A novel Sry-downstream cellular event which preserves the readily available energy source of glycogen in mouse sex differentiation

Shogo Matoba1, Yoshiakira Kanai1,*, Tomohide Kidokoro1, Masami Kanai-Azuma2, Hayato Kawakami2, Yoshihiro Hayashi3 and Masamichi Kurohmaru1

1Department of Veterinary Anatomy, 2Department of Global Agricultural Sciences, The University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113-8657, Japan
2Department of Anatomy, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan
*Author for correspondence (e-mail: aykanai@mail.ecc.u-tokyo.ac.jp)

Accepted 18 January 2005
Journal of Cell Science 118, 1449-1459 Published by The Company of Biologists 2005
doi:10.1242/jcs.01738

Summary

Sry is transiently activated in pre-Sertoli cells of the gonadal ridge to initiate testis differentiation in mice. In pre-Sertoli cells, however, the cellular events induced immediately after the onset of Sry expression remain largely unknown. Here we show that testis-specific glycogen accumulation in pre-Sertoli cells is one of the earliest cellular events downstream of Sry action. In developing XY gonads, glycogen accumulation starts to occur in pre-Sertoli cells from around 11.15 dpc (tail somite 14 stage) in a center-to-pole pattern similar to the initial Sry expression profile. 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 stage. In vitro analyses using various culture conditions suggest that testis-specific glycogen deposition is a tissue-autonomous event that can be induced even in serum-free conditions and in a culture of gonadal explants without adjacent mesonephros. Moreover, glycogen accumulation in pre-Sertoli cells was significantly inhibited in vitro by the PI3K inhibitor LY294002, but not by the MEK inhibitor PD98059. 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. Therefore, these findings suggest that immediately after the onset of Sry expression, activation of the PI3K-AKT pathway promotes testis-specific glycogen storage in pre-Sertoli cells. To the best of our knowledge, this is a novel Sry-downstream cellular event which preserves this readily available energy source in Sertoli cells for testis-specific morphogenesis and hormone production.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/118/7/1449/DC1

Key words: Sry, Sox9, Glycogen, Sertoli cell, PI3K-AKT signaling, Sex differentiation

Introduction

Sry (Sex-determining region of the Y) is essential for initiating male sex differentiation in mammals (Sinclair et al., 1990; Koopman et al., 1991). Sry is transiently expressed in a center-to-pole wave along the anteroposterior (AP) axis of the XY gonad for a very short period just before testis formation in mice (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001). Thus, Sry is likely to act as a trigger for a cascade of molecular and cellular events that direct the bipotential gonad to differentiate into a testis in a center-to-pole manner. Sox9, a Sry-related gene, is one of the candidate genes directly upregulated by Sry. Shortly after the onset of Sry expression, Sox9 is also upregulated in a center-to-pole pattern similar to that of the initial Sry expression profile (Morais da Silva et al., 1996; Kent et al., 1996; Schepers et al., 2003). 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 and activation of testis-specific markers Mit and P450sc (Chaboissier et al., 2004). Moreover, miss-expression of Sox9 in mouse XX gonads results in testis development, as demonstrated by the findings from two transgenic lines (Bishop et al., 2000; Vidal et al., 2001). Therefore, these reports suggest that Sry directly or indirectly promotes testis-specific Sox9 activation, and that Sox9 is involved in the initiation and maintenance of Sertoli cell differentiation during early phases of testis differentiation.

In mouse sex differentiation, three testis-specific cellular events: cell proliferation, cell migration and vasculogenesis, are known to direct early testicularogenesis. 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 may give rise to a certain population of pre-Sertoli cells early on and to interstitial cells throughout this period (Karl and Capel, 1998). The cells contributing to the interstitium, including vascular endothelial cells and peritubular myoid cells, migrate into the testis from the adjacent mesonephros (Buehr et al., 1993; Martineau et al., 1997; Capel et al., 1999; Brennan et al., 2002). These cells are also required for testicular cord formation (Martineau et al., 1997).
1997; Tilmann and Capel, 1999). 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 the coelomic epithelium, endothelial cells or mesonephric cells (Morais da Silva et al., 1996; Kent et al., 1996; Bullejos and Koopman, 2001; Albrecht and Eicher, 2001; Moreno-Mendoza et al., 2004). Although pre-Sertoli cells have been shown to aggregate in the testicular cord at late phases of testis differentiation (from around 12.0 dpc), the cellular events induced immediately after Sry expression in pre-Sertoli cells remain largely unknown.

