Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis

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Establishment of the steroid-producing Leydig cell lineage is an event downstream of *Sry* that is critical for masculinization of mammalian embryos. Neither the origin of fetal Leydig cell precursors nor the signaling pathway that specifies the Leydig cell lineage is known. Based on the sex-specific expression patterns of *Desert Hedgehog* (*Dhh*) and its receptor *Patched 1* (*Ptch1*) in XY gonads, we investigated the potential role of DHH/PTCH1 signaling in the origin and specification of fetal Leydig cells. Analysis of $Dhh^{-/-}$ XY gonads revealed that differentiation of fetal Leydig cells was severely defective. Defects in Leydig cell differentiation in $Dhh^{-/-}$ XY gonads did not result from failure of cell migration from the mesonephros, thought to be a possible source of Leydig cell precursors. Nor did DHH/PTCH1 signaling appear to be involved in the proliferation or survival of fetal Leydig precursors in the interstitium of the XY gonad. Instead, our results suggest that DHH/PTCH1 signaling triggers Leydig cell differentiation by up-regulating *Steroidogenic Factor 1* and *P450 Side Chain Cleavage* enzyme expression in *Ptch1*-expressing precursor cells located outside testis cords.

[*Key Words*: *Desert Hedgehog*; *Patched 1*; Leydig; mesonephros; testis; organogenesis]

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A critical event in testis organogenesis is the specification of somatic cell lineages including Sertoli cells, peritubular myoid cells, and Leydig cells. Specification of these lineages is crucial for the establishment of testis morphology and the production of hormones. A single gene on the Y chromosome, *Sry* (sex-determining region of the Y chromosome), is believed to induce a cascade of signaling pathways for the differentiation of these somatic cell lineages (Gubbay et al. 1990; Koopman et al. 1991). Autonomous expression of *Sry* in somatic cells in the XY gonad leads to differentiation of Sertoli cells (Albrecht and Eicher 2001). Differentiating gonadal cells induce migration of cells from the mesonephros into the gonad. The migrating cells contribute to precursors of the peritubular myoid and vascular cell lineages (Martineau et al. 1997; Capel et al. 1999; Tilmann and Capel 1999). Differentiation of peritubular myoid cells and the consequent formation of testis cords are regulated by Desert hedgehog (DHH), a signaling protein produced by Sertoli cells (Clark et al. 2000; Pierucci-Alves et al. 2001). Fetal Leydig cells are first identifiable within the interstitium of the XY gonad (between testis cords) when they express *P450 Side Chain Cleavage* (*Scc*) enzyme and other steroidogenic enzymes required for the production of androgens.

The specification of adult Leydig cells has been studied extensively (Habert et al. 2001). Adult Leydig cells are believed to be a separate population of steroidogenic cells that arise from adult peritubular mesenchymal cells (Ariyaratne et al. 2000). They are believed to be completely independent of the population of fetal Leydig cells responsible for initial masculinization of the embryo. The origin of fetal Leydig cells is unknown. During fetal life, Leydig cell precursors could arise from one or both of two possible sources: the mesonephros or the coelomic epithelium. When gonads from 11.5 days postcoitum (dpc) embryos were grafted to mesonephroi from mice carrying transgenic markers such as β-galactosidase (β-gal) or GFP, the markers were found in some of the peritubular myoid cells and other interstitial cells of the testis (Buehr et al. 1993; Merchant-Larios et al. 1993; Nishino et al. 2000). Some migratory mesonephric cells acquired ultrastructural features of steroidogenic Leydig cells (Merchant-Larios and Moreno-Mendoza 1998). A small population of these migrating cells differentiated into Leydig cells when cultured in vitro (Nishino et al. 2001). However, when the XY gonad was separated from

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the mesonephros at 11.5 dpc and cultured alone (Merchant-Larios et al. 1993) or when XY gonads were grafted to embryonic hind limbs at 11.5 dpc and subsequently cultured (Moreno-Mendoza et al. 1995), differentiation of Leydig cells proceeded normally. The results of these two experiments suggest that most Leydig precursors are already present in the gonad by 11.5 dpc. Another possible source of Leydig cell precursors is the coelomic epithelium that covers the entire coelomic surface of the gonad. Both proliferation studies (Schmahl et al. 2000) and DiI lineage tracing experiments (Karl and Capel 1998) revealed that coelomic epithelial cells in XY gonads proliferate rapidly between 11.5 and 12.5 dpc and contribute many interstitial cells to the developing testis. The fate of these cells has not been defined. The signals that induce differentiation of fetal Leydig cells are also unknown. At present only a negative regulator of Leydig cell differentiation (*Wnt4*) has been identified (Vainio et al. 1999). Expression of the hedgehog receptor, *Patched 1* (*Ptch1*), throughout the cells of the interstitium in 12.5 dpc XY gonads suggested that DHH/PTCH1 signaling might function in Leydig cell differentiation in addition to its role in signaling between Sertoli and peritubular myoid cells (Bitgood et al. 1996). To determine the role of DHH/PTCH1 signaling in Leydig cell differentiation, we explored the temporal and spatial expression patterns of *Dhh*, *Ptch1*, and *Scc*, and analyzed gonads from $Dhh^{-/-}$ XY embryos. Here we show that disruption of DHH/PTCH1 signaling in $Dhh^{-/-}$ mice results in defects of fetal Leydig cell differentiation, whereas it has no effect on mesonephric cell migration or on the establishment of the interstitial cell population. These results suggest that DHH/PTCH1 signaling does not affect the origin of fetal Leydig precursors, but instead, operates later to specify the Leydig cell lineage by up-regulating *Steroidogenic Factor 1* (*Sf1*) and *Scc* expression in *Ptch1*-expressing precursor cells located outside testis cords.

