Journal of Reproduction and Development, Vol. 54, No. 6, 2008

—Full Paper—

Endocrine Status and Development of Porcine Testicular Tissues in Host Mice

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Abstract. Clarification of the endocrine status of host mice provides us with basic knowledge with which we can manipulate the growth and function of xenografted testicular tissues. We investigated the hormonal profiles of castrated mice grafted with porcine immature testicular tissues from 30 to 210 or more days after grafting (day 0= castration and grafting). The serum follicle stimulating hormone (FSH) concentrations of the host mice declined ($P<0.05$) from day 60 compared with those of the castrated, ungrafted mice. The serum inhibin and testosterone levels were higher ($P<0.05$) than those in the castrated, ungrafted mice from days 30 and 90 days, respectively. The inhibin levels further increased ($P<0.05$) from day 90, during which time the levels were higher ($P<0.05$) than those in the intact male mice. In the grafts, formation of lumens occurred in the seminiferous cords on day 90 and spermatozoa appeared in the lumens from day 120. However, spermatogenesis in the grafts did not reach the qualitatively normal levels observed in adult boars. The intensity of the immune reaction to inhibin α subunits in the Sertoli cells of the grafts decreased with differentiation of the seminiferous tubules. The present findings indicate that a feedback loop was established between the mouse hypothalamo-pituitary axis and the grafted porcine tissues from 60 days post-grafting. The results also indicate that the serum inhibin levels in the host mice remained high even after the appearance of lumens in the seminiferous tubules of the grafted tissues; this is strikingly different to the situation in normal male animals, in which the serum inhibin levels decline at around the time of tubular differentiation. The lack of efferent ducts in the tubules of the grafted tissues probably caused the accumulation of inhibin to be released into the lumens, resulting in high concentrations of circulating inhibin. These high levels of inhibin may directly affect spermatogenic activity and suppress FSH secretion.

Key words: Hormonal profile, Host mouse, Porcine testis, Testis development, Xenografting

(**J. Reprod. Dev. 54:** 480–485, 2008)

Grafting of testicular tissues into immunodeficient mice is a potential way of enabling us to utilize immature germ cells present in the neonatal mammalian testis. Testicular tissues have been prepared from species phylogenetically distant from mice, including cattle [1, 2], goats [3], pigs [3], horses [4], cats [5] and rhesus monkeys [6], and then xenografted into immunodeficient mice. Successful recovery of spermatozoa from the grafts has been proven in several species (pigs [3], cats [5] and rhesus monkeys [6]). However, no progeny have been obtained from spermatozoa isolated from the xenografts of these animals. Clarification of the endocrine status of the host mouse would likely help us to improve spermatogenesis in grafted testicular tissues, since development of the testis is controlled by the balance between the pituitary hormones, which stimulate testicular function, and testicular hormones, which alter the secretion of the pituitary hormones [7]. To date, the hormonal characteristics of host mice have been elucidated only after grafting of murine testicular tissues [8]; the study indicated that a feedback loop was established between the graft and the host's hypothalamo-pituitary 2 weeks after grafting, when the graft acquired the ability to produce testosterone. However, the time required for testicular maturation is much longer in large animals, such as pigs, cattle and horses than in rodents. In addition, functional changes in the Leydig cells of grafts have been assessed by measuring the serum testosterone levels of host mice [1, 8, 9], but the functions of Sertoli cells after grafting have not been reported. Inhibin is a glycoprotein that is produced mainly by the Sertoli cells of male animals and has the ability to suppress FSH secretion by the pituitary in male rats [10, 11], primates [12], Shiba bucks [13], rams [14] and bulls [15, 16].

Our objectives were to 1) clarify the hormonal relationships between the mouse hypothalamus-pituitary axis and the porcine grafted testis as a model for xenografting of the testes of large animals and 2) evaluate the functional changes in the Sertoli cells of porcine grafts by examining inhibin production.

