Introduction

Cell lineage determination is a complex process involving developmental decisions that gradually restrict the ability of stem cells to differentiate into tissue-specific cell types. During development, the pluripotent stem cells from the inner cell mass differentiate into multipotent stem cells in various tissues (Eckfeldt et al., 2005). The pluripotent stem cells from the inner cell mass differentiate into tissue-specific cell types. During development, the pluripotent stem cells from the inner cell mass differentiate into multipotent stem cells in various tissues (Eckfeldt et al., 2005). The tissue-specific stem cells, under the influences of the cellular environment or external cues, then acquire characteristics and functions of organ-specific cell lineages (Rossant, 2001). The embryonic gonad uniquely can differentiate into two distinct organs (testis or ovary) and therefore is a powerful model for studying cell fate determination. In mouse gonadal primordium, somatic cell precursors derived from a pool of undifferentiated cells expressing transcription factors such as Steroidogenic factor 1 (SF1), Wilms’ tumor 1 (WT1), and Gata4 (Hatano et al., 1996; Luo et al., 1994). In response to sex-specific genetic and signaling cues (Brennan and Capel, 2004), these somatic cell precursors give rise to either testicular (Sertoli, Leydig, and peritubular myoid cells) or ovarian (granulosa and theca) somatic cells. Regardless of the sex chromosomes of the embryo, expression of either Sry (Sex-determining region of the Y chromosome) or Sox9 (Sry-related HMG-box gene 9) is sufficient to direct a subpopulation of somatic cell precursors to become Sertoli cells (Kanai et al., 2005; Sekido and Lovell-Badge, 2008). Sertoli cells, in turn, initiate testis morphogenesis and induce the appearance of fetal Leydig cells and other somatic cell lineages.

Although Sertoli cells trigger the program of testis morphogenesis and produce anti-Müllerian hormone (AMH) that causes regression of the female reproductive tract or the Müllerian ducts, Sertoli cells are not sufficient for full masculinization of the embryos. Instead, fetal Leydig cells control the maintenance and differentiation of the Wolffian ducts, secondary sexual characteristics, testis descent, and differentiation of the brain (Haider, 2004). The absence of Sry and Sox9 expression in fetal Leydig cells suggests that their development is facilitated by Sertoli cells, which produce paracrine factor(s) to specify the Leydig cell lineage. Multiple genes have been involved in the specification of fetal Leydig cell lineage, such as X-linked aristless-related homeobox gene (Arx), Desert Hedgehog (Dhh), and platelet-derived growth factor receptor alpha (Pdgfrα) genes (Bitgood et al., 1996; Brennan et al., 2003; Habert et al., 2001; Kim and Capel, 2006; Kitamura et al., 2002). However, loss of any of these genes did not completely prevent Leydig cell differentiation. In the Arx−/− fetal testis, the Leydig cell population was severely diminished. Arx, a transcription factor, was barely detectable in fetal...
Leydig cells, suggesting that the effects of Arx could be indirect, probably from the fibroblast-like cells in the testis interstitium where Arx is highly expressed. Pdgfra was expressed in the coelomic border and interstitium of the fetal testis. In Pdgfra−/− fetal testis, mesonephric cell migration, Sertoli cell proliferation, and fetal Leydig cell differentiation were all reduced (Brennan et al., 2003). Consequently, the Leydig cell defects in Pdgfra−/− testis could be secondary to Sertoli cell problems as well as mesonephric cell migration, a potential source of fetal Leydig cell precursors. Contrary to observations in Pdgfra−/− testes, loss of Dhh resulted in a decrease in fetal Leydig cell numbers without affecting migration or proliferation of precursor cells or differentiation of Sertoli cells (Pierucci-Alves et al., 2001; Yao et al., 2002). The decrease, instead of absence, of fetal Leydig cells in the Dhh−/− testis was proposed to result from a possible compensation of other Hh ligands. This notion was further supported by the finding that culturing fetal testes in the presence of a general Hh inhibitor, cyclopamine, abolished the appearance of fetal Leydig cells, a phenotype more severe than that in the Dhh−/− testis (Yao et al., 2002; Yao and Capel, 2002).