Testis-specific cellular events, such as proliferation, migration, vasculogenesis and cord morphogenesis, 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 with 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; Sinclair et al., 2003; Gruetter, 2003; Ferrer et al., 2003). Our previous study has shown that embryonic testis is one of the glycogen-rich tissues in mouse organogenic embryos, and that glycogen accumulation predominantly occurs in Sertoli cells within newly formed testicular cords at 12.5 dpc (Canai et al., 1989). Moreover, the glycogen deposits in Sertoli cells rapidly disappear shortly after testicular cord formation, suggesting that the glycogen granules in Sertoli cells are involved as an energy source in the dynamic morphogenesis of the testis and active hormonal production at around 12.5 dpc. However, any further information including the timing of the onset of glycogen accumulation and its mechanism in developing XY gonads is not available at present.

In this study, we examined the spatiotemporal patterns of glycogen accumulation and its signaling pathways during early phases of mouse sex differentiation in order to analyze a 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. We also demonstrated that such glycogen deposition is mediated via the PI3K/AKT signaling pathway, which is activated in gonadal somatic cells in a testis-specific manner. Therefore, these findings clearly illustrate that immediately after the onset of Sry expression, activation of the PI3K-AKT pathway 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 somite (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. 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, we 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. The PI3K (phosphoinositide 3-kinase) inhibitor LY294002 (15 µM; Calbiochem) and the 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 ISOPOR membrane filter (3.0 µm; Millipore), floated on DMEM (Dulbecco’s Modified Eagle’s Medium; Sigma) containing 10% horse serum and penicillin/streptomycin (Gibco-BRL), and cultured at 37°C for 6 to 48 hours as described previously (Canai 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 histochemical and immunoblot analyses.

Histological and histochemical analyses

For periodic acid Schiff (PAS) reaction, the isolated embryos were fixed in 10% formaldehyde containing 2% Ca (CH3COO)2 at 4°C for 12 hours, and then dehydrated in ethanol, and embedded in paraffin. Serial sagittal sections (approximately 5 µm in thickness) were cut and stained with PAS reagent (Canai et al., 1989). For transmission electron microscopy, the genital ridges at 14-18 ts were postfixed in 1% OsO4-0.05 M potassium ferricyanide in 0.05 M PB containing 0.05 M sucrose at 4°C for 4 hours. After washing with 0.05 M PB containing 0.05 M potassium ferricyanide, the samples were postfixed in 1% OsO4-0.05 M potassium ferricyanide in 0.05 M PB at 4°C for 2 hours. 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 1010 transmission electron microscope at 80 kV.

Immunohistochemistry

For immunohistochemistry, both embryos and cultured explants were fixed in 4% PFA-PBS at 4°C for 4 hours, dehydrated, and then
embedded in paraffin. Deparaffinized sections were subjected to immunohistochemistry, using anti-SOX9 antibody (Ab) (10 ng/µl; kindly provided by Peter Koopman, University of Queensland, Australia) (Kent et al., 1996), anti-SF-1/Ad4BP Ab (1/1000 dilution; kindly provided by Ken-ichirou Morohashi, the National Institute for Basic Biology, Japan) (Hatano et al., 1994; Ikeda et al., 2001), and anti-phospho-AKT Ab (Ser473; 1/50 dilution; Cell Signaling Technology). The sections were incubated with each rabbit primary antibody at 4°C for 12 hours. For comparative staining with PAS reagent and anti-SOX9 or anti-SF-1/Ad4BP Ab, two consecutive 4 µm-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 using PAS, while another mirror section was incubated with anti-SOX9 or anti-SF-1/Ad4BP Ab at 4°C for 12 hours. Thereafter, the immunoreaction with each first antibody was visualized by HRP-labelled anti-rabbit IgG Ab using a Tyramide Signal Amplification kit (NEN Life Science Product). 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-SF-1/Ad4BP or anti-phospho-AKT Ab.

