Study of Sex Dimorphic Energy Metabolism

in Mouse Gonadal Sex Differentiation

(マウス性分化期生殖腺のエネルギー代謝に関する研究)

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GENERAL INTRODUCTION

An “organ” is a group of cells or tissues that perform a specific function or group of functions in a matured organism. When we observe the detailed anatomy/histology of an organ, we see an intricate and precise arrangement of many types of cells. In the process of embryonic development, an organ originates from a primordium, which is defined as the initial founder group of cells that give rise to a tissue, involving differentiation of cells and organization into multicellular arrangements. This dynamic cellular differentiation and organization process as a whole is called “organ morphogenesis” or “organogenesis” in developmental biology. During the decades, the explosive progress of developmental biology, especially at a molecular level, such as transgenic and knockout mouse models, and genome sequence analyses, unveiled the well organized underlying molecular mechanisms of organogenesis. As a result, however, I think that researchers have forgotten several notions that ever mentioned before the invention of molecular approach technique. Among these notions, I focused on “energy metabolism” (Spielmann, 1973; New, 1978; Cockroft, 1979). Based on the facts that organogenesis involves numerous dynamic cellular events and that each biological step requires comparable amounts of energy in general, I hypothesize that there must exist something well organized regulatory mechanisms of energy metabolism in organogensis. However, to the best of my knowledge, there is no any systematically-studied information concerning energy metabolism, including both [energy supply] and [energy demand] aspects, in organogenesis. Therefore, here I set the purpose of this study to systematically investigate the involvement of energy metabolism in organogenic process, and finally integrate the concept of “energy metabolism” into “organogenesis” at a molecular level.

The gonad, or genital ridge, is unique among all organ primordia, because of its bipotential ability to differentiate into either a testis or an ovary. In mice, gonadal primordia develop at around 10.5 days *post coitum* [dpc] as a thickening of epithelial layer facing the body cavity, i.e. coelomic epithelium, in the region that overlays the ventral surface of mesonephros (Byskov, 1986). The genital ridges of each sex are morphologically indistinguishable until 11.5 dpc (Fig. 1). In male gonads, a portion of gonadal somatic cells starts to differentiate into Sertoli cells (precursor of Sertoli cell; pre-Sertoli cell), those are essential in subsequent testis differentiation, at around 11.5 dpc. Following the differentiation of pre-Sertoli cells, various testis-specific morphogenetic events such as cell proliferation, migration, vasculogenesis and cord formation are known to occur and direct early testiculogenesis. Increased proliferation of the coelomic epithelium of gonads occurs between 11.3 and 12.0 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 the formation of various somatic cell types in testicular interstitium (Buehr *et al*., 1993; Martineau *et al*., 1997; Capel *et al*., 1999; Brennan *et al*., 2002). This mesonephric migration which is essential for proper testis cord formation also occurs in a testis-specific manner (Martineau *et al*., 1997; Tilmann and Capel, 1999). The male-specific reorganization of vasculature just beneath coelomic epithelia is coincident with cord formation between 11.5 and 12.5 dpc (Combes *et al*., 2008). In female gonads, on the other hand, no clearly-defined morphogenesis is detected in ovarian differentiation, and the onset of folliculogenesis occurs shortly before birth (18.0 dpc in mice). Thus, the testis-specific morphogenetic events during sex differentiation period suggest that male gonads have a higher energy metabolism than female ones (Fig. 1; Mittwoch, 2007). Therefore, in this study, I focused on gonadal

differentiation just after sex determination as a particularly interesting model to investigate the relation between energy metabolism and organogenesis.

Sex of individual, in most mammals, is genetically determined depending on its chromosomal composition XX: female or XY: male. That is ultimately dependent on the absence or presence of Y-chromosome (See review by Brennan and Capel, 2004; Wilhelm *et al*., 2007). The development of secondary sex characteristics, including the external genitalia, male or female ductal systems and sex dimorphic behaviors, depends on whether the gonad develops as a testis or an ovary. That is, the differentiation of the testis or ovary determines the sex of the whole embryo. After the long controversy, the testis-determining gene on Y chromosome was identified in humans and mice by the mutation analyses in 1990, and named as *Sry* (Sex-determining region on Y chromosome) (Sinclair *et al*., 1990). Following mouse model experiments, XY mice with no functional *Sry* develop ovaries; conversely, the addition of *Sry* to XX mice triggers the testis pathway, proved that *Sry* is the long-sought “master gene” of testis determination (Koopman *et al*., 1991). *Sry* encodes a putative transcription factor that contains a high mobility group (HMG)-box DNA-binding domain (Sinclair *et al*., 1990; Koopman *et al*., 1991), and is conserved in most mammalian species, but not among vertebrates. Since the discovery of *Sry*, a considerable progress has been made over the last 18 years. Several genes involved in various aspects of mammalian sex determination have been identified, including *Sox9* (Foster *et al*., 1994; Wagner *et al*., 1994), which is one of HMG-box containing *Sry*-related genes, steroidogenic factor 1 (*SF1/Ad4Bp*) (Hatano *et al*., 1994; Ikeda *et al*., 2001), Wilms’ tumor suppresser 1 (*Wt1*) (Hammes *et al*., 2001), GATA-binding protein 4 (*GATA4*) (Tevosian *et al*., 2002), fibroblast growth factor 9 (*Fgf9*) (Colvin *et al*., 2001), forkhead box L2 (*Foxl2*) (Loffler *et al*., 2003), *Wnt4* (Vainio *et al*., 1999; Jeays-Ward *et al*., 2004), a member of the wingless family genes, and

R-spondin 1 (*Rspo1*) (Parma *et al*., 2006). However, the ever identified direct target gene of SRY is *Sox9* alone (Sekido and Lovell-Badge, 2008). SRY directly binds to multiple gonad-specific enhancers of *Sox9* and together with SF1/Ad4Bp, cooperatively upregulates *Sox9* expression in mice (Sekido and Lovell-Badge, 2008).

In mice, *Sry* is transiently expressed in a center-to-pole wave along the anteroposterior (AP) axis of XY gonads for a very short period, from 11.0 dpc to 12.0 dpc, just before testis formation (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001; Kidokoro *et al*., 2005). Shortly after the onset of *Sry* expression, 11.2 dpc; 4 hours [h] later, *Sox9* is also up-regulated in pre-Seroli cells in a center-to-pole pattern, similar to that of the initial *Sry* expression profile (Kent *et al*., 1996; Morais da Silva *et al*., 1996; Schepers *et al*., 2003). As described above, *Sox9* is verified as the direct target of SRY in mice (Sekido and Lovell-Badge, 2008). Human *SOX9* mutation causes XY sex reversal in most cases (Foster *et al*., 1994; Wagner *et al*., 1994). Homozygous deletion of *Sox9* in mouse XY gonads interferes with testis differentiation (Chaboissier *et al*., 2004). Moreover, miss-expression of *Sox9* in mouse XX gonads results in testis development, even in the absence of *Sry*, as demonstrated by the findings from two transgenic lines (Bishop *et al*., 2000; Vidal *et al*., 2001; Qin and Bishop, 2005). These facts demonstrate that *Sox9* can act as a testis-determining gene in mammalian sex differentiation as well as *Sry*. Unlike *Sry*, however, *Sox9* continues to be expressed in pre-Sertoli cells throughout testis development (Kent *et al*., 1996; Morais da Silva *et al*., 1996; Schepers *et al*., 2003). Therefore, 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.

Every vital process of animal cell requires comparable amounts of energy, and

cellular energy is provided in catabolic processes of several nutrients including carbohydrates, amino acids and fatty acids. Basically, all these nutrients are provided to peripheral tissues/cells through circulating blood flow. Additionally, for preparing excessive energy consumptions, cells deposit secondary energy in cytoplasm, such as glycogen granules and lipid droplets. Each energy storage has its own distinctive characteristics in several aspects. For example, glycogen is a large branched polysaccharide of glucose, and can be quickly broken down to glucose molecules when energy is needed for sudden, strenuous activity. Lipid droplet, on the other hand, consists of neutral lipid, or triacylglyceride, and is slowly hydrolyzed to provide energy for prolonged excessive consumptions (van der Vusse and Reneman, 1996; McArdle *et al.*, 2001).

The regulatory mechanism of cytoplasmic glycogen accumulation, i.e. glycogenesis, is well studied in livers, skeletal muscles and adipose tissues of matured animals (Thong and Graham, 2002; Ferrer *et al.*, 2003; Gruetter, 2003; Sinclair *et al.*, 2003). In these tissues, extracellular glucose is imported by glucose transporter family (GLUT) into cytoplasm (Wood and Trayhurn, 2003), and the imported glucose is phosphorylated and converted into glucose-6-phosphate (G6P). Thereafter, glycogen synthase (GS) catalyzes the addition of glucose residue to the nascent glycogen chain using the phosphorylated intracellular glucose as a substrate. GS activity is highly regulated through phosphorylation at multiple sites and allosteric effectors (Bollen *et al.*, 1998). Although the reaction catalyzed by GS is rate limiting for glycogen synthesis in most organs (Roach *et al.*, 1998), the regulatory mechanism of GS activity varies among each organ (Ferrer *et al.*, 2003). In livers, for example, extracellular concentration of glucose, i.e. blood-glucose level, is critical to regulate GS activity in hepatocytes. In muscles and adipose tissues, on the other hand, insulin/IGFs secreted from pancreatic

beta cells play a pivotal role in its regulation (Lawrence and Roach, 1997). In addition, in embryonic period, glycogenesis is also observed in various tissues/organs (Cabrini, 1961; Scott and Glimcher, 1971; Ohshima *et al*., 1999). However, the regulatory mechanisms of glycogenesis, ultimately GS activity, in these embryonic tissues are poorly understood at present.

A previous study has shown that embryonic testis is one of glycogen-rich tissues in mouse organogenic embryos, and that glycogen accumulation predominantly occurs in differentiating Sertoli cells within newly-formed testis cords at 12.5 dpc (Kanai *et al*., 1989). Moreover, the glycogen deposits in pre-Sertoli cells rapidly disappear shortly after testis cord formation, suggesting that the glycogen granules in pre-Sertoli cells are involved as an energy source in the dynamic morphogenesis of testis and active hormonal production at around 12.5 dpc. However, no further information concerning the mechanisms initiating glycogen accumulation and its functional significance in developing XY gonads, is available at present.

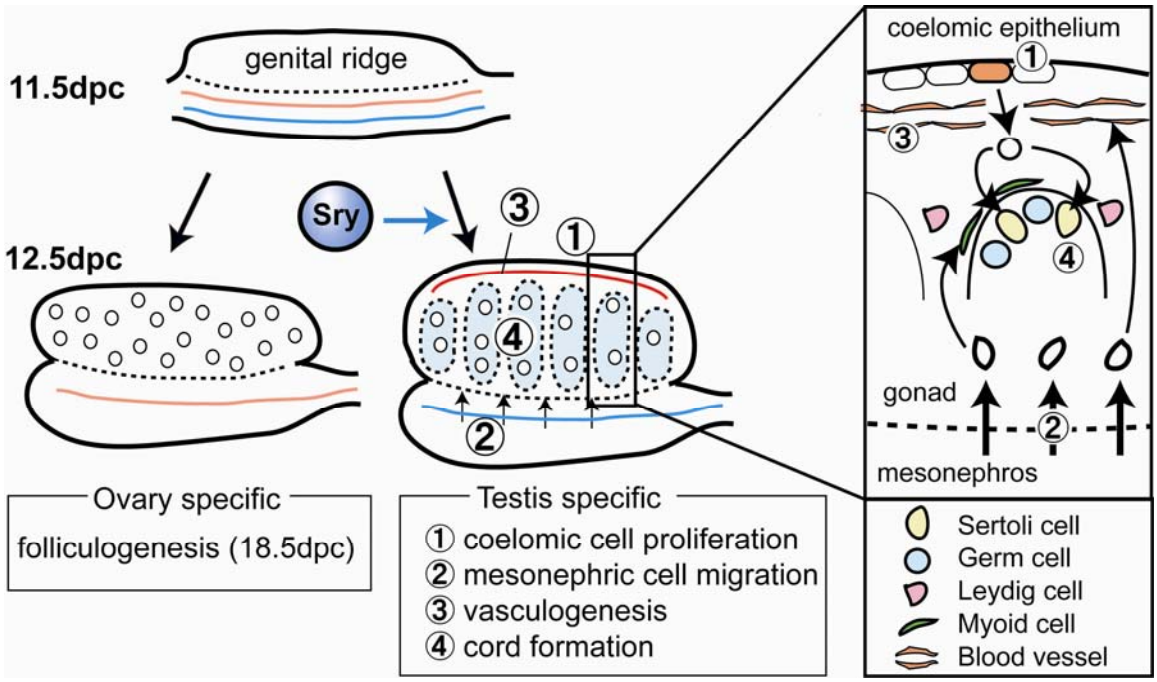
Therefore, in order to reveal the regulatory mechanism of energy metabolism and to investigate the significance of energy metabolic regulation for organogenesis, both at a molecular level, here I started this study using the gonads of sex differentiation period as a model. In chapter 1, to reveal the mechanisms regulating glycogen accumulation in male gonads, I performed detailed histological and genetic analyses and *in vitro* organ culture experiments. As a result, I report that glycogen is stored in pre-Sertoli cells, and it is regulated by direct downstream of *Sry* action through PI3K-AKT pathway. In chapter 2, in order to investigate the functional significance of glycogen or high-glucose condition on gonadal sex differentiation, *in vitro* glucose-deprivation (GD) experiments and detailed histological and molecular analyses

are conducted. Consequently, I demonstrate that in developing pre-Sertoli cells, the high-glucose metabolic state is required for establishment of SOX9 expression through an ECM (extracellular matrix)-mediated feedforward pathway. These results demonstrate the novel role of *Sry* to regulate both glycogenesis [energy supply] and high-glucose requiring events [energy demand] simultaneously. To the best of my knowledge, this is the first evidence at the molecular level that one transcription factor regulates both organ determination and energy metabolism simultaneously in mammalian organogenesis.

Fig. 1. Schematic representation of male-specific dynamic morphogenesis in mouse gonadal sex differentiation induced by *Sry*.

In mice, the genital ridges of each sex are morphologically indistinguishable until 11.5 dpc. In male gonads, shortly after the expression of *Sry*, several testis-specific dynamic cellular events, including 1) coelomic epithelial cell proliferation, 2) mesonephric cell migration into gonad, 3) vasculogenesis just beneath coelomic epitherium and 4) testis cord formation, are induced by the action downstream of *Sry*, and turns to be morphologically distinctive from female gonad until at least 12.5 dpc. In female gonads, on the other hand, no clearly-defined morphogenesis is detected in ovarian differentiation until perinatal folliculogenesis (18.0 dpc in mice). These testis-specific dynamic cellular events indicate a difference in energy metabolism between male and female gonads during sex differentiation.

Fig. 1


11.5dpc
genital ridge
Sry
12.5dpc
Ovary specific
folliculogenesis (18.5dpc)
Testis specific
① coelomic cell proliferation
② mesonephric cell migration
③ vasculogenesis
④ cord formation
coelomic epithelium
gonad
mesonephros
Sertoli cell
Germ cell
Leydig cell
Myoid cell
Blood vessel

CHAPTER 1

"A Novel Sry-Downstream Cellular Event which Preserves the Readily Available Energy Source of Glycogen in Mouse Sex Differentiation"

INTRODUCTION

In mouse sex differentiation, three testis-specific cellular events: cell proliferation, cell migration and vasculogenesis, are known to direct early testiculogenesis. These early cellular events are likely to be controlled indirectly by *Sry* and/or *Sox9*, because both *Sry* and *Sox9* are expressed specifically in pre-Sertoli cell lineage, but not in coelomic epithelium, endothelial cells, or mesonephric cells (Kent *et al*., 1996; Morais da Silva *et al*., 1996; Albrecht and Eicher, 2001; Bullejos and Koopman, 2001; Moreno-Mendoza *et al*., 2004). Although pre-Sertoli cells have been shown to aggregate in the testis cord at late phases of testis differentiation (from around 12.0 dpc), the cellular events induced immediately after *Sry* expression (starts from 11.0 dpc, peaks at 11.5 dpc and disappears until 12.0 dpc) in pre-Sertoli cells remain largely unknown.

Testis-specific cellular events clearly indicate a difference in energy metabolism between male and female gonads during sex differentiation (Mittwoch, 2004). This further suggests that XY gonads require a higher energy metabolic rate for the dynamic morphogenesis of male gonads, compared to XX gonads which exhibit no appreciable histological changes. Glucose, which plays a major role in energy metabolism, is stored as a readily available energy source, glycogen, in cells during various developmental and physiological states (Thong and Graham, 2002; Ferrer *et al*., 2003; Gruetter, 2003; Sinclair *et al*., 2003). The previous study has shown that embryonic testis is one of glycogen-rich tissues in mouse organogenic embryos, and that glycogen accumulation predominantly occurs in Sertoli cells within newly-formed testis cords at 12.5 dpc (Kanai *et al*., 1989). However, the timing of the onset and its mechanism of glycogen accumulation in developing XY gonads are still unknown.