The goal of this study was to investigate whether activation of the Hh pathway alone is sufficient to induce the appearance of fetal Leydig cells. By using a gain-of-function approach, we ectopically activated the Hh pathway in the developing ovary, where the Hh signaling is normally inactive (Yao et al., 2002). This model also enables us to explore the possibility that Leydig cell differentiation can occur in the absence of other testicular components such as Sertoli cells.

**Materials and methods**

**Generation of Sf-1/Cre:SmoYFP animals**

SmoYFP transgenic mice (The Jackson Laboratory, Maine USA; Jeong et al., 2004) was crossed to the Sf-1/Cre transgenic mice, in which the Cre recombinase is under the control of Sf-1 promoter (Bingham et al., 2006). SmoYFP females were housed with Sf-1/Cre males and plug-checked next morning. Detection of a vaginal plug was considered as embryonic day 0.5. Embryos of pregnant females were harvested at the desired dates. Embryos were genotyped as described (Henrique et al., 1995). We used alkaline phosphatase-conjugated digoxigenin-labeled RNA probes for Insl3, Wnt4, and Fst.

**Immunohistochemistry**

Gonads were collected at the desired stages, fixed in 4% paraformaldehyde at 4 °C overnight, and stored in methanol at −20 °C. Upon embedding, samples were rehydrated through a sucrose/OCT gradient and cryosectioned. Primary antibodies used were: rabbit anti-SOX9 (1:1000), rabbit anti-β3HSD (1:1000), and rabbit anti-SF1 (1:500; above three antibodies were gifts from Dr. Morohashi at National Institute of Natural Science, Japan), rabbit anti-CYP17 (1:100 from Dr. Buck Hales, University of Illinois, Chicago, USA), rabbit anti-Laminin (1:200, Sigma, USA), and goat anti-AMH (1:1000, Santa Cruz, USA). Secondary antibodies used were FITC-, Rhodamine- or Cy3-conjugated donkey anti-Rabbit and FITC- or Rhodamine-conjugated donkey anti-Goat (all 1:200, Jackson Immuno Research, USA). When two primary antibodies from the same species were used, tyramide amplification combined with sequential immunofluorescence was performed following the technique described in Buki et al. (2000). Fluorescent images were captured using a Fast1394 QImaging Camera (Qimaging, Canada) installed on a Leica Dmi 4000B microscope (Leica, Germany).

**In situ hybridization**

Samples were fixed overnight in 4% paraformaldehyde in PBS at 4 °C and processed as described (Henrique et al., 1995). We used alkaline phosphatase-conjugated digoxigenin-labeled RNA probes for Insl3, Wnt4, and Fst.

**Plastic sections for ultrastructure**

Samples were fixed in 4% glutaraldehyde overnight and kept in 2% glutaraldehyde at RT or 4 °C. Tissue processing and staining (Toluidine blue/basic Fuchsin staining) were based on previously published procedures (Hoffman EO, 1983; Login and Dvorak, 1993; Miller, 1982).

**Fig. 1.** Activation of the Hedgehog pathway in Sf-1/Cre:SmoYFP ovary. (A, B) Cells positive for cytoplasmic Smo/YFP protein (green, arrows) counterstained with nuclear DAPI (red) were found in Sf-1/Cre: SmoYFP ovary, but not in the control ovary. White arrowheads indicate autofluorescence in red blood cells. Scale bar = 20 μm. (C, D) Presence of Gli1 mRNA (dark purple deposits) was detected by whole mount in situ hybridization at E13.5. Gli1 mRNA was normally present in the mesonephros (m) but not in the ovary (o) in the control. In the Sf-1/Cre:SmoYFP ovary, Gli1 expression was upregulated.
Fig. 2. Appearance of fetal Leydig cells in the Sf-1/Cre:SmoYFP ovary at E16.5. (A–C) CYP17 protein (green staining, higher magnification shown in insets) and (D–F) Insl3 mRNA (dark purple deposits) via whole mount in situ hybridization were present in control and Sf-1/Cre:SmoYFP testes at E16.5. These two markers were negative in the control ovary. Scale bar=100 μm. (G–I) Toluidine blue/basic Fuchsin staining was performed on plastic sections of newborn testis. Clusters of fetal Leydig cells with characteristic cytoplasmic lipid droplets are outlined with dashed red lines. (J, K) Colocalization of YFP and 3βHSD in Sf-1/Cre:SmoYFP ovary. (J) Cells positive for cytoplasmic Smo/YFP protein (green). (K) Cells positive for cytoplasmic 3βHSD (red). (L) Merge of A and B. White arrows indicate autofluorescence in red blood cells. gc = germ cell; m = mesonephros; tc = testis cord; scale bar=10 μm.
Results

