Failure of normal adult Leydig cell development in androgen-receptor-deficient mice

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Summary

During testicular development, fetal and adult populations of Leydig cells arise sequentially. Previous studies have shown that androgen action is required for normal steroidogenic activity in the mouse testis. Therefore, to determine the role of androgens in regulating fetal and adult Leydig cell differentiation and function, Leydig development has been measured in mice lacking functional androgen receptors (AR-null). The Leydig cell number was normal on day 5 after birth in AR-null mice but failed to increase normally thereafter and was about 30% of the control level on day 20 and about 60% of control level in adult animals. Levels of 15 different mRNA species expressed specifically in Leydig cells were measured by real-time PCR in AR-null and control animals. Expression levels of all mRNA species were normal on day 5 when only fetal Leydig cells are present. In older animals, which contain predominantly adult Leydig cells, five of the mRNA species (3β-hydroxysteroid dehydrogenase (3βHSD) type 1, cytochrome P450scc, renin, StAR protein and luteinising hormone receptor) were expressed at normal or increased levels in AR-null mice. All other mRNA species measured showed significantly reduced expression in older animals, and three of these mRNA species (17β-hydroxysteroid dehydrogenase type III, prostaglandin D (PGD)-synthetase and 3βHSD type VI), which are only expressed in the adult population of Leydig cells, were barely detectable in the adult AR-null mouse. The results show that in the absence of androgen receptors, fetal Leydig cell function is normal, but there is a developmental failure of adult Leydig cell maturation, with cells only aquiring partial characteristics of the adult population.

Key words: Androgen-receptor, Testis, Leydig cell, Development, Gene expression, Cell number, Puberty

Introduction

During normal testicular development, two separate populations of Leydig cells arise in a sequential manner. In the mouse the first or 'fetal' population of cells starts to appear about 12.5 days postcoitum, soon after testicular differentiation, whereas the second or 'adult' population begins to differentiate shortly after birth (Vergouwen et al., 1991; Baker et al., 1999; Nef et al., 2000). Factors regulating the differentiation and development of the two populations of cells remain uncertain. Luteinising hormone (LH) is essential for the normal function of the adult Leydig cells but is not required for fetal Leydig cell function before birth (O'Shaughnessy et al., 1998) and may not be required for differentiation of the adult cell population (Ariyaratne et al., 2000). We have shown previously that in mice with androgen-receptor deficiency (AR-null) there is a marked reduction in circulating testosterone levels, with an associated increase in progesterone production (Murphy and O'Shaughnessy, 1991). These changes occur despite an increase in circulating LH and are related to loss of 17α-hydroxylase and 17-ketosteroid reductase enzyme activity in Leydig cells of the adult animal (Murphy and O'Shaughnessy, 1991; Murphy et al., 1994). Although these data show that androgens are required for normal adult Leydig cell development, it is not clear whether loss of steroidogenic function in AR-null animals is caused by a failure of adult Leydig cell differentiation, a failure to establish normal adult Leydig cell number or a failure of normal adult Leydig cell development. It is also not known whether androgens play a role in the differentiation of the fetal Leydig cell population. In recent studies we have characterised the normal pattern of Leydig cell proliferation and gene expression during testicular development in the mouse (Baker and O'Shaughnessy, 2001b; O'Shaughnessy et al., 2002). To determine the underlying cause of steroidogenic failure in AR-null mice we have now compared the pattern of Leydig cell development in these animals with appropriate normal controls. The results show that in the absence of functional androgen receptors, fetal Leydig cell development is normal although there is a failure of adult Leydig cell maturation, with cells only developing partial characteristics of the adult population.

Materials and Methods

Animals

Normal and AR-null (*Tfm*) mice bred on a C3H/HeH-101/H genetic background were derived from stock animals obtained originally from the MRC Radiobiology Unit (now the MRC, Mammalian Genetics Unit, Harwell, UK). Animals were maintained as required under the UK Home Office regulations and were used for study at the ages indicated in the text. The testes of normal mice undergo final descent to the scrotum at about 25 days, whereas testes in AR-null mice remain intra-abdominal into adult life. To control for the failure of testicular descent in AR-null mice, normal animals were surgically rendered cryptorchid at 21 days and used for experiments when adult

(Murphy and O'Shaughnessy, 1991). Animals were killed at the appropriate age, and testes were (a) frozen in liquid N_2 for subsequent study of specific mRNA levels, (b) fixed overnight in Bouin's fluid and stored in 70% ethanol for subsequent measurement of cell number or (c) homogenised immediately to measure steroidogenic enzyme activity.