Results

Temporal and spatial expression of Dhh, Ptch1, *and* Scc *in testis organogenesis*

To determine whether fetal Leydig cells might be targets of DHH signaling, we first detailed the expression patterns of *Dhh*, its receptor, *Ptch1*, and a Leydig cell marker *Scc* (Rouiller et al. 1990) in XY gonads from 11.5 to 13.5 dpc, the period during which the differentiation of fetal Leydig cells occurs. Expression of *Dhh* began at 11.5 dpc and continued afterward in the Sertoli cell lineage as previously described (Fig. 1; Bitgood et al. 1996). Analyzing β-galactosidase activity in $Ptch^{tm1Mps}$ ($Ptch^{LacZ}$) XY gonads, we found that $Ptch^{LacZ}$ was not expressed at 11.5 dpc XY gonads, but was prominently expressed in the interstitial space between testis cords in 12.5 and 13.5 dpc XY gonads (Fig. 1). $Ptch^{LacZ}$ expression was also found around the mesonephric tubules in the anterior part of the mesonephros from 11.5 to 13.5 dpc. We compared $Ptch^{LacZ}$ expression with *Scc* expression to determine whether $Ptch^{LacZ}$-expressing cells became *Scc*-positive. At 12.5 dpc, the majority of interstitial cells were $Ptch^{LacZ}$-positive and only a small population of them expressed *Scc* (Fig. 1). In 13.5 dpc XY gonads, a much larger percentage of $Ptch^{LacZ}$-expressing cells were also expressing *Scc* (Fig. 1, bottom panels). Neither $Ptch^{LacZ}$ nor *Scc* was expressed in the coelomic epithelium of XY gonads (Fig. 1, bottom panels) or in endothelial cells of the vasculature (data not shown). *Patched 2*, another mammalian hedgehog receptor (Carpenter et al.

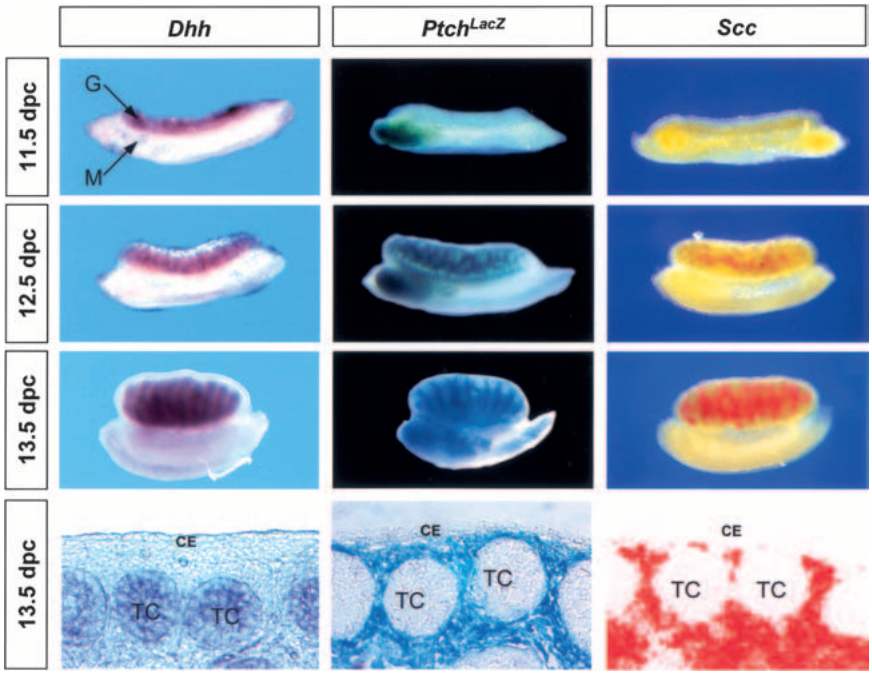

Figure 1. Expression patterns of *Dhh* (dark purple), *Ptch1* (blue), and the Leydig cell marker *Scc* (red) in XY gonads from 11.5 dpc to 13.5 dpc. Expression of *Dhh* and *Scc* were detected by whole-mount in situ hybridization. *Ptch1* expression was detected by analyzing β-galactosidase activity in the $Ptch^{tm1Mps}$ XY gonads. Sections of 13.5 dpc whole-mount samples are shown at the bottom to confirm the cell-specific expression patterns of *Dhh*, *Ptch1*, and *Scc* in the gonads. CE, coelomic epithelium; G, gonad; M, mesonephros; TC, testis cords.

