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Cell proliferation is necessary for the determination of male fate in the gonad

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Abstract

Cell proliferation has been shown to have multiple functions in development and pattern formation, including roles in growth, morphogenesis, and gene expression. Previously, we determined that the earliest known morphological event downstream of the male sex determining gene, *Sry*, is the induction of proliferation. In this study, we used proliferation inhibitors to block cell division during early gonad development, at stages before the XY gonad has committed to the testis pathway. Using the expression of sex-specific genes and the formation of testis morphology as markers of testis determination, we found that proliferation within a specific 8-h window was critical for the establishment of the male pathway and the formation of the testis. Inhibition of proliferation before or after this critical period led to smaller gonads, but did not block testis formation. The critical period of proliferation coincides with the initiation of *Sry* expression and is essential for the differentiation of Sertoli cells, suggesting that proliferation is a vital component of the initiation of the male pathway by *Sry*. We believe these studies suggest that proliferation is involved not only in the elaboration of organ pattern, but also in the choice between patterns (male and female) in the bipotential gonad.

Keywords: *Sry*; Sex determination; Cell proliferation; Organogenesis; Testis; Gonad; Sertoli cell

Introduction

In embryonic development, much is known about how genes and pattern formation initiate and control cellular processes, such as cell division, migration, and differentiation. However, the reciprocal effect of these cellular processes on gene expression and pattern formation is often overlooked. Studies in a variety of systems indicate that cell division has a role in many aspects of organogenesis, not only in mediating growth and shape of organs, but also in the generation of cell diversity through asymmetric division, the timing of biological clocks, and the regulation of distances between signaling centers (Horvitz and Herskowitz, 1992; Pourquie, 1998; Tabin, 1998). It has been theorized that, for some cell types, the process of DNA replication remodels chromatin structure and allows access to previously inactive regulatory domains, thereby changing the expression patterns of specific genes in dividing cells (Holtzer et al., 1975, 1983).

In support of this theory, inhibition of cell proliferation in many systems has been shown to block differentiation of specific cell types and change the expression of some genes. Studies in the developing limb have shown that cell division and pattern formation are functionally integrated, as alterations in pattern formation affect regions of proliferation and, reciprocally, alterations in proliferation and cell cycle lengths affect pattern formation (French et al., 1976; Bryant and Gardiner, 1992; Ohsugi et al., 1997; Schaller et al., 2001). Many key molecules in the limb, such as SHH, FGFs, BMPs, and RA, are involved in both outgrowth and pattern formation and may provide links between the regulation of proliferation and limb morphogenesis. In support of these ideas, inhibition of cell proliferation in the limb by various mechanisms has been shown to cause alterations in gene expression and leads to diverse effects on limb morphology, such as supernumerary digits or the loss of proximal elements (Stephens, 1988; Ohsugi et al., 1997).

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Studies in another system, the early gonad, have suggested that proliferation and related organ growth may not only be involved in the acquisition and elaboration of organ pattern, but also in the choice between patterns (male and female) in the bipotential gonad. In the mouse, the gonad forms at 10.0 days postcoitum (dpc) as a ridge of cells on the ventromedial surface of the mesonephros. Initially, the gonad is morphologically identical in both sexes and is considered bipotential, as it can be induced to become either a testis or an ovary. Early studies in several mammalian species suggested that the choice between testis and ovarian patterns in the gonad was potentially controlled by cell proliferation and gonad growth. At equivalent gestational ages, it was observed that, on average, male embryos are more developmentally advanced than females (Scott and Holson, 1977; Pedersen, 1980; Seller and Perkins-Cole, 1987). This difference between the sexes precedes the formation of the gonad and led to the hypothesis that growth rate may influence the sex of the embryo. The observation that the early male gonad is larger than the female gonad in many vertebrates supported the view that the differential growth rate between the sexes somehow determined the sex of the gonad, such that gonads with more rapid growth rates became testes, while gonads which failed to reach a certain size by a given stage developed into ovaries (Mittwoch, 1969, 1989; Mittwoch and Buehr, 1973; Hunt and Mittwoch, 1987). However, many studies comparing gonad size between the sexes did not take into account the accelerated development of male versus female embryos. Additionally, the more rapid growth rate of male embryos is not an absolute distinction: some male embryos are smaller and develop more slowly than their female littermates, suggesting that growth rate and/or developmental acceleration are not the primary determinants of the sex of the embryo.

In the 1990s, it was discovered that sex is genetically determined in mammals by the presence or absence of a single gene on the Y chromosome, *Sry* (Gubbay et al., 1990; Lovell-Badge and Robertson, 1990). Expression of this gene is sufficient to initiate the testis pathway in XX gonads (Koopman et al., 1991; Eicher et al., 1995), thereby overriding any bias of the smaller XX gonads for the ovarian pathway. Additionally, it has been shown that the acceleration of development in postimplantation male embryos is an effect of the X chromosome constitution, not the Y chromosome, and therefore is not the primary sex-determining mechanism (Thornhill and Burgoyne, 1993; Burgoyne et al., 1995, 2001). However, as we have shown previously, one of the earliest effects of *Sry* expression is an increase in cell proliferation (Schmahl et al., 2000). A corresponding increase in the size of the male gonad is also observed within 24 h of the peak of *Sry* expression. Thus, proliferation and differential growth rates may yet have an important role in testis formation and sex determination downstream of *Sry*.

In this study, we used proliferation inhibitors both in vivo and in culture to investigate whether cell proliferation is necessary for aspects of sex determination and the development of the testis. We show that a specific 8-h period of early proliferation is necessary for the differentiation of male-specific cell types, the expression of male-specific genes, and the formation of testis morphology. This period occurs within hours of the initiation of *Sry* expression, suggesting that proliferation within this period is an early part of the mechanism by which *Sry* initiates the male pathway.

Materials and methods

Proliferation inhibition in staged prenatal gonads

Timed matings were produced by housing CD-1 female mice with males of the same strain overnight and checking for vaginal plugs. Noon on the day a vaginal plug was recorded was considered 0.5 days postcoitum (dpc). To reduce variability in the age of the embryos, most matings set up for the injection of proliferation inhibitors were set up for a short period of time (between 9 PM and 2 AM). 5-Flurouracil (5-FU; 30 mg/kg) or methotrexate (MTX; 40 mg/kg) was administered by intraperitoneal injection into pregnant females between 10.5 and 11.5 dpc, at the times indicated in Table 1. Three to five pregnant mice were injected at each time point to achieve a minimum of 15 XY embryos per time point. The litters of these injected mice were dissected at 12.5 dpc (or later as indicated), the gonads were removed from each embryo, and the presence or absence of testis cords was noted using a dissecting microscope (Leica MZFLIII). The sex of these embryos was determined by staining of X chromatin Barr bodies in the amniotic sac (Palmer and Burgoyne, 1991). An embryo that was XY by amniotic staining but had no visible testis cords was considered a cordless XY (Table 1). The percent cordless XY was calculated for each litter, and the mean cordless XY was calculated for each time point by averaging the percent cordless XY from each litter within that time point. The appearance of litters and individual embryos within each litter injected with 5-FU varied (see Results for description). Some litters had severely affected individuals, with many large or burst blood vessels, the presence of blood within the body cavity, and large, fluid-filled edemas in the head, body, and limbs. The organs of these individuals were much smaller than normal and exhibited signs of deterioration, such as many loose cells and a rough appearance. Gonads were not dissected form these embryos. Instead, these embryos were classified as severely affected, and the number of severely affected embryos was added to the number of dead or reabsorbed individuals in each litter, divided by the total number of embryos in the litter and recorded as percent lethality in Table 1.