Immunoblot analysis

The genital ridges at 9-11 ts were cultured at 37°C for 24 hours in 10% horse serum-DMEM with or without inhibitors. 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), anti-AKT (1:1000 dilution; Cell Signaling Technology), anti-phospho-ERK (Thr202/Tyr204; 1:1000 dilution; Cell Signaling Technology) or anti-ERK (1:1000 dilution; Cell Signaling Technology) Ab at 4°C for 12 hours. The immunoreaction was visualized with HRP-labelled goat anti-rabbit secondary Ab using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) as described previously (Kanai et al., 1996).

Results

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

Our previous study showed that embryonic testis is a glycogen-rich tissue in mouse embryogenesis, and that glycogen accumulation predominantly occurs in Sertoli cells within newly formed testicular cords (Kanai et al., 1989) (Fig. 5C in this study). Fig. 1 shows a sex-dimorphic distribution of glycogen deposits in mouse embryos at 11.5 dpc [18 tail somite (ts) stage] using periodic acid Schiff (PAS) staining (red staining). In both XY and XX embryos, several tissues including notochord (nc), skeletal muscle (sm) and the area close to dorsal aorta (da) show positive PAS staining. In the XY gonad, PAS-positive reactions are observed only in the gonadal region of XY, but not XX, embryos. In the XY gonad, PAS-positive cells are located close 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; da; dorsal aorta; ms, mesonephros; 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 the XY (F), but not the XX, gonad (E). G, germ cell; Gly, glycogen granule. Bar, 1 µm. (G,H) Two serial sections of the 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 the XY gonad. The insets show higher magnification of the cells indicated by arrows. asterisk, germ cell; ms, mesonephros. Bar, 50 µm.

To examine the timing of the onset of glycogen deposition
in developing XY gonads, we 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. 2A). 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 (Fig. 2B, right panel). This is clearly in contrast to the absence of PAS-positive cells in wildtype XY gonads at all stages examined (Fig. 2A,B, left panels). Moreover, we separately performed the PAS reaction and immunohistochemical staining with anti-SF-1/Ad4BP (a marker for precursor cells of both Sertoli and Leydig cells) or anti-SOX9 (pre-Sertoli cell marker) Ab using two consecutive mirror sections of the same PFA-fixed specimens. A comparative analysis of PAS reaction and anti-SF-1/Ad4BP staining revealed that PAS-positive cells clearly expressed SF-1/Ad4BP in their nucleus (Fig. 3A,B), although PAS reaction was markedly reduced in PFA-fixed gonads compared with those fixed with 10% formalin/2% Ca(CH3COO)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. 3C,D). Because Sry expression is first detected in XY gonads at around 12 ts (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001), 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 the XY gonad before their expression domains expand to both anterior and posterior ends during early phases of testis differentiation (Bullejos and Koopman, 2001; Albrecht and Eicher, 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. 4A) 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. 4C; also see Fig. S1 in supplementary material). 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

An interesting finding from the present histochemical observations is that PAS-positive cells were frequently found near germ cells in XY gonads even at 14-15 ts (right panels in Fig. 4A). 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. 4B). 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, we 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. 5). It was shown that there was no appreciable
difference between busulfan-treated gonads and the control

---

**Fig. 3.** PAS reaction and anti-SF-1/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-SF-
1/Ad4BP (a marker for precursor cells of both Sertoli and Leydig
cells; A,B) or anti-SOX9 (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 the boxed area in the corresponding
left plate. PAS-positive cells clearly overlap with gonadal somatic cells expressing SF-1/Ad4BP (arrows in B) or SOX9 (arrows in D) in the XY
gonads. ms, mesonephros. Bar, 50 µm.

---

**Fig. 4.** 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 the 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 the boxed area in the 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 the 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). The vertical axis represents the PAS-positive cell number per
gonad, whereas the horizontal axis represents regions I-V of the
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 the anterior (region I) and posterior
(region V) edges from 15 to 16 ts.
either at 11.5 or 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 (A), despite the drastic reduction in number of germ cells. In the testes at 12.5 dpc, the testicular cords are formed in the 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. 5. 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 the testes at 12.5 dpc, the testicular cords are formed in the 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.