1998), was not expressed in XY gonads during this time period (data not shown). Other hedgehog genes such as *Sonic Hedgehog* and *Indian Hedgehog* are not expressed in the gonad (Bitgood and McMahon 1995).

Defects in differentiation of fetal Leydig cells in Dhh$^{-/-}$ *XY gonads*

The expression patterns of *Dhh* and its receptor, *Ptch1*, indicated that DHH signaling could be involved in the early development of Leydig cells. To investigate whether differentiation of fetal Leydig cells was affected by loss of DHH signaling, we analyzed the expression of *Scc* in 13.5–14.5 dpc *Dhh*$^{+/+}$, *Dhh*$^{+/-}$, and *Dhh*$^{-/-}$ XY gonads (Clark et al. 2000). No differences were noted between *Dhh*$^{+/+}$ and *Dhh*$^{+/-}$ samples. Representative *Dhh*$^{+/-}$ samples are shown in Figures 2 and 3. At 13.5 dpc, expression of *Scc* appeared in the center of all *Dhh*$^{+/+}$ and *Dhh*$^{+/-}$ gonads, whereas *Scc* expression was completely absent in 70% (7/10) of *Dhh*$^{-/-}$ gonads (Fig. 2). By 14.5 dpc, *Scc* expression reached its peak in interstitial cells in *Dhh*$^{+/+}$ and *Dhh*$^{+/-}$ gonads. However, only sparse staining for *Scc* was seen in the majority of 14.5 dpc *Dhh*$^{-/-}$ gonads (Fig. 2). It is known that the expression of *Scc* is under the regulation of SF1 (Clemens et al. 1994; Hatano et al. 1994). We performed immunocytochemistry for SF1 on 13.5 dpc XY gonads after in situ hybridization for *Scc* to verify that *Scc*-expressing cells were also SF1-positive. We found that all *Scc*-expressing cells (Fig. 3A, red cells outside of testis cords) showed strong nuclear staining for SF1 (Fig. 3A, green stain). In *Dhh*$^{-/-}$ gonads, the number of interstitial Leydig cells with strong nuclear SF1 staining was dramatically decreased compared to *Dhh*$^{+/+}$ and *Dhh*$^{+/-}$ gonads (Fig. 3B,C, arrows). However, interstitial cells with weak nuclear SF1 staining were still present in *Dhh*$^{-/-}$ gonads in normal numbers (Fig. 3C, arrowheads). Expression of

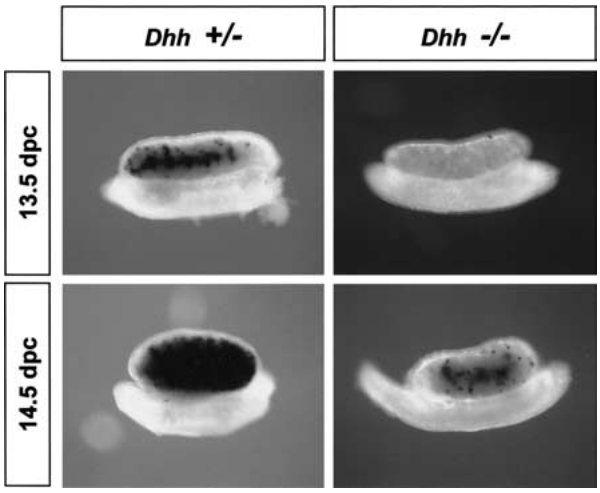

Figure 2. Expression of *Scc* in *Dhh*$^{+/-}$ and *Dhh*$^{-/-}$ XY gonads. The mRNA for the Leydig cell marker *Scc* (black stain) is present at 13.5 and 14.5 dpc in *Dhh*$^{+/+}$ (data not shown) and *Dhh*$^{+/-}$ but is reduced or absent in *Dhh*$^{-/-}$ XY gonads.