Table 1A
Inhibition of proliferation at a specific stage blocks testis cord formation (10.5–11.0 dpc)

Time 5-FU Inj[a]	Condition of litter[b]	% lethality	Total XY	# Cord-less XY	% Cord-less XY
1–2 PM (10.5 dpc)	mild-mod	0%	4	0	0%
	mod-sev	25.0%	5	0	0%
	mod-sev	54.5%	1	0	0%
	mod-sev	78.6%	3	0	0%
	mod-sev	78.6%	2	0	0%
		47.3 ± 17.2%[c]	**15**[d]	**0**[d]	**0%**[c]
2–3 PM	mod	0%	5	0	0%
	mod	14.3%	6	0	0%
	mod-sev	20.0%	5	0	0%
	mod-sev	40.0%	3	0	0%
		18.6 ± 9.6%	**19**	**0**	**0%**
3–4 PM	mod-sev	10.0%	5	0	0%
	mod-sev	18.2%	5	0	0%
	mod-sev	25.0%	7	0	0%
		17.7 ± 5.3%	**17**	**0**	**0%**
4–5 PM	mild	0.0	7	0	0%
	mod-sev	33.3	4	0	0%
	mod-sev	44.4	3	0	0%
	mod-sev	78.6	1	0	0%
		39.1 ± 18.7%	**15**	**0**	**0%**
5–6 PM	mild	nd	7	0	0%
	mod	0%	3	0	0%
	mod-sev	17%	5	0	0%
		8.3 ± 11.8%	**15**	**0**	**0%**
6–7 PM	mild	nd	3	0	0%
	mild	9.1	6	0	0%
	mod	0.0%	7	0	0%
		4.5 ± 6.4%	**16**	**0**	**0%**
7–8 PM	mod	7.7%	4	0	0%
	mod	14.3%	3	0	0%
	mod	27.7%	3	0	0%
	mod-sev	33%	5	0	0%
		20.7 ± 6.8%	**15**	**0**	**0%**
8–9 PM	mild	nd	6	3	50.0%
	mild	0.0%	8	3	37.5%
	mod-sev	8.3%	5	2	40.0%
	mod-sev	16.7%	7	0	0.0%
		8.3 ± 5.9%	**26**	**8**	**31.9 ± 12.7%**
9–10	mild	0%	7	2	28.6%
	mild	9%	6	4	66.7%
	mod	7%	6	0	0.0%
		5.4 ± 3.4%	**19**	**6**	**31.8 ± 23.6%**
10–11 PM	mild	7.7%	5	0	0.0%
	mod	nd	4	3	75.0%
	mod-sev	16.7%	5	5	100.0%
	mod-sev	33.3%	5	5	100.0%
		19.2 ± 9.2%	**19**	**13**	**68.8 ± 27.3%**
11–12 PM (11.0 dpc)	mild	20.0%	5	2	40%
	mod	0.0%	6	0	0.0%
	mod-sev	0.0%	3	2	66.7%
	mod-sev	33.3%	1	0	0.0%
		13.3 ± 9.4%	**15**	**4**	**26.7 ± 32.6%**

[a] Injections of the proliferation inhibitor 5-FU were administered to pregnant females between 10.5 and 11.0 dpc at the times indicated.
[b] Embryos were dissected at 12.5 dpc and the condition of the litter was noted. See text for description of mild, moderate (mod), and severely (sev) affected.
[c] Mean for that time point, ± SEM.
[d] Total for that time point.

Table 1B
Inhibition of proliferation at a specific stage blocks testis cord formation (11.0–11.5 dpc)

Time 5-FU Inj[a]	Condition of litter[b]	% lethality	Total XY	# Cord-less XY	% Cord-less XY
12–1 AM (11.0 dpc)	mild	nd	4	0	0.0%
	mod	nd	8	4	50.0%
	mod	0.0%	9	1	11.1%
	mod	9.1%	6	2	33.3%
	mod-sev	15.4%	6	1	16.7%
		8.1 ± 5.5%[c]	**33**[d]	**8**[d]	**22.2 ± 9.8%**[c]
1–2 AM	mild	0.0%	9	0	0.0%
	mild	7.7%	8	1	12.5%
	mild	14.3%	5	1	20%
		7.3 ± 5.1%	**22**	**2**	**10.8 ± 7.1%**
2–3 AM	mod	0.0%	7	0	0.0%
	mod	0.0%	6	0	0.0%
	mod	16.7%	5	1	20.0%
		5.6 ± 6.8%	**18**	**1**	**6.7 ± 8.2%**
3–4 AM	mild	14.3%	7	0	0%
	mod	14.3%	5	0	0%
	mod	27.3%	4	2	50%
		18.6 ± 5.3%	**16**	**2**	**16.7 ± 20.4%**
4–5 AM	mild	0.0%	5	0	0%
	mild	7.7%	3	0	0%
	mod	0.0%	3	0	0%
	mod-sev	0.0%	5	0	0%
		1.9 ± 2.2%	**16**	**0**	**0%**
5–6 AM	mild	0.0%	5	0	0%
	mod	0.0%	8	0	0%
	mod	8.3%	7	0	0%
		2.8 ± 3.4%	**20**	**0**	**0%**
6–7 AM	mild	7.7%	7	0	0%
	mild-mod	0.0%	4	0	0%
	mild-mod	0.0%	5	0	0%
		2.6 ± 3.1%	**16**	**0**	**0%**
7–8 AM	mild	11.1%	5	0	0%
	mild-mod	0%	4	0	0%
	mod	10.0%	6	0	0%
		7.0 ± 4.3%	**11**	**0**	**0%**
8–9 AM	mild	0.0%	8	0	0%
	mild	7.1%	8	0	0%
	mild-mod	0.0%	2	0	0%
		2.4 ± 2.8%	**18**	**0**	**0%**
9–10 AM	mild	0.0%	6	0	0%
	mild-mod	10%	2	0	0%
	mod	0.0%	7	0	0%
		3.3 ± 4.1%	**15**	**0**	**0%**
10–11 AM	mild	0.0%	8	0	0%
	mild	0.0%	9	0	0%
	mild	6.7%	6	0	0%
		2.2 ± 2.7%	**23**	**0**	**0%**
11–12 AM (11.5 dpc)	mild	0.0%	4	0	0%
	mild	nd	3	0	0%
	mod	nd	3	0	0%
	nd	nd	8	0	0%
		0.0%	**18**	**0**	**0%**

[a] Injections of the proliferation inhibitor 5-FU were administered to pregnant females between 11.0 and 11.5 dpc at the times indicated.
[b] Embryos were dissected at 12.5 dpc and the condition of the litter was noted. See text for description of mild, moderate (mod), and severely (sev) affected.
[c] Mean for that time point, ± SEM.
[d] Total for that time point.

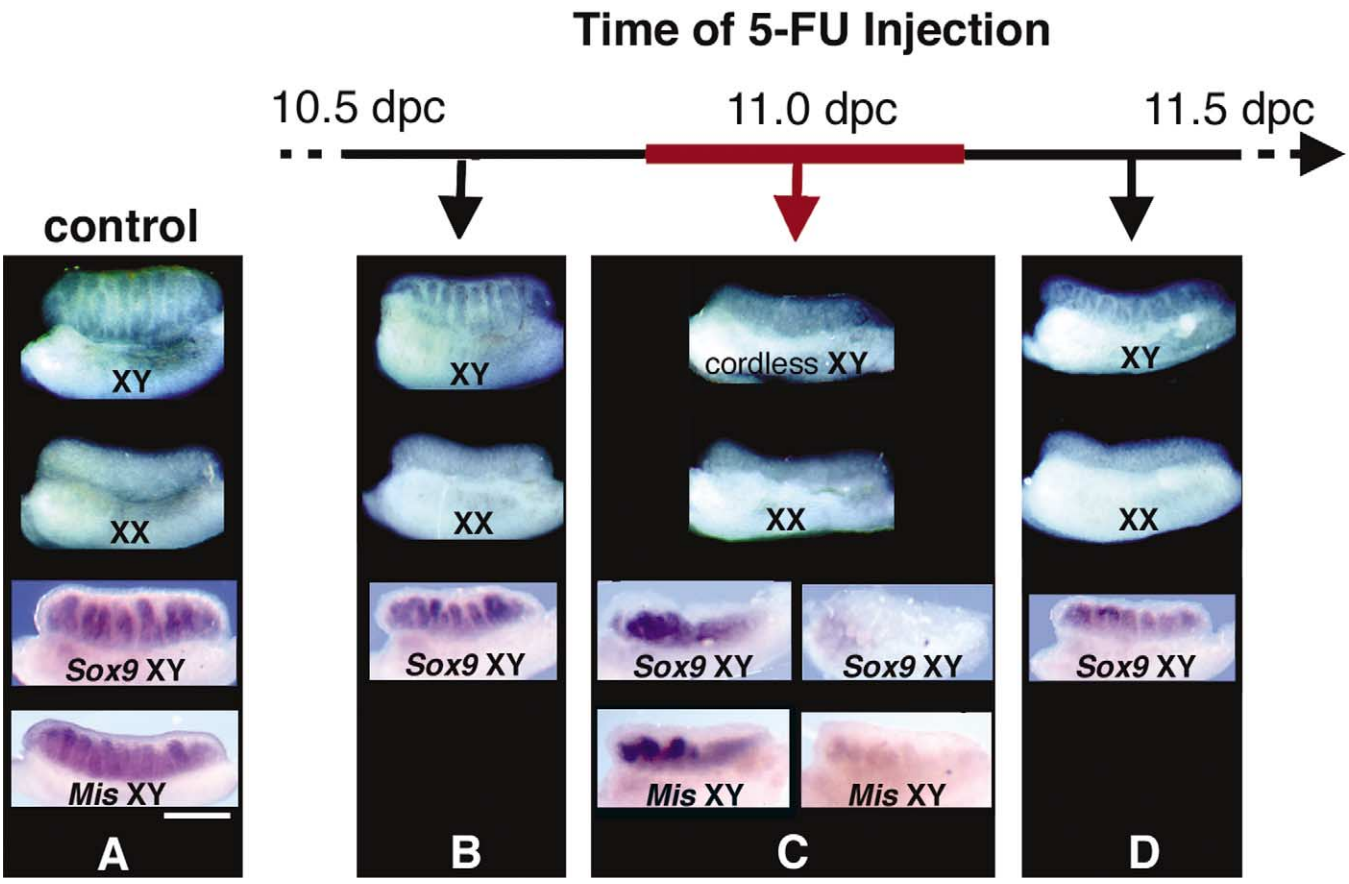
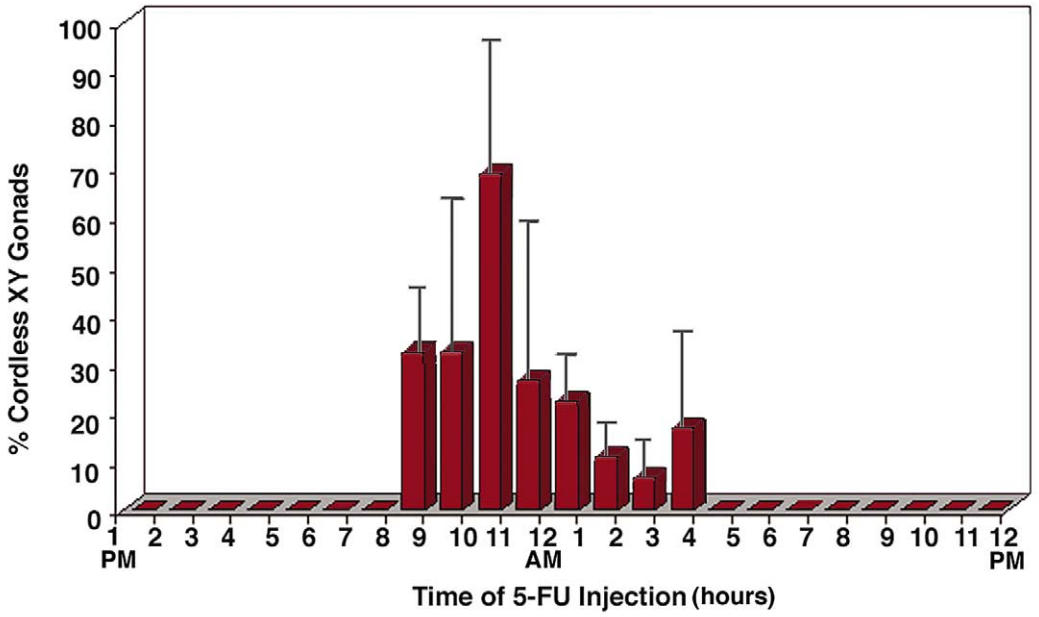