Accepted for publication: September 4, 2008
Published online in J-STAGE: October 15, 2008
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Materials and Methods

Testicular xenografting and sampling

The protocols for the use of animals were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences (Tsukuba, Japan). Testes were obtained from 5- to 10-day-old piglets from a local abattoir. All piglets were crossbreeds of Landrace × Large White or Landrace × Large White × Duroc produced at the National Institute of Livestock and Grassland Science, Tsukuba, Japan. Testes were minced into pieces of approximately 1 × 1 × 1 mm in size in saline supplemented with 668 units/ml penicillin (Sigma-Aldrich Chemical, St. Louis, MO,

USA) and 0.2 mg streptomycin sulfate (Sigma). As recipients, 5- to 6-week-old male immunodeficient mice (Crlj:CD1-*Foxn1*^{nu}; Charles River Japan, Yokohama, Japan), kept in an environmentally controlled room illuminated daily from 0500 to1900 h, were anesthetized with a combination of Somnopentyl (pentobarbital sodium; Kyoritsu Pharmaceuticals, Tokyo, Japan) and ether and then castrated. Immediately after castration, 20 pieces of porcine testicular tissue were inserted under the skin of the back. Testicular grafts obtained from 20 piglets were transplanted into 131 mice between 2003 and 2007. Nine testis fragments were excised from three piglets (three fragments/piglet) just before the xenografting and served as histological data for Day 0 (day 0=grafting).

The host mice were anesthetized and bled by heart venipuncture between 0700 and 0900 h at 30 (n=5 mice/group), 60 (n=7), 90 (n= 11), 120 (n=13), 150 (n=18), 180 (n=11) or 210 or more (n=18) days after grafting (day 0=grafting); 48 mice died before sampling. Sera were stored at –30 C until assayed for inhibin, testosterone and FSH. After blood sampling, the grafts were immediately recovered. Three fragments were randomly excised from different grafts in each mouse and were subjected to histological examination. The remaining portions were used to recover sperm. For comparison, blood samples were collected from 8 castrated, ungrafted mice 30 days after castration and from 10 intact 6-week-old nude mice. We also collected a single testicular fragment from three 7-month-old boars for histological comparison with grafted tissues.

Recovery of sperm

Grafts from each mouse were minced in Dulbecco's phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5 mg/ml bovine serum albumin (BSA; Sigma).

Histological analysis of testicular grafts

Fragments excised from the grafts and from testes of the piglets just before grafting were fixed in Bouin's solution, embedded in paraffin and then sectioned at 5 μm. Sections were stained with hematoxylin and eosin. The seminiferous tubule cross-sections were sorted into the following categories: 1) no germ cells present (tubule cross-sections with Sertoli cells only); 2) gonocytes/spermatogonia; 3) spermatocytes present as the most advanced germ cells; 4) round spermatids present as the most advanced germ cells; 5) elongated spermatids present as the most advanced germ cells; and 6) mature spermatozoa present in the lumens of the tubules. Nine fragments obtained from three mice (three fragments/mouse) from each group were subjected to histological examination. All seminiferous tubules observed in each cross-section of each fragment were examined. The data obtained from three grafts for each mouse were pooled, and the number of tubule cross-sections in each category as a percentage of the total number of tubule cross-sections examined was calculated. The same analyses were performed on testicular fragments obtained from 7-month-old boars.

Immunohistochemistry of inhibin α subunits in testicular grafts

For antigen retrieval, the sections were autoclaved in 10 mmol/l sodium citrate buffer (pH 6.0) at 121 C for 15 min. The other immunohistochemical procedures, performed with purified inhibin α subunit antibody (lot GB), have been described elsewhere [16, 17]. The immune reaction was visualized with a VectaStain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine. Previous results of staining using antibody preabsorbed with excessive inhibin indicate that the immune reaction in the porcine testis is specific to the inhibin α subunit antibody [18].