In this chapter, I examined the spatiotemporal patterns of glycogen accumulation

and its signaling pathways during the early phases of mouse sex differentiation, in order to analyze the possible mechanism of sex-dimorphic glycogenesis in mammalian sex differentiation. As a result, the present study is the first to show that testis-specific glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of *Sry*. I also demonstrate that such glycogen deposition is mediated via PI3K-AKT signaling pathway, which is activated in gonadal somatic cells in a testis-specific manner. Therefore, these findings clearly illustrate that immediate activation of the PI3K-AKT pathway after the onset of *Sry* expression may promote testis-specific glycogen accumulation in pre-Sertoli cells. This will shed light on a potential link between *Sry* action and sex-dimorphic energy metabolism in gonadal sex differentiation of mammals.

MATERIALS AND METHODS

Animals and busulfan treatment

Embryos were obtained from pregnant female mice (ICR strain) from 10.5 to 12.5 dpc (7-30 tail somites [ts]). In some cases, the XX sex-reversal transgenic line carrying the *Sry* transgene (Kidokoro *et al*., 2005) was also used in this study. After counting the tail somite number and separating the head tissues in each embryo for sex determination, genital ridges (i.e., gonad plus mesonephros) were used for the experiments described below. As for busulfan treatment, busulfan (40 mg/kg body weight) was injected intraperitoneally into pregnant female mice at 9.5 dpc (McClive *et al*., 2003), and then the embryos were isolated at 11.5 and 12.5 dpc. In addition, genomic DNAs were prepared from the head region of each embryo, and the sex of each embryo was determined by PCR using Zfy-specific primers as described previously (Bowles *et al*., 1999).

Organ culture

To examine the effects of culture conditions and inhibitors for signaling molecules on glycogen accumulation in developing XY gonads, I performed organ cultures using genital ridges isolated at 9-14 ts. One of each pair of genital ridges was cultured in a medium containing an inhibitor, while the other was used as a control. PI3K (phosphatidylinositol 3-kinase) inhibitor LY294002 (15 μM; Calbiochem) and MEK (mitogen-activated or extracellular signal-regulated protein kinase) inhibitor PD98059 (50 μM; Calbiochem) were mainly used in this study. To analyze a possible contribution of the adjacent mesonephros on glycogen accumulation in XY gonads, the

genital ridges were isolated at 14 ts, and the gonads were separated from the mesonephros under a dissecting microscope using a sharp needle. All explants were placed onto an ISOPORE membrane filter (3.0 μm; Millipore, MA, USA), floated on DMEM (Dulbecco's Modified Eagle's Medium; Sigma-Japan, Tokyo, Japan) containing 10% horse serum and penicillin/streptomycin (GIBCO BRL/Invitrogen, CA, USA), and cultured at 37^{o}C for 6 to 48 h (Kanai *et al*., 1992; Hiramatsu *et al*., 2003). Some genital ridges were also cultured with DMEM alone (i.e., without 10% horse serum). All cultured explants were used for the following analyses.

Histological and histochemical analyses

For periodic acid Schiff (PAS) reaction, the isolated embryos were fixed in 10% formaldehyde containing 2% Ca $(CH_3COO)_2$ at 4^{o}C for 12 h, then dehydrated in ethanol, and embedded in paraffin. Serial sagittal sections (approximately 5 μm in thickness) were cut and stained with PAS reagent. To clarify the histochemical specificity for glycogen, two serial sections were pretreated with or without 0.1% α-amylase (Sigma) at 37^{o}C for 1 h before PAS staining. All gonadal images of serial sagittal sections (five embryos at 14 ts; four embryos each at 15 and 16 ts) were photographed. After five regions along the anteroposterior (AP) axis (regions I~V in Fig. 5A) were selected in each gonadal image, I calculated the number of PAS-positive cells located in each region and those located adjacent to germ cells. Moreover, each gonadal area (regions I~V) was separately measured using the Scion image program (Ver. 4.02). In each embryo, I estimated 1) total number of PAS positive cells per gonad, 2) total number of PAS-positive cells located in each region, 3) relative number of PAS-positive cells per unit area (cell number per mm^2) in each region, and 4) ratio of PAS-positive cells located

adjacent to germ cells.

For transmission electron microscopy, the genital ridges at 14-18 ts were fixed in 3% glutaraldehyde-0.05 M phosphate buffer (PB) containing 0.05 M sucrose at 4^{o}C for 4 h. After washing with 0.05 M PB containing 0.05 M potassium ferricyanide, the samples were postfixed in 1% OsO_4-0.05 M potassium ferricyanide in 0.05 M PB at 4^{o}C for 2 h. The specimens were then dehydrated in ethanol, and embedded in Araldite M. Ultrathin sections were cut using an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and then observed under a JEM 1200EX transmission electron microscope at 80 kV.

Immunohistochemistry

For immunohistochemistry, both embryos and cultured explants were fixed in 4% PFA-PBS at 4^{o}C for 4 h, dehydrated, and then embedded in paraffin. Deparaffinized sections were subjected to immunohistochemistry, using anti-SOX9 antibody (Ab) (10 ng/μl; Kent *et al*., 1996; kindly provided by Dr. Peter Koopman, University of Queensland, Australia), anti-SF1/Ad4BP Ab (1/1000 dilution; Hatano *et al*., 1994; Ikeda *et al*., 2001; kindly provided by Dr. Ken-ichirou Morohashi, Kyushu University, Japan), and anti-phospho-AKT Ab (Ser473; 1/50 dilution; Cell Signaling Technology Japan, Tokyo, Japan). The sections were incubated with each rabbit primary antibody at 4^{o}C for 12 h. For comparative staining with PAS reaction, anti-SOX9 or anti-SF1/Ad4BP Ab, two consecutive 4μm-thick mirror sections (i.e., two serial sections with the same cutting plane were put on separate slide glasses) were prepared from paraffin blocks of 4%-PFA-fixed samples. One section was stained with PAS staining, while another

mirror section was incubated with anti-SOX9 or anti-SF1/Ad4BP Ab at 4^{o}C for 12 h. Thereafter, the immunoreaction with each first antibody was visualized by HRP-labelled anti-rabbit IgG Ab using a Tyramide Signal Amplification Kit (NEN/PerkinElmer, MA, USA). In addition, non-specific reactions could not be detected in germ cells or gonadal somatic cells, when the sections were incubated with control rabbit IgG instead of anti-SOX9, anti-SF1/Ad4BP or anti- phospho-AKT Ab.

Whole-mount in situ hybridization

The genital ridges and their cultured explants were fixed in 4% PFA-PBS for 4 h and then dehydrated in methanol. The samples were rehydrated, pretreated with 10 μg/ml proteinase K in PBST for 30 minutes [min], and then hybridized with digoxigenin (DIG)-labelled antisense *Sry* RNA probes (Bullejos and Koopman, 2001; kindly provided by Dr. Peter Koopman) in a solution containing 50% formamide, 10% dextran sulfate, 5x SSC, 1% SDS, 50 μg/ml heparin and 50 μg/ml denatured yeast RNA at 70^{o}C for 16 h. After treatment with RNase A (100 μ g/ml; Sigma) at 37^{o}C for 15 min, they were washed twice with 2x SSC/5xSSC at 65^{o}C for 1 h. The signals were detected by an immunological method using alkaline phosphatase-conjugated anti-DIG antibody (Roche Molecular Biochemicals, Germany) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) / nitro blue tetrazolium (NBT) (Roche) as the chromogen.

Immunoblot analysis

The genital ridges at 9-11 ts were cultured in 10% horse serum-DMEM with or

without inhibitors at 37^{o}C for 24 h. Then, the explants were dissolved in non-reduced SDS sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue). After each protein sample was electrophoresed and transferred to nylon membranes, the blots were incubated with anti-phospho-AKT (1:1000 dilution) or anti-AKT Ab (1:1000 dilution; Cell Signaling Technology). The immunoreaction was visualized with HRP-labelled goat anti-rabbit secondary Ab using an Enhanced Chemiluminescence Detection Kit (GE Healthcare Bio-Sciences KK, Tokyo, Japan).

RESULTS

Testis-specific glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of *Sry* action

The previous observation showed that embryonic testis is a glycogen-rich tissue in mouse embryogenesis, and this glycogen accumulation predominantly occurs in Sertoli cells within newly-formed testis cords at 12.5 dpc (Kanai *et al.*, 1989; Fig. 6C in this study). In order to confirm the sex-dimorphic distribution of glycogen deposits in mouse embryo around sex differentiation period, 11.5 dpc (18 ts stage), I performed periodic acid Schiff (PAS) staining which specifically visualizes mucous glycoconjugates such as glycogen. PAS-positive reactions were found in skeletal muscles, notochord, and the area surrounding the dorsal aorta in both XY and XX embryos at similar levels. In the gonadal region, however, PAS-positive reactions were detected in XY, but not XX, embryos (Fig. 2A-D). These reactions were predominantly observed in gonadal somatic cells near germ cells, i.e., presumptive pre-Sertoli cells (Fig. 2D). Such reactions completely disappeared when pre-incubated with α-amylase (Fig. 2G, H). Electron microscopic observations showed that glycogen granules were frequently found in the cytoplasm of gonadal somatic cells closely associated with germ cells in XY, but not XX, gonads (Fig. 2E, F), indicating that testis-specific glycogen accumulation occurs from at least 11.5 dpc (18 ts).

To examine the timing of the onset of glycogen deposition in developing XY gonads, I compared the spatial patterns of PAS reactions and immunostaining against SOX9 protein, one of the first factors induced by *Sry*, during early phases of mouse testis differentiation (Fig. 3A). SOX9-positive cells were first detected in the XY gonad isolated at 14 ts, and then increased in number at later stages (from 16 to 18 ts). Similar

to the temporal pattern of SOX9 expression, PAS-positive cells were first detected in the XY gonad isolated at 14 ts, and then rapidly increased in number to aggregate into cord-like structures at 18 ts. In addition to XY gonads, PAS reactions were also observed in gonads isolated from XX male embryos carrying the *Sry* transgene (right panel in Fig. 3B). This is clearly in contrast to the absence of PAS-positive cells in wildtype XX gonads at all stages examined (left panels in Fig. 3A, B). Moreover, I separately performed the PAS reaction and immunohistochemical staining with anti-SF1/Ad4BP (a marker for precursor cells of both Sertoli and Leydig cells) or anti-SOX9 (a pre-Sertoli cell marker) Ab using two consecutive mirror sections of the same PFA-fixed specimens. A comparative analysis of PAS reaction and anti-SF1/Ad4BP staining revealed that PAS-positive cells clearly expressed SF1/Ad4BP in their nucleus (Fig. 4A, B), although PAS reaction was markedly reduced in PFA-fixed gonads as compared to those fixed with 10% formalin - 2% $Ca(CH_3COO)_2$ (probably due to reduced preservation of glycogen in PFA-fixed samples). Moreover, in XY gonads, the nucleus of PAS-positive cells was positive for anti-SOX9 staining (when their nucleus was in the section; Fig. 4C, D). Since *Sry* expression is first detected in XY gonads at around 12 ts (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001; Kidokoro *et al*., 2005), these findings clearly indicate that glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of *Sry* during early phases of testis differentiation.

A center-to-pole pattern of glycogen accumulation in developing XY gonads

Previous studies have demonstrated that the expression of both *Sry* and *Sox9* is first detected in the central region of XY gonads, before their expression domains expand to

both anterior and posterior ends during early phases of testis differentiation (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001; Schepers *et al*., 2003). In order to examine the spatial pattern of glycogen accumulation along the AP axis of developing XY gonads, consecutive sagittal sections spanning from the lateral to medial edges were stained with PAS reaction, and the number of PAS-positive cells in each gonadal region (regions I~V, equally divided along the AP axis of the gonad; left plates of Fig. 5A) was determined. The total number of PAS-positive cells per gonad increased rapidly from 14 to 16 ts (cell number per gonad ± standard error: 4.0 ± 0.9 at 14 ts [n=5]; 26.3 ± 8.7 at 15 ts [n=4]; 419.5 ± 43.3 at 16 ts [n=4]). PAS-positive cells were found to be positioned predominantly in the middle regions (regions II to IV) at 14 ts (Fig. 5C). This center-restricted distribution expanded into the anterior (region I) and posterior (region V) edges from 15 to 16 ts.

Glycogen accumulation in pre-Sertoli cells is likely to be independent of germ cells

The interesting finding from the present histochemical observations was that PAS-positive cells were frequently found near germ cells in XY gonads even at 14-15 ts (right panels in Fig. 5A). Electron microscopic analysis of XY gonads at 15 ts also confirmed that glycogen-rich cells were found to be directly associated with germ cells (Fig. 5B). A quantitative analysis of PAS-stained sections revealed that even at 14-15 ts, approximately 75% of PAS-positive cells were located in the area adjacent to germ cells at the sectioning level (ratio of PAS-positive cells closely associated with germ cells per total PAS-positive cells ± standard error: 81.7 ± 5.5% at 14 ts [n=5]; 73.0 ± 8.0% at 15 ts, 76.3 ± 4.2% at 16 ts [n=4]). In order to investigate a possible involvement of germ cells in glycogen accumulation in pre-Sertoli cells, I experimentally produced gonads with a

severely reduced number of germ cells by administration of busulfan to 9.5 dpc pregnant mice (McClive *et al*., 2003), and examined the effects of germ cell loss on PAS-staining pattern in XY gonads at 11.5 (18 ts) and 12.5 (30 ts) dpc (Fig. 6). It was shown that there was no appreciable difference between busulfan-treated gonads and control either at 11.5 or at 12.5 dpc. In short, in XY gonads treated with busulfan, PAS-positive cells were properly aggregated into slender cord-like structures similar to those in control XY gonads at the same stage, despite the drastic reduction in number of germ cells (Fig. 6A, B). In the testes at 12.5 dpc, the testicular cords were formed in the testes without germ cells, and all Sertoli cells showed positive PAS staining (Fig. 6C, D).

Testis-specific glycogen deposition in genital ridge organ cultures: Effects of developmental stage at culture initiation, serum withdrawal, and absence of adjacent mesonephros

In the previous study, 3-day cultures of XY gonadal explants initiated at the stages prior to 11 ts (approximately 11.0 dpc) failed to induce either testis cord formation or testis-specific *Sox9* expression (Hiramatsu *et al*., 2003). This is in contrast to the proper induction of Leydig cell differentiation (Hiramatsu *et al*., 2003) and *Sry* expression (Fig. 7A) in the cultured explants initiated at 9-11 ts. In order to examine the effect of gonadal stage at culture initiation on sex-dimorphic glycogen accumulation, I cultured genital ridges isolated at 9-10 and 12-13 ts in 10% horse serum-DMEM, and examined the time course of accumulation patterns of glycogen deposits in developing gonads *in vitro* (Fig. 7B). In XY explants at 12-13 ts, PAS-positive cells were first detected at 6 h, and the cells rapidly increased from 12 to 24 h. No PAS-positive cells were detected in XX explants at 12-13 ts, even after 48 h, suggesting that the present

culture condition is capable of inducing sex-dimorphic glycogen accumulation in developing gonads. Interestingly, in all cultures of genital ridges isolated at 9-10 ts, PAS-positive cells were observed in the gonadal region in a testis-specific manner, suggesting that glycogen accumulation in pre-Sertoli cells is not directly associated with subsequent testis cord formation and Sertoli cell differentiation. Most interestingly, when I cultured 12 ts genital ridges in serum-free DMEM medium (i.e., without 10% horse serum), glycogen deposition was induced in XY, but not in XX, explants, despite obvious growth defects in both XY and XX genital ridges (four out of four in each sex; Fig. 7C).

It has been shown that *Sry*-dependent mesonephric cell migration is crucial for Sertoli cell differentiation and *Sox9* expression during early stages of mouse testis differentiation (Buehr *et al*, 1993; Martineau *et al*., 1997; Tilmann and Capel, 1999). In order to analyze a possible contribution of mesonephric cells to testis-specific glycogen accumulation, I next examined the PAS-staining pattern in culture explants with or without adjacent mesonephros isolated at 14 ts (Fig. 8). In 24 h cultured gonads with adjacent mesonephros (i.e., genital ridge), glycogen accumulation was induced in all five XY explants, but not in any XX explants (Fig. 8B, E). Similar to the pattern of glycogen accumulation in the control cultures, PAS-positive cells were observed in all XY, but not in any XX, explants without mesonephros (four out of four in each sex; Fig. 8C, F). The present finding on testis-specific induction of glycogen accumulation even in serum-free cultures suggests that this sex-dimorphic glycogen deposition is a tissue-autonomous cellular event downstream of *Sry*.