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

In our previous study, 3-day cultures of XY gonadal explants initiated at 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 (see Fig. S2 in supplementary material) 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, we 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. 6). In XY explants at 12-13 ts, PAS-positive cells were first detected at 6 hours, and the cells rapidly increased from 12 to 24 hours. No PAS-positive cells were detected in XX explants at 12-13 ts even after 48 hours, which suggests 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 testicular cord formation and Sertoli cell differentiation. Most interestingly, when we cultured 12 ts genital ridges in serum-free DMEM medium (i.e. without 10% horse serum), it was shown that 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. 6).

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, we next examined the PAS-staining pattern in culture explants with or without adjacent mesonephros isolated at 14 ts (Fig. 7). In 24-hour 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. 7B,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. 7C,F). With regards to the present finding of testis-specific induction of glycogen accumulation even in serum-free cultures, this finding 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 fibroblast growth factor 9 (FGF9), platelet derived growth factor (PDGF) and insulin growth factor (IGF), are known to activate two major signaling pathways: the extracellular-signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, and the phosphoinositide 3-kinase (PI3K)/AKT pathway. To analyze a possible role of the PI3K-AKT pathway in sex-dimorphic glycogen accumulation in developing gonads, we examined the effects of the PI3K inhibitor, LY294002, on glycogen accumulation in 10-11ts genital ridges in vitro (Fig. 8; 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. 8C). XY genital ridges treated with 5 µM of LY294002 exhibited a partial inhibition of glycogen deposition, especially in the surface area of the XY gonadal explants (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. By contrast, the 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. 8B), despite the fact that administration of PD98059
drastically reduced the phosphorylation levels of the MEK substrate, ERK, in these explants (see Fig. S3 in supplementary material). 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. 8D-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 its substrate AKT in gonads, we examined the phosphorylation level of XY and XX genital ridges by anti-phospho-AKT (active form of AKT) staining (Fig. 9). In the 48-hour control culture, anti-phospho-AKT immunohistochemical reactions were frequently observed in the gonadal area of XY explants (Fig. 9A). 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. 9A). Immunoblot analysis also confirmed that AKT was phosphorylated at a higher level in the XY explants than in XX explants in 24-hour control cultures of the genital ridges (cont in Fig. 9B). Moreover, in both XY and XX genital ridges, phosphorylation levels were repressed by the administration of LY294002 (+LY in Fig. 9B). 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 ganglion at similar levels in both sexes, but their reactions in the gonadal region were higher in XY than in XX embryos in vivo (Fig. 9C). 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 (Fig. 9A,C, right panels).

Discussion

In mouse sex differentiation, Sry expression is first detected in the central region of XY gonads at around 12 ts (11.0 dpc), with its expression subsequently expanding to the entire gonadal area from 14 to 15 ts in a center-to-pole wave (Bullejos and Koopman, 2001; Albrecht and Eicher, 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 hours) 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, one of the first factors downstream of Sry. 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, the present study shows that testis-specific glycogen deposition can be induced even in serum-free conditions and in a culture of gonadal explants without adjacent mesonephros, which suggests 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;
induced via a distinct pathway from that of glycogen accumulation in developing XY gonads (Puglianiello et al., 2004). This further suggests the possibility that 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 show the genital ridge after being cultured for 0 (A,D) or 24 (B,C,E,F) hours. In the 24-hour 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), whereas no PAS-positive cells are detected in XX explants without mesonephros (F). ms, mesonephros. Bar, 50 µm.

Testis-specific glycogen accumulation does not require or that it occurs earlier than testis-specific Sox9 expression 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, which is one of the major signaling pathways downstream of tyrosine kinase receptors for many polypeptide growth factors, including PDGF, FGF and insulin/IGFs (reviewed by Alessi and Cohen, 1998). This suggests that one or more of these growth factors are involved in testis-specific glycogenesis in pre-Sertoli cells. To date, it has been assumed that several growth factors are downstream of Sox9 and are important in testis differentiation. For example, the XY gonads of Pdgfra-α knockout mice display disruptions in the organization of vasculature and in the partitioning of interstitial and testicular cord compartments (Brennan et al., 2003). In addition, studies have shown that Fgf-9 is involved in testis-specific mesonephric migration and cell proliferation during testis differentiation (Colvin et al., 2001; Schmalh et al., 2004). By contrast, inhibition by the PI3K inhibitor LY294002 to the medium drastically reduced glycogen accumulation in the gonadal area of XY explants (C). By contrast, the 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. 7. 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 show the genital ridge after being cultured for 0 (A,D) or 24 (B,C,E,F) hours. In the 24-hour 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), whereas no PAS-positive cells are detected in XX explants without mesonephros (F). ms, mesonephros. Bar, 50 µm.

