In vivo evidence for the crucial role of SF1 in steroid-producing cells of the testis, ovary and adrenal gland

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SUMMARY
Adrenal and gonadal steroids are essential for life and reproduction. The orphan nuclear receptor SF1 (NR5A1) has been shown to regulate the expression of enzymes involved in steroid production in vitro. However, the in vivo role of this transcription factor in steroidogenesis has not been elucidated. In this study, we have generated steroidogenic-specific Cre-expressing mice to lineage mark and delete Sf1 in differentiated steroid-producing cells of the testis, the ovary and the adrenal gland. Our data show that SF1 is a regulator of the expression of steroidogenic genes in all three organs. In addition, Sf1 deletion leads to a radical change in cell morphology and loss of identity. Surprisingly, sexual development and reproduction in mutant animals were not compromised owing, in part, to the presence of a small proportion of SF1-positive cells. In contrast to the testis and ovary, the mutant adult adrenal gland showed a lack of Sf1-deleted cells and our studies suggest that steroidogenic adrenal cells during foetal stages require Sf1 to give rise to the adult adrenal population. This study is the first to show the in vivo requirements of SF1 in steroidogenesis and provides novel data on the cellular consequences of the loss of this protein specifically within steroid-producing cells.

KEY WORDS: Leydig cell differentiation, Adrenal development, Gonad development, Steroidogenic factor 1, Steroidogenic gene regulation, Mouse

INTRODUCTION
Endogenous steroid hormones are powerful small molecules produced in vertebrates through the metabolic process of steroidogenesis that impact a large number of normal homeostatic processes and disease development. In mammals, the sex steroids testosterone and oestradiol, principally produced in the testis and ovary, respectively, are crucial for reproduction, metabolism and behaviour. Adrenal glands produce corticosteroids that have numerous physiological impacts on, for example, stress and the immune response, metabolism, electrolyte levels and behaviour. Specific cells within each of these steroidogenic organs carry out the crucial function of converting cholesterol, the precursor to all steroids, into sex and corticosteroids by expression of a suite of cytochrome P450 and hydroxysteroid dehydrogenase enzymes, some of which are shared and others organ specific. Shared enzymes include CYP17, 3βHSD and P450 Side Chain Cleavage (P450SCC). The latter protein, which is encoded by the Cyp11a1 gene, catalyses the conversion of cholesterol to pregnenolone, the first rate-limiting enzymatic step in the biosynthesis of all steroid hormones.

The orphan nuclear hormone receptor steroidogenic factor 1 (SF1; also known as NR5A1 or Ad4BP) has been proposed to be a key regulator of steroidogenesis. It, and its family member LRH-1 (also known as NR5A2), bind specific DNA sequences found in the transcriptional regulatory promoters of many steroidogenic genes and can activate reporter constructs in vitro (reviewed by Omura and Morohashi, 1995; Parker and Schimmer, 1995; Hammer and Ingraham, 1999; Val et al., 2003). Consistent with this proposal, in vivo deletion of the ‘P’ site within the Cyp11a1 gene, to which SF1 or LRH-1 can bind, leads to a dramatic decrease in Cyp11a1 levels in the adult adrenal gland and testis (Shih et al., 2008). SF1 has been shown to have other functions, particularly during the development of the gonads and adrenal glands. SF1-deficient mice exhibit complete adrenal and gonadal agenesis owing to apoptosis in the developing genital ridge (Luo et al., 1994) and defects within the pituitary and ventral medial hypothalamus (Ingraham et al., 1994; Ikeda et al., 1995; Sadovsky et al., 1995; Shinoda et al., 1995). Although no humans bearing homozygous null mutations have been identified, a vast array of heterozygous alleles have been associated with adrenal failure, testis dysgenesis, androgen synthesis defects, hypoplasias, anorchia and male factor infertility in 46,XY individuals (reviewed by Ferraz-de-Souza et al., 2011).

SF1 expression is first found in the adrenal and gonad anlagen, the adrenogonadal primordium (AGP), and continues in multiple differentiated cell types within the steroidogenic organs that are descendants of this embryological structure (Hatano et al., 1995; Ikeda et al., 1994; Hatano et al., 1996). Foetal and adult testes express high levels of SF1 in the testosterone-synthesizing Leydig cell and the non-steroidogenic, germine-supporting Sertoli cell. Analogous to the testes, ovaries express SF1 in non-steroidogenic (follicle or granulosa cells) and steroidogenic (stromal and theca cells) cell populations. Similar to the testis, the adrenal gland has a transient steroidogenic foetal population that is replaced by a definitive adult steroidogenic population and both express SF1 (reviewed by Kim and Hammer, 2007; Morohashi and Zubair, 2011).