Reverse transcription and real-time PCR

For quantification of the content of specific mRNA species in testes during development, a real-time PCR approach was used that utilised the TaqMan PCR method following reverse transcription of the isolated RNA (Bustin, 2000). To allow specific mRNA levels to be expressed per testis and to control for the efficiency of RNA extraction, RNA degradation and the reverse transcription step, an external standard was used (Baker and O'Shaughnessy, 2001a). The external standard was luciferase mRNA (Promega UK, Southampton, UK), and 5 ng was added to each testis at the start of the RNA extraction procedure. Testis RNA was extracted using Trizol (Life Technologies, Paisley, UK), and residual genomic DNA was removed by DNAse treatment (DNA-free, Ambion Inc, supplied by AMS biotechnology, UK). The RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript II, Life Technologies, UK) as described previously (O'Shaughnessy and Murphy, 1993; O'Shaughnessy et al., 1994).

With the exception of renin 1 (Ren 1), primers and probes for use in the TaqMan method have been described previously (O'Shaughnessy et al., 2002). The primers used to amplify Ren1 had sequences CCGAGCTGCCCCTGATC and GGGAAAGCCCATGC-CTAGAA, whereas the probe had sequence CTTTCATGCTGG-CCAAGTTTGACGG; these were all based on GenBank sequence NM_031192. The real-time PCRs were carried out in a 25 μl volume using a 96-well plate format. Components for real-time PCR were purchased from Applied Biosystems (Warrington, UK) apart from the primers and probes, which came from MWG Biotech (Milton Keynes, UK). Each PCR well contained reaction buffer (with passive reference), 5 mM $MgCl_2$, 200 μM dNTPs, 300 nM each primer, 200 nM probe and 0.02 U/μl enzyme (Amplitaq Gold). Reactions were carried out and fluorescence detected on a GeneAmp 5700 system (Applied Biosystems, Warrington, Cheshire, UK). For each sample a replicate was run omitting the reverse transcription step, and a template negative control was run for each primer-probe combination. The quantity of each measured cDNA was then expressed relative to the internal standard luciferase cDNA in the same sample. This method allows direct comparison of expression levels per testis between different samples (Baker and O'Shaughnessy, 2001a).

Enzyme activity

The activity of 5α-reductase activity in testes of normal and AR-null mice was measured by determining the conversion of a saturating concentration of tritiated substrate (testosterone) by homogenates of whole testes (O'Shaughnessy, 1991). Substrate and product were separated by thin layer chromatography, and enzyme activity was expressed as pmol/minute/testis.

Stereology

Testes were embedded in Technovit 7100 resin, cut into sections (20 μm thickness) and stained with Harris' haematoxylin. Total testis volume was estimated using the Cavalieri principle (Mayhew, 1992), and the slides used to count the number of cells were also used to measure testis volume. The optical disector technique (Wreford, 1995) was used to count the number of Leydig cells in each testis. The numerical density of Leydig cells was estimated using an Olympus BX50 microscope fitted with a motorized stage (Prior Scientific Instruments, Cambridge, UK) and Stereologer software (Systems Planning Analysis, Alexandria, VA, USA).

Statistics

Results were analysed by analysis of variance. Differences between AR-null animals and the appropriate control group at each age (normal animals when aged 5 and 20 days and cryptorchid animals when adult) were assessed by t-tests using the pooled variance. Differences between adult normal, cryptorchid and AR-null animals were determined using analysis of variance followed by the Neuman-Keul test. Where heterogeneity of variance occurred values were log-transformed before analysis.