Fig. 8. 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 the 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 hours and stained with PAS. In XY explants, the addition of LY294002 to the medium drastically reduced glycogen accumulation in the gonadal area of XY explants (C). By contrast, the 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.

Puglianiello et al., 2004). This further suggests the possibility that glycogen accumulation in developing XY gonads is induced via a distinct pathway from that of glycogen accumulation in developing XY gonads (Puglianiello et al., 2004). This further suggests the possibility that testis-specific glycogen accumulation does not require or that it occurs earlier than testis-specific Sox9 expression 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, which is one of the major signaling pathways downstream of tyrosine kinase receptors for many polypeptide growth factors, including PDGF, FGF and insulin/IGFs (reviewed by Alessi and Cohen, 1998). This suggests that one or more of these growth factors are involved in testis-specific glycogenesis in pre-Sertoli cells.

To date, it has been assumed that several growth factors are downstream of Sox9 and are important in testis differentiation. For example, the XY gonads of Pdgfra-α receptor (Pdgfra)-knockout mice display disruptions in the organization of vasculature and in the partitioning of interstitial and testicular cord compartments (Brennan et al., 2003). In addition, studies have shown that Fgf-9 is involved in testis-specific mesonephric migration and cell proliferation during testis differentiation (Colvin et al., 2001; Schmalh et al., 2004). By contrast, 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. have shown that insulin/IGF signaling is required for testis differentiation in mice (Nef et al., 2003). They demonstrated that XY mice with mutations for all three insulin receptor family members (Ir, Igf1r and Irf) developed ovaries and showed a completely female phenotype. Because it has been shown that insulin/IGF signaling generally stimulates glucose metabolism in target organs via the PI3K-AKT pathway (Salit 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, our preliminary in vitro experiments using the insulin/IGF inhibitor AG1024 (Calbiochem; 20 µM) and the PDGF inhibitor AG1295 (Calbiochem; 50 µM) showed that the insulin/IGF inhibitor AG1024, but not the PDGF inhibitor AG1295, significantly inhibited testis-specific glycogen accumulation in developing XY gonads, despite a similar level of reduction in phopho-AKT expression by both inhibitors (S.M. and Y.K., unpublished). However, Nef et al., also showed that the level of Sry expression was severely reduced in XY mice with mutations for all three insulin receptor family members, which suggests a possible role for insulin signaling upstream of Sry rather than a role in its downstream cascade (Nef et al., 2003). Therefore, further studies to identify growth factors that induce Sry-dependent glycogen accumulation and to determine how Sry activates the PI3K-AKT pathway in pre-Sertoli cells are warranted.