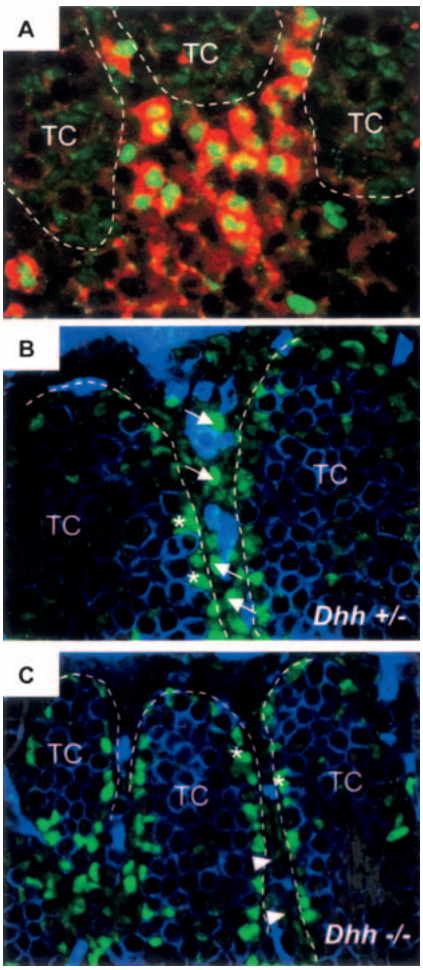

Figure 3. Expression of SF1 and *Scc* in 13.5 dpc *Dhh*$^{+/-}$ and *Dhh*$^{-/-}$ XY gonads. (*A*) Colocalization of SF1 (green nuclear staining) and *Scc* (red cytoplasmic staining) in Leydig cells in normal 13.5 dpc XY gonads by immunocytochemistry for SF1 and in situ hybridization for *Scc*. (*B*) Strong nuclear SF1 staining (arrows) in Leydig cells in *Dhh*$^{+/-}$ gonads. (*C*) Absence of strong nuclear SF1 staining in Leydig cells in *Dhh*$^{-/-}$ gonads. Weak nuclear SF1 staining was still present (arrowheads). SF1 staining was also detected in Sertoli cells (asterisks) in testis cords (TC, outlined by dotted lines). Germ cells and endothelial cells were stained with an anti-PECAM antibody (blue staining in *B* and *C*).

SF1 in Sertoli cells in testis cords was not affected by disruption of DHH signaling (Fig. 3B,C, asterisks).

Normal mesonephric cell migration in Dhh$^{-/-}$ *XY gonads*

One of the cellular events downstream of *Sry* is migration of interstitial cells from the mesonephros into the gonad between 11.5 and 12.5 dpc (Capel et al. 1999; Tilmann and Capel 1999). Because most interstitial cells express *Ptch*^{LacZ} at 12.5 dpc (Fig. 1), we investigated whether Dhh signaling regulates mesonephric cell migration. *Ptch*^{LacZ} expression showed a unique pattern

during the period when mesonephric cell migration occurs. At 11.5 dpc, *Ptch*^{LacZ} expression was observed only around the mesonephric tubules at the anterior part of the mesonephros but not in gonads of either sex (Fig. 1). As the development of gonads proceeded to 12.0 dpc, *Ptch*^{LacZ} expression appeared in the interstitium in the anterior part the XY gonad close to the mesonephric tubules (Fig. 4A). At 12.25 dpc, *Ptch*^{LacZ} expression in the XY gonad extended anteriorly and posteriorly (Fig. 4A). By 12.5 dpc, the entire interstitium of the XY gonad expressed *Ptch*^{LacZ}, except for the most posterior tip of the gonad (Fig. 1). No *Ptch*^{LacZ} expression was found in XX gonads at any stage examined (data not shown).

This unique pattern of *Ptch*^{LacZ} expression (Fig. 4A) suggested that the DHH/PTCH1 signaling pathway might induce migration of *Ptch1*-expressing cells from the mesonephros into the interstitium of the XY gonad, beginning near the anterior end of the gonad. To test this hypothesis, we assembled two different recombinant organ cultures at 11.25 dpc. In the first recombinant culture (Fig. 4B), we assembled a wild-type gonad with a *Ptch*^{LacZ} mesonephros. We reasoned that if *Ptch*^{LacZ}-expressing cells derive from the mesonephros, we should observe β-gal-positive cells in the wild-type gonad after migration has taken place. In the second recombinant culture (Fig. 4C), we assembled the reciprocal combination with a *Ptch*^{LacZ} gonad apposed to a wild-type mesonephros. After culture for 30 h (corresponding to ~12.5 dpc in vivo), samples were stained for β-gal. We found no β-gal staining in the interstitium of the wild-type gonad in the first recombinant culture (Fig. 4B), suggesting that few if any cells that have migrated from the mesonephros during this period of culture express *Ptch*^{LacZ}. In the second recombinant culture with a *Ptch*^{LacZ} gonad and a wild-type mesonephros, β-gal staining appeared in the interstitium of all *Ptch*^{LacZ} gonads (Fig. 4C), suggesting that *Ptch*^{LacZ} expression is induced in cells already present in the gonad by 11.25 dpc.