The complete adrenal and gonadal agenesis and the perinatal lethality in Sf1−/− mutant mice have prevented in vivo analyses of this factor in the different differentiated steroidogenic cell types in the foetus and adult. Previous studies with the Amhr2tm3(cre)Bhr targeted allele and the Sf1 conditional allele attempted to circumvent the AGP apoptosis to study the role of SF1 in Leydig and follicle cells (Jeyasuria et al., 2004). Unfortunately, this genetic model deleted Sf1
too early to allow normal testis development, as exemplified by the reduced cell proliferation one day prior to Leydig cell appearance. More importantly, recent data has shown that this Cre strain also induces abundant recombination in Sertoli cells, another crucial testis cell type (Tanwar et al., 2010). Furthermore, Amhr2<sup>tm1Cre</sup> is not expressed in the adrenal gland or the steroidogenic stromal and theca cells in the ovary, precluding the in vivo characterization of SFI in these steroidogenic cell types. Finally, these studies failed to genetically mark the SFI mutant cells, which prevented the assessment of the fate of these cells in vivo. Therefore, no systematic study has addressed the in vivo function of SFI in different differentiated steroidogenic cells of the testis, ovary and adrenal gland.

We have overcome this by creating a novel mouse transgenic line, which, when combined with an SFI conditional mutation, has allowed us to test the in vivo function of SFI in the steroidogenic cell types residing in the three primary steroidogenic tissues in mammals. Importantly, we simultaneously lineage mark the mutant steroidogenic cells and provide the first report of the outcome of these cells in their native context. We find that SFI acts as an in vivo major regulator of all steroidogenic cells of the testis whereas SFI dependence in ovarian steroidogenic cells is cell type specific. In addition, in the testis, ovary and foetal adrenal gland, SFI deletion leads to a radical change in cell morphology. In contrast to the testis and ovary, the mutant adult adrenal gland shows a lack of SFI-deleted cells, suggesting that foetal Cyp11a1-expressing adrenal cells require SFI to give rise to the adult adrenal population.

**MATERIALS AND METHODS**

**Mouse strains and breeding**

The SFI<sup>Fl</sup> conditional mutant mice were kindly provided by Dr Keith Parker (Zhao et al., 2001b). The Gt(Rosa26)Sor<sup>Tm1TgJ</sup> (R26<sup>R<sup>Cre</sup></sup>) and Gt(Rosa26)Sor<sup>Tm1TgJ-GFP<sup>Cua</sup></sup> (R26<sup>R<sup>YFP</sup></sup>) R26 Cre reporter strains were developed by Drs Philippe Soriano and Frank Costantini, respectively (Soriano, 1999; Srinivas et al., 2001). SFI<sup>Fl<sup>Cre</sup></sup>;Tg(Cyp11a1-<i>iCre</i>) animals are shown as controls as initial experiments did not show any phenotypic difference between SFI<sup>Fl<sup>Cre</sup></sup> and SFI<sup>Fl<sup>+/+</sup></sup> mice carrying the Tg(Cyp11a1-iCre) transgene.

**Bacterial artificial chromosome (BAC) construct**

The Cyp11a1-<i>iCre</i> construct was generated using a previously characterized BAC containing the Cyp11a1 transcriptional regulatory elements (Jeays-Ward et al., 2003). We used the previously described homologous recombination method in DY380 cells (Jeays-Ward et al., 2003). For the recombination fragment, the iCre coding region plus the rabbit β-globin polyadenylation signal sequences were introduced at 14 bp upstream of the Cyp11a1 ATG codon, within the 5′ untranslated region of the mRNA. The resulting construct contained a 2.8 kb Cyp11a1 fragment with the iCre + polyadenylation signal sequence insertion and a 100 bp deletion of Cyp11a1 sequences, starting at the site of insertion and including the ATG codon. This fragment was introduced into DY380 cells containing the unmodified Cyp11a1 BAC and homologous recombination was induced. For pronuclear injections, BAC DNA was prepared using the Qiagen unmodified -galactosidase (R26R lacZ) in cells that have been exposed to a functional Cre recombinase protein and their descendants. Foetal testes and adrenal glands, but not ovaries, are steroidogenic and express Cyp11a1. We observed robust Cre-mediated reporter expression in E14.5 testes and adrenal glands derived from foetuses carrying the Cyp11a1-Cre transgene and R26<sup>R<sup>YFP</sup></sup> (Fig. 1A) or R26<sup>R<sup>Cre</sup></sup> reporter strains. YFP or β-galactosidase expression could initially be detected in a few cells of E13.5 testes whereas foetal ovaries failed to show reporter expression (data not shown). Adult testis, ovary and adrenal gland all express Cyp11a1 and revealed high level of Cre-mediated recombination (Fig. 1B-D). Sections through foetal and adult tissues revealed reporter expression restricted to Cyp11a1-
expressing steroidogenic cell types. Foetal testes showed reporter expression in interstitial cells with morphological characteristics of Leydig cells (Fig. 1E,F). Adult ovaries showed reporter expression in thin cells surrounding the growing follicles, where theca cells reside, and in the stromal region (Fig. 1G). Reporter expression was prevalent in the foetal adrenal gland, prior to the formation of different cortical layers and was found in the steroidogenic cortical layer at later stages (Fig. 1H). Finally, reporter-expressing cells (YFP) co-expressed steroidogenic markers in all of these organs as assayed by co-labelling methods (shown in later figures).