A testis-specific activation of the PI3K-AKT pathway mediates a sex-dimorphic

storage of glycogen in the pre-Sertoli cell lineage

Several growth factors essential for testis differentiation (such as FGF9, PDGF-α, and insulin/IGF) are known to activate two major signaling pathways: extracellular-signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, and phosphatidylinositol 3-kinase (PI3K)/AKT pathway. To analyze a possible role of these two pathways in sex-dimorphic glycogen accumulation in developing gonads, I examined the effects of each inhibitor [MEK (MAPK/ERK) inhibitor; PD98059, PI3K inhibitor; LY294002] on glycogen accumulation in 10-11ts genital ridges *in vitro* (Fig. 9; Table 1). It was shown that the addition of LY294002 (15 μM) to the medium drastically reduced glycogen accumulation in the gonadal area of XY explants (Fig. 9C). XY genital ridges treated with 5 μM of LY294002 exhibited a partial inhibition of glycogen deposition, especially in the surface area of XY gonadal explants (figure not shown). In all XY explants treated with 25 μM of LY294002, no PAS-positive cells were detected in their gonadal area (six out of six), suggesting that LY294002 dose-dependently inhibited glycogen accumulation in developing XY gonads. In contrast, MEK (MAPK/ERK) inhibitor PD98059 did not have any obvious effect on glycogen accumulation in XY explants even at a concentration of 50 μM (Fig. 9B), despite that administration of PD98059 drastically reduced the phosphorylation levels of the MEK substrate, ERK, in these explants (data not shown). Moreover, no appreciable difference was detected in XX explants in which no PAS-positive cells were found in the gonadal area of explants treated with LY294002 or PD98059 (Fig. 9D-F). These findings clearly suggest a possible involvement of the PI3K-AKT pathway in glycogen accumulation in developing XY gonads. To confirm whether or not the PI3K inhibitor LY294002 represses phosphorylation of PI3K downstream effector AKT in gonads, I examined the phosphorylation level of XY and XX genital ridges by anti-phospho-AKT

(active form of AKT) staining (Fig. 10). In the 48 h control culture, anti-phospho-AKT immunohistochemical reactions were frequently observed in the gonadal area of XY explants (Fig. 10A). However, positive signals were only weakly detected in the gonadal area of XX explants, even though some positive signals were detected in their mesonephric regions. Moreover, the addition of the PI3K inhibitor LY294002 clearly reduced the intensity of anti-phospho-AKT staining in XY explants ("XY+LY" in Fig. 10A). Immunoblot analysis also confirmed that AKT was phosphorylated at a higher level in the XY explants than in XX explants in 24 h control cultures of genital ridges ("cont" in Fig. 10B). Moreover, in both XY and XX genital ridges, phosphorylation levels were repressed by administration of LY294002 ("+LY" in Fig. 10B). By using anti-phospho-AKT staining of transverse sections of 11.5 dpc embryos, anti-phospho-AKT reactions were found in the ventral region of neural tubes and dorsal root ganglia at similar levels in both sexes, but their reactions in the gonadal region were higher in XY than in XX embryos *in vivo* (Fig. 10C). Anti-phospho-AKT reactions in XY gonads, both *in vivo* and *in vitro*, were found in somatic cells located near germ cells, i.e., presumptive Sertoli cells (right panels in Fig. 10A, C).

DISCUSSION

In mouse sex differentiation, *Sry* expression is first detected in the central region of XY gonads at around 12 ts (11.0 dpc), and subsequently expands to the entire gonadal area from 14 to 15 ts in a center-to-pole wave (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001). The present study clearly demonstrates that glycogen accumulation starts to occur in pre-Sertoli cells in developing XY gonads from around 14 ts in a center-to-pole pattern similar to the *Sry* expression profile, suggesting a delay of only 2 ts (approximately 4 h) after the onset of *Sry* expression. Moreover, the timing of the onset of accumulation is quite similar to that of testis-specific expression of *Sox9*, the direct target of SRY (Sekido and Lovell-Badge, 2008). Therefore, these data indicate that testis-specific glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of *Sry* action. Moreover, in the present study, testis-specific glycogen deposition can be induced even in serum-free conditions and in a culture of gonadal explants without adjacent mesonephros, suggesting that sex-dimorphic glycogen deposition in developing gonads is tissue-autonomous. This is clearly in contrast to the essential roles of the gonad-mesonephros interaction and exogenous serum factors in testis-specific *Sox9* expression and/or maintenance in pre-Sertoli cells *in vitro* (Taketo and Koide, 1981; Tilmann and Capel, 1999; Puglianiello *et al*., 2004). This further suggests that glycogen accumulation in developing XY gonads is induced via a distinct pathway from *Sox9* regulation by SRY in the sex-determining cascade.

The present *in vitro* analyses also demonstrate that the sex-dimorphic storage of glycogen granules in pre-Sertoli cells is mediated via the PI3K-AKT pathway. The PI3K-AKT pathway is one of the major signaling pathways downstream of tyrosine kinase receptors for many polypeptide growth factors, including PDGF, FGF, and

insulin/IGFs (see review, Alessi and Cohen, 1998), suggesting the possible involvement of some growth factor(s) in testis-specific glycogenesis in pre-Sertoli cells. To date, it has been assumed that several growth factors are downstream of *Sry* and are important in testis differentiation. For example, the XY gonads of Pdgfr-α (Pdgf-α receptor) knockout mice display disruptions in the organization of vasculature and in the partitioning of interstitial and testicular cord compartments (Brennan *et al*., 2003). *Fgf9* has also shown to be involved in testis-specific mesonephric migration and cell proliferation during testis differentiation (Colvin *et al*., 2001; Schmahl *et al*., 2004). On the other hand, it has also been shown that inhibition by the PI3K inhibitor LY294002 drastically disrupts cord formation and testis-specific mesonephric migration *in vitro* (Uzumcu *et al*., 2002). Although mesonephric migration does not make an appreciable contribution to sex-dimorphic glycogen deposition, these findings suggest that these growth factors induce glycogen accumulation in pre-Sertoli cells by activating the PI3K-AKT pathway in an autocrine and/or paracrine manner.

Most interestingly, Nef *et al*. (2003) have shown that insulin/IGF signaling is required for testis differentiation in mice. They demonstrated that XY mice with mutations for all three insulin receptor family members (Ir, Igf1r and Irr) developed ovaries and showed a completely female phenotype. Since it has been shown that insulin/IGF signaling generally stimulates glucose metabolism in target organs via the PI3K-AKT pathway (Saltiel and Kahn, 2001; Pirola *et al*., 2004), insulin/IGF is a strong candidate factor to directly regulate testis-specific glycogenesis in pre-Sertoli cells downstream of *Sry*. In fact, my preliminary *in vitro* experiments showed that the insulin/IGF receptor inhibitor [AG1024] significantly inhibited testis-specific glycogen accumulation in XY gonads and that the forced activation of insulin receptor signal, by phospho-tyrosine phosphatase inhibitor [bpv(phen)], resulted in an ectopic glycogen

deposit in XX gonadal somatic cells. However, the addition of insulin itself did not induce glycogenesis in XX gonads. These observations suggest that the intracellular signaling cascade downstream of insulin is expected to be regulated by SRY. Here, I propose two possible mechanisms regulating glycogen accumulation by SRY; SRY possibly 1) activating a male-specific factor positively regulating the PI3K-AKT signal or 2) repressing a female-specific factor negatively regulating the PI3K-AKT pathway through transcriptional regulation ([Y] or [X] in Fig. 19, respectively). Although Nef *et al*. (2003) proposed that insulin signaling acts upstream of *Sry*, the possibility of insulin action downstream of *Sry* cannot be ruled out at present. Therefore, further studies to identify growth factors which induce *Sry*-dependent glycogen accumulation and to determine how *Sry* activates the PI3K-AKT pathway in pre-Sertoli cells are warranted.

SUMMARY

In chapter 1, in order to reveal the mechanisms regulating glycogen accumulation in male gonads, I performed detailed histological and genetic analyses, and *in vitro* organ culture experiments. In developing XY gonads, glycogen accumulation starts to occur in pre-Sertoli cells from around 11.2 dpc, only 4 h after the initiation of *Sry* expression. This phenomenon progresses in a center-to-pole pattern, similar to the spatio-temporal expression profile of *Sox9*, which is the only one gene verified as a direct target gene of SRY. Glycogen accumulation was also found in XX male gonads of *Sry*-transgenic embryos, but not in XX female gonads of wildtype embryos at any developmental stages. These results imply that glycogenesis in pre-Sertoli cells is one of the earliest cellular events direct downstream of *Sry* action. Moreover, glycogen accumulation in pre-Sertoli cells was significantly inhibited by PI3K inhibitor LY294002, but not by MEK inhibitor PD98059 *in vitro*. In addition, active phospho-AKT (PI3K effector) showed a high degree of accumulation in gonadal somatic cells of genital ridges in a testis-specific manner, both *in vitro* and *in vivo*. Taken together, these findings suggest that immediately after the onset of *Sry* expression, extracellular signaling factors, possibly insulin/IGFs, may promote glycogen accumulation in pre-Sertoli cells via PI3K-AKT pathway (Fig. 19 [p.98]). Above all, these data clearly raise an interesting hypothesis of a potential link between *Sry* action and sex-dimorphic energy metabolism in mammalian gonadal sex determination.

FIGURES & TABLE

Fig. 2. A sex-dimorphic distribution of glycogen deposits in gonads at 11.5 dpc (18 tail somite [ts] stage)

(A-D) Transverse sections of 11.5 dpc XX (A, C) and XY (B, D) embryos. Periodic acid Schiff (PAS) staining (red staining). In both XY and XX embryos, several tissues including notochord (nt), skeletal muscle (sm) and the area close to dorsal aorta (da) show positive PAS staining. However, in developing genital ridges, PAS reactions are observed only in the gonadal region of XY, but not XX, embryos. In XY gonad, PAS-positive cells are located closely adjacent to germ cells (asterisks) (D), whereas no positive cells are detected in XX gonad (C). Plates C and D show higher magnification images, indicated by the broken rectangle in plates A and B, respectively. asterisk, germ cell; ce, coelomic epithelium; ms, mesonephros; da; dorsal aorta; nc, notochord; nt, neural tube; sm, skeletal muscle. Bar, 50 μm. (E, F) Transmission electron micrographs showing an accumulation of glycogen granules (arrows; a massive glycogen deposit is indicated by "Gly") in the cytoplasm of gonadal somatic cells closely associated with germ cells in XY (F), but not XX, gonad (E). G, germ cell; Gly, glycogen granule. Bar, 1 μm. (G, H) Two serial sections of 11.5 dpc XY gonad were pretreated with (H) or without α-amylase (G) before PAS staining. α-amylase digestion results in a complete loss of PAS reaction in XY gonad. The insets show higher magnification of the cells indicated by arrows. asterisk, germ cell; ms, mesonephros. Bar, 50 μm.

Fig. 2

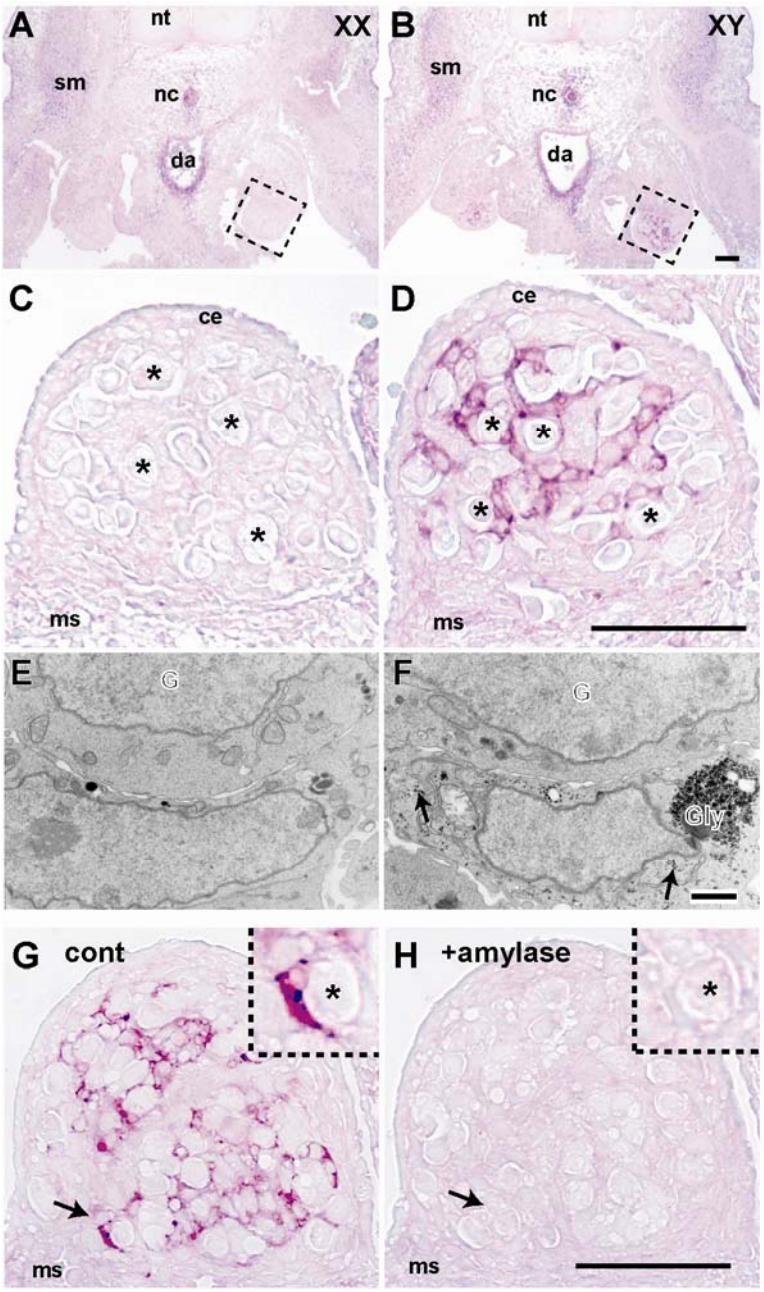

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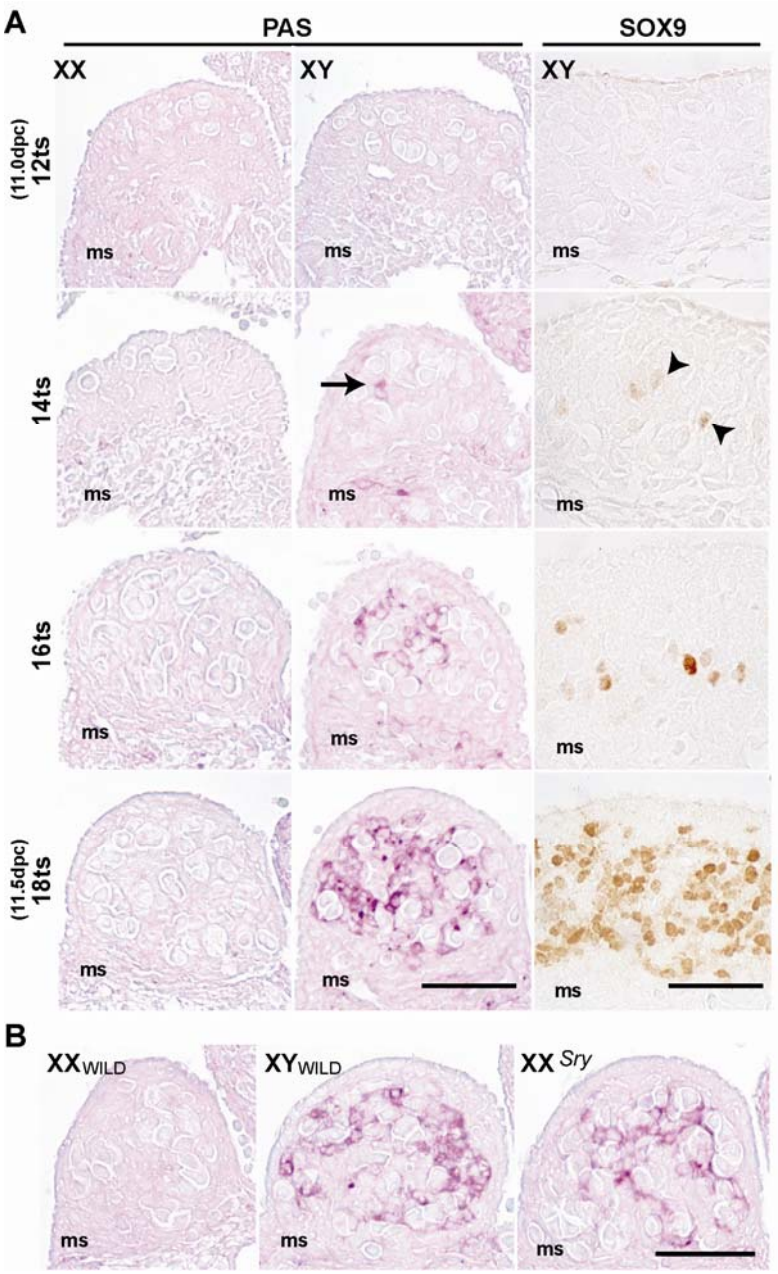
Fig. 3. Timing of onset of glycogen accumulation in developing XY gonads and its accumulation in 11.5 dpc XX male gonad of sex-reversal *Sry* transgenic mice

(A) Temporal patterns of PAS reactions (red staining; transverse sections) and immunostaining against SOX9 protein (brown staining; sagittal sections) in XX (left) and XY (middle and right) gonads during early phases of sex differentiation. Similar to the temporal pattern of SOX9 expression, PAS-positive cells are first detected in XY gonad isolated at 14 ts (arrow for PAS; arrowheads for SOX9). These cells rapidly increase in number, and then aggregate into cord-like structures at 18 ts. No PAS-positive cells are detected in XX gonads at any stages examined. Embryos at approximately 11.0 and 11.5 dpc show 12 and 18 ts, respectively. (B) Transverse sections of 11.5 dpc (18 ts) embryos of XX wildtype, XY wildtype and XX *Sry* trangenic embryos. PAS staining. As well as PAS reactions in XY wildtype gonads, the same reactions are also observed in the XX gonads of male embryos carrying the *Sry* transgene. ms, mesonephros. Bar, 50 μm.

