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 gonad (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; Barriendo 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, 209 versus 235 females in XX).
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 (Reombinant human fibroblast growth factor 9, Sigma; 100 ng/ml) and/or sFRP2 (Reombinant 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(CH3COO)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 subject was identified by the characteristic pattern of cells in the seminiferous tubules. For quantitative analysis of the number of SOX9-positive cells, the cell number per area (cell number per mm2) was calulated in three longitudinal sagittal sections per explant, as described previously (Kidokoro et al., 2005). For quantitative analysis of the number of BrdU-positive cells in the coelomic epithelial cells 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 mesonephroi 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-
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 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
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-YDOM 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 expression.

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

<table>
<thead>
<tr>
<th>Tail somite stage at HS treatment</th>
<th>10</th>
<th>11</th>
<th>12-13 (11.0 dpc)</th>
<th>14</th>
<th>15 (11.25 dpc)</th>
<th>16-17</th>
<th>18-21 (11.5 dpc)</th>
</tr>
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<tbody>
<tr>
<td>Number of XX/testis sex-reversed explants/total explant number</td>
<td>0/8 (0%)</td>
<td>1/10 (10%)</td>
<td>19/23 (83%)</td>
<td>9/11 (82%)</td>
<td>3/11 (27%)</td>
<td>0/15 (0%)</td>
<td>0/12 (0%)</td>
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*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.

1Embryos at approximately 11.0, 11.25 and 11.5 dpc show 12, 15 and 18 ts, respectively.

2Two explants showing ovotestis-like structures were excluded.
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 glycosgenesis 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 glycosgenesis 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 XX Tg gonads (18 ts) (Fig. 4C, upper panels). Quantification of this staining revealed no significant difference between these two treatments (Fig. 4D, third and fourth bars). This is clearly in contrast to the high contribution of GFP-positive mesonephric cells to the gonadal area after 2 days in culture (n=4; Fig. 4B, upper panels). This is likely 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.

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
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, 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 in all explants (C). However, in explants at 18-19 ts, there was a 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.

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
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).
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 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-XYPOS 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
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

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 testsis cord formation in XX 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 testsis-specific induction of early morphogenetic events [CE (coelomic epithelial cell) proliferation and mesonephric cell migration; blue arrows]. In XX Tg gonads at 16-21ts, 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 testsis 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) (Net 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 testsis development is limited to a time window of approximately 12-15 ts, corresponding to only 6 hours after the normal onset of Sry