Fig. 1. Inhibition of proliferation at specific stages can block testis cord formation and alter the expression of male-specific genes. Testis cords are visible in normal XY gonads dissected at 12.5 dpc (the gonad is pictured on top of the attached mesonephros). At this stage, XY gonads express the male-specific genes *Sox*9 and *Mis* within testis cords (column A). Cords are not visible in the XX gonad. During most stages, injection of a proliferation inhibitor (5-FU) into pregnant females led to smaller gonads, but XY gonads still formed testis cords and expressed *Sox9* (XY gonads in column B, D). However, when proliferation was blocked between 10.8 and 11.2 dpc (indicated by red box on timeline), many of the XY gonads did not form cords and did not express normal levels of *Sox9* or *Mis* (column C). Scale bar indicates 250 μM.

The time course of proliferation inhibition and apoptosis in vivo

To determine the time course of proliferation inhibition, we injected pregnant mice with 30 mg/kg 5-FU at 10:30 PM on the 10th day postcoitum and dissected litters 2, 4, 14, or 24 h after injection. Gonads were removed from half of the XY embryos in these litters, and dividing cells were detected by using a 1:500 dilution of a rabbit polyclonal antibody against phospho-Histone H3 (Upstate #06-570). Germ cells and vasculature were visualized by using a 1:200 dilution of a rat IgG antibody against mouse platelet endothelial cell adhesion molecule (PECAM; Pharmagen, cat. #553370). For immunofluorescence, XY gonads from each time point were pooled, fixed in 4% paraformaldehyde overnight, rinsed three times in PBS, incubated for 1 h at room temperature in 10% goat serum, 0.01% Triton X-100 in PBS, incubated overnight at 4°C in primary antibodies diluted in 1% goat serum, 0.01% Triton X-100, and then rinsed three times in PBS. The samples were incubated in 1:1000 dilutions of the appropriate secondary antibodies overnight (rhodamine-conjugated anti-rat IgG and fluorescein-conjugated anti-rabbit from Jackson ImmunoResearch), rinsed three times in PBS, and mounted for whole-mount confocal imaging as described in Karl and Capel (1998).

To determine whether 5-FU treatment caused an increase in apoptosis in the gonad, the remaining XY embryos from the above 5-FU time course were treated with the vital lysosomal dye, LysoTracker Red (Molecular Probes, OR), using a protocol modified from Zucker et al. (1999). Briefly, 3 μl of LysoTracker Red was added to 1 ml of DMEM with 10% fetal calf serum and 5% CO_2. Embryos were incubated in this labeling media for 30 min at 37°C. After labeling, embryos were washed three times in PBS, then three times in PBS containing 0.1% Tween 20 for 30 min each wash. These samples were fixed in 4% paraformaldehyde, embedded in 20% PBS:OCT, and cryosectioned. Sections were blocked in 10% goat serum, 0.1% Triton X-100 in PBS, and incubated with primary and secondary antibodies by using the protocol described above. Laminin was detected with a rabbit polyclonal antibody generously donated by Harold Erickson.



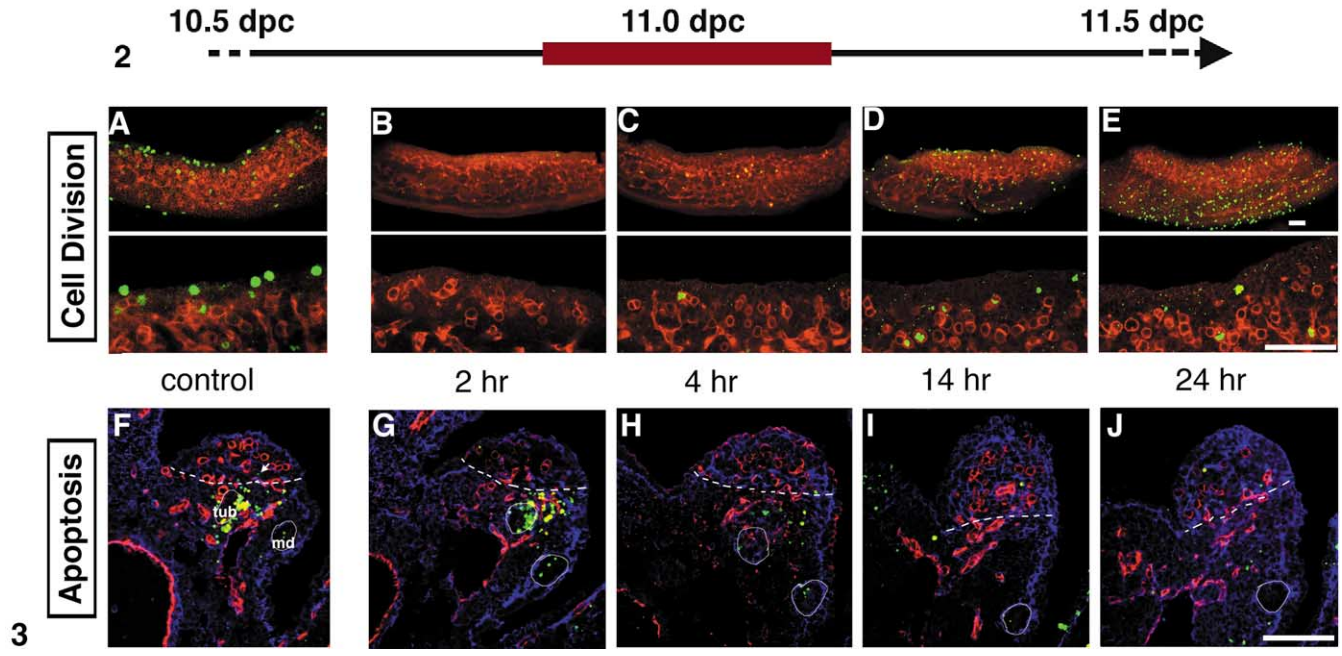


Fig. 2. Graph of the percent cordless XY gonads observed per litter versus the time of 5-FU injection. Injection of a proliferation inhibitor (5-FU) into pregnant females between 8 PM on the 10th gestational day and 4 AM on the 11th gestational day (10.8–11.2 dpc) blocked testis cord formation in a percentage of XY gonads (red bars). Gray lines indicate SEM. Injection of 5-FU before or after this period did not block cord formation.