To further test the possibility that DHH/PTCH1 signaling was involved in mesonephric cell migration, we assembled an 11.5 dpc *Dhh*$^{+/+}$, *Dhh*$^{+/-}$, or *Dhh*$^{-/-}$ XY gonad apposed to an 11.5 dpc mesonephros expressing GFP and compared the migration of GFP-expressing cells in the presence and absence of DHH signaling. We found that GFP-expressing cells migrated from the mesonephros into the XY gonad in a similar pattern in *Dhh*$^{+/+}$ (data not shown), *Dhh*$^{+/-}$, and *Dhh*$^{-/-}$ gonads (Fig. 4D, red arrows). Analysis of *Scc* expression in these samples revealed that despite normal mesonephric cell migration, expression of *Scc* is completely absent in *Dhh*$^{-/-}$ XY gonads compared to *Dhh*$^{+/+}$ and *Dhh*$^{+/-}$ gonads (Fig. 4D, red staining).

Stage-specific effects of the hedgehog inhibitor cyclopamine on Leydig cell differentiation

To determine whether DHH/PTCH1 signaling regulates the earliest stages of Leydig cell differentiation or later

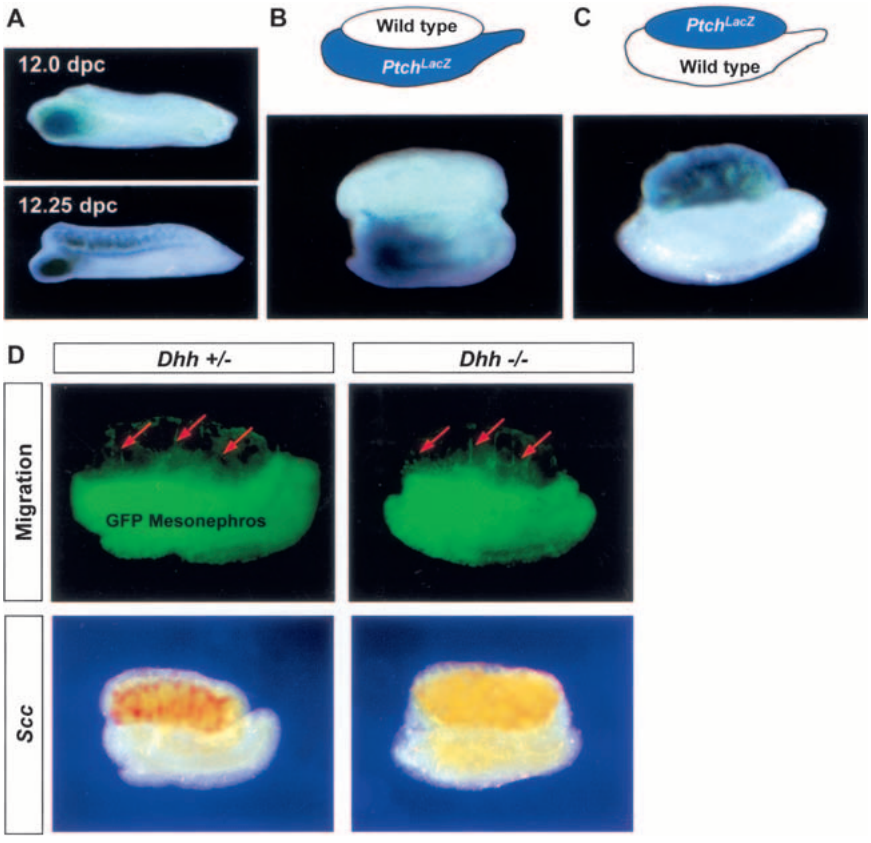

Figure 4. DHH/PTCH1 signaling is not responsible for induction of mesonephric cell migration into XY gonads. (*A*) Analysis of *Ptch*^{LacZ} expression in 12.0 and 12.25 dpc XY gonads. (*B*) Recombinant organ culture using a wild-type gonad apposed to a *Ptch*^{LacZ} mesonephros. (*C*) Reciprocal organ culture with a *Ptch*^{LacZ} gonad apposed to a wild-type mesonephros. Recombinant organ cultures in both *B* and *C* were assembled at 11.25 dpc, cultured for 48 h, and assayed for *Ptch*^{LacZ} expression. (*D*) Mesonephric cell migration and *Scc* expression in *Dhh*$^{+/-}$ and *Dhh*$^{-/-}$ gonads: Recombinant gonad cultures were assembled with an 11.5 dpc *Dhh*$^{+/-}$ or *Dhh*$^{-/-}$ XY gonad apposed to an 11.5 dpc mesonephros expressing GFP. Cell migration (green cell, red arrows) was detected 48 h after culture. Samples were fixed, and then expression of *Scc* was detected by in situ hybridization (red staining in gonads).

maintenance or expansion of the Leydig cell population, we examined *Scc* expression in gonad organ cultures in the presence and absence of a DHH signaling inhibitor, cyclopamine, introduced at 11.5 dpc or 12.5 dpc. Cyclopamine inhibits hedgehog signaling by inactivating Smoothened, the first downstream signaling molecule after binding of hedgehog protein to its receptor, PTCH1 (Taipale et al. 2000). *Scc* was expressed normally in both 11.5 and 12.5 dpc gonads after 24-h culture in the absence of cyclopamine. When cyclopamine was added at 11.5 dpc, the expression of *Scc* in Leydig cells was completely inhibited. In contrast, addition of cyclopamine to cultures at 12.5 dpc or 13.5 dpc had no effect on *Scc* expression in Leydig cells (Fig. 5, black stain; 13.5 dpc data not shown).

