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 post-coitum [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 mesenchymal 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
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 Dil 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 [Vainio et al. 1999]. Expression of the hedgehog receptor, Patched 1 [Ptc1], 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, Ptc1, 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 Ptc1-expressing precursor cells located outside testis cords.

Results

Temporal and spatial expression of Dhh, Ptc1, 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, Ptc1, 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 Ptc1tm1Mps [Ptc1lacZ] XY gonads, we found that Ptc1lacZ 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]. Ptc1lacZ expression was also found around the mesonephric tubules in the anterior part of the mesonephros from 11.5 to 13.5 dpc. We compared Ptc1lacZ expression with Scc expression to determine whether Ptc1lacZ-expressing cells became Scc-positive. At 12.5 dpc, the majority of interstitial cells were Ptc1lacZ-positive and only a small population of them expressed Scc [Fig. 1, bottom panels]. Neither Ptc1lacZ 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].
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, Ptc1, 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 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; Tillmann and Capel 1999). Because most interstitial cells express Ptc1LacZ at 12.5 dpc (Fig. 1), we investigated whether Dhh signaling regulates mesonephric cell migration. Ptc1LacZ expression showed a unique pattern
During the period when mesonephric cell migration occurs. At 11.5 dpc, Ptch\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{LacZ}, except for the most posterior tip of the gonad (Fig. 1). No Ptch\textsuperscript{LacZ} expression was found in XX gonads at any stage examined (data not shown).

This unique pattern of Ptch\textsuperscript{LacZ} expression suggested that the DHH/PTCH1 signaling pathway might induce migration of Ptch\textsuperscript{1}-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\textsuperscript{LacZ} mesonephros. We reasoned that if Ptch\textsuperscript{LacZ}-expressing cells derive from the mesonephros, we should observe \(\beta\)-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\textsuperscript{LacZ} gonad apposed to a wild-type mesonephros. After culture for 50 h (corresponding to \(\sim\)12.5 dpc in vivo), samples were stained for \(\beta\)-gal. We found no \(\beta\)-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\textsuperscript{LacZ}. In the second recombinant culture with a Ptch\textsuperscript{LacZ} gonad and a wild-type mesonephros, \(\beta\)-gal staining appeared in the interstitium of all Ptch\textsuperscript{LacZ} gonads (Fig. 4C), suggesting that Ptch\textsuperscript{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\textsuperscript{+/-}, Dhh\textsuperscript{+/-}, or Dhh\textsuperscript{-/-} 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\textsuperscript{+/-} (data not shown), Dhh\textsuperscript{+/-}, and Dhh\textsuperscript{-/-} 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\textsuperscript{-/-} XY gonads compared to Dhh\textsuperscript{+/-} and Dhh\textsuperscript{+/-} 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...
maintenance or expansion of the Leydig cell population, we examined \textit{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). \textit{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 \textit{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 \textit{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).

**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 Wolffian 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. \textit{Dhh} is expressed downstream of \textit{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, \textit{Ptch1}, was known to be expressed in interstitial cell populations (Bitgood et al. 1996). Original generation of \textit{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 \textit{Ptch1} (Bitgood et al. 1996). However, transfer of the \textit{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.

\textit{Ptch1} is first expressed around the mesonephric tubules at the anterior end of the mesonephros. At 12.0 dpc, interstitial cells toward the anterior end of the gonad begin to express \textit{Ptch1} under the positive regulation of DHH. Expression of \textit{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 \textit{Dhh} appear to exert a detrimental effect on Sertoli differentiation, as MIS and \textit{Sox9} expression in \textit{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

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**Figure 5.** Stage-specific effects of the hedgehog inhibitor cyclopamine on expression of \textit{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 \textit{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.
along specific pathways. For example, it has been shown that DHH influences the differentiation of peritubular myoid cells in Ptc1-expressing cells most proximal to the DHH signal [Clark et al. 2000; Pierucci-Alves et al. 2001]. Here we show that DHH signals the Ptc1-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 Ptc1, not all Ptc1-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 et al. 1999; Strigini and McMahon 1996; Neumann and Cohen 1997; Strigini et al. 1998). 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; Charron 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 up-regulation 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 Ptc1tm1Mps 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% CO2/95% 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, Boehler Mannheim) were used for color development.
Double whole-mount in situ hybridization and immunocytochemistry

To double-label Scn [mRNA] and SFI [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 SFI. After fast red color development (1–5 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 SFI [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 Ptc1-lacZ embryos were separated. A CD1 XY gonad was assembled with a GFP or a Ptc1-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 MgCl₂, 0.02% Nonidet P-40 in PBS), incubated overnight at 37°C in β-gal stain [1 mg/mL X-gal, 200 mM K₃Fe(CN)₆, 200 mM K₄Fe(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. Gonad explants were washed 3 times in PBS, fixed overnight in 4% paraformaldehyde in PBS at 4°C, and stained immunocytochemically for a proliferation marker, phosphorylated His-tion 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, 1:1000. Jackson Immunochemicals. Samples were washed 3 times for 10 min each in washing solution and mounted for confocal microscopy.

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