Fig. 3. Timeline of proliferation inhibition and apoptosis in vivo. Dividing somatic cells (green in A–E) are concentrated at the surface of the male gonad between 10.5 and 11.0 dpc. Some germ cells and vascular cells (red) also divide during this period (A). 5-FU blocks most cell division in the genital ridge within 2 h of injection (B). Cell division resumes between 4 and 14 h after injection (C, D) and is observed in somatic and germ cells through at least 24 h after injection (E). In (A–E), the gonad and attached mesonephros was disected and viewed by using a 10× lens. Sagittal confocal sections of the gonad at higher magnification (40×) are shown below. Apoptosis (green in F–J) was examined in transverse sections of male embryos. In these sections, an antibody against laminin was used to visualize embryo structures (blue in F–J). Apoptotic cells are normally observed in and associated with the mesonephric duct (md) and tubules (tub) of the mesonephros (outlined in F–J). In the gonad (above the dashed line), few apoptotic cells were observed at this stage. After 5-FU injection, apoptosis does not substantially increase in the gonad and few apoptotic cells were observed even 14 (I) or 24 h (J) after injection. Scale bars indicate 100 μM.

Proliferation inhibition in culture

To block proliferation in culture, gonads were removed from embryos between 11.0 and 11.5 dpc and cultured in shallow grooves on agar blocks as previously described (Martineau et al., 1997). Affi-gel blue beads (Biorad) were absorbed in a 10-μg/ml solution of aphidicolin in PBS, or a control of PBS for 1 h at room temperature. Two to three beads were then cultured adjacent to XX or XY gonads for 36–48 h. The gonads were fixed in 4% paraformaldehyde and processed for in situ hybridization as previously described (Henrique et al., 1995). The in situ probe for *Sox9* was provided by Peter Koopman (Kent et al., 1996), the probe for *Mis* was provided by Robin Lovell-Badge, and the probe for *Dax-1* was provided by Amanda Swain (Swain et al., 1996). To determine the extent of proliferation inhibition with aphidicolin beads, gonads were cultured for 12 h as above, with affi-gel blue beads absorbed with aphidicolin or PBS, in media containing 10 μM 5′ bromo-2-deoxyuridine (BrdU). BrdU is a thymidine analog that is incorporated into the DNA of dividing cells and will label cells that pass through S-phase during this 12-h labeling period. Gonads were fixed after 12 h in 4% paraformaldehyde, and BrdU was detected with an antibody as described in Schmahl et al. (2000). To determine the affects of aphidicolin on gonad morphology, somatic cells were detected with a 1:200 dilution of an antibody against steroidogenic factor 1 (SF1), a rabbit polyclonal antibody donated by Ken-ichirou Morohashi. Germ cells and vasuclature were detected with an antibody against PECAM.

Results

Blocking proliferation at specific stages blocks cord formation

To investigate the role of proliferation in gonad organogenesis, we administered an intraperitoneal injection of 30 mg/kg of the proliferation inhibitor 5-FU to staged pregnant mice during the early, bipotential stages of gonad formation (between 10.5 and 11.5 dpc). This timeline includes stages just before the initiation of *Sry* expression, through the first signs of male morphology and differentiation. Gonads injected with proliferation inhibitor were dissected after testis morphology is normally apparent (12.5 dpc). At this stage, testis cords are visible using a light microscope and consist of groups of germ cells surrounded by a layer of Sertoli cells (male-specific cells that act as supporting cells for the germ cells), covered by a thick basal lamina. As expected, the gonads of both sexes were much smaller than controls after the injection of a proliferation inhibitor (Fig. 1), linking a size increase of both the ovary and the testis to proliferation. However, when proliferation was blocked between 8 PM on the 10th gestational day and 4 AM on the 11th gestational day, many of the XY gonads in each litter did not develop testis cords (Figs. 1C and 2; Table 1A and B). Inhibition of proliferation before or after this critical period led to smaller testes and reduced numbers of cords, but did not completely block testis cord formation.

A large amount of variation in the percentage of cordless gonads was recorded at each time point. This was probably due to variation in the exact stage of each litter and the individuals within the litter at the time of injection, as litters could only be staged based on the assumption that mating occurred at midnight on the day of the post coital plug. Most matings were set up for only 5 h (between 9 PM and 2 AM) to reduce variability in the age of the embryos; however, differences in the time of ovulation may also affect the time of conception. As the window of cordless gonads is small, even a 5-h difference in the stage of the litter could put it outside of the window of susceptibility. More accurate ways of staging the litter (such as counting tail somites) could not be done on the day of injection since the embryos were not sacrificed for several days. Proliferation inhibitors affected tail somite numbers on the day of dissection. Despite this inability to exactly stage the embryos, cordless gonads were not observed when injected before 8 PM on the 10th gestational day (Table 1A), or after 4 AM on the 11th gestational day (Table 1B), indicating that the ability of proliferation inhibitors to block cord formation is restricted to a stage within this 8-h period.

To determine the concentration of 5-FU needed to block cord formation, we tested a range of doses for each inhibitor. Previous studies in pregnant mice or rats at similar stages show a decrease in fetal weight, an accumulation of cells in S-phase, and decrease of DNA synthesis between doses of 20 and 40 mg/kg 5-FU, with malformations and percent lethality increasing as the dose increases (Skalko and Jacobs, 1978; Elstein et al., 1993; Shuey et al., 1994). We tested concentrations of 5-FU within and above this range (20, 30, 40, and 50 mg/kg). A dose of 50 mg/kg 5-FU resulted in 100% lethality by 13.5 dpc. Doses of both 30 and 40 mg/kg 5-FU blocked cord formation when injected between 8 PM on the 10th gestational day and 4 AM on the 11th gestational day. We choose to use the lower dose (30 mg/kg) in this study due to its higher survival rate and less severe effects on embryo morphology. Doses of 20 mg/kg at 8 PM and midnight on the 10th day of gestation did not affect cord formation, but a complete time course was not done for this concentration.

The block of cord formation seen in 5-FU-treated mice is due to the inhibition of cell proliferation

To verify that 5-FU blocks cell division and to determine the time course of this block, we injected 30 mg/kg 5-FU into pregnant mice at 11 PM on the 10th day postcoitum. Litters were dissected 2, 4, 14, or 24 h after injection, and dividing cells were detected by using an antibody against the mitotic marker phospho-Histone H3 (Hendzel et al., 1997). In controls during this period, this antibody detects a

population of dividing cells at the surface of the male gonad (Fig. 3A). Within 2 h of 5-FU injection, a loss of cell proliferation was observed in the gonad and the adjacent mesonephros (Fig. 3B). This inhibition of proliferation continued for at least 4 h after injection. Between 4 and 14 h after injection, cell proliferation resumed in the embryo, and dividing cells (both somatic and germ cells) were once again observed in the gonad (Fig. 3C–E).