Results

Testis morphology and Leydig cell number

Compared with normal animals, the seminiferous tubule diameter was reduced in both AR-null mice and in cryptorchid mice, although the effect was more marked in the AR-null group (Fig. 1). There was, in addition, apparent hyperplasia of the interstitial tissue in both AR-null and cryptorchid testes, although this was combined with a marked reduction in testis size (control, 112±5 mm^3; AR-null, 4.2±0.3 mm^3; cryptorchid 21.6±2.6 mm^3). The apparent interstitial hyperplasia in AR-null and cryptorchid mice could be caused by increased Leydig cell number or by the reduced tubule size with no change in interstitial cell number. To determine whether the Leydig cell number was changed in AR-null mice, stereological techniques were used to measure the cell number directly. On day 5 after birth, Leydig cell number in AR-null mice was normal but failed to show the usual pre-pubertal increase and was about 30% of control values on day 20 (Fig. 2). Between day 20 and adulthood, there was a similar increase in Leydig cell number in both AR-null and control animals so that in the adult, animal cell numbers were about

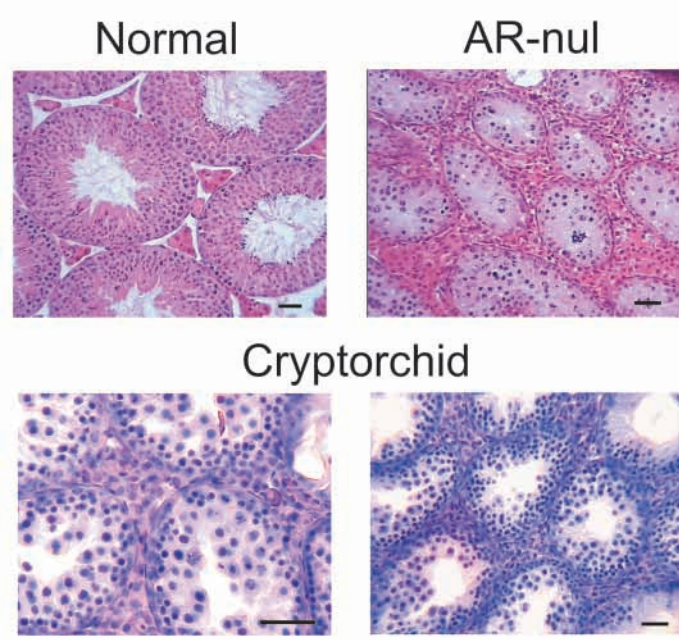

Fig. 1. Light micrographs showing morphology of testes from normal, AR-null and cryptochid adult mice. Bar, 50 μm.

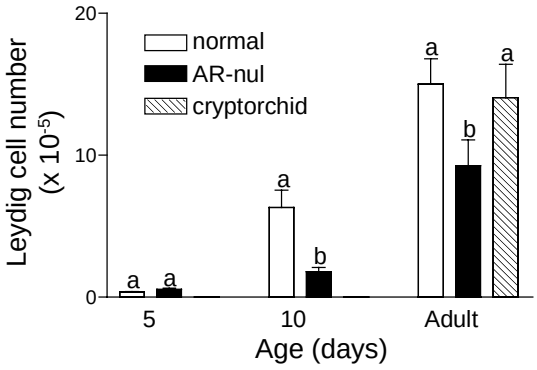

Fig. 2. Leydig cell number during development in normal mice and in AR-null animals. Cryptorchid controls are included for the adult group. Within each age group different letter superscripts indicate a significant difference in cell number (P<0.05). Results show means±s.e.m. of between three and five animals in each group.

60% of normal. There was no effect of cryptorchidism on Leydig cell number.

Expression of Leydig-cell-specific mRNA species

Genes expressed at a normal or increased level in AR-null animals

Of the 14 Leydig-cell-specific mRNA species measured in this study, six [3β-hydroxysteroid dehydrogenase type I (3βHSD I), cytochrome P450 side chain cleavage (P450scc), luteinising hormone receptor (LH-R), steroidogenic acute regulatory (StAR) protein, thrombospondin 2 (TSP-2) and Ren 1] showed normal or increased levels of expression in AR-null mice (Fig. 3A). Expression levels in this group were all normal on days 5 and 20 in AR-null mice apart from P450scc, which showed a small but significant increase in expression on day 5. In the adult animal, levels of mRNA encoding StAR protein and LH-receptor were normal in AR-null mice, but levels of the other three mRNA species (3βHSD I, P450scc and Ren 1) were all increased relative to cryptorchid controls. Cryptochidism per se reduced expression of 3βHSD I and P450scc, but had no significant effect on Ren1, StAR protein and LH-R mRNA levels.