Appearance of fetal Leydig cells in the Sf-1/Cre; Smo<sup>YFP</sup> fetal ovaries

We used the Cre/loxP system to activate the Hh pathway in the SF1-positive somatic cells of the fetal ovary by targeting Smoothened (Smo), a gene that encodes a transmembrane protein responsible for transducing the intracellular signaling pathway induced by Hh ligands. When the Sf-1/cre transgenic line is crossed to the Smo/YFP (Smo<sup>YFP</sup>) line, Cre recombinase under the control of the Sfi promoter removes the STOP sequence upstream of the Smo<sup>YFP</sup> transgene. Removal of the STOP sequence allows the transcription of a constitutively active form of mutated Drosophila Smoothened (smo) fused with yellow fluorescent protein gene (YFP) (Jeong et al., 2004).

Fig. 3. Maintenance and differentiation of the Wolffian duct derivatives in the Sf-1/Cre;Smo<sup>YFP</sup> newborn female. (A–C) Wholemount images of the urogenital system from control male, control female, and Sf-1/Cre;Smo<sup>YFP</sup> female mice were taken at the time of birth. a = adrenal; k = kidney; o = ovary; t = testis; Arrow = position of the gonad. (D, E) Higher magnification images of the gonads were taken from A and C, respectively. Histological sections of different parts of the Wolffian duct were obtained and stained with H&E. epi = epididymis; o = ovary; ov = oviduct; t = testis; ut = uterine horn; v = vas deferens. Scale bar = 500 μm.
The Smo<sup>YFP</sup> transgene then activates the Hh pathway regardless of the presence or absence of the Hh ligands. The Sf-1/cre: Smo<sup>YFP</sup> model restricts constitutive activation of the Hh pathway in the SF1-positive somatic cell population in the fetal ovary. We first confirmed that Smo/YFP expression was indeed activated in this model. Cytoplasmic YFP fluorescence was seen in the Sf-1/Cre; Smo<sup>YFP</sup> ovaries (Fig. 1B) but not in the control ovary (Sf-1/Cre or Smo<sup>YFP</sup> only, Fig. 1A). Consistent with activation of the Hh pathway in these cells, we observed increased expression of Gli1 (Figs. 1C and D) and Ptch1 (data not shown), two immediate downstream targets of Smo activation (Koebernick and Pieler, 2002). These results indicate that the Sf-1/Cre; Smo<sup>YFP</sup> transgenic strategy activates the Hh pathway in the fetal ovary.