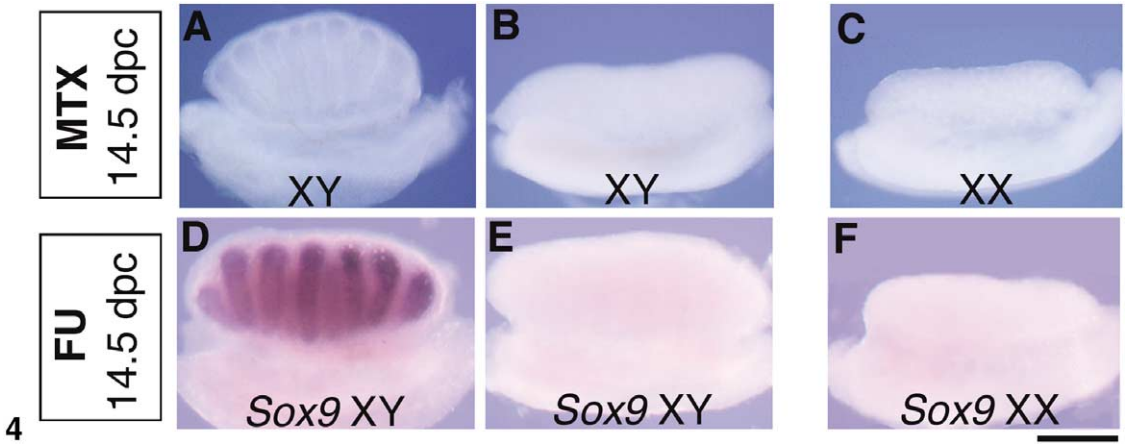
5-FU can induce either cell cycle arrest or apoptosis in dividing cells. This apoptosis occurs via a process known as "thymineless death" and is generally observed within 12–24 h after 5-FU treatment. Studies have shown that the sensitivity to thymineless death varies in different cell types and tissues and is dependent on many factors, including the cell cycle stage, bcl/bax ratio, microenvironment, and quantity of 5-FU administered (Houghton and Houghton, 1996; Mirjolet et al., 2000; Inomata et al., 2002). To determine whether 5-FU induces apoptosis in the gonad, we used the vital lysosomal dye, LysoTracker Red. In control embryos not injected with 5-FU, LysoTracker Red detected apoptosis in and associated with the mesonephric tubules (tub) and duct (md; outlined in Fig. 3F–J). This pattern of apoptosis is consistent with the known degradation and remodeling of these structures during this period, which eventually leads to the formation of the epididymus in the male (Smith and Mckay, 1991). In the gonads of control embryos, a few (1–3) apoptotic cells were observed per section (arrow in Fig. 3F). These cells were often, but not always, found near the boundary of the gonad and mesonephros (dashed line). Following 5-FU injection, LysoTracker Red detected an increase in the number of apoptotic cells in other tissues, particularly in and around the somites, and limb buds 14 and 24 h after injection (data not shown). However, in the mesonephros, the number of apoptotic cells actually appeared to decrease overtime (Fig. 3G–J). This was presumably due to the normal progressive loss of the tubules from all but the caudal portions of the mesonephros during this phase in development. In the gonad, few apoptotic cells were observed even up to 14 or 24 h after 5-FU injection, with roughly one to three labeled cells observed per section. As in control gonads, these cells were frequently associated with the mesonephric boundary, and no concentrations of apoptotic cells or areas of intense staining were observed in the gonad itself, indicating that this concentration of 5-FU does not induce apoptosis in this tissue at this time.

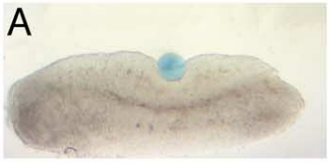
5-FU is a pyrimidine analogue that is known to inhibit thymidylate synthase, a key enzyme in the production of dTTP (Santi et al., 1974; Christopherson and Lyons, 1990). This results in the inhibition of DNA synthesis. However, 5-FU has been shown to have effects other than the inhibition of DNA replication. Problems in pre-mRNA synthesis, splicing, and translation have been observed in 5-FU-treated cells, possibly due to the incorporation of 5-FU into pre-mRNA or a more direct inactivation of components of splicing machinery (Heidelberger et al., 1983; Ghoshal and Jacob, 1997). To verify that the block of cord formation seen in 5-FU-treated mice was due to the inhibition of proliferation and not other effects of 5-FU, we injected mice with another proliferation inhibitor, methotrexate (MTX). MTX also blocks cell division through the decrease of dTTP production. However, MTX is a folic acid analog and a potent inhibitor of a different enzyme in the thymidylate synthesis pathway (dihydrofolate reductase; reviewed in Genestier et al., 2000). The cytotoxic and differentiating effects of MTX can be prevented by the addition of thymidine (Burres and Cass, 1987; Schwartz et al., 1992; O'Neill, 1998), suggesting that the effects of MTX are specific to the inhibition of de novo thymidylate synthesis and DNA replication. Previous studies indicated that doses of MTX between 20 and 50 mg/kg were likely to affect proliferation in the embryo (Skalko and Gold, 1974; Darab et al., 1987). We tested doses within and above this range (20, 30, 40, and 60 mg/kg). Doses of 20 and 30 mg/kg resulted in no visible effects on embryo development by 14.5 dpc and did not appear to affect cord formation. At doses of 40 and 60 mg/kg MTX, some malformations of the embryo were observed and cord formation was inhibited within the sensitive period in some gonads. (Fig. 4A and B), supporting the theory that the loss of testis cords is due to the inhibition of cell proliferation and the loss of dividing cells in the early gonad.

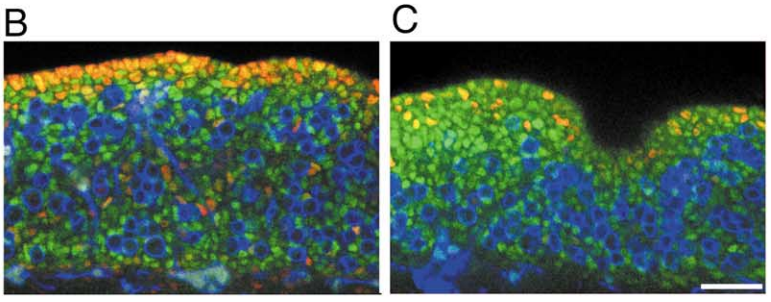
The ability of the proliferation inhibitor to block cord formation did not correspond to the severity of the malformations induced by the inhibition

Litters (and individual embryos within each litter) exposed to proliferation inhibitors varied in their general appearance. Some litters showed very few ill effects, beyond a shortening of the limbs and tail. These litters generally had low lethality (ranging from 0 to 14.3%) and were classified as mild in Table 1. Many litters showed more characteristics of proliferation inhibition, such as a much shorter or kinked tail, cranial/facial abnormalities, small edemas in the midbrain, and a few swollen or burst blood vessels. These litters exhibited higher lethality (0– 33.3%) and were classified as moderately affected (mod) in Table 1. Litters that had a mix of appearances between moderate and more severely affected were also observed and classified as mod-sev in Table 1. (Gonads from the most severely affected individuals were eliminated from the study, see materials and methods for description.) Mod-sev litters had a higher range of lethality (0–78.6%).

Litters with mildly affected appearances were more likely to result from 5-FU injections at later time points, while mod-sev litters generally, but not exclusively, resulted from 5-FU injections during the first 12-h range of this study (10.5–11.0 dpc). This difference in the severity of the general effects of 5-FU injection could be due to the difference in the time that these litters were allowed to develop after injection. (For example, litters injected at noon on 10.5 dpc developed for 48 h before dissection at 12.5 dpc, while







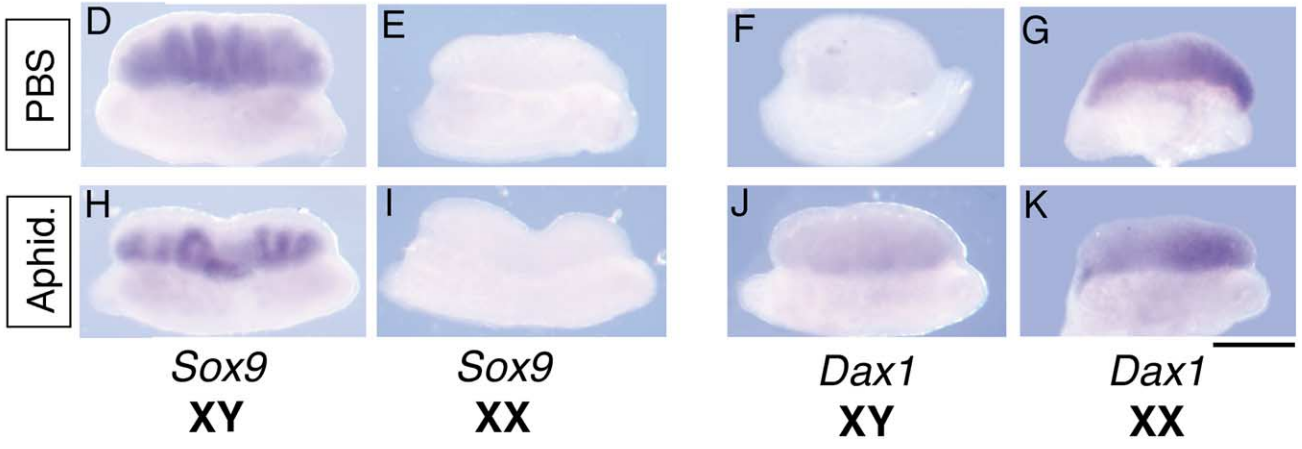


Fig. 4. Cordless XY gonads are observed at later stages. The proliferation inhibitors 5-FU and MTX were injected into pregnant mice during the sensitive period (8 PM and 10 PM, respectively), then dissected at 14.5 dpc. As observed in litters dissected at 12.5 dpc, both XY gonads with cords (A, D) and XY cordless gonads (B, E) were observed at 14.5 dpc. Cords were detected by using light microscopy (A–C) and *Sox9* expression (D–F). Scale bar indicates 250 μM.