Glycogen accumulation is transiently observed in several tissues and cell types in various aspects of development. In the chick, for example, primordial germ cells contain an abundance of glycogen granules in their cytoplasm during the migratory phase, but they rapidly disappear shortly after entering the gonads (Clawson and Domm, 1963). The precursor cells of osteoblasts possess an accumulation of
glycogen during fetal (Cabrini, 1961; Scott and Glimcher, 1971) and experimentally induced bone (Decker et al., 1995) formation, and the loss of glycogen in differentiated osteoblasts is closely correlated to calcification (Harris, 1932). During tooth morphogenesis in mice, Ohshima et al. have suggested that glycogen deposition in the enamel organ cells from the cap to early bell stages (E14-E15) is associated with the active secretion of glycosaminoglycan components into extracellular spaces and the formation of the stellate reticulum (Ohshima et al., 1999). These reports clearly suggest that cell-type-specific glycogen depositions are closely associated with cell state, such as active migration, protein production, and secretion during tissue morphogenesis and differentiation. The testis differentiation is generally accompanied by dynamic morphogenesis and active production of hormones such as Mullerian inhibiting substance (Byskov, 1986). By contrast, no clearly defined morphogenesis is detected in ovarian morphogenesis and active production of hormones such as Mullerian inhibiting substance (Byskov, 1986). By contrast, no clearly defined morphogenesis is detected in ovarian differentiation, and the onset of folliculogenesis occurs shortly before birth (18.0 dpc in mice). It is, therefore, reasonable to conclude that the preservation of energy stock as glycogen granules in differentiating pre-Sertoli cells reflects the active cell state associated with dynamic testis morphogenesis and the active production of testis-specific growth factors and structural components. This is clearly consistent with our previous observation that showed a rapid reduction in glycogen depositions in Sertoli cells after 12.5 dpc (Kanai et al., 1989). These reports clearly suggest that cell-type-specific glycogen depositions are closely associated with cell state, such as active migration, protein production, and secretion during tissue morphogenesis and differentiation. The testis differentiation is generally accompanied by dynamic morphogenesis and active production of hormones such as Mullerian inhibiting substance (Byskov, 1986). By contrast, no clearly defined morphogenesis is detected in ovarian morphogenesis and active production of hormones such as Mullerian inhibiting substance (Byskov, 1986). By contrast, no clearly defined morphogenesis is detected in ovarian differentiation, and the onset of folliculogenesis occurs shortly before birth (18.0 dpc in mice). It is, therefore, reasonable to conclude that the preservation of energy stock as glycogen granules in differentiating pre-Sertoli cells reflects the active cell state associated with dynamic testis morphogenesis and the active production of testis-specific growth factors and structural components. This is clearly consistent with our previous observation that showed a rapid reduction in glycogen depositions in Sertoli cells after 12.5 dpc (Kanai et al., 1989). These reports clearly suggest that cell-type-specific glycogen depositions are closely associated with cell state, such as active migration, protein production, and secretion during tissue morphogenesis and differentiation. The testis differentiation is generally accompanied by dynamic morphogenesis and active production of hormones such as Mullerian inhibiting substance (Byskov, 1986). By contrast, no clearly defined morphogenesis is detected in ovarian morphogenesis and active production of hormones such as Mullerian inhibiting substance (Byskov, 1986).
whether or not such sex-dimorphic energy metabolism in gonadal sex differentiation is a conserved event in other vertebrate and invertebrate species.

We thank B. Capel and J. Bowles for their critical reading and comments on the manuscript, and M. Fukuda and I. Tsugiyama for their technical and secretarial assistance. This work was supported by Grant-in-Aids for Scientific Research Fund from the Ministry of Education, Science, Sports and Culture of Japan to Y.K.

References

Albrecht, K. H. and Eicher, E. M. (2001). Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. Dev. Biol. 240, 92-107.

Alessi, D. R. and Cohen, P. (1998). Mechanism of activation and function of protein kinase B. Curr. Opin. Genet. Dev. 8, 55-62.

Bishop, C. E., Whitworth, D. J., Qin, Y., Agoulnik, A. I., Agoulnik, I. U., Harrison, W. R., Behringer, R. R. and Overbeek, P. A. (2000). A transgenic insertion upstream of sox9 is associated with dominant XX sex reversal in the mouse. Nat. Genet. 26, 490-494.

Bowles, J., Cooper, L., Berkman, J. and Koopman, P. (1999). Sry requires a CAG repeat domain for male sex determination in Mus musculus. Nat. Genet. 22, 405-408.

Brennan, J., Karl, J. and Capel, B. (2002). Divergent vascular mechanisms downstream of Sry establish the arterial system in the XY gonad. Dev. Biol. 244, 418-428.

Brennan, J., Tilmann, C. and Capel, B. (2003). Pdgfr-alpha mediates testis cord organization and fetal Leydig cell development in the XY gonad. Genes. Dev. 17, 800-810.

Buehr, M., Gu, S. and McLaren, A. (1993). Mesonephric contribution to testis differentiation in the fetal mouse. Development 117, 273-281.

Bullejos, M. and Koopman, P. (2001). Spatially dynamic expression of Sry in mouse gonadal ridges. Dev. Dyn. 221, 201-205.

Byskov, A. G. (1986). Differentiation of mammalian embryonic gonad. Physiol. Rev. 66, 71-117.