To define the functional role of SF1 in Cyp11a1-expressing cells, we produced mice that contained the Cyp11a1-Cre transgene, the Sf1 conditional loss-of-function mutation (hereafter referred to as Sf1Ft) (Zhao et al., 2001b) and the R26R YFP reporter allele. This genetic design allows cells to first become steroidogenic before SF1 is deleted. Furthermore, our aim was to identify, through YFP expression, cells in which Sf1 deletion was likely to have taken place and analyse their phenotype. Mice with the Cyp11a1-Cre transgene and homozygous for Sf1Ft (mutant) were viable and showed no decrease in body mass (Fig. 1I). Furthermore, male mutants showed no fertility phenotypes as assayed by testis mass, epididymal sperm counts, impregnation rate or litter size (Fig. 1I; data not shown). Consistent with this observation, intratesticular testosterone levels were similar between mutant and control animals (9509±863 versus 9262±1585 ng testosterone/g tissue). Histological analysis of sections from the adult testis, ovary and adrenal gland showed no major organ abnormalities, although subtle effects were found, which will be discussed below (Fig. 1J-L).

Testes with Sf1-deleted Leydig cells show defects in steroidogenic gene expression

Mammalian testes have a remarkable capability to compensate for developmental defects, prompting us to investigate steroidogenic development during the foetal phase when Leydig cells are first arising. Morphologically, E14.5 mutant testes are indistinguishable from controls. However, whole-mount RNA in situ hybridization (WISH) revealed numerous gene expression changes associated with the steroid-producing foetal Leydig cell population. Sf1 is expressed at high levels in foetal Leydig cells but is observed at lower levels in Sertoli cells, which reside in the testis cords (Fig. 1E; Fig. 2A). As expected, mutant testes showed a notable Sf1 reduction in the high-expressing interstitial Leydig cells but no change in the Sertoli cell Sf1 expression (Fig. 2A). Cyp11a1,
in mutants, as expected, as the Cyp11a1-Cre transgene is not expressed in Sertoli cells (Fig. 2B). These data suggest that loss of SF1 in foetal Leydig cells reduces their steroidogenic capacity in the foetal tests.

**Foetal Leydig cells require SF1 cell-autonomously for steroidogenesis**

In order to investigate the mutant foetal testis phenotype further, we performed co-marker immunohistochemical analyses on sections to study individual SF1-deleted cells. Two steroidogenic enzymes were analysed, P450SCC and 3βHSD, together with YFP. Double immunostaining of control E14.5 testes, which express a nuclear and cytoplasmic localized YFP after exposure to Cre, showed that 100% of the interstitial YFP cells were positive for the mitochondrial and endoplasmic reticulum localized markers P450SCC and 3βHSD, respectively (Fig. 3A; supplementary material Fig. S1). These data confirm that the Cyp11a1-driven Cre activity targets foetal Leydig cells. In contrast to control foetal Leydig cells, mutants showed a marked reduction in P450SCC (68% of YFP+ cells were P450SCC+) whereas 3βHSD levels were more subtly reduced (78% of YFP+ cells were 3βHSD+) (Fig. 3C; supplementary material Fig. S1). The continued expression of steroidogenic markers in E14.5 mutant Leydig cells raised the possibility that the Cre activity was not efficiently deleting both SF1 conditional alleles. Therefore, we analysed SF1 expression in mutant testes. All YFP-positive Leydig cells in control testes express SF1 (Fig. 3B). By contrast, 88% of mutant Leydig cells (YFP positive) were deficient in SF1, whereas the SF1 expression in the non-Cre expressing Sertoli cells remained unchanged (Fig. 3B,C). These data are consistent with our previous RNA expression studies.