Fluoroimmunoassays (FIAs) for inhibin, FSH and testosterone

The concentrations of inhibin (total inhibin) in the sera of the host mice were determined by a competitive FIA with europium (Eu)-labeled inhibin A as a probe [19, 20]. In the FIA of inhibin, anti-bovine-inhibin serum (TNDH-1 [21]) was used as a primary antibody. Bovine 32-kDa inhibin A was used for Eu labeling and as a reference standard. Anti-inhibin serum was provided by Dr. K. Taya (Tokyo University of Agriculture and Technology, Tokyo, Japan) and bovine 32-kDa inhibin was provided by Dr. Y Hasegawa (Kitasato University, Towada, Japan). The detection limit of the FIA was 0.078 ng/ml. The intra-assay and interassay coefficients of variations (CVs) were 9.4 and 15.0%, respectively.

The concentrations of follicle stimulating hormone (FSH) in the sera of the host mice were determined by a competitive FIA with Eu-labeled rat FSH as a probe [22]. In the FIA for FSH, anti-rFSH-S-11 was used as a primary antibody, rFSH-I-9 was used for Eu labeling and rFSH-RP-2 was used as a reference standard (as assay material; a Rat FSH RIA kit was provided by Dr. AF Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA). The detection limit of the FIA was 0.78 ng/ml. The intraassay and interassay CVs were 10.3 and 13.5%, respectively.

The concentrations of testosterone were determined with a commercial competitive FIA kit (Delfia testosterone kits; PerkinElmer Japan, Yokohama, Japan). Testosterone was extracted from the serum with 2 ml ether before being applied to the kit. Cross-reactivity to androgens in the FIA kit was 27.2% for 5α-dihydrotestosterone, less than 1% for other androgens and 100% for testosterone. The detection limit of the FIA was 1.0 pg/ml.

Statistical analysis

Percentages of tubule cross-sections in each category were expressed as means ± SEM for three mice in each group. All data, including hormone levels, were subjected to ANOVA. When a significant effect was obtained with ANOVA, the significance of the difference between means was determined by Duncan's multiple range test. Regression analyses between FSH and inhibin or testosterone were also performed. The General Linear Models or REG Procedure of Statistical Analysis Systems (SAS/STAT) [23] was used for the analyses. Differences at $P<0.05$ were considered to be significant.

Results

Changes in serum concentrations of inhibin, testosterone and FSH in host mice

Thirty days after grafting, the inhibin levels in the host mice were significantly greater ($P<0.05$) than in the castrated, ungrafted

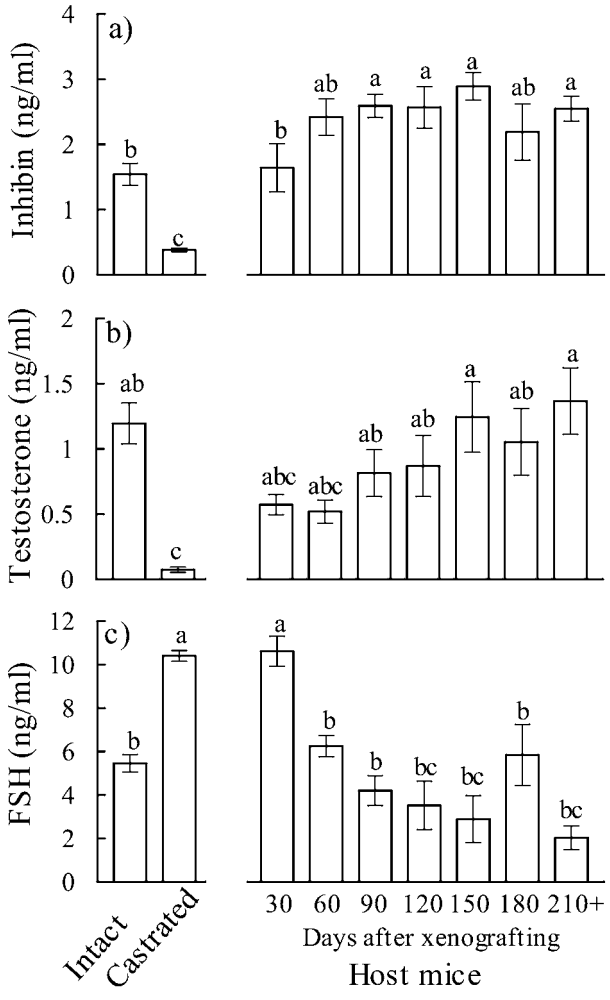

Fig. 1. Circulating levels of (a) inhibin, (b) testosterone and (c) FSH in castrated host mice after grafting of porcine immature testicular tissues. Blood samples were also collected from castrated, ungrafted mice and intact male mice. Values are means ± SEM of 5 to 18 mice. [a–c]Values without common superscripts are significantly different from each other.