Genes with a reduced level of expression in AR-null mice

Nine of the fifteen mRNA species measured showed a reduced level of expression in adult testes of AR-null mice. Three of these mRNA species [17β-hydroxysteroid dehydrogenase type III (17βHSD III), prostaglandin D (PGD)-synthetase and 3β-hydroxysteroid dehydrogenase type VI (3βHSD VI)] were barely detectable in the adult AR-null testis (Fig. 3B), whereas the other six mRNA species [relaxin-like factor (RLF), glutathione S-transferase 5-5 (GST5-5), cytochrome P450 17α-hydroxylase (P450c17), epoxide hydrolase (EH), 5α-reductase type I and estrogen sulfotransferase (EST)] showed a significant reduction in expression relative to the cryptorchid controls (Fig. 3C). With one exception (17βHSD III), expression of all mRNA species was normal on day 5 in AR-null mice, and all were reduced on day 20 apart from GST5-5. The overall pattern of expression, therefore, is one of normal mRNA expression levels on day 5 in AR-null mice but with reduced expression on day 20 and a relatively more marked reduction in expression in the adult animal. Cryptorchidism reduced expression in five of the eight mRNA species, with a marked effect on the expression of EST.

5α-reductase enzyme activity

Changes in 5α-reductase enzyme activity during development in normal and AR-null mice are shown in Fig. 4. In normal animals, there was a pubertal peak of activity around day 25 followed by a decline in the adult animal. In the AR-null mouse, levels of 5α-reductase activity were generally lower than in normal animals but there was a similar developmental pattern of enzyme activity. There was no apparent effect of cryptorchidism on testicular 5α-reductase activity.

Discussion

Androgen-receptor-null mutations have a major effect on the phenotype of affected males, causing pseudohermaphrodism, loss of internal reproductive duct systems and failure of testicular descent. Spermatogenesis is disrupted with a loss of normal germ cell development, although it remains to be determined how much of this is due to the cryptorchid state of the animals and how much is due directly to loss of androgen receptors. In addition to these defects, results from this study show that Leydig cell number is reduced in AR-null mice and that there is a highly abnormal pattern of Leydig cell gene expression during development. These defects are consistent with a failure of normal adult Leydig cell development and maturation in the absence of functional androgen receptors.

The fetal Leydig cell population is responsible for masculinisation of the fetus during development in utero. In studies reported here, Leydig cell function was normal in AR-null mice on day 5, indicating that fetal Leydig cell development and function is not dependent on androgens. In mice the fetal Leydig cell population persists after birth but becomes subordinate to the adult population, which begins to differentiate sometime between days seven and 10 after birth (Vergouwen et al., 1991; Baker et al., 1999; Nef et al., 2000). The stimulus leading to adult Leydig cell differentiation around day 10 is not clear, but in the absence of desert hedgehog (Dhh) or platelet-derived growth factor (PDGF)-A the adult Leydig cell population fails to develop, indicating a role for these growth factors in this process (Clark et al., 2000; Gnessi et al., 2000). Once differentiation has started, development of the adult Leydig cell population is critically dependent on LH (O'Shaughnessy, 1991; Zhang et al., 2001; Baker and O'Shaughnessy, 2001b), but the highly abnormal pattern of Leydig cell gene expression in AR-null mice reported here shows that androgens are also required for this process. From the data reported, two alternative hypotheses can be advanced to explain the role of androgens in post-natal Leydig cell development. Firstly, androgens may be required for maturation and maintenance of the adult Leydig cell population; alternatively, in the absence of androgens, the adult Leydig cell population may fail to differentiate and Leydig cells present in adult AR-null mice may be derived predominantly from the fetal population.