We next investigated whether ectopic Hh activation in the fetal ovary was sufficient to induce the differentiation of fetal Leydig cells. Cytochrome P450 17-hydroxylase (CYP17), a key steroidogenic enzyme for testosterone production, was expressed in fetal Leydig cells of developing testes at E16.5 (Fig. 2A) but not in ovaries (Fig. 2B). We found that ectopic activation of the Hh pathway induced the appearance of CYP17-positive cells in the Sf-1/Cre; Smo<sup>YFP</sup> ovaries (Fig. 2C). To confirm that these steroidogenic cells are bona fide fetal Leydig cells rather than ectopic adrenal cells, we examined the expression of Leydig cell-specific marker Insl3. Insl3 mRNA was expressed in the testis but not the control ovary at E16.5 (Figs. 2D and E). Clusters of Insl3-positive cells (Fig. 2F) were observed in Sf-1/Cre; Smo<sup>YFP</sup> fetal ovaries, indicating that these ectopic steroidogenic cells were indeed fetal Leydig cells. In normal testes, fetal Leydig cells, which cluster in the interstitial region, contain abundant cytoplasmic lipid droplets (Fig. 2G, dotted red lines). Similar cells were found in the Sf-1/Cre; Smo<sup>YFP</sup> ovaries, while the control ovaries were devoid of these cells (Figs. 2I and H respectively). In addition, these ectopic fetal Leydig cells co-expressed YFP/SMO fusion protein and the steroidogenic enzyme, 3-beta hydroxy-delta-5-steroid dehydrogenase (3βHSD; Figs. 2K–J), indicating that Hh activation directly induces the appearance of ectopic fetal Leydig cells in the ovary.

**Masculinization of the Sf-1/Cre;Smo<sup>YFP</sup> female embryos**

Testosterone produced by fetal Leydig cells transforms the Wolffian ducts into the male internal genitalia (i.e., epididymis and vas deferens, Fig. 3D) (Jost, 1947, 1953; Wattenberg, 1958), while both androgens and INSL3 are required for descent of the testis (Kubota et al., 2001; Kumagai et al., 2002; Spiess et al., 1999) (Fig. 3A). In female embryos, which lack androgens and INSL3, the Wolffian ducts degenerate and the ovaries at birth remain attached posterior to the kidneys (Fig. 3B). In the Sf-1/Cre; Smo<sup>YFP</sup> newborn female, however, the ovaries descended to the position lateral to the bladder, closely resembling the testes in newborn male (Fig. 3C). Moreover, the Wolffian ducts in Sf-1/Cre; Smo<sup>YFP</sup> newborn female differentiated into epididymis and vas deferens indistinguishable from those in the normal male (Figs. 3D and E). The Sf-1/Cre; Smo<sup>YFP</sup> newborn females also had virilized external genitalia (data not shown), again providing functional evidence for the production of testosterone. Unlike the normal males, however, female internal genitalia (i.e., the oviducts and uterus) persisted in the Sf-1/Cre; Smo<sup>YFP</sup> newborn female (Fig. 3E). Taken together, these data demonstrate that ectopic activation of the Hh pathway in fetal

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**Fig. 4.** Absence of Sertoli cells markers in the Sf-1/Cre;Smo<sup>YFP</sup> ovary. Immunohistological analysis for (A–C) laminin, (D–F) SOX9, and (G–I) AMH, were performed on sections from control testis, control ovary, and Sf-1/Cre;Smo<sup>YFP</sup> ovary. Green cells in E and F were auto-fluorescent red blood cells. tc = testis cord; scale bar = 100 μm.
ovaries led to appearance of fetal Leydig cells, which produced androgens and INSL3 that caused female pseudohermaphroditism.

**Absence of Sertoli cells and testicular structure in the Sf-1/Cre;SmoYFP ovary**

The persistence of Müllerian duct-derived structure in the Sf-1/Cre;SmoYFP females argued that the ectopic activation of Hh signaling did not cause the differentiation of Sertoli cells or the expression of Sertoli cell marker AMH. To address this more directly, we examined expression of the Sertoli cell markers SOX9 and AMH and the testis cord component laminin. Normally, the fetal testes organize into testes cords (Fig. 4A), which are surrounded by a basement membrane consisted of laminin. Sertoli cells that express both nuclear SOX9 (Fig. 4D) and cytoplasmic AMH (Fig. 4G) are enclosed inside the testis cords. In contrast, the fetal ovary lacks cords (Fig. 4B) and expresses neither SOX9 (Fig. 4E) nor AMH (Fig. 4H) at any stages examined (E11.5 to birth, only E13.5 samples shown here as a representative). These findings are consistent with the model that the differentiation of fetal Leydig cells in Sf-1/Cre;SmoYFP ovaries was a direct effect of Hh activation independent of Sertoli cells.