To determine whether the loss of DHH signaling affected proliferation or maintenance of Leydig precursors, we examined cell proliferation using an antibody against phosphorylated Histone H3 (pHH3; Paulson and Taylor 1982; Hendzel et al. 1997; Saka and Smith 2001), and apoptosis, using LysoTracker reagent (Zucker et al. 1998, 1999), in 11.5 dpc gonad explants cultured for 40 h in the presence or absence of cyclopamine. We found a similar total number of pHH3-positive cells (cell counts from 10 serial sections) in gonads cultured in the absence or presence of cyclopamine (Fig. 6, arrows). Although normal apoptotic cells were detected in the Müllerian duct in the mesonephros at this stage (Roberts et al. 1999), no apoptotic cells were found in the gonadal region of samples cultured in the presence or absence of cyclopamine (Fig. 6, the gonad is outlined by a dotted line).

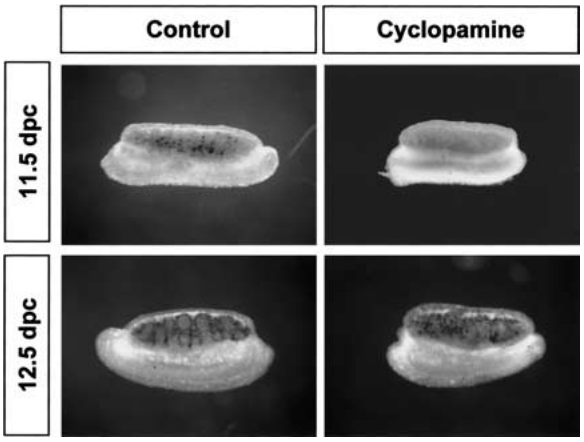

Figure 5. Stage-specific effects of the hedgehog inhibitor cyclopamine on expression of *Scc* mRNA in Leydig cells. XY gonads (11.5 or 12.5 dpc) were cultured in the presence or absence of cyclopamine (25 μM) for 24 h followed by whole-mount in situ hybridization for *Scc* (black staining in gonads).

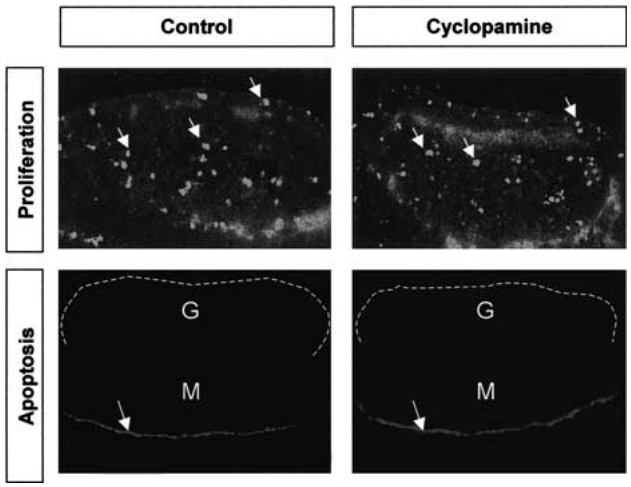

Figure 6. Effects of the hedgehog inhibitor cyclopamine on proliferation and apoptosis in 11.5 dpc gonads. Gonads (11.5 dpc) were cultured for 24 h in the presence or absence of cyclopamine (25 μM) followed by immunocytochemistry for phosphorylated Histone H3 (arrows) or LysoTracker staining for apoptosis (arrows indicate position of the Müllerian duct). G, gonad (outlined by a dotted line); M, mesonephros.

Discussion

It has been more than five decades since Jost first discovered that testosterone synthesized by the fetal testis is essential for differentiation of the Wölffian duct and development of male secondary sex characteristics (Jost 1947). Here we report that DHH/PTCH1 signaling is a positive regulator of the differentiation of steroid-producing Leydig cells in the fetal testis. *Dhh* is expressed downstream of *Sry*, specifically in Sertoli cells inside testis cords (Bitgood et al. 1996), and is the only known mammalian hedgehog protein expressed in the gonad between 11.5 and 13.5 dpc. One of the hedgehog receptors, *Ptch1*, was known to be expressed in interstitial cell populations (Bitgood et al. 1996). Original generation of *Dhh*-null mice on a *129/Sv* genetic background resulted in defects in spermatogenesis but no defects in testis organogenesis and Leydig cell differentiation despite down-regulation of *Ptch1* (Bitgood et al. 1996). However, transfer of the *Dhh* mutation to another genetic background resulted in discrete defects in development of the peritubular myoid cell lineage, leading to abnormal cord organization and loss of adult Leydig cells (Clark et al. 2000; Pierucci-Alves et al. 2001). We show here that it also results in a defect in differentiation of fetal Leydig cells.

