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Establishment of testis-specific SOX9 activation requires high-glucose metabolism in mouse sex differentiation

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ABSTRACT

In mouse sex differentiation, SRY promotes Sertoli cell differentiation via SOX9 action, resulting in testis formation. SRY/SOX9 also initiates various testis-specific morphogenic events including glycogenesis in pre-Sertoli cells, suggesting the importance of glucose storage for certain SRY/SOX9-downstream events in gonadal sex determination. However, it remains unclear which cell types and what molecular/cellular events require sex-dimorphic high-energy metabolic rate. Here we show that the establishment of SOX9 activation itself is a metabolically active process with sex-dimorphic high-energy requirements in gonadal sex differentiation. The glucose-deprivation and metabolic rescue experiments using genital ridge cultures of the XY/XX-wildtype and XX/*Sry* transgenic embryos demonstrated that, among the various somatic cell types, pre-Sertoli cells are the most sensitive to glucose starvation despite the differences between XX/*Sry* and XY genotypes. Moreover, our data showed that, in developing pre-Sertoli cells, the high-glucose metabolic state is required for the establishment of SOX9 expression through an ECM (extracellular matrix)-mediated feed-forward pathway. In contrast, the expression of SRY, SF1/Ad4Bp, GATA4 and WT1, as well as initiation of early SOX9 expression, is properly maintained in the glucose-deprived condition. Therefore, our results imply the metabolic importance of the high-glucose condition for the establishment of SOX9 activation in testis differentiation.

Introduction

The development of the bipotential gonad as a testis or ovary is a particularly interesting model of organ determination involving two physiologically and metabolically distinct tissues. *Sry* (*Sex-determining region on the Y*) and *Sox9*, another SRY-box containing gene, are essential for initiating testis determination in mammals (Sinclair et al., 1990; Koopman et al., 1991; Bishop et al., 2000; Vidal et al., 2001). *Sry* is transiently activated in pre-Sertoli cells only for a short period just before testis formation in mice. Shortly after *Sry* initiation, *Sox9* is up-regulated in pre-Sertoli cells in developing XY gonads but, unlike *Sry*, *Sox9* continues to be expressed in pre-Sertoli cells throughout testis development (Morais da Silva et al., 1996; Kent et al., 1996; Schepers et al., 2003). Since *Sox9* can substitute for *Sry* function in male sex differentiation (Bishop et al., 2000; Vidal et al., 2001; Qin et al., 2005), it is likely that *Sry* promotes testis-specific *Sox9* activation in pre-Sertoli cells and that the maintenance of sufficiently high levels of SOX9 expression is crucial for subsequent testis formation. Moreover, recent studies have shown that the maintenance of *Sox9* expression may be regulated by positive-feedback loops with several signaling factors such as FGF9/extracellular matrix (ECM) (Colvin et al., 2001; Schmahl et al., 2004; Kim et al., 2006) and prostaglandin D2 (Malki et al., 2005; Wilhelm et al., 2005, 2007). A detailed understanding of the molecular mechanisms involved, though, is still lacking.

Shortly after transient *Sry* activation, various testis-specific morphogenetic events (i.e., cell proliferation, migration, vasculogenesis and cord formation) are known to direct early testiculogenesis. Increased proliferation of the coelomic epithelium of gonads occurs between 11.3 and 12.0 days post coitus [dpc] (Schmahl et al., 2000; Schmahl and Capel, 2003). This proliferation is crucial for testis cord formation and may also give rise to a population of Sertoli cells (Karl and Capel, 1998; Colvin et al., 2001). Mesonephric cells contribute to various somatic cell types of the testicular interstitium (Buehr et al., 1993; Martineau et al., 1997; Capel et al., 1999; Brennan et al., 2002). This mesonephric migration also occurs in a testis-specific manner, which is essential for proper testis cord formation (Martineau et al., 1997; Tilmann and Capel, 1999). Finally, testis-specific storage of glycogen, a readily available energy source of glucose, has also been shown to be an early cellular event downstream of *Sry* action (Matoba et al., 2005). In developing mouse XY gonads, glycogen accumulation starts to occur in pre-Sertoli cells shortly after the onset of *Sry* expression and coincides approximately with the initial *Sox9* activation. Glycogen storage occurs in XX/*Sry* transgenic male gonads, but

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not in XX-wildtype female gonads at any developmental stage, indicating a potential link between *Sry* action and sex-dimorphic energy metabolism in mammalian gonadal sex determination. Testis-specific morphogenetic events suggest that male gonads have a higher energy requirement than female ones (Mittwoch, 2007). However, it remains unclear which cell types and what molecular/cellular events in mammalian sex differentiation require a high-energy metabolic rate. Moreover, the biological significance of testis-specific glycogen storage in pre-Sertoli cells is also unknown.

It is well known that metabolically active cells with high-energy requirements are susceptible to structural and functional impairment when deprived of glucose and oxygen, major factors for generating ATP (Lee et al., 2000; Dzeja and Terzic, 2003). In order to determine the biological significance of sex-dimorphic energy requirements and male-specific glycogen storage just before testis formation, we examined the influence of glucose starvation on mouse gonadal sex differentiation *in vitro*. Our study demonstrated for the first time that, of the various somatic cell types in the male and female genital ridges, pre-Sertoli cells are the most sensitive to glucose deprivation. Our results also indicate that the establishment of sustained high levels of SOX9 expression is the first molecular event with sex-dimorphic high-energy requirements in pre-Sertoli cells.

Materials and methods

Animals

Embryos were obtained from pregnant female mice (ICR strain) and *ROSA26* mice (Jackson Labs) at 11.0 dpc (12–13 tail somite stage [ts]; Hacker et al., 1995) and 11.5 dpc (17–19 ts). In some cases, the XX sex-reversal transgenic embryos carrying the *Sry* transgene (Kidokoro et al., 2005) were also used in this study. After counting the tail somite number and separating the head tissues in each embryo for sex determination and genotype, genital ridges (i.e., gonad plus mesonephros) were used for the organ culture experiments described below. The sex and genotype of each embryo were determined by PCR (Kidokoro et al., 2005). Animal experiments were conducted in accordance with the Guidelines for Animal Use and Experimentation as set out by the University of Tokyo.

Organ culture

Genital ridges were cultured on ISOPORE membrane filters (Millipore) in the high-glucose (control; final concentration: 410 mg/dl) or glucose-deprived (GD; final concentration: 5 mg/dl) medium for 6 to 72 h as described previously (Hiramatsu et al., 2003). The GD medium consisted of 10% horse serum (50 mg/dl glucose; GIBCO BRL) and glucose-free DMEM (Dulbecco's Modified Eagle's Medium; Sigma). The culture medium containing 50, 185, or 410 mg/dl glucose (final concentrations) was prepared by adding corresponding amounts of glucose (cell culture grade, Sigma) to glucose-free DMEM medium supplemented with 10% horse serum. The glucose levels in the horse serum and each culture medium were also examined using the Fuji DRI-CHEM system (Glu-PIII and Dri-Chem 5500 autoanalyzer; Fujifilm Medical, Japan).

For the cell proliferation assay, BrdU (final 10 μM; Sigma) was added to the high-glucose (control) or GD medium. For rescue experiments, pyruvate was added to the GD medium as an alternative energy source. Since one molecule of glucose is converted to two molecules of pyruvate, sodium pyruvate (550 mg/dl [50 mM]; cell culture grade, Wako Pure Chem) was added to glucose-free DMEM instead of glucose (450 mg/dl [25 mM]). FGF9 (100 ng/ml; Sigma), PGD2 (500 ng/ml; Cayman Chemical) and Matrigel (basement membrane matrix, BD Bioscience) were also added to the GD medium. Matrigel was dialyzed overnight against cold glucose-free DMEM.

The genital ridges were embedded in Matrigel on the filter, and then floated on the GD medium in the presence or absence of FGF9 (100 ng/ml). In some genital ridges, the anterior and posterior edges were excised by using a sharp needle to directly expose pre-Sertoli cells to Matrigel (see Fig. 7A).

All cultured explants were used for the following histological and immunohistochemical analyses.

Histological, histochemical and immunohistochemical analyses

Cultured explants were fixed in 4% paraformaldehyde (PFA) or 10% formalin containing 2% $Ca(CH_3COO)_2$, and routinely embedded in paraffin. Serial sections (4 μm in thickness) were subjected to conventional histological (periodic acid Schiff [PAS]) and immunohistochemical staining. The histochemical specificity for glycogen was confirmed by reduced PAS reaction in the sections pre-treated with α-amylase. For control and GD explants in each experiment, fixation, paraffin embedding, sectioning and staining were performed as carefully as possible under the same conditions in order to evaluate and compare the relative signal intensities.

