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A critical time window of *Sry* action in gonadal sex determination in mice

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In mammals, the Y-linked sex-determining gene *Sry* cell-autonomously promotes Sertoli cell differentiation from bipotential supporting cell precursors through SRY-box containing gene 9 (*Sox9*), leading to testis formation. Without *Sry* action, the supporting cells differentiate into granulosa cells, resulting in ovarian development. However, how *Sry* acts spatiotemporally to switch supporting cells from the female to the male pathway is poorly understood. We created a novel transgenic mouse line bearing an inducible *Sry* transgene under the control of the *Hsp70.3* promoter. Analysis of these mice demonstrated that the ability of *Sry* to induce testis development is limited to approximately 11.0-11.25 dpc, corresponding to a time window of only 6 hours after the normal onset of *Sry* expression in XY gonads. If *Sry* was activated after 11.3 dpc, *Sox9* activation was not maintained, resulting in ovarian development. This time window is delimited by the ability to engage the high-FGF9/low-WNT4 signaling states required for Sertoli cell establishment and cord organization. Our results indicate the overarching importance of *Sry* action in the initial 6-hour phase for the female-to-male switching of FGF9/WNT4 signaling patterns.

KEY WORDS: *Sry*, *Sox9*, *Fgf9*, *Wnt4*, Sertoli cells, Sex differentiation, Mouse

INTRODUCTION

The development of a testis or an ovary is a particularly interesting model of organ determination, involving the bipotential gonad differentiating into one of two physiologically and metabolically distinct tissues. In mammals, *Sry*, which encodes a high mobility group (HMG) domain transcription factor, is essential for initiating Sertoli cell differentiation cell-autonomously from the bipotential supporting cells, thereby initiating testis formation (Sinclair et al., 1990; Gubbay et al., 1990; Koopman et al., 1991). Without *Sry* action the supporting cells differentiate into granulosa cells (Albrecht and Eicher, 2001), resulting in ovarian development. During mouse embryogenesis, *Sry* is transiently expressed from 12 to 24 tail somites (ts) – around 11.0-12.0 days post-coitum (dpc) – in the supporting cell lineage. This expression occurs in a center-to-pole wave pattern along the anteroposterior (AP) axis of the indifferent XY gonads (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001). An autosomal gene, *Sox9*, also required for testis determination (Bishop et al., 2000; Vidal et al., 2001; Chaboissier et al., 2004; Barrionuevo et al., 2006), is upregulated in the supporting cells in the same center-to-pole pattern shortly after the onset of *Sry* expression, apparently in response to *Sry* (Sekido et al., 2004; Kidokoro et al., 2005; Wilhelm et al., 2005; Sekido and Lovell-Badge, 2008). However, unlike *Sry*, *Sox9* continues to be expressed in Sertoli cells throughout testis development (Morais da Silva et al., 1996; Kent et al., 1996). It is believed that continuous *Sox9* expression is involved in directing subsequent testis differentiation and development (for reviews, see Brennan and Capel, 2004; Kanai et al., 2005; Polanco and Koopman, 2007).

Recently, we demonstrated that induced, ubiquitous expression of an *Sry* transgene in the entire gonadal area earlier than normal *Sry* expression does not result in any advance in timing or ectopic activation of *Sox9* expression (Kidokoro et al., 2005). This finding indicates that the testis-initiation program immediately downstream of *Sry* action is tightly regulated and that supporting cells need to achieve a competent state to respond to *Sry*. However, the molecular mechanism and the time window within which SRY must act to induce a switch from the female to the male pathway in gonadal supporting cells are unknown.

In order to resolve these questions, we established a heat shock-inducible *Sry* transgenic (Tg) mouse system that allows the induction of testis development in cultured XX genital ridges at various time points during development. By using this *Sry*-inducible system, we demonstrate for the first time that the ability of *Sry* to determine the testis fate is limited to approximately 12 to 15 ts (11.0-11.25 dpc), a time window of only 6 hours. We found that this time window is delimited by the competing actions of FGF9 and WNT4 signaling. Our findings indicate an unexpectedly narrow time window during which *Sry* must act to initiate and maintain *Sox9* expression in developing XY gonads to induce testis formation.

MATERIALS AND METHODS

HSP-Sry transgenic mouse line

The construction of the *HSP-Sry* transgene by replacing the entire 5′-flanking region of the murine *Sry* gene with the mouse heat-inducible *Hsp70.3* promoter sequences was previously reported (Kidokoro et al., 2005). Although most XX transgenic (Tg) mice with the *HSP-Sry* transgene showed an XX-male sex reversal in normal breeding (e.g. #40 and #46), we successfully established one *HSP-Sry* transgenic line (#44; ICR background) in which all XX Tg mice develop into normal fertile females (all Tg mice were viable and fertile with no obvious abnormalities). We refer to #44 line of *HSP-Sry* as ‘Tg’ in this paper. In this line, the transgene was transmitted at an expected Mendelian ratio in both matings between the Tg males and wild-type females (*HSP-Sry* versus wild type: 219 versus 245 males in XY,

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252 versus 229 females in XX, total 945) and matings between wild-type males and Tg females (*HSP-Sry* versus wild type: 14 versus 13 males in XY, 11 versus 14 females in XX, total 52). We also obtained *Wnt4*$^{+/-}$ mice from the Jackson Laboratory (Bar Harbor, ME). They were crossed with this *HSP-Sry* Tg line, and then maintained on a mixed 129/ICR genetic background. The sex and genotype of each animal were determined by PCR as described previously (Kidokoro et al., 2005; Mizusaki et al., 2003). Animal experiments were conducted in accordance with the Guidelines for Animal Use and Experimentation set out by the University of Tokyo.

Heat-shock (HS) treatment and organ culture

Embryos at 9.5-16.5 dpc were collected from pregnant female mice pre-treated with aspirin (Fawcett et al., 1997). From 10.5 to 12.5 dpc, the tail somites of each embryo were counted for accurate staging. Using tail somite stages, 10.5 dpc corresponds to approximately 8 ts, 11.5 dpc to 18 ts, and 12.5 dpc to 30 ts (Hacker et al., 1995). Genital ridges were isolated in cold Dulbecco's Modified Eagle's Medium (DMEM; Sigma). In all samples, one genital ridge (left) of each pair was treated with heat shock (HS; 43°C for 10 minutes), while the other genital ridge (right) was used as a control. HS treatment did not exert any appreciable negative effects on testis formation in the wild-type XY genital ridges in vitro [number of the abnormal testis explants (e.g. atrophy and defective cord formation) per total HS-treated XY explants: 4/37 explants at 12-14 ts and 0/40 explants at 15-18 ts]. In some experiments, we HS-treated whole embryos and other various organs (43°C for 10-15 minutes in thin-wall PCR tubes). All samples except for whole embryos were cultured on ISOPORE membrane filters (Millipore) in DMEM containing 10% horse serum or fetal calf serum at 37°C for appropriate periods (2 hours to 4 days), as described previously (Hiramatsu et al., 2003). Some genital ridges were cultured in 10% horse serum-DMEM supplemented with FGF9 (Recombinant human fibroblast growth factor 9, Sigma; 100 ng/ml) and/or sFRP2 (Recombinant mouse secreted frizzled-related protein 2, R&D Systems; 1.5 μg/ml; medium change every 24 hours). Whole embryos were cultured using a rotating-bottle system in DMEM containing 50% rat serum at 37°C. All explants were subjected to histological, immunohistochemical and RT-PCR analyses as described below.