Cabrini, R. L. (1961). Histochemistry of ossification. Int. Rev. Cytol. 11, 283-306.

Capel, B., Albrecht, K. H., Washburn, L. L. and Eicher, E. M. (1999). Migration of mesonephric cells into the mammalian gonad depends on Sry. Mech. Dev. 84, 127-131.

Chaboissier, M. C., Kobayashi, A., Vidal, V. L., Lutzkendorf, S., van de Kamps, J., Veenstra, M. de Rooij, D. G., Behringer, R. R. and Schell, A. (2004). Functional analysis of Sox8 and Sox9 during sex determination in the mouse. Development 131, 1891-1901.

Clawson, R. C. and Domm, L. V. (1963). Developmental changes in glycogen content of primordial germ cells in chick embryo. Proc. Soc. Exp. Biol. Med. 112, 533-537.

Colvin, J. S., Green, R. P., Schmahl, J., Capel, B. and Ornitz, D. M. (2001). Male-to-female sex reversal in mice lacking fibroblast growth factor 9. Cell 104, 875-889.

Decker, B., Bartels, H. and Decker, S. (1995). Relationships between endothelial cells, pericytes, and osteoblasts during bone formation in the sheep femur following implantation of tricalciumphosphate-ceramic. Anat. Rec. 242, 310-320.

Ferrer, J. C., Favre, C., Comn, R. R., Fernandez-Novell, J. M., Garcia-Rocha, M., de la Iglesia, N., Cid, E. and Guinovart, J. J. (2003). Control of insulin action. Diabetologia 47, 170-184.

Fujisawa, M., Kainai-Maeda, H., Fujii, K., Kraft, J., de la Iglesia, N., Cid, E. and Guinovart, J. J. (2004). Expression and role of PDGF-BB and PDGFR-beta during testis morphogenesis in the mouse embryo. J. Cell Sci. 117, 1151-1160.

Fujii, K., Acampora, I., Fasella, J. D., Frestadiatis, A., Accili, D. and Parada, L. F. (2003). Testis determination requires insulin receptor family function in mice. Nature 426, 291-295.

Gogos, B., Verma-Kurvari, S., Merenmies, J., Vassalli, J. D., Efstratiadis, A., Nef, S., Schepers, G., Wilson, M., Wilhelm, D. and Koopman, P. (1999). Expression during early phases of testis differentiation in mice. Dev. Biol. 258, 271-281.

Hiramatsu, R., Kanai, Y., Mizukami, T., Ishii, M., Matoba, S., Kanai-Azuma, M., Kurohmaru, M., Kawakami, H. and Hayashi, Y. (2003). Regionally distinct potencies of mouse XY genital ridge to initiate testis differentiation dependent on anteroposterior axis. Dev. Dyn. 228, 247-253.

Ikeda, Y., Takeda, Y., Shikayama, T., Muki, T., Hisano, S. and Morohashi, K. I. (2001). Comparative localization of Dax-1 and Ad4BP/SF-1 during development of the hypothalamic-pituitary-gonadal axis suggests their closely related and distinct functions. Dev. Dyn. 220, 363-376.

Ishii, M., Matoba, S., Kurohmaru, M., Hayashi, Y. and Nishida, T. (1989). Formation of male and female sex cords in gonadal development of C57BL/6 mouse. Jpn. J. Vet. Sci. 51, 7-16.

Kidokoro, T., Matoba, S., Hiramatsu, R., Fujisawa, M., Kanai-Azuma, M., Tay, C., Kurohmaru, M., Kawakami, H., Hayashi, Y. and Kanai, Y. et al. (2005). Influence on spatiotemporal patterns of a male-specific sox9 activation by ectopic Sry expression during early phases of testis differentiation in mice. Dev. Biol. 281, 511-525.

Kooiman, P., Gubbay, J., Vivian, V., Goodfellow, P. and Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for Sry. Nature 351, 117-121.

Koopman, P., Gubbay, J., Vivian, V., Goodfellow, P. and Lovell-Badge, R. (1996). Sry expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat. Genet. 14, 62-68.

Koosan, M., de la Iglesia, N., Cid, E. and Guinovart, J. J. (2003). Subtractive hybridisation screen identifies sexually dimorphic gene expression in the embryonic mouse gonad. Genesis 37, 84-90.