For immunohistochemical staining, sections were incubated with anti-BrdU antibody (1/100 dilution; Dako Cytomation), anti-GATA4 antibody (1/250 dilution; Santa Cruz), anti-GRP78 (glucose-regulated protein-78) antibody (1/200 dilution; ABR-Affinity BioReagents), anti-Laminin antibody (1/400 dilution; ICN Pharmaceuticals), anti-MIS antibody (1/100 dilution; Santa Cruz), anti-phospho-Histone H3 antibody (1/200 dilution; Cell Signaling Technology), anti-SF1/Ad4Bp antibody (1/1000 dilution; Ikeda et al., 2001; kindly provided by Dr. K. Morohashi, Kyushu University, Japan), anti-SOX9 antibody (10 ng/μl; Kent et al., 1996; Kidokoro et al., 2005), anti-SRY antibody (1:50 dilution; Wilhelm et al., 2005; kindly provided by Drs. D. Wilhelm, P. Koopman, The University of Queensland, Australia) and anti-WT1 antibody (1/50 dilution; Dako Cytomation). Thereafter, the immunoreaction with each first antibody was visualized using biotin-labeled secondary antibodies with an ABC kit (Vector Laboratories). For whole mount immunohistochemistry, the PFA-fixed explants were incubated with monoclonal anti-PECAM1 antibody (1/200 dilution; Pharmingen). The reactions were visualized with biotin-labeled secondary antibodies in combination with alkaline phosphatase (AP)-conjugated streptavidin (Vector Laboratories).

For transmission electron microscopy, the explants were fixed in 2.5% glutaraldehyde–0.1 M phosphate buffer (PB) at 4 °C for 4 h. After washing with 0.1 M PB, the samples were postfixed in 1% OsO_4 in 0.1 M PB at 4 °C for 2 h. The specimens were then dehydrated in ethanol and embedded in Araldite M. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and then observed under a JEM 1010 transmission electron microscope at 80 kV.

Migration assay and beta-gal staining

Gonads and mesonephroi from wildtype or LacZ-positive *ROSA26* embryos at 11.5 dpc were separated using a sharp needle in cold PBS. An XY gonad was assembled with a *ROSA26* mesonephros, and then cultured in control or GD medium as described above. These 3-day cultured explants were fixed in 1% PFA–0.2% glutaraldehyde–0.02% NP40-PBS, and subjected to whole mount X-gal staining. After the stained explants were photographed under a dissecting microscope, transverse frozen sections were prepared for histological analysis. The mesonephric migration activity was estimated as the contribution of the LacZ-positive cells inside the gonadal area under a dissecting microscope.

Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed as previously described by Hiramatsu et al. (2003). In order to compare the relative

signal intensities of control and GD explants, fixation, hybridization and staining were performed under the same conditions, mainly using an automatic *in situ* hybridization system (AIH-201; Aloka, Tokyo). Briefly, the cultured explants were fixed in 4% PFA-PBS for 4 h and dehydrated in methanol. Using the automatic *in situ* hybridization system, the samples were rehydrated, pre-treated with 10 μg/ml proteinase K for 60 min, and then hybridized with digoxigenin (DIG)-labeled RNA probes at 70 °C for 16 h. After treatment with RNase A, they were finally washed with 2× SSC at 65 °C for 1 h. The signals were detected by an immunological method using alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche Molecular Biochemicals). RNA probes for *Sox9* (Kent et al., 1996), *Pgds* (Wilhelm et al., 2005; 2007), *Col9a3* (McClive and Sinclair, 2003), *Wnt4* (Mizusaki et al., 2003) and *Bmp2* (Chaboissier et al., 2004) 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 min, each RNA was reverse-transcribed using random primers with a Superscript III cDNA synthesis kit (Invitrogen Life Technologies, CA) following the manufacturer's instructions. For real-time quantitative RT-PCR for *Sox9* expression, specific primers and the fluorogenic probe were used as follows: *Sox9* forward primer, 5′-CGT GGA CAT CGG TGA ACT GA-3′; *Sox9* reverse primer: 5′-GGT GGC AAG TAT TGG TCA AAC TC-3′; Taqman probe, 5′-AGC GAC GTC ATC TCC AAC ATT GAG ACC T-3′. For *Fgf9* and *Pgds* 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 with 40 cycle amplifications of 95 °C for 15 s, 60 °C for 1 min followed by enzyme activation at 95 °C for 2 min. All reagents for real-time PCR were purchased from Applied Biosystems. The expression levels represented the relative expression levels of each marker gene per *Gapdh* amplicon ratio (mean ± standard error).

Results

Pre-Sertoli cells are the most sensitive cell type to glucose starvation, with failure of testis formation evident in glucose-deprived (GD) genital ridge cultures

In order to clarify the glucose requirement for gonadal somatic cells during sex differentiation, we first histologically examined the influence of glucose starvation on testis differentiation *in vitro* using the 11.5-dpc genital ridges (18–19 ts) in which both glycogen accumulation and SOX9 expression in pre-Sertoli cells had already reached high levels. The genital ridges were cultured for 2–3 days in glucose-deprived (GD, 5 mg/dl) or high-glucose (control; 410 mg/dl) medium.

Dissecting microscopic observations showed no gross differences in size or shape between GD and control explants of both XY and XX

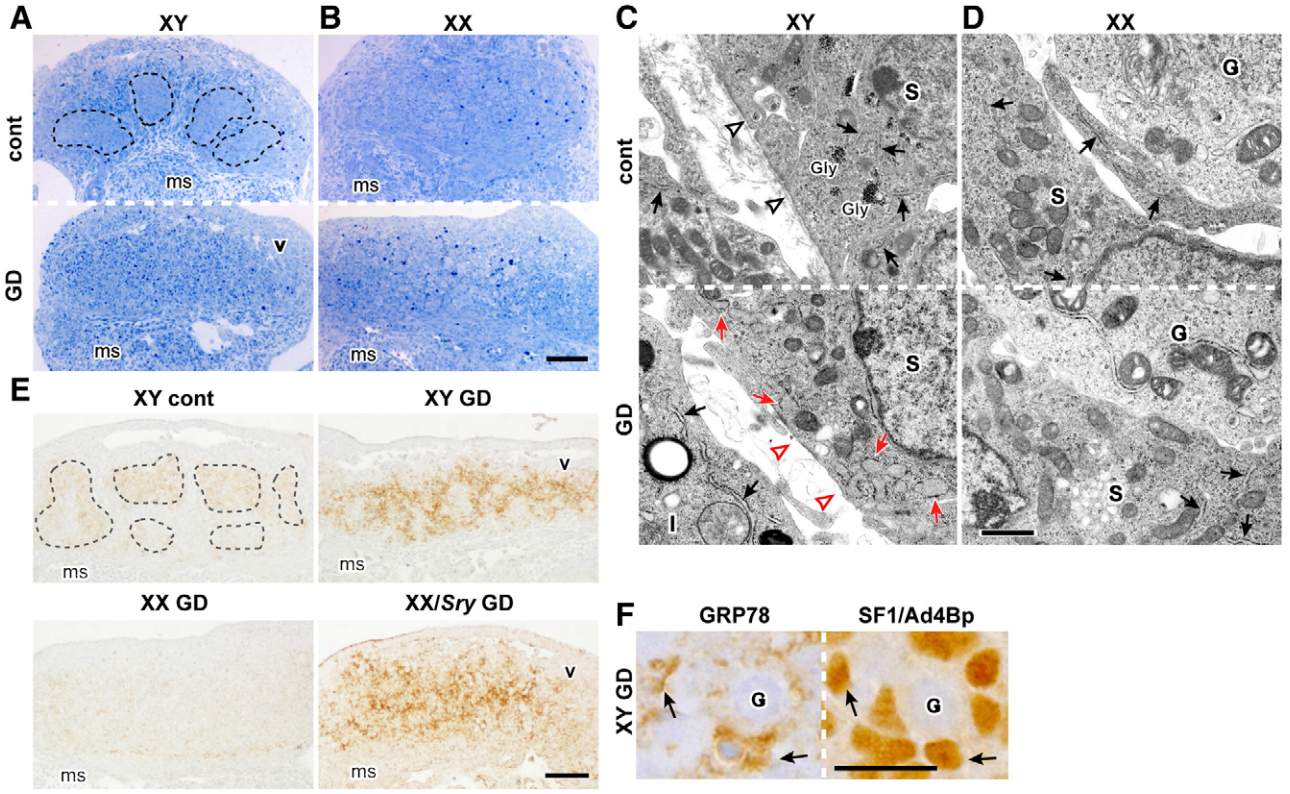

Fig. 1. Influence of glucose starvation on gonadal sex differentiation *in vitro* using 11.5-dpc genital ridges. XY male (A, C, upper two plates in E; F), XX female (B, D, lower left in E) or XX/*Sry* male (*Sry* transgenic line; lower right in E) genital ridges were isolated at 11.5 dpc (17–19 ts [tail somite stage]), and cultured for 3 days in high-glucose (control; 410 mg/dl) or glucose-deprived (GD, 5 mg/dl) medium. (A–D) Semi-thin sectioning (toluidine blue; A, B) and electron microscopic (C, D) analyses show defective cord formation in XY GD explants (A), where disorganized presumptive Sertoli cells have no basal lamina formation along their basal surface (open red and black arrowheads in C). No histological defects by GD are detectable in XX explants (B, D). Glucose starvation specifically induces enlargement of rough endoplasmic reticulum (ER) in pre-Sertoli cells in XY GD explant (red arrows in C; normal ER morphology indicated by black arrows in C, D). (E) Anti-GRP78 immunostaining (brown) demonstrates that glucose starvation up-regulates GRP78-positive signals in the gonadal parenchyma of male explants isolated from XY wildtype and XX/*Sry* transgenic embryos at 11.5 dpc. No positive signals are detectable in female XX explants cultured in the same GD conditions. Weak positive signals are also noticeable even in XY control explants (broken lines, testis cords). (F) Anti-GRP78 (left) and SF1/Ad4Bp (right) staining of two consecutive sections, showing GRP78-positive signals in the cytoplasm of some SF1/Ad4Bp-positive cells in the XY GD explant (arrows). G, germ cells; Gly, glycogen granules; I, interstitial cells; ms, mesonephros; S, Sertoli or presumptive supporting cells; v, blood vessel. Bars, 100 μm in B and E; 1 μm in D; 10 μm in F.