Histology and immunohistochemistry

Cultured explants were fixed in Bouin's solution, 4% paraformaldehyde (PFA) or 10% formaldehyde containing 2% $Ca(CH_3COO)_2$, and routinely embedded in paraffin. De-paraffinized sections (4 μm in thickness) were subjected to conventional histological (Hematoxylin-Eosin, Periodic Acid Schiff) and immunohistochemical staining. Testis cord formation in the XX Tg explants was histologically evaluated at each tail somite stage (10-24 ts).

For immunohistochemical staining, sections were incubated with anti-SRY (1:50 dilution) (Wilhelm et al., 2005), anti-SOX9 (1:250 dilution) (Kent et al., 1996; Kidokoro et al., 2005), anti-SCP3 (1:250 dilution) (Chuma and Nakatsuji, 2001) (kindly provided by Drs Shinichiro Chuma and Norio Nakatsuji, Kyoto University, Japan), anti-SF1/Ad4Bp, anti-3βHSD (1:1000 and 1:2000 dilution, respectively) (Ikeda et al., 2001), anti-laminin (1:400 dilution; ICN Pharmaceuticals) or anti-MIS (1:100 dilution; sc-6886; Santa Cruz Biotechnology, CA) antibody at 4°C for 12 hours. The reaction was visualized with biotin-conjugated secondary antibody in combination with the Elite ABC Kit (Vector Laboratories, CA).

For quantitative analysis of the number of SOX9-positive cells, the cell number per area (cell number per mm^2) was calculated in three longitudinal sagittal sections per explant, as described previously (Kidokoro et al., 2005).

Proliferation assay and immunofluorescence

The genital ridges were isolated at 11.0 dpc (12-13 ts) and 11.5 dpc (18-19 ts), and then treated with or without HS. To detect proliferating cells, all explants were cultured in the presence of BrdU (bromodeoxyuridine, 10 μM; Sigma) for 3 hours following the pre-culture for 9 hours as described above (total 12-hour culture). The PFA-fixed explants were then used for double staining of BrdU and SF1/Ad4Bp. BrdU labeling was first visualized by using mouse anti-BrdU antibody (Dako Cytomation) in combination with anti-mouse IgG-AF488 (Molecular Probes). Explants were then stained with anti-SF1/Ad4Bp antibody in combination with anti-rabbit IgG-AF594 (Molecular Probes). After being counterstained with DAPI, the numbers of BrdU-positive cells in the coelomic epithelium were calculated in three longitudinal sagittal sections per explant. Finally, the proliferation index (%) of coelomic epithelial cells was estimated in the explants (n=5).

Mesonephric cell migration assay

Gonads were separated from the XX Tg or XY/XX wild-type genital ridges treated with HS at 11.5 dpc (18-19 ts). GFP-positive mesonephroi were dissected from the XY embryos at 11.5 dpc (Green mice; SLC, Japan). HS-treated gonads were assembled with GFP-positive mesonephros and cultured on 1.5% agar blocks in 10% horse serum-DMEM for 48 hours (Martineau et al., 1997).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed on 4% PFA-fixed cultured explants at 70°C for 16 hours as described previously (Hiramatsu et al., 2003). RNA probes for *Sry* (Bullejos and Koopman, 2001), *Fgf9* (Colvin et al., 2001) and *Wnt4* (Mizusaki et al., 2003) were used in this study.

Quantitative RT-PCR analyses

Total RNA was extracted from the genital ridges using Trizol reagent (Invitrogen Life Technologies, CA). After treatment with DNase I for 30 minutes, RNA was reverse transcribed using random primers with a Superscript III cDNA Synthesis Kit (Invitrogen Life Technologies, CA), following the manufacturer's instructions. A reverse-transcriptase-free reaction was performed as control.

In RT-PCR for *Sry* expression, *Sry* and *Gapdh* (control) were amplified as described previously (Kidokoro et al., 2005). For *Sox9*, *Fgf9* and *Wnt4* expression, specific primers and fluorogenic probes were purchased from Applied Biosystems (Assays-on-Demand, Applied Biosystems, CA). Amplification of the *Gapdh* gene was used to standardize the amount of RNA in each reaction mixture (Taqman control reagents). PCR was performed using an ABI Prism 7900HT sequence detector as described previously (Kidokoro et al., 2005). The expression levels represented the relative expression levels of each marker gene per *Gapdh* amplicon ratio (mean±s.e.m.).

Statistical analysis

Quantitative data of both gene expression and cell number were analyzed by Student's t-test. For the stage-dependent differences in frequency of XX/testis formation, statistical analysis was based on the Fisher exact test (two tailed) using the StarView statistical program.

RESULTS

Establishment of an *Sry*-inducible transgenic mouse line

To allow precise experimental control over the timing of onset of *Sry* expression, we established a transgenic (Tg) mouse system that allows the induction of *Sry* expression in cultured genital ridges by heat shock (HS) treatment at 43°C for 10 minutes. Under normal breeding conditions, this Tg line is non-sex reversing, with all XX mice being normal fertile females (Kidokoro et al., 2005). In situ hybridization analysis of XX Tg embryos showed no detectable expression of the *Sry* transgene in various tissues, including the genital ridges (left, Fig. 1A). HS treatment of whole embryo and organ cultures promoted strong *Sry* expression within several hours in whole embryos/various organs of this line (right, Fig. 1A). Anti-SRY immunohistochemical analyses confirmed that the transgene-derived SRY protein was ubiquitously expressed in the somatic cells of various HS-treated tissues (Fig. 1B), whereas only weak signals were detectable in germ cells (arrowheads; Ov and Ts, Fig. 1B).

Next, we examined in detail the consequences of inducing *Sry* at different time points in ex vivo gonad cultures. In wild-type genital ridges explanted at 12 ts (approximately 11.0 dpc), endogenous *Sry* expression started in the center of the gonad, expanded throughout the whole gonadal area after 12 hours (12 h), and disappeared by 24 hours (24 h) in culture (Fig. 1C), which is consistent with the center-