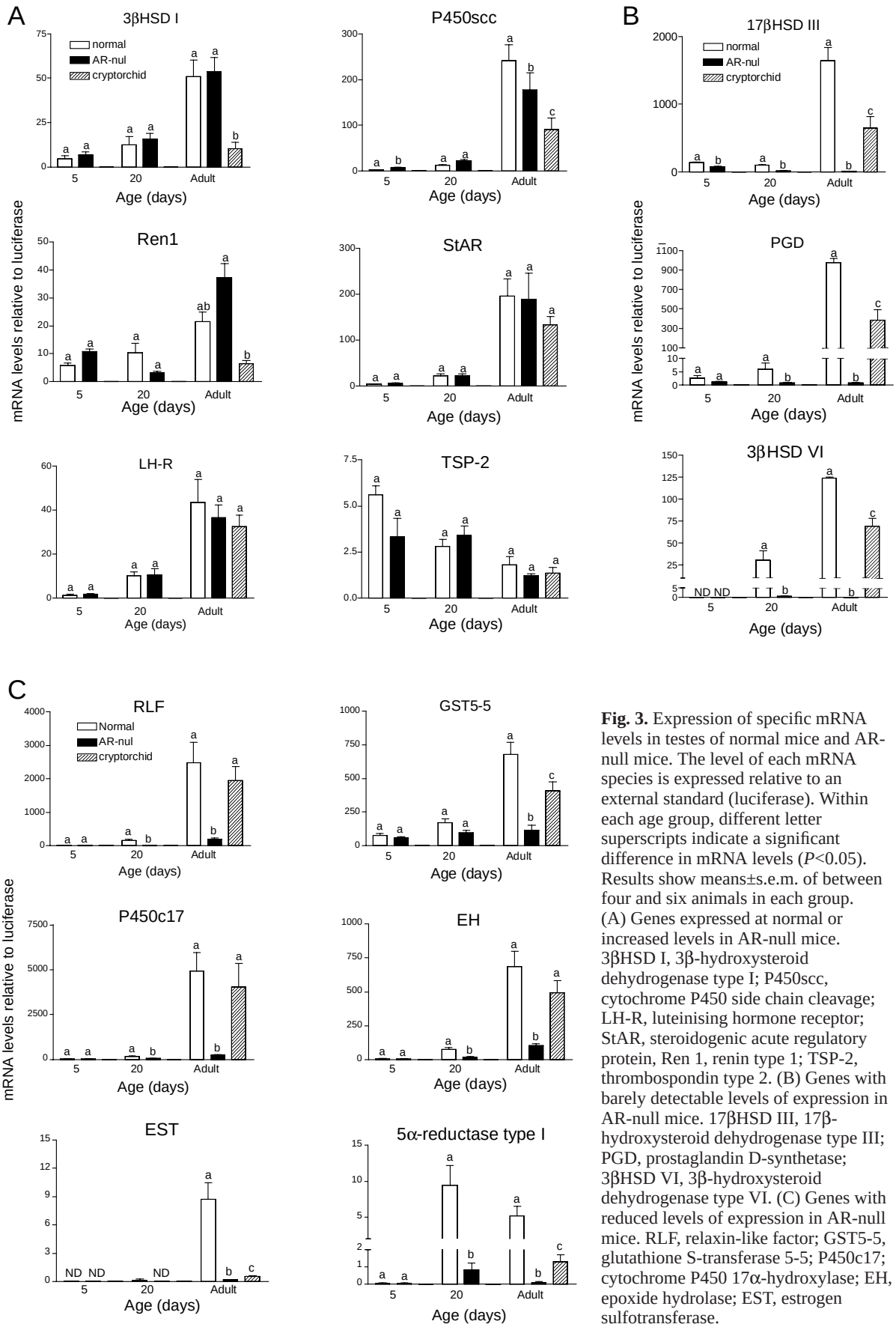

Fig. 3. Expression of specific mRNA levels in testes of normal mice and AR-null mice. The level of each mRNA species is expressed relative to an external standard (luciferase). Within each age group, different letter superscripts indicate a significant difference in mRNA levels ($P<0.05$). Results show means±s.e.m. of between four and six animals in each group. (A) Genes expressed at normal or increased levels in AR-null mice. 3βHSD I, 3β-hydroxysteroid dehydrogenase type I; P450scc, cytochrome P450 side chain cleavage; LH-R, luteinising hormone receptor; StAR, steroidogenic acute regulatory protein, Ren 1, renin type 1; TSP-2, thrombospondin type 2. (B) Genes with barely detectable levels of expression in AR-null mice. 17βHSD III, 17β-hydroxysteroid dehydrogenase type III; PGD, prostaglandin D-synthetase; 3βHSD VI, 3β-hydroxysteroid dehydrogenase type VI. (C) Genes with reduced levels of expression in AR-null mice. RLF, relaxin-like factor; GST5-5, glutathione S-transferase 5-5; P450c17; cytochrome P450 17α-hydroxylase; EH, epoxide hydrolase; EST, estrogen sulfotransferase.

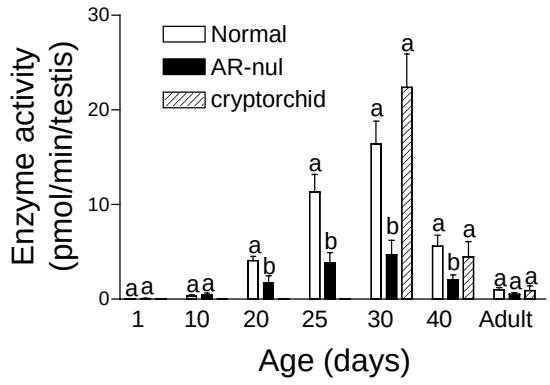

Fig. 4. Activity of 5α-reductase in testes from normal or AR-null mice of different ages. Enzyme activity is expressed per testis as pmol/minute. Within each age group different letter superscripts indicate a significant difference in enzyme activity (P<0.05). Results show means±s.e.m. of between three and six animals in each group.

In some respects the evidence available fits the second hypothesis more clearly. Of the 14 mRNA species measured in this study, four (17βHSD III, PGD-synthetase, 3βHSD VI and EST) are expressed only in the adult Leydig cell population and not in the fetal cell population and could, therefore, act as markers for adult Leydig cell differentiation (O'Shaughnessy et al., 2002). In the AR-null mice all four of these mRNA species showed barely detectable expression in the adult testis. Whereas this may be linked to the inherent cryptorchidism of the animals as far as EST is concerned, failure of 17βHSD III, PGD-synthetase and 3βHSD VI expression would be consistent with failure of adult Leydig cell differentiation. Other