McClive, P. J., Hurley, T. M., Saeraj, M. A., van den Bergen, J. A. and Sinclair, A. H. (2003). Modulation of the coelomic epithelium in male development. Genes 136, 228-239.

Mittal, Y. and Capel, B. (1998). Sertoli cells of the mouse testis originate from the coelomic epithelium. Dev. Biol. 203, 323-333.

Morr,is da Silva, S., Hacker, A., Harley, V., Goodfellow, P., Swain, A. and Lovell-Badge, R. (1996). Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat. Genet. 14, 62-68.

Morais da Silva, S., Hacker, A., Harley, V., Goodfellow, P., Swain, A. and Lovell-Badge, R. (2004). Modulation of Sox9 expression in male fetal gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat. Genet. 351, 117-121.

Nef, S., Verma-Kurvari, S., Merenmies, J., Vassalli, J. D., Frestaditatis, A., Testa, D. and Parada, L. F. (2003). Testis determination requires insulin receptor family function in mice. Nature 426, 291-295.

Nef, S., Verma-Kurvari, S., Merenmies, J., Vassalli, J. D., Frestaditatis, A., Testa, D. and Parada, L. F. (2003). Testis determination requires insulin receptor family function in mice. Nature 426, 291-295.

Ohashima, H., Wartiovaara, J. and Thesleff, I. (1999). Developmental regulation and ultrastructure of glycogen deposits during murine tooth morphogenesis. Cell. Tissue Res. 297, 271-281.

Pirola, L., Johnson, A. M. and Van Obberghen, E. (2004). Modulation of insulin action. Diabetologia 47, 170-184.

Puglianiello, A., Campagnolo, L., Farini, D., Cipollone, D., Russo, M. A., Bruni, D. and Vassalli, J. D., Efstratiadis, A., Nef, S., Schepers, G., Wilson, M., Wilhelm, D. and Koopman, P. (2003). SOX8 is expressed during testis differentiation in mice and synergizes with SF1 to activate the Amp promoter in vitro. J. Biol. Chem. 278, 28101-28108.

Schmahl, J. and Capel, B. (2003). Cell proliferation is necessary for the determination of male fate in the gonad. Dev. Biol. 258, 264-276.

Schmahl, J., Eicher, E. M., Washburn, L. L. and Capel, B. (2000). Sry induces cell proliferation in the mouse gonad. Development 127, 65-73.

Schmahl, J., Kim, Y., Colvin, J. S., Ornitz, D. M. and Capel, B. (2004).
Fgf9 induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination. Development 131, 3627-3636.
Scott, B. L. and Glimcher, M. J. (1971). Distribution of glycogen in osteoblasts of the fetal rat. J. Ultrastruct. Res. 36, 565-586.
Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A. M., Lovell-Badge, R. and Goodfellow, P. N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346, 240-244.
Sinclair, K. D., Rooke, J. A. and McEvoy, T. G. (2003). Regulation of nutrient uptake and metabolism in pre-elongation ruminant embryos. Reprod. Suppl. 61, 371-385.
Taketo, T. and Koide, S. S. (1981). In vitro development of testis and ovary from indifferent fetal mouse gonads. Dev. Biol. 84, 61-66.
Thong, F. S. and Graham, T. E. (2002). The putative roles of adenosine in insulin- and exercise-mediated regulation of glucose transport and glycogen metabolism in skeletal muscle. Can. J. Appl. Physiol. 27, 152-178.
Tilmann, C. and Capel, B. (1999). Mesonephric cell migration induces testis cord formation and Sertoli cell differentiation in the mammalian gonad. Development 126, 2883-2890.
Uzumcu, M., Westfall, S. D., Dirks, K. A. and Skinner, M. K. (2002). Embryonic testis cord formation and mesonephric cell migration requires the phosphotidylinositol 3-kinase signaling pathway. Biol. Reprod. 67, 1927-1935.
Vidal, V. P., Chaboissier, M. C., de Rooij, D. G. and Schedl, A. (2001). Sox9 induces testis development in XX transgenic mice. Nat. Genet. 28, 216-217.
Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F. D., Keutel, J., Hustert, E. et al. (1994). Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 79, 1111-1120.