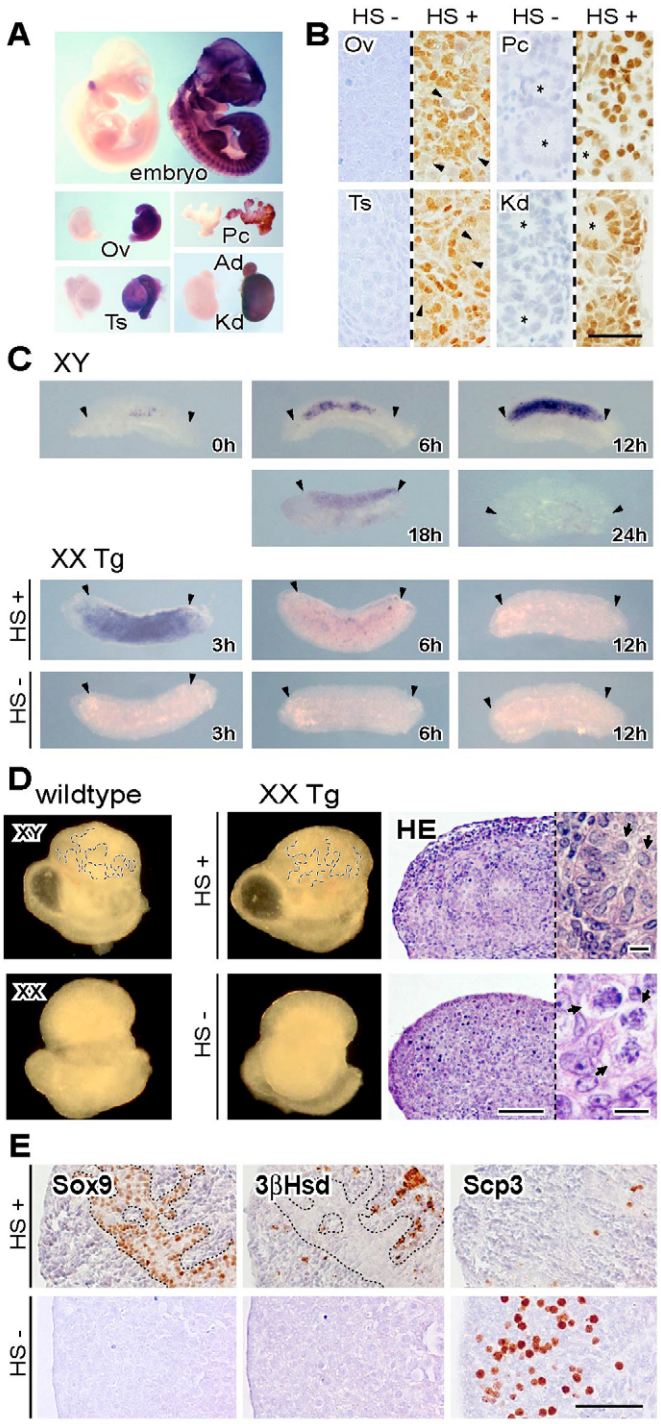

Fig. 1. Characterization of the inducible *Sry* Tg mouse line. (**A**,**B**) Whole-mount in situ hybridization (A) and immunohistochemical (B) analyses showing HS-dependent *Sry* induction at both mRNA and protein levels in *HSP-Sry* Tg mice 2 hours after HS treatment (43°C for 10 minutes; right of each set in both A and B); control non-heat-shocked *HSP-Sry* Tg mice are shown on the left. (A) Ectopic *Sry* induction was detected in 10.5 dpc whole embryos and 16.5 dpc organs, such as ovary (Ov), testis (Ts) pancreas (Pc), adrenal (Ad) and kidney (Kd; right). (B) Anti-SRY immunohistochemical staining confirmed the ubiquitous expression of SRY proteins in the HS-treated somatic cells of the 16.5 dpc organs (note nuclear localization; asterisks, tubular lumens; arrowheads, germ cells). No signal is detectable in non-treated samples (left). Scale bar: 50 μm. (**C**) Whole-mount in situ hybridization, showing *Sry* expression patterns in organ cultures [0-24 hours (h) after *Sry* induction] of XY wild-type (upper) and XX Tg (lower) genital ridges at 12-13 ts (tail somite stage; approximately 11.0 dpc). Anterior/posterior edges of the gonadal area are indicated by arrowheads. (**D**,**E**) Four-day cultured explants of XY and XX wild-type genital ridges (left in D) and the XX Tg genital ridges with or without HS treatment (right in D; E) at 12-13 ts. Testis cords are indicated by dashed lines. Hematoxylin and Eosin (HE) staining shows testis cords in the left genital ridge (HS+) and the presence of the meiotic germ cells in the right genital ridge (HS–) of the same XX Tg embryo (arrows in insets in D indicate germ cells). Immunohistochemical staining with anti-SOX9, anti-3βHSD and anti-SCP3 antibodies demonstrates the differentiation of testicular Sertoli and Leydig cells in the HS-treated XX explants. Scale bars: 100 μm (bars in insets in D, 10 μm).

to-pole wave pattern of endogenous *Sry* expression in vivo (Bullejos and Koopman, 2001). Similarly, genital ridges of XX Tg embryos were explanted at 12 ts and either left untreated or exposed to HS treatment. Control cultures without HS treatment showed no *Sry* expression at any time point investigated. By contrast, HS treatment induced strong *Sry* expression within 3 hours (3h; Fig. 1C, left panel). This expression was rapidly reduced to barely detectable levels by 6 hours after HS treatment (6h; Fig. 1C, middle panel).

To assess morphological consequences of the induced *Sry* expression, we isolated a pair of genital ridges from each XX Tg embryo at 12-13 ts, the time of onset of endogenous *Sry* expression in XY genital ridges. We HS-treated the left genital ridge and used the right one as a non-treated control, and cultured both for 4 days to observe gonadal sex differentiation ex vivo (Fig. 1D,E). Most XX Tg explants treated with HS formed well-defined testis cords in the gonadal parenchyma, similar to XY control genital ridges (Table 1; Fig. 1D, upper panels). In these sex-reversed explants, Sertoli cells differentiated, as shown by SOX9 (Fig. 1E) and MIS (data not shown) expression within the testis cords, and 3βHSD-positive Leydig cells were found in the testicular interstitium (Fig. 1E). By contrast, normal ovarian differentiation was observed in all non-treated (control) XX Tg explants (Fig. 1D), with oocytes staining positive for anti-SCP3, a meiotic germ cell marker (Fig. 1E; see also Fig. S1 in the supplementary material). Although the HS-dependent *Sry* induction showed temporal and spatial patterns of *Sry* expression that were distinct from the endogenous ones (Fig. 1C), these results indicate that this transient *Sry* activation by HS treatment is sufficient to induce testis formation in cultured XX genital ridges of this mouse line. This Tg line is the first *Sry*-inducible system in mammals that allows the induction of testis formation from XX ovaries by HS treatment.

The critical time window of SRY action is limited to approximately 6 hours

To define the critical time window of *Sry* expression that is required for testis formation, genital ridges were isolated from XX Tg embryos at a range of developmental stages, HS treated, and cultured for four days to evaluate testis differentiation (Table 1). Among the XX Tg explants HS treated at 12 to 14 ts, during and immediately after the onset of endogenous Sry expression in XY wild-type gonads, approximately 80% displayed testis differentiation as assessed by laminin, SOX9 and SCP3 immunohistochemistry (Fig. 2A). By contrast, the frequency of XX

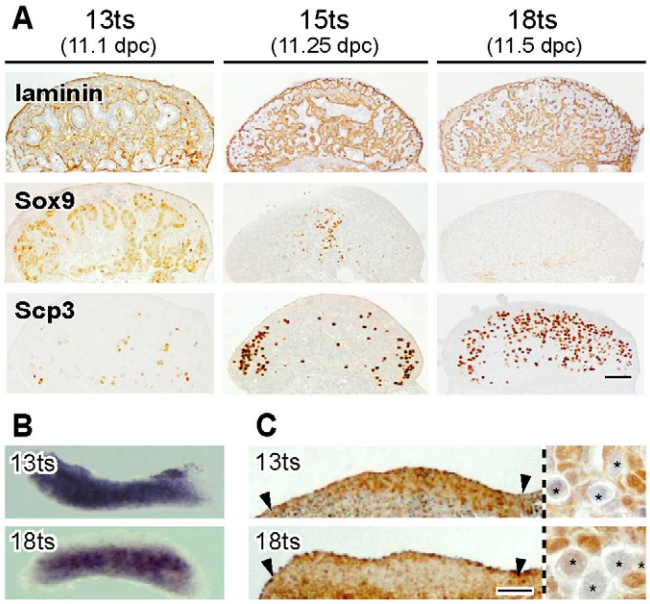

Fig. 2. Artificial delay of *Sry* expression by 6 hours leads to the failure of proper testis formation. (**A**) Immunohistochemical staining with anti-laminin, SOX9 and anti-SCP3 antibodies, showing 4-day-cultured explants of XX Tg genital ridges HS treated at 13, 15 and 18 ts (tail somite stage). In contrast to the testis formation observed in the XX Tg explants HS treated at 13 ts, some explants HS treated at 15 ts display ovotestis development with a central testicular area. Beyond this stage, HS treatment is not capable of inducing XX/testis sex reversal in the XX Tg genital ridges (18 ts). (**B**,**C**) Whole-mount in situ hybridization (B) and immunohistochemical (C) analyses of the XX Tg genital ridges at 13 and 18 ts (3 hours after HS treatment), showing no appreciable difference between the 13 and 18 ts stages in the signal intensities for HS-dependent *Sry* expression at both mRNA and protein levels. Insets in C show higher magnification, with nuclear localization of SRY protein in the presumptive supporting cells directly associated with germ cells (asterisk) at both stages. In B and C, anterior/posterior edges of the gonadal area are indicated by arrowheads. Scale bars: 100 μm.