Ptch1 is first expressed around the mesonephric tubules at the anterior end of the mesonephros. By 12.0 dpc, interstitial cells toward the anterior end of the gonad begin to express *Ptch1* under the positive regulation of DHH. Expression of *Ptch1* gradually extends toward both anterior and posterior ends of the gonad. Despite the implications of this expression pattern, we find no evidence that DHH is involved in signaling for mesonephric cell migration. Nor does loss of *Dhh* appear to exert a detrimental effect on Sertoli differentiation, as MIS and *Sox9* expression in $Dhh^{-/-}$ gonads and in cyclopamine treated gonads (Yao and Capel 2002) are normal.

Instead, this and previous data suggest that DHH is involved in signaling proximal cells to differentiate

along specific pathways. For example, it has been shown that DHH influences the differentiation of peritubular myoid cells in *Ptch1*-expressing cells most proximal to the DHH signal (Clark et al. 2000; Pierucci-Alves et al. 2001). Here we show that DHH signals the *Ptch1*-expressing cells located slightly further away from the DHH-producing Sertoli cells to differentiate as Leydig cells. Although it appears that all Leydig cells express *Ptch1*, not all *Ptch1*-expressing cells differentiate as Leydig cells. This likely means that other signals combine with DHH signals to specify Leydig cell fate.

Leydig precursors responsive to the DHH signal may be set aside earlier by their lineage origin, or they may be specified among cells of the interstitium by the intersection of multiple signals. Some evidence suggests that Leydig cells and steroid cells of the adrenal share a common origin at 10.5 dpc near the anterior end of the mesonephros (Hatano et al. 1996). If this is true, they must move into the gonad prior to 11.25 dpc under the control of signals other than DHH or they would have been detected in our recombinant organ culture system. Another possibility is that Leydig cells do not have a discrete lineage origin: pluripotent cells may derive from the coelomic epithelium between 11.5 and 12.5 dpc whose differentiation is under the control of combinatorial signals that intersect in the field of the gonad. This type of paradigm could suggest that the interstitial cells of the gonad are equivalent and plastic in the sense that, regardless of where they originate, they may follow one of several cell fates in the gonad. This decision could depend not on their lineage origin, but on their distance from other signaling cells or their spatial relationship to the vasculature or to other structural features of the gonad. Hedgehog signaling effects related to distance from the signal have been noted in many systems (Bumcrot and McMahon 1996; Neumann and Cohen 1997; Strigini and Cohen 1999; Vervoort 2000).

DHH does not regulate the size of the precursor population. We found that interstitial cells with low SF1 expression were still present in the $Dhh^{-/-}$ gonads, which may account for morphological identification of fetal Leydig cells in electron micrographs in $Dhh^{-/-}$ gonads (Clark et al. 2000). In previous work, we showed that low SF1-expressing cells derived from a second wave of proliferation in the coelomic epithelium (Schmahl et al. 2000). No difference in proliferation or apoptosis was observed in gonads cultured with the hedgehog inhibitor cyclopamine, suggesting that DHH/PTCH1 signaling does not regulate proliferation or survival of fetal Leydig cell precursors as has been shown to occur in other systems (Cann et al. 1999; Oppenheim et al. 1999; Charrier et al. 2001). The time at which DHH affects Leydig differentiation, based on in vitro experiments using cyclopamine to block hedgehog signals, suggests that DHH/PTCH1 signaling specifies Leydig cell fate by early upregulation of SF1 and its target, *Scc*.

The failure of fetal Leydig cell differentiation provides an explanation for the feminized external genitalia phenotype of $Dhh^{-/-}$ XY mice (Clark et al. 2000) and a 46,XY partial gonad dysgenesis patient with a *Dhh* mutation (Umehara et al. 2000). Both cases developed premature female external genitalia with a blind vagina. The internal accessory sex glands and ducts, whose development depends upon the proper amount of testosterone from fetal Leydig cells, are decreased in size, and the testes were undescended. The appearance of a few Leydig cells in $Dhh^{-/-}$ gonads at later stages is not sufficient to rescue differentiation of secondary sex characteristics in $Dhh^{-/-}$ mice; however, it does suggest that other signaling pathways may partially compensate for loss of the DHH/PTCH1 signaling pathway. Alternatively, a subpopulation of Leydig cells may derive independent of DHH/PTCH1 signaling. We are conducting more experiments to explore the origin of Leydig cell precursors and the interaction between DHH/PTCH1 and other signaling pathways.

