

Studies on the Autologous Transplantation of Testicular Cells in Microminipigs

(マイクロミニピッグにおける精巣細胞の自家移植に関する研究)

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Abbreviations

cm	Centimeter
BW	Body weight
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DBA	Lectin dolichos biflorus agglutinin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
g	Gram
Gy	Gray
HE	Hematoxylin and eosin
kg	Kilogram
μm	Micrometer
μl	Microliter
MeV	Mega-electron
min	Minute
mg	Milligram
mM	Millimole
mm ³	Cubic Millimeter
ml	Milliliter
mo	Month
PBS	Phosphate buffered saline
POU5F1	POU class 5 homeobox 1

PGCs	Primordial germ cells
SEM	Standard error of mean
SD	Seminiferous tubule diameter
SSCs	Spermatogonial stem cells
TC	Testicular circumference
UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1
vol	Volume
ZBTB16	Zinc finger and BTB domain containing 16
°C	Degree Celsius
1-mo-old	1 month old
2-mo-old	2 months old
30-days-old	30 days old
45-days-old	45 days old
80-days-old	80 days old

General Introduction

Spermatogonial Stem cells (SSCs), are defined as self-renewing stem cells that generate daughter cell lines and ultimately produce spermatozoa. SSCs are responsible for spermatogenesis throughout life [39], and include two types of stem cells. One is gonocytes, primordial male germ cells, which gradually migrate from the center of the seminiferous tubules to epithelium and differentiate into undifferentiated spermatogonia. Another is undifferentiated spermatogonia, which further differentiate into differentiated spermatogonia [7, 9, 34]. These SSCs generate early phase of spermatogenesis and testicular development in early stage of life.

In 1994, Brinster and colleagues introduced the SSC transplantation method in the mouse; SSCs from a fertile donor male transplanted into the seminiferous tubules of infertile recipient testes, where donor SSCs initiated donor-derived gametes production [2, 3]. SSC transplantation is a technology that not only provide knowledge about spermatogenesis and the biology of the seminiferous tubule, but also open a new field of research for the infertility treatments, and the production of transgenic animals [21, 44].

In SSC transplantation, there are three alternative methods: heterologous, homologous, and autologous SSC transplantations. Heterologous transplantation is the transplantation of donor SSCs from one fertile male to other animals of other species [6], but transplantation from a species that has evolutionary distance does not result in successful recovery of spermatogenesis [11, 12]. Homologous transplantation is the transplantation of donor SSCs from one fertile male to other

animals of the same species [16]. This technique has further applications in the conservation of genetic of rare or valuable livestock [28, 56]. Homologous transplantation has been performed in some domestic species [16, 18, 19, 35, 55], but offspring production by homologous transplantation was successful only in goat and genetic infertile pigs [23, 40]. Homologous transplantation low efficiency is most likely due to immunorejection [28]. Autologous transplantation is the transplantation of donor SSCs from one male testis to another testis of the same individual. This technique require only one animal and does not produce immunological rejection, enabling a potentially technique for production of donor sperm with a high efficiency [16]. Autologous transplantation has further applications for generation of transgenic farm animals [25].

Microminipigs which were developed by Fuji Micra Inc. (Shizuoka, Japan), are one of the smallest miniature pig breed; they are 1/10 BW of domestic pigs and 1/2 BW of other miniature pigs; their body weight is 10 kg at 6 months of age [31]. Since they are small in size, microminipigs are thought to overcome disadvantage of pigs: uneasy manageable size and require much cost for rearing [4, 62], and to make use on advantage being pigs: anatomical, physiological, and biomedical characteristics similar to humans. Consequently, microminipigs are a strong candidate for a non-rodent animal model in biomedical research [41, 42, 45, 50, 67].

Transgenic animals play an important role in biomedical research. In rodents, many transgenic strains are established and used for various field of science. However, strains of transgenic animals are limited in other species, and development of such animal models are required to proceed applied biomedical sciences [4]. For the development of transgenic animals, efficient method for its production is needed, but only a few techniques are applied in other species [33]. Especially in pigs, somatic cell nuclear transfer and others are main techniques, and its efficiency of transgenic animal production is not very high.

Autologous testicular cell transplantation in microminipigs could be a powerful tool for transgenic animal production [16, 28]. However, the percentage of colonization and donor-derived

sperm production after the SSC transplantation is rather low, and two fundamental procedures involved in the transplantation; preparation and selection of the donor SSCs and preparation of recipient testis should be developed. In this study, I clarified the suitable conditions to improve these two procedures (Figure 1).

An adequate isolation and selection of suitable donor cells have a direct effect on success of the transplantation, because enlarged number of transplanted SSCs increases possibility of the successful transplantation [15, 29, 44, 58, 68]. For obtaining much number of SSCs, choosing a donor animal with a relatively high proportion of SSCs is an option. In immature testes, SSCs are present in a higher proportion, and therefore SSC isolation should be performed in pre-pubertal period. However, as number of SSCs in the pre-pubertal testes abruptly changes [17], identification and characterization of SSC population is needed in the testes during pre-pubertal period. Consequently in chapter I, for a first step for the successful SSC transplantation, characterization of SSCs in microminipigs is performed to determine suitable age for isolation of donor SSCs.

The next step in the SSC transplantation is to prepare the recipient testis. Since transplanted SSCs competed to endogenous SSCs, depletion of SSCs in the recipient testis to create biological niche is crucial for colonization, migration, and spermatogenesis of donor SSCs [18, 43, 49, 59, 60]. Thus, in chapter II, suitable irradiation dose for creation of biological niche was determined.

Based on acquired knowledge from Chapter I and II, autologous transplantation of testicular cells in microminipig was performed in Chapter III; a neonatal microminipig was irradiated with a single dose of 6 Gy and hemi-castrated; isolated testicular cells were injected into recipient testis after the irradiation; and ejaculates were analyzed at 23 weeks after the injection. Finally, this protocol for autologous SSC transplantation aiming at development of new transgenic technique in microminipigs was evaluated.

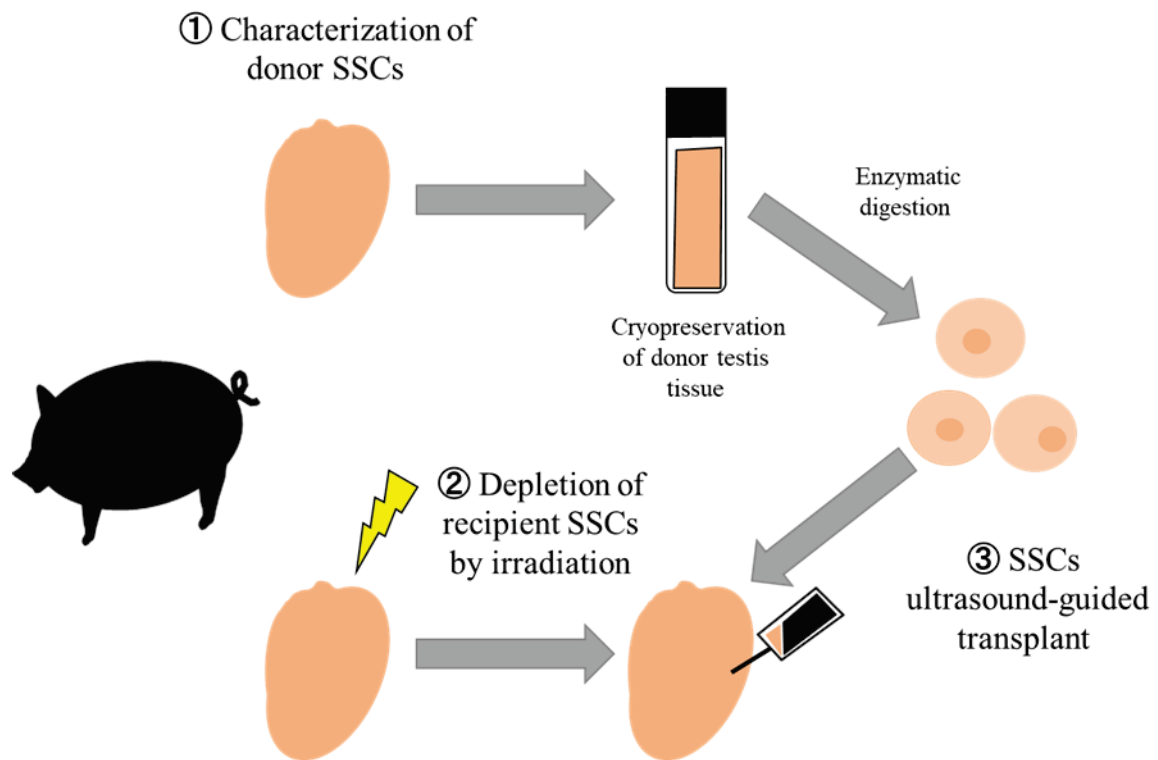


Figure 1. A schematic procedure of autologous SSC transplantation in microminipigs.

Chapter I

Characterization of SSC populations in microminipigs in preparation for the donor cells

1-1 Introduction

Spermatogenesis is the process by which spermatozoa are produced by way of meiosis from SSCs. These cells are defined as self-renewing stem cells that generate daughter cells before differentiation into sperm. SSCs which include gonocytes and undifferentiated spermatogonia are present in large number in neonatal testes [4]. Before the puberty, gonocytes gradually differentiate into undifferentiated spermatogonia, and the spermatogonia migrate onto the basal membrane of the seminiferous tubule, where they further differentiate into differentiated spermatogonia [2, 9].

In pigs, SSCs, as well as somatic cells populations in the seminiferous tubules, change in morphology, location, and number in the first 2 months of life [13, 17]. For successful autologous SSC transplantation, it is necessary to isolate the largest number possible of SSCs [30, 34], and the selection of the donor testes with a high density of SSCs before first two month of life. However, distribution of SSCs in microminipigs, even its distribution in domestic pigs, is yet to be clarified, and therefore prediction of suitable timing to collect donor testicular cells in microminipigs is difficult. Thus, in this chapter, characterization of domestic pig SSCs for acquiring general information of SSC development in pigs was performed in section I. Then, based on the reference from domestic pigs, characterization of microminipigs SSCs for determining suitable age for the isolation as donor cells was performed in section II.

To understand spermatogenesis in the early stages of life, SSCs are classically identified based on size, morphology, and location [48, 53, 65]. The SSCs are larger than somatic cells, and therefore identification of SSCs is not difficult. However, further identification of the developmental

stage of SSCs based only on classical identification is inaccurate. To identify SSCs in neonatal and pre-pubertal testis, it is necessary to use pluripotent markers [1, 13, 14, 17].

1-2 Section I: Characterization of domestic pig spermatogenesis using SSC markers in the early months of life

In domestic pigs, SSCs are present in large number before 2 months of life [26]. During this period, SSC populations change drastically in morphology and number [13, 14], in addition to changes in the intensity of the affinity to pluripotent markers as the piglet grows. However, knowledge about this transition from gonocytes to undifferentiated spermatogonia is still limited [9], and the precise changes in SSCs, reflecting changes in the intensity of affinity to the markers, are unclear. In the first section of this chapter, I anatomically, histologically, and immunohistologically characterized domestic pig SSCs in neonatal, 1-mo-old, 2-mo-old, and adult testes using the pluripotent markers: DBA, UCHL1, ZBTB16, and POU5F1. Changes in expression of these markers have been evaluated, combined with cell size, morphology, and location in the early stages of life.

1-2-1 Materials and Methods

Testes samples

To clarify pig spermatogenesis in the first two months of life, testicular sections were evaluated at four developmental stages: neonatal (n=6), 1-mo-old (n=6), 2-mo-old (n=6), and adult (n=6). The ages of the neonatal, 1-mo-old, 2-mo-old, and the adult stages were <7 days, 30.0 ± 0.6 days, 57.3 ± 1.7 days, and >6 months, respectively. Testes from Duroc and crossbred (Landrace \times Large White Yorkshire \times Duroc) piglets were obtained from the Gifu Prefecture Livestock Research Center and local farms, and adult testes from crossbred boar were obtained from a local

slaughterhouse. Piglets were castrated according to the operation manual for animal husbandry management of the farms. The protocols for the experiment were approved by the Committee for Animal Research and Welfare of Gifu University (#16032).

Sample processing and staining

After castration, samples were washed with PBS. Then, samples were cut into the equatorial region and fixed in 4% paraformaldehyde, embedded in paraffin, processed routinely, sectioned, and stained with HE. Immunohistochemical analysis was performed for the additional tissue sections by using the polymer immunocomplex method (Envision+; Dako, Glostrup, Denmark). Details regarding the immunohistochemical studies and antibodies are shown in Table 1-1. The sections with primary antibodies were incubated overnight at 4°C. For visualization, the sections were developed in a 3,3'-diaminobenzidine solution (Liquid DAB+ Substrate-Chromogen System; Dako) and then counterstained with Mayer's hematoxylin.

Table 1-1 Antibodies used for immunohistochemical staining

Antigen	Clone	Dilution	Pretreatment	Manufacturer ^c
DBA ^a		50	HIAR ^b	EY lab
UCHL1	Polyclonal	100	HIAR	Abgent
ZBTB16	Polyclonal	100	HIAR	SCB
POU5F1	Polyclonal	100	HIAR	SCB
Vimentin	V9	Prediluted	HIAR	DAKO

a) DBA, DBA-conjugated horseradish peroxidase

b) HIAR, heat-induced antigen retrieval treatment using Target Retrieval Solution, pH 6.0 (DAKO)

c) EY lab: EY laboratories Inc. CA, U.S.A.; Abgent: a WuXi AppTec Company, CA, U.S.A.; SCB: Santa Cruz Biotechnology, TX, U.S.A.; DAKO: Dako Cytomation, Glostrup, Denmark.

Evaluation of testicular development

Testicular development was assessed anatomically and histologically. Anatomically, TC was measured at each development stage. Histologically, the presence or absence of seminiferous lumen was confirmed, and SD was measured.

Immunohistochemistry

Immunohistochemical analyses were conducted for, DBA, UCHL1, ZBTB16, POU5F1 and vimentin, at each developmental stage. To evaluate the total number of positive cells, I counted the positive cells for DBA, UCHL1, ZBTB16, and vimentin per 150 round seminiferous tubules (DBA+/tubule, UCHL1+/tubule, ZBTB16+/tubule, and Vimentin+/tubule) of cross sections from each testis. As testes grew month after month, the number of positive cells increased. Therefore, it was necessary to correct the data to compare the different developmental stages, and the positive cells per 100 Sertoli cells, so I calculated DBA+/Sertoli, UCHL1+/Sertoli, and ZBTB16+/Sertoli.