observations would be consistent with a predominance of fetal Leydig cells in the adult AR-null testes. For example, RLF mRNA levels are reduced in fetal Leydig cells (Balvers et al., 1998; O'Shaughnessy et al., 2002), and renin levels are increased (Perera et al., 2001). In addition, LH levels are increased in adult AR-null mice (Scott et al., 1992; Murphy et al., 1994), and normal expression of LH-receptor mRNA in the presence of increased circulating LH is consistent with the presence of fetal-type Leydig cells (Pakarinen et al., 1990; Pakarinen et al., 1994).

There are, however, a number of observations that would run contrary to this hypothesis. Firstly, expression and activity of the 5α-reductase enzyme and *5α-reductase type 1* gene shows a characteristic peak in adult Leydig cells around puberty in rodents (Ficher and Steinberger, 1971; Rosness et al., 1977; Viger and Robaire, 1995). In this study, a similar peak of activity and expression was seen in AR-null mice, although levels were lower than normal. Secondly, the expression pattern of TSP-2 during development in normal mice is consistent with predominant expression in the fetal population (O'Shaughnessy et al., 2002), but overall expression in the adult AR-null mouse testis was no different from control animals. Thirdly, in transgenic mice in which the adult Leydig cell population fails to develop (Dhh-null and PDGF-A-null animals), the fetal Leydig cells do not proliferate and populate the adult interstitial tissue, which is left largely devoid of Leydig cells (Clark et al., 2000; Gnessi et al., 2000). This suggests that, even in the absence of adult Leydig cells, the fetal population is unable to proliferate post-natally, although it is possible, of course, that this is affected by the absence of Dhh or PDGF. Lastly, whereas levels of mRNA encoding 3βHSD VI, PGD-synthetase, 17βHSD III and EST are extremely low in the adult AR-null mouse, they are not undetectable, particularly in the case of EST. In addition, taking into account changes in Leydig cell number, the expression of 3βHSD VI on day 20 is about 7% of normal (as opposed to 0.15% of normal in the adult), showing that there is initial differentiation that is not maintained.