Materials and methods

Mouse strains

The generation of *Dhh*-null mice was described previously, and original breeding mice for the Curis colony were kindly provided by Dr. Andrew McMahon (Harvard University, Cambridge, MA). Mice were bred on a mixed background of *129/Sv*, *C57BL/6*, and Swiss Webster. The *Dhh* genotype was determined by polymerase chain reaction (PCR) of tail DNA. CD1 random-bred mouse strains (Charles River) were used for organ culture, immunocytochemistry, and in situ hybridization. GFP transgenic mice (Hadjantonakis et al. 1998) were used for migration studies. The $Ptch^{tm1Mps}$ mice were generated as described by Goodrich et al. (1997) and were kindly provided by Dr. Matthew Scott of Stanford University.

Organ culture

Genital ridges (gonad plus mesonephros) from 11.25–11.5 dpc embryos (0.5 dpc represents noon of the day when the vaginal plug was detected) were obtained for organ culture. To determine the sex of 11.25–12.5 dpc embryos, we used a staining method (Palmer and Burgoyne 1991) to detect the presence of XX-specific Barr bodies in the amnion of individual embryos. Genital ridges were cultured at 37°C with 5% CO_2/95% air on a 1.5% agar block for 48 h in Dulbecco's Minimal Eagle Medium (DMEM), supplemented with 10% fetal calf serum (Hyclone), and 50 μg/mL ampicillin. Cyclopamine (25 μM, TRC Biomedical Research Chemicals) was added to the culture medium to inhibit the hedgehog signaling pathway. This concentration of cyclopamine represented the minimal concentration resulting in disruption of testis cord formation as determined previously (Yao and Capel 2002). An equivalent volume of methanol (solvent for cyclopamine) was added to other organ cultures as controls.

Whole-mount in situ hybridization

Samples were fixed overnight in 4% paraformaldehyde in PBS at 4°C and processed according to the method of Henrique et al. (1995). We used alkaline phosphatase-conjugated digoxigenin-labeled RNA probes for *Dhh* and *Scc*. Two different alkaline phosphatase substrates (NBT/BCIP for *Dhh*, Fast Red for *Scc*, Boehringer Mannheim) were used for color development.

Double whole-mount in situ hybridization and immunocytochemistry

To double-label *Scc* (mRNA) and SF1 (protein) in the gonads, whole-mount in situ hybridization was performed as described above using Fast Red as the substrate for alkaline phosphate followed by immunocytochemistry against SF1. After fast red color development (~5 h at room temperature), samples were washed in PBS for 10 min and blocked in the blocking solution (10% heat-inactivated goat serum and 0.1% Triton X-100 in PBS) for 1 h at room temperature. A rabbit polyclonal antibody against SF1 (1:200) was added to the blocking solution and samples were incubated overnight at 4°C. Samples were then washed 3 times for 10 min each in washing solution (1% heat-inactivated goat serum and 0.1% Triton X-100 in PBS) followed by incubation in the blocking solution with the secondary antibody (FITC-conjugated goat anti-rabbit antibody, 1:1000, Jackson Immunochemicals). Samples were washed 3 times for 10 min each in washing solution and mounted for confocal microscopy.

Migration assay

Gonads and mesonephroi from 11.5 dpc CD1 or GFP or $Ptch^{LacZ}$ embryos were separated. A CD1 XY gonad was assembled with a GFP or a $Ptch^{LacZ}$ mesonephros and cultured on an agar block for 48 h as described (Martineau et al. 1997). Images were obtained using a Leica MZFLIII dissecting microscope with a GFP filter.

β-*gal stain*

Samples were washed in PBS and fixed in 2% paraformaldehyde for 20 min at room temperature. Samples were then rinsed in washing solution (2 mM $MgCl_2$, 0.02% Nonidet P-40 in PBS), incubated overnight at 37°C in β-gal stain (1 mg/mL X-gal, 200 mM $K_3Fe(CN)_6$, 200 mM $K_4Fe(CN)_6$), washed, and postfixed in 4% paraformaldehyde.

Assay for proliferation and apoptosis

To assay proliferation, gonad explants were fixed overnight in 4% paraformaldehyde in PBS at 4°C immediately after culture. Samples were processed and cut into 10-µm frozen serial sections as described (Karl and Capel 1998) and stained immunocytochemically for a proliferation marker, phosphorylated Histone H3 (pHH3). The primary antibody was a rabbit polyclonal antibody against pHH3 (1:1000; Upstate Biotechnology) and the secondary was an FITC-conjugated goat anti-rabbit antibody (1:500, Jackson Immunochemicals). pHH3-positive cells from 10 serial sections of each gonad ($n = 5$) were counted and subjected to statistical analysis. To assay apoptosis, gonad explants were cultured in 1 mL medium with 2 µL of LysoTracker Red DND-99 (Molecular Probes) for an additional 30 min at the end of 24-h of culture. Gonad explants were washed 3 times in PBS, fixed overnight in 4% paraformaldehyde in PBS at 4°C, and mounted for confocal imaging.

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