Fig. 3


A
PAS
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XX
XY
XY
(11.0dpc)
12ts
14ts
16ts
(11.5dpc)
18ts
ms
B
XX WILD
XY WILD
XX Sry

Fig. 4. PAS reaction and anti-SF1/Ad4BP or anti-SOX9 staining of two consecutive mirror sections of 11.5 dpc XY gonads demonstrating that glycogen accumulation occurs in pre-Sertoli cell lineage

PAS reaction (red staining) and immunohistochemical staining (brown staining) with anti-SF1/Ad4BP (a marker for precursor cells of both Sertoli and Leydig cells; A, B) or anti-SOX9 (a pre-Sertoli cell marker; C, D) Ab were performed using two consecutive mirror sections of the same PFA-fixed specimens. For comparison, images of immunostained mirror sections are computationally reversed. Plates B and D are the merged images of PAS reaction (left) and immunoreaction (right; reversed images) in plates A and C, respectively. Plates B and D also include higher magnification images (right), indicated by broken rectangle in the corresponding left plate. PAS-positive cells clearly overlap with gonadal somatic cells expressing SF1/Ad4BP (arrows in B) or SOX9 (arrows in D) in XY gonads. ms, mesonephros. Bar, 50 μm.

Fig. 4

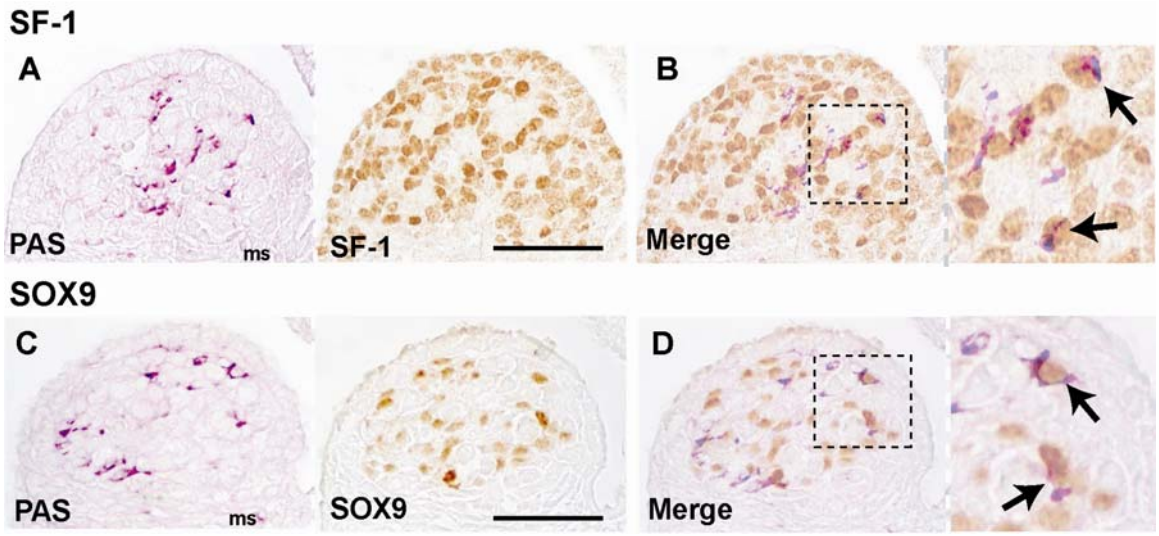
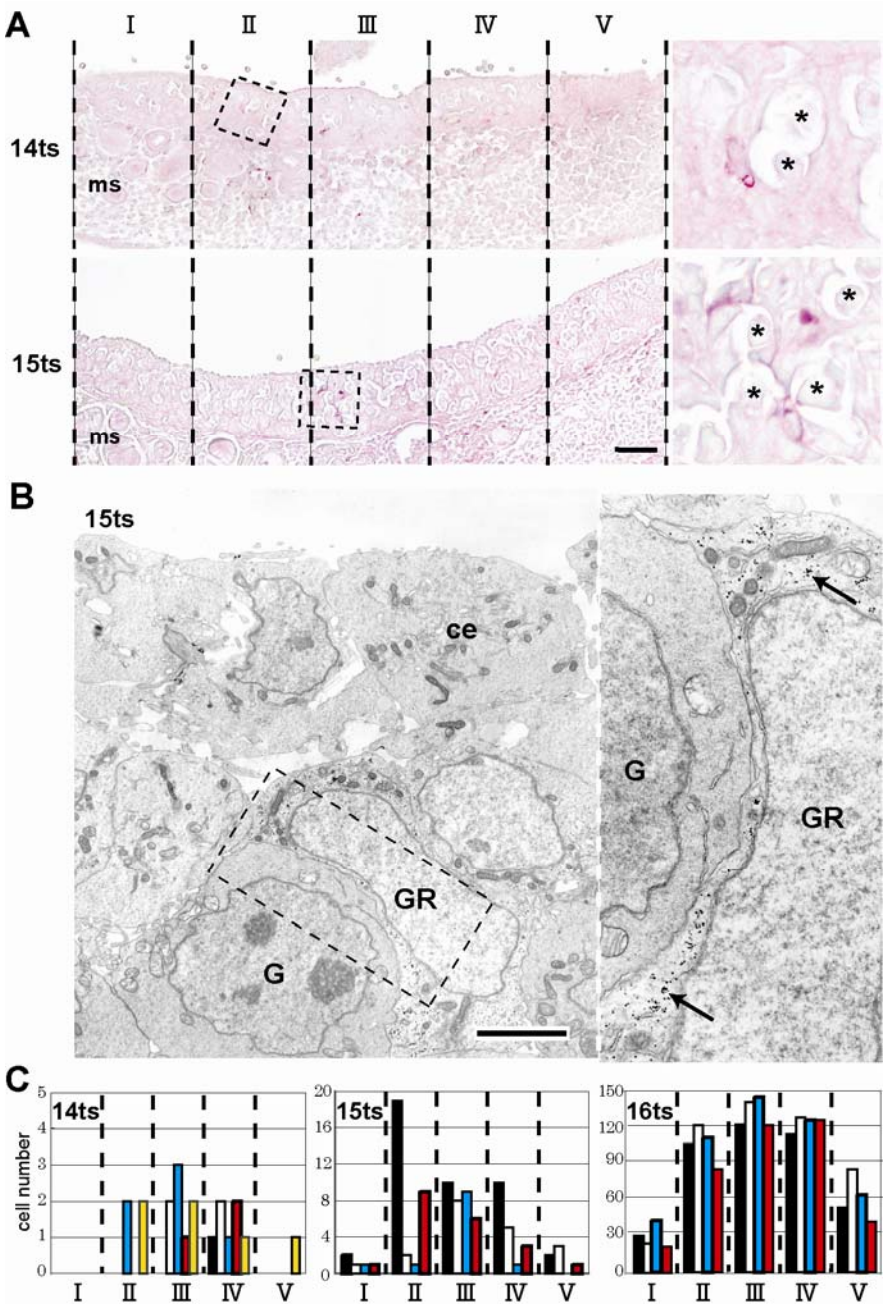


Fig. 5. A center-to-pole pattern of glycogen accumulation along anteroposterior (AP) axis of developing XY gonads and close association between glycogen-rich cells and germ cells during early phases of testis differentiation

(A) Sagittal sections of XY gonads isolated at 14 ts (upper) and 15 ts (lower) stages. PAS staining. Regions I~V are equally divided along the AP axis of gonad. PAS-positive cells at 14 and 15 ts are located in regions II and III, respectively. Interestingly, they are frequently found in an area near germ cells even at 14 and 15 ts stages (right panel). Right panels show higher magnification images, indicated by broken rectangle in corresponding left panel. Asterisk, germ cell; ms, mesonephros. Bar, 50 μm. (B) Transmission electron micrographs showing a direct association between glycogen-positive cells and germ cells in XY gonad at 15 ts. Right panel shows a higher magnification image, indicated by broken rectangle in left panel. Arrows indicate glycogen granules. ce, coelomic epithelial cells; G, germ cells; GR, glycogen-rich cells. Bar, 5 μm. (C) All consecutive sagittal sections were stained with PAS reaction, and then the total number of positive cells in each gonad was measured separately in regions I~V which were equally divided along the AP axis of the gonad (broken lines in A). Vertical axis represents PAS-positive cell number per gonad, while horizontal axis represents regions I~V of gonads. In each graph, bars of the same color show the cell number in each region of the same gonad (14 ts, five gonads; 15 and 16 ts, each four gonads). PAS-positive cells are positioned predominantly in the middle regions (regions II to IV) at 14 ts. This center-restricted distribution clearly expands into anterior (region I) and posterior (region V) edges from 15 to 16 ts.

Fig. 5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cell number
I
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IV
V

Fig. 6. Glycogen accumulation occurs in XY gonads with severely-reduced germ cells that are experimentally induced by busulfan treatment

Sagittal sections showing the effects of germ cell loss on PAS-staining pattern in XY gonads at 11.5 (18 ts; upper plates) and 12.5 (30 ts; lower plates) dpc. PAS staining. No germ cells are detected in these sections of busulfan-treated XY gonads shown in plates B and D. In XY gonads treated with busulfan, PAS-positive cells are properly aggregated into slender cord-like structures (B), similar to those in control XY gonads at the same stage (A), despite the drastic reduction in number of germ cells. In testes at 12.5 dpc, testis cords are formed in differentiated testes without germ cells, and Sertoli cells show positive PAS staining in both control (C) and busulfan-treated (D) testes. asterisk, germ cell; ms, mesonephros. Bar, 50 μm.

Fig. 6

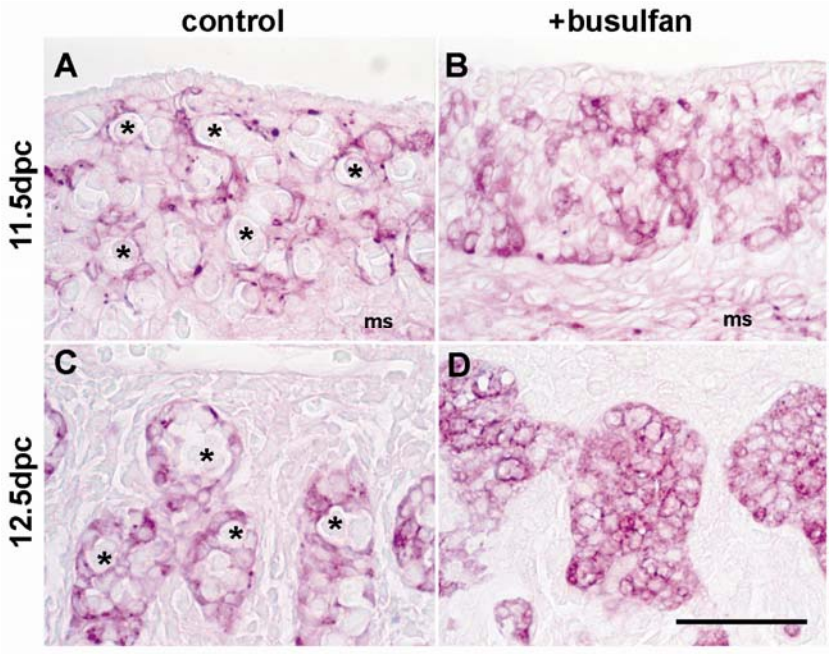
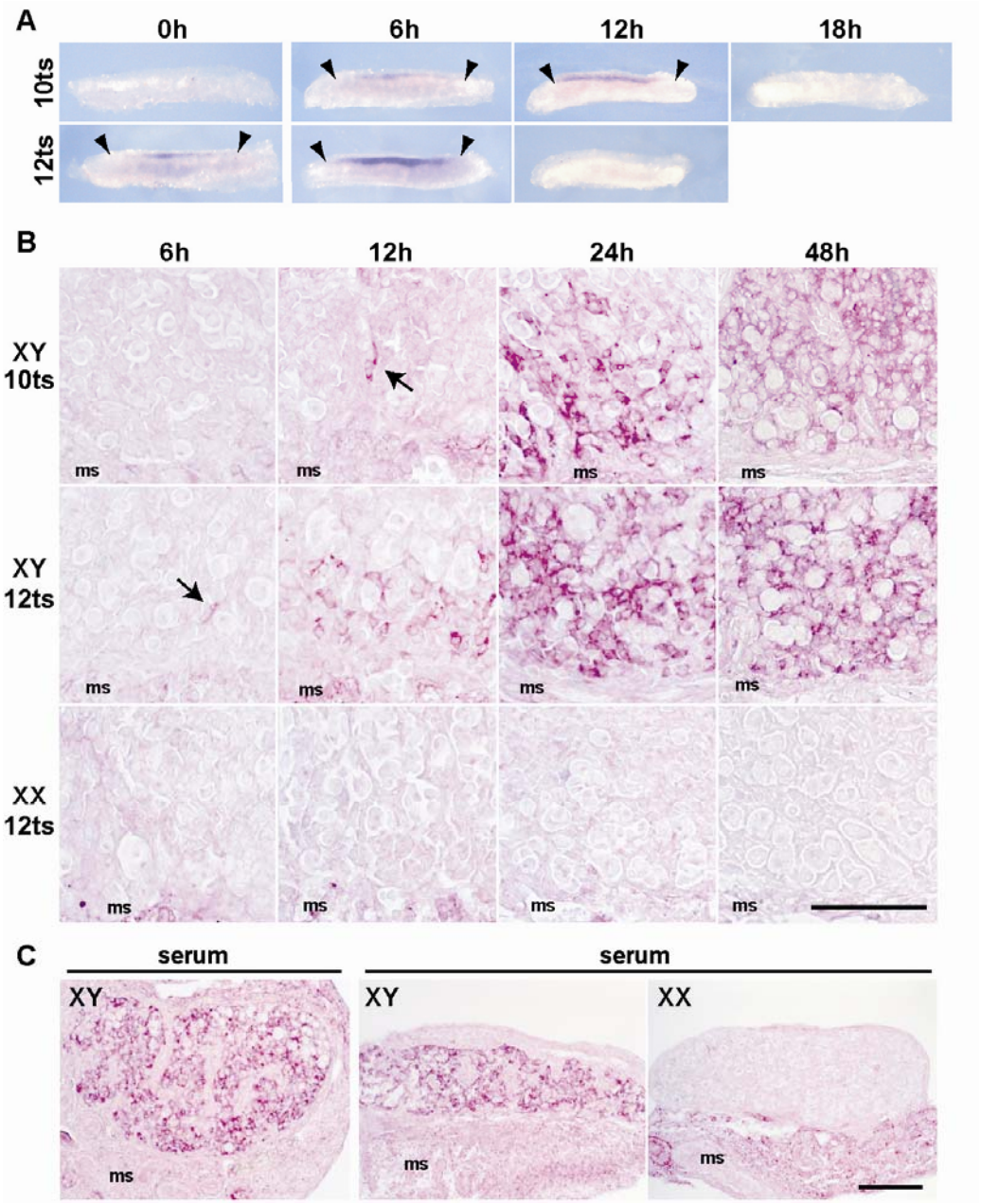


Fig. 7. Genital ridge organ cultures showing the time course of *Sry* expression *in vitro* (A) and testis-specific glycogen accumulation (B) which is independent of serum (C)

(A) Whole mount *in situ* hybridization showing *Sry* expression in developing XY genital ridges *in vitro*. *Sry* expression is first detected in the central region of XY gonads at 12 ts (most left plates; “0h”). In XY explants isolated at 12 ts, *Sry* expression is increased after being cultured for 6 h, and then its expression is rapidly reduced to the undetectable level until 12 h. In XY explants isolated at 10 ts, *Sry* expression is properly induced in the central region of gonadal area, and then its expression domain is expanded to their whole gonadal area until 12 h. Anterior is shown on the left in each plate, while anterior and posterior edges of gonadal area are indicated by arrowheads. (B) Sagittal sections of genital ridges cultured with 10% horse serum-DMEM for 6, 12, 24 and 48 h. PAS staining. In XY explants isolated at 12 ts, PAS-positive cells are detected after being cultured for 6 h (arrow in "XY 12ts"), and then rapidly increased in gonadal area near germ cells from 12 to 24 h. In XY genital ridges isolated at 10 ts, PAS-positive cells are induced in the gonadal region at 12 - 24 h (arrow in "XY 10 ts"). In contrast, no PAS-positive cells are detected in gonadal region of XX genital ridges, even after being cultured for 48 h ("XX 12 ts"). (C) Sagittal sections of XY (left and middle) and XX (right) genital ridges (12 ts) after 48-h culture in medium with or without 10% horse serum. PAS staining. Despite severe growth defects in genital ridges, PAS-positive cells are found to be induced in gonadal area of the XY, but not XX, genital ridge, suggesting no appreciable effect of serum withdrawal on sex-dimorphic glycogen deposition *in vitro*. ms, mesonephros. Bar, 50 μm.