The lack of concordance between the number of SF1-deficient YFP cells (88%) in comparison with the number of cells lacking the steroidogenic proteins (32% of mutant cells were P450SCC negative) suggested that the SF1 protein turnover might be more rapid than that of steroidogenic enzymes, which reside in the mitochondria and endoplasmic reticulum. To investigate this hypothesis, we looked at testes from later stages. Antibody staining at postnatal day (P) 1 revealed notably more mutant YFP-positive cells lacking P450SCC (82%) and 3βHSD (80%), compared with observations in E14.5 testis, confirming a slower decay of these proteins (Fig. 3C). In addition, SF1/P450SCC co-staining at this stage showed that all mutant cells that had lost SF1 had also lost P450SCC (80%) and 3βHSD (80%) (data not shown). Our data therefore show that Leydig cells require cell-autonomous SF1 function for steroidogenic gene expression. The delayed decay of steroidogenic enzymes in foetal Leydig cells is likely to account for the lack of an overt feminization phenotype during foetal development.

**SF1 loss leads to steroidogenic cell shape changes without cell death**

SF1 deletion has been shown to induce apoptosis in the early adrenal gland and gonad (Luo et al., 1994). The loss of Leydig cells through apoptosis could account for the reduction in Leydig cells-specific gene expression. However, we failed to detect increased apoptosis in E14.5 mutant testes using Lysotracker and TUNEL assays (supplementary material Fig. S1C; data not shown). Although the YFP intensity in the mutant cells was not as strong as in control Leydig cells, we did not observe a reduced number of mutant YFP cells, supporting the conclusion that cell death is not induced in the SF1-deficient foetal Leydig cells (Fig. 3D). However, we did observe that mutant Leydig cells exhibited a

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**Fig. 2. The steroidogenic gene expression pathway is reduced in Cyp11a1-Cre:Sf1flox/flox foetal testes. (A)** Whole mount in situ hybridization of representative E14.5-15 testes from control and mutant mouse embryos were analysed for the expression of steroidogenic genes as indicated. Anterior is to the left with the testis (T) positioned above the mesonephros (M). Steroidogenic Leydig cells (black arrows) reside between seminiferous tubules, which contain Sf1-expressing Sertoli cells (white arrowhead). (B) Quantitative RT-PCR on RNA from E14.5 control (CON) and mutant (MUT) testes for steroidogenic genes as indicated. *P<0.05. Error bars represent s.e.m.

Cyp11a1 and Hsd3b1 are steroidogenic genes that are crucial for testosterone production and have been shown to be direct transcriptional targets of SF1 in vitro. Mutant testes showed dramatic reductions in all three genes compared with controls (Fig. 2A). Furthermore, the more recently recognized adrenal-like steroidogenic genes that reside in the foetal testis (Val et al., 2006) were also compromised in mutant testes, as indicated by the reduction in Cyp21 gene expression (Fig. 2A). Quantitative (q)RT-PCR analysis confirmed our WISH observations and showed Cyp11a1, Cyp17a1 and Hsd3b1 to be reduced 48%, 49% and 53% relative to controls, respectively (Fig. 2B). Importantly, the direct transcriptional target of SF1 in Sertoli cells, Sox9, was unaffected
major cell shape change. Control Leydig cells express YFP in the nuclear and cytoplasmic compartments and typically appear as large round or ovoid cells (Fig. 3A,B). In striking contrast, mutant Leydig cells are not only smaller, but exhibit a dramatically different cell morphology and appear flat or crescent-shaped, as seen most clearly in the YFP-stained samples (Fig. 3A,B).