sex reversal was significantly ($P<0.05$, two-tailed Fisher's exact test; Table 1) reduced to approximately 25% of the explants treated with HS at 15 ts, the time point at which endogenous Sry expression normally just reaches the poles (Bullejos and Koopman, 2001; Kidokoro et al., 2005). At this stage, about half of the explants displayed ovarian development, while the remainder developed into typical ovotestes with testicular tissue, shown by SOX9-positive Sertoli cells, in the central region and ovarian tissue, with SCP3-positive oocytes, at the poles (Fig. 2A, middle panel), similar to that seen in B6-Y^{DOM} sex reversal models (Eicher et al., 1982).

In genital ridges explanted and HS treated at and beyond 16 ts, no well-defined testis cords were detected in any of the HS-treated XX Tg explants (Fig. 2A, right panel), even though *Sry* expression was induced throughout the whole gonadal area (Fig. 2B,C; Fig. 1A,B). Moreover, anti-SRY immunostaining displayed no differences in signal intensity in the gonadal somatic cells between the XX Tg explants induced at 12-13 ts and those induced at 18-19 ts (Fig. 2C; see also Fig. S2 in the supplementary material). Therefore, we conclude that the critical time window of SRY action required to induce testis formation is limited to approximately the first 6 hours after the onset of endogenous *Sry* expression, that is, the period from 12 to 15 ts (approximately 11.0-11.25 dpc).

Sox9 expression is induced but not maintained by ectopic *Sry* beyond the critical time window

In order to gain further insight into the cause of this narrow window of SRY action required to determine the testis fate, the time course of SOX9 expression was examined in SRY-induced XX Tg gonads within and beyond this critical time point (15 ts; Fig. 3). In the SRY-induced gonads at 12 ts (on time), SOX9-positive cells were first detected after 6 hours in culture, had rapidly increased in number by 9 hours, and were maintained at similar numbers 12 and 24 hours after *Sry* induction (Fig. 3A, left panels). In explants at 24 ts (~24 hours after endogenous *Sry* induction), no appreciable SOX9 expression was detected throughout the culture period (Fig. 3A, right panels). These results support the above data showing testis formation in explants induced for *Sry* expression at 12 ts, but ovarian development in explants initiated after the critical period (see Table 1).

Surprisingly, in genital ridges explanted at 18 to 21 ts, a stage at which all explants display ovarian development at later stages, SOX9-positive cells were detectable after 6 hours in culture and had increased in number by 9 hours (Fig. 3A, middle panels). However, SOX9 expression levels were rapidly reduced to an undetectable level, resulting in no SOX9-positive cells after 24 hours. Quantitative real-time RT-PCR analysis confirmed transient *Sox9* upregulation 6 hours after *Sry* induction ($P<0.01$, Student's *t*-test; Fig. 3B). This 6-hour interval between HS-dependent *Sry* induction and *Sox9* upregulation reflects the in vivo expression patterns of these genes (approximately a 4-hour-time lag) (Sekido et al., 2004; Kidokoro et al., 2005; Wilhelm et al., 2005), taking into account a time lag for the recovery from HS stress. Immunohistochemistry demonstrated that the transient SOX9 expression occurred in the presumptive supporting cells, as shown by SF1/Ad4Bp (NR5A1) staining (Fig. 3C), even though transgenic *Sry* is expressed ubiquitously. These data demonstrate that *Sry* expression in itself does not necessarily result in the upregulation of *Sox9*, but that the cellular environment plays an important role. *Sox9* is induced only in the supporting cells of the XX Tg gonads at 12 to 21 ts by ectopic *Sry* expression, which coincides roughly with the end of *Sry*

Table 1. Stage-dependent testis induction by heat shock (HS) treatment in the XX genital ridges of *Hsp-Sry* #44 Tg embryos in vitro*

	Tail somite stage at HS treatment						
	10	11	12-13 (11.0 dpc)†	14	15 (11.25 dpc)†	16-17	18-21 (11.5dpc)†
Number of XX/testis sex-reversed explants/total explant number	0/8 (0%)	1/10 (10%)	19/23 (83%)	9/11 (82%)	3/11 (27%)‡	0/15 (0%)	0/12 (0%)

*Genital ridges were isolated from XX Tg embryos at each tail somite stage (ts), heat shocked (43°C for 10 minutes) in medium, and then cultured for four days to histologically evaluate testis formation.
†Embryos at approximately 11.0, 11.25 and 11.5 dpc show 12, 15 and 18 ts, respectively.
‡Two explants showing ovotestis-like structures were excluded.