mice (Fig. 1a). A further increase in inhibin levels was noted from day 90 (day 0=grafting) onward, during which time the levels were significantly higher ($P<0.05$) than those in the intact male mice, except on day 180. The serum testosterone levels in the grafted mice rose gradually and were significantly greater ($P<0.05$) than in the castrated, ungrafted mice from day 90 onward; however the levels after day 90 were not significantly different from those in the intact mice (Fig. 1b). From day 60 onward, the concentrations of serum FSH were significantly ($P<0.05$) lower in the grafted mice than in the castrated, ungrafted mice; they remained low and did not differ significantly from those in the intact mice (Fig. 1c). An inverse relationship was found between the levels of FSH and inhibin (r=–050. $P<0.001$) or testosterone (r=–0.43, $P<0.001$).

Histology of testicular grafts and recovery of spermatozoa

Testicular tissues before grafting contained seminiferous cords consisting of only gonocytes/spermatogonia and Sertoli cells (Table 1 and Fig. 2a). On day 90, formation of lumens was recorded in the seminiferous cords. Spermatocytes first appeared on day 60 (Table 1 and Fig. 2b) in the grafts, and spermatids on day 90. Spermatozoa were detected in the tubules from day 120 (Fig. 2c). Significant ($P<0.05$) increases in the percentages of tubule cross-sections containing elongated spermatids or spermatozoa were noted on days 150 and 210 or more compared with each value on day 120 (Table 1). However, the proportion of tubule cross-sections containing one or both of these cell types was lower in the grafted testicular tissues (16.4% on day 150 and 40.4% on day 210 or more) than that (81.4%) observed in the 7-month-old boar testis. The interstitium contained morphologically normal Leydig cells (Figs 2a–c). Spermatozoa were recovered from the grafts from 4 out of 13 mice on day 120 (Fig. 2d). The recovery rate increased as time after grafting passed; spermatozoa were recovered from 13/18 mice on day 150, 9/11 mice on day 180 and 16/18 mice on day 210.

Immunohistochemistry of inhibin in testicular grafts

Strong immunostaining for inhibin α subunits was found in the cytoplasm of Sertoli cells in the tissues obtained from neonatal pigs (Fig. 3a). The immune reaction gradually weakened after grafting (Figs. 3a–d), but Sertoli cells remained positive for inhibin α antibody on day 150 (Fig. 3d). No clear immunostaining was observed in the cytoplasm of germ cells (Figs. 3a to d). The immune reaction in the interstitial cells was much weaker than that in the Sertoli cells (Figs. 3a to c).

Discussion

The endocrine status of the castrated host mice that received immature porcine testicular tissues was characterized by 1) the establishment of a feedback loop between the mouse hypothalamo-pituitary axis and the grafted testicular tissues from about day 60 after grafting and 2) the continued elevation of circulating inhibin levels after differentiation of seminiferous tubules in the grafts, which is different from the characteristics of intact males in many species.

From day 60 after grafting, the serum FSH levels in the grafted mice were significantly lower than those in the castrated ungrafted males, whereas the inhibin and testosterone levels remained significantly higher from 30 and 90 days, respectively, after grafting. The results indicate that the testicular xenografts were able to exert inhibitory effects on the hypothalamo-pituitary axis of the adult host mice and that this interrelationship became active at about day 60. The timing of establishment of the feedback loop in our mice was delayed compared with that in mice grafted with murine testicular tissues (2 weeks) [8], probably because of the longer time required for acquisition of full endocrine functions by the porcine testicular tissues compared with the murine tissues. To our knowledge, there is little data available on the endocrine role of inhibin in male mice; however, in male rats, circulating inhibin levels decline towards sexual maturation [10, 24], whereas testosterone levels increase. In addition, inhibin immunization of male rats induces