Fig. 7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ms
C
serum
serum
XY
XY
XX
ms

Fig. 8. Testis-specific glycogen accumulation does not require adjacent mesonephros *in vitro*

Sagittal sections of genital ridges ("gonad + mesonephros"; A, B, D, E) and genital ridges without adjacent mesonephros ("gonad alone"; C, F) isolated at 14 ts. PAS staining. Plates (A and D) and (B, C, E, F) show genital ridge after being cultured for 0 and 24 h, respectively. In 24 h cultures, glycogen accumulation is induced in XY explants (A, B), but not in XX explants (D, E), of genital ridges. Similar to the pattern of glycogen accumulation in genital ridge cultures, PAS-positive cells are observed in XY explants without mesonephros (C), while no PAS-positive cells are detected in XX explants without mesonephros (F). ms, mesonephros. Bar, 50 μm.

Fig. 8

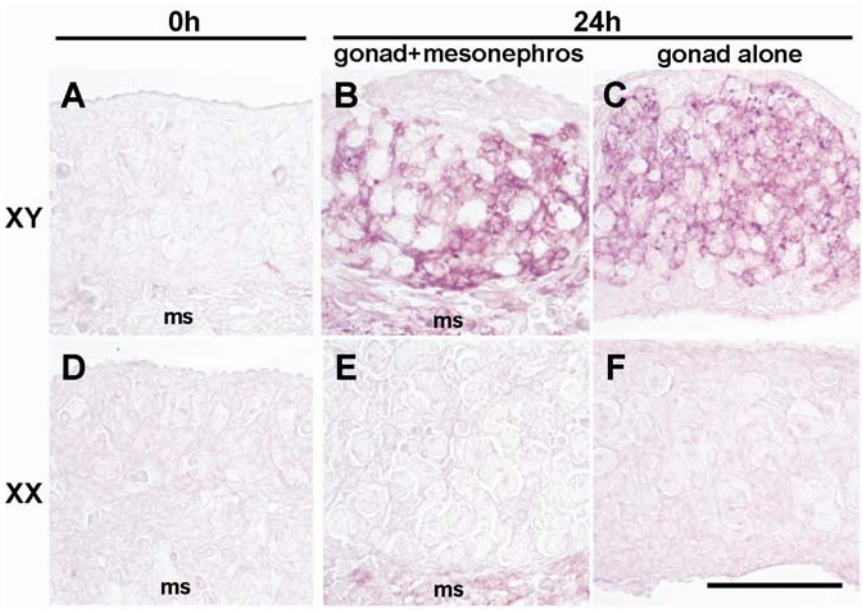

Fig. 9. Inhibition of PI3K inhibitor (+LY), but not MEK inhibitor (+PD), on testis-specific glycogen accumulation in developing XY genital ridges *in vitro*

Sagittal sections of genital ridges (10-11 ts) cultured in absence (A, D) or presence of MEK inhibitor (50 μM, PD98059; B, E) or PI3K inhibitor (15 μM; LY294002; C, F) with 10% horse serum-DMEM for 48 h. PAS staining. In XY explants, addition of LY294002 to the medium drastically reduced glycogen accumulation in the gonadal area of XY explants (C). In contrast, MEK inhibitor PD98059 did not exert any obvious effect on glycogen accumulation in XY explants (B). Moreover, no appreciable glycogen accumulation is observed in XX genital ridges treated with these inhibitors (D-F). ms, mesonephros. Bar, 50 μm.

Fig. 9

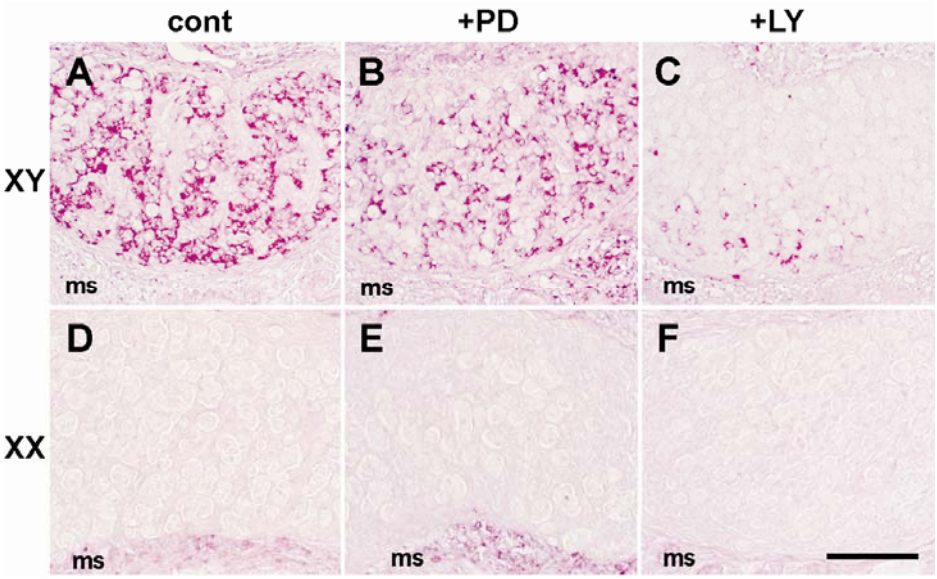

Fig. 10. Phosphorylation levels of AKT (a downstream effector of PI3K) in developing genital ridges *in vitro* and *in vivo*

(A) Sagittal sections of genital ridges (10-11 ts) cultured in the absence (cont) or presence of PI3K inhibitor (15 μM; LY294002; "+LY") for 48 h. Anti-phospho-AKT staining. Positive signals for anti-phospho-AKT staining are detected in gonadal somatic cells located near germ cells, with a strong signal detected in XY explant (XY cont) but only a weak signal in XX explant (XX cont). Anti-phospho-AKT signals are clearly reduced in XY explants cultured in the presence of PI3K inhibitor (XY + LY). (B) Immunoblot analyses of phosphorylation levels of AKT in XY and XX genital ridges (10-11 ts) cultured for 24 h in the presence or absence of LY294002. In control XY explants, AKT phosphorylation level is higher than that in XX control explants, and the level is clearly reduced by addition of LY294002 both in XY and XX explants. Repeat experiments were performed three times, and similar results were obtained each time. (C) Transverse sections of 11.5 dpc embryos (18 ts). Anti-phospho-AKT staining. Anti-phospho-AKT reactions are found in the ventral region of neural tubes and dorsal root ganglia at similar levels in both sexes, but the reactions in gonadal region are higher in XY than in XX embryos *in vivo*. These positive reactions in XY gonads are found in somatic cells near germ cells (asterisks). In plates A and C, right panels show higher magnification images, indicated by broken rectangle in the corresponding left panel. asterisk, germ cell; drg, dorsal root ganglion; ms, mesonephros; nt, neural tube. Bar, 50 μm.

Fig. 10

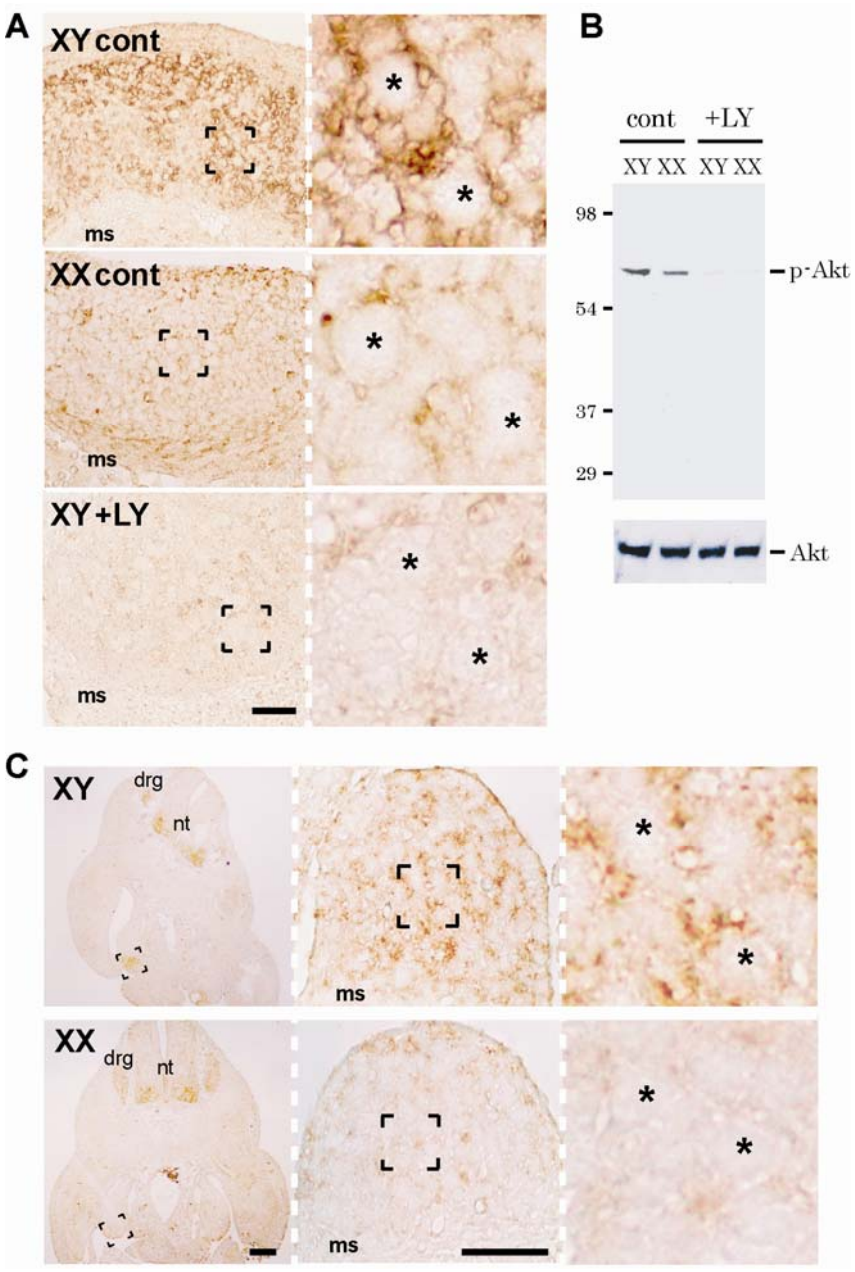
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Table 1. Effects of MEK inhibitor, PD98059, and PI3K inhibitor, LY294002, on testis-specific glycogen accumulation in developing gonads *in vitro* [a]

Inhibitor	Sex	Glycogen accumulation in gonadal area [b]				Total number of explants
		++	+	+/-	-	
Control	XY	32	0	0	0	32
	XX	0	0	0	17	17
PD98059	XY	5	2	0	0	7
	XX	0	0	0	5	5
LY294002	XY	0	4	8	13	25
	XX	0	0	0	12	12

[a] Effects of each inhibitor (PD98059, 50 μM; LY294002, 15 μM) on glycogen accumulation were examined by histochemical analysis of PAS reaction in a 48 h culture of genital ridges isolated at 9-11 ts.

[b] Number of explants showing i) no PAS-positive cells ("-"), ii) some PAS-positive cells detected in parts of gonadal area ("+/-"), or iii) PAS-positive cells detected throughout whole gonadal area, but the number of cells or PAS reactivity was reduced ("+") as compared with those in control XY explants ("++").

CHAPTER 2

"Establishment of Testis-Specific SOX9 Activation Requires High-Glucose Metabolism in Mouse Sex Differentiation"

INTRODUCTION

The development of the bipotential gonad as a testis or an ovary is a particularly interesting model of organ determination involving two physiologically and metabolically distinct tissues. *Sry* and *Sox9* 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 of developing XY gonads, but unlike *Sry, Sox9* continues to be expressed in pre-Sertoli cells throughout testis development (Kent *et al*., 1996; Morais da Silva *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-feedforward 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, however, 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 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 the formation of 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. It 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 (in chapter 1; Matoba *et al*., 2005). 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, I examined the influence of glucose starvation on mouse gonadal sex differentiation *in vitro*. This study demonstrated for the first time that of various somatic cell types in male and female genital ridges, pre-Sertoli cells are the most sensitive to glucose deprivation. The 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 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 high-glucose (control; final concentration: 410 mg/dl) or glucose-deprived (GD; final concentration: 5 mg/dl) medium for 6 to 72 h (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 10μM; 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. 17A).

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

Histological 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. 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), 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) and anti-WT1 antibody (1/50 dilution; Dako Cytomation). Thereafter, the immunoreaction with each first antibody was visualized using biotin-labelled 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-labelled 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^{o}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^{o}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 in chapter 1. 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, pretreated with 10 μg/ml proteinase K for 60 min, and then hybridized with digoxigenin (DIG)-labelled RNA probes at 70^{o}C for 16 h. After treatment with RNase A, they were finally washed with 2x SSC at 65^{o}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 genital ridges using Trizol reagent (Invitrogen Life Technologies, CA, USA). 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) following the manufacturer's instructions. For real-time quantitative RT-PCR for *Sox9* expression, specific primers and 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, USA). 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^{o}C for 15 seconds, 60^{o}C for 1 min followed by enzyme activation at 95^{o}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 in glucose-deprived (GD) genital ridge cultures

In order to clarify the glucose requirement for gonadal somatic cells during sex differentiation, I first histologically examined the influence of glucose starvation on testis differentiation *in vitro* using 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 genital ridges (figure not shown). Histological analyses revealed no appreciable defects in XX GD explants after 3 days of culture (Fig. 11B). 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. 11A; upper column in Table 2). 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. 11C). Moreover, GD treatment induced enlargement of rough endoplasmic reticulum (ER) in most pre-Sertoli cells (red arrows in Fig. 11C), reflecting morphological evidence for 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 somatic cells of XY or XX GD explants within the first 3 days of culture (Fig. 11C,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. 11E). No positive signals were detected in control XX explants or XX and XY genital ridges isolated at 11.5 dpc before culture initiation (figure not shown), suggesting a weak activation of ER stress in pre-Sertoli cells even in control (high-glucose) conditions. All XY GD explants clearly displayed considerably high levels of GRP78 expression in SF1/Ad4Bp-positive somatic cells of gonadal area, but not in cells located at coelomic epithelia and mesonephros ("XY GD" in Fig. 11E,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 GD medium (XX GD vs XX/*Sry* GD in Fig. 11E). These findings indicate that of 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 at 12 h after initiation of GD culture

Next, I carried out time-course histochemical analyses of *in vitro* testis differentiation in control and GD explants (Fig. 12). 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. 12B). PAS staining also revealed that glycogen storage was maintained at 24 h (left in Fig. 12A). During testis cord formation by

pre-Sertoli cells, PAS reaction in pre-Sertoli cells appeared to be reduced at 48 h (left lowest plates in Fig. 12A,C), and 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 completely disappeared at 12 h after culture initiation (right in Fig. 12A). Interestingly, based on the glycogen consumption in 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. 12B). 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 (Fig. 12C,E). 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 gonadal somatic cells) in GD explants at 48 h (Fig. 12D,F). 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, I 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 the anti-SOX9 immunostaining data shown in Fig. 12B, glucose starvation clearly reduced the signal intensity for *Sox9 in situ* hybridization (Fig. 13A).

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 feedforward 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. 13B). In contrast to normal *Pgds* expression in XY GD explants, the expression of *Col9a3*, another possible SOX9 target, was severely repressed by glucose starvation (Fig. 13C), 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 high-glucose explants ("cont" in Fig. 13C), 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 not appear to be affected in XX GD explants ("XX" in Fig. 13C), 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), I 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. 14).

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. 14A). However, no appreciable differences in the mitotic activity were observed between control and GD explants. Anti-phospho-Histone-H3 staining also showed no difference between high-glucose and GD conditions (right in Fig. 14A), indicating no appreciable defects in testis-specific cell proliferation of coelomic epithelia in GD explants.

Next, a mesonephric cell migration assay was performed using wild type gonads and Rosa26-derived (LacZ-positive) mesonephroi isolated from 11.5 dpc embryos. After being recombined and cultured in control or GD medium (Fig. 14B), 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. 14B), as was the case in 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 XY genital ridges (n=4; solid arrows in “XY cont” of Fig. 14C), which is in contrast to the poor vascular development in the corresponding area of XX genital ridges (broken arrows in “XX cont” of Fig. 14C). Even in GD conditions, XY explants displayed testis-specific vascular development just beneath the coelomic epithelium (solid arrows in “XY GD” of Fig. 14C), 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. 11A, 11E, 12D, 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. 15; upper column in Table 2).

In GD explants (5 mg/dl), neither high-level SOX9 expression nor cord formation was detected in XY explant cultures (Figs. 11A, 12B,C, 15A). 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 (Fig. 15B,C). Interestingly, in explants at 185 mg/dl glucose, SOX9-positive cells and their cord-like structures were found 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. 15D).

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. 15E, the addition of pyruvate restored both high levels of SOX9 expression and testis cord formation, as was the case in control XY explants (Fig. 15D). Therefore, reduced SOX9 expression and defective cord formation in GD explants are likely to be due to the lower energy availability of 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), I 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. 16; lower column in Table 2).