Fig. 5. Inhibiting proliferation in culture alters the expression of sex-specific genes. Gonads were dissected between 11.0 and 11.5 dpc and cultured 36 h with beads treated with PBS or the proliferation inhibitor, aphidicolin (gonad with bead shown in A). Using BrdU to label all dividing cells within a 12-h period, we observed that proliferation was reduced under the aphidicolin-treated bead and in the gonad as a whole (dividing cells are red in B and C). Most somatic cells were detected with an antibody against SF1 (green), and germ cells and vasculature were detected with an antibody against PECAM (blue). Using in situ hybridization to determine the expression of sex-specific genes in XY gonads, it was observed that aphidicolin-treated beads reduced the expression of the male-specific gene *Sox9* expression under the area of the bead (H) and induced or maintained expression of the female-specific gene, *Dax1* (J). In XX gonads, aphidicolin did not affect the expression of *Sox9* (I) or *Dax1* (K). The black scale bar indicates 250 μM in D–K.

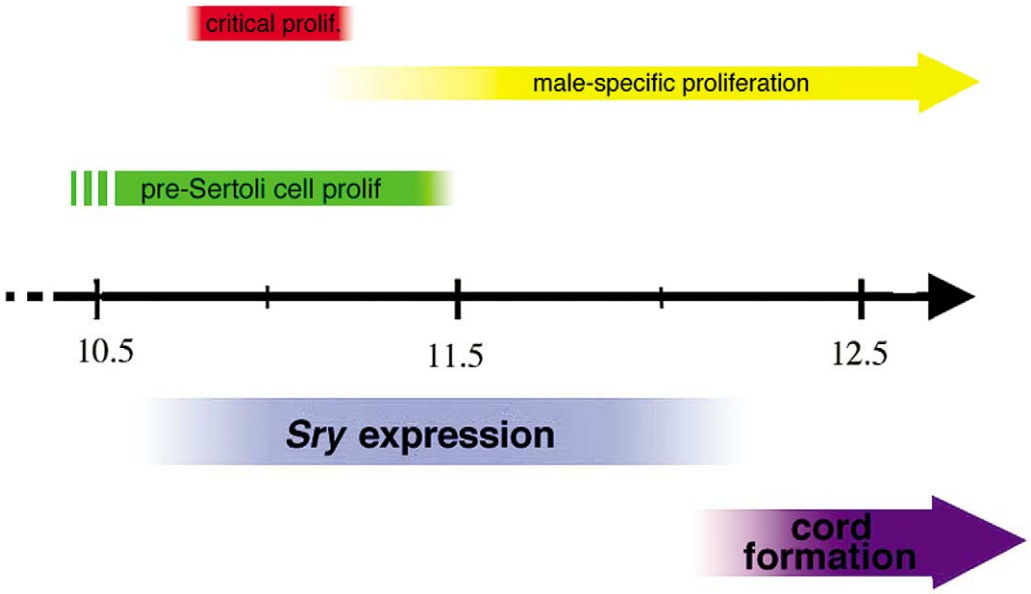


Fig. 6. Stages of testis development. *Sry* (light blue) is expressed between roughly 10.5 and 12.4 dpc (Hacker et al., 1995). Testis cords (purple) become apparent between 12.0 and 12.5 dpc. BrdU pulse-chase studies have indicated that pre-Sertoli cells (light green) proliferate between 10.5 and 11.5 dpc (Schmahl et al., 2000). Pre-Sertoli cells cease proliferation after 11.5 dpc. Sertoli cell proliferation resumes after 12.0, when dividing Sertoli cells are observed within the nascent cords. Blocking experiments in this study have identified a stage of proliferation (red) that appears to be critical for testis cord formation and Sertoli differentiation. This stage is between 10.8 and 11.2 dpc, just before BrdU labeling detects the beginnings of a wave of male-specific proliferation (yellow), and is coincident with the early proliferation of pre-Sertoli cells.

litters injected at noon on 11.5 dpc developed for only 24 h before dissection at 12.5 dpc.) However, litters injected during the last 12 h (11.0–11.5 dpc) and dissected 48 or even 72 h later (at 13.5 and 14.5 dpc) still showed a mild appearance. This suggests that the difference in the general effects of the proliferation inhibitors is linked to the sensitivity of the stage of the litter, not the length of the interval between injection and dissection, an effect well documented with teratogens during pregnancy (Persaud et al., 1985). Cordless XY gonads were observed in all classifications of litters (mild, mod, and mod-sev; see Table 1A and B), and a Chi-squared test indicated that there was no significant difference between any of the classifications in the percent of cordless XY gonads during the sensitive period ($\chi^2 = 1.53$, $P > 0.45$). Thus, the ability of the proliferation inhibitor to block cord formation was not dependent on the severity of the general effects of the proliferation inhibitor.

The expression of male-specific genes is altered when cord formation is blocked by proliferation inhibitors

The expression of male-specific genes was investigated in gonads treated with proliferation inhibitors. Under normal conditions, *Sox9* and *Mis* have sex-specific expression patterns at 12.5 dpc and are detected in XY gonads, not XX gonads. At this stage, *Sox9* and *Mis* are expressed within Sertoli cells in the testis cords and are considered hallmarks of Sertoli differentiation. In XY gonads that appeared cordless after exposure to proliferation inhibitors, the expression of both *Sox9* and *Mis* was abnormally localized, reduced, or even absent (Fig. 1C), indicating that Sertoli differentiation is also disrupted in these gonads. Like the block of cord formation, this effect on gene expression was stage-dependent. Inhibition of proliferation outside the critical period (before 8 PM or after 4 AM) did not affect the expression pattern of these genes. Interestingly, when *Sox9* and *Mis* were detected in cordless XY gonads, they were invariably detected most strongly at the anterior of the gonad.

To determine whether the block of cord formation and male-specific gene expression seen in cordless XY gonads at 12.5 dpc is due to a permanent alteration of the male pathway, or merely a delay in testis formation, we examined gonads from litters dissected at a latter stage. At 14.5 dpc, 2 days after cord formation is normally visible by light microscopy, litters injected with 5-FU or MTX during the critical period still showed both corded (Fig. 4A and D) and cordless (Fig. 4B and E) XY gonads. *Sox9* expression was absent in cordless XY gonads at this stage as well (Fig. 4E), indicating that injection of proliferation inhibitors at earlier stages produces a long-lasting block of cord formation and Sertoli differentiation.

We also used aphidicolin-treated beads to inhibit proliferation in cultured gonads. Using BrdU to label proliferating cells over a 12-h period, it was observed that aphidicolin-treated beads blocked proliferation under the bead (see Fig. 5B and C). In cultured XY gonads, *Sox9* expression is normally associated with areas of cord formation, and this expression was not disrupted by culture with control beads (Fig. 5D). However, when XY gonads were cultured with aphidicolin-treated beads, the extent of *Sox9* expression was

decreased in the gonad as a whole, and nearly eliminated from the area under the bead (Fig. 5H), suggesting that proliferation inhibition blocked the differentiation of Sertoli cells in this region of the gonad.

Another gene with a sex-specific expression pattern, *Dax1,* is expressed in both XX and XY gonads before sex determination, but is downregulated in XY gonads by 12.5 dpc, and its expression becomes specific to XX gonads (Swain et al., 1998). *Dax1* was not observed in XY gonads cultured with control beads (Fig. 5F). However, when XY gonads were cultured with beads soaked in aphidicolin, *Dax1* expression was observed (Fig. 5J), indicating that the reduction of proliferation induced or maintained the expression of this female-specific gene in the XY gonad. Interestingly, *Dax1* was not upregulated specifically in the area under the bead, and its expression was more uniform than *Sox9* repression. Using BrdU labeling, it was observed that aphidicolin-treated beads decreased proliferation in the gonad as a whole, not just under the bead (Fig. 5B and C). Thus, the expression of *Dax1* in the XY gonad may be an effect of an overall decrease of proliferation in the gonad. Aphidicolin beads had no effect on the expression of *Sox9* (Fig. 5E and I) or *Dax1* (Fig. 5G and K) in cultured XX gonads.

Discussion

The mammalian sex-determining gene, *Sry*, is expressed in the XY gonad during a very short period of time (less than 48 h in the mouse; Hacker et al., 1995), yet during this period, it initiates a cascade of events that divert the development of the early gonad from the ovarian to the testis pathway. One of the first morphological changes initiated by *Sry* is an increase in the size of the XY gonad. This increase in size is due at least in part to an increase in cell proliferation in the XY gonad that is not observed in the XX gonad. This male-specific increase in proliferation is observed before any other morphological changes in the testis pathway (such as the formation of testis cords) and occurs in at least two stages, with pre-Sertoli cells (the cell type that expresses *Sry*) proliferating during the first stage, but not the second (Karl and Capel, 1998; Schmahl et al., 2000). Using XX/*Sry* transgenic mice, it has been shown that the proliferation increase is dependent on *Sry* and independent of other genetic differences between the sexes (such as X chromosome dosage, or other genes on the Y chromosome).