**Adult Leydig cells require SF1 cell-autonomously for steroidogenesis**

Foetal Leydig cells are removed from the prepubertal testis by an ill-defined mechanism referred to as regression, with the de novo development of adult Leydig cells occurring during the prepubertal phase (reviewed by Habert et al., 2001). We therefore examined the adult Leydig cell phenotype of the Cyp11a1-Cre;Sf1<sup>YFP<sup>Cre</sup></sup> animals. Adult Leydig cells, are large pyramidal shaped P450<sup>SCC</sup>-positive cells positioned in the interstitial space between several seminiferous tubule cross sections. Similar to the foetal Leydig cell findings, we observed that all YFP-positive cells from control animals expressed P450<sup>SCC</sup>, confirming the specificity of this transgene (Fig. 4A). However, YFP–/P450<sup>SCC</sup> cells were observed in control testes, suggesting that a small percentage of adult Leydig cells did not express the Cyp11a1-Cre transgene (Fig. 4A). Mutant testes showed an obvious reduction in P450<sup>SCC</sup> staining with numerous YFP-positive cells lacking P450<sup>SCC</sup> expression (Fig. 4A). Notably, their shape and position were dramatically impacted (Fig. 4B). As in control animals, a low number of P450<sup>SCC</sup>-expressing cells in the mutant testes did not express YFP, probably representing fully functioning Leydig cells that were not targeted by the Cre transgene (Fig. 4A). To confirm that SF1 had been deleted in the mutant testis, we carried out double immunostaining for SF1 and YFP. All YFP cells in control testes, which expressed P450<sup>SCC</sup>, were positive for SF1 (Fig. 4B). By contrast, mutant testes contained an abundance of YFP cells that were negative for SF1 (Fig. 4B). These cells showed the same aberrant cell shape and inter-tubule distribution as the YFP-positive cells that were negative for P450<sup>SCC</sup> expression. As expected, SF1 expression in Sertoli cells in mutant animals was not affected. Mutant testes do occasionally contain YFP cells that continue to express SF1 and exhibit the cell shape and inter-tubule position indicative of fully functioning Leydig cells, probably representing Cyp11a1-expressing Leydig cells that did not delete both SF1 alleles. Analysis of 3βHSD expression showed a similar phenotype to P450<sup>SCC</sup>, with all YFP cells in adult control testes being positive for 3βHSD but an abundance of YFP cells in mutant testes showed no detectable 3βHSD and had atypical cell shapes and/or distributions (Fig. 4B). To confirm the immunohistochemical studies, qRT-PCR was performed for Cyp11a1, Cyp17a1 and Hsd3b1 expression and the levels of these genes were found to be reduced in the mutant testes to 21%, 24% and 16% of control testes levels, respectively (Fig. 4C). The level of the Sertoli cell-specific Sox9 gene, was unaffected in mutant testes and supports the previous finding that Sertoli cell gene expression is not disturbed (Fig. 4C). These data clearly show that the steroidogenic gene programme and cellular morphology of adult Leydig cells require SF1 in a cell-autonomous manner.

**Steroidogenic cells of the adult ovary show differential SF1 dependence**

The mammalian adult ovary is responsible for the production of numerous steroids that are required for female reproduction. Unlike the adult testis, several different cell types produce P450<sup>SCC</sup> and allow for the production of oestrogens in the ovarian follicle or progesterone from the corpus luteum. Steroidogenic P450<sup>SCC</sup>-positive cells that express detectable levels of SF1 include the
interstitial stromal cells and the theca cells, but not the P450<sup>SCC</sup>-expressing cells within the corpus luteum (Fig. 5B,C). Based on our findings from the mutant testes, we decided to analyse the control and mutant ovaries in a similar manner to the mutant males to determine whether SF1 deficiency impacts P450<sup>SCC</sup> cells differentially. Double immunostaining for the Cre recombinase reporter (YFP) and P450<sup>SCC</sup> in adult control ovaries revealed that Cre activity was restricted specifically to the three prominent P450SCC-expressing cells: thecal, stromal, and corpora luteal cells (Fig. 5A). Like the testis, all control YFP cells were P450<sup>SCC</sup>
positive (Fig. 5A). However, occasional P450SCC single positive cells were observed, suggesting that not all steroidogenic cells expressed the Cyp11a1-Cre transgene (Fig. 5A). In contrast to controls, mutant ovaries showed an obvious reduction in P450SCC expression within the stromal and thecal compartment, although YFP expression indicated that the cells were still resident within the interstitial regions (Fig. 5A). However, we did observe YFP+/P450SCC+ stromal cells in mutant ovaries, which probably represent cells that did not delete both Sf1 conditional alleles but activated the reporter transgene (Fig. 5A). Similar to the mutant testis, YFP+/P450SCC− cells in the mutant ovary had a change in cell shape, being flatter and more elongated, which was particularly obvious in the stromal compartment (Fig. 5A, inset). Strikingly, no reduction in P450SCC levels was observed in the corpora luteal cells, which is consistent with the lack of SF1 in this compartment. Double immunostaining with SF1 and YFP showed that mutant ovaries had an obvious loss of SF1-positive cells in the steroidogenic stromal and theca cell compartments (Fig. 5B). Control and mutant ovaries express SF1 in the granulosa cells of the follicle, a cell population that never expresses P450SCC and, as expected, SF1 level was not altered in this compartment in mutant ovaries. Consistent with previous results, YFP-positive mutant cells in positions where stromal and theca cells reside have an altered cell shape and do not co-express SF1 (Fig. 5B). SF1/P450SCC co-staining showed that all mutant cells that had lost P450SCC had also lost SF1 and was consistent with the mutant YFP+/P450SCC− stromal and theca cells also being deficient in SF1 (Fig. 5C). Importantly, we did observe YFP+/SF1+ cells in mutant ovaries, suggesting that not all Cyp11a1-expressing stromal and theca cells delete both Sf1 alleles (Fig. 5B, C). Our data shows that, in contrast to the adult testis, not all P450SCC-positive ovarian cell types require SF1, as shown by the continued expression of P450SCC in corpora luteal cells that have been exposed to Cre activity. However, like adult Leydig cells, the stromal and theca cells require SF1 cell-autonomously for their steroidogenic phenotype and cell morphology.

