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From SRY to SOX9: Mammalian Testis Differentiation

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***Sry* (sex-determining region on the Y chromosome) is a master gene that initiates testis differentiation of the bipotential indifferent gonad in mammals. In mice, *Sry* expression is transiently activated in a center-to-pole wave along the anteroposterior (AP) axis of developing XY gonads. Shortly after the onset of *Sry* activation, *Sox9* (*Sry-related HMG box-9*), a fundamental testis-differentiation gene common to all vertebrates, is also activated in a center-to-pole pattern similar to the initial *Sry* expression profile. Several male-specific cellular events, such as glycogenesis, coelomic epithelium proliferation, mesonephric migration and vasculogenesis, are induced in XY gonads following the onset of *Sry* and *Sox9* expression. This paper mainly focuses on recent advances in elucidating the regulatory mechanisms of *Sry* and *Sox9* expression and male-specific cellular events immediately downstream of SRY action during the initial phases of testis differentiation.**

Key words: genital ridge, sex differentiation, *Sox9*, *Sry*, testis.

Abbreviations: AP, anteroposterior; dpc, day post coitum; EGFP, enhanced green fluorescent protein; HMG, high mobility group; hPLAP, human placental alkaline phosphatase; Sry, Sex-determining region on the Y chromosome; Sox, Sry-related HMG box; ts, tail somite.

Sry (Sex-determining region on the Y chromosome), which encodes a high mobility group (HMG) box transcription factor, is essential for initiating male sex differentiation in mammals (*1*, *2*). Since *Sry* is activated for a very short period in gonadal somatic cells (*3*, *4*), SRY may up-regulate testis-specific genes (and/or repress ovarian genes) to initiate testis differentiation of bipotential gonads. Unfortunately, the targets of SRY have yet to be identified. It has been speculated, however, that a *Sry-related HMG box-9* (*Sox9*) gene is a candidate target gene. In mouse sex differentiation, *Sox9* is up-regulated in developing gonads in a testis-specific manner shortly after the onset of *Sry* expression (*5*, *6*). Human *SOX9* mutation causes XY female sex-reversal with abnormal skeletal development in most cases (*7*, *8*), while duplication of *SOX9* leads to XX male sex-reversal (*9*). Homozygous deletion of *Sox9* in mouse XY gonads interferes with testis differentiation (*10*), while misexpression of *Sox9* in XX gonads results in testis development, as demonstrated by the findings in *Odsex* (*Ods*) mutant mice with a transgene inserted upstream of *Sox9* (*11*, *12*) and transgenic mice ectopically expressing *Sox9* driven by the *Wt1* promoter (*13*). These findings indicate that *Sox9* can substitute for all functions of *Sry* (*14*). Since both *Sry* and *Sox9* genes are specifically expressed in pre-Sertoli cells of developing XY gonads (*5*, *6*, *15–17*), these reports clearly suggest that *Sry* directly promotes male-specific *Sox9* activation and that *Sox9* is mainly involved in the initiation and maintenance of Sertoli cell differentiation during testis differentiation.

Sry, a Y-linked sex-determining gene, is conserved only in mammals, while *Sox9* may act as a fundamental testis-differentiating gene common to all vertebrates. Therefore, for the last decade, many researchers have investigated the molecular mechanisms by which *Sry* regulates *Sox9* expression in developing XY gonads. This information is vital to our understanding of the role of *Sry* in mammalian sex determination. However, to date, neither the direct linkage between *Sry* and *Sox9* nor the cellular events in pre-Sertoli cells immediately downstream of *Sry* actions has been clarified. In this review, we will focus mainly on recent advances in elucidating the regulatory mechanisms of *Sry* and *Sox9* expression and cellular events immediately downstream of *Sry* actions during the initial phases of sex differentiation. The history of the discovery and the molecular and functional aspects of *Sry*, *Sox9* and other sex-specific genes have previously been covered by several excellent in-depth reviews (*18–22*).