Table 1
Summary of testis cord formation in XY genital ridges cultured in various glucose concentrations and their metabolic rescue by pyruvate in glucose-deprived (GD) culture

Stage at culture initiation	Glucose/pyruvate concentration in medium (mg/dl)	Testis cord formation[a]				
		++	+	+/−	−	Total no. of explants
11.5 dpc (17–19 ts)	Glucose 410 (high-glucose)	56	0	0	0	56
	185	2	10	0	0	12
	50	0	2	7	0	9
	5 (GD)	0	0	5	41	46
	Glucose 5+pyruvate 495	9	19	0	0	28
11.0 dpc (12–13 ts)	Glucose 410 (high-glucose)	3	7	0	0	10
	5 (GD)	0	0	0	14	14
	Glucose 5+pyruvate 495	2	8	0	0	10

Effects of glucose/pyruvate concentration on testis cord formation were histologically estimated in 2–3-day cultures of XY genital ridges.

[a] Number of explants showing i) no cord structures (−), ii) definite cord-like structures partially detected in the gonadal area (+/−), iii) well-defined cords in some parts of the gonadal area or well-defined, but slender/irregular cords formed throughout whole gonadal area (+), or iv) well-defined cords throughout whole gonadal area (++).

genital ridges (figure not shown). Histological analyses revealed no appreciable defects in the XX GD explants after 3 days of culture (Fig. 1B). In contrast, most of XY GD explants showed no testis cord formation in the gonadal area, though well-defined cords were seen in all XY control explants (Fig. 1A; upper column in Table 1). Transmission electron microscopic analyses of XY GD explants confirmed a disorganized arrangement of presumptive Sertoli cells, in which no basal lamina formation was detectable along the basal surface (red open arrowheads in Fig. 1C). Moreover, GD treatment induced enlargement of the rough endoplasmic reticulum (ER) in most pre-Sertoli cells (red arrows in Fig. 1C), reflecting morphological evidence for the glucose starvation-induced ER-stress response (Ma and Hendershot, 2002). Except for the enlargement and defective formation of basal lamina, no appreciable ultrastructural defects were found in the somatic cells of XY or XX GD explants within the first 3 days of culture (Figs. 1C, D).

Anti-GRP78 (an ER-stress marker; see review by Lee, 2001) staining showed that GRP78-positive signals were weakly detected in Sertoli cells of newly-formed testis cords in control XY explants (XY cont in Fig. 1E). No positive signals were detected in control XX

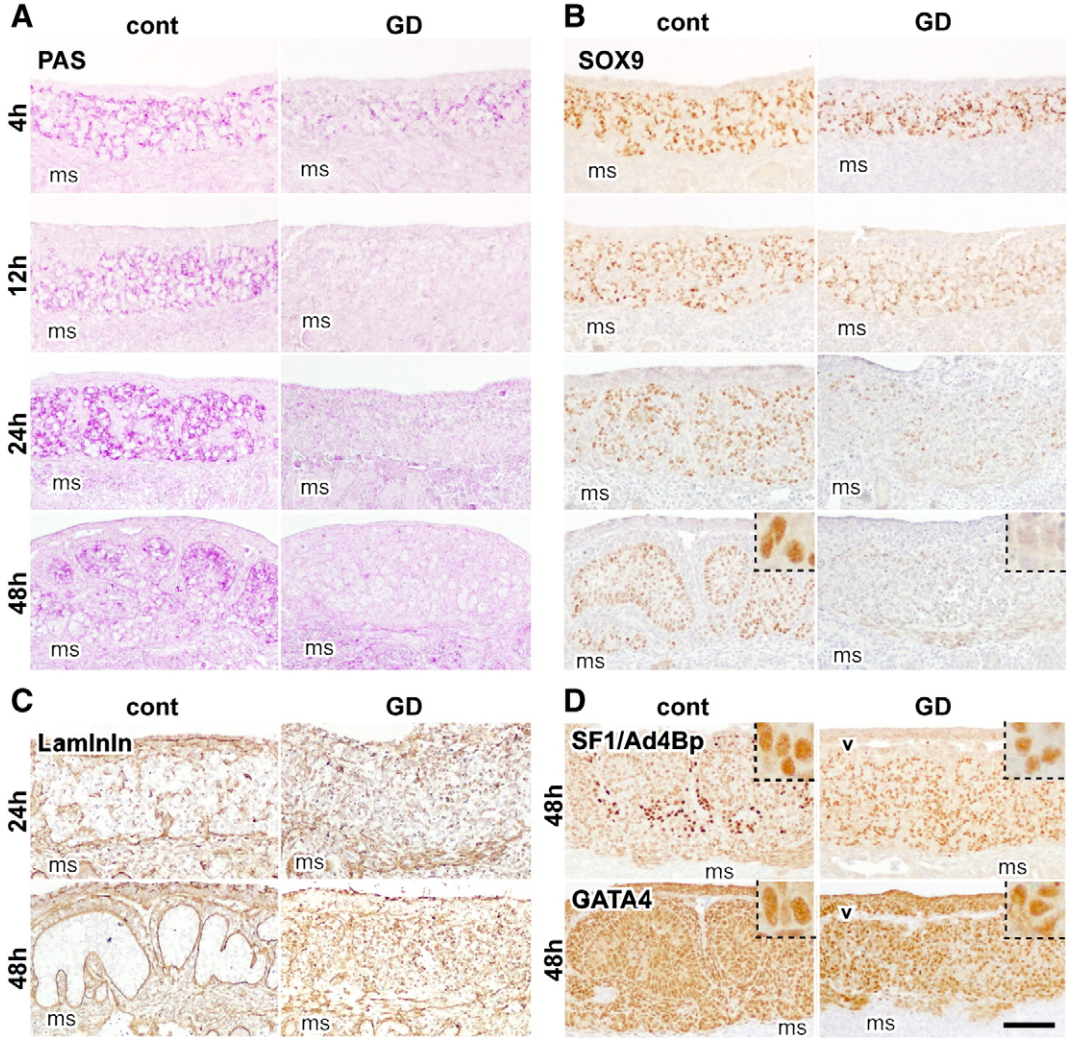

Fig. 2. Time course of glycogen consumption and reduced SOX9 expression in 11.5-dpc XY genital ridges under glucose-deprived (GD) culture conditions. XY genital ridges were isolated at 11.5 dpc (17–19 ts), cultured for 4–48 h in high-glucose (control; 410 mg/dl) or glucose-deprived (GD; 5 mg/dl) medium, and then comparatively examined by histological PAS staining (A; red) and anti-SOX9 (B), anti-Laminin (C), anti-SF1/Ad4Bp or anti-GATA4 (D) immunostaining (brown). In both control and GD explants (24 and 48 h), serial sections were used for immunostaining for SOX9, Laminin, SF1/Ad4Bp and GATA4 (lower in B; C, D). Glucose starvation rapidly induces both glycogen consumption and reduced SOX9 expression in pre-Sertoli cells within 12 h after culture initiation (A, B), leading to defective cord formation at 48 h (C). However, even at 48 h of GD culture, high levels of expression of both SF1/Ad4Bp and GATA4 are maintained in the gonadal region (D), in contrast to only weak SOX9 expression in the same explant (48 h in B). Insets show high magnified images of the nuclear staining. ms; mesonephros, v; blood vessel. Bar, 100 μm.

explants (figure not shown) or XX and XY genital ridges isolated at 11.5 dpc before culture initiation (see Supplementary Information, Fig. S1), suggesting a weak activation of ER stress in pre-Sertoli cells even in the control (high-glucose) conditions. All XY GD explants clearly displayed considerably high levels of GRP78 expression in SF1/Ad4Bp-positive somatic cells of the gonadal area, but not in cells located at coelomic epithelia and mesonephros ("XY GD" in Figs. 1E, F). Glucose starvation also induced high levels of GRP78 expression in male genital ridges isolated from XX/*Sry* embryos. This result is in sharp contrast to the absence of any GRP78-positive signals in all wildtype XX explants cultured in the GD medium (XX GD vs XX/*Sry* GD in Fig. 1E). These findings indicate that, of the various types of XY, XX and XX/*Sry* gonadal somatic cells, pre-Sertoli cells are the most sensitive to glucose starvation, despite having stored glycogen before culture initiation.