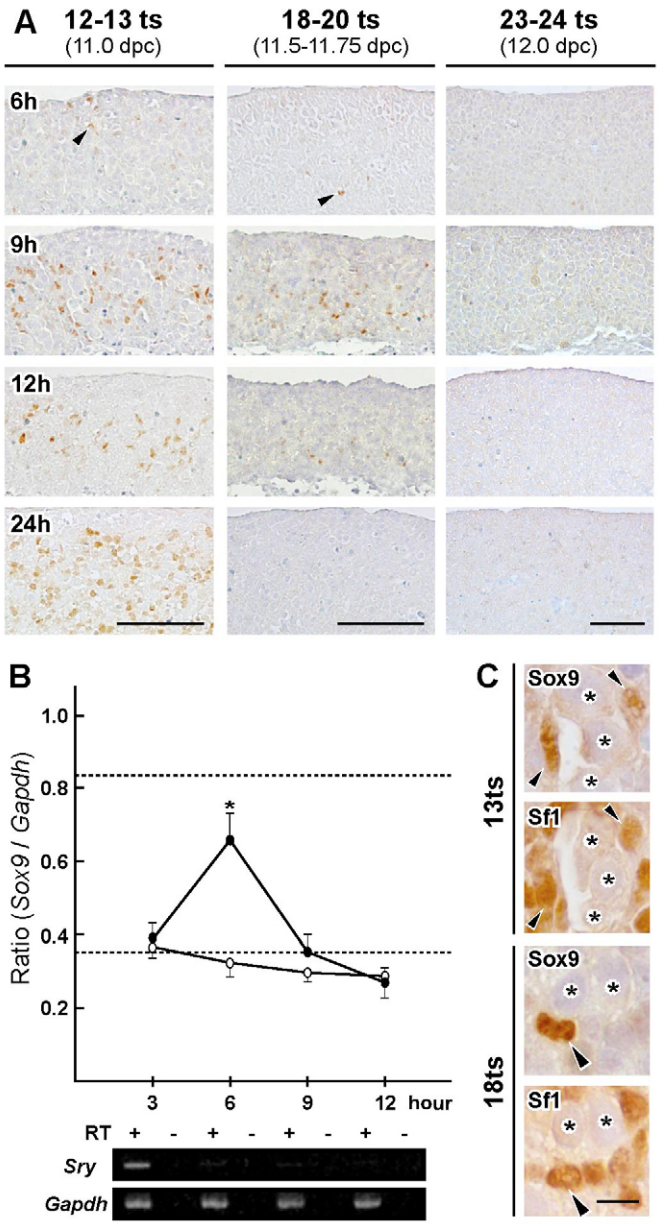

Fig. 3. Time course of SOX9 expression in *Sry*-induced XX Tg gonads. (**A**) Immunostaining of sagittal sections of XX Tg gonads initiated at 12-13, 18-20 and 23-24 ts, showing SOX9 expression (brown staining) at 6, 9, 12 and 24 hours (h) after *Sry* induction. Similar to the SOX9 expression pattern in the XX Tg gonads at 12-13 ts, SOX9-positive cells are detected at 6 hours (arrowheads) and increased at 9 hours in the explants at 18-20 ts. However, in the explants at 18-20 ts, SOX9 signals were decreased at 12 hours after *Sry* initiation, in contrast to the maintained SOX9 expression seen in many cells of the explants at 12-13 ts. In the explants at 23-24 ts, no appreciable signals were detected throughout the culture period. Scale bars: 100 μm. (**B**) Real-time RT-PCR analysis showing changes of *Sox9* expression levels in the XX Tg genital ridges (18-20 ts) treated with (black circle) or without (white circle) HS over a 12-hour-culture period. Circles represent mean values±s.e.m. (n=5 at each point). Vertical axis represents the *Sox9*/*Gapdh* amplicon ratio, whereas the horizontal axis represents the culture period after HS treatment. Asterisk indicates significantly (P<0.01, Student's t-test) higher expression of *Sox9* transcripts, as compared with all other values in both SRY-induced and control XX Tg explants. The two dashed lines indicate the *Sox9* expression levels in the XX (0.36±0.04, lower line) and XY (0.85±0.04, upper line) wild-type genital ridges isolated at 23-24 ts (n=4). The RT-PCR analysis below shows the HS-dependent *Sry* expression at 3 hours. (**C**) Comparative immunohistochemistry for SOX9 and SF1/Ad4Bp were performed using two consecutive sections of one *Sry*-induced XX gonad at 13 and 18 ts, respectively (9 hours after HS treatment). SOX9-positive cells overlap with presumptive supporting cells expressing SF-1/Ad4Bp at both stages (arrowheads). Asterisk, germ cells. Scale bar: 10 μm.

expression in developing XY gonads. *Sry* expression that has been artificially delayed by more than 6 hours is not capable of maintaining sufficiently high levels of *Sox9* expression, which consequently results in ovarian development.

Delayed *Sry* expression does not induce early testis-specific cellular events that are required for Sertoli cell establishment and subsequent testis cord formation

The present data indicate the importance of SRY action during a critical period (12-15 ts) for the establishment of SOX9 expression. At present, little information is available for genes with sex-dimorphic expression (i.e. possible SRY-target genes except for *Sox9*) during this time period. However, several *Sry*-downstream cellular events, i.e. the glycogenesis of pre-Sertoli cells (~14 ts) (Matoba et al., 2005), mesonephric cell migration (~15 ts) (Tilmann and Capel, 1999) and the proliferation of coelomic epithelial cells (~14-15 ts) (Schmahl et al., 2000; Schmahl et al., 2004), were shown to occur just at or immediately after this period and to play crucial roles in the subsequent establishment of SOX9 expression in pre-Sertoli cells.

First, the testis-specific glycogenesis of pre-Sertoli cells, which is likely to occur cell-autonomously immediately downstream of SRY action (Matoba et al., 2005), was examined in SRY-induced and non-induced XX Tg gonads at 18 ts (11.5 dpc; 12-hour delay). A comparative analysis of PAS (Periodic Acid Schiff) reaction and anti-SOX9 staining revealed that glycogen accumulation is properly induced in the SOX9-positive supporting cells in SRY-induced explants (Fig. 4A).

Next, we performed a mesonephric cell migration assay (Martineau et al., 1997) by combining HS-treated XX Tg gonad and GFP-positive XY mesonephros at 18 ts. No explants of non-induced, as well as SRY-induced, XX Tg gonads showed an appreciable contribution of GFP-positive mesonephric cells to the gonadal area after 2 days in culture (n=4; Fig. 4B, upper panels). This is clearly in contrast to the high contribution of GFP-positive mesonephric cells in XY wild-type gonads at 18 ts and in SRY-induced XX Tg gonads explanted at 13 ts (Fig. 4B, bottom panels).

Finally, anti-BrdU immunohistochemistry demonstrated proliferating somatic cells at and near the coelomic epithelium of both SRY-induced and non-induced XX Tg explants at 18 ts (Fig. 4C, upper panels). Quantification of this staining revealed no significant difference between these two treatments (Fig. 4D, third and fourth bars). Moreover, the numbers of proliferating cells were similar to those seen in the non-induced XX Tg gonads at 12-13 ts (Fig. 4C, bottom left panel; Fig. 4D, first bar). By contrast, the XX Tg gonads that were isolated and HS-treated at 12-13 ts (Fig. 4C, bottom right panel; Fig. 4D, second bar) showed a significantly higher number of proliferating cells at the coelomic epithelium than