I. A center-to-pole wave of *Sry* expression in developing XY gonad of mice

Previous RT-PCR and RNase protection analyses have revealed that the window for *Sry* expression in the mouse developing genital ridge is very narrow, extending from 10.5 dpc (day post coitum) to 12.5 dpc (*4*, *23*). Bullejos and Koopman (Ref. *24*) have succeeded in visualizing endogenous *Sry* expression in mouse genital ridges by whole mount in situ hybridization, finding that *Sry* is expressed in a dynamic center-to-pole wave along the anteroposterior (AP) axis in developing XY gonads (Fig. 1). In brief, *Sry* expression is first detected in the central region of the XY gonad at 11.0 dpc (12–14 tail-somite [ts] stage) and extends to both anterior and posterior ends by 11.5 dpc (approximately 18 ts). Thereafter, its expression is rapidly down-regulated in the middle region, becoming restricted to the posterior pole before it completely disappears at around 12.5 dpc (approximately 30 ts) (*24*). This finding indicates that the male-specific program starts in

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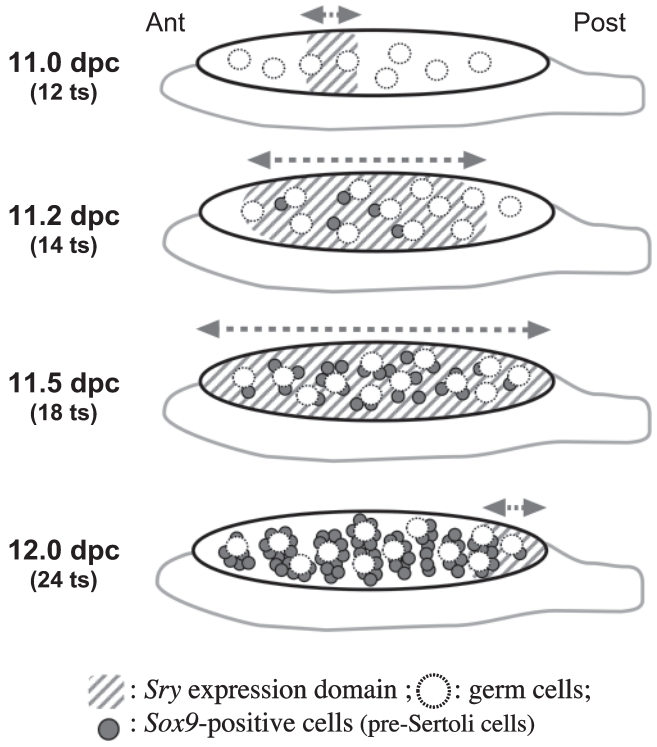

Fig. 1. **Dynamic center-to-pole patterns of *Sry* and *Sox9* expression along the anteroposterior (AP) axis of developing XY gonads.** *Sry* expression (shaded area) is first detected in the central region of the XY gonad at 11.0 dpc and extends to both anterior (Ant) and posterior (Post) ends by 11.5 dpc. Thereafter, its expression is rapidly down-regulated in the middle region, becoming restricted to the posterior pole before it completely disappears at around 12.5 dpc. *Sox9* expression is also first detected in the central region of XY gonads at 11.1–11.2 dpc (13–14 ts) and subsequently, expands to both anterior and posterior ends until 11.5 dpc. *Sox9*-positive cells begin to form the testicular cords from 12.0 dpc onward. The arrows above each gonad indicate the *Sry* expression domain at each developmental stage. Embryos at approximately 11.0, 11.5 and 12.0 dpc show 12, 18 and 24 ts (tail-somite), respectively.

the central region of the gonads. This is also consistent with our previous report demonstrating a similar center-to-pole pattern in the potencies of both Sertoli cell differentiation and testis cord formation in the cultures of anterior, middle and posterior segments of the XY genital ridge (*25*).