A time-course immunohistochemical analysis showing defective maintenance of SOX9 expression in pre-Sertoli cells 12 h after initiation of GD culture

Next, we carried out time-course histochemical analyses of *in vitro* testis differentiation in the control and GD explants (Fig. 2). In XY 11.5-dpc genital ridges, high levels of both glycogen accumulation and SOX9 expression were attained in pre-Sertoli cells before culture initiation (figure not shown). In the control (high-glucose) medium culture, pre-Sertoli cells displayed high levels of SOX9 expression in their nucleus throughout the culture period (left in Fig. 2B). PAS staining also revealed that glycogen storage was maintained at 24 h (left in Fig. 2A). During testis cord formation of pre-Sertoli cells, the PAS reaction in pre-Sertoli cells appeared to be reduced at 48 h (left lowest plates in Figs. 2A, C) and had disappeared completely by 72 h after culture initiation (figure not shown). In 11.5-dpc XY genital ridges cultured in GD medium (5 mg/dl), glycogen storage was rapidly reduced at 4 h, and had completely disappeared at 12 h after culture initiation (right in Fig. 2A). Interestingly, based on the glycogen consumption of pre-Sertoli cells, signal intensities for anti-SOX9 staining were clearly decreased at 12 h, and only weakly detectable at 24 h after culture initiation (right in Fig. 2B). This reduction in SOX9 expression after 12 h resulted in reduced expression of MIS (a direct SOX9 target) and Laminin and defective formation of testis cords at 48 h (right in Fig. 2C; see Supplementary Information, Fig. S2). Defective SOX9 expression in pre-Sertoli cells is in sharp contrast to the sustained expression of high levels of SF1/Ad4Bp (a marker specific for Sertoli and Leydig cells), GATA4 and WT1 (markers for

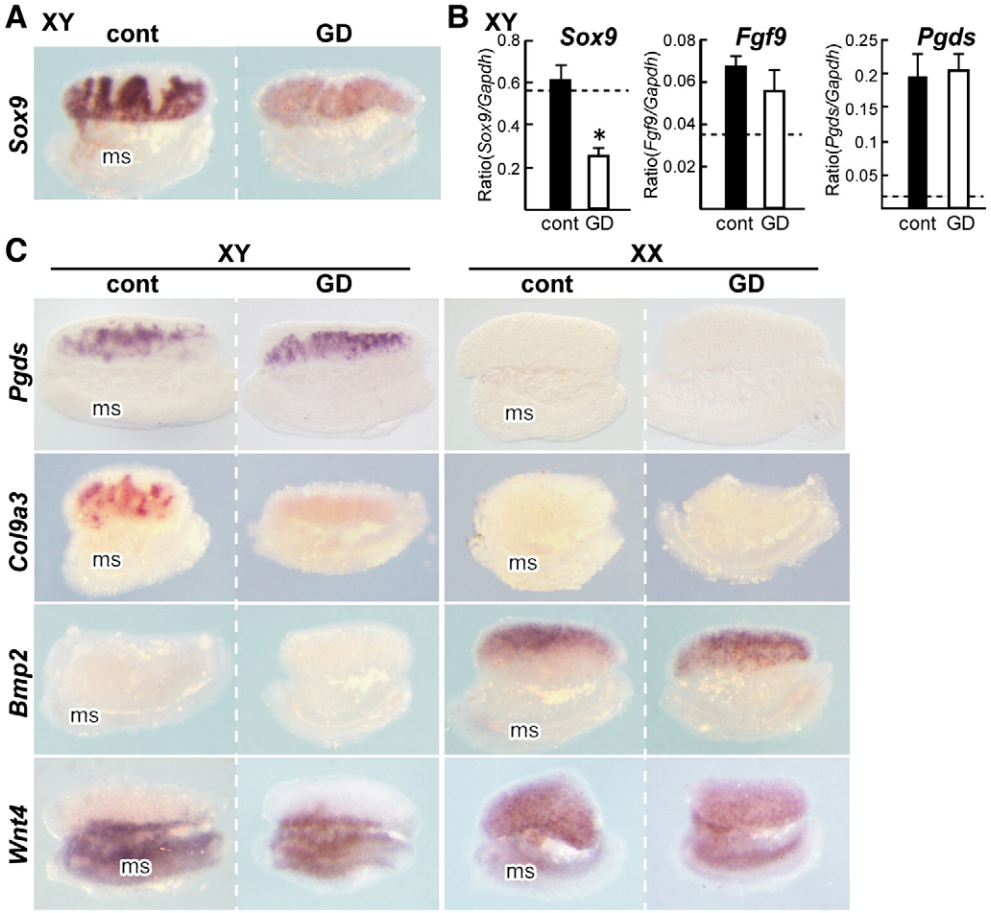

Fig. 3. Influence of glucose starvation on expression of *Sox9* and its downstream male- and female-specific genes in developing XY and XX gonads. Genital ridges were isolated from XY and XX embryos at 11.5 dpc (17–19 ts) and cultured for 48 h in high-glucose (control) or glucose-deprived (GD) medium. The explants were comparatively examined by whole mount *in situ* hybridization and real-time RT-PCR analyses. (A, C) Whole mount *in situ* hybridization analysis showing the effect of glucose starvation on expression of *Sox9* (A), *Pgds*, *Col9a3*, *Wnt4* and *Bmp2* (C) transcripts. In XY genital ridges, glucose starvation markedly represses expression of both *Sox9* (A) and *Col9a3* (the second row from the top in C) transcripts, but does not affect *Pgds* expression (the first row in C). In XX genital ridges, high levels of expression of both *Bmp2* and *Wnt4* are maintained in the gonadal area of GD explants (the third and fourth rows in C). ms; mesonephros. (B) Real-time RT-PCR analysis showing *Sox9* (left), *Fgf9* (middle) and *Pgds* (right) transcript levels in XY control (solid bar) and GD (open bar) explants. Vertical axis represents *Sox9*, *Fgf9* or *Pgds* expression level relative to *Gapdh*. The data represent the mean values ± standard error ($n=4$). Glucose starvation induces the significant reduction of *Sox9* expression in XY GD explants (asterisk: $p<0.05$, Student's *t*-test), although there is no significant difference in either *Fgf9* or *Pgds* expression level between control and GD explants. Horizontal broken lines indicate the expression levels of *Sox9* (0.54 ± 0.07), *Fgf9* (0.034 ± 0.001) and *Pgds* (0.016 ± 0.002) in the XY genital ridges isolated at 11.5 dpc (before culture initiation), respectively ($n=4$).

gonadal somatic cells) in the GD explants at 48 h (Fig. 2D; see Supplementary Information, Fig. S3). Therefore, these findings indicate that glucose starvation specifically causes defective maintenance of high levels of SOX9 expression in pre-Sertoli cells.

Influence of glucose starvation on expression of Sox9 and its downstream male- and female-specific genes in developing XY and XX gonads

Next, we examined the effect of glucose starvation on expression of *Sox9* and its downstream genes, *Fgf9* (Kim et al., 2006), *Pgds* (prostaglandin D synthase; Wilhelm et al., 2005, 2007) and *Col9a3* (McClive and Sinclair, 2003), in addition to early ovarian somatic cell markers, *Wnt4* and *Bmp2* (Mizusaki et al., 2003; Chaboissier et al., 2004).

Consistent with our anti-SOX9 immunostaining data shown in Fig. 2B, glucose starvation clearly reduced signal intensity for *Sox9 in situ* hybridization (Fig. 3A). Real-time RT-PCR analyses also confirmed the significant reduction of *Sox9* mRNA expression in XY GD explants. However, the expression levels of both *Fgf9* and *Pgds* that are crucial for the maintenance of *Sox9* expression in a positive feed-forward manner (Wilhelm et al., 2005; Malki et al., 2005; Kim et al., 2006) were not significantly altered in the same cDNA samples of XY GD explants (Fig. 3B). In contrast to normal *Pgds* expression in the XY GD explants, the expression of *Col9a3*, another possible SOX9 target, was severely repressed by the glucose starvation (Fig. 3C), suggesting the distinct influences by GD in expression among various *Sox9*-downstream genes.

The ovarian somatic cell markers, *Bmp2* and *Wnt4*, clearly showed the female-specific high expression in the gonadal area of the high-glucose explants ("cont" in Fig. 3C), which roughly mirrored their *in vivo* expression patterns in the ovary at 12.5 dpc (data not shown). Interestingly, the female-specific high levels of *Bmp2* and *Wnt4* expression appear to be not affected in XX GD explants ("XX" in Fig. 3C), suggesting no appreciable defects in ovarian development under the same GD conditions.

No appreciable defects in testis-specific cell proliferation, mesonephric cell migration or vasculogenesis under GD culture conditions

In order to clarify the influence of glucose starvation on testis-specific cellular events other than glycogenesis and testis cord formation (see reviews by Capel, 2000; Kanai et al., 2005), we examined coelomic epithelial cell proliferation, mesonephric cell migration and vasculogenesis in GD and control cultures using XY genital ridges isolated at 11.5 dpc (Fig. 4).