Statistics

The results are shown as mean \pm SEM. Variations in the number of testicular cells, TCs, and SDs were evaluated using a one-way ANOVA, the Tukey-Kramer test, and the Steel-Dwass test. The level of significance was set at $P < 0.05$.

1-2-2 Results

Testicular development

The testes grew and became larger with increasing age (Table 1-2). The TCs in neonatal, 1-mo-old, 2-mo-old, and adult testes were 3.4 ± 0.1 cm, 4.9 ± 0.2 cm, 7.7 ± 0.2 cm, and 20.9 ± 0.2 cm, respectively. There were significant differences in TC among all four developmental stages.

Histologically, piglet testes developed with age (Table 1-2). The SDs increased as piglets grew. The SD in neonatal, 1-mo-old-old, 2-mo-old, and adult testes were 52.1 ± 0.7 μ m, 55.2 ± 1.4 μ m, 61.6 ± 2.7 μ m, and 161.2 ± 24.6 μ m, respectively. However, there was no statistically significant difference among neonatal, 1-mo-old, and 2-mo-old testes. In the neonatal, 1-mo-old, and 2-mo-old testes, there was no seminiferous lumen, and only a single layer of cell was observed within the seminiferous tubules. On the other hand, the adult testes were significantly larger than the others, and the seminiferous lumen was present.

Table 1-2 Testicular maturity of domestic pigs in each developmental stage.

Stage	Testis Circumference (cm) (mean \pm SEM)	Seminiferous Tubule Diameter (mm) (mean \pm SEM)	Seminiferous Lumen
Neonatal	3.4 ± 0.1^a	52.1 ± 0.7^a	Absence
1-mo-old	4.9 ± 0.2^b	55.2 ± 1.4^a	Absence
2-mo-old	7.7 ± 0.2^c	61.6 ± 2.6^a	Absence
Adult	20.9 ± 0.2^d	161.2 ± 24.6^b	Presence

^a Different letters indicate significant differences within a column ($P < 0.05$)

Immunohistochemistry

In neonatal and 1-mo-old testes, DBA expression was confirmed in the cytoplasm, and the positive cells were located in the central region and occasionally attached to the basal membrane (Figure 1-1). In 2-mo-old testes, few DBA positive gonocytes were observed, but there were DBA negative gonocytes that were morphologically identified. In adult testes, DBA expression was

observed in the apical cytoplasm and around the nucleus of somatic cells, Sertoli cells, and Leydig cells.

The average DBA+/tubule in neonatal testes was 1.4 ± 0.1 cells and increased significantly to 1.7 ± 0.1 cells in 1-mo-old testes (Figure 1-2A). The total number of DBA positive cells had increased from the neonatal stage to the 1-mo-old stage. After one month of age, DBA positive cells were not observed. The average DBA+/Sertoli in neonatal and in 1-mo-old testes was 11.4 ± 0.9 cells and 10.4 ± 0.5 cells, respectively; however, this was not statistically different (Figure 1-3A).

UCHL1 expression, located on the basal cytoplasm and nucleus, was observed in the seminiferous tubules at all developmental stages (Figure 1-4). The positive cells were located in the central and basal regions of the tubule in neonatal testes. In 1-mo-old and 2-mo-old testes, occasionally positive cells were observed in the central region. In the adult testes, the positive cells were only in the basal region of the tubules. In all stages, the expression was also observed in the Leydig cells.

The average number of UCHL1+/tubule in neonatal, 1-mo-old, 2-mo-old, and adult testes was 2.3 ± 0.2 cells, 2.3 ± 0.1 cells, 2.7 ± 0.1 cells, and 9.7 ± 0.7 cells, respectively (Figure 1-2B). There was no statistical difference among neonatal, 1-mo-old, and 2-mo-old testes. Moreover, the average UCHL1+/Sertoli in neonatal, 1-mo-old, 2-mo-old, and adult testes was 18.9 ± 1.7 cells, 14.6 ± 0.4 cells, 17.1 ± 1.2 cells, 56.3 ± 4.8 cells, respectively. There was no statistical difference among neonatal, 1-mo-old, and 2-mo-old testes (Figure 1-3B).

ZBTB16 expression was observed in the nucleus and cytoplasm of cells inside the seminiferous tubules in neonatal testes, but was seen in gonocytes and spermatogonia in 1-mo-old testes (Figure 1-5). Moreover, ZBTZ16 immunoreactivity in some gonocytes was not intense in 1-mo-old testes. In the 2-mo-old testes, strongly ZBTB16-positive stained spermatogonia was observed. In contrast to piglet testes, the expression of ZBTB16 was weak in adult testes.

The average number of ZBTB16+/tubule in neonatal, 1-mo-old, 2-mo-old, and adult testes was 1.4 ± 0.1 cells, 1.3 ± 0.1 cells, 1.8 ± 0.1 cells, 6.7 ± 0.6 cells, respectively (Figure 1-2C), and there was statistical difference between neonatal and 2-mo-old testes. Moreover, the average number of ZBTB16+/Sertoli in neonatal, 1-mo-old, 2-mo-old, and adult testes was 11.4 ± 0.8 cells, 8.2 ± 0.4 cells, 11.4 ± 1.0 cells, and 39.0 ± 4.0 cells, respectively (Figure 1-3C), and there was no statistical difference among piglet testes.

The average number of DBA+/Sertoli and UCHL1+/Sertoli in neonatal, 1-mo-old, and 2-mo-old testes were compared. The average number of DBA+/Sertoli was significant lower than that of UCHL1+/Sertoli in neonatal testes, although the average number of UCHL1+/Sertoli was the same as neonatal and 1-mo-old testes, the average number of DBA+/Sertoli decreased rapidly to an undetectable level.

The average number of DBA+/Sertoli and ZBTB16+/Sertoli in neonatal and 1-mo-old testes was compared (Figure 1-3). In neonatal testes, the average numbers of DBA+/Sertoli and ZBTB16+/Sertoli were similar. Together with the morphology (Figure 1-5), ZBTB16 positive cells were identified as gonocytes in neonatal testes. However, although the numbers of ZBTB16+/Sertoli and DBA+/Sertoli in neonatal testes were similar, the number of ZBTB16+/Sertoli in 1-mo-old testes was significantly lower than that of DBA+/Sertoli, suggesting that ZBTB16 did not identify some cells that expressed DBA.

POU5F1 expression was observed in the cytoplasm of gonocytes and spermatogonia but was weak in neonatal, 1-mo-old, and 2-mo-old testes (Figure 1-6). In 1-mo-old and 2-mo-old testes, weak POU5F1 expression was also observed in Leydig cells. In adult testes, POU5F1 expression was observed in spermatogonia, spermatocytes, and other cells. Consequently, it was difficult to perform accurate SSC identification and counting using POU5F1.

Vimentin clearly defined Sertoli cells (Figure 1-7). The average number of Vimentin+/tubule in neonatal, 1-mo-old, 2-mo-old, and adult testes was 12.1 ± 0.6 cells, 16.2 ± 0.3

cells, 15.7 ± 0.5 cells, and 17.5 ± 0.4 cells, respectively (Figure 1-8). The average number of Vimentin+/tubule in neonatal testes was less than at other stages.

1-2-3 Discussion

The results supported the theory that the first two months of life in pigs is a transition period of SSCs. During this period, the TC became large, but the SD did not change significantly. Moreover, only a single cell layer of seminiferous tubules was observed in the testes and the seminiferous lumen was absent. In addition, the total number of SSCs, that were UCHL1 positive cells, did not change during this period, and the number of DBA positive cells decreased from one month after birth. Therefore, only somatic cells increased during this period, and the SSCs had been differentiated from gonocytes into undifferentiated spermatogonia, meaning that it was a transition period.

Consistent with other species [54], DBA is considered a specific marker for pig gonocytes and primordial germ cells [36]. It has been suggested that DBA expression in pigs decreases due to the maturation and differentiation of gonocytes during the first month of life. However, results acquired in this study have clarified that the number of DBA positive cells and the number of DBA+/Sertoli in neonatal testes did not change until one month of age. Then, DBA positive cells decreased, and few DBA positive cells were observed in 2-mo-old testes. Therefore, DBA was not a useful gonocyte marker after two months of age. However, there were some DBA negative gonocytes, which were morphologically identified, in 2-mo-old testes. Therefore, in pig testes after two months of age, gonocytes or gonocytes expressing DBA might have decreased. Considering this phenomenon, although more data are needed to confirm this, I suggest another possibility that pig gonocytes might have changed to gonocytes known as “emergency gonocytes”, which are complementary gonocytes without DBA expression preparing for the loss of SSCs, after one month of age [9].

Because UCHL1 is a landmark of total pig SSCs [38], and DBA positive cells are defined as gonocytes, it is possible to estimate the differentiation of gonocytes into SSCs (undifferentiated

spermatogonia) by comparing the average numbers of DBA+/Sertoli and UCHL1+/Sertoli. The results clarified that spermatogenesis and SSC transition started at birth, and that the differentiation of gonocytes into undifferentiated spermatogonia was accelerated at one month of age, as the number of DBA positive cells rapidly decreased during this stage but not UCHL1 positive cells. After two months of age, few DBA positive cells were observed, but UCHL1 positive cells remained, and therefore early pig spermatogenesis, that is, the transition of gonocytes into undifferentiated spermatogonia, might be completed at two months of age.

ZBTB16 has been suggested as a marker for gonocytes only in neonatal pig testes [13], and my results support this suggestion. Together with exhibiting the morphology of ZBTB16 positive cells, ZBTB16 positive cells were identified as gonocytes in neonatal testes. However, ZBTB16 is a transcriptional repressor of cyclin A that suppresses cell growth by inhibiting entry and progression to the S-phase of the cell cycle [8, 57, 69], and therefore proliferating gonocytes may not show its expression in 1-mo-old testes. This may be the reason why the number of ZBTB16+/Sertoli was significantly lower than that of DBA+/Sertoli and some gonocytes identified by way of their morphology did not express ZBTB16. Since ZBTB16 may be a marker for stable gonocytes or spermatogonia, the multiplication of pig SSCs in neonatal testes may not be very vigorous, and this may be accelerated around one month of age manifesting as DBA expression. Consequently, I conclude that ZBTB16 is a gonocyte marker in neonatal testes and a spermatogonial marker in pig testes after 2 months of age.

In addition, POU5F1 was found to be a pig SSC marker but identification of SSCs using this marker is not recommended. Although POU5F1 expression increased with age, and this marker was adaptable for SSCs after two months of age (earlier than after five months as previously described) its expression was weak, and it was difficult to distinguish SSCs at all stages of testes development. For this reason, I will not use this marker in the microminipig SSCs characterization.

Sexual maturity in pigs is much more rapid than in other domestic animals [26], and this remains an evolutionary question as to why pigs develop so quickly, both physically and sexually. The data supported this, because differentiation of pig SSCs starts during the fetal period and then the transition of SSCs from gonocytes to undifferentiated spermatogonia was accelerated at one month of age. Differentiation of pig SSCs starts during the fetal period and then the transition of SSCs from gonocytes to undifferentiated spermatogonia was accelerated at one month of age. However, individual differences bring about difficulties in clarifying the serial steps of differentiation and classification of SSCs in animals with rapid growth. Therefore, precise evaluation with additionally shorter intervals is needed to further understand the biology of pig SSCs in the early stage of life.

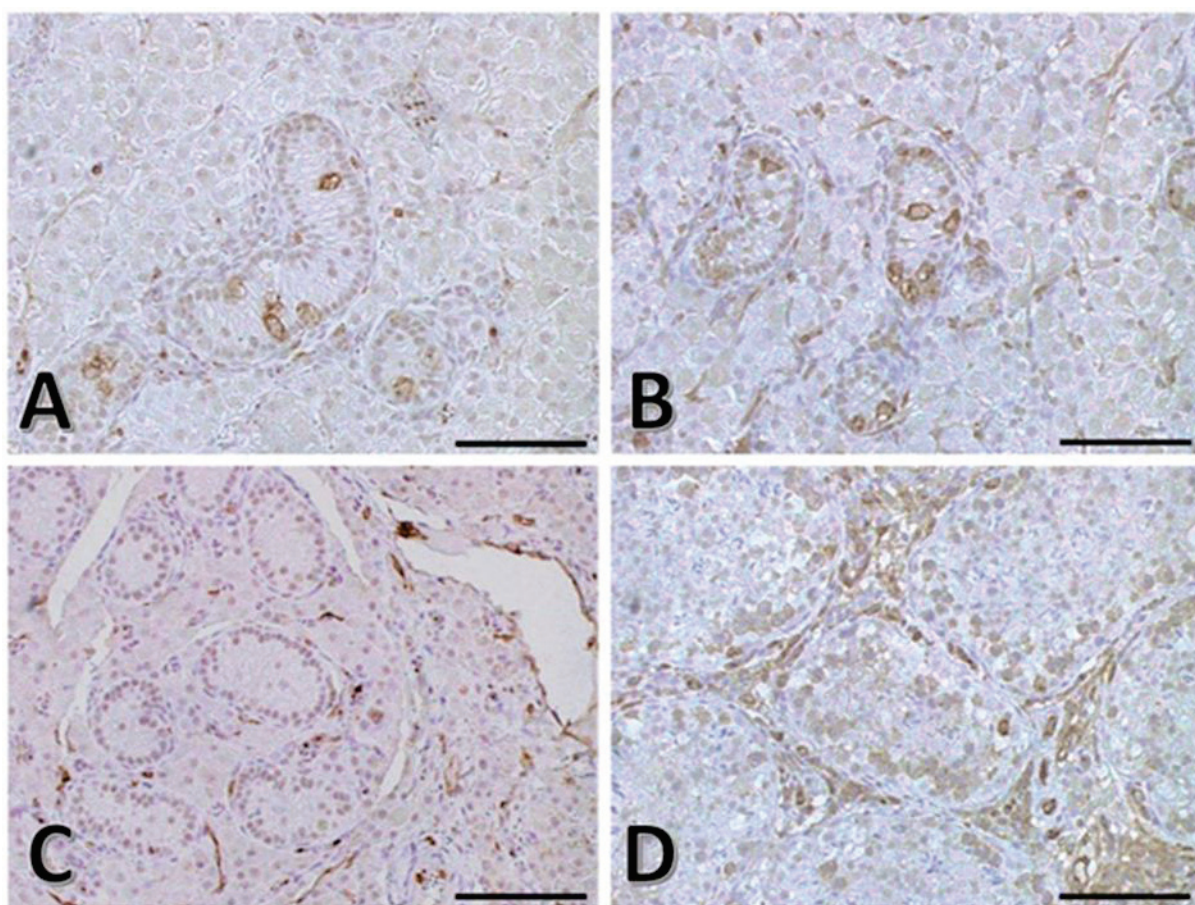


Figure 1-1. Domestic pig testicular sections stained with DBA.