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 high-glucose explants of 11.0 dpc XY genital ridges (“cont” of Fig. 16A,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 11.5 dpc XY explants (Fig. 16C). In 11.0 dpc XY genital ridges under GD

conditions, SRY and SOX9 expressions were properly induced within 12 h after culture initiation (“GD” in Fig. 16A, data not shown), but SOX9 expression was rapidly reduced to a lower level after 24 h (“GD” in Fig. 16B). In contrast to proper initial SOX9 activation even in GD conditions, glycogen accumulation in pre-Sertoli cells was not detectable throughout the culture period (“GD” in Fig. 16A,B). This implies that although glucose starvation clearly represses testis-specific glycogenesis, glucose starvation, 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. 15E), I 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 (5mg/dl, “GD + pyruvate” in Fig. 16; lower column in Table 2). 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 of GD cultures. This finding in turn suggests that endogenous glucose storage in pre-Sertoli cells may not be essential for subsequent testis formation, assuming that 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 previous data demonstrated that 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, I finally carried out the rescue experiments in 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 feedforward loop (Malki *et al*., 2005; Wilhelm *et al*., 2005; 2007; Kim *et al*., 2006). First, I added exogenous FGF9 (100 ng/ml) or PGD2 (500 ng/ml) into the GD medium, and cultured 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 (Fig. 17A). All explants treated with FGF9 or PGD2 also showed defective formation of well-defined testis cords in the gonadal area (n= 10 for each additive; Fig. 17B). This is clearly consistent with the present real-time RT-PCR data showing no significant reduction in both *Fgf9* and *Pgds* expression in XY GD explants (Fig. 14B).

The present data also demonstrated the defective formation of basal lamina (Fig. 11C) and the drastically reduced expression of ECM molecules (such as Laminin and *Col9a3*) in pre-Sertoli cells of XY GD explants (Figs. 12C; 13C). 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, 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. 18; Table 3). In parts of 11.5 dpc XY genital ridges, I excised the anterior and posterior edges to directly expose pre-Sertoli cells to Matrigel (Fig. 18A). The segmented or whole explants were embedded in Matrigel on filters, and then cultured in GD medium (5 mg/dl).

In segment cultures, both SOX9 expression and cord formation were induced in control (high-glucose) medium, but defective in GD medium (lower plates, Fig. 18B), as was the case in control and GD explants using whole genital ridges (upper plates, Fig. 18B; see also Figs. 12B, 12C, 15). 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. 18B; upper column in Table 3), suggesting non-utilization of the additives as an alternative metabolic energy source to glucose. Interestingly, in 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. 18B; lower column in Table 3). A similar recovery of both SOX9 expression and cord formation was also observed in segment explants treated with ECM gel alone (without FGF9 addition) (Fig. 18C). Unfortunately, I could not find any appreciable positive effects of exogenous FGF9 addition in segment explants embedded in ECM gel (Table 3), but SOX9 expression appeared to be higher in the cells directly attached on ECM gel in the gonadal area near the cutting edges, as compared with those near the area with sparse ECM deposits (Fig. 18C). 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 feedforward pathway of SOX9 expression is likely to be a rate-determining step in glucose-energy metabolism during gonadal sex differentiation.

DISCUSSION

It was previously shown in chapter 1 that testis-specific glycogenesis in pre-Sertoli cells occurs immediately after the onset of *Sry* expression, 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*, 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 (Fig. 11C-F). Even in control (high-glucose) explants, GRP78 expression was detectable, albeit weak, in Sertoli cells inside testis cords, suggesting an inadequate supply of glucose or other energy source even in 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; Kidokoro *et al*., 2005; Sekido and Lovell-Badge, 2008). In contrast to transient *Sry* expression, *Sox9* expression in pre-Sertoli cells continues throughout life, suggesting a distinct difference between the mechanisms which underlie 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 developing testes (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. My data demonstrated that glucose starvation severely affects maintenance of SOX9 expression in pre-Sertoli cells, while SF1/Ad4Bp, GATA4 and WT1 expressions continue 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 (Fig. 16). 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 (Malki *et al*., 2005; Wilhelm *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 feedforward 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, the rescue experiments demonstrated that the external supply of ECM gel could restore the maintenance of SOX9 expression in XY GD explants (Table 3, Fig. 18). These findings possibly suggest that some ECM components are involved in the positive feedforward 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 well known that various growth factors and cytokines are also known to be embedded in 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 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 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).

SUMMARY

In chapter 2, in order to investigate the functional significance of glycogen or high-glucose condition on gonadal sex differentiation, I performed a series of glucose-deprivation (GD) experiments *in vitro*, and conducted detailed histological and molecular analyses. My data demonstrated that, among various somatic cell types in XY and XX gonads, pre-Sertoli cells are the most sensitive to glucose starvation in sex differentiating period. Although GD did not affect the initiation of Sox9 expression in XY genital ridges, it resulted in a markedly defective maintenance of SOX9 expression in pre-Sertoli cells, leading to the failure of testis cord formation and severely reduced expression of several extracellular matrix (ECM) components including Laminin and *Col9a3*. Addition of FGF9 (SOX9-maintenance factor)/ECM gel (a mediator of FGF signal) restored SOX9 expression and subsequent cord formation in XY genital ridges of GD. However, *Fgf9* expression was not altered in GD XY explants, and addition of FGF9 alone did not rescue defective SOX9 expression or cord formation in GD. These findings indicate that the establishment of SOX9 maintenance mechanism via ECM-mediated positive feedforward pathway in pre-Sertoli cells is a metabolically active process with high-energy requirements. This further suggests the importance of high-glucose condition assured by glycogen in the establishment of SOX9 maintenance mechanism in testis differentiation. 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 types, developmental stages and molecular/cellular events in mammalian organogenesis.

FIGURES & TABLES

Fig. 11. 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 transmission 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 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.

Fig. 11

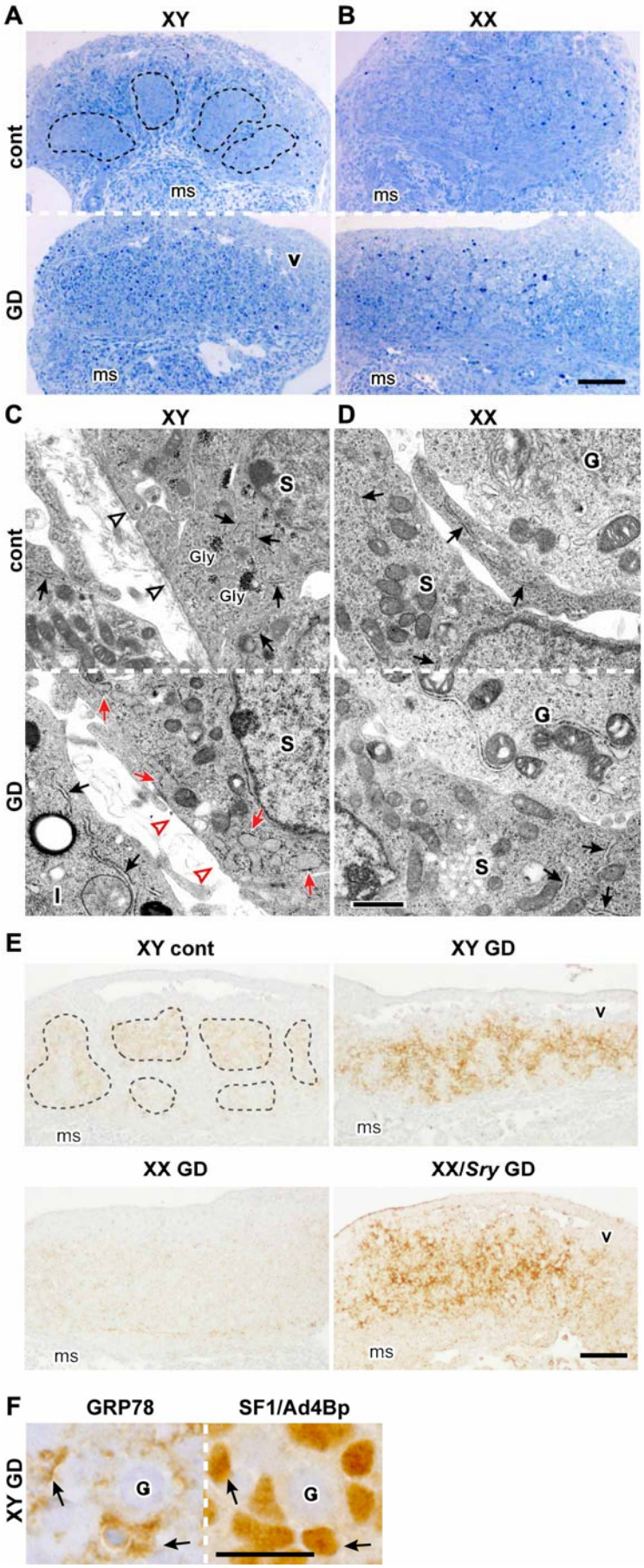

A
XY
B
XX
cont
GD
ms
v
C
XY
D
XX
S
Gly
G
I
E
XY cont
XY GD
XX GD
XX/Sry GD
F
GRP78
SF1/Ad4Bp
XY GD

Fig. 12. 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 4~72 h in high-glucose (control; 410 mg/dl) or glucose-deprived (GD; 5 mg/dl) medium, and then comparatively examined by PAS staining (A; red), and anti-SOX9 (B), anti-Laminin (C), anti-SF1/Ad4Bp or anti-GATA4 (D), anti-MIS (E) and anti-WT1 (F) 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 glycogen consumption and reduces 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). Especially for WT1, its expression was maintained even in GD explants cultured for 72 h (F). Insets show high magnified images of the nuclear staining. ms; mesonephros, v; blood vessel. Bar, 100 μm.

Fig. 12

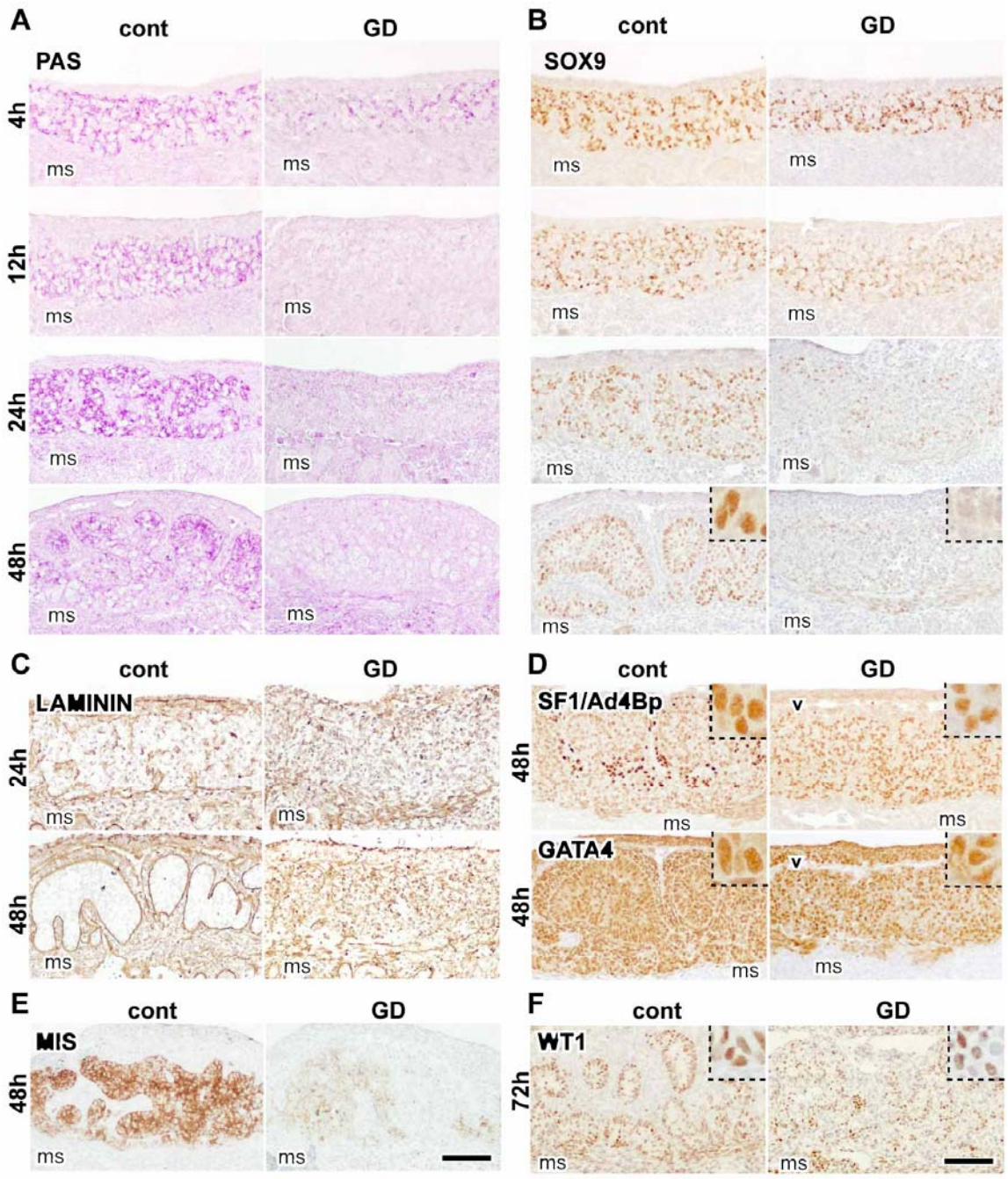

A
cont
GD
PAS
4h
12h
24h
48h
ms
B
cont
GD
SOX9
ms
C
cont
GD
LAMININ
24h
48h
ms
D
cont
GD
SF1/Ad4Bp
48h
v
ms
GATA4
E
cont
GD
MIS
48h
ms
F
cont
GD
WT1
72h
ms

Fig. 13. 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 dose 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 XY genital ridges isolated at 11.5 dpc (before culture initiation), respectively (n= 4).

Fig. 13

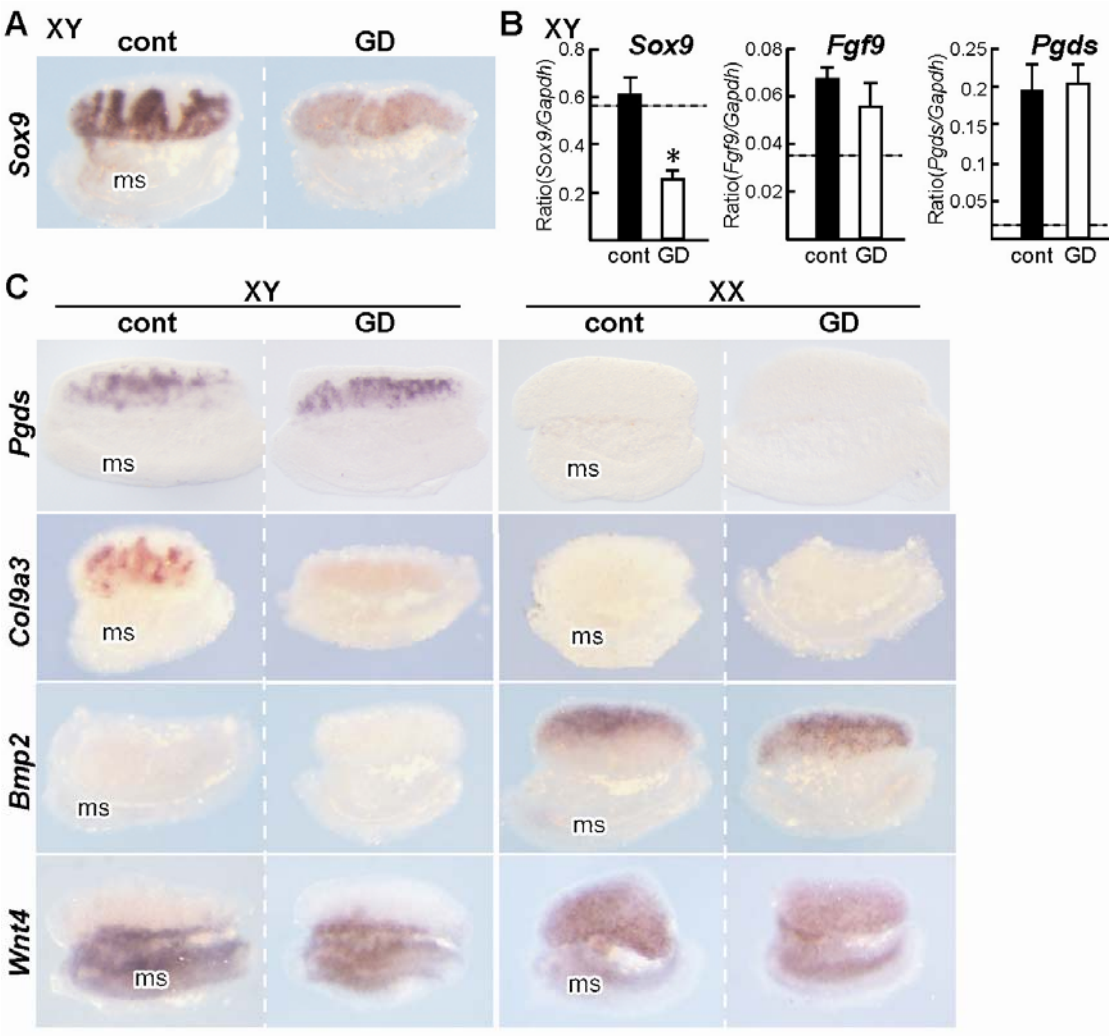

A
XY
cont
GD
Sox9
ms
B
XY
Sox9
Fgf9
Pgds
Ratio(Sox9/Gapdh)
Ratio(Fgf9/Gapdh)
Ratio(Pgds/Gapdh)
0.8
0.6
0.4
0.2
0.08
0.06
0.04
0.02
0.25
0.2
0.15
0.1
0.05
*
cont GD
C
XY
XX
cont
GD
Pgds
Col9a3
Bmp2
Wnt4
ms

Fig. 14. 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 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 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 are 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 coelomic epithelia in XX genital ridges (broken arrows). Asterisks, testis cords; ms, mesonephros. Bar, 100 μm.