First, anti-BrdU immunohistochemistry demonstrated that cell proliferation activities were high in somatic cells at and near the coelomic epithelium of XY genital ridges of both control and GD conditions (left in Fig. 4A). However, no appreciable differences in the mitotic activity were observed between control and GD explants. Anti-

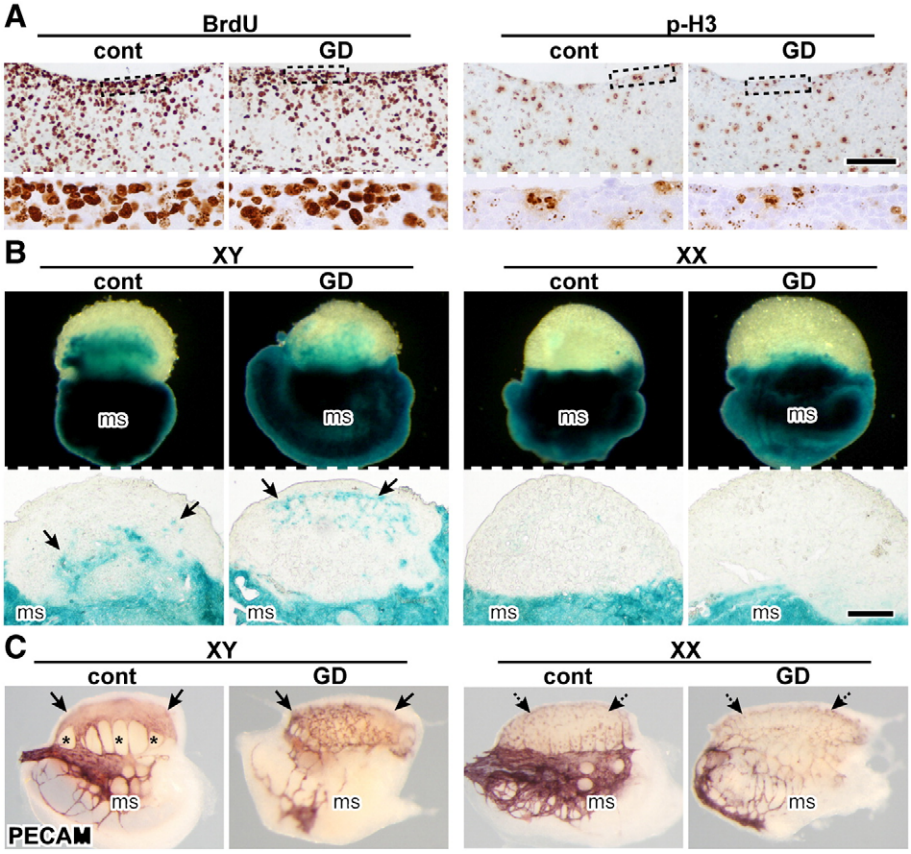

Fig. 4. Testis-specific morphogenetic events of cell proliferation (A), mesonephric cell migration (B) and vasculogenesis (C) in XY genital ridges under GD culture conditions. (A) Anti-BrdU and phospho-Histone-H3 (p-H3) immunostaining (brown) showing cell proliferation activity in 11.5-dpc XY explants cultured in high-glucose (cont) or glucose-deprived (GD) medium (12 h). In the experiments using anti-BrdU (left) and p-H3 (right) staining, there is no appreciable difference in cell proliferation activity between control and GD explants. Lower plates show higher magnified images of the coelomic epithelia indicated by broken lines in upper plates. (B) Mesonephric cell migration assay (upper, whole mount view; lower, sagittal frozen sections; LacZ [blue] staining) using wildtype XY or XX gonads and *Rosa26*-derived (LacZ-positive) XY mesonephroi isolated from 11.5-dpc embryos. The wildtype gonad and LacZ-positive mesonephros were re-combined and cultured in control and GD medium for 3 days. LacZ-positive mesonephric cells were frequently found in the gonadal area of both control and GD XY explants (arrows, left in B), while few positive cells are detectable in the gonadal area of the XX explants (right in B). (C) Whole mount anti-PECAM staining (brown) of XY and XX genital ridges cultured in control or GD medium for 3 days. In both control and GD XY explants, well-developed vasculatures are newly formed just beneath the coelomic epithelia (solid arrows). This is in contrast to fewer PECAM-positive cells seen just beneath the coelomic epithelia in the XX genital ridges (broken arrows). Asterisks, testis cords; ms, mesonephros. Bar, 100 μm.

phospho-Histone-H3 staining also showed no difference between the high-glucose and GD conditions (right in Fig. 4A), indicating no appreciable defects in testis-specific cell proliferation of coelomic epithelia in GD explants.

Next, a mesonephric cell migration assay was performed using wildtype gonads and *Rosa26*-derived (*LacZ*-positive) mesonephroi isolated from 11.5-dpc embryos. After being re-combined and cultured in control or GD medium (Fig. 4B), visualization of mesonephric cells by LacZ staining showed that migration of mesonephric cells into the gonadal area was induced in control cultures of XY, but not XX, gonadal explants. This observation is consistent with the data obtained in previous studies (Martineau et al., 1997). In all XY explants cultured in GD medium, LacZ-positive mesonephric cells migrated into the gonadal area in a testis-specific manner ($n=5$; "XY GD" in Fig. 4B), as was the case in the high-glucose XY explants.

Finally, anti-PECAM staining of high-glucose explants revealed that testis-specific vasculogenesis occurred in the gonadal area just beneath the coelomic epithelia of the XY genital ridges ($n=4$; solid arrows in "XY cont" of Fig. 4C), which is in contrast to the poor vascular development in the corresponding area of the XX genital ridges (broken arrows in "XX cont" of Fig. 4C). Even in GD conditions, XY explants displayed testis-specific vascular development just beneath the coelomic epithelium (solid arrows in "XY GD" of Fig. 4C), although vascularization in the gonadal area appeared to be more complex and mesh-like due to the lack of testis cords. This finding was supported by other histological analyses in which well-developed vasculatures beneath the coelomic epithelium were frequently found in XY glucose-deprived explants (see "v" in Figs. 1A, E, 2D etc).

These data suggest that morphogenetic cellular events occur properly in other somatic cell types in XY GD explants.

Glucose concentration-dependent defects in SOX9 expression/cord formation and their metabolic rescue by pyruvate in XY GD explants

XY genital ridges (at 11.5 dpc) were cultured in medium at intermediate glucose levels (final concentrations: 50 and 185 mg/dl) for 3 days, and histochemically compared with GD (5 mg/dl) and control (410 mg/dl) explants (Fig. 5; upper column in Table 1).

In GD explants (5 mg/dl), neither high-level SOX9 expression nor cord formation was detected in XY explant cultures (Figs. 1A, 2B,C, 5A). In XY explants at both 50 and 185 mg/dl glucose, however, recovery of both SOX9 expression and cord formation was observed in a glucose-concentration-dependent manner (Figs. 5B, C). Interestingly, in explants at 185 mg/dl glucose, SOX9-positive cells and their cord-like structures were formed partially in the gonadal area near the adjacent mesonephros, but not in the gonadal area on the coelomic side. This observation is in contrast to the well-defined testis cords formed throughout the gonadal area of control XY gonads (410 mg/dl; Fig. 5D).

To ascertain whether the sensitivity to glucose starvation is due to ATP deprivation, pyruvate (550 mg/dl; 50 mM) was added to GD medium (final concentration: 5 mg/dl glucose+495 mg/dl pyruvate) as an alternative energy source to 450 mg/dl (25 mM) glucose, and the testis cord formation was histologically estimated at 3 days after culture initiation. As shown in Fig. 5E, the addition of pyruvate restored both high levels of SOX9 expression and testis cord formation, as was the case in control XY explants (Fig. 5D). Therefore, reduced SOX9 expression and defective cord formation in GD explants are likely to be due to the lower energy availability of the GD medium.

Influence of glucose starvation on initial SOX9 activation and glycogenesis in XY 11.0-dpc genital ridges

The present glucose-deprivation experiments using genital ridges at 11.5 dpc (17–19 ts) demonstrated that the maintenance of SOX9 activation in pre-Sertoli cells is one of the processes most sensitive to glucose starvation in gonadal sex differentiation. In order to clarify the influence of glucose starvation on initial SOX9 activation and glycogenesis in early testis differentiation (11.2–11.5 dpc), we examined SOX9 expression, glycogen accumulation and cord formation in GD (5 mg/dl) and high-glucose (410 mg/dl) cultures using XY genital ridges isolated at 11.0 dpc (12–13 ts, at onset of SRY expression) (Fig. 6; lower column in Table 1).

A time-course histochemical analysis revealed that both glycogen accumulation and SOX9 activation started to occur within 12 h, and were maintained for 2 days after culture initiation in the high-glucose explants of 11.0-dpc XY genital ridges ("cont" of Figs. 6A, B). In control 11.0-dpc XY explants, testis cords were formed within the first 3 days of culture. However, testis cords appeared to be more irregular and slender in shape than those in the 11.5-dpc XY explants (Fig. 6C). In 11.0-dpc XY genital ridges under GD conditions, SRY and SOX9 expression were properly induced within 12 h after culture initiation ("GD" in Fig. 6A; see Supplementary Information, Fig. S4), but their expression was rapidly reduced to a lower level after 24 h ("GD" in Fig. 6B). In contrast to proper initial SOX9 activation even in