Table 1. Percentages of seminiferous cord or tubule cross-sections, as classified by the most advanced type of germ cell present, in porcine testicular tissues grafted into host mice

Days after grafting	No germ cells	Percentage of seminiferous tubule or cord cross-sections[a]				
		Gonocytes/ spermatogonia	Spermatocytes	Round spermatids	Elongated spermatids	Spermatozoa
0[b]	20.8 ± 3.9^{de}	79.2 ± 3.9^{d}	0^{g}	0^{f}	0^{f}	0^{f}
30	38.1 ± 0.7^{d}	61.9 ± 0.7^{de}	0^{g}	0^{f}	0^{f}	0^{f}
60	42.2 ± 17.0^{d}	57.1 ± 16.9^{def}	0.5 ± 0.1^{g}	0^{f}	0^{f}	0^{f}
90	47.2 ± 8.3^{d}	37.3 ± 6.9^{f}	13.8 ± 2.5^{fg}	1.5 ± 0.7^{f}	0.2 ± 0.1^{f}	0^{f}
120	33.6 ± 14.1^{d}	10.7 ± 1.5^{g}	47.4 ± 12.8^{d}	4.6 ± 2.1^{f}	3.0 ± 0.9^{f}	0.7 ± 0.2^{f}
150	34.4 ± 5.8^{d}	7.9 ± 4.3^{g}	31.4 ± 3.9^{de}	9.8 ± 2.9^{e}	10.6 ± 1.1^{e}	5.8 ± 1.8^{e}
210+	26.2 ± 6.7^{de}	1.2 ± 0.2^{g}	23.0 ± 3.9^{ef}	9.2 ± 1.4^{e}	19.2 ± 6.7^{e}	21.2 ± 6.1^{d}
Adult boars[c]	0^{e}	0^{g}	1.6 ± 0.9^{g}	17.0 ± 0.2^{d}	52.6 ± 1.3^{d}	28.8 ± 1.8^{d}

[a]Data obtained from three animals in each group. [b]Day 0 data were obtained from testicular tissues excised from 5- to 10-day-old piglets before grafting. [c]Data obtained from 7-month-old boars. [d–g]Values (mean ± SEM) in the same column with no common superscript are significantly different ($P<0.05$) from each other.

hypersecretion of FSH during infancy but not after puberty [10, 11]. The above findings seem to suggest that testosterone, not inhibin, is a major regulator of FSH secretion in adult male rats. However, our current host mice showed elevations of both inhibin and testosterone levels from days 30 and 90, respectively, suggesting that FSH secretion in these mice was regulated by both inhibin and testosterone.

In the grafted tissues, Leydig cells appeared to be morphologically normal, and the ability to produce testosterone was restored as previously reported [1, 8, 9]. On the other hand, the intensity of the immune reaction to inhibin α in the Sertoli cells in the grafted tissues decreased with differentiation of the seminiferous tubules; this is similar to previous findings in the seminiferous cords/tubules of developing boars [18], bulls [16, 22, 25] and rats [10]. These results strongly suggest that the levels of inhibin produced by the Sertoli cells in the grafted tissues declined with tubular differentiation, as occurs in normal developing male animals [10, 16, 18, 22, 25]. However, the profile of circulating inhibin in the host mice was quite different from that in normal animals, in which inhibin levels decline as the male reaches sexual maturation (rats [10, 24], pigs [18], bulls [25, 26] and rams [27]). In normal males, the total inhibin content per testis progressively increases as the animal reaches puberty [18, 24, 26], owing to an increase in the total number of Sertoli cells [24, 28, 29] despite the decline in inhibin levels per Sertoli cell. Coincidently with the differentiation of germ cells, the lumens of the seminiferous tubules appear, along with evidence of fluid secretion and formation of the blood-testis barrier. After formation of the blood-testis barrier, inhibin is released into the seminiferous tubules and excreted into the seminal plasma, but some of it reaches the general circulation by resorption from the testis fluid via the lymphatic vessels [30–32]. This probably explains the decrease in circulating inhibin levels as sexual maturation approaches. In our host mice, lumens formed in the seminiferous tubules of the subcutaneous grafted tissues from day 90, and the inhibin level per Sertoli cell was estimated to be in decline with differentiation of seminiferous tubules as in normal boars [18, 33]. However, as a matter of course, the tubules lacked efferent ducts. Accumulation of inhibin in the seminiferous tubules because of the absence of efferent ducts may account for the high levels of circulating inhibin in the host mice.