**Foetal adrenal cells require SF1 for steroidogenesis**

The adrenal primordium is first apparent at E10.5 in the mouse embryo (Hatano et al., 1996). However, steroidogenic cells, as detected by Cyp11a1 expression, are not obvious until E11.5. We confirmed that the Cyp11a1-Cre transgene was specifically inducing recombination in P450SCC-positive cells in the E14.5 foetal adrenal gland (Fig. 1A). Double immunostaining of control foetal adrenal glands showed that, like the foetal testis, all YFP cells were positive for P450SCC (Fig. 6A, inset). In contrast to the foetal testis, however, we observed many YFP−/P450SCC+ cells in control adrenal glands, suggesting reduced activity of the Cyp11a1-Cre transgene in this foetal organ (Fig. 6A). Mutant foetal adrenal glands contained YFP+/P450SCC− cells, a marker combination type never observed in control adrenal glands (Fig. 6A, inset). Some of these cells showed a more elongated and flat shape, as seen in the foetal mutant testis (Fig. 5A, inset). Co-staining for YFP and SF1 showed that all YFP-positive cells in control adrenal glands express SF1 (Fig. 6B, inset). However, most mutant YFP-positive cells did not express SF1, suggesting the Cyp11a1-Cre transgene was deleting both Sf1 alleles in the targeted adrenal cortical cells (Fig. 6B, inset). The large number of YFP+/P450SCC+ cells in the mutant adrenal glands suggests that the P450SCC protein has a slow decay rate, as is the case in the foetal Leydig cell. An increase in apoptosis due to the lack of SF1 could account for the relatively lower number of YFP+/P450SCC− cells in the mutant foetal adrenal gland compared with the testis. However, Lysotracker staining on E14.5 adrenal glands did not show any differences between control and mutant tissue (supplementary material Fig. S2A).
Mutant adult adrenal cortical cells maintain SF1 expression

As in the case of Leydig cells, adrenal cortical cells have a foetal and adult (or definitive) population. Therefore, we analysed the phenotype of the adult adrenal gland of Cyp11a1-Cre;Sf1fl/fl animals to investigate whether SF1 had a similar role to that in adult Leydig cells. Double immunohistochemistry on control animals showed that all YFP-positive cells expressed P450scc and, in contrast to foetal stages, only a few P450scc-positive cells failed to show evidence of Cre recombinase activity, confirming that the Cyp11a1-Cre transgene was effective in the cells that contribute to the adult adrenal gland (Fig. 6C). In the mutant adrenal gland, in contrast to all other mutant steroidogenic adult tissues that express SF1, P450scc expression was not reduced and was found throughout the cortex. YFP expression analysis revealed that some P450scc-positive cells were positive for YFP and others were not (Fig. 6C). The number of YFP-positive cells was variable and tended to occur in patches, suggesting a clonal origin (see supplementary material Fig. S2C for other examples of mutant adrenal staining). Immunohistochemistry analysis revealed that SF1 had not been lost in the mutant adrenal cortex as most cells retained the protein (Fig. 6D), which is consistent with these cells being positive for P450scc expression. Therefore, these studies suggest that lack of SF1 is incompatible with adult adrenal cortical cell development.

Characterization of SF1-deficient adult Leydig cells

The cell shape and peritubular association of many mutant adult Leydig cells suggested that the cells had transdifferentiated into a different cell type. Therefore, we investigated the expression of various markers in YFP-positive cells within the mutant testes. Like controls, mutant Leydig cells continue to express the androgen receptor (AR) (Fig. 7A). Peritubular myoid (PTM) cells express AR and smooth muscle actin (SMA), and have flat nuclei and thin cell bodies around the basement membrane of the seminiferous tubules. The cell morphology, nuclear shape and expression of AR in mutant cells raised the possibility they had taken on a PTM cell fate. Immunostaining for YFP and SMA predictably showed that control Leydig cells express YFP but do not co-express SMA (Fig. 7B). Although mutant cells exhibit numerous hallmarks of PTMs, we never observed SMA in mutant YFP cells but found them in tight juxtaposition (Fig. 7B). Vascular smooth muscle cells (VSMCs) and pericytes (PCs) have been proposed to be precursors to adult Leydig cells (Davidoff et al., 2004). To test whether the SF1-deficient Leydig cells had reverted back to a more ‘primitive’ developmental state that would cause them to express VSMC and PC markers, we analysed the expression of the pan-endothelial marker PECAM (CD31) (Fig. 7C). Even though mutant Leydig cells can exhibit similar cell morphology shapes to endothelial cells, we failed to detect any co-expression of YFP and PECAM (Fig. 7C). Mutant Leydig cells also lacked neurofilament heavy chain expression, which was identified as a marker of a transient intermediate state of adult Leydig cell differentiation (data not shown) (O’Shaughnessy et al., 2008).