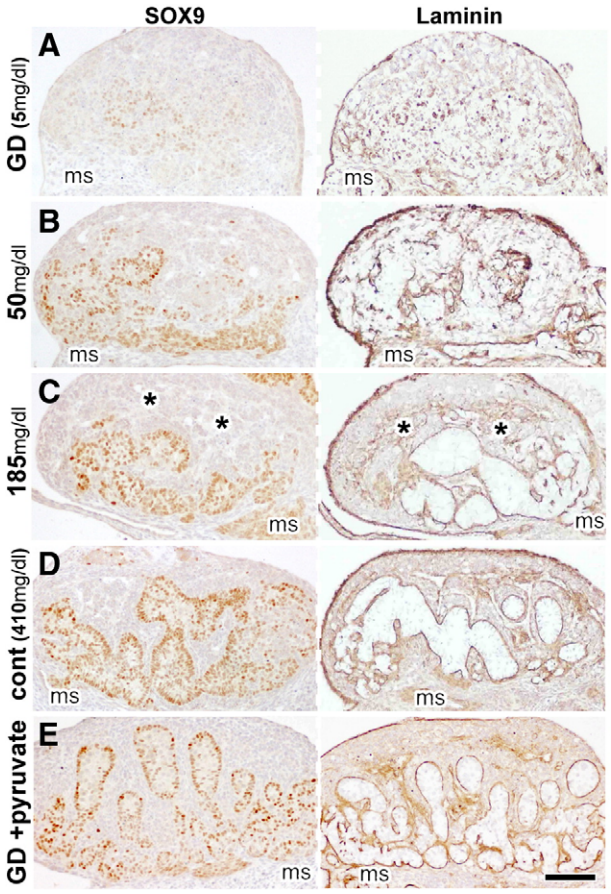

Fig. 5. Glucose concentration-dependent influence and metabolic rescue by pyruvate in 11.5-dpc XY genital ridges under GD culture conditions. XY genital ridges at 11.5 dpc (17–19 ts) were cultured for 3 days in medium at various glucose concentrations (final concentrations: 5 [GD], 50, 185 or 410 [cont] mg/dl) or GD medium plus pyruvate (final concentration: 495 mg/dl). Then the ridges were comparatively examined by anti-SOX9 (left) and anti-Laminin (right) staining. In GD (5 mg/dl) explants, neither SOX9 expression nor cord formation is detected in XY explants cultures (A), but both SOX9 expression and cord formation were clearly restored in a glucose-concentration-dependent manner in XY explants cultured with 50 or 185 mg/dl glucose (B, C). In explants cultured with 185 mg/dl glucose, SOX9-positive cells and their cord-like structures are formed partially in the gonadal area on the mesonephric side but not on the coelomic side (asterisks in C). The addition of pyruvate completely restores both high levels of SOX9 expression and testis cord formation (E). ms, mesonephros; Bar, 100 μm.

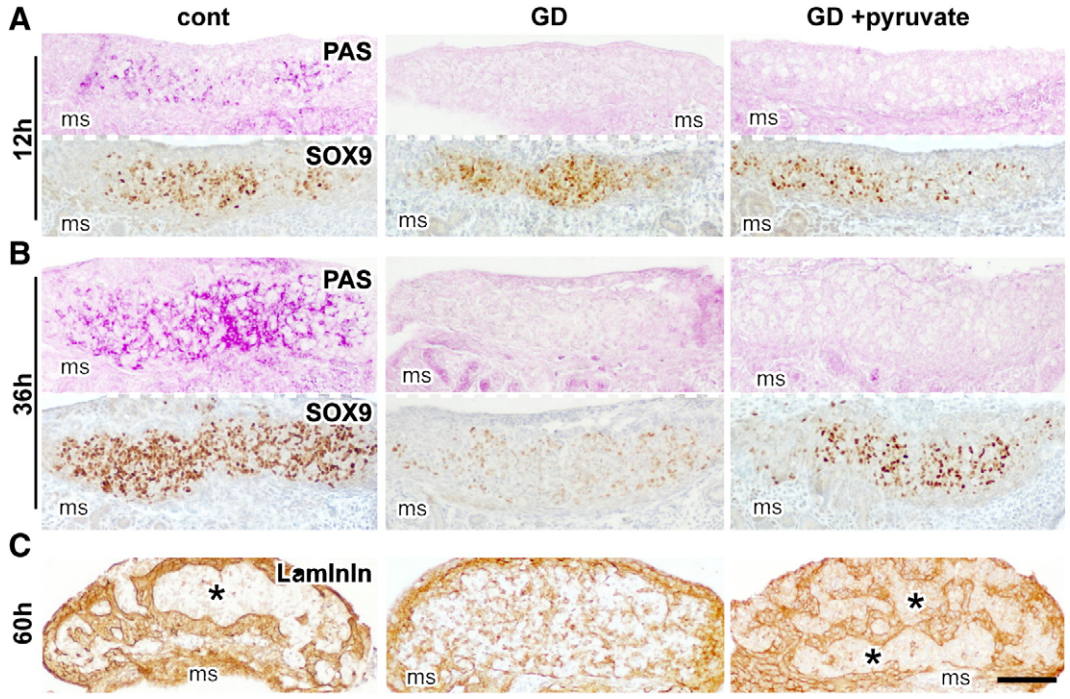

Fig. 6. Initial SOX9 activation and glycogenesis in 11.0-dpc XY genital ridges under GD culture conditions. XY genital ridges at 11.0 dpc (12–13 ts) were cultured for 12–60 h in the high-glucose (control; 410 mg/dl) or glucose-deprived (GD; 5 mg/dl) medium in the presence or absence of pyruvate (495 mg/dl). They were comparatively examined by histological PAS staining (upper in A, B; red) and anti-SOX9 (lower in A, B) or anti-Laminin (C) immunostaining (brown). Testis-specific glycogenesis (PAS), SOX9 expression and subsequent cord formation (Laminin) were properly induced in control explants using 11.0-dpc genital ridges (cont). Glucose starvation properly induces initial SOX9 expression, but its expression is not maintained at 36 h after culture initiation (B). Neither glycogenesis nor testis cord formation is detectable in GD explants. Addition of pyruvate to GD medium clearly restores the maintenance of SOX9 expression, but shows no PAS reaction in the gonadal region throughout the culture period. Asterisks in C indicate testis cords. ms; mesonephros. Bar, 100 μm.

GD conditions, glycogen accumulation in pre-Sertoli cells was not detectable throughout the culture period ("GD" in Figs. 6A, B). This implies that, although glucose starvation clearly represses testis-specific glycogenesis, glucose starvation properly, albeit transiently (12 h after GD cultures), does not prevent induction of initial SOX9 activation in pre-Sertoli cells of XY gonads at 11.0 dpc.

Since the addition of pyruvate restored both SOX9 expression and cord formation in 11.5-dpc GD explants (see Fig. 5E), we also examined the time-course patterns of SOX9 expression and glycogenesis in 11.0-dpc XY explants cultured in pyruvate (495 mg/dl)-added GD medium (5 mg/dl, "GD+pyruvate" in Fig. 6; lower column in Table 1). The addition of pyruvate clearly restored the maintenance of SOX9 expression and subsequent testis cord formation, with SOX9 expression being at a similar level to that seen in control explants. However, no glycogen deposits were detectable in the gonadal area of XY genital ridge explants throughout the culture period. This clearly demonstrates that the addition of pyruvate can rescue both SOX9 expression and subsequent cord formation, but not restore testis-specific glycogenesis, in pre-Sertoli cells in GD cultures. This finding in turn suggests that endogenous glucose storage of pre-Sertoli cells may not be essential for subsequent testis formation, assuming developing XY gonads are sufficiently supplied with exogenous glucose from blood vessels (Brennan et al., 2002).

External supply of extracellular matrix (ECM) gel is able to rescue the defective maintenance of SOX9 expression by glucose starvation

The present data demonstrate that the sustained high-level expression of SOX9 in pre-Sertoli cells is a metabolically active process with sex-dimorphic high-energy requirements. In order to define the metabolically active step, we finally carried out the rescue experiments in the GD culture of 11.5-dpc genital ridges by the external supply of several candidate factors required for the establishment of SOX9 expression in Sertoli cell differentiation.

It was previously reported that both FGF9 and PGD2 can help the maintenance of *Sox9* expression by forming a positive feed-forward loop (Malki et al., 2005; Wilhelm et al., 2005, 2007; Kim et al., 2006). First, we added exogenous FGF9 (100 ng/ml) or PGD2 (500 ng/ml) into the GD medium, and cultured the XY genital ridges in the presence or absence of these additives under GD condition for 2 days. The addition of either FGF9 or PGD2 could not rescue the defective maintenance of SOX9 expression in pre-Sertoli cells under GD condition (see Supplementary Information, Fig. S5A). All explants treated with FGF9 or PGD2 also showed defective formation of the well-defined testis cords in the gonadal area ($n=10$ for each additive). This is clearly consistent with the present real-time RT-PCR data showing no significant reduction in both *Fgf9* and *Pgds* expression in the XY GD explants (Fig. 3B).

The present data also demonstrated the defective formation of basal lamina (Fig. 1C) and the drastically reduced expression of ECM molecules (such as Laminin and *Col9a3*) in pre-Sertoli cells of the XY GD explants (Figs. 2C, 3C). It was previously reported that several members of ECM molecules are directly up-regulated by SOX9 itself (Bell et al., 1997; Liu et al., 2007, Hanley et al., 2008), and that certain ECM molecules can act as a possible mediator of FGF9 signaling in pre-Sertoli cells (Schmahl et al., 2004). Finally, to clarify a possible contribution of reduced ECM molecules and FGF9 signaling to GD-dependent dysfunction of pre-Sertoli cells, the 11.5-dpc genital ridges were cultured with ECM gel (a Matrigel solution that had been dialyzed overnight against cold glucose-free DMEM) in the presence or absence of FGF9 (100 ng/ml) under GD condition (5 mg/dl) for 2 days (Fig. 7; Table 2). In parts of the 11.5-dpc XY genital ridges, we excised the anterior and posterior edges to directly expose pre-Sertoli cells to Matrigel (Fig. 7A). The segmented or whole explants were embedded in Matrigel on the filters, and then cultured in GD medium (5 mg/dl).