Complete spermatogenesis was observed in several tubule cross-sections in the porcine grafted tissues, confirming the previous findings in grafted tissues prepared from several species (cattle [1, 2], pigs [3], cats [5] and rhesus monkeys [6]). The timing of appearance of spermatocytes or spermatozoa in the xenografts was similar to those observed in developing boars [33, 34]. Zeng *et al.* [35] demonstrated that the length of the spematogenic cycle in porcine xenografts implanted into immunodeficient mice was about 9 days, which is similar to that observed in boars [33, 36]. However, spermatogenesis in the grafted tissues did not reach the quantitatively normal levels observed in young adult boars. Approximate thirty percent of tubule cross-sections contained Sertoli cells only in the grafted tissues even after day 120. Previous studies have also indicated the occurrence of defectiveness in the differentiation of tubules [1, 2]. At present, the reasons for this are unclear. Anemia after subcutaneous grafting may be the primarily factor affecting the development of grafted tissues. The accumulation of fluid in the seminiferous tubules may also induce disturbance of spermatogenesis in the grafts [8]. The high serum inhibin levels in our host mice suggest that inhibin accumulated in the seminiferous tubules. High levels of inhibin may reduce spermatogenetic activity, since intratesticular injection of bovine follicular fluid or inhibin preparations reduce the number of spermatogonia in mice and hamsters [37] and the number of round spermatids in rats [38]. *In vitro* inhibin treatment of rat seminiferous tubule segments reduces DNA synthesis in spermatogonia [39].

From day 120, we were able to recover spermatozoa from the xenografts; the proportion of seminiferous tubule cross-sections containing elongated spermatids and/or spermatozoa in the grafts and the rate of recovery of spermatozoa from the host mice increased with time after xenografting. Some of the spermatozoa retrieved from the grafts on day 180 or later likely accumulated for over 60 days in the seminiferous tubules. We do not know the best timing for recovery of porcine spermatozoa with the ability to sup-