The consistent and dramatic change in size and morphology prompted us to assess the mTOR pathway owing to its known role in regulating cell growth (Laplante and Sabatini, 2012). The serine-phosphorylated form of the ribosomal subunit S6 (RPS6) protein (P-S6) has been shown to mark cells with mTOR activation and its target S6 kinase. YFP-positive adult Leydig cells in control testes exhibited high levels of P-S6, showing that the mTOR pathway is active in this cell population in vivo (Fig. 7D). By contrast, YFP-positive cells in mutant testes are negative for P-S6 (Fig. 7D). These data show that the role of SF1 in differentiated steroidogenic cells is not restricted to regulating the transcription of steroid-producing genes. Consistent with this, we analysed by (q)RT-PCR the expression of Leydig cell markers that were not associated with the production of steroids from cholesterol, such as Insl3, Hmgcs1, which encodes the enzyme HMG CoA synthase, and Lhcgr, which encodes the luteinizing hormone receptor, and found that their levels were significantly reduced in the mutant (Fig. 7E). These data suggest that Sf1 deletion leads to a loss of steroidogenic cell identity.
DISCUSSION
It has been 20 years since the identification of SF1 and the proposal that it is a transcriptional activator of steroidogenic genes based on in vitro cell culture transcription assays and its in vivo expression profile. Our analysis of mutant animals provides the first in vivo genetic and molecular evidence that SF1 is necessary for the steroidogenic gene programme in foetal and adult Leydig cells, ovarian theca and stromal cells and in foetal adrenal cortical cells. All genes associated with steroid production from cholesterol that were analysed showed a consistent decrease in expression in mutant tissues. As SF1 deletion occurred after the targeted cells started expressing Cyp11a1, we are effectively investigating the role of SF1 in the maintenance of expression of steroidogenic genes within these cells. Therefore, the half life of mRNA and protein need to be considered when analysing the changes occurring in mutant tissues. Our finding that mutant foetal Leydig cells have reduced steroidogenic protein levels that progressively decrease during development argues that loss of SF1 leads to a cessation of transcriptional activation and degradation of associated transcripts and proteins over time.

The viability and reproductive ability of SF1 mutant animals was an unexpected phenotype. Nevertheless, this proved to benefit our analysis as it allowed us to study SF1 loss within well developed and healthy organs. A ‘masculinization programming window’ (MPW) exists in early foetal development, prior to the definitive or adult adrenal cortical cells. The phenotype of mutant adrenal glands was variable and healthy organs. A ‘masculinization programming window’ (MPW) exists in early foetal development, prior to the MPW. The phenotype of mutant adrenal glands was variable and in some cases they were found to be reduced in size (supplementary material Fig. S2C). However, all cortical cells expressed SF1 and P450scC, providing an explanation for the lack of an adrenal failure. The most likely explanation for the continued reproductive performance in both male and female Cyp11a1Cre;Sf1F/F mice is the residual steroidogenesis carried out by the Leydig, stromal and theca cells that did not have both SF1 alleles deleted by the Cre recombinase or that did not express the Cyp11a1-Cre transgene. A reminiscent finding was observed when Zhao et al., conditionally ablated SF1 in the pituitary gland (Zhao et al., 2001a). They observed a hypomorphic phenotype due to incomplete Cre-mediated recombination in the target cells. Our mutant animals produced normal levels of testosterone in the adult and exhibited no spermatogenic phenotype, although the levels of steroidogenic enzymes were reduced to ~20% of controls. This phenotype is very similar to that of mice with the Cyp11a1 hypomorph allele (Shih et al., 2008).

We failed to detect increased apoptosis in mutant foetal adrenal glands and testes, consistent with the observation that the number of YFP-positive cells was not significantly different between wild-type and mutant organs. This differs from the apoptosis induced within the pre-steroidogenic adrenal and gonad of Sf1−/− embryos and suggests that the differentiated steroidogenic cell types do not require SF1 for survival. In contrast to most cell types in which SF1 has been studied, Leydig cells have been shown to be post-mitotic (Habert et al., 2001). One possibility is that the role of SF1 in cell survival is dependent on whether the cells have exited the cell cycle.