The data, therefore, fit more closely with the hypothesis that adult Leydig cell differentiation occurs in the AR-null mouse except that there is a failure of cell development. Morphological and functional development of the adult Leydig cell lineage has recently been reviewed (Mendis-Handagama and Ariyaratne, 2001). Leydig cell precursor cells are found in the peritubular region and possibly around the vessels of the interstitium and differentiate initially to progenitor cells in the same region. Further development to 'newly formed' and 'immature' adult Leydig cells is associated with development of the typical Leydig cell morphology and movement of the cells to the central interstitial region (Mendis-Handagama and Ariyaratne, 2001). In an earlier developmental study of adult Leydig cell gene expression, we showed that EST, PGD-synthetase and 17βHSD III were expressed relatively late in Leydig cell development (O'Shaughnessy et al., 2002), which would be consistent with a failure of cell maturation in AR-null mice. This would not be consistent, however, with failure of 3βHSD VI expression, which starts early in adult Leydig cell development (O'Shaughnessy et al., 2002). It appears likely, therefore, that in the absence of androgen action, adult Leydig cells will differentiate but fail to develop the characteristics of the adult cells. Most of the mRNA species that are expressed normally in the Leydig cells of the adult AR-null mouse are fundamental to Leydig cell function (e.g. StAR protein, LH-R, P450scc and 3βHSD I) but are not specific to the adult population of cells. It is likely, therefore, that these mRNA species are either constitutively expressed in the Leydig cells [e.g. 3βHSD (O'Shaughnessy, 1991)] or are under the control of LH alone [e.g. P450scc (O'Shaughnessy, 1991)]. This correlates with morphological studies in AR-null mice, which have shown that Leydig cells in these animals lack the characteristic growth in smooth ER and surface specialisations associated with adult Leydig cell development (Russo and De Rosas, 1971; Blackburn et al., 1973).

During normal testis development, adult Leydig cells first appear shortly before day 10, and there is a marked, LH-dependent, increase in adult Leydig cell number between days 10 and 20 (Baker and O'Shaughnessy, 2001b). This is followed by a further increase between day 20 and adulthood, which establishes the normal adult cohort of cells. In AR-null mice, Leydig cell number was normal on day 5, showing that establishment of the fetal Leydig cell number is not androgen dependent. The normal pre-pubertal rise in Leydig cell number was significantly attenuated, however, in AR-null mice, showing that this process is partially androgen dependent. After day 20, Leydig cell number increased by a similar number in both normal and AR-null mice, indicating that only the early part of the developmental process, which establishes Leydig cell number, is androgen dependent. Results from this study also highlight the importance of measuring Leydig cell number directly rather than inferring changes from apparent

interstitial hyperplasia, which can arise simply through shrinkage of the seminiferous tubules.

Although it is clear from results reported here and from other studies that androgens are required for normal adult Leydig cell development, the mechanisms by which androgens regulate this process are uncertain. There has been limited study of androgen-receptor distribution in the mouse testis during development. Zhou et al. have reported immunohistochemical localisation of the androgen receptor only in Sertoli cells, myoid cells and germ cells of the mouse testis two weeks after birth with immunoreactivity present in the Leydig cells after three weeks (Zhou et al., 1996). This suggests that newly formed adult Leydig cells lack androgen receptors and that the effects of androgens on early Leydig cell differentiation and development are mediated through the myoid cells or Sertoli cells. Later effects on Leydig cell function and development may be mediated directly through receptors on the cells themselves or may continue to be mediated through other cells in the testis. In cultured mouse Leydig cells, androgens acting through the androgen receptor inhibit the synthesis of the P450c17 enzyme (Hales et al., 1987). This direct effect of androgens is opposite to what is seen in the AR-null mouse and suggests that at least some of the effects of the AR-null mutation in the adult are mediated through another cell type.

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