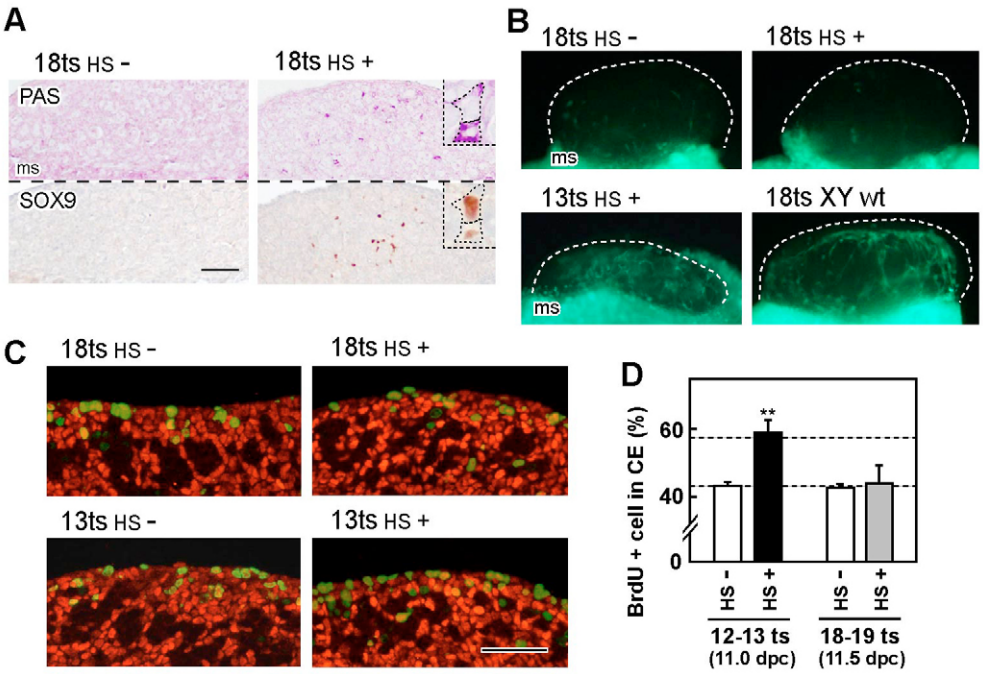

Fig. 4. Defective induction of early non-cell-autonomous morphogenic events caused by delayed SRY activation.
(**A**) Immunohistochemistry for SOX9 and PAS staining in the *Sry*-induced (HS+) and non-induced (HS–) XX Tg explants at 18-19 ts (12-hour culture). PAS reaction and anti-SOX9 staining of two consecutive sections show that testis-specific glycogenesis is induced in the SOX9-positive cells of the *Sry*-induced XX Tg gonads (HS+). Insets show higher magnified images of the positive cells. (**B**) Mesonephric cell migration assay using the XX Tg gonad and GFP-positive XY mesonephros isolated at 13 ts and 18-19 ts. Gonads were isolated from HS-treated or non-treated genital ridges, assembled with GFP-positive mesonephros, and then cultured for 48 hours. No contribution of GFP mesonephric cells is detectable in both *Sry*-induced (HS+) and non-induced (HS–) explants at 18 ts, in contrast to the higher contribution of GFP-positive cells in both SRY-induced XX Tg explants at 13 ts (13ts, HS+) and control XY wild-type explants at 18 ts (18 ts, XY wt). Dashed lines indicate the gonadal surface area of reconstituted explants. (**C**,**D**) Cell proliferation assay of double immunohistochemistry using anti-BrdU (green) and anti-SF1/Ad4Bp (red) antibodies in SRY-induced (HS+) and non-induced (HS–) XX Tg explants at 12-13 ts and 18-19 ts (12-hour culture). The gonadal explants were labeled for BrdU (green) for 3 hours after the 9-hour pre-culture. The BrdU-positive cells are highly detected in the SF1-positive cells (red) in and near the coelomic epithelium in all explants (C). However, in explants at 18-19 ts, there was no significant difference between SRY-induced and non-induced XX Tg gonads in their mitotic indices (D; the mean percentage of BrdU-positive cells in coelomic epithelium ±s.e.m.; n=5). The mitotic index of coelomic epithelial cells in the *Sry*-induced explants at 12-13 ts is significantly higher, when compared with those in the other three explants (**P<0.01). In D, the two dashed lines indicate the mitotic index of the coelomic epithelial cells in the XX (43.3±1.0%, lower line) and XY (57.8±1.5%, upper line) wild-type explants at 12-13 ts (n=5), respectively. ms, mesonephros. Scale bars: 100 μm.

did those of all other treatments. These findings indicate that delayed *Sry* induction is not capable of inducing the early testis-specific mesonephric cell migration and increased proliferation within the coelomic epithelium.

Delayed *Sry* induction results in a tilt of the balance between FGF9 and WNT4 signals towards the female pathway

It was previously shown that FGF9 signaling is crucial for mediating mesonephric migration and cell proliferation, in addition to its important roles in Sertoli cell differentiation and subsequent testis cord formation (Colvin et al., 2001; Schmahl et al., 2004). Moreover, recent genetic analysis has indicated that the balance between two opposing pathways involving *Fgf9* and *Wnt4* could affect the establishment of SOX9 expression in supporting cells at later stages (Kim et al., 2006). Although both *Fgf9* and *Wnt4* are expressed in similar patterns in XX and XY gonads at early phases of the sex-determining period, their sex-dimorphic expression (i.e. high *Fgf9*/low *Wnt4* expression in males, but low *Fgf9*/high *Wnt4* in females) becomes evident around at 18 ts (Mizusaki et al., 2003; Schmahl et al., 2004). In XX Tg gonads HS treated at 12-13 ts (on time), *Sry* induction resulted in the male-specific pattern of *Fgf9* and *Wnt4* expression (Fig. 5A, left panel). By contrast, in *Sry*-induced XX Tg gonads dissected at 18 ts (12-hour delay), we could not find any changes in either *Fgf9* or *Wnt4* expression when compared with expression in the non-induced control explants (Fig. 5A, right panels). No significant changes between SRY-induced and non-induced gonads at 18 ts were confirmed by quantitative real-time RT-PCR (Fig. 5B). Our data indicate that delayed *Sry* induction is not capable of switching the XX gonad from the female- to the male-specific expression patterns of *Fgf9* and *Wnt4*.

A forced reversal from the female- to the male-specific patterns of FGF9/WNT4 signaling states can rescue the defective maintenance of SOX9 expression caused by delayed SRY induction

In order to clarify the possible contribution of female-type *Fgf9*/*Wnt4* expression in defining this critical time window of SRY action, we next examined the effects of the exogenous addition of FGF9 and/or a WNT4 antagonist, the secreted frizzled-related protein 2 (sFRP2) (Lee et al., 2000), on the maintenance of SOX9 expression in XX Tg gonads *Sry* induced at 18 ts (Fig. 5C,D). The addition of FGF9 and sFRP2 did not cause any appreciable changes in the SOX9 expression pattern in control XY and XX wild-type gonads at 18 ts (see Fig. S3

