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Wolffian duct differentiation by physiological concentrations of androgen delivered systemically

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ABSTRACT

In developing mammalian males, conversion of the Wolffian ducts into the epididymides and vasa deferentia depends on androgen secretion by the testes, whereas in females these ducts remain in a vestigial form or regress. However, there is continuing uncertainty whether the androgen needs to be delivered locally, either by diffusion from the adjacent testis or, by secretion into the lumen of the duct, or whether circulating androgens maintain and virilize the Wolffian ducts. To resolve this uncertainty, we transplanted either day 0–2 or day 8–9 post-partum testes beneath the flank skin of three groups of neonatal (days 0–1) female tammar wallabies, where they developed and secreted physiological levels of hormones. The Wolffian ducts of all these females were retained and had formed extensive epididymides when examined at days 25, 34 and 87 after birth. In the two older groups of females, sampled after the time of prostatic bud formation, the urogenital sinus was virilized and there was extensive prostatic development similar to that of normal males of the same age, showing that androgen secretion had occurred. Virilization of the Wolffian ducts occurred during an early but short-lived window of sensitivity. This study provides the first clear evidence that under physiological conditions virilization can be mediated by circulating androgen.

Introduction

Virilization of the male urogenital tract during mammalian development involves two processes—formation of the epididymides, vasa deferentia, and, in some species, seminal vesicles, from the Wolffian ducts, and conversion of the urogenital tubercle and urogenital sinus into the phallus, male urethra, and prostate. In all mammalian species—eutherian and marsupial—these processes are mediated by androgens secreted by the developing testes (George and Wilson, 1994; Wilson et al., 1995).

In all species studied to date virilization of the urogenital sinus and external genitalia is mediated by the 5α-reduced androgen dihydrotestosterone, which is formed by one of two mechanisms in the tissues of the urogenital tract—either the 5α-reduction of testosterone of testicular origin to dihydrotestosterone or by the oxidation of testicular 5α-androstanediol to dihydrotestosterone (George and Wilson, 1994, Wilson et al, 2003). In both instances circulating androgens are converted to the more potent dihydrotestosterone which acts via the androgen receptor to initiate the virilization process (George and Wilson, 1994; Wilson et al., 2002).

The process by which the Wolffian ducts are converted to the male ejaculatory system is less well understood in two regards. First, in eutherians and in some marsupial species testosterone itself is believed to be the responsible intracellular hormone (George and Wilson, 1994), whereas in the tammar wallaby, *Macropus eugenii* the process appears to be mediated by dihydrotestosterone formed from circulating androstanediol (Shaw et al. 2006). Two mechanisms have been considered for delivery of androgen to the developing ducts. Based on a single rabbit embryo in which a testis was grafted onto the mesosalpinx of a female fetus, Jost (1953a; 1953b) proposed that testicular androgen diffused directly down the Wolffian ducts and promotes virilization of the tissue ipsilaterally. This concept is in keeping with the fact that in many humans with 46,XY/45,X mosaicism or with true hermaphroditism, the Wolffian ducts virilize only on the side with a testis (Donahoe et al., 1978; McKelvie et al., 1987). Further evidence in favour of this concept was obtained by Wilson (1973) who showed that the Wolffian ducts of the rabbit can concentrate radioactive testosterone against a concentration gradient, by Veyssiere et al. (1982) who demonstrated an increase of testosterone in rabbit Wolffian ducts during their virilization, and by Tong et al. (1996) who microinjected embryonic (E) day 14 mouse urogenital ridges in organ culture with testosterone–albumin–fluorescein isothiocyanate and found fluorescence distributed throughout the Wolffian ducts that became maximal in the caudal end within 48 h. Androgen receptors are localized in the

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mesenchymal cells at the medial side of the Wolffian duct and only subsequently in the epithelial cells (Bentvelsen et al, 1995). Thus these various types of evidence supported the concept that the Wolffian duct is virilized locally by androgens either passing through the lumen of the duct or diffusing along the tissue around the duct. Nevertheless, circulating androgens can virilize the Wolffian ducts: administration of androgen to pregnant mice (Goldstein and Wilson, 1972) and rats (Schultz and Wilson, 1974) induces formation of epididymides, vasa deferentia, and seminal vesicles in female offspring. However, the latter phenomenon are more likely to be a result of pharmacological rather than physiological effects due to the doses administered.

The former experiments cannot discount the possibility that circulating androgens may be involved while the latter experiments may involve pharmacological rather than physiological effects as well as the possible metabolism of the administered hormone by the placenta. We decided to reinvestigate the mechanism of Wolffian duct virilization by taking advantage of the fact that xenotransplantation well before maturation of the immune system after days 90–120 post-partum (pp) (Basden et al., 1997) can be performed in tammar wallaby pouch young (Tyndale-Biscoe and Hinds, 1989; Whitworth et al, 1996).