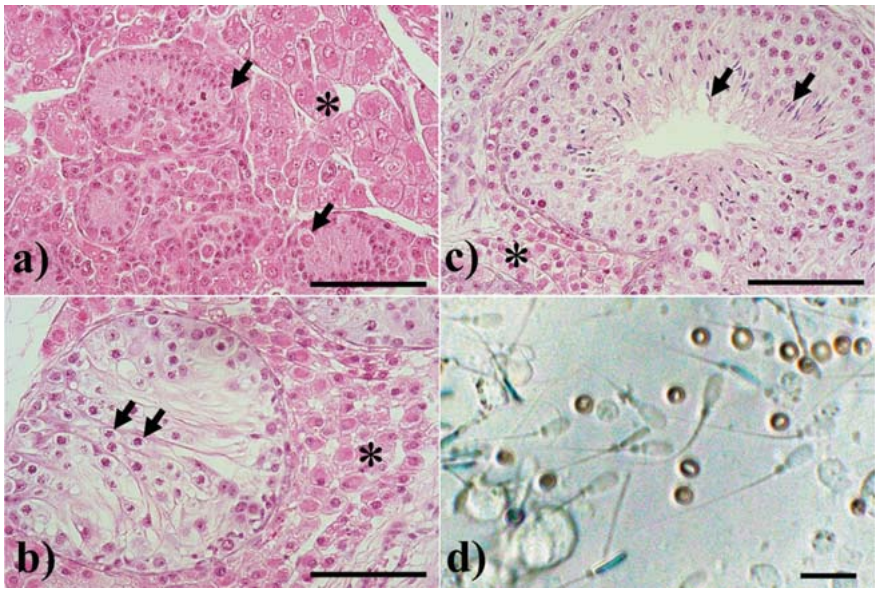

Fig. 2. Histological appearance of testicular tissues before and after xenografting and of recovered spermatozoa. a) Tissue from an 8-day-old piglet contains gonocytes/spermatogonia (arrows) in the seminiferous cords. Tissues recovered from grafted mice b) on day 60 (day 0=grafting) contain spermatocytes (arrows) in the seminiferous cords, and c) those recovered on day 120 have elongated spermatids and spermatozoa (arrows) in the tubules. The interstitial tissue contains Leydig cells (asterisks). Bars=50 μm. d) Spermatozoa retrieved from xenografts on day 120. Bar=10 μm.

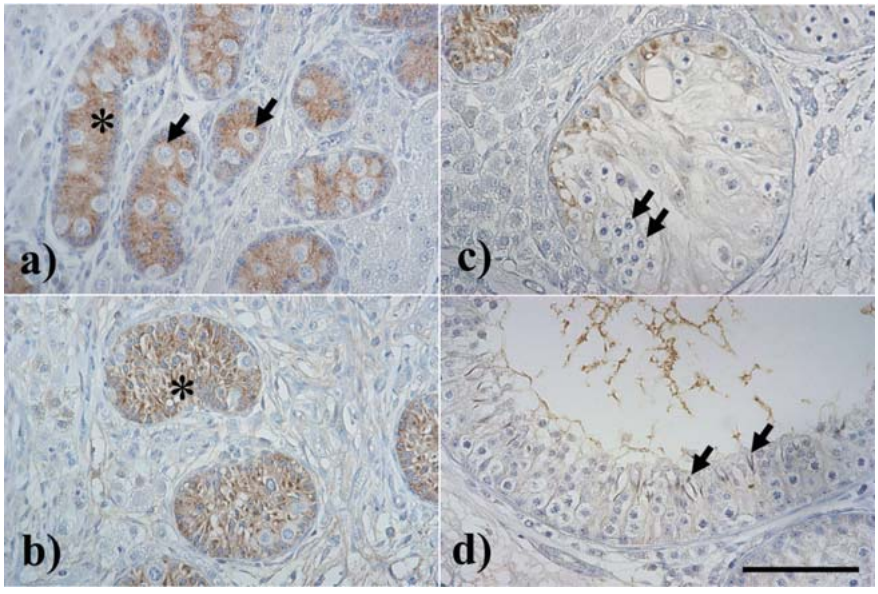

Fig. 3. Immunohistochemical localization of inhibin α subunits in testicular tissues before and after xenografting. a) Tissue from an 8-day-old piglet contains gonocytes/spermatogonia (arrows) in the seminiferous cords. Tissues recovered from grafted mice b) on day 30 (day 0=grafting) and c) day 60 contain spermatocytes (arrows) in the seminiferous cords. Tissue recovered on d) day 150 contains elongated spermatids (arrows) in the tubules. The Sertoli cells in the seminiferous cords/tubules on days 30 and 60 show intense immunostaining for inhibin α (asterisks). Bar=50 μm.

port full embryonic development, but blastocysts can be obtained by recovering porcine spermatozoa between days 120 and 210 and injecting them into the cytoplasm of porcine oocytes [40].

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific

Research from the Japanese Society for the Promotion of Science to HK (17380170). We thank Drs. K Taya (Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, Fuchu 183-0054, Japan) for providing anti-bovine inhibin serum (TNDH-1) and Y Hasegawa (Kitasato University, Towada 034-8628, Japan) for providing bovine 32-kDa inhibin A. We also thank Dr. AF Parlow (National Hormone and Peptide Program of National Institute of Diabetes and Digestive and Kidney Diseases, Harbor-UCLA Medical Center, Torrance, CA, USA) for providing the rat FSH immunoassay kit.

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