**Normal progression of the ovarian program in the Sf-1/Cre;SmoYFP ovaries**

We next investigated whether the ovarian program was affected by activation of the Hh pathway. Expression of Wnt4 and follistatin (Fst), both ovary-specific genes critical for maintaining ovarian identity (Figs. 5A, B, D, and E), was not altered in Sf-1/Cre;SmoYFP ovary (Figs. 5C and F) compared to the control (Figs. 5B and E). These observations indicate that appearance of fetal Leydig cells in the Sf-1/Cre;SmoYFP ovaries did not require or result in Sertoli cells or major changes in expression ovarian genes.

**Upregulation of SF1 in the Sf-1/Cre;SmoYFP ovaries**

SF1 is expressed in somatic cell precursors of the bipotential gonads in both sexes (Ikeda et al., 1994). As sex differentiation unfolds, SF1 expression decreases in the ovary and remains very low until after birth. In contrast, as the testis program progresses, SF1 expression is downregulated in Sertoli cells and increased significantly in fetal Leydig cells. This increased SF1 expression in fetal Leydig cells was partially linked to Sertoli cell-derived DHH (Park et al., 2007; Yao et al., 2002). Moreover, without SF1, Leydig cell differentiation does not occur (Koskimies et al., 2002; Leers-Sucheta et al., 1997; Morohashi et al., 1992; Reinhart et al., 1999; Val et al., 2003). We therefore speculated that the Hh pathway directs the undifferentiated somatic cell precursors in the fetal ovary toward a fetal Leydig cell fate by inducing the expression of SF1. Immunohistochemical detection of SF1 and the steroidogenic enzyme CYP17, a known target gene of SF1, showed that ectopic activation of the Hh pathway induced the co-expression of SF1 (nuclear) and CYP17 (cytoplasmic) (Fig. 5I) in a manner that closely resembled the normal testis (Fig. 5G). In contrast, the control ovary lacked cells expressing either SF1 or CYP17. These

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**Fig. 5.** Expression of Wnt4 (A–C) and Fst (D–F) mRNA was detected by wholemount in situ hybridization in control male, control female, and Sf-1/Cre;SmoYFP female gonads. Brown-purple deposits indicate specific staining. (G–I) Double immunohistochemistry was performed on gonads using SF1 (green) and CYP17 (red) antibodies. m = mesonephros; o = ovary; t = testis; scale bar = 20 μm.
data provide evidence that SF1 is a molecular link between the Hh pathway and fetal Leydig cell differentiation.

**Discussion**

**Activation of the Hh pathway alone is sufficient for the differentiation of fetal Leydig cells**

Defects in fetal Leydig cell establishment have been found in mice lacking functional Arx, Dihh, or Pdgfalpha genes (Bitgood et al., 1996; Brennan et al., 2003; Habert et al., 2001; Kim and Capel, 2006; Kitamura et al., 2002; Yao et al., 2002). However, the loss of any of these genes did not completely abolish fetal Leydig cell population. In this study we demonstrate that activation of the Hh pathway alone is sufficient to transform the SF1-positive ovarian cells into functional fetal Leydig cells without the presence of any testicular components. The ectopic fetal Leydig cells in the ovary were functional, leading to masculinization of the female embryos or female pseudohermaphroditism. These results prove conclusively that fetal Leydig cells can arise independent of Sertoli cells—although Sertoli cells normally play a key role in this process by releasing the morphogen that activates Hh signaling in Leydig cell precursors.

Fetal Leydig cell numbers increase dramatically from E12.5 to 15.5 in mouse embryo despite that fetal Leydig cells are mitotically inactive during this period (Orth, 1982). This observation suggests that the appearance of fetal Leydig cells is probably a result of transformation and/or migration of precursor cells. Different sources of fetal Leydig cell precursors have been proposed, including cells from the neighboring mesonephros (Merchant-Larios and Moreno-Mendoza, 1998), migrating neural crest cells (Mayerhofer et al., 1996), the coelomic epithelium (Karl and Capel, 1998; Schmahl et al., 2000), or SF1-positive somatic cells (Hatanoko et al., 1996). In this study, we reveal that the SF1-positive somatic cells are definitive precursor cells for fetal Leydig cells. However, our results did not exclude the possibility that other sources may contribute to the fetal Leydig cell population.