A specific stage of proliferation is necessary for the establishment of the testis pathway

In this study, we used proliferation inhibitors both in vivo and in culture to investigate the function of proliferation in the establishment of the testis pathway. We have shown that inhibition of proliferation during a specific 8-h period of gonad development blocked the formation of testis cords, and reduced or blocked the expression of male-specific genes in XY gonads. Proliferation inhibition also increased or maintained the expression of a female-specific gene (*Dax1*) in the male, yet did not affect *Dax1* expression in the female. Thus, proliferation is not only necessary for elements of the male pathway, but may also be involved in the inhibition of the female pathway in the XY gonad. Inhibiting proliferation before or after this 8-h period did not block cord formation or male-specific gene expression, indicating that only this specific stage of proliferation is essential for aspects of male pattern formation. The critical period of proliferation corresponds to roughly 10.8 through 11.2 dpc (Fig. 6) and coincides with the initiation of *Sry* expression, which is first detected in the gonad between 10.5 and 10.8 dpc (Hacker et al., 1995; Bullejos and Koopman, 2001), suggesting that proliferation within this period is an early part of the initiation of the male pathway by *Sry*.

Based on expression of Sertoli cell markers *Sox9* and *Mis*, proliferation inhibition during this window either decreased or blocked differentiation of Sertoli cells, implying that proliferation within this critical period occurs in Sertoli precursors. This critical period occurs slightly earlier than a male-specific increase in proliferation is detected at the coelomic surface of the XY gonad (between 11.2 and 11.8 dpc; Schmahl et al., 2000). This timing suggests that detectable proliferation differences between XY and XX gonads may be secondary to earlier stages of proliferation in the XY gonad. Despite the fact that proliferation rates are not noticeably different in males and females before 11.2 dpc, BrdU pulse chase studies demonstrate that precursors of Sertoli cells proliferate in the male throughout this stage (Fig. 4; Schmahl et al., 2000). Also, the earliest signs of Sertoli differentiation, such as cell hypertrophy and the upregulation of *Sox9*, occur by 11.0 dpc (Magre et al., 1980; Bullejos and Koopman, 2001), suggesting that the establishment of this lineage begins earlier, during the critical stage of proliferation.

How does cell proliferation act to establish the male pathway?

It is not yet clear how proliferation is involved in the establishment of the male pathway. Much evidence argues against Mittwoch's original idea (Mittwoch, 1969) that an increase in size alone determines maleness, but the importance of proliferation in the male pathway suggests that we should consider a number of possibilities that link proliferation with the molecular control of Sertoli cell specification or lineage expansion downstream of *Sry*.

Although *Sry* contains an HMG-type DNA binding domain, its DNA targets are still unknown. There is evidence that HMG-type DNA binding domains may be involved in establishing chromatin domains (Ferrari et al., 1992; Suda et al., 1996). It has been suggested that a mitotic cell division is required to reset chromatin domain architecture (Holtzer et al., 1975, 1983), providing a possible link between the

function of *Sry* and cell proliferation. In *Drosophila*, the link between cell division and gene expression is dependent on the *polycomb group* genes, which are responsible for local compaction of chromatin domains at specific sites (Weigmann and Lehner, 1995). Interestingly, deletion of a mouse homologue of these genes (M33) results in decreased gonadal growth and male-to-female sex reversal (Katoh-Fukui et al., 1998), suggesting a role for both chromatin domains and proliferation in the determination of the male pathway.

In this study, inhibition of proliferation during the critical period eliminated Sertoli cells, a cell type known to have critical roles in testis development. At early stages, Sertoli cell precursors express *Sry*, which appears to act through this cell type to divert the gonad from the ovarian to the testis pathway (reviewed in Burgoyne and Palmer, 1993; Albrecht and Eicher, 2001). Asymmetric division has been shown to control fate specification in a number of systems (Horvitz and Herskowitz, 1992). It is possible that cell division in the early gonad is required to allocate cellular components asymmetrically in the process of Sertoli lineage specification in the XY gonad. Thus, blocking this division could prevent the differentiation of Sertoli cells. Another function of cell proliferation during organogenesis is to increase the number of precursors for terminally differentiated cell types. Multiple lines of evidence indicate that a minimum number (threshold) of *Sry*-expressing pre-Sertoli cells is necessary to drive sex determination: (1) Analysis of XX-XY chimeras in mice and humans indicate that, if more than 30% of the cells of the gonad are XY, XY cells become Sertoli cells and testis development proceeds; whereas if less than 20% of the cells are XY, ovary differentiation is initiated (reviewed in Burgoyne and Palmer, 1993). (2) In humans, the translocation of *Sry* onto a preferentially inactivated X chromosome is expected to result in fewer cells that are capable of expressing *Sry* (and forming Sertoli cells). These individuals do not form testes, but instead develop ovaries or gonads with mixed ovarian and testis tissue, despite the presence of a functional *Sry* gene (Fechner et al., 1994; Kusz et al., 1999). (3) An increase in the proliferation of a population that contains Sertoli cell precursors is downstream of *Sry* and is one of the earliest morphological differences between the sexes (Schmahl et al., 2000). In the context of previous work, our data suggests that an early function of proliferation in the XY gonad may be to increase the number of Sertoli cell precursors to a number above the threshold necessary to direct testis determination. The present results don't distinguish between these possible roles of cell proliferation in the gonad, but strongly indicate that proliferation is a necessary part of the male pathway.

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References

Albrecht, K.H., Eicher, E.M., 2001. Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. Dev. Biol. 240, 92–107.

Bryant, S.V., Gardiner, D.M., 1992. Retinoic acid, local cell–cell interactions, and pattern formation in vertebrate limbs. Dev. Biol. 152, 1–25.

Bullejos, M., Koopman, P., 2001. Spatially dynamic expression of *Sry* in mouse genital ridges. Dev. Dyn. 221, 201–205.

Burgoyne, P.S., Lovell-Badge, R., Rattigan, A., 2001. Evidence that the testis determination pathway interacts with a non-dosage compensated, X-linked gene. Int. J. Dev. Biol. 45, 509–512.

Burgoyne, P., Palmer, S., 1993. Cellular basis of sex determination and sex reversal in mammals, in: Hiller, S.G. (Ed.), Gonadal Development and Function. Raven Press, New York, pp. 17–29.

Burgoyne, P.S., Thornhill, A.R., Boudrean, S.K., Darling, S.M., Bishop, C.E., Evans, E.P., 1995. The genetic basis of XX-XY differences present before gonadal sex differentiation in the mouse. Philos. Trans. R. Soc. Lond. B Biol. Sci. 350, 253–260, discussion 260–261.

Burres, N.S., Cass, C.E., 1987. Inhibition of methotrexate-induced differentiation of cultured human choriocarcinoma (BeWo) cells by thymidine. Cancer Res. 47, 5059–5064.

Christopherson, R.I., Lyons, S.D., 1990. Potent inhibitors of de novo pyrimidine and purine biosynthesis as chemotherapeutic agents. Med. Res. Rev. 10, 505–548.

Darab, D.J., Minkoff, R., Sciote, J., Sulik, K.K., 1987. Pathogenesis of median facial clefts in mice treated with methotrexate. Teratology 36, 77–86.

Eicher, E.M., Shown, E.P., Washburn, L.L., 1995. Sex reversal in C57BL/6J-Y^{POS} mice corrected by a *Sry* transgene. Philos. Trans. R. Soc. Lond. B Biol. Sci. 350, 263–269.

Elstein, K.H., Zucker, R.M., Andrews, J.E., Ebron-McCoy, M., Shuey, D.L., Rogers, J.M., 1993. Effects of developmental stage and tissue type on embryo/fetal DNA distributions and 5-fluorouracil-induced cell-cycle perturbations. Teratology 48, 355–363.

Fechner, P.Y., Rosenberg, C., Stetten, G., Cargile, C.B., Pearson, P.L., Smith, K.D., Migeon, C.J., Berkovitz, G.D., 1994. Nonrandom inactivation of the Y-bearing X chromosome in a 46, XX individual: evidence for the etiology of 46, XX true hermaphroditism. Cytogenet. Cell Genet. 66, 22–26.

Ferrari, S., Harley, V.R., Pontiggia, A., Goodfellow, P.N., Lovell-Badge, R., Bianchi, M.E., 1992. *SRY*, like HMG1, recognizes sharp angles in DNA. EMBO J. 11, 4497–4506.

French, V., Bryant, P.J., Bryant, S.V., 1976. Pattern regulation in epimorphic fields. Science 193, 969–981.