In the segment cultures, both SOX9 expression and cord formation were induced in the control (high-glucose) medium, but defective in

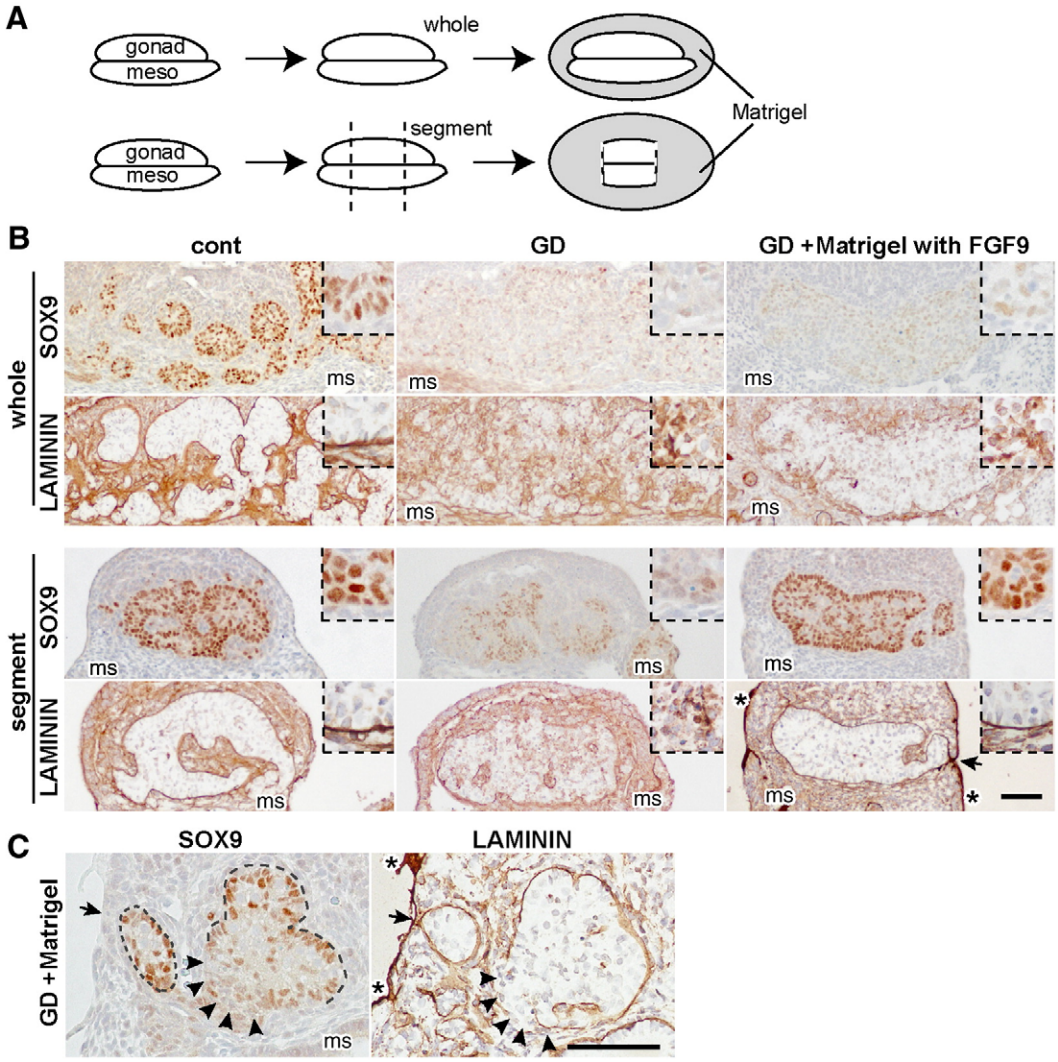

Fig. 7. External supply of extracellular matrix (ECM) gel around pre-Sertoli cells is able to restore SOX9 expression and subsequent testis cord formation under GD culture conditions. (A) A schematic representation showing the whole (upper) or segment (lower) culture experiment of 11.5-dpc genital ridges embedded in Matrigel on the filter (GD medium; 2 days). In parts of the genital ridges, the anterior and posterior edges were excised (lower in A) before embedding in Matrigel. (B, C) Anti-SOX9 and anti-Laminin immunostaining of two serial sections showing the whole (upper in B) or segment (lower in B; C) explants cultured in high-glucose (cont; left) or glucose-deprived (GD; middle) medium for 2 days. Immunostaining images of the XY GD explants embedded in Matrigel with FGF9 (100 ng/ml) are shown in right plates of B (GD+Matrigel with FGF9), while higher magnified images of the gonadal area near the cutting edges in the XY GD segment explant in Matrigel (without FGF9) are shown in C (GD+Matrigel). In the segment cultures, both high-level SOX9 expression and testis cord organization are properly induced in the control (high-glucose) medium (left lower in B), but defective in the glucose-deprived medium (middle lower in B), as was the case for the whole explants (left and middle upper in B). Regardless of the presence or absence of exogenous FGF9 addition, the external supply of Matrigel can restore both high-level SOX9 expression and testis cord organization in the segment explants (right lower in B; C), but not in the whole explant (right upper in B) under the same GD conditions. In the XY GD explants embedded in Matrigel, SOX9 expression appears to be higher in the Sertoli cells directly attached on the ECM in the gonadal area near the cutting edge (arrows in C). Insets in B show higher magnified images of pre-Sertoli cells. In B (right bottom plate) and C, asterisks show anti-Laminin positive signals in the exogenous Matrigel, while arrows indicate the cutting edges of the explants. In C, arrowheads or broken lines mark the region corresponding to the sparse broken or strong continuous signals for anti-Laminin staining, respectively. ms; mesonephros. Bar, 100 μm.

the GD medium (lower plates, Fig. 7B), as was the case in the control and GD explants using whole genital ridges (upper plates, Fig. 7B; see also Figs. 2B, C, 5). In GD cultures using whole genital ridges, the addition of either ECM gel alone or ECM gel/FGF9 did not compensate for the defective SOX9 expression and cord formation (right upper plates in Fig. 7B; upper column in Table 2), suggesting non-utilization of the additives as an alternative metabolic energy source to glucose. Interestingly, in the segment explants cultured in GD medium, the external supply of ECM gel with FGF9 markedly restored both SOX9 expression and testis cord formation (right lower plates in Fig. 7B; lower column in Table 2). A similar recovery of both SOX9 expression and cord formation was also observed in the segment explants treated with ECM gel alone (without FGF9 addition) (Fig. 7C; see Supplementary Information, Fig. S5B). Unfortunately, we could not find any appreciable positive effects of exogenous FGF9 addition in the segment explants embedded in ECM gel (Table 2), but SOX9 expression appeared to be higher in the cells directly attached on the ECM gel in the gonadal area near the cutting edges, as compared with those near the area with sparse ECM deposits (Figs. 7C, S5B). These findings, therefore, indicate that the external supply of ECM gels around pre-Sertoli cells can restore and maintain a high-level SOX9 expression under GD condition. This finding in turn suggests that the ECM-mediated feed-forward pathway of SOX9 expression is likely to be a rate-determining step in glucose-energy metabolism during gonadal sex differentiation.

Discussion

It has previously been shown that testis-specific glycogenesis in pre-Sertoli cells occurs immediately after the onset of *Sry* expression,

Table 2
Summary of testis cord formation in glucose-deprived (GD) cultures using the whole or segmented genital ridges that are embedded in Matrigel with or without FGF9

	Glucose concentration in medium (mg/dl)	Additives (Matrigel [dialyzed], FGF9 [100 ng/ml])	Testis cord formation[a]				
			++	+	+/−	−	Total no. of explants
Whole explant	410 (high-glucose)	None	19	0	0	0	19
	5 (GD)	None	0	0	3	13	16
	5 (GD)	Matrigel	0	0	0	10	10
	5 (GD)	Matrigel+FGF9	0	0	1	8	9
Segment explant	410 (High-glucose)	None	19	0	0	0	19
	5 (GD)	None	0	0	3	11	14
	5 (GD)	Matrigel	11	3	0	0	14
	5 (GD)	Matrigel+FGF9	10	3	0	0	13

Effects of the external supply of Matrigel (with or without FGF9) on testis cord formation were histologically estimated in the segmented or whole explants of XY genital ridges at 11.5 dpc (3-day culture).

[a] Number of explants showing i) no cord structures (−), ii) certain cord-like structures partially detected in the gonadal area (+/−), iii) well-defined cords in some parts of the gonadal area or well-defined, but slender/irregular cords formed throughout whole gonadal area (+), or iv) well-defined cords throughout whole gonadal area (++).

indicating the presence of sex-dimorphic energy metabolism in mouse gonadal sex determination (Matoba et al., 2005). However, which cell types and what cellular/molecular events require a high-energy metabolic rate in gonadal development was unknown. The present study demonstrated that, despite high levels of glycogen storage in pre-Sertoli cells, glucose starvation severely reduces SOX9 expression in pre-Sertoli cells, leading to defective testis cord formation. No appreciable defects were found in other testis-specific cellular events such as coelomic epithelial cell proliferation, mesonephric cell migration and vasculogenesis. Moreover, high levels of expression of *Bmp2* and *Wnt4*, both female-specific somatic cell markers, were properly induced and maintained in developing XX gonads under the same glucose-deprived conditions. These data suggest that pre-Sertoli cells are the most sensitive to glucose starvation in gonadal sex differentiation.