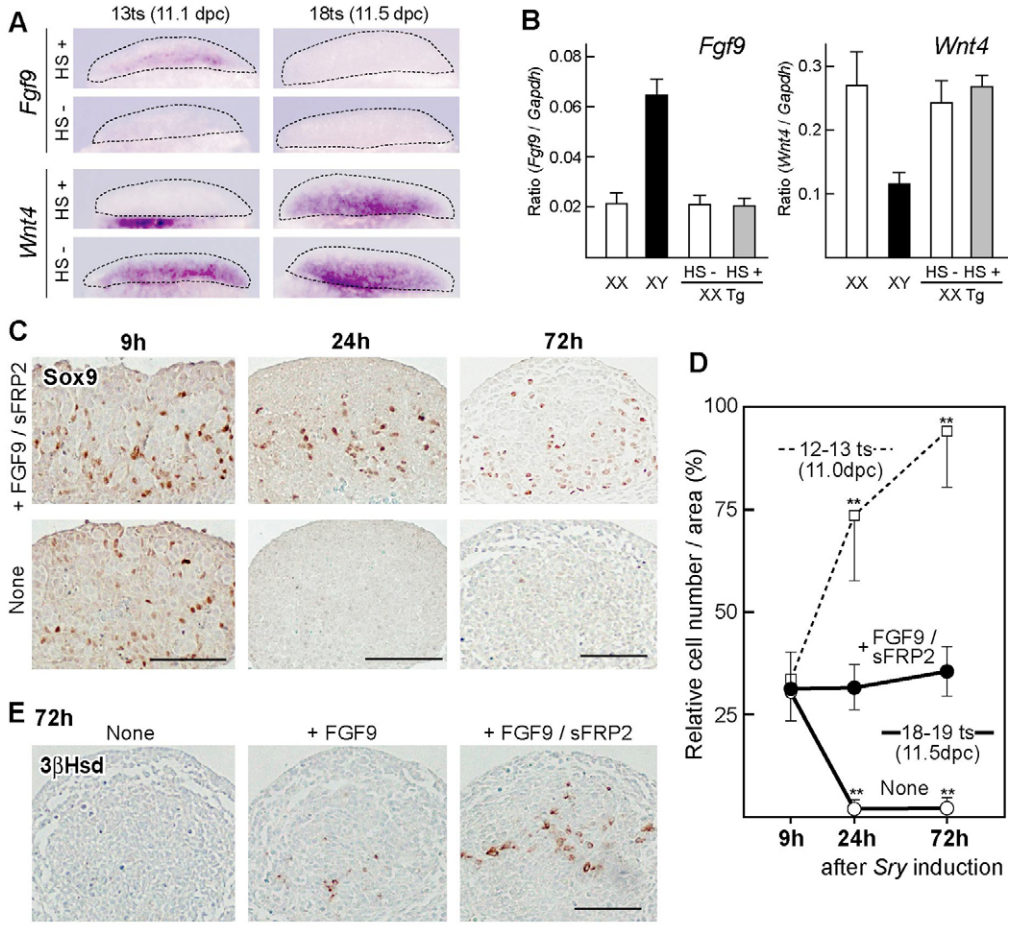

Fig. 5. Female-specific patterns of *Fgf9*/*Wnt4* signaling states lead to the defective testis induction caused by delayed *Sry* expression. (**A**) Whole-mount in situ hybridization analyses showing *Fgf9* and *Wnt4* expression in *Sry*-induced (HS+) and non-induced (HS–) XX Tg gonads at 13 and 18 ts (18-hour culture). The gonadal area is indicated by a dashed line. (**B**) Real-time RT-PCR analysis showing *Fgf9* and *Wnt4* transcript levels in the *Sry*-induced and non-induced XX Tg explants initiated at 18 ts (18-hour culture). The transcript levels in the wild-type XY (black bar) and XX (white bar) explants (HS+) of the same littermates at the same stage are also shown. Vertical axis represents *Fgf9* or *Wnt4* expression level relative *Gapdh*. The data represent mean values ±s.e.m. (*n*=4). (**C-E**) Time-course immunohistochemical analyses with anti-SOX9 (C,D) and anti-3βHSD (E) antibodies, showing effects of exogenous FGF9 (100 ng/ml) and sFRP2 (1.5 μg/ml) on the initiation (9-hour culture) and maintenance (24-hour and 72-hour culture) of Sertoli cells (C,D) and subsequent Leydig cell differentiation (72-hour culture; E) in XX Tg explants *Sry*-induced at 18-19 ts. (D) Quantification of the relative number per gonadal area of SOX9-positive cells in the immunostained sections (C) of these XX Tg explants cultured in the presence (black circle) or absence (white circle) of FGF9 and sFRP2 (mean values of cell number ±s.e.m.; *n*=4). The value in the XY wild-type explants of the same littermates (18-19 ts; 72-hour culture) is set as 100% ($18.3\pm1.5\times10^3$ cell number per mm^2). Asterisks indicate a significant difference (**P<0.01) as compared with the data from the FGF9/sFRP2-treated XX Tg explants initiated at 18-19 ts. The relative numbers of SOX9-positive cells in the XX Tg explants *Sry*-induced at 12-13 ts are also plotted in D (white square). (E) The addition of FGF9 and sFRP2 together appears to induce an increase of 3βHSD-positive cell number in the *Sry*-induced XX Tg explants (18-19 ts) at 72 hours in culture, as compared with those of the *Sry*-induced explants non-treated (None) or treated with FGF9 alone (+FGF9). Scale bars: 100 μm.

in the supplementary material), or in the XX Tg gonads explanted at 18 ts and incubated for 9 hours after *Sry* induction (9h; Fig. 5C,D). After 24 hours, the WNT4 inhibitor sFRP2 alone could not restore the defective maintenance of SOX9 expression in XX Tg gonads, although the addition of FGF9 alone resulted in a few SOX9-positive cells (see Fig. S4 in the supplementary material). By contrast, the addition of FGF9 and sFRP2 together restored the maintenance of SOX9 expression for up to 72 hours in culture (Fig. 5C,D), although the number of SOX9-positive cells was approximately one-third of those in SRY-induced XX Tg gonads at 12-13 ts (Fig. 5D; see also Fig. S3B in the supplementary material). Moreover, prolonged SOX9 expression led to subsequent Leydig cell differentiation in all of the XX Tg explants *Sry* induced at 18 ts, as judged by 3βHSD expression (Fig. 5E). These data indicate that a forced male-specific pattern of the imbalance between FGF9 and WNT4 signals can counteract the defective maintenance of SOX9 expression caused by delayed *Sry* activation, leading to the establishment of Sertoli and Leydig cells at later stages.

Both Sertoli cell establishment and subsequent testis cord formation are properly induced by delayed *Sry* expression in XX *Wnt4*$^{+/-}$ gonads

Finally, in order to assess the direct contribution of WNT4 activity in defining this critical time window of SRY action, we isolated each pair of genital ridges from XX Tg(*Hsp-Sry*);*Wnt4*$^{+/-}$ double mutant embryos at 18-19 ts, HS treated the left genital ridge and used the right one as a non-treated control, and then cultured them for 72 hours (Fig. 6).

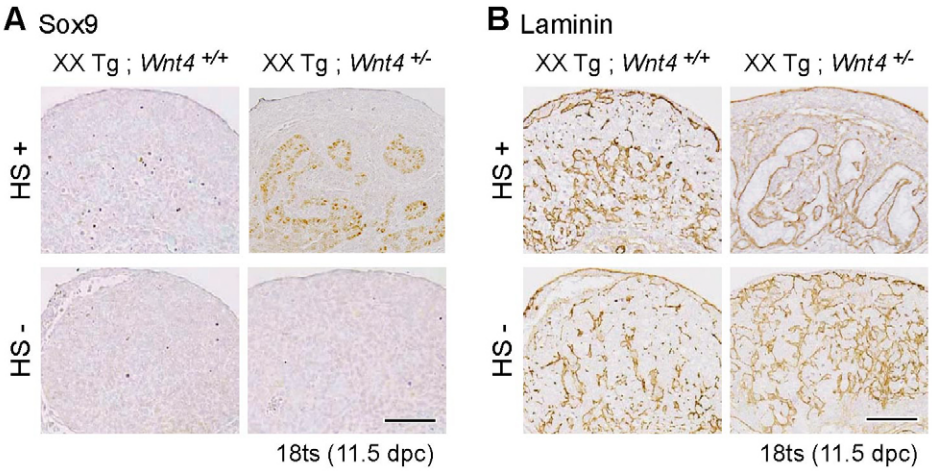

Fig. 6. Delayed *Sry* expression can induce testis development in XX *Wnt4*$^{+/-}$ genital ridges. (**A**,**B**) The genital ridges were isolated from the XX Tg *Wnt4*$^{+/+}$ (wild type) or *Wnt4*$^{+/-}$ embryos at 18 ts. The left genital ridges were treated with HS (HS+), while the right ones were used as non-treated control (HS–). After culture for 72 hours, sagittal serial sections of these XX Tg explants were comparatively analyzed by anti-SOX9 (A) and anti-laminin (B) staining (brown staining). Both maintenance of SOX9 expression and testis cord formation are properly induced in the XX *Wnt4*$^{+/-}$ explant *Sry*-induced at 18 ts (right upper panels in A,B). In the non-treated (HS–) *Wnt4*$^{+/-}$ explant from the same embryo, neither SOX9 expression nor cord formation is detectable (right lower panels in A,B), which is similar to non-treated and *Sry*-induced XX Tg explants of the *Wnt4*$^{+/+}$ wild-type littermate. Scale bars: 100 μm.

In non-treated (HS–) *Wnt4*$^{+/-}$ explants dissected at 18-19 ts, neither SOX9 expression nor cord formation was detected after 72 hours in culture (Fig. 6A,B; right lower panels), which was similar to what was observed in non-treated and *Sry*-induced XX Tg explants of the *Wnt4*$^{+/+}$ wild-type littermates (Fig. 6; left panels). Interestingly, in all XX *Wnt4*$^{+/-}$ explants *Sry*-induced at 18-19 ts (12-hour delay), SOX9 expression was maintained at 72 hours in culture (n=3; Fig. 6A; right upper panel). In these *Wnt4*$^{+/-}$ explants, well-defined testis cords were also induced in their gonadal region (Fig. 6B; right upper panel). These data indicate that the loss of one allele of the *Wnt4* gene can rescue the failure of the Sertoli cell establishment and testis cord formation caused by delayed *Sry* induction. This further implies that the reduced *Wnt4* activity prolongs the critical time window of SRY action that is required to determine the testis fate in developing XX gonads.

