males by day 13 p.p.. The testes enter the internal inguinal ring at about day 25 p.p., about the time that prostatic buds first appear in the urogenital sinus, and are in the inguinal canal from days 25 to 36 p.p.. They enter the scrotum at around day 36 p.p. and testicular descent is complete by days 65–72 p.p.. Melanin develops in the tunica vaginalis 72 days after birth. The overall development of the urogenital system in this marsupial is similar to that of eutherians but the sequence of events differs, with some aspects of genital differentiation preceding gonadal differentiation, apparently because they are directly controlled by X-linked genes, rather than indirectly controlled by gonadal steroids.

Key words Marsupial · Gonad · Scrotum · Pouch · Mammary primordia

Introduction

In marsupials, as in eutherians, gonadal differentiation is determined by the presence or absence of the testis-determining gene SRY on the Y-chromosome (Foster et al. 1992). Many similarities are evident between marsupials and eutherians. The testes of developing marsupials produce testosterone (George et al. 1985; Fadem and Harder 1992; Renfree et al. 1992a) and Müllerian-inhibiting substance (MIS) (Hutson et al. 1988). Testosterone stimulates the Wolffian ducts to form the epididymis and
vas deferens, and some male secondary sex characters such as the prostate (Shaw et al. 1988). MIS induces Mullerian duct regression (Hutson et al. 1988). In females the ovaries do not produce significant quantities of any steroid (Renfree et al. 1992a) or MIS (Hutson et al. 1988) in the neonatal period.

Despite these similarities one important difference between eutherian and marsupial mammals is that the differentiation of the scrotum, the mammary primordia, the pouch, the gubernaculum and the processus vaginalis are hormone-independent. Working on the American opossum, McCrady (1938), Burns (1939a, b, 1961) and earlier, Bresslau (1912) missed the significance of these essential marsupial-eutherian differences. In the tammar, *M. eugenii*, scrotal bulges only develop in genetic males, and primordia of the mammary glands only develop in genetic females: both structures differentiate several days before birth (Alcorn 1975; Renfree et al. 1987; O et al. 1988). Likewise, neonatal males of *Antechinus* (Bolton 1983), *Didelphis* and *Monodelphis* (Renfree et al. 1990) and *Trichosurus* (Ullmann 1993) also have scrotal bulges visible on or before the day of birth. These early sexual dimorphisms appear to be under direct genetic control determined by X-linked genes (O et al. 1988; Shaw et al. 1988, 1995; Renfree and Short 1988; Cooper et al. 1990; Sharman et al. 1990; Cooper 1993; Renfree et al. 1995).

Another difference in gonadal and internal genital tract development between marsupials and eutherians is in the timing of these events relative to birth. Newborn marsupials are extremely altricial and most sexual differentiation occurs postnatally, whereas in eutherians it occurs prenatally. McCrady (1938) provided a substantial description of the embryology of the North American opossum *D. virginiana*, but only a brief account of postnatal development. Subsequent studies have been largely confined to limited stages of pre-natal or postnatal development. This study provides the first detailed gross morphological, scanning electron and light microscope description of sexual differentiation in any marsupial during fetal and early postnatal life. By using the best understood marsupial, the tammar wallaby, *M. eugenii*, it also establishes a sound morphological basis for concurrent studies of the molecular and endocrine control of sexual differentiation.

**Materials and methods**

**Animals**

Tammar wallabies (*M. eugenii*) of Kangaroo Island origin were obtained from our breeding colony maintained at Monash University as previously described (Renfree et al. 1989). For prenatal samples, pregnancies were initiated by removal of pouch young (RPY) from females known to have blastocysts in lactational diapause. The gestation period after RPY is 26.4 ± 1.0 days (Tyndale-Biscoe and Renfree 1987; Fletcher et al. 1988). The day of RPY is designated day 0 RPY. Animals were checked for births between 08:00 and 10:00 h each day, and for postnatal samples, the day of birth was recorded as day 0 post partum (p.p.). Some additional young were used from untimed pregnancies. The young were removed from the mother’s pouch at appropriate times p.p. All embryos and fetuses were weighed, and head length (HL) recorded, as well as fetal crown rump length (CRL) (Renfree 1973). Pouch young were weighed and HL measured. Where date of birth was unknown, ages were determined from the growth curves of Poole et al. (1991).

Care and treatment of the animals conformed to the National Health and Medical Research Council guidelines (National Health and Medical Research Council of Australia 1990) and all experiments were approved by Institutional Animal Experimentation Ethics Committees.