Fig. 14

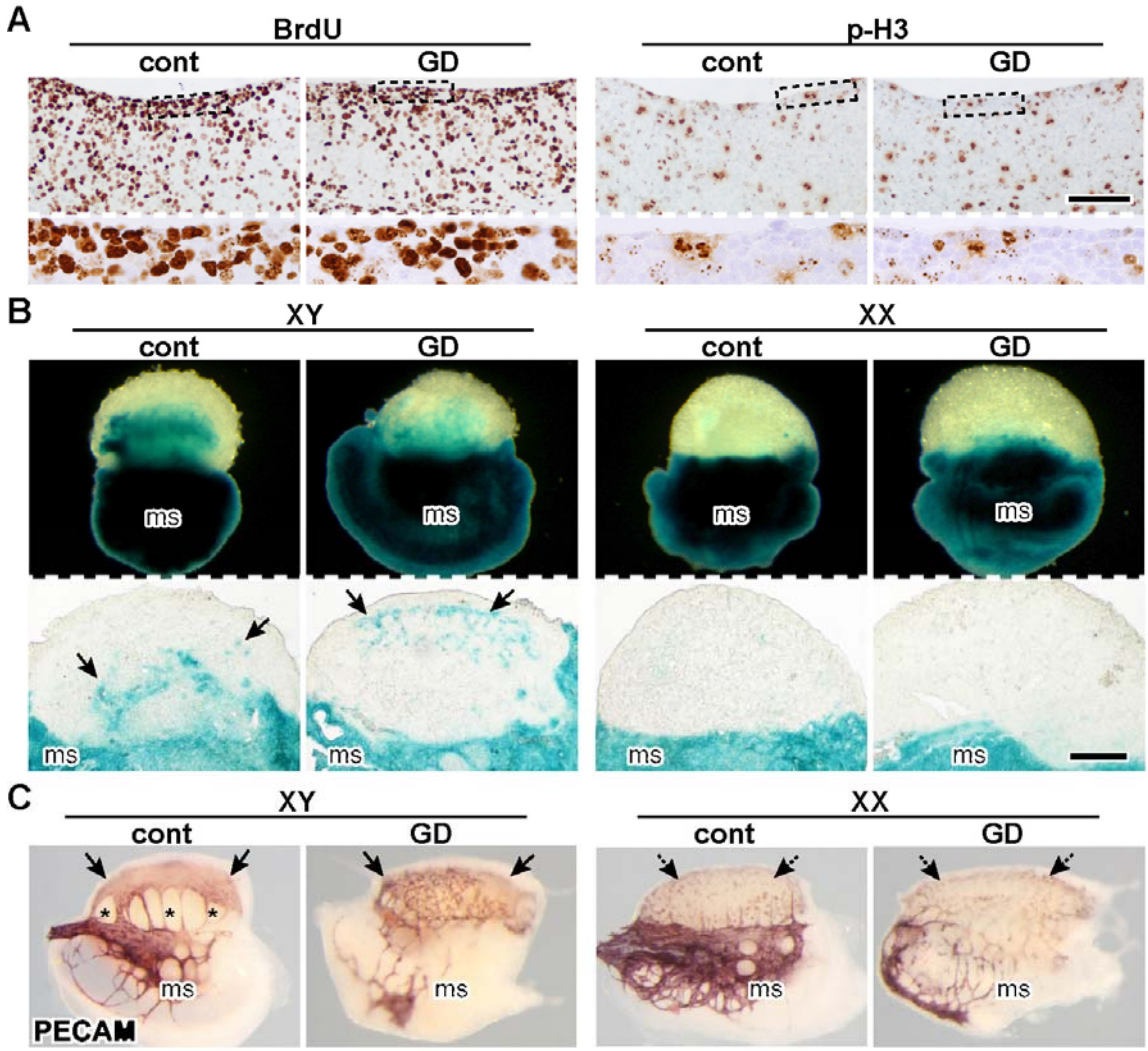

A
BrdU
cont
GD
p-H3
cont
GD
B
XY
cont
GD
XX
cont
GD
ms
C
XY
cont
GD
XX
cont
GD
ms
PECAM

Fig. 15. 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 (5mg/dl) explants, neither SOX9 expression nor cord formation is detected in XY explants cultures (A), but both SOX9 expression and cord formation are 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. 15

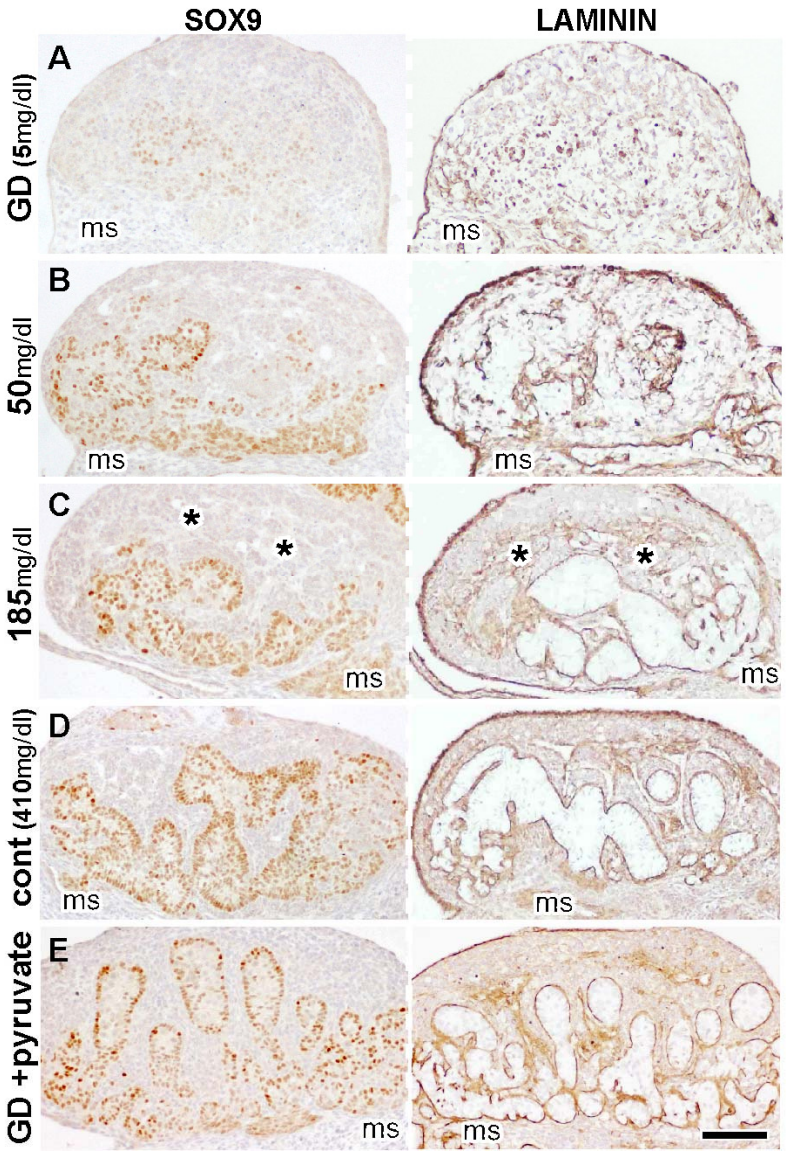

SOX9
LAMININ
A
GD (5mg/dl)
ms
ms
B
50mg/dl
ms
ms
C
185mg/dl
*
*
*
*
ms
ms
D
cont (410mg/dl)
ms
ms
E
GD +pyruvate
ms
ms

Fig. 16. 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) are 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.

Fig. 16

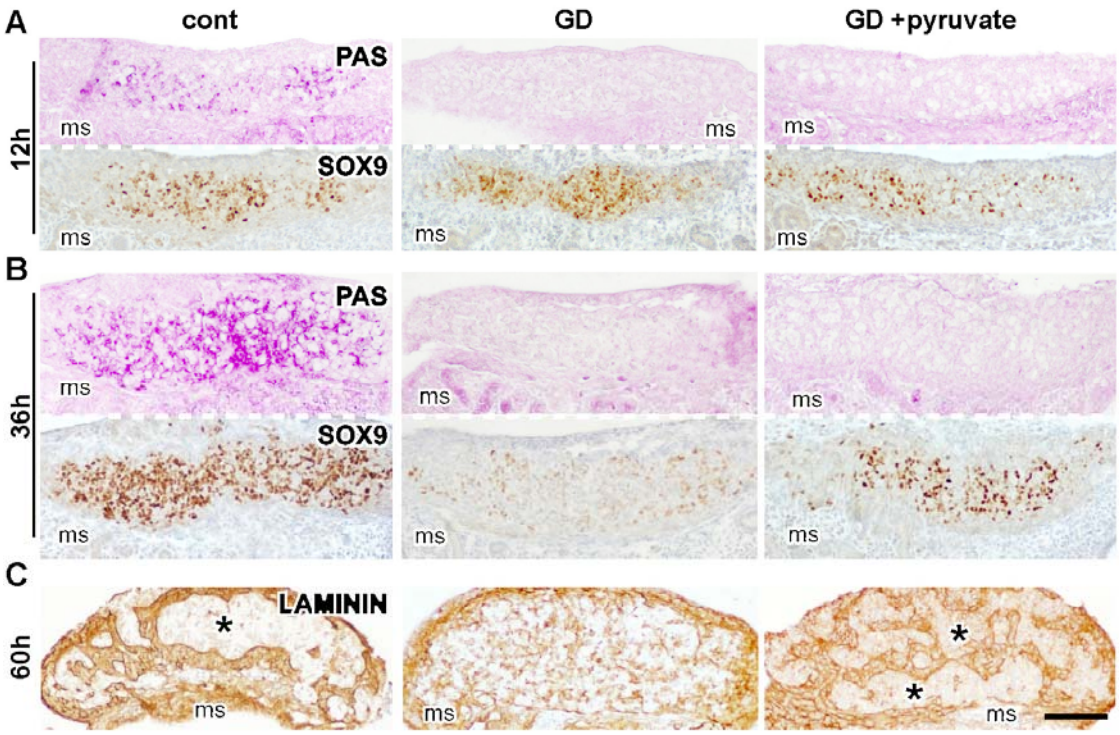


Fig. 17. Addition of FGF9 or PGD2 alone cannot restore SOX9 expression (A) and subsequent testis cord formation (B) under GD culture conditions

XY genital ridges at 11.5 dpc (18-19ts) were cultured in high-glucose (control; 410 mg/dl) or glucose-deprived (GD; 5 mg/dl) medium in the presence or absence of FGF9 (100ng/ml) or PGD2 (500ng/ml) for 48 h. Then, the cultured explants were comparatively examined by immunohistochemistry using anti-SOX9 (A) and anti-Laminin (B) antibodies. Addition of FGF9 or PGD2 alone can restore neither high-level expression of *Sox9* nor testis cord formation under GD condition. ms; mesonephros. Bar, 100 μm.

Fig. 17

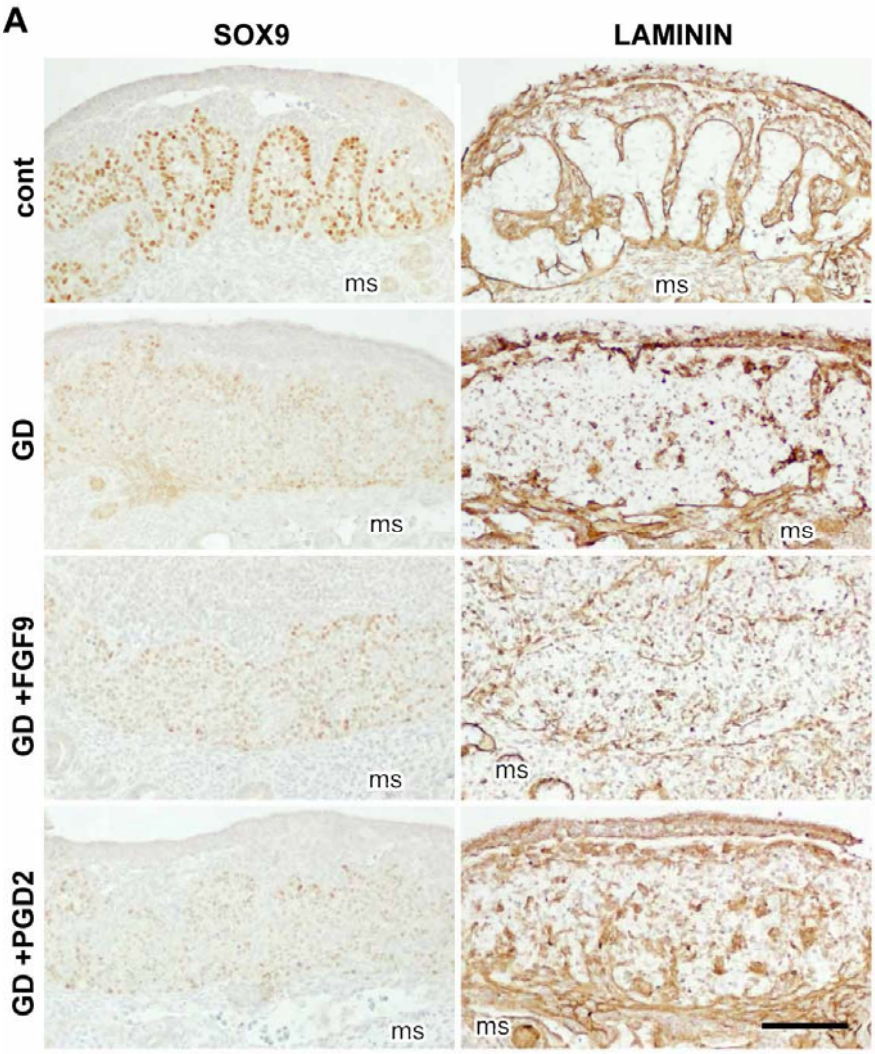


Fig. 18. External supply of extracellular matrix (ECM) gel around pre-Sertoli cells is able to restore SOX9 expression and subsequent testis cord formation under GD culture conditions

(A) A schematic representation showing the whole (upper) or segment (lower) culture experiment of 11.5 dpc genital ridges embedded in Matrigel on filter (GD medium; 2 days). In parts of genital ridges, 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 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 cutting edges in 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 formation are properly induced in control (high-glucose) medium (left lower in B), but defective in glucose-deprived medium (middle lower in B), as was the case for whole explants (left and middle upper in B). Regardless of the presence or absence of exogenous FGF9 addition, external supply of Matrigel can restores both high-level SOX9 expression and testis cord formation in segment explants (right lower in B; C), but not in whole explant (right upper in B) under the same GD conditions. In XY GD explants embedded in Matrigel, SOX9 expression appears to be higher in Sertoli cells directly attached on ECM in the gonadal area near 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 exogenous Matrigel, while arrows indicate cutting edges of explants. In C, arrowheads or broken lines mark the region corresponding to sparse broken or strong continuous signals for anti-Laminin staining, respectively. ms; mesonephros. Bar, 100 μm.