As noted by Albrecht and Eicher (Ref. *15*) and Bullejos and Koopman (Ref. *24*), the center-to-pole wave of *Sry* expression may explain the typical histology (i.e., testicular material in the central region with ovarian tissue at the poles) of ovotestes in B6-Y^{DOM} sex reversal models, which occurs when certain variants of the *Mus domesticus* Y chromosome are crossed onto the genetic background of the C57BL/6J (B6) inbred mouse strain (*e.g.*, B6-Y^{POS}, B6-Y^{TIR}). Recently, the onset of a center-to-pole wave of *Sry* expression was shown to be delayed in B6-Y^{POS} sex reversal gonads, and the downstream molecular event, *Sox9* activation, is similarly delayed and detected only in the central region of B6-Y^{POS} gonads (*26*). Similar delayed onset of SRY expression and center-restricted pattern of *Sox9* activation have also been noted in B6-Y^{TIR} fetal gonads (*27*, *28*). Interestingly, Y^{POS}-derived *Sry* transcripts are expressed at low levels only in the central region at the stage when other alleles are at or close to their maximal levels of expression along the entire region of the genital ridge (*26*). This finding suggests that the threshold of *Sry* expression level is reached only in the central region, but not in pole area, of the B6-Y^{POS} gonad, which may possibly result in testis induction at the central region, but ovarian differentiation at the poles, of these gonads.

II. Possible regulatory mechanisms of *Sry* expression

Using transgenic mice carrying the *EGFP* (*enhanced green fluorescent protein*) gene driven by the 5′-flanking region of mouse *Sry* promoter (*Sry-EGFP* in Fig. 2), Albrecht and Eicher (Ref. *15*) have demonstrated that a center-to-pole pattern of initial *Sry* expression is reproduced by reporter gene expression. Some EGFP signals, however, were detectable in the gonads until late developmental stages when endogenous *Sry* expression is extinguished. Sekido *et al.* (Ref. *16*) generated two types of transgenic lines containing most of the 14.6-kb murine *Sry* genomic sequence (*Sry-Myc* and *Sry-hPLAP* in Fig. 2) and demonstrated that both transgenes are specifically expressed in all pre-Sertoli cells within the genital ridge at the more correct timing (strong at 18 ts, weak at 24 ts, but not at 30 ts). A comparison of the results obtained from *Sry-EGFP* and *Sry-Myc* (*Sry-hPLAP*) constructs indicates that the 7.8-kb 5′-flanking region (+542–+8304 in Fig. 2) contains *cis*-regulatory elements which directly regulate the cell-specific and center-to-pole expression patterns in developing XY gonads. Moreover, it is likely that the 6.3 kb 3′-flanking sequences of *Sry* (+8304–+14625 in Fig. 2) may contain important information for its more correct regulation. The importance of the 3′-flanking sequences for *Sry* regulation was also suggested by our recent study showing that the *Sry* construct driven by a weak basal promoter of the *Hsp70.3* gene (*Hsp-Sry* in Fig. 2) is capable of efficiently inducing XX sex reversal (*17*). This is consistent with the previous finding that the *Sry* construct with the deletion of 5′-sequences at 57 bp from the transcriptional start site is capable of efficiently inducing sex reversal (*19*). Interestingly, despite completely lacking 5′-upstream *Sry* sequences, transgenic expression in *Hsp-Sry* transgenic lines was detected strongly in developing gonads from 7 ts (10.5 dpc) by whole mount in situ hybridization (*17*). In two independent lines, positive signals indicating transgenic expression appear to be stronger in the gonadal area than in mesonephric tissue, and stronger in Sertoli cells than in interstitial stromal cells. These data suggest that the 5.5-kb 3′-flanking sequences (+8288–+13780 in Fig. 2) may contain important information for *Sry* expression in the pre-Sertoli cell lineage during the sex determination period.