**Scanning electron microscopy**

Embryos or fetuses were removed from the uterus at days 18 (n=3) 20 (1), 21 (3), 24 (1), 25 (1) after RPY as previously described (Renfree 1973) after the mother was killed with an overdose of sodium pentobarbitone (Abbott Laboratories, Kurnell, NSW) in 0.9% saline. Pouch young were collected at days 0 (n=3), 1 (2), 2 (1), 4 (2), 5 (1), 6 (2), 7 (3), 8 (1) 9 (2), 10 (3), 11 (1), 13 (1) p.p.. After weighing and measuring, embryos and pouch young were killed by decapitation and carefully examined externally, then dissected to display the developing gonads, Wolffian and Mullerian ducts, mesonephric kidneys, metanephric kidneys, adrenals, and the urogenital sinus. A small piece of liver and/or lung was taken for karyotyping to determine genetic sex.

After dissection the body was fixed in Superfix (370 ml 0.2 M sodium cacodylate + 75 ml 12% parafomaldehyde, 0.5 g picric acid, 65 ml glutaraldehyde) and held at 5°C until critical point drying for scanning electron microscopy. After drying, specimens were coated with gold and photographed in a Cambridge stereoscopic scanning electron microscope (SEM).

**Light microscopy**

Fetuses were removed from the uterus at days 21 (n=4) 22 (4), 23 (4), 24 (3), 25 (4) after RPY. Pouch young were collected at days 0 (n=18), 2 (3), 7 (2), 8 (2), 10 (2), 11 (1), 12 (1), 13 (1), 15 (2), 18 (2), 21 (2), 25 (11) p.p. as described above. Fixation was in buffered formal-saline (100 ml of 40% formaldehyde; 900 ml distilled water + 4 g NaH2PO4·2H2O + 6.5 g Na2HPO4) for 7–14 days before birth (c male at day 1 p.p. d male at day 2 p.p. See also Fig 10. At birth, c male at day 1 p.p. d male at day 2 p.p. See also Fig 10. After weighing and measuring, embryos and pouch young were killed by decapitacion and carefully examined externally, then dissected to display the developing gonads, Wolffian and Mullerian ducts, mesonephric kidneys, metanephric kidneys, adrenals, and the urogenital sinus. A small piece of liver and/or lung was taken for karyotyping to determine genetic sex.

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**Abbreviations for figures**

*Ad* Adrenal  *All* allantoidis  *Am* amnion  *Bl* bladder  *BW* body wall  *C* cords  *Co* colon  *Coe* coelom  *Cor* cortex  *DA* dorsal aorta  *DI* dorsal ligament  *DM* dorsal mesentery  *G* gut  *Go* gonad  *GR* gonadal ridge  *Gub* gubernaculum  *HL* hind limb  *HG* hind gut  *Ing* inguinal canal  *Kid* kidney  *M* mammary gland  *MD* Mullerian duct  *Med* medulla  *Mes* mesonephros  *Mi* metabolite  *Mb* metanephric bud  *Os* ostium  *Ov* ovary  *P* papilla  *Ph* phallicus  *Pou* pouch  *PO* pouch opening  *PS* pouch scale  *PV* processus vaginals  *S* scrotum  *SB* scrotal bulges  *SE* surface epithelia  *ST* seminiferous tubules  *T* testis  *Ta* tunica albuginea  *Ti* tail  *Ur* urachus  *Uc* urogenital cord  *UGS* urogenital sinus  *Umb* umbilicus  *Ur* ureter  *Vv* vitelline vein  *WD* Wolffian duct  *YS* yolk sac

**Fig. 1a–d**  Gross morphology of the urogenital system of wallaby fetuses and pouch young during early sexual differentiation. Scanning electron micrographs show females (top) and males (bottom): a Female fetus at 24 days of gestation, b female neonate on day of birth, c male at day 1 p.p. d male at day 2 p.p. See also Fig 10. The mesonephros is a prominent ridge of tissue with the gonad lying medially, and the urogenital cord, containing the Wolffian and Mullerian ducts, lying laterally. The adrenal and developing metanephric kidney lie medially and dorsal to the gonads. The cut end of the colon and bladder are in the midline. By day 2 p.p. the testis has rounded up and by day 8–10 p.p. there is a marked difference between the rounded testis and the glandular-shaped ovary. The gubernaculum is visible between the base of the mesonephros and the inguinal region. The diaphragmatic ligament attaches the cranial portion of the urogenital cord to the diaphragm close to the first rib. Bars 1 mm