*The Hh pathway induces fetal Leydig cell differentiation by upregulating SF1*

SF1 is essential for Leydig cell differentiation by controlling expression of steroidogenic enzymes and INSL3 (Koskimies et al., 2002; Leers-Sucheta et al., 1997; Morohashi et al., 1992; Reinhart et al., 1999; Val et al., 2003). Based on the current study, the ectopic activation of the Hh pathway induced the upregulation of SF1 in the somatic cells of the fetal ovary where SF1 is normally downregulated. The presence of SF1 in the ovarian somatic cells overlapped with CYP17 expression, a steroidogenic enzyme known to be regulated by SF1. In addition, SF1 expression was downregulated in the Dhh−/− fetal testis (Yao et al., 2002) and loss of one SF1 allele in the Dihh−/− background (SF1+/−; Dihh−/−) completely abolished fetal Leydig cell population compared to the reduced fetal Leydig cell population in SF1+/−; Dihh+/− males (Park et al., 2007). Together, these data suggest that Hh signaling works through SF1 to transform precursor cells into the fetal Leydig cell lineage. We are currently investigating the regulatory sequences of SF1 gene for Hh responsive elements.

**Critical ovarian genes are maintained despite the presence of fetal Leydig cells**

The model in this study introduced an ectopic population of steroidogenic Leydig cells in the ovarian environment. It provides a unique opportunity to test whether the presence of fetal Leydig cells could steer the fetal ovarian program towards the testis identity. Both Wnt4 and Fst are known to be ovarian-specific and antagonistic to the testicular pathway (Yao et al., 2006). WNT4 acted through FST to maintain germ cell survival in the ovary (Yao et al., 2004). Our results showed that both Wnt4 and Fst expressions are not affected by the appearance of Leydig cells in the ovary, indicating that ectopic appearance of fetal Leydig cells was neither the cause nor the result of defects in ovarian development.

Leydig cells and their ovarian counterpart theca cells are believed to derive from the same precursor lineage based on similar androgen-producing ability. To exclude the possibility that the androgen-producing cells in the Hh activated ovary are premature appearance of theca cells, we have compared the cellular characteristics of ectopic steroidogenic cells in the Hh activated ovary and theca cells in the adult ovary. The lipid droplets in the ectopic steroidogenic cells in the Hh activated ovary were patterned in 1–2 clusters similar to that of fetal Leydig cells, but different from the scattered cytoplasmic lipid droplets of theca cells and adult Leydig cells (data not shown). Also these ectopic steroidogenic cells were able to express steroidogenic enzymes and INSL3 as early as E15.5 when luteinizing hormone (LH) has not been produced (O’Shaughnessy et al., 1998). This ability to produce steroids and INSL3 in a LH-independent manner is similar to fetal Leydig cells instead of theca cells, which require LH to become steroidogenically active (Erickson et al., 1985; Kawamura et al., 2004; Magoffin, 2002). To our knowledge, molecular markers capable of distinguishing fetal Leydig cells from theca cells are not available. Based on the morphological characteristics and their LH-independent ability to produce androgens, we conclude that the steroidogenic cells in the Hh activated ovary are fetal Leydig cells.

In this study we demonstrated that activation of the Hh pathway alone is sufficient to induce the fetal Leydig cell lineage without the contribution of Sry and Sertoli cells. The ectopic appearance of fetal Leydig cells in the ovary led to masculinization of the female embryos while maintaining the female reproductive tract. These results provide not only conclusive evidence for the specification of a testicular somatic cell lineage other than Sertoli cells, but also possible mechanisms for female pseudohermaphroditism.

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**References**