The process of sexual differentiation is well documented in the tammar (O et al., 1988; Renfree and Short, 1988, Shaw et al., 1988; 1990; Renfree et al., 1992; 1995; 1996; Wilson et al., 1995; 2003). The scrotum, mammary glands, pouch, gubernaculum and processus vaginalis all differentiate before the gonad is differentiated and are controlled by a gene or genes on the X chromosome, not steroids (O et al, 1988; Renfree and Short, 1988; Shaw et al., 1988). The testis is undifferentiated on the day of birth, and early seminiferous cords do not develop until 2 days after birth. The ovary remains undifferentiated for longer, and develops cortical and medullary regions by day 8 pp (Renfree et al., 1996). Testicular testosterone is unmeasurable on the day of birth and contains less than 0.2 ng/mg up to day 4 but between days 5–10 it has reached 1 ng/mg. This concentration is sustained until day 40 pp in the testes but there is no measurable testosterone in ovaries between day of birth and day 70 pp (Renfree et al., 1992).

The circulating androgen in the tammar is 5α-androstane-3α,17β-diol (androstanediol), since plasma concentrations of 5α-adiol were 3 times higher in day 20–40 pp male plasma pools (averaging 1.9 ng ml^{-1}) than in female pools (averaging 0.6 ng ml^{-1}) (Shaw et al., 2000) and there is no sexual dimorphism in the levels of plasma testosterone or DHT at these ages (Renfree et al., 1992). In the tammar, dihydrotestosterone is formed by an alternate pathway during virilization of the urogenital tract (Wilson et al., 2003). Androstanediol is the principal androgen secreted by the testis at the time when virilization of the Wolffian ducts commence around days 8–10 of pouch life (Shaw et al., 2006), when prostate development is initiated between days 20–40 (Shaw et al., 1988; Renfree et al, 1996; Lucas et al, 1997), and when the phallus differentiates after day 40, the latter two as a result of a window of sensitivity to androgen (androgen imprinting) (Leihy et al, 2002, 2004). We therefore transplanted testes from male young aged less than 2 days old that had not yet begun to synthesize androgens (Renfree et al., 1992), into the flank of neonatal female pouch young, a stage at which the Wolffian ducts are undifferentiated. The recipient females' urogenital tracts were examined at day 34 and day 87 after birth. In a third group of recipient females, day 8 pp testes, that are already secreting androgens (Renfree et al., 1992) were transplanted into females aged day 0–1, and the recipient urogenital tracts were examined at day 25 post-partum. Our findings demonstrate that virilization of the Wolffian ducts in this species can be mediated by physiological levels of systemically circulating androgens.

Materials and methods

Animals

Tammar wallabies (*Macropus eugenii*) were held in open grassy yards with shelters provided. Diet was supplemented with compressed lucerne cubes and water *ad libitum*. Females were checked daily for births during the breeding season (Tyndale-Biscoe and Renfree, 1987). On the day of birth (day 0) neonates were sexed by the presence of scrotal bulges (male) or mammary primordia (female) (O et al, 1988). Care and treatment of animals conformed to the National Health and Medical Research Council of Australia (2004) guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committees.

Experimental design

Neonatal young were either given testis grafts or allowed to grow normally (Table 1). These animals were killed at day 34 or day 87 to assess the degree of virilization. In a third group, testes from day 8 males when testicular testosterone levels are much higher were transplanted into neonatal females (Table 1). This group was killed at day 25 after transplant and compared to young from previous studies that had been allowed to grow normally (Shaw et al. 1988, Lucas et al., 1997; Ryhorchuk et al, 1997).

Testicular grafts

Transplantation from male neonate to female neonate

Both testes from males aged 0–2 days pp were transplanted under the flank skin of neonatal (days 0–1) females as previously described (Whitworth et al., 1996). Briefly, males were removed from the pouch, sedated by cooling on ice and then killed by decapitation. The testes attached to the mesonephroi were removed and placed in saline while the recipient female was prepared. The recipient female young was anesthetized by hypothermia (Renfree 2002). A small incision was made in the abdominal skin on the left hand side. The two testes with mesonephroi attached were inserted subcutaneously

Table 1
Status of reproductive tissues of female pouch young carrying testicular grafts at autopsy.

	Day 25 transp. female	Day 34 control female	Day 34 control male	Day 34 transp. female	Day 87 control female	Day 87 control male	Day 87 transp. female
Number surviving to autopsy	5/6	4/4	5/5	3/5	3/3	3/3	4/5
Testes present and developed	5/5[a]	–	5/5	3/3[b]	–	3/3	2/4
Both Wolffian ducts virilized	5/5	0/4	5/5	3/3	0/3	3/3	4/4
Both Müllerian ducts regressed	0/5	0/4	5/5	0/3	0/3	3/3	0/4
Abnormal ipsilateral[c] ovary	5/5	0/3	–	2/3	0/3	–	1/4
Abnormal contralateral[c] ovary	0/5	0/3	–	0/3	0/3	–	1/3
Urogenital sinus virilized	–	0/4	5/5	3/3	0/3	3/3	4/4

[a] One testicular graft in this group had stopped growing by the time of autopsy.
[b] Two testicular grafts in this group had stopped growing by the time of autopsy.
[c] Relative position of ovary to testes graft shown.