DISCUSSION

This study is the first to define two distinct critical time windows of SRY action: (1) to initiate Sertoli cell differentiation; and (2) to secure testis development in mammalian embryogenesis (see Fig. 7; blue and purple arrows). The time window of SRY action required to initiate Sertoli cell differentiation (i.e. the initial SOX9 upregulation) coincides roughly with the period of endogenous *Sry* expression in developing XY gonads (approximately 24 hours). By contrast, the ability of *Sry* to secure the testis fate is limited to approximately only 6 hours after the normal onset of *Sry* expression. This unexpectedly narrow time window of testis determination is non-cell-autonomously defined by the availability of the FGF9/WNT4 signaling states, which lead to the early morphogenic events required for Sertoli cell establishment and testis cord formation (Colvin et al., 2001; Schmahl et al., 2004; Kim et al., 2006). These findings, therefore, indicate that SRY action during the initial 6-hour phase is crucial to switch from the female- to the male-specific patterns of FGF9/WNT4 signaling in developing gonads. The overarching importance of SRY action during this initial phase (12 to 15 ts) is corroborated by the finding that a delay in *Sry* expression during this critical period in B6-XY^{POS} mice results in XY sex reversal (Bullejos and Koopman, 2005).

In this study, we demonstrated that this narrow critical time window required to determine testis fate is non-cell-autonomously defined by the ability to engage the FGF9 signaling state required for Sertoli cell establishment (Colvin et al., 2001; Schmahl et al., 2004) and by the competing action of WNT4 signaling that promotes the female pathway (Vainio et al., 1999; Kim et al., 2006; Ottolenghi et al., 2007). We showed that delayed SRY induction is not capable of switching the gonad from the female- to the male-specific expression patterns of *Fgf9* and *Wnt4*. A forced male-specific pattern of the imbalance between these two signals can counteract the defective maintenance of SOX9 expression, leading to the establishment of Sertoli cells at later stages. Moreover, we genetically demonstrated that reduced *Wnt4* activity can rescue the failure of the Sertoli cell establishment and testis cord formation caused by delayed *Sry* induction. Because the sex-dimorphic expression of *Fgf9* and *Wnt4* becomes evident in the gonadal area by 11.5 dpc (Mizusaki et al., 2003; Schmahl et al., 2004), the FGF9/WNT4 signal state that progresses the female pathway is likely to define the end of the time window of SRY action required to determine testis fate in developing XX gonads. These data also provide clear evidence to support the hypothesis by Kim et al. that sex determination is controlled by mutually antagonistic signals between FGF9 and WNT4 in the gonadal field of mouse embryos (Kim et al., 2006).

In contrast to the narrow time window of *Sry* required to ensure testis development, the present data showed the wider time window of cell-autonomous SRY action that is required to initiate pre-Sertoli cell differentiation in developing XX gonads (Fig. 6, purple arrow). In *Sry*-induced XX gonads, SOX9 was shown to be, although transiently, activated in the presumptive supporting cells during 12 to 21 ts (11.0-11.75 dpc), which coincides roughly with the period of endogenous *Sry* expression in developing XY gonads (Bullejos and Koopman, 2001). These findings indicate that XX gonads, as well as XY gonads, maintain the ability to initiate *Sox9* activation

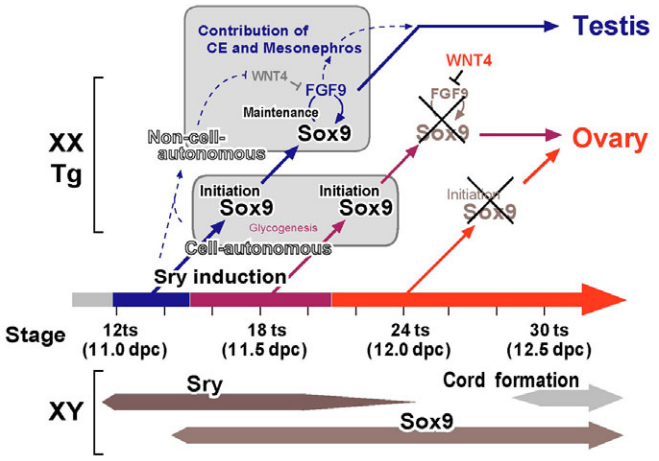

Fig. 7. Schematic showing two distinct critical time windows of SRY action for the initiation and maintenance of *Sox9* expression in developing XX gonads. The horizontal bar represents the bipotential period (blue), and the early (purple) and late (red) ovarian differentiation phases of developing XX gonads. The initiation and maintenance patterns of endogenous *Sox9* expression in *Sry*-induced XX Tg gonads at each developmental stage are shown in the upper part (XX Tg). The timings of *Sry* and *Sox9* expression and testis cord formation in XY wild-type gonads are shown in the lower part (XY). In XX Tg gonads at 12-14 ts, artificial *Sry* induction cell-autonomously induces initial *Sox9* activation and glycogenesis in pre-Sertoli cells. Such SRY/SOX9 expression leads to high-FGF9/low-WNT4 expression patterns in the gonadal area, which results in the maintenance of *Sox9* expression and the testis-specific induction of early morphogenic events [CE (coelomic epithelial cell) proliferation and mesonephric cell migration; blue arrows]. In XX Tg gonads at 16-21 ts, the delayed *Sry* induction is capable of promoting initial *Sox9* activation and glycogenesis in pre-Sertoli cells. However, a lack of SRY action before 16 ts results in the female-specific high *Wnt4* expression that leads to failure of the maintenance of SOX9/FGF9 expression, resulting in the ovarian development at later stages (purple arrows). Beyond 22 ts, neither testis formation nor transient *Sox9* activation was detected in *Sry*-induced XX gonads (red arrows).

upon *Sry* expression during the sex differentiation period. Although recent microarray data has revealed the initiation of a robust female-specific genetic program, including high *Wnt4* expression, as early as 11.5 dpc (~18 ts) (Nef et al., 2005; Beverdam and Koopman, 2006), it was shown that *Foxl2*, a granulosa cell marker gene implicated in ovarian determination (Crisponi et al., 2001; Schmidt et al., 2004; Uda et al., 2004; Ottolenghi et al., 2005; Ottolenghi et al., 2007), starts to be upregulated in a female-specific manner at around 12.5 dpc (~30 ts) (Loffler et al., 2003; Schmidt et al., 2004). Taken together, the present results suggest that the major population of XX supporting cells maintains the sexually undifferentiated and bipotential states by 21 ts. The rapid loss of the potency to initiate SRY-dependent *Sox9* activation at around 23-24 ts is one of the earliest cellular events of pre-granulosa cell differentiation in developing XX gonads (Fig. 6, red arrow).

In conclusion, we have established a novel *Sry*-inducible system that permits switching the differentiation of supporting cells from the female to the male pathway. By using this model system, we have demonstrated for the first time that the ability of *Sry* to induce testis development is limited to a time window of approximately 12-15 ts, corresponding to only 6 hours after the normal onset of *Sry* expression in XY gonads. These data also provide direct evidence showing that, in developing XX gonads, ovarian differentiation starts to occur at around 6 hours after the onset of the male program in XY gonads. To our knowledge, this is the first study to define the critical time window of a master gene required to determine the organ fate in mammalian organogenesis.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/1/129/DC1

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