Fig. 18

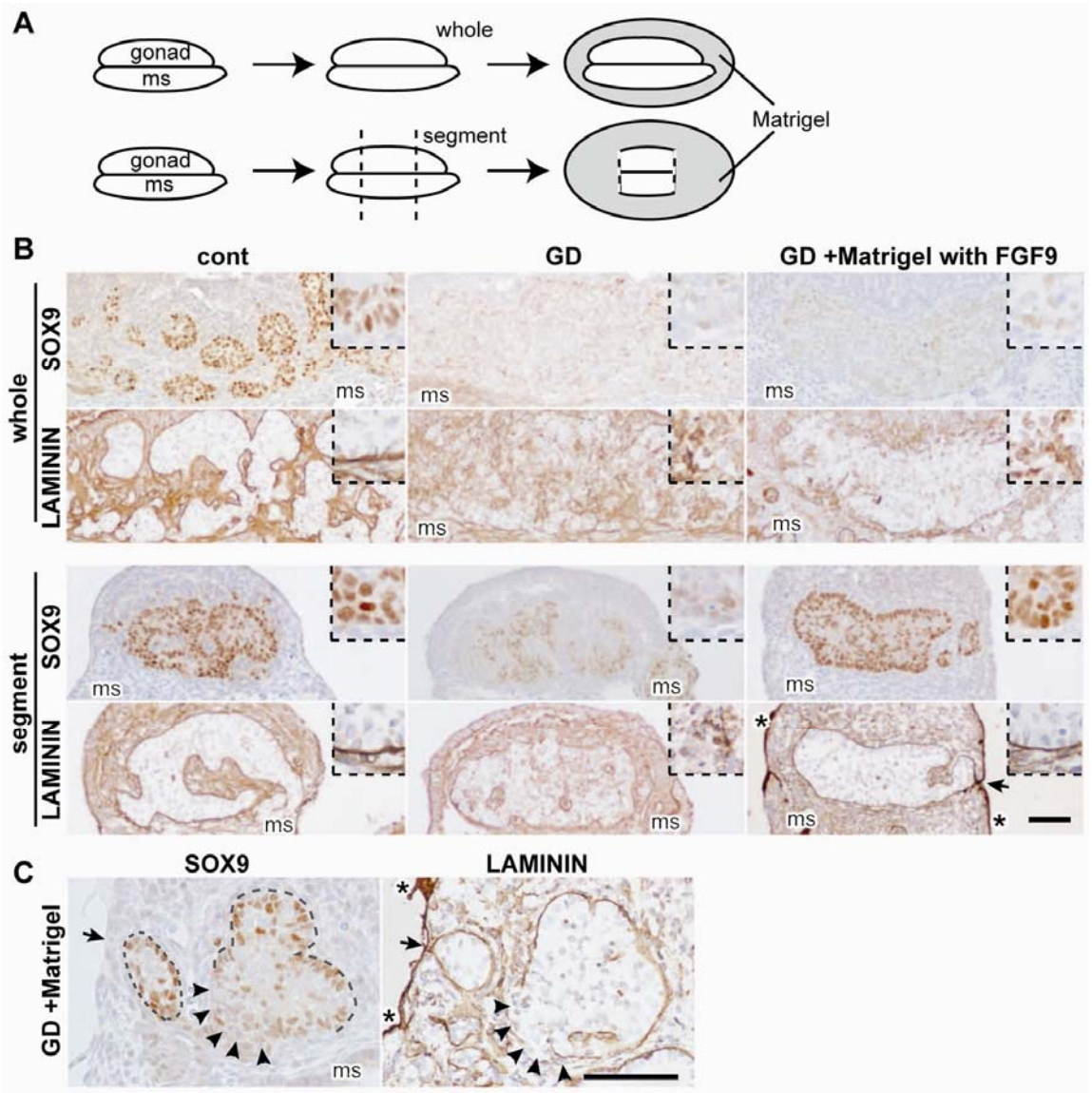

A
gonad
ms
whole
Matrigel
segment
gonad
ms
B
cont
GD
GD +Matrigel with FGF9
whole
SOX9
LAMININ
segment
SOX9
LAMININ
ms
C
SOX9
LAMININ
GD +Matrigel

Table 2. 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 [a]

Stage at culture initiation	Glucose / Pyruvate concentration in medium (mg/dl)				Testis cord formation [b]				
					++	+	+/-	-	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

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

b 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 (++).

Table 3. Summary of testis cord formation in glucose-deprived (GD) cultures using whole or segmented genital ridges that are embedded in Matrigel with or without FGF9 [a]

	Glucose concentration in medium (mg/dl)	Additives (Matrigel [dialyzed], FGF9 [100 ng/ml])	Testis cord formation [b]				
			++	+	+/-	-	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

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

b 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 (++).

GENERAL DISCUSSION

The purpose of this study was to reveal energy metabolism regulation system and to investigate the significance of energy metabolic regulation for organogenesis using the gonads of sex differentiation period as a model, and finally to integrate the notion of “energy metabolism” into “organogenesis”.

Sex determination mechanism widely varies among taxa. Many sex determining systems have evolved in vertebrates, including environmental sex determination in fishes and reptiles, and genetic sex determination in birds and mammals (Western and Sinclair, 2001; Yao and Capel, 2005). In a molecular level, *Sry* is conserved only in mammals, while *Sox9* is a fundamental testis-determining gene in vertebrates including fishes, reptiles, Amphibia, birds and mammals (Morais da Silva *et al*., 1996; Morrish and Sinclair, 2002). Although the identification of *Sry* as a master gene of testis-determination in mammals in 1990 (Sinclair *et al*., 1990) led to the enthusiastic analyses to find the target gene of SRY (McElreavey *et al*., 1993; Pontiggia *et al*., 1994; Ohe *et al*., 2002; Thevenet *et al*., 2005), the ever identified target of SRY is *Sox9* alone (Sekido and Lovell-Badge, 2008). Since *Sox9* is a widely conserved gene as described above, the identification of other target gene regulated by SRY is required to comparatively analyze the molecular evolution of mammalian sex determination mechanism. In this study, I demonstrated that testis-specific glycogen accumulation in pre-Sertoli cells is initiated at the same timing of the onset of SOX9 expression, and it is regulated by downstream of *Sry* action. These results strongly suggest that a candidate factor(s), which plays pivotal role in the regulation of glycogenesis in pre-Sertoli cells, can be one of the direct targets of SRY, other than *Sox9* (Fig. 19). Further analyses at a molecular level may lead to the identification of a novel direct target of SRY and result in

providing a good tool to elucidate the molecular evolution of mammalian sex determination mechanism.

Testis-specific glycogenesis in pre-Sertoli cells is transiently observed from 11.2 dpc (14ts) when gonads are unable to distinguish male from female by its morphology, to 13.5 dpc, just after the completion of testis-specific cord formation. Similarly, transient glycogen accumulation is observed when the precursors of osteoblasts differentiate and induce born formation involving calcification *in vivo* (Cabrini, 1961; Scott and Glimcher, 1971) and *in vitro* (Harris, 1932; Decker *et al*., 1995). In addition, during tooth morphogenesis in mice, Ohshima *et al*. (1999) have suggested that glycogen deposition in enamel organ cells from the cap to early bell stages (E14-E15) is associated with the active secretion of glycosaminoglycan components into extracellular spaces. These reports clearly suggest that high-glucose conditions attributed to glycogen depositions are closely associated with cellular protein production and secretion during tissue morphogenesis and differentiation. Consistent with these reports, I showed that in testis differentiation, high-glucose metabolic state is required for the production of ECM, which eventually contributes to the establishment of SOX9 maintenance mechanism and subsequent testis cord formation. To the best of our knowledge, this is the first proposal of molecular model that explains the relation between high-glucose state and its functional significance in mammalian development.

This study further provides a novel concept for the molecular action of master gene in organ determination. “Master gene” is a concept of a single gene whose expression is both necessary and sufficient to trigger the activation of many other genes in a coordinated fashion, leading to the development of a specific tissue or organ. In this regard, *Sry* and *Sox9* both meet the above condition. Other than them, several master

genes, although little, are known to be the conductor of organogenesis. For instance, *Pax6* (paired-box-containing gene 6) which encodes a transcription factor having a paired-domain as a DNA binding domain, is a highly conserved gene that controls eye morphogenesis in all species where it has been tested (Pichaud and Desplan, 2002; Tsonis and Fuentes, 2006). *Pdx1* (pancreatic duodenal homeobox 1), encoding a homeo-domain containing transcription factor, is the well known determinant of pancreas morphogenesis in mammals (McKinnon and Docherty, 2001; Kaneto *et al*., 2008). However, the past functional analyses of these master genes were restricted in its fate-determining function, of course not performed in terms of energy metabolism regulator. Although PPARγ is famous for both as a master gene of adipose tissue differentiation (Tontonoz *et al.*, 1994; Barak *et al.*, 1999) and as a metabolic regulator of lipid homeostasis (Lowell, 1999; Anghel *et al.*, 2007), its function as the regulator of metabolism is investigated only in adult tissues, but not in the process of embryonic organogenesis (Tontonoz and Spiegelman, 2008). In this study, I demonstrated that *Sry* acts not only on testis-fate determination but also on energy metabolism as the regulator of glycogenesis which may contribute to the subsequent testis development. To the best of my knowledge, this is the first evidence that one master gene governs organ determination and energy metabolism required for subsequent organogenesis, simultaneously. Considering that development of each organ accompanies dynamic morphogenesis, such energy-supply mechanism may underlie in various organogenesis and might be regulated by the master gene of each tissue.

From a pathophysiological viewpoint, the relation between the high-glucose state possibly provided by glycogen deposits and the completion of proper mammalian organogenesis has been shown to be important. Hypoglycemia, or low blood glucose, is a common clinical condition in human and livestock as a result of disease states such as

starvation or tumors (Senior and Sadeghi-Nejad, 1989), or particularly as a side effect of diabetes mellitus therapy (McAulay *et al*., 2001). Hypoglycemia has been demonstrated to interfere with normal embryogenesis in laboratory animals *in vivo* (Smithberg and Runner, 1963; Ellington, 1980; Buchanan *et al*., 1986) and *in vitro* (Ellington, 1980; Akazawa *et al*., 1987; Ellington, 1987; Sadler and Hunter, 1987; Akazawa *et al*., 1989; Smoak and Sadler, 1990). And it is also known as one possible cause of intrauterine growth retardation (IUGR), which is a term for a fetus who is smaller than expected in mammals (Abell *et al*., 1976; Woodall, 1996; Ogata *et al*., 1999; Hay, 2006). Characteristic pathological phenotype of gestational hypoglycemia is observed in heart and kidney in mouse, rat and sheep models (Amri *et al*., 1999; Smoak, 2002; Boyce *et al*., 2007). Even brief period of exposure, hypoglycemia during gestation can alter morphology, function and metabolism of the heart in postnatal mice (Smoak, 1997). *In vitro* glucose deprivation experiment obtained impaired metanephros growth and differentiation in rats (Amri *et al*., 1999). Considering the facts that heart and kidney are the major tissues which deposits a huge amount of glycogen in embryonic period (Gutierrez-Correa *et al*., 1991), and that glycogen depletion in embryonic heart induces abnormal cardiac development in a glycogen synthase knockout mouse model (Pederson *et al*., 2004), it is suggested that glycogen accumulating cells are metabolically active and therefore sensitive to hypoglycemia. This is clearly consistent with the results of this study that glycogen accumulating pre-Sertoli cells are most sensitive to glucose deprivation among gonadal somatic cells in sex differentiating period, and that testis morphogenesis is impaired in gonads exposed to glucose deprived condition at the timing of glycogen accumulation *in vitro*. With regard to these evidences, it can be hypothesized that testicular morphogenesis might have some defects when exposed to hypoglycemia during critical period in gestation *in vivo*, resulting in possible congenital malformations

in postanatal animal or human testis. Therefore, my study highlights the importance of glycogen reservation in testis differentiation of mammals, and in turn suggests the existence of unrecognized defects of male reproductive function in the offspring of starved or diabetic females. *In vivo* analyses evaluating the effects of hypoglycemia on testis differentiation should be required.

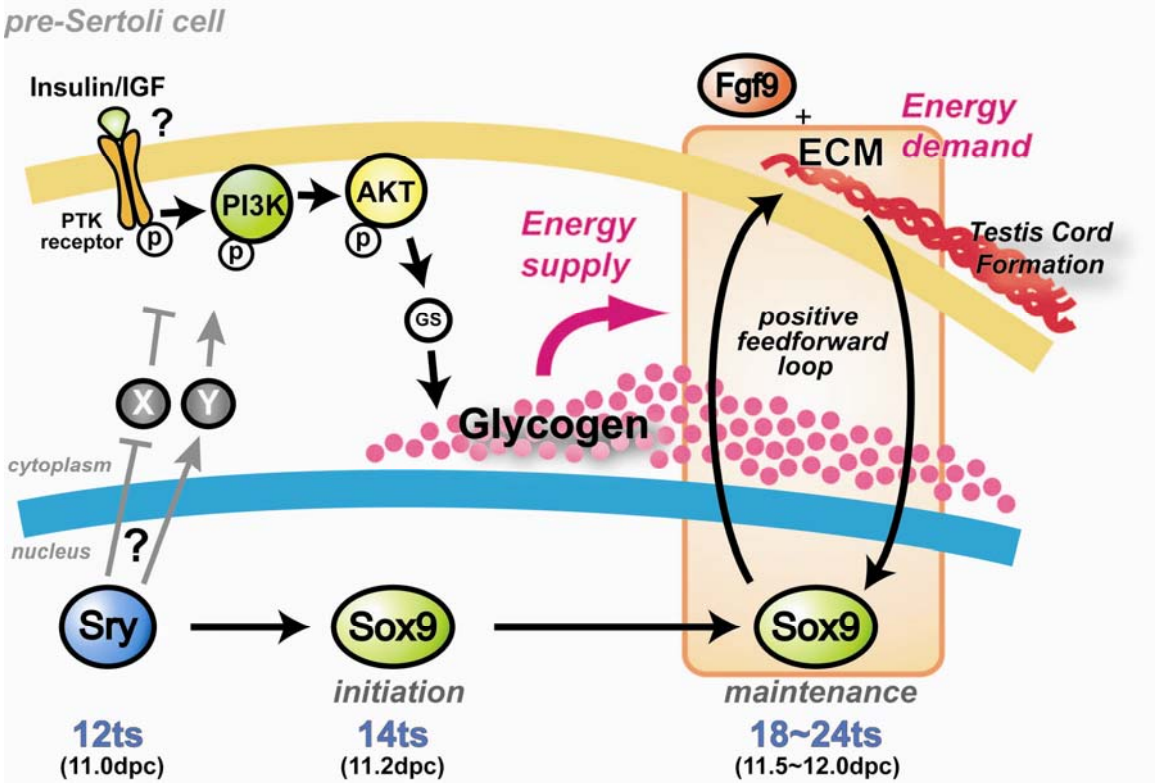
Why glycogen, not lipid droplet? As described above, glycogen transiently accumulates in pre-Sertoli cells probably to serve energy for subsequent testis differentiation, and glycogen is observed several tissues in embryogenesis, such as heart, kidney, bone, etc. However, transient accumulation of lipid droplet, another intracellular energy storage, is hardly observed in any tissue in embryonic organogenesis period of mammals (Hull, 1975), even though lipid can store more than double energy per mass compared with glycogen (approximately 9 Kcal/g (39 kJ/g) for fatty acids and 4 Kcal/g (17 kJ/g) for carbohydrates). There are two possible explanations for this, 1) oxygen and 2) timing. 1) Glucose/glycogen can be catabolized even under anaerobic condition through glycolysis, but lipid requires oxygen for its catabolism in mammals. Since it is known that embryonic blood circulation is poorer for oxygen than mother (approximately 80% in embryo: >98% in mother in humans), embryos might be unable to catabolize lipid efficiently. 2) Lipid requires much more time to be catabolized than glucose. In particular, the breakdown rate for lipid storage is 30 to 50% slower than that of glycogen to produce ATPs in human muscles (van der Vusse and Reneman, 1996; McArdle *et al.*, 2001). Since every organogenesis events occur with rapidity, lipid may not be able to serve an appropriate amount of energy on the timing of demand. Anyway, it is interesting to investigate the spatio-temporal relationships of fetal physiology and surrounding maternal conditions in developmental process at a molecular level.

In summary, here I propose a novel model of molecular mechanism regulating energy metabolism by *Sry* from both supply and demand aspects in pre-Sertoli cells in gonadal sex differentiation (Fig. 19). In this model, SRY regulates glycogenesis [energy supply] by directly controlling the transcription of intracellular signaling factor(s), resulting in activation of PI3K-AKT pathway in pre-Sertoli cells. Simultaneously, SRY also initiates a downstream cascade requiring high-energy metabolism [energy demand], involving the establishment of SOX9 expression-maintenance mechanism through an ECM-mediated feedforward pathway. Glycogen storage in pre-Sertoli cells induced by *Sry* is likely to act as a backup energy source for subsequent establishment of SOX9 maintenance mechanism and probably for subsequent cord formation. Taken together, I demonstrated a novel role of *Sry*, other than as a testis-determining factor, as a regulator of the energy metabolism in gonadal sex differentiation. Above all, this study opened up the new field of mammalian embryogenesis with the notion of “energy metabolism” integrated into “organogenesis”.

Fig. 19. A schematic model of male-specific glucose supply and demand in differentiating pre-Sertoli cell

In testis differentiation, *Sry* expression is first initiated at 12ts (11.0 dpc), and immediately after *Sry* expression both glycogen accumulation and initial SOX9 expression start to occur at 14ts (11.2 dpc) in pre-Sertoli cells. Such glycogen storage is induced through PI3K-AKT pathway which is probably activated by insulin/IGF signals. SRY may regulate glycogenesis by repressing transcription of female-specific repressor (X) or by activating transcription of male-specific regulator (Y), resulting in activation of PI3K-AKT pathway in pre-Sertoli cells. After decline of *Sry* expression and initiation of *Sox9* expression by SRY, *Sox9* expression is maintained through positive feedforward system of several signaling during 11.5 to 12.0 dpc. Among SOX9-maintenace feedforward pathways, ECM-mediated step is likely to be 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 around 11.75 dpc and probably for subsequent cord formation after 12.0 dpc. GS: glycogen synthase, PTK: protein tyrosine kinase, ECM: extra-cellular matrix.

Fig. 19


pre-Sertoli cell
Insulin/IGF
?
PTK
receptor
p
PI3K
p
AKT
p
GS
Fgf9
+
ECM
Energy
demand
Testis Cord
Formation
Energy
supply
positive
feedforward
loop
Glycogen
X
Y
cytoplasm
nucleus
?
Sry
Sox9
initiation
12ts
(11.0dpc)
14ts
(11.2dpc)
Sox9
maintenance
18~24ts
(11.5~12.0dpc)

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