Both *Hsp-Sry* and *Sry-EGFP* constructs lead to prolonged expression of their transgenes in XY gonads, even after 13.5 dpc when endogenous *Sry* expression is extinguished. Since the *Sry-Myc* construct containing the whole 14.6-kb murine *Sry* genomic sequence exhibits proper extinction of its expression at 12.5 dpc (*16*), the remaining 5′- and 3′-flanking sequences (+0–+542 bp; +13780–+14625 bp in Fig. 2) may be important for the

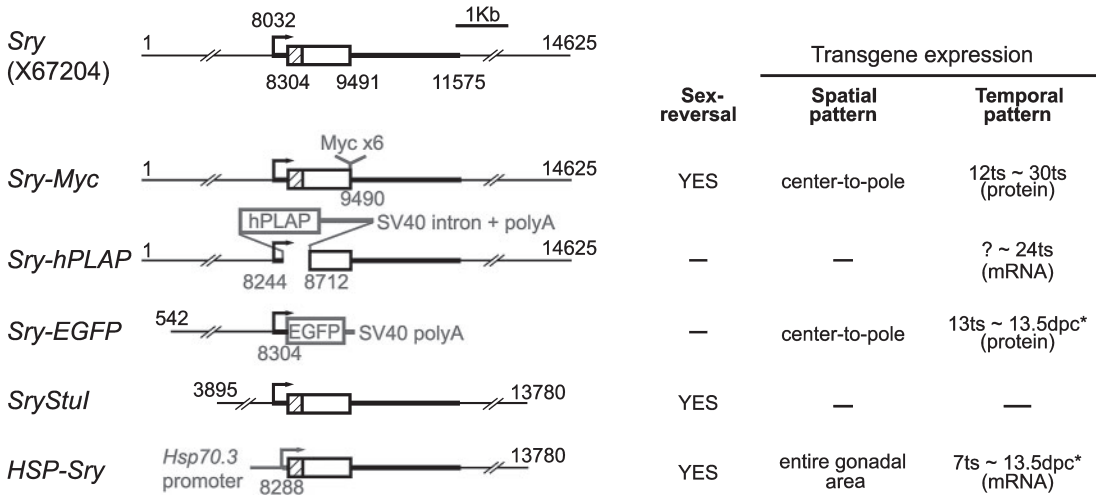

Fig. 2. **Schematic representations of *Sry* transgenes (left) and their spatiotemporal expression patterns (right).** The 14.6-kb mouse *Sry* genomic region and its nucleotide numbers (accession no. X67204; Ref. *2*) are shown in the top of the figure. *Sry-Myc* was constructed by inserting six copies of the Myc-epitope tag in a 14.6-kb *Sry* construct as an in-frame fusion immediately before the stop codon, while *Sry-hPLAP* was constructed by replacing half of the coding region with the *hPLAP* (human placental alkaline phosphatase) gene with SV40 splice and polyadenylation (polyA) signals (Ref. *16*). *Sry-EGFP* was the EGFP (enhanced green fluorescent protein) reporter construct driven by the 5′-flanking *Sry* promoter region (Ref. *15*). *Sry-StuI* was a shortened version (9.9 kb) of the 14.6-kb genomic fragment, which is capable of efficiently inducing XX sex reversal (Ref. *69*). *Hsp-Sry* was constructed by replacing the entire 5′-flanking region of *Sry-StuI* with the mouse *Hsp70.3* promoter sequences (Ref. *17*). The box indicates the open-reading frame (hatched box, HMG box), while the solid bold bars indicate the noncoding region of *Sry* cDNA. The ability to induce XX sex reversal and the spatiotemporal expression patterns of each transgene are shown at right. An asterisk means the extended expression found at 13.5 dpc and later stages. Embryos at approximately 11.0, 11.5, 12.0 and 12.5 dpc show 12, 18, 24 and 30 ts (tail-somite), respectively.