Neonatal testes (A), 1-mo-old testes (B), 2-mo-old testes (C), and adult testes (D). Bar=100 μm.

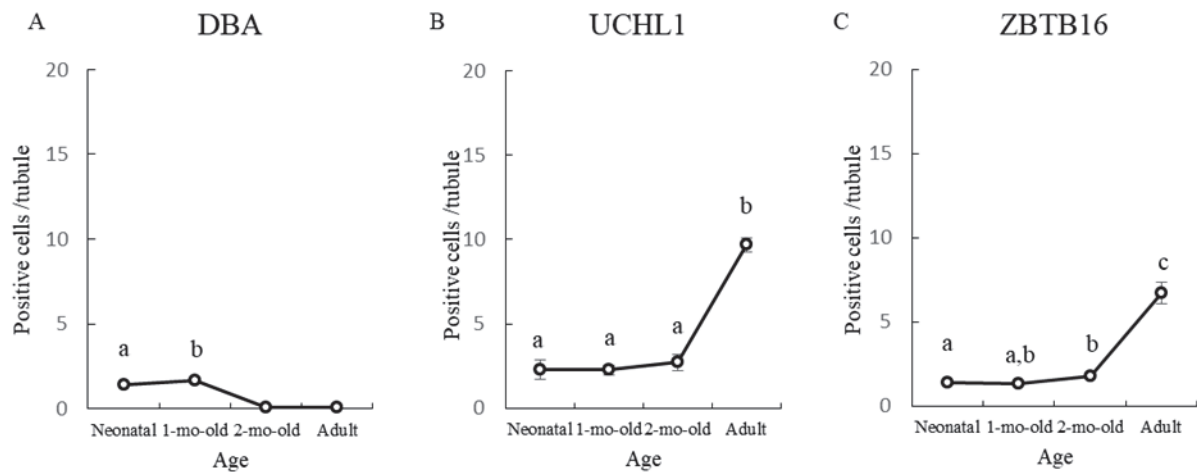


Figure 1-2. Changes in DBA, UCHL1, and ZBTB16 positive cells per seminiferous tubule in domestic pigs.

A: The average DBA+/tubule have significantly increased from neonatal to 1-mo-old testes, and no positive cells are observed from 2-mo-old testes. B: The average UCHL1+/tubule in piglets are not different, but are significantly different from the adult testes. C: The average ZBTB16+/tubule in piglets are significantly different between neonatal and 2-mo-old testes, and significantly different from the adult testes. Different letters indicate significant differences within a graphic.

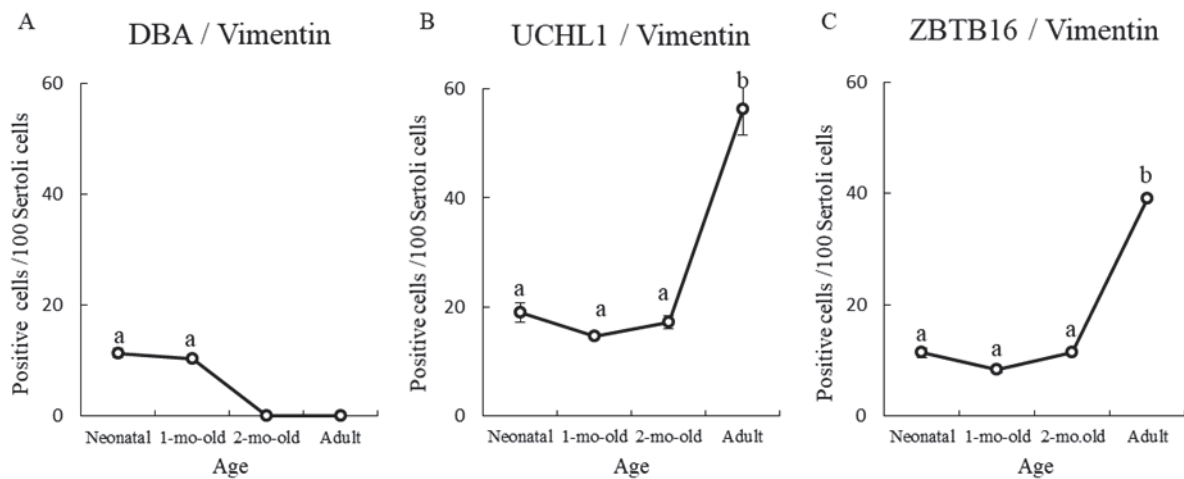


Figure 1-3. Changes in DBA, UCHL1, and ZBTB16 positive cells per 100 Sertoli cells in domestic pigs.

A-C: The average DBA+/Sertoli in neonatal and 1-mo-old testes are not different, but decrease to undetectable levels from 2 months. The average DBA+/Sertoli in neonatal testes is significantly lower than that of the UCHL1+/Sertoli. The average UCHL1+/Sertoli and ZBTB16+/Sertoli positive cells in piglets are not different but are significantly different from the adult testes. The average ZBTB16+/Sertoli in 1-mo-old testes is lower than that of the DBA+/Sertoli. Different letters indicate significant differences within a graphic.

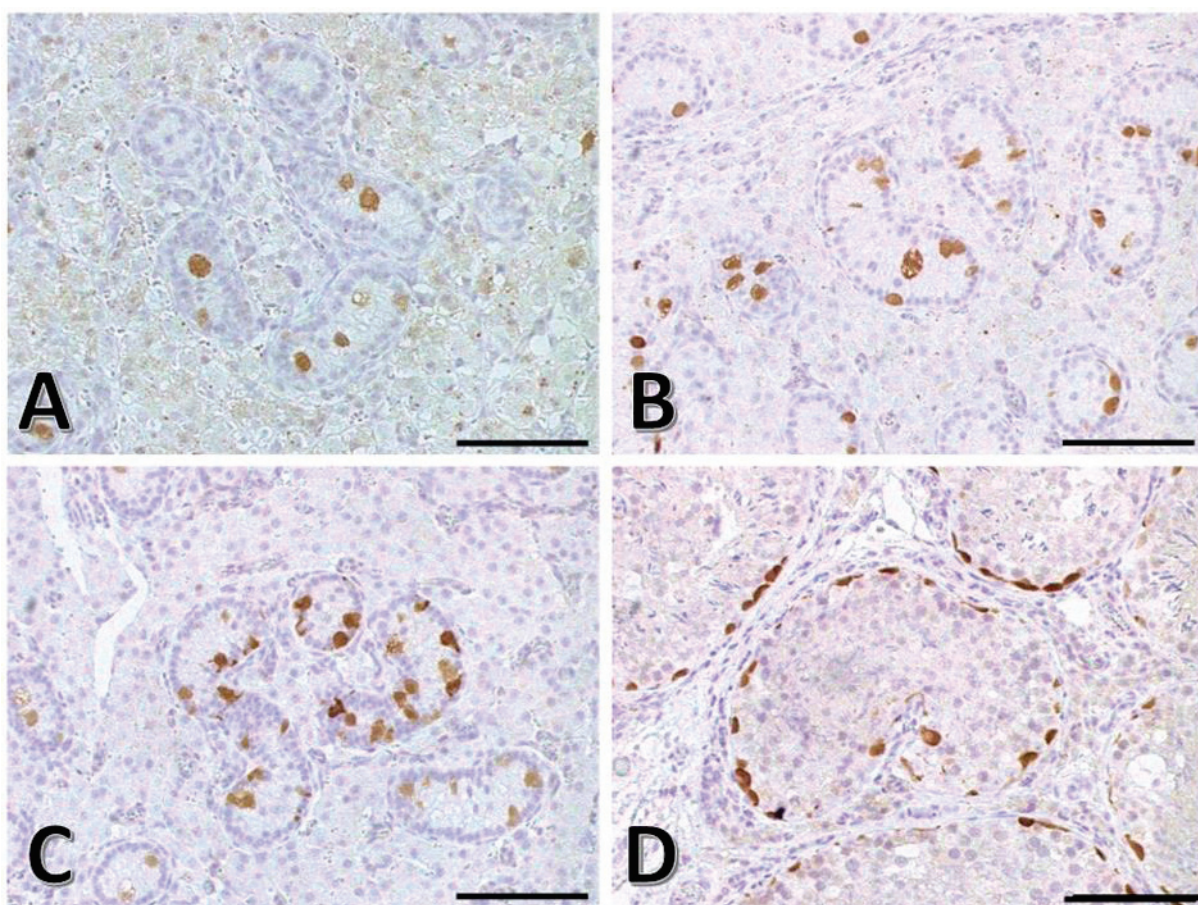


Figure 1-4. Domestic pig testicular sections stained with UCHL1.

Neonatal testes (A), 1-mo-old testes (B), 2-mo-old testes (C), and adult testes (D). Bar=100 μm.

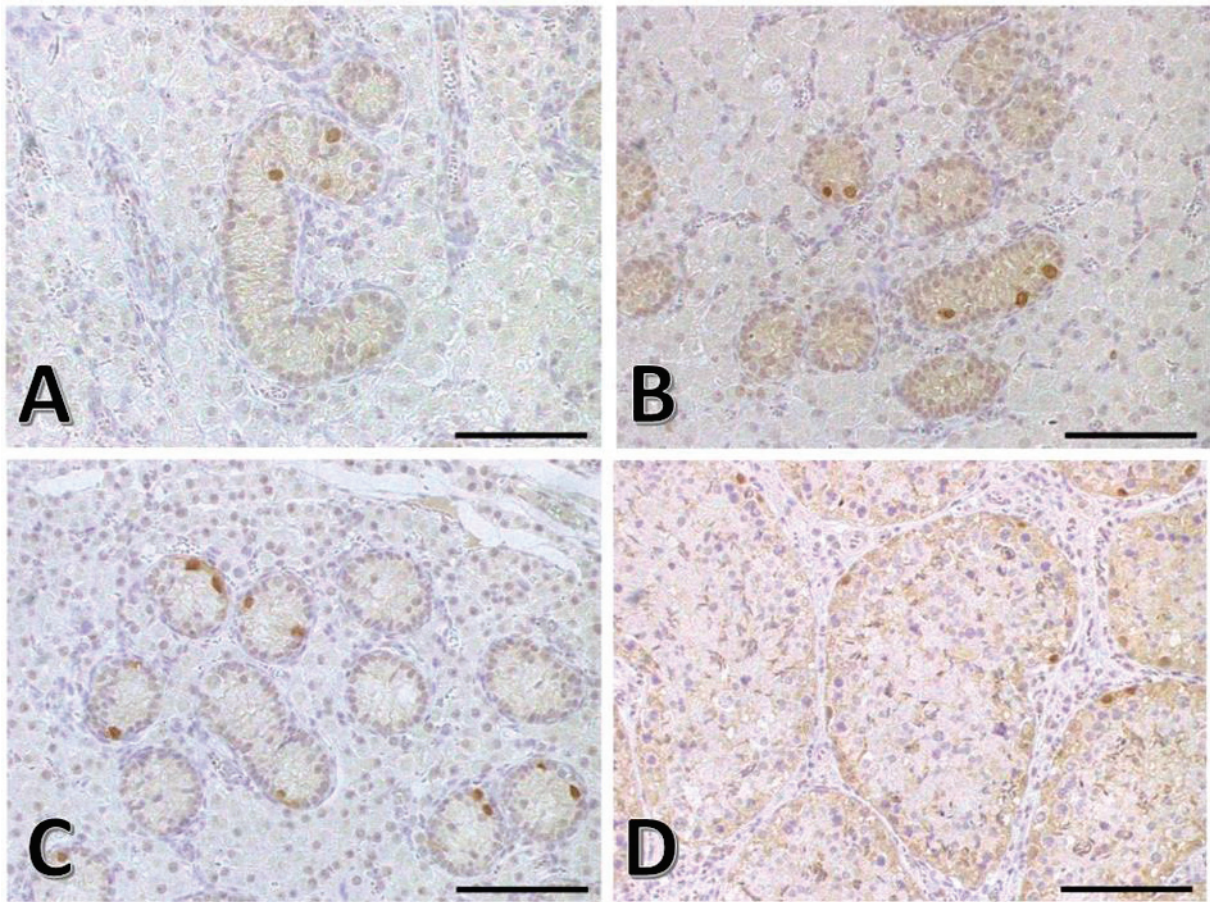


Figure 1-5. Domestic pig testicular sections stained with ZBTB16.

Neonatal testes (A), 1-mo-old testes (B), 2-mo-old testes (C), and adult testes (D). Bar=100 μ m.

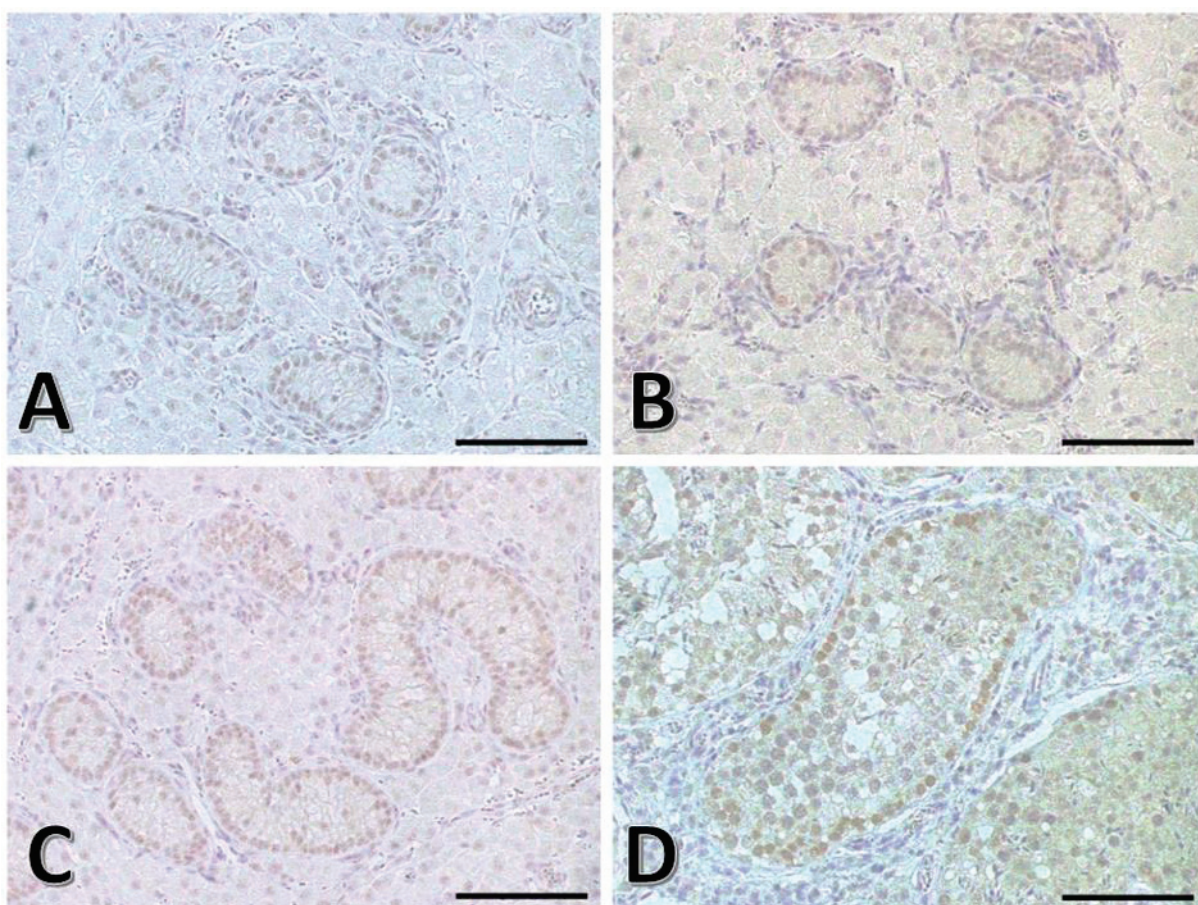


Figure 1-6. Domestic pig testicular sections stained with POU5F1.

Neonatal testes (A), 1-mo-old testes (B), 2-mo-old testes (C), and adult testes (D). Bar=100 μm.

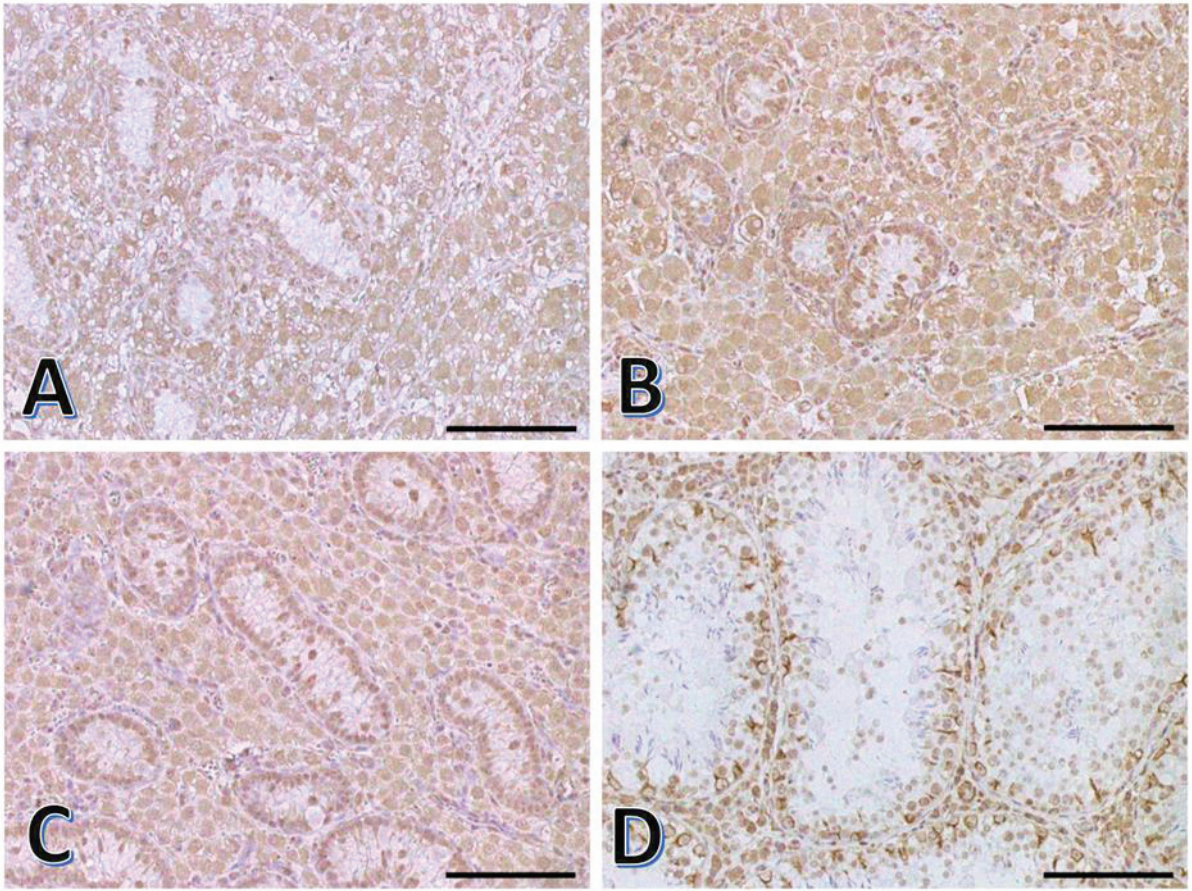


Figure 1-7. Domestic pig testicular sections stained with vimentin.

Neonatal testes (A), 1-mo-old testes (B), 2-mo-old testes (C), and adult testes (D). Vimentin clearly defines Sertoli cells. Bar=100 μ m.

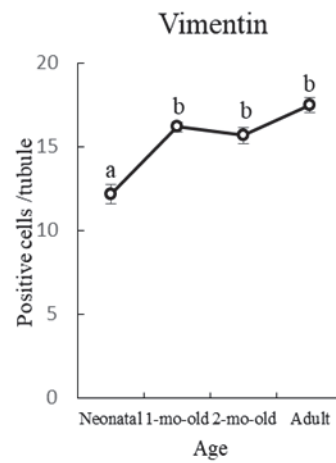


Figure 1-8. Changes in vimentin positive cells per seminiferous tubule in domestic pigs.

The average number of Vimentin+/tubule in neonatal testes is significantly lower than that in 1-mo-old, 2-mo-old, and adult testes. Different letters indicate significant differences within a graphic.

1-3 Section II: Characterization of SSC populations in microminipigs in preparation for autologous transplantation

According to the characterization of domestic pig SSCs, I characterized SSCs in microminipigs with shorter intervals at neonatal, 30-days-old, 45-days-old, 80-days-old and adult testes using DBA, UCHL1 and ZBTB16, to determine the age range when gonocytes and undifferentiated spermatogonia are abundant in the seminiferous tubules in the second section of this chapter.

1-3-1 Materials and Methods

Samples

To characterize pre-pubertal microminipig SSC populations, seminiferous tubules were evaluated at five developmental stages: neonatal (n=4), 30-days-old (n=4), 45-days-old (n=4), 80-days-old (n=4), and adult (n=4). The ages of castrated neonatal, 30-days-olds, 45-days-old, 80-days-old and adult stages were <7 days, 30.0 ± 0.3 days, 45.0 ± 0.3 days, 80.0 ± 0.7 days and <24 months, respectively. Well-developed stillborn microminipigs without any macroscopic findings were used as the neonatal. Testes obtained from retired pigs for the breeding were used as the adult. Other testes at different developmental stages were castrated by a veterinarian under isoflurane anesthesia (ISOFUL®; Zoetis Japan, Tokyo, Japan). The protocols for the experiment were approved by the Committee for Animal Research and Welfare of Gifu University (#17158).

Sample processing, staining, testicular development analysis, immunohistochemistry

The sample processing was performed following the method used in domestic pigs. Moreover, the analysis of testicular development was performed following the method used in domestic pigs.

Immunohistochemical analysis were conducted for DBA, UCHL1, ZBTB16 and vimentin, using the same protocol in the domestic pigs.

Statistics

The results are shown as mean \pm SEM. Variations in the number of testicular cells, TCs, and SDs were evaluated using a one-way ANOVA, the Tukey-Kramer test, and the Steel-Dwass test. The level of significance was set at $P < 0.05$.

1-3-2 Results

Testicular development

The testes grew and became larger with increasing age (Table 1-3). The TCs in neonatal, 30-days-old, 45-days-old, 80-days-old and adult testes were 1.5 ± 0.1 cm, 4.2 ± 0.2 cm, 6.8 ± 0.2 cm, 7.7 ± 0.1 cm and 8.9 ± 0.8 cm, respectively. There were no significant differences in the TC between 45-days-old and 80-days-old or between 80-days-old and adult testes.

Histologically, SDs increased significantly as the piglets grew (Table 1-3). The SD in neonatal, 30-days-old, 45-days-old, 80-days-old and adult testes were 53.1 ± 0.7 μ m, 92.6 ± 1.5 μ m, 130.2 ± 1.8 μ m, 156.9 ± 2.4 μ m and 193.6 ± 2.5 μ m, respectively. Although there was only a single layer of cells within the seminiferous tubules without any visible lumen at the neonatal testes, lumens were observed in 21.0% and 75.0% of the tubules in 30-days-old and 45-days-old testes, respectively. In the 80-days-old and adult testes, 100% of tubules showed a visible lumen (Figure 1-9).

Table 1-3 Testicular maturity of microminipigs in each developmental stage.

Stage	Testis Circumference (cm) (mean \pm SEM)	Seminiferous Tubule Diameter (mm) (mean \pm SEM)	Seminiferous Lumen
Neonatal	1.5 \pm 0.1 ^a	53.1 \pm 0.7 ^a	Absence
30-days-old	4.2 \pm 0.2 ^a	92.6 \pm 1.5 ^b	Presence
45-days-old	6.8 \pm 0.2 ^{a,b}	103.2 \pm 1.8 ^c	Presence
80-days-old	7.7 \pm 0.1 ^{b,c}	156.9 \pm 2.4 ^d	Presence
Adult	8.9 \pm 0.8 ^c	193.6 \pm 2.5 ^e	Presence

^a Different letters indicate significant differences within a column ($P < 0.05$)

Immunohistochemistry

In the neonatal and 30-days-old testes, DBA expression was confirmed in the cytoplasm, and DBA positive cells were observed in the central region and occasionally attached to the basal membranes. In the 45-days-old testes, few DBA positive gonocytes were observed, but DBA negative gonocytes were morphologically identified. In the 80-days-old and adult testes, DBA expression was observed in the apical cytoplasm and around the nucleus of somatic cells, Sertoli cells, and Leydig cells (Figure 1-10).

The average DBA+/tubule in neonatal testes was 1.9 ± 0.1 cells and decreased significantly to 1.6 ± 0.1 cells in 30-days-old testes (Figure 1-11A). After the 30-days-old testes, DBA positive cells were not observed. The average DBA+/Sertoli in neonatal testes was 15.9 ± 0.3 cells and decreased significantly to 13.5 ± 0.4 cells in 30-days-old testes (Figure 1-12A).

UCHL1 expression was located on the SSCs basal cytoplasm and nucleus at all developmental stages. Positive SSCs were observed in the central and basal regions of the seminiferous tubules in neonatal testes. From 30-days-old to the adult testes, positive cells were observed only in the basal region of the tubules. UCHL1 expression was also observed in Leydig cells at all stages (Figure 1-13).

The average number of UCHL1+/tubule in neonatal, 30-days-old, 45-days-old, 80-days-old and adult testes was 4.3 ± 0.1 cells, 6.2 ± 0.1 cells, 6.3 ± 0.1 cells, 7.2 ± 0.1 cells, and 10.1 ± 0.1 cells,

respectively (Figure 1-11B). There were no statistical significant difference between 30-days-old and 45-days-old testes. The number of UCHL1+/Sertoli indicating the number of SSCs/Sertoli in neonatal, 30-days-old, 45-days-old, 80-days-old, and adult testes was 32.3 ± 0.5 cells, 43.2 ± 0.6 cells, 46.1 ± 0.7 cells, 47.8 ± 0.8 cells and 63.3 ± 0.8 cells, respectively (Figure 1-12B), and the SSCs/Sertoli did not change between 45-days-old and 80-days-old stages.

ZBTB16 expression was observed in the nucleus and cytoplasm of gonocyte in the neonatal testes. In the 30-days-old and 45-days-old testes, ZBTB16 expression was observed in some gonocytes and spermatogonia, but a large number of ZBTB16 negative gonocytes were morphologically identified within the tubules. Moreover, in contrast to domestic pigs, the expression of ZBTB16 was weak in the 80-days-old and adult testes (Figure 1-14).

The average number of ZBTB16+/tubule in neonatal, 30-days-old, 45-days-old, 80-days-old and adult testes was 1.6 ± 0.1 cells, 3.8 ± 0.1 cells, 5.2 ± 0.1 cells, 7.1 ± 0.1 cells and 9.6 ± 0.1 cells, respectively (Figure 1-11C), and was significantly increased as the piglets grew. The number of ZBTB16+/Sertoli in neonatal, 30-days-old, 45-days-old, 80-days-old and adult testes was 15.5 ± 0.4 cells, 26.7 ± 0.5 cells, 37.9 ± 0.5 cells, 57.2 ± 0.8 cells and 60.3 ± 0.7 cells, respectively (Figure 1-12C), and also increased significantly as the piglets grew.

Vimentin immunoreactivity was observed in Sertoli cells (Figure 1-15). The average number of Vimentin+/tubule in neonatal, 30-days-old, 45-days-old, 80-days-old and adult testes was 12.1 ± 0.1 cells, 14.4 ± 0.1 cells, 13.8 ± 0.1 cells, 15.1 ± 0.1 cells, and 16.1 ± 0.2 cells, respectively, and the number of Sertoli cells increased significantly as the piglets grew (Figure 1-16).

1-3-3 Discussion

In the second section of the chapter, I characterized the microminipig SSC populations, with the objective of determining the optimal age of donor SSCs isolation for the autologous transplantation. In microminipigs, differentiation of gonocytes to undifferentiated spermatogonia was already started at birth and proliferation of SSCs was intense in the early stage of life. Their early phase of spermatogenesis was completed by 45 days of life in contrast to that in domestic pigs where differentiation begins at around 30 days of life and the early phase of spermatogenesis is completed at 60 days of life. Consequently, sexual precocity in male microminipigs was characterized by early differentiation of gonocytes as well as active development and proliferation of SSCs, which shortened the early phase of spermatogenesis. Based on these results, I concluded that 30 days or younger age is the appropriate age for isolation donor cells to perform the SSC transplantation in microminipigs.

The histological findings confirmed sexual precocity in microminipigs. The SD is a good indicator of testicular maturation because the diameter of tubules expands as the number of SSCs and volume of tubular fluid increases [7, 31, 32]. In microminipigs, SD was increased to $92.6 \pm 2.6 \mu\text{m}$ at 30-days-old testes, and seminiferous lumens were observed. Moreover, spermatids appeared at the 45-days-old stage, suggesting that the SSC maturation was completed around this stage. In domestic pigs, the SD was $55.2 \pm 1.4 \mu\text{m}$ and even $61.6 \pm 2.7 \mu\text{m}$ at one and two months after birth, respectively, and no seminiferous lumen was observed during the period. Thus, it is apparent that male microminipigs are sexually precocious.

Focusing on the DBA+/Sertoli and UCHL1+/Sertoli at the neonatal and 30-days-old stages, the differentiation of gonocytes to undifferentiated spermatogonia and the proliferation of SSCs was clarified. The number of DBA+/Sertoli was significantly lower than the UCHL1+/Sertoli in neonatal testes, suggesting that the differentiation of gonocytes had already started at birth. However, the differentiation of gonocytes in domestic pigs had not changed until 30 days of life, whereas it was

increased significantly in microminipigs by 30 days of life, suggesting that the differentiation of gonocytes in microminipigs was much intense than that in domestic pigs in the early days of life.

As UCHL1+/Sertoli increased until the 45-days-old stage, SSCs, gonocytes, and undifferentiated spermatogonia proliferated until the 45-days-old testes, and the duration of the early phase of spermatogenesis was around 45 days after birth. Because the total number of SSCs in domestic pigs did not increase significantly in number before 60 days after birth, the duration of early phase of spermatogenesis was around 60 days after birth. Consequently, the proliferation of SSCs in microminipigs were earlier and much accelerated than that in domestic pigs, and a shorter duration of early spermatogenesis was characteristic of the precocity in microminipigs.

The results of the ZBTB16+/Sertoli supported the result of UCHL1+/Sertoli, indicating active proliferation of SSCs in microminipigs at the early phase of spermatogenesis. ZBTB16 is a marker for proliferating SSCs [13]. In domestic pigs, ZBTB16 expression was first observed in gonocytes and was gradually changed to undifferentiated spermatogonia until 60 days of life. In microminipigs, ZBTB16 expression was similar to DBA expression in neonatal testes, suggesting that gonocytes were proliferating, and ZBTB16 expression was much more intense in the 30-days-old testes suggesting that undifferentiated spermatogonia were proliferating increasingly. Together with the UCHL1 results, this result suggests that an accelerated proliferation of SSCs from as early as the first weeks of life is characteristic of the sexual precocity in microminipigs.

The immunohistological results clarify the physiology and development of early phase of spermatogenesis in microminipigs. A shorter duration of SSC transition was found to be a characteristic of their precocity. However, I only clarified the phenomenon that microminipigs showed early differentiation and active development and proliferation of SSCs in the early phase of spermatogenesis. Therefore, further studies are needed to clarify the physiology of their precocity. Interestingly, precocity is sometimes observed in miniature breeds, and therefore, this phenomenon may be a nature of evolution or adaptation to the environment. As microminipigs are precocious, the

best age to isolate donor SSCs in microminipig is before 30 days of age, when gonocytes are much abundant. In the next chapter, I determine the suitable conditions to prepare for the recipient testis in microminipigs.

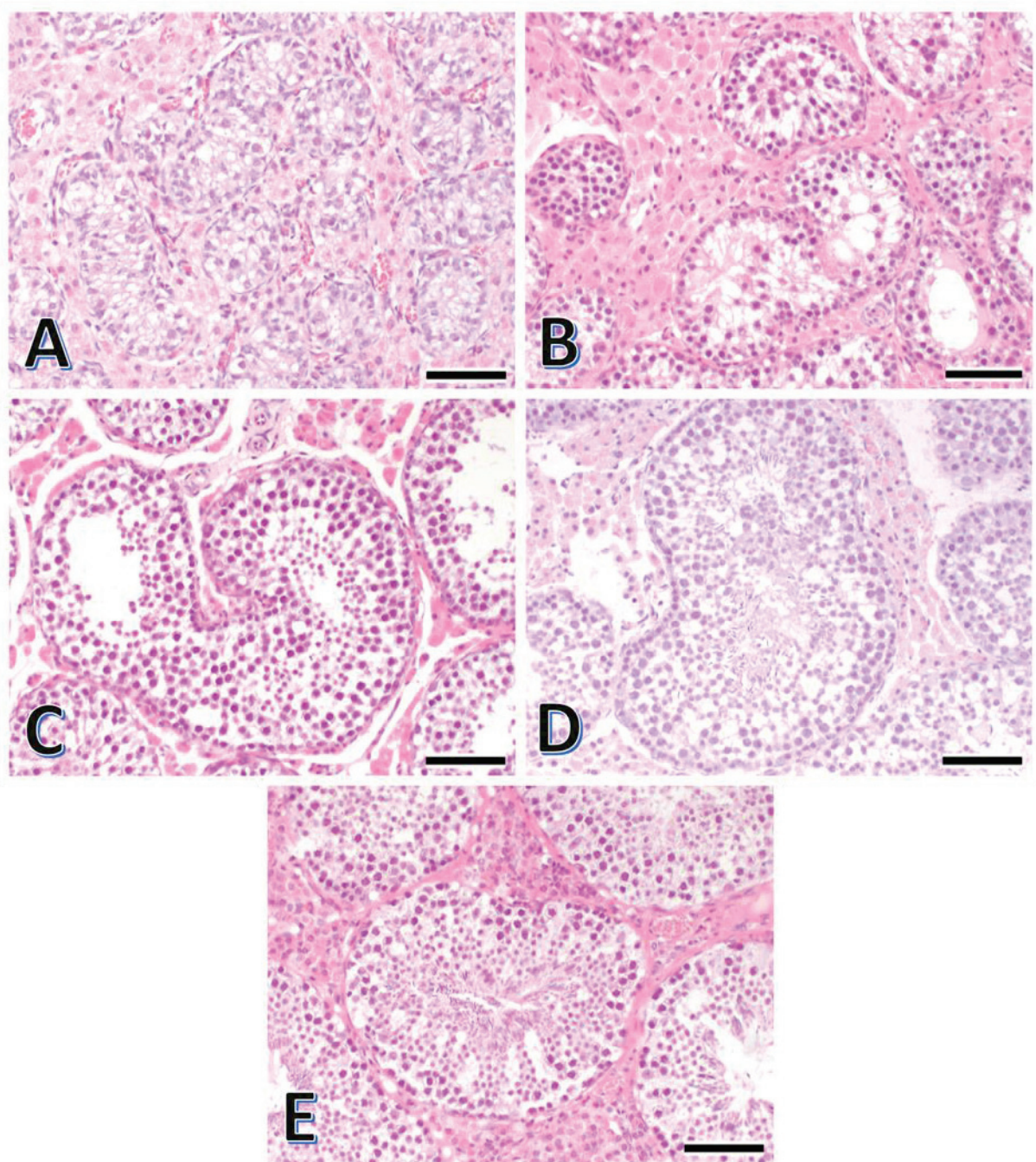


Figure 1-9. Microminipig testicular sections stained with HE.

Neonatal testes (A), 30-days-old testes (B), 45-days-old testes (C), 80-days-old testes (D) and adult testes (E). Bar=100 μm .

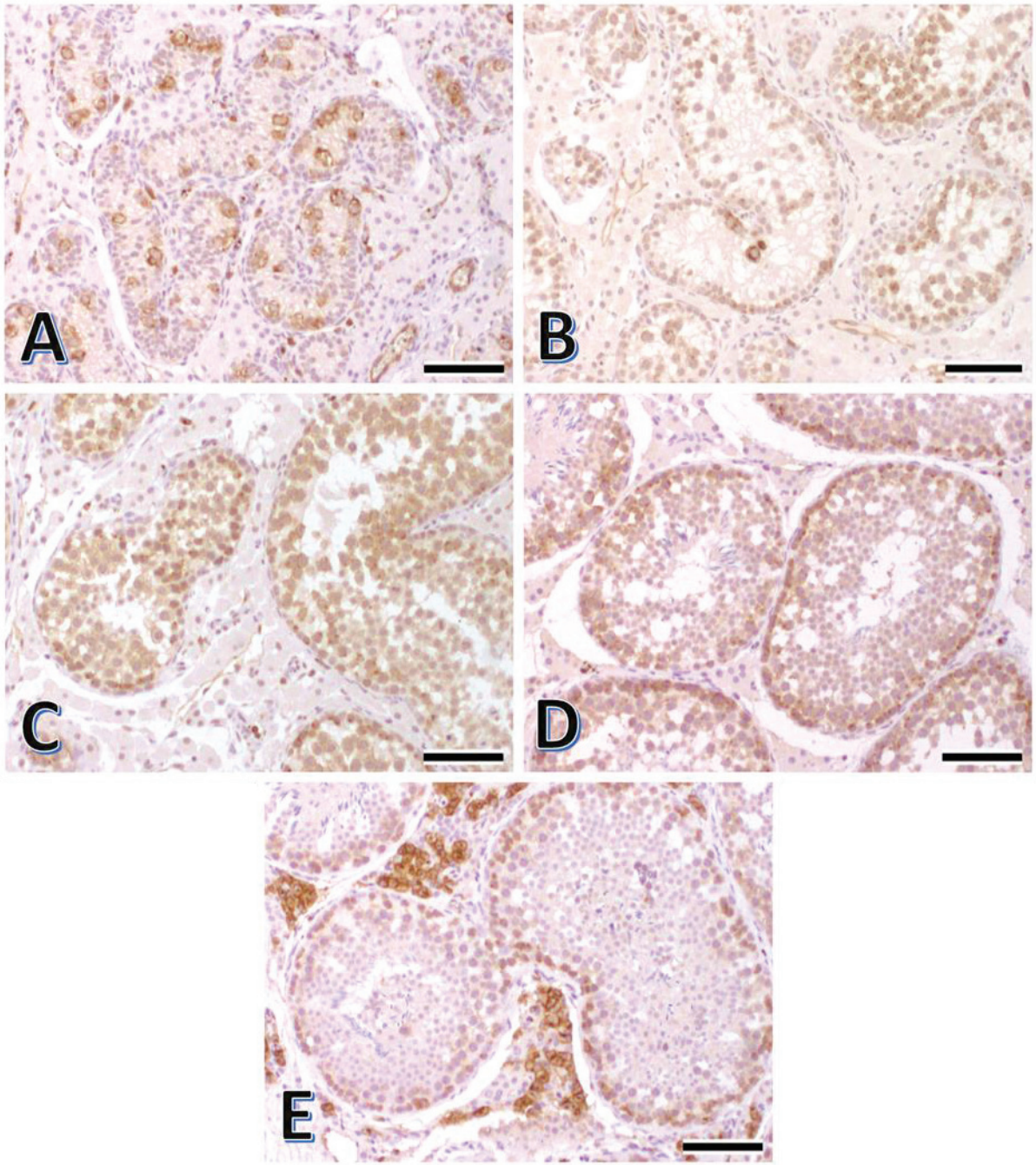


Figure 1-10. Microminipig testicular sections stained with DBA.

Neonatal testes (A), 30-days-old testes (B), 45-days-old testes (C), 80-days-old testes (D) and adult testes (E). Bar=100 µm.

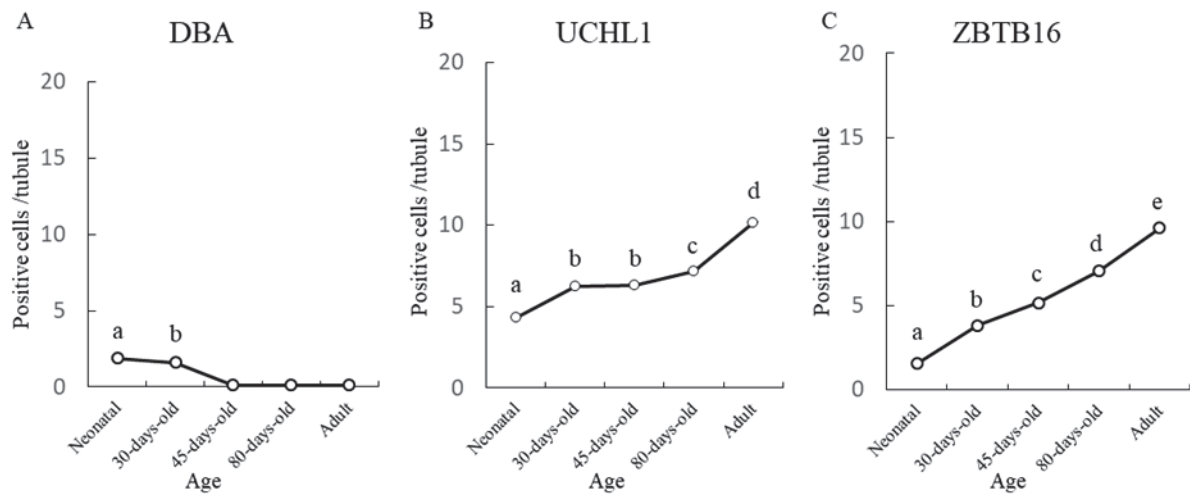


Figure 1-11. Changes in DBA, UCHL1, and ZBTB16 positive cells per seminiferous tubule in microminipigs.

A: The average DBA+/tubule have significantly decreased from neonatal to 30-days-old testes. B: The average UCHL1+/tubule have significantly increased from neonatal to 30-days-old. C: The average ZBTB16+/tubule in piglets are significantly different between all stages. Different letters indicate significant differences within a graphic.

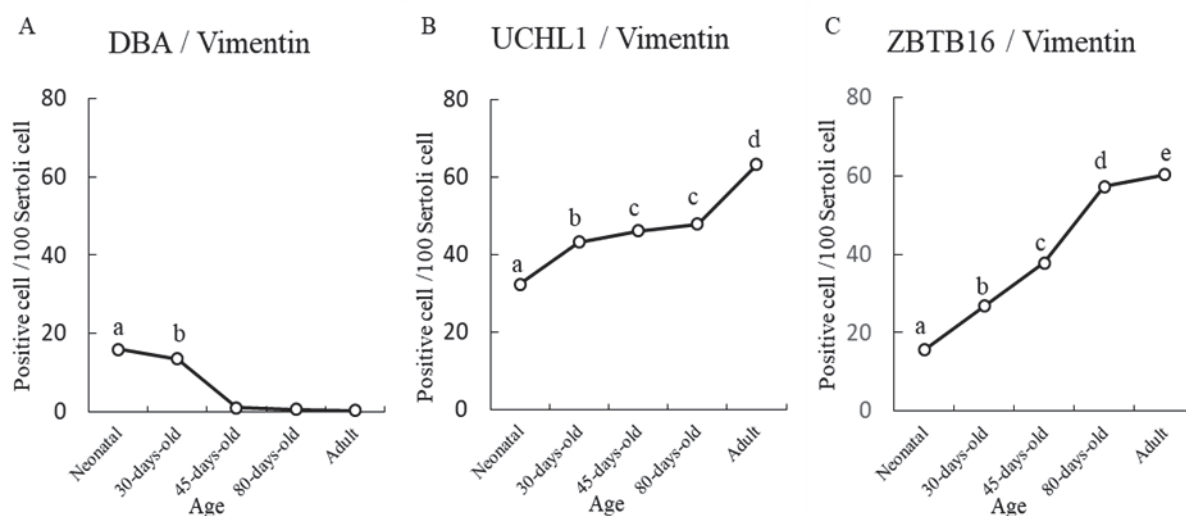


Figure 1-12. Changes in DBA, UCHL1, and ZBTB16 positive cells per 100 Sertoli cells in microminipigs.

A: The average DBA+/Sertoli have significantly decreased from neonatal to 30-days-old testes, and decrease to undetectable levels from 45-days-old. The average DBA+/Sertoli significantly decreased from neonatal to 30-days-old testes. B: The average UCHL1+/Sertoli are significantly different between all groups, except between 45-days-old and 80-days-old. C: The average ZBTB16+/Sertoli are significantly different between all groups. Different letters indicate significant differences within a graphic.

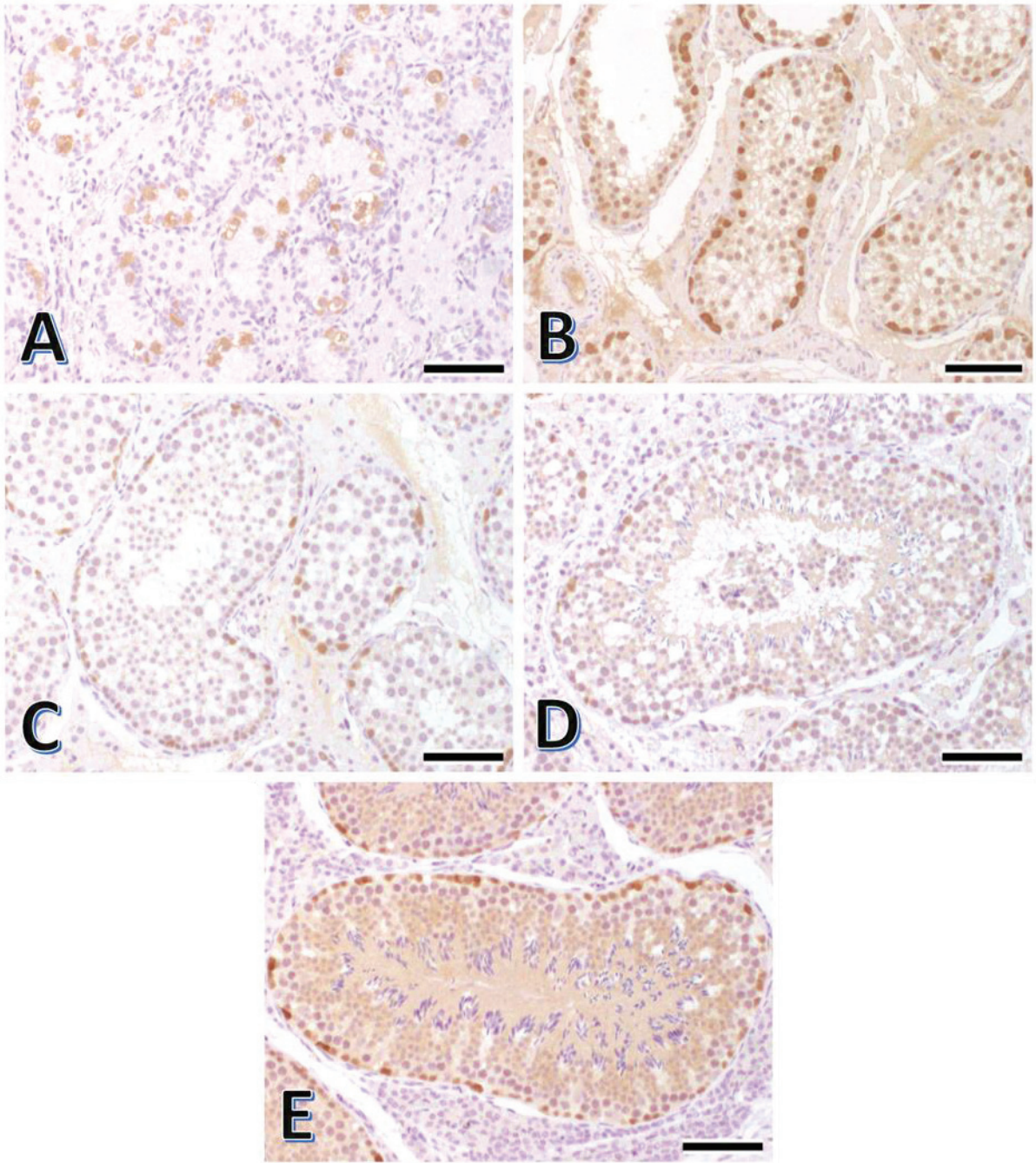


Figure 1-13. Microminipig testicular sections stained with UCHL1.

Neonatal testes (A), 30-days-old testes (B), 45-days-old testes (C), 80-days-old (D) and adult testes (E).

Bar=100 μ m.

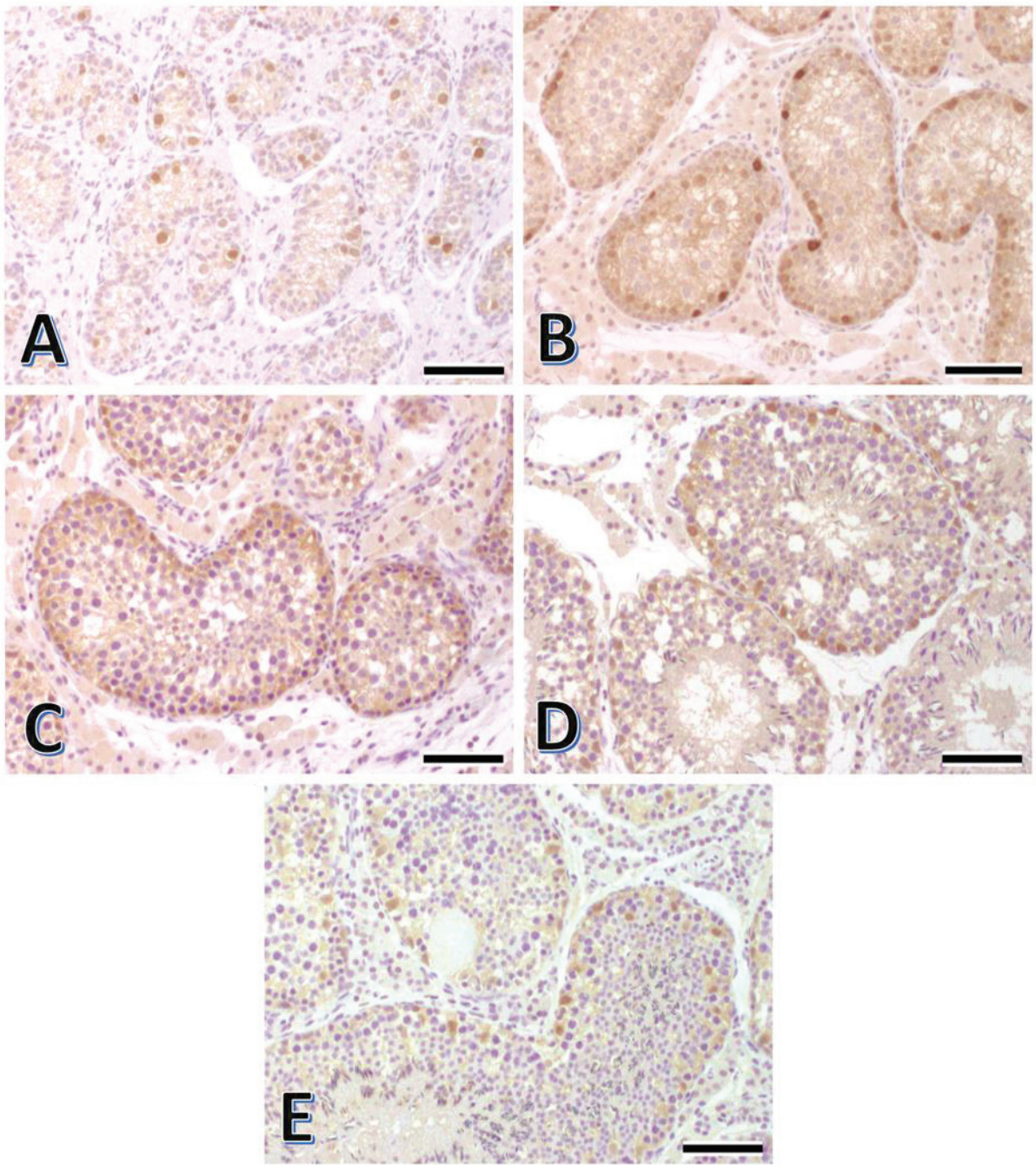


Figure 1-14. Microminipig testicular sections stained with ZBTB16.

Neonatal testes (A), 30-days-old testes (B), 45-days-old testes (C), 80-days-old testes (D) and adult testes (E). Bar=100 μ m.

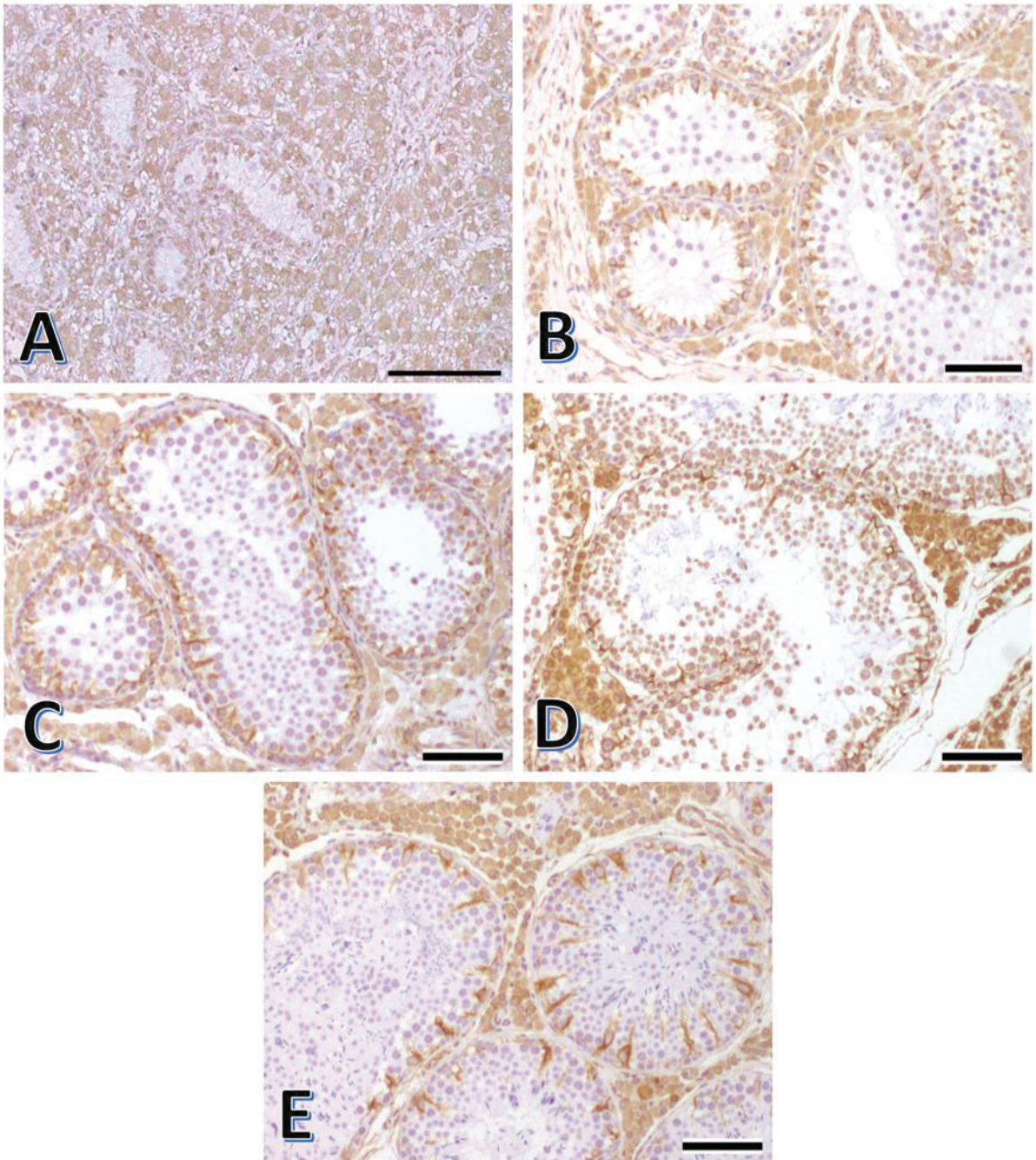


Figure 1-15. Microminipig testicular sections stained with vimentin.

Neonatal testes (A), 30-days-old testes (B), 45-days-old testes (C), 80-days-old testes (D) and adult testes (E). Vimentin clearly defines Sertoli cells. Bar=100 μ m.

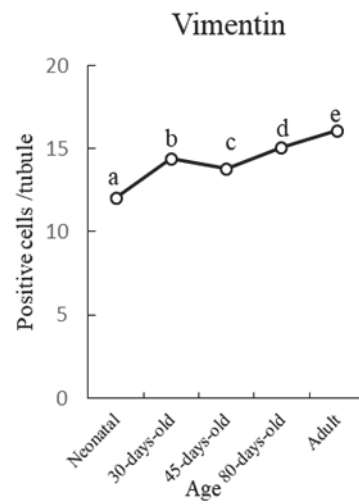


Figure 1-16. Changes in vimentin positive cells per seminiferous tubule in microminipigs.

The average Vimentin+/tubule are significantly different between all groups. Different letters indicate significant differences within a graphic.

Chapter II

Depletion of SSCs in microminipigs by irradiation to create a biological niche in preparation for recipient testis

2-1 Introduction

After autologous transplantation, the transplanted cells compete with endogenous cells to colonize the testicular niche in the basal membrane of the seminiferous tubules [51, 60, 61]. Therefore, depletion of endogenous cells to produce a suitable niche for the transplanted cells is a prerequisite for successful transplantation [18].

For this purpose, busulfan, a chemotherapeutic drug, or irradiation treatments are applied to domestic animals [24]. However, chemotherapeutic treatment is easy to perform, but is highly toxic and sometimes lethal to animals. In contrast, although the equipment is not always available, irradiation treatment is apparently safe, localized, rapid, and easy to apply, and therefore, is preferred for the depletion.

Appropriate irradiation is needed to create the testicular niche for SSC transplantation. However, different sensitivities to irradiation were observed in different stages of development and between species. Therefore, for SSC transplantation, understanding a suitable radiation dose for the target animal testes is essential. Based on information from sheep, cattle, goats, monkeys and rodents [10, 20, 24, 70], a dose of 6–9 Gy can completely deplete the SSCs, while preserving the somatic cells, such as Sertoli cell, and the structure of testicular tubules. However, in pigs, irradiation to deplete the testicular cells to create a niche has yet to be performed, and suitable irradiation doses for the testes are unknown.

For SSC transplantation, in order to deplete endogenous SSCs while maintaining the structure and function of testicular somatic cells, an appropriate dose of radiation must be used. A reduction in

testicular volume of more than 40% and reduction in the diameter of the seminiferous tubules by more than 30% leads to difficulty in recovering spermatogenesis of the irradiated testes [20]. Moreover, a reduction of Sertoli cell per tubule to 25-30% reduces the efficiency of transplantation [61]. In addition, pathological changes, such as fibrosis, edema, and hemorrhage obviously have a negative impact on donor SSCs.

The purpose of this chapter was to determine a suitable radiation dose to create a biological niche in the testes of pre-weaning microminipigs. In this study, I irradiated the pig testes with 0 Gy, 6 Gy, and 9 Gy doses. All testes were evaluated histologically and immunohistochemically 6 weeks after irradiation, when inflammation is supposed to be absent in the tissue [19].

2-2 Materials and Methods

Microminipigs

Nine male microminipigs, 30 days old, were used in this study. The piglets were randomly assigned to 3 groups: control testes (n=3), testes irradiated with a single dose of 6 Gy (n=3), and testes irradiated with a dose of 9 Gy (n=3). The protocols for the experiment were approved by the Committee for Animal Research and Welfare of Gifu University (#17044).

Radiation procedure

Microminipigs, weighing 2.2 ± 0.4 kg, were sedated with an intramuscular administration of 0.015 mg/kg medetomidine (Dorbene vet; Kyoritsu Seiyaku Corporation, Tokyo, Japan), 0.15 mg/kg midazolam (Dormicum injection 10 mg; Astellas, Tokyo, Japan), and 0.12 mg/kg butorphanol (Vetorphale; Meiji Seika Pharma Co., Tokyo, Japan). The radiation was performed following the method described by Takahashi *et al.*, 2018 with minor modification [63]. Briefly, the animals were placed in dorsal decubitus and the testes were positioned with packing material to provide a more uniform dose. The radiation was delivered using a linear accelerator (PRIMUS Mid energy 4 MeV

X-ray; Toshiba Medical Systems, Tochigi), with X-ray energy outputs of 4 MeV and dose rates of 1.0 Gy/min.

Sample processing and staining

Because 6 weeks is the preferable interval between irradiation and SSC transplantation [19], I castrated piglet testes at 72 days old, body weights 5.8 ± 0.7 kg. The testis samples were washed with PBS and TC was measured. Then, samples were cut into the equatorial region, examined for any pathological changes, fixed in 4% paraformaldehyde, embedded in paraffin, processed routinely, sectioned, and stained with HE. Immunohistochemical analysis was performed for the additional tissue sections by using the polymer immunocomplex method (Envision+; Dako, Glostrup, Denmark). Details regarding the immunohistochemical studies and antibodies are shown in Table 2-1. The sections were incubated overnight at 4°C with the primary antibodies. For visualization, the sections were developed with 3,3'-diaminobenzidine solution (Liquid DAB+ Substrate-Chromogen System; Dako) and then counterstained with Mayer's hematoxylin.

Table 2-1 Antibodies used for immunohistochemical staining

Antigen	Clone	Dilution	Pretreatment	Manufacturer ^b
UCHL1	Polyclonal	100	HIAR ^a	Abgent
Vimentin	V9	Prediluted	HIAR	DAKO

a) HIAR, heat-induced antigen retrieval treatment using Target Retrieval Solution, pH 6.0 (DAKO)

b) Abgent: a WuXi AppTec Company, CA, U.S.A.; DAKO: Dako Cytomation, Glostrup, Denmark.

Histological and immunohistological analyses

The development of seminiferous tubules was observed in histological sections and the SD were measured. To determine the total number of SSCs and Sertoli positive cells after irradiation, I counted the cells that were positive for UCHL1, and vimentin per 100 round seminiferous tubules, (UCHL1+ cells/tubule and Vimentin+ cells/tubule) in the cross sections from each testis.

Statistics

The results are shown as means \pm SEM. Variations in the number of testicular cells, TCs, and SDs were evaluated using a one-way ANOVA and the Steel-Dwass test. The level of significance was set at $P < 0.05$.

2-3 Results

In the control testes, TC increased consistently from 4.9 ± 0.2 to 7.9 ± 0.2 cm after 6 weeks. In the irradiated testes, the TC increased from 5.2 ± 0.1 to 6.4 ± 0.4 cm in 6-Gy testes and from 4.9 ± 0.4 to 5.4 ± 0.3 cm in 9-Gy testes after 6 weeks. At the end of 6 weeks, the TC was significantly decreased in the irradiated testes compared with the TC in the control testes. However, there was no statistically significant difference in the TC values between the two irradiation doses. In comparison with the control, an irradiation dose of 6 Gy and 9 Gy resulted in TC reductions of 27.0% and 33.0%, respectively.

The SDs in the control, 6-Gy, and 9-Gy testes were 156.8 ± 1.3 μ m, 127.4 ± 1.1 μ m, and 126.2 ± 1.0 μ m, respectively. The SD was significantly decreased in the irradiated testes in comparison with that of the control. However, there was no statistically significant difference in the SDs between the two irradiation doses.

On histopathologic evaluation, seminiferous tubules in both 6-Gy and 9-Gy testes were maintained in morphology. The cell density in the seminiferous tubules of testis irradiated with two-irradiation doses is lower than that of the control. The SSCs such as spermatogonia were poor, but Sertoli cells were observed (Figure 2-1).

In SSCs, UCHL1 immunoreactivity was located in the cytoplasm and nucleus of SSCs (Figure 2-2), and vimentin immunoreactivity was located in the perinuclear cytoplasm of Sertoli cells (Figure 2-3). UCHL1-positive cells were localized to the basement membrane of the tubules. In irradiated testes, the number of UCHL1+ cells/tubule was reduced significantly compared with that

in the control testes. However, there was no significant difference between the two irradiation doses. The average number of UCHL1+ cells/tubule in the control, 6-Gy, and 9-Gy testes was 7.4 ± 0.2 cells, 0.8 ± 0.1 cells, and 0.7 ± 0.1 cells, respectively. The reduction of UCHL1+ cells/tubule was 89.0% in the 6-Gy testes and 90.0% in the 9-Gy testes relative to the control testes. At least one UCHL1-positive cell was observed in 30.3% and 21.8% of the seminiferous tubules in the 6-Gy and 9-Gy testes, respectively. The percentage of the tubules with recovered spermatogenesis or containing differentiated cells from spermatogonia was 9.0% and 7.0% in the 6-Gy and 9-Gy testes, respectively.

To investigate the effect of both doses on Sertoli cells populations, the number of vimentin-positive cell per tubule was counted. In irradiated testes, the number of Sertoli cell per tubule was significantly reduced compared with that in the control testes. There was significant difference between irradiated testes. The average number of Vimentin+ cells/tubule in the control, 6-Gy, and 9-Gy testes was 15.4 ± 0.2 cells, 13.7 ± 0.1 cells, and 11.9 ± 0.2 cells, respectively. The reduction in positive vimentin cells per tubule was 11.1% and 22.7% in the 6-Gy and 9-Gy testes, respectively, relative to the control value.

The testes were examined to search for any type of alteration. In the 6-Gy testes, clots and hemorrhages were microscopically observed in the peripheral region of one testis (Figure 2-4A). However, other abnormalities or inflammation was not observed. In the 9-Gy testes, abnormalities were observed in all testes, mainly consisting of different sizes of clots, the absence of Leydig cells, the presence of fibrous lesions throughout the testis parenchyma and hyalinization (Figure 2-4B-D). The seminiferous tubules exhibited other abnormalities, such as centrally located Sertoli cells and tubules devoid of cells.

2-4 Discussion

The present study suggests that a 6-Gy dose of focal testicular irradiation is suitable for niche creation for SSC transplantation in microminipigs. The 6-Gy dose satisfied two opposing demands for the depletion treatment: 1) to deplete large numbers of endogenous SSCs, and 2) to maximize survival of the somatic cells and minimize chronic tissue damage.

Inflammation and fibrosis are the principal factors that restrict recuperation of the tissue, survival of the somatic cells, and proliferation of the transplanted cells [19]. In 6 Gy testes, no fibrosis or other chronic damages on the testis tissue were observed. However, 9 Gy severely damaged the testicular cell structure; I observed tissue destruction between the tubules, an absence of the cell layer of the seminiferous tubules, blood clots, and localized fibrosis, all of which would affect the colonization efficiency of transplanted SSCs in the recipient testis. Consequently, considering this histological damage, a dose of 9 Gy appears to be excessive, and a 6-Gy dose seems appropriate for niche creation.

Both 6- and 9-Gy irradiation treatments depleted the SSCs in the testes. Thus, both radiation treatments achieved sufficient elimination of the SSCs to produce a recipient testis, enabling the transplanted SSCs to establish without competition from endogenous SSCs for space and nutrients. For successful SSC transplantation, more than 80% of endogenous SSCs should be eliminated [20]. In this study, both irradiation doses reduced endogenous SSCs (UCHL1+ cells/tubule) by more than 89.9%. Moreover, with both irradiation doses, spermatogenesis was observed in less than 10.0% of the seminiferous tubules. Consequently, from the perspective of depletion of endogenous SSCs, both doses were appropriate for niche creation.

Both irradiation doses did not damage somatic cell populations critically. The Sertoli cell population is crucial for the successful outcome of SSCs transplantation. Sertoli cells and Leydig cells are regarded as less radiosensitive than SSCs [64]. Greater than 70% of the Sertoli cells should survive the irradiation [20]. In the 6-Gy testes, 88.9% of the Sertoli cells survived after the irradiation,

without any reduction or absence of Leydig cells. With 9 Gy irradiation, however, only 77.3% of the Sertoli cells survived. The number of viable Sertoli cells was significantly lower in the 9-Gy testes than in the 6-Gy testes. Moreover, an extensive area without Leydig cells was observed in the 9-Gy testes. Consequently, from the perspective of survival of endogenous somatic cells, a dose of 9 Gy appears excessive and a dose of 6 Gy seems appropriate for niche creation.

The TC and SD are important indicators of the suitability of the irradiation, and a large reduction in these parameters lowers the probability of spermatogenic recuperation after SSC transplantation [19]. However, a reduction in TC of approximately 20-30% and a reduction in SD of approximately 17-35% were not impediments to the successful transplantation and production of donor sperm in previous studies [5, 19, 20, 56]. In this study, the reduction in TC relative to that of control was 27.0% and 33.0% in the 6-Gy and 9-Gy testes, respectively, and the reduction in SD was 19.7% and 20.4%, respectively. Consequently, from the perspective of reduced TC and SD, a dose of 9 Gy might be excessive; therefore, a 6-Gy irradiation dose appears appropriate for niche creation.

Considering the depletion of endogenous SSCs, survival of endogenous somatic cells, histological damage, and reduction in TC and SC, a 6-Gy dose to 30-day-old microminipig testes may be suitable to create a biological niche as a recipient for SSC transplantation. However, it is necessary to corroborate these results performing the transplantation of SSCs into irradiated testis. In the final chapter, I test the autologous transplantation protocol into irradiated testis, with the objective of confirm the spermatogenesis recovery capacity and sperm quality.

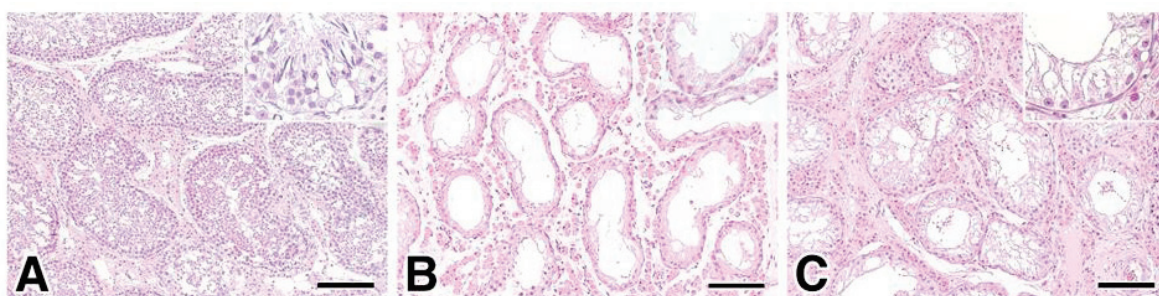


Figure 2-1 Testicular sections of irradiated testes stained with HE.

HE staining of the control (A), 6-Gy (B), and 9-Gy testes (C). Seminiferous tubules in both 6-Gy and 9-Gy irradiated testes were maintained in morphology. In irradiated seminiferous tubules, SSCs were depleted in a large number of tubules, but Sertoli cells were preserved. Bar=200 μ m.

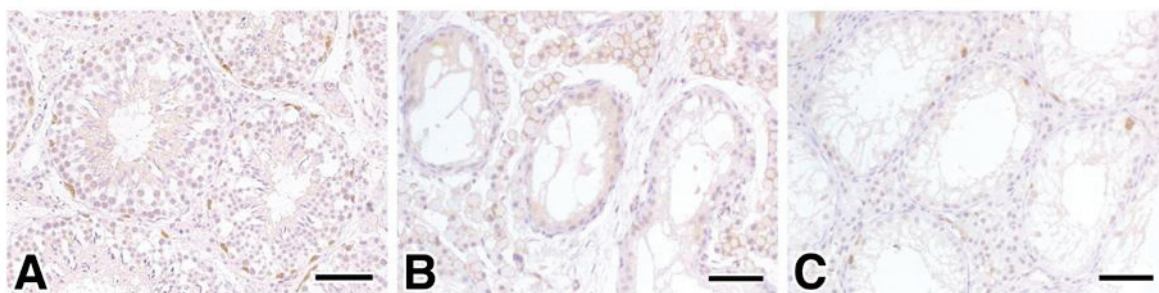


Figure 2-2. Testicular sections stained with UCHL1.

In the control testes, UCHL1-positive cells were observed in the seminiferous tubules (A), in the 6-Gy (B) and 9-Gy testes (C). UCHL1-positive cells were rarely identified in the seminiferous tubules. Bar=100 μ m.

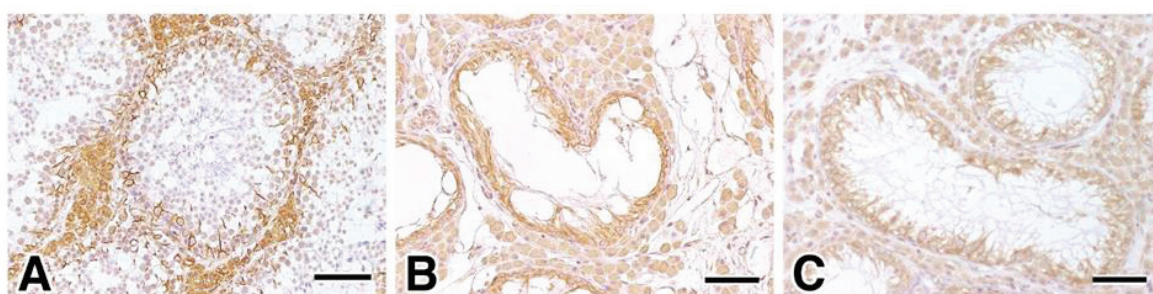


Figure 2-3. Testicular sections stained with vimentin.

Testicular sections stained with vimentin. In the control testes, vimentin-positive cells were observed in the seminiferous tubules (A), in the 6-Gy (B) and 9-Gy testes (C). Vimentin-positive cells were observed in large number in most of the seminiferous tubules. Bar=100 μ m.

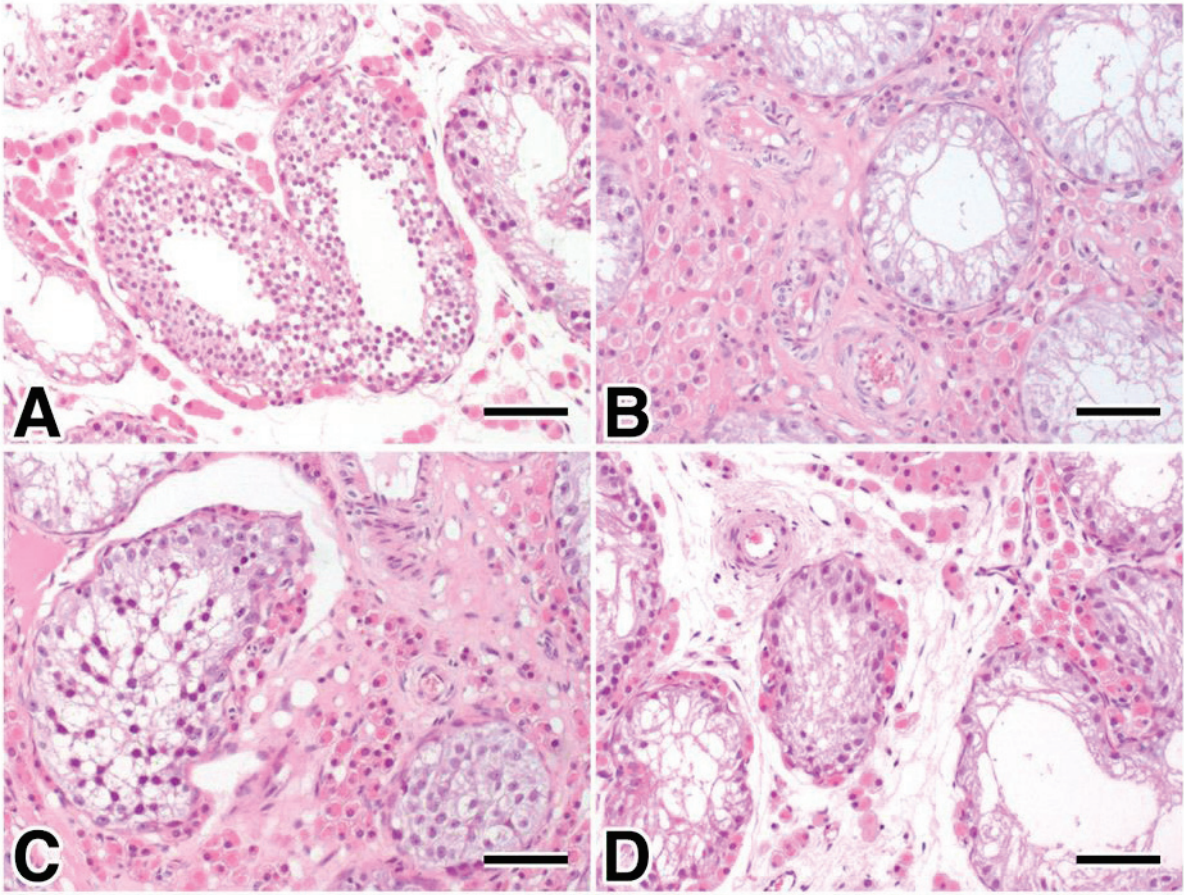


Figure 2-4. Testicular sections of 6-Gy (A) and 9-Gy (B-D) irradiated testes stained with HE.

In the irradiated testes parenchyma destruction of tissue structure and absence of Leydig cells (A, D), centrally fibrous lesions throughout the testis parenchyma (B, D), hyalinization (B, C), centrally located Sertoli cells and tubules devoid of cells (C, D) were observed. Bar=100 μ m.

Chapter III

Autologous transplantation of testicular cells in microminipigs

3-1 Introduction

In the first two chapters, the basic conditions to perform the autologous transplantation were clarified. Based on the results of the first chapter, it was clarified that donor cells should be isolated from a microminipig less than 30 days old to obtain donor cells with a high density of SSCs. Based on the results of chapter two, it was clarified that 6 Gy was the ideal dose to deplete the cells of the recipient testis.

The autologous transplantation could be successful in microminipigs if collection of testicular cells is performed before 30 days old and depletion of the SSCs is performed by 6 Gy irradiation treatment. However, six weeks after the irradiation treatment is necessary to avoid inflammation that interferes with the migration and colonization of the donor cells. Thus, donor SSCs must be stored adequately until the time of transplantation.

In pigs, different types of freezing media increase the survivability of the SSCs, resulting in 55-73% [27]. The most effective technique for the tissue cryopreservation is to use a freezing media with trehalose [37]. This method improved the cryopreservation efficiency of pig testicular cells; with a viability of 73%. Moreover, the porcine testis cell recovery and the SSC proliferation capacity were high compared to other cryopreservation techniques.

In this final chapter, I performed the autologous transplantation of testicular cells in one pre-pubertal microminipig using the conditions studied in the first two chapters (Figure 3-1). Here, I castrated one testis from 25 days old microminipig which has a high density of SSCs. Then, I cryopreserved the testicular tissue using a freezing medium containing trehalose. At 30 days old, I irradiated the recipient-side testis with a single 6-Gy dose. Finally, six weeks after irradiation

treatment, I thawed the tissue, enzymatically digested, and transplanted the donor SSCs using an ultrasound-guided cannulation to the rete testis. Five months after the transplantation, I collected the ejaculates, and evaluated sperm quality.

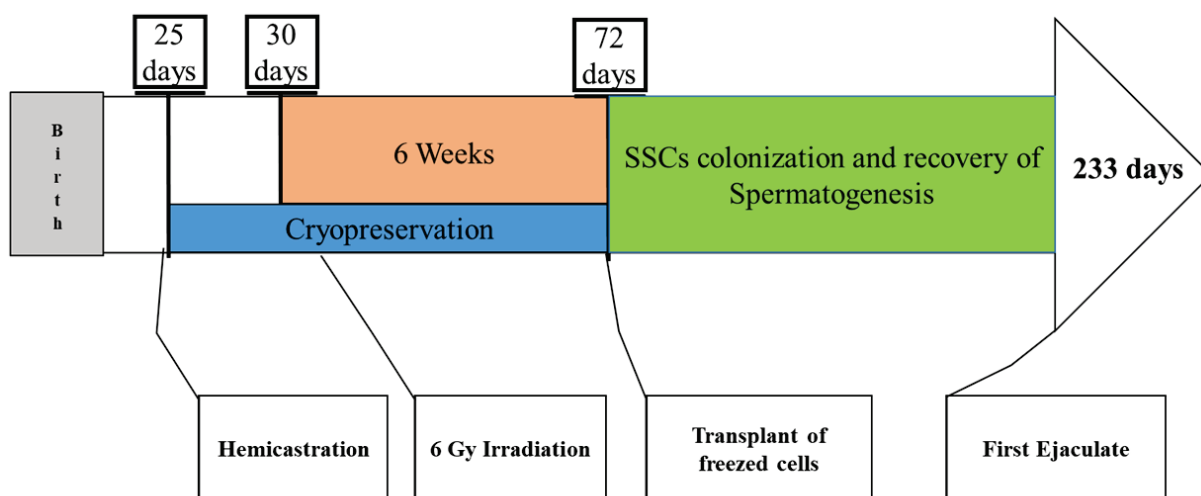


Figure 3-1. A schematic representation of procedures involved in SSCs autologous protocol.

The castrated testis tissue was cryopreserved from day 25 to day 72. At 23 weeks after transplantation, microminipigs ejaculates were obtained and analyzed.

3-2 Materials and Methods

Donor testicular tissue storage

The protocols for the experiment were approved by the Committee for Animal Research and Welfare of Gifu University (#17151). To perform the autologous transplantation, one microminipig piglet aging 25 days old was hemi-castrated. No abnormalities were observed in the castrated testis. Testis tissue was cryopreserved using medium containing trehalose [37]. Testis was measured, decapsulated, minced in 2-3 mm³ pieces, and washed three times with PBS. Testis tissue were divided in cryopreservation vials, 1 g of tissue per vial. Freezing media consisted of DMEM with 10% DMSO, 10 % FBS and 100 mM trehalose. Prior to cryopreservation, tissue were suspended in freezing media and placed in 1.8 ml cryovials (Thermo Fisher Scientific, Japan). The vials were placed in a freezing container (Nihon freezer co., Japan) and placed at -80°C for overnight. The next morning, the vials were placed in liquid nitrogen.

Depletion SSCs of recipient-side testis

When the microminipig was 30 days old weighting 2.2 kg, recipient-side testis was received 6 Gy irradiation treatment. The radiation procedure was same as described in chapter II.

Autologous transplantation

Six weeks after the irradiation treatment, the autologous transplantation was performed. Before the transplantation, the vials were thawed in a 37°C water bath. Thawed tissue were enzymatic digested following the method described by Yang *et al.*, 2010 with minor modification [68]. Briefly, thawed tissue was digested with 0.2% w/v collagenase IV, 0.1% hyaluronidase and 0.01% w/v DNase I (Sigma-Aldrich, Japan) at 37°C for 10 min. Digestion was stopped with the addition of 100% FBS (Life technologies, Canada). Cell suspension was filtered through a 40-µm cell strainers (FALCON,

USA), and washed with PBS. The erythrocytes were then removed with a Red blood cell lysing buffer (Sigma-Aldrich, Japan).

The piglet of 72 days old, body weight 5.1 kg, was anesthetized under general anesthesia. Prior to the transplantation, donor cells were diluted to 5.4×10^5 cells/ml in DMEM medium. The transplantation was performed using an ultrasonography (Figure 3-2A), following the method described by Honaramoov *et al.*, 2002 with minor modification [22]. Briefly, after successful placement of the needle in the rete testis, 0.53 ml of cell suspension was injected over 10-15 min. The injection finished when the total cell suspension was injected in the testis, using 0.50 ml DMEM medium to distribute cell solution along the rete testes (Figure 3-2D).

Semen collection

The training for semen collection was started 3 months after the donor cell transplantation, when the microminipig achieved sexual maturity [31]. Semen samples were collected three times from the piglet of 233 days old, manually while boar mounted on sow in estrus. Semen gel fraction was filtered out during collection using a sterile gauze and collected into a thermos (Figure 3-3). Sperm motility, concentration, and morphology were microscopically examined. The viability of spermatozoa was assessed with Eosin/Nigrosin staining (Sigma-Aldrich, Japan). Briefly, fraction of a drop of semen was mixed with a drop of mix stain solution and over the surface of a glass slide. The semen collection and evaluation was repeated three times.

Statistics

The results are shown as mean \pm SEM.

3-3 Results

Sperm quality analysis

No abnormal smell, consistency, nor color were observed in the ejaculates. The volume of ejaculates was 46.5 ± 3.3 ml. None of erythrocytes, large number of cells, nor other types of artifacts in the ejaculates were observed. Spermatozoa concentration, motility, and viability were $1.0 \times 10^7 \pm 0.1$ cells/total, $95.0 \pm 2.0\%$, and $80.0 \pm 9.1\%$, respectively. The morphological abnormality was $13.1 \pm 2.7\%$; $6.6 \pm 1.3\%$ of spermatozoa with abnormalities in the tail and $8.5 \pm 3.1\%$ of spermatozoa with head abnormalities (Table 3-1).

Table 3-1 Semen quality parameters in transplanted pig

	Volume (ml)	Concentration ($\times 10^8$ cell/ml)	Motility (%)	Viability (%)	Morphological Abnormality (%)
Domestic Pigs ^a	200-300	0.5-10	75	80	0.2-10
Minipigs ^b	120-150	1.5	-	-	2.3
Irradiated microminipig	46.5 ± 3.3	0.1 ± 0.1	95.0 ± 4.3	80.0 ± 7.5	13.1 ± 4.3

^aWolf & Smital (2009)

^bNakanishi et al (1985)

3-4 Discussion

In this chapter, I confirmed the feasibility of the autologous transplantation protocol. As a result, the pig ejaculated normally; viability of sperm was fine; concentration of sperm was moderate for pig species; less percentage of abnormal spermatozoa was observed.

The ejaculated semen quality seemed to be adequate, because the sperm collected from transplanted microminipig showed a normal color; few blood cells and somatic cells were observed; abnormal sperm was less in number; and vitality and motility were within a normal range for pigs. The sperm concentration was lower than the standard values, but this was an expected result as a logical consequence of the hemicastration.

The origin of sperm after the autologous transplantation was difficult to identify. However, as a result from Chapter II, even though a few spermatogenesis was observed in 6 Gy irradiated testes in microminipig aged 72 days, no spermatid nor sperm were observed in the seminiferous tubules. Since spermatid were normally observed from 45 days old testes (Chapter I), 6 Gy irradiated testes without any spermatid nor sperm at 72 days old may be eternal infertility. Consequently, these results suggested that most of sperm collected from the transplanted pig was derived from transplanted SSCs.

All stage of the protocol had been correctly performed in the microminipig, and it was possible to acquire adequate sperm derived from possibly transplanted SSCs. However, only a quality of the transplanted sperm was assessed, and its fertility was not confirmed. Therefore, breeding trial should be done before it is concluded to be successful. When it is done, the autologous transplantation is established, and a new transgenic technique via autologous SSCs transplantation comes one step closer to reality.