Genestier, L., Paillot, R., Quemeneur, L., Izeradjene, K., Revillard, J.P., 2000. Mechanisms of action of methotrexate. Immunopharmacology 47, 247–257.

Ghoshal, K., Jacob, S.T., 1997. An alternative molecular mechanism of action of 5-fluorouracil, a potent anticancer drug. Biochem. Pharmacol. 53, 1569–1575.

Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P., Lovell-Badge, R., 1990. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature 346, 245–250.

Hacker, A., Capel, B., Goodfellow, P., Lovell-Badge, R., 1995. Expression of *Sry*, the mouse sex determining gene. Development 121, 1603–1614.

Heidelberger, C., Danenberg, P.V., Moran, R.G., 1983. Fluorinated pyrimidines and their nucleosides. Adv. Enzymol. Relat. Areas Mol. Biol. 54, 58–119.

Hendzel, M.J., Wei, Y., Macini, M.A., Van Hooser, A., Ranalli, T., Brinkley, B.R., Bazett Jones, D.P., Allis, C.D., 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106, 348–360.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., Ish-Horowicz, D., 1995. Expression of a delta homologue in prospective neurons in the chick. Nature 375, 787–790.

Holtzer, H., Biehl, J., Antin, P., Tokunaka, S., Sasse, J., Pacifici, M., Holtzer, S., 1983. Quantal and proliferative cell cycles: how lineages generate cell diversity and maintain fidelity. Prog. Clin. Biol. Res. 134, 213–227.

Holtzer, H., Rubinstein, N., Fellini, S., Yeoh, G., Chi, J., Birnbaum, J., Okayama, M., 1975. Lineages, quantal cell cycles, and the generation of cell diversity. Q. Rev. Biophys. 8, 523–557.

Houghton, J.A., Houghton, P.J., 1996. Cellular responses to antimetabolite anticancer agents: cytostasis versus cytotoxicity. Prog. Cell Cycle Res. 2, 175–185.

Horvitz, R.H., Herskowitz, I., 1992. Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. Cell 68, 237–255.

Hunt, S.E., Mittwoch, U., 1987. Y-chromosomal and other factors in the development of testis size in mice. Genet. Res. 50, 205–211.

Inomata, A., Hori, I., Suzuke, K., 2002. 5-Flurouracil-induced intestinal toxicity: what determines the severity of damage to murine intestinal crypt epithelia? Tox. Lett. 133, 231–240.

Karl, J., Capel, B., 1998. Sertoli cells of the mouse testis originate from the coelomic epithelium. Dev. Biol. 203, 323–333.

Katoh-Fukui, Y., Tsuchiya, R., Shiroishi, T., Nakahara, Y., Hashimoto, N., Noguchi, K., Higashinakagawa, T., 1998. Male to female sex reversal in M33 mutant mice. Nature 393, 688–692.

Kent, J., Wheatley, S.C., Andrews, J.E., Sinclair, A.H., Koopman, P., 1996. A male-specific role for *SOX9* in vertebrate sex determination. Development 122, 2813–2822.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., Lovell-Badge, R., 1991. Male development of chromosomally female mice transgenic for *Sry*. Nature 351, 117–121.

Kusz, K., Kotecki, M., Wojda, A., Szarras-Czapnik, M., Latos-Bielenska, A., Warenik-Szymankiewicz, A., Ruszczynska-Wolska, A., Jaruzelska, J., 1999. Incomplete masculinisation of XX subjects carrying the *SRY* gene on an inactive X chromosome. J. Med. Genet. 36, 452–456.

Lovell-Badge, R., Robertson, E., 1990. XY female mice resulting from a heritable mutation in the murine primary testis determining gene, *Tdy*. Development 109, 635–646.

Magre, S., Agelopoulou, R., Jost, A., 1980. Sertoli cells and organogenesis of the fetal testi. Ann. Endocrinol. (Paris) 41, 531–537.

Martineau, J., Nordqvist, K., Tilmann, C., Lovell-Badge, R., Capel, B., 1997. Male-specific cell migration into the developing gonad. Curr. Biol. 7, 958–968.

Mirjolet, J.F., Barberi-Heyob, M.C., Didelot, M., Peyrat, J.P., Abecassis, J., Millon, R., Merlin, J.L., 2000. Bcl-2/Bax protein ratio predicts 5-fluorouracil sensitivity independently of p53 status. Br. J. Cancer 83, 1380–1386.

Mittwoch, U., 1969. Do genes determine sex? Nature 221, 446–448.

Mittwoch, U., 1989. Sex differentiation in mammals and tempo of growth: probabilities vs. switches. J. Theor. Biol. 137, 445–455.

Mittwoch, U., Buehr, M., 1973. Gonadal growth in embryos of sex reversed mice. Differentiation 1, 291–224.

O'Neill, C., 1998. Endogenous folic acid is essential for normal development of preimplantation embyros. Hum. Reprod. 13, 1312–1316.

Ohsugi, K., Gardiner, D.M., Bryant, S.V., 1997. Cell cycle length affects gene expression and pattern formation in limbs. Dev. Biol. 189, 13–21.

Palmer, S.J., Burgoyne, P.S., 1991. The *Mus musculus domesticus Tdy* allele acts later than the *Mus musculus musculus Tdy* allele: a basis for XY sex reversal in C57B1/6-YPOSmice. Development 113, 709–714.

Pedersen, J.F., 1980. Ultrasound evidence of sexual difference in fetal size in first trimester. Br. Med. J. 281, 1253.

Persaud, T., Chudley, A., Skalko, R. (Eds.), 1985. Basic Concepts in Teratology. Liss, New York.

Pourquie, O., 1998. Clocks regulating developmental processes. Curr. Opin. Neurobiol. 8, 665–670.

Santi, D.V., McHenry, C.S., Sommer, H., 1974. Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. Biochemistry 13, 471–481.

Schaller, S.A., Li, S., Ngo-Muller, V., Han, M.J., Omi, M., Anderson, R., Muneoka, K., 2001. Cell biology of limb patterning. Int. Rev. Cytol. 203, 483–517.

Schmahl, J., Eicher, E.M., Washburn, L.L., Capel, B., 2000. *Sry* induces cell proliferation in the mouse gonad. Development 127, 65–73.

Schwartz, P.M., Barnett, S.K., Atillasoy, E.S., Milstone, L.M., 1992. Methotrexate induces differentiation of human keratinocytes. Proc. Natl. Acad. Sci. USA 89, 594–598.

Scott, W.J., Holson, J.F., 1977. Weight differences in rat embryos prior to sexual differentiation. J. Embryol. Exp. Morphol. 40, 259–263.

Seller, M.J., Perkins-Cole, K.J., 1987. Sex difference in mouse embryonic development at neurulation. J. Reprod. Fertil. 79, 159–161.

Shuey, D.L., Buckalew, A.R., Wilke, T.S., Rogers, J.M., Abbott, B.D., 1994. Early events following maternal exposure to 5-fluorouracil lead to dysmorphology in cultured embryonic tissues. Teratology 50, 379–386.

Skalko, R.G., Jacobs, D.M., 1978. The effect of 5-fluorouracil on [3H]nucleoside incorporation into the DNA of mouse embryos and maternal tissues. Exp. Mol. Pathol. 29, 303–315.

Smith, C., Mackay, S., 1991. Morphological development and fate of the mouse mesonephros. J. Anat. 174, 171–184.

Stephens, T.D., 1988. Proposed mechanisms of action in thalidomide embryopathy. Teratology 38, 229–239.

Suda, T., Mishima, Y., Takayanagi, K., Asakura, H., Odani, S., Kominami, R., 1996. A novel activity of HMG domains: promotion of the triple-stranded complex formation between DNA containing (GGA/TCC)11 and d(GGA)11 oligonucleotides. Nucleic Acids Res. 24, 4733–4740.

Swain, A., Narvaez, S., Burgoyne, P., Camerino, G., Lovellbadge, R., 1998. DAX1 antagonizes SRY action in mammalian sex determination. Nature 391, 761–767.

Swain, A., Zanaria, E., Hacker, A., Lovell-Badge, R., Camerino, G., 1996. Mouse *Dax1* expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. Nat. Genet. 12, 404–409.

Tabin, C.J., 1998. A developmental model for thalidomide defects. Nature 396, 322–323.

Thornhill, A., Burgoyne, P., 1993. A paternally imprinted X chromosome retards the development of the early mouse embryo. Int. Ann. Indexes 118, 171–174.

Weigmann, K., Lehner, C.F., 1995. Cell fate specification by even-skipped expression in the Drosophila nervous system is coupled to cell cycle progression. Development 121, 3713–3721.

Zucker, R.M., Hunter 3rd, E.S., Rogers, J.M., 1999. Apoptosis and morphology in mouse embryos by confocal laser scanning microscopy. Methods 18, 473–480.