The present ultrastructural and anti-GRP78 immunohistochemical analyses also confirmed that glucose-deprived characters (i.e., ER enlargement and GRP78 up-regulation) were specifically detected in presumptive pre-Sertoli cells of the genital ridge (Figs. 1C–F). Even in control (high-glucose) explants, GRP78 expression was detectable, albeit weak, in Sertoli cells inside the testis cords, suggesting an inadequate supply of glucose or other energy source even in the high-glucose (control) medium. Since XX/*Sry* pre-Sertoli cells were also sensitive to glucose starvation, it is likely that certain cellular/molecular events downstream of *Sry* action require a higher metabolic rate in pre-Sertoli cells. This notion is consistent with previous reports which have shown that pre-Sertoli cells play a pivotal role in the induction of testis formation such as cell proliferation, vascularization, and testis cord formation (Burgoyne et al., 1988, also see reviews by Capel, 2000; Kanai et al., 2005).

It is well known that SRY action induces testis-specific *Sox9* up-regulation in early testis differentiation (Sekido et al., 2004, 2008; Kidokoro et al., 2005). In contrast to transient *Sry* expression, *Sox9* expression in pre-Sertoli cells continues throughout life, suggesting a distinct difference between the mechanisms which underly the initiation and maintenance of *Sox9* expression in gonadal supporting cells. It is most likely that continuous *Sox9* expression is crucial for both testis formation and its subsequent maintenance in the developing testis (Chaboissier et al., 2004; Barrionuevo et al., 2006). This finding is also supported by the observation that, although the initial *Sox9* expression is induced properly in several mutant gonads without proper cord formation, such as *Fgf9* (Colvin et al., 2001), *Wt1* (Gao et al., 2006) and *Wnt4* (Kim et al., 2006) mutants, its subsequent maintenance is defective. Our data demonstrated that glucose starvation severely affects maintenance of SOX9 expression in pre-Sertoli cells while SF1/Ad4Bp, GATA4 and WT1 expression continues to be maintained at a high level. Moreover, both initial SOX9 activation and SRY expression appear to be properly induced in the same GD culture conditions using 11.0-dpc XY genital ridges (Figs. 6, S4). Therefore, the maintenance mechanism of SOX9 expression is one of the processes most sensitive to glucose deprivation in differentiating pre-Sertoli cells. This in turn implies that the establishment of sustained SOX9 expression is a metabolically active process with sex-dimorphic high-energy requirements in gonadal sex differentiation.

In mouse testis development, several recent studies have demonstrated that sustained high levels of SOX9 expression in pre-Sertoli cells are mediated through several signaling molecules such as FGF9 (Schmahl et al., 2004; Kim et al., 2006), PGD2 (Wilhelm et al., 2005; Malki et al., 2005) and Vinexin (Matsuyama et al., 2005). In particular, FGF9 signaling has been shown to be essential for SOX9 expression and subsequent testis cord formation (Colvin et al., 2001; Schmahl et al., 2004). In pre-Sertoli cells, it has also been shown that FGF9 acts in a positive feed-forward loop to directly or indirectly stabilize SOX9 expression through specific ECM components (Schmahl et al., 2004). Interestingly, the present data showed that both basal lamina formation and production of several ECM molecules were severely affected in XY GD explants, in contrast to the proper maintenance of *Fgf9* expression under the same GD condition. Moreover, our rescue experiments demonstrated that the external supply of ECM gel could restore the maintenance of SOX9 expression in XY GD explants (Table 2, Fig. 7). These findings possibly suggest that some ECM components are involved in the positive feed-forward regulation of SOX9 expression by stabilizing endogenous FGF9 signals, although the external supply of FGF9 did not exert any appreciable effects on the SOX9 expression in XY GD explants. However, it is possible that ECM gel itself did directly restore the reduced SOX9 expression and defective epithelial morphogenesis in pre-Sertoli cells, as demonstrated in the *in vitro* model system of epithelial morphogenesis using the embryonic stem cells with a dominant negative *Fgfr2* mutation (Li et al., 2001). Moreover, it is well known that various growth factors and cytokines are also known to be embedded in the Matrigel. Therefore, the following possibilities cannot be excluded at this stage: rescue by 1) the contribution of growth factor contamination, 2) the trophic compensatory mechanism in pre-Sertoli cells by external ECM supply, and 3) the extracellular microenvironment surrounded by the basal lamina to the restored SOX9 expression in XY GD explants. Further analyses on the maintenance of SOX9 expression through ECM molecules are needed to clarify the mechanisms involved.

Another interesting point is that glucose starvation exerted the distinct influences in expression among various *Sox9*-downstream genes: no significant changes in either *Fgf9* or *Pgds* expression, but severely reduced expression of *Col9a3* and Laminin in XY GD explants. Since both *Fgf9* and *Pgds*, as well as *Sox9*, are already highly expressed in the XY 11.5-dpc genital ridges before culture initiation (Mizusaki et al., 2003; Schmahl et al., 2004), this finding suggests that such reduced SOX9 expression levels by glucose starvation are sufficient for the maintenance of *Fgf9* and *Pgds* expression, or that the expression of ECM molecules such as *Col9a3* requires high-level SOX9 expression in testis differentiation. This is consistent with the previous report showing the dosage-dependent reduced expression of certain SOX9-target genes such as *Col2a1* by *Sox9* haploinsufficiency (Bi et al., 2001).

How significant is glycogen storage in pre-Sertoli cells just before testis formation? During mouse testis differentiation *in vivo*, both glycogen accumulation and SOX9 expression start to occur immediately after *Sry* expression (approximately 11.2 dpc), with the level of glycogen storage in pre-Sertoli cells reaching a peak by 11.5 dpc, just before the onset of the rapid decline in *Sry* expression (see Fig. 8). The glycogenesis in pre-Sertoli cells is likely to be mediated through insulin/IGF signals that are required for *Sry* expression and testis differentiation in mice (Nef et al., 2003). Moreover, it is possible that this glycogenesis (approximately 11.0–11.5 dpc) may reflect an excessive glucose/energy state around and in pre-Sertoli cells. This is

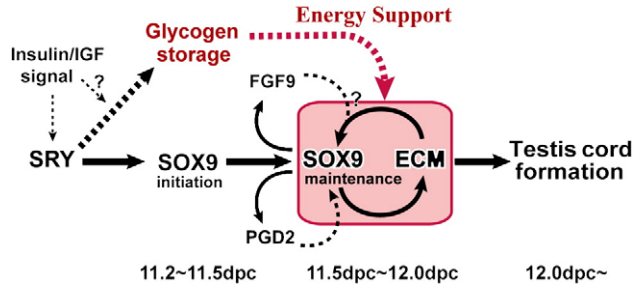

Fig. 8. A schematic representation showing testis-specific glycogen storage and glucose metabolism in differentiating Sertoli cells. In testis differentiation, both glycogen accumulation and SOX9 expression start to occur immediately after *Sry* expression (11.2 dpc), with the level of glycogen storage in pre-Sertoli cells reaching a peak by 11.5 dpc, just before the onset of the rapid decline in *Sry* expression. Such glycogen storage is likely to be induced through insulin/IGF signals that are required for *Sry* expression. After a decline in *Sry* expression, the maintenance of SOX9 expression is mediated through a positive-feedback system of several signaling (FGF9 and PGD2) and ECM molecules during 11.5 to 12.0 dpc. Among these SOX9-maintenance feed-forward pathways, the ECM-mediated step is likely to be the rate-determining step in glucose-energy metabolism. Glycogen storage in pre-Sertoli cells is a backup energy source for the maintenance and establishment of SOX9 expression at 11.5–12.0 dpc and probably for subsequent cord formation after 12.0 dpc.

consistent with our data that showed no appreciable defects in either SRY expression or initial SOX9 activation within 12 h after the initiation of GD culture of 11.0-dpc genital ridges. During 11.5 to 12.0 dpc, it is likely that the maintenance of SOX9 expression is mediated through a positive-feedback system of several signaling/ECM molecules instead of SRY action (Schmahl et al., 2004; Malki et al., 2005; Wilhelm et al., 2005; Kim et al., 2006). The present study demonstrated that the ECM-mediated step of these maintenance pathways is most likely to require a high-glucose energy metabolic rate in pre-Sertoli cells. This further suggests that glycogen storage in pre-Sertoli cells is a backup energy source for this step of the maintenance regulation of SOX9 expression at 11.5–12.0 dpc. Since testis-specific vasculogenesis occurs at around 11.5–12.0 dpc (Brennan et al., 2002), pre-Sertoli cells may receive a testis-specific energy supply from both exogenous blood-derived glucose/oxygen and endogenous glucose from the glycogen storage in their cytoplasm. After 12.0 dpc, the maintenance mechanism for the high levels of SOX9 expression appears to become stabilized in the differentiating Sertoli cells because, in organ cultures using 12.0-dpc XY genital ridges, both SOX9 expression and cord formation are properly maintained in most XY GD explants for 2–3 days (Matoba and Kanai, unpublished).

In conclusion, our results suggest that testis-specific glycogen storage of pre-Sertoli cells is available as an energy source to establish SOX9 expression in pre-Sertoli cells at 11.5–12.0 dpc. Moreover, to the best of our knowledge, this study is the first to demonstrate the existence of a distinct glucose metabolic state that is dependent upon cell type, developmental stage and molecular/cellular events in mammalian organogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.09.004.

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