proper extinction of *Sry* after testis determination is initiated. Recently, it was reported that the 5′-flanking region of the *Sry* gene is hypermethylated in tissues that do not express *Sry*, while this region is specifically hypomethylated in the mouse gonad at 11.5 dpc (*29*). Since *in vitro* methylation of the *Sry* promoter region causes suppression of reporter activity (*29*), this finding suggests that the DNA methylation-mediated gene-silencing mechanism may contribute to the proper extinction of *Sry* expression in mouse testis development. In addition, WT1, SF1 and SOX9 have also been shown to transactivate the *SRY* promoter of the pig and human *SRY* genes (*30–33*). It has also been shown that GATA4, its co-factor FOG2 (*34*), and WT1 (+KTS) isoform (*35*) are required for proper *Sry* expression in the developing mouse XY genital ridge.

III. Regulation of SRY activity in developing gonads

Both importin β1 and calmodulin have been shown to have an important role in the nuclear localization of SRY and SOX9 (*21*, *36*). The control of nuclear export and/or import may be one of the regulatory mechanisms of SRY activity. It has also been reported that phosphorylation by the cyclic AMP-dependent protein kinase modulates the DNA-binding ability of human SRY protein (*37*). Recently, Thevenet *et al.* (Ref. *38*) showed that human SRY interacts with histone acetyltransferase p300 and histone deacetylase-3 (HDAC3) *in vitro*. They also found that p300 and HDAC3 expression in somatic cells of human and mouse genital ridges coincides with *Sry* expression, suggesting a possible regulation of SRY activity by acetylation and deacetylation during early phases of testis differentiation. It has also been shown that mouse SRY is associated indirectly with KAP1 and heterochromatin protein 1 (HP1) through its interaction with KRAB-O, a novel protein containing only a Kruppel associated box (KRAB) domain (*39*). They speculated that mouse SRY could utilize the KRAB-KAP1-HP1 organized transcriptional regulatory complex to regulate its yet-to-be-identified downstream target genes.

IV. Possible SRY actions in pre-Sertoli cells

Up-regulation of *Sox9* expression. The expression of both *Sry* and *Sox9* is initiated in pre-Sertoli cells in a center-to-pole pattern in developing XY gonads, with the time lag between the onset of expression of each gene being only 4 h (*i.e.*, approximately 2 tail-somite stages) (*17*, *26*) (Fig. 1). All SRY-positive cells become SOX9 positive (*16*). Delayed expression of *Sry* leads to a synchronous delay in *Sox9* activation in the B6-Y^{POS} model (*26*). *Sry* up-regulates the level of *Sox9* expression dosage-dependently (*17*). These data support the notion that SRY directly regulates the initial *Sox9* activation in pre-Sertoli cells. Unfortunately, the mechanism by which SRY regulates *Sox9* expression remains obscure, despite numerous intensive studies. This may be mainly due to the regulatory region for *Sox9* possibly spanning an interval of over 1 Mb in both human and mouse (*11*, *12*, *40–43*). In the mouse, the sex-reversal *Ods* transgenic line shows an insertional mutation at 980 kb upstream from *Sox9*, causing misexpression of *Sox9* in 11.5 dpc XX Ods/+ fetal gonads (*11*, *12*). Although the mechanism underlying such long-range alterations of *Sox9* expression is still unclear, the insertional mutation may possibly lead to conformational changes of the chromatin structure around the 5′-flanking promoter region of *Sox9*

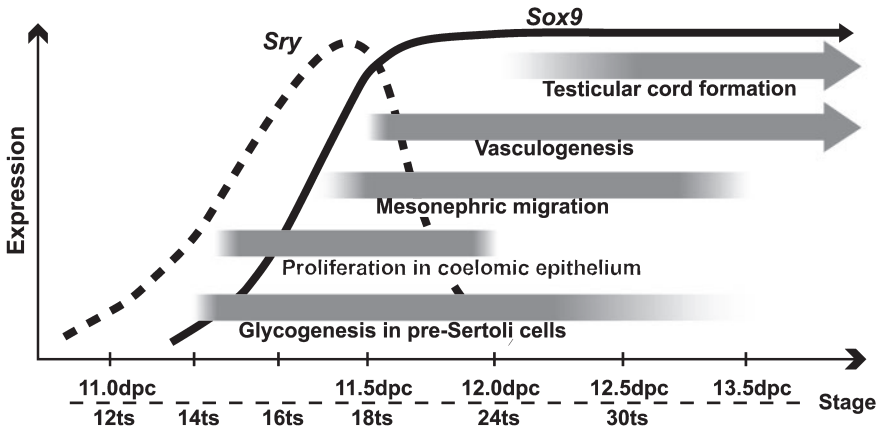

Fig. 3. **Schematic representation of the timing of male-specific cellular events in mouse gonadal sex differentiation.** The horizontal scale represents the developmental stages of ts (tail somite) and dpc (day post coitum). The vertical axis represents the expression levels of *Sry* (broken line) and *Sox9* (solid line) transcripts, while the gradient bar indicates the timing of the onset of each testis-specific cellular event.

(*44*). This raises the possibility that SRY may directly or indirectly induce a similar conformational change to that observed in this *Ods* mutation. In contrast, Lovell-Badge *et al.* (Ref. *20*) demonstrated that the reporter construct of the mouse *Sox9* gene containing approximately 70 kb 5′- and 30 kb 3′-flanking sequences can mimic its Sertoli cell–specific expression within the gonads of transgenic mice. Although a direct link between SRY and this gonad-specific transgene expression has not been established at present, further analyses of this region may enable us to define the SRY-acting *cis*-elements.

In our previous study, we have also tried another approach to examine the possibility that *Sry* directly induces a center-to-pole pattern of *Sox9* expression during initial phases of mouse testis differentiation. Using a *Hsp-Sry* sex-reversal transgenic line in which transgene-derived *Sry* transcripts are ectopically expressed throughout the entire gonadal area along the AP axis from early stages (*17*), the effects of *Sry* expression on spatiotemporal patterns of *Sox9* expression were investigated by misexpression of *Sry*. It was shown that misexpression of *Sry* transcripts from earlier stages does not promote any advance in the timing or any appreciable ectopic up-regulation of endogenous *Sox9* expression. Moreover, in *Hsp-Sry* genital ridges, *Sox9* activation was found in SF1/Ad4Bp-positive somatic cells located in the inner gonadal area only and not in cells within or immediately beneath the coelomic epithelium (including precursors of both Sertoli and interstitial cells [Ref. *45*]). This was despite a sufficiently high level of expression of transgene-derived *Sry* transcripts in these cells. Although we cannot exclude the possibility of translational and post-translational regulation of *Sry* in these ectopic sites, this finding indicates that *Sry* expression alone is not capable of inducing *Sox9* activation in these precursors before they have made the cell-fate decision to become a supporting cell type. These observations also suggest that a male-specific *Sox9* up-regulation by SRY is dependent on some co-factors that are expressed or activated in a center-to-pole pattern similar to the initial expression profile of *Sry*. This is clearly consistent with the general belief that SOX proteins require a partner protein to exert a regulatory function on their target genes (see reviews by Kamachi *et al.* [Ref. *46*]; Wilson and Koopman [Ref. *47*]).

It has also been speculated that the regulation of *Sox9* is more complex than a simple activation through SRY, as several factors such as WNT4 (*48*) and DAX1 (*49*) have been shown to be involved in testis initiation downstream of *Sry*, but upstream of *Sox9*. Although *Wnt4* and *Dax1* have previously been shown to be important in female sexual development (*50*, *51*), mice lacking *Dax1* on the Y^{POS} background do not express *Sox9*, despite apparently normal levels of *Sry* (*49*). Similarly, a lack of *Wnt4* gives rise to a defect in Sertoli cell differentiation which occurs downstream of *Sry* but upstream of *Sox9* in the initial stages of mouse testis determination (*48*). These findings suggest that both WNT4 and DAX1 may induce a testis-specific up-regulation of *Sox9* in cooperation with SRY, or downstream of *Sry*. Moreover, recent studies on the regulatory mechanisms of *Sox9* expression in chondrogenesis have indicated that *Sox9* expression is controlled or affected by various signaling molecules such as MAP-kinase (*52*, *53*) and RhoA/ROCK (*54*) pathways. Post-transcriptional regulation of *Sox9* mRNA stability has also been shown to be important in the regulation of the level of *Sox9* expression during chondrocyte differentiation (*55*). Therefore, it is likely that some, but not all, of these mechanisms are involved in a sex-dimorphic activation of *Sox9* in the developing fetal gonad in a similar manner to that in chondrocyte differentiation.

Glycogenesis in pre-Sertoli cells immediately downstream of *Sry* actions. In mouse sex differentiation, several testis-specific cellular events, including cell proliferation, cell migration, vasculogenesis and testicular cord formation, are known to direct early testiculogenesis (see review by Brennan and Capel [Ref. *22*]; Fig. 3). Increased proliferation of the coelomic epithelium of gonads occurs between 11.3 and 12.0 dpc (*56*, *57*). This proliferation may give rise to a certain population of pre-Sertoli cells in early phases of testis differentiation and to interstitial cells throughout this period (*45*). The cells contributing to the interstitium, including vascular endothelial cells and peritubular myoid cells, migrate into the testis from the adjacent mesonephros (*58–61*). These cells are also required for testicular cord formation (*59*, *62*).

Such testis-specific cellular events indicate a difference in energy metabolism between male and female gonads during sex differentiation (*63*). This also suggests that, compared to XX gonads which exhibit no appreciable histological changes, XY gonads require a higher rate of energy metabolism for the dynamic process of testis morphogenesis. Recently, we have discovered a novel *Sry*-downstream cellular event which preserves the readily

available energy source of glycogen in pre-Sertoli cells for testis-specific morphogenesis and hormone production (*64*). In developing XY gonads, glycogen accumulation starts to occur in pre-Sertoli cells from around 11.2 dpc (*i.e.*, tail-somite 14 stage) in a center-to-pole pattern similar to the initial *Sry* expression profile. We also found glycogen accumulation in XX male gonads of *Sry*-transgenic embryos, but not in XX female gonads of wildtype embryos at any developmental stage. These findings suggest a potential link between *Sry* action and sex-dimorphic energy metabolism in mammalian gonadal sex determination. Moreover, this sex-dimorphic storage of glycogen in the pre-Sertoli cell lineage is mediated by a testis-specific activation of the PI3K-AKT pathway (*64*). Since insulin/IGF signaling generally stimulates glucose metabolism in target organs via the PI3K-AKT pathway (*65*, *66*), it is likely that insulin/IGF induces testis-specific glycogenesis in pre-Sertoli cells through PI3K-AKT activation immediately after the onset of *Sry* expression. This is clearly consistent with the finding that XY mice with mutations for all three insulin receptor members (*Ir*, *Igf1r* and *Irr*) developed ovaries and showed a completely female phenotype (*67*).

Future prospects

In this review, we have mainly discussed recent findings regarding molecular and cellular events which are most likely to occur immediately downstream of *Sry* actions in mouse gonadal sex differentiation. However, despite the discovery of the *Sry* gene 15 years ago, there is still no evidence of the target genes for SRY or of its direct actions. In order to resolve these questions, it will be necessary to develop novel experimental models which will allow evaluation of molecular events from *Sry* to *Sox9* during the initial phases of testis differentiation. It should be noted that the present paper omits an important finding regarding the nuclear localization of FGFR2 in pre-Sertoli cells as one of the cellular events downstream of SRY actions (*68*). This mechanism will be discussed by Drs. Yao and Capel in the current review series of JB.

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