The only gonadal steroidogenic cell type independent of SF1 function for the maintenance of steroidogenesis is the corpora lutea cell. Low levels of SF1 have been previously reported in corpora lutea; however, we failed to detect staining above background in control and mutant animals. The related nuclear hormone receptor LHR-1 (NR5A2), which can activate steroidogenic gene transcription in vitro, has been shown to be expressed in corpora lutea cells (Falender et al., 2003; Hinshelwood et al., 2003), suggesting that it could be the primary steroidogenic regulator in this cell population. Unfortunately, Nr5a2−/− mice are embryonic lethal and corpora lutea are missing from Amhr2Δ/Δ;Nr5a2−/− ovaries owing to ovulation defects, preventing the functional assessment of this related molecule in this third steroidogenic cell type of the ovary (Duggavathi et al., 2008). The use of our new Cyp11a1-Cre strain in combination with the Nr5a2 conditional allele could test this model.

Cell lineage marker studies in the mouse have suggested that the definitive or adult adrenal cortical cells are derived from the foetal population and appear prior to birth (Zubair et al., 2008; Wood and Hammer, 2011). The mutant adrenal gland showed a similar dependence on SF1 for steroidogenic gene expression at E14.5 to the other tissues analysed. However, in the adult adrenal cortex, SF1 and P450scC expression were maintained in most cells, including the YFP-positive cells. These data suggest that the only cells that were able to give rise to adult cortical cells were those in which either the Cyp11a1-Cre transgene was not expressed or in which Cre recombinase had failed to delete both SF1 alleles. The patchy nature of P450scC expression in some mutant adrenal glands (see supplementary material Fig. S2B) is consistent with the model of clonal expansion of SF1-containing cells. These results also suggest that steroidogenic Cyp11a1-expressing cells can serve as precursors of adult cortical cells. As we were not able to detect a significant increase in apoptosis during foetal stages, the fate of the SF1-deleted cells was not clear. Careful analysis of the adult adrenal gland revealed a few YFP-positive cells that did not express P450scC at the border between the cortex and medulla, particularly in virgin females (supplementary material Fig. S2C). This pattern was reminiscent of the lacZ expression observed in adult transgenic mice containing the SF1 foetal adrenal enhancer driving this marker (Zubair et al., 2006). These cells also had a more elongated shape, as seen in SF1-deleted cells of the other organs.

An unexpected finding from these studies was the dramatic morphological cell shape changes and loss of expression of most Leydig cell markers in the mutant testis. Even though the cell shape and position of the mutant cells in the adult testis resembled endothelial or PTM cells, our marker studies ruled out a transdifferentiation process to these cell fates taking place. A second possibility is that SF1 deletion triggered a de-differentiation programme to a more progenitor-like state. Adult Leydig cells originate from non-steroidogenic stem cells during the prepubertal phase (Habert et al., 2001). However, YFP cells in the mutant adult testis did not consistently express the stem cell markers c-kit or leukemia inhibitory factor receptor (Ge et al., 2006) suggesting that these cells had not acquired a stem or precursor cell fate (data not shown). A third possibility is that these cells represent a recessed or involuted state, as observed with foetal Leydig cells that extinguish their steroidogenic activity in the maturing pubertal testis (Habert et al., 2001). Control foetal Leydig cells are permanently marked by YFP in our experimental design and should continue to express YFP, even if they lose their steroidogenic activity (e.g. loss of Cyp11a1 expression), change their shape and/or reposition themselves in the intertubular space. Careful analyses of adult testes from control Cyp11a1-Cre mice with the R26R-YFP Cre reporter showed that all YFP interstitial cells were steroidogenic and suggests that the non-steroidogenic foetal Leydig cells are removed.

Our study highlights the all-encompassing role of SF1 in the gonad and adrenal gland, where it is required for their initial
development and also serves as a major regulator of the differentiatiated steroid-producing cells within these organs. In steroidogenic cells, our data show that it not only regulates the expression of genes involved in steroid synthesis but also controls cell morphology, cell signalling and the expression of non-steroidogenic markers of these cells.

Acknowledgements
We thank Jaime Carvajal and Kevin Taylor for performing the pronuclear injections; Ian Mason, Ken Morohoshi and David Robertson for generous provision of antibodies; and Blanche Capel for critical reading of the manuscript. We are indebted to Keith Parker for generously providing the SF1 mutant mice for this study.

Funding
This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) [BB/C517825/1 to F.W.B.]; and the Medical Research Council (MRC) [G0901645 to J.R.G.]. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/supp/doi:10.1242/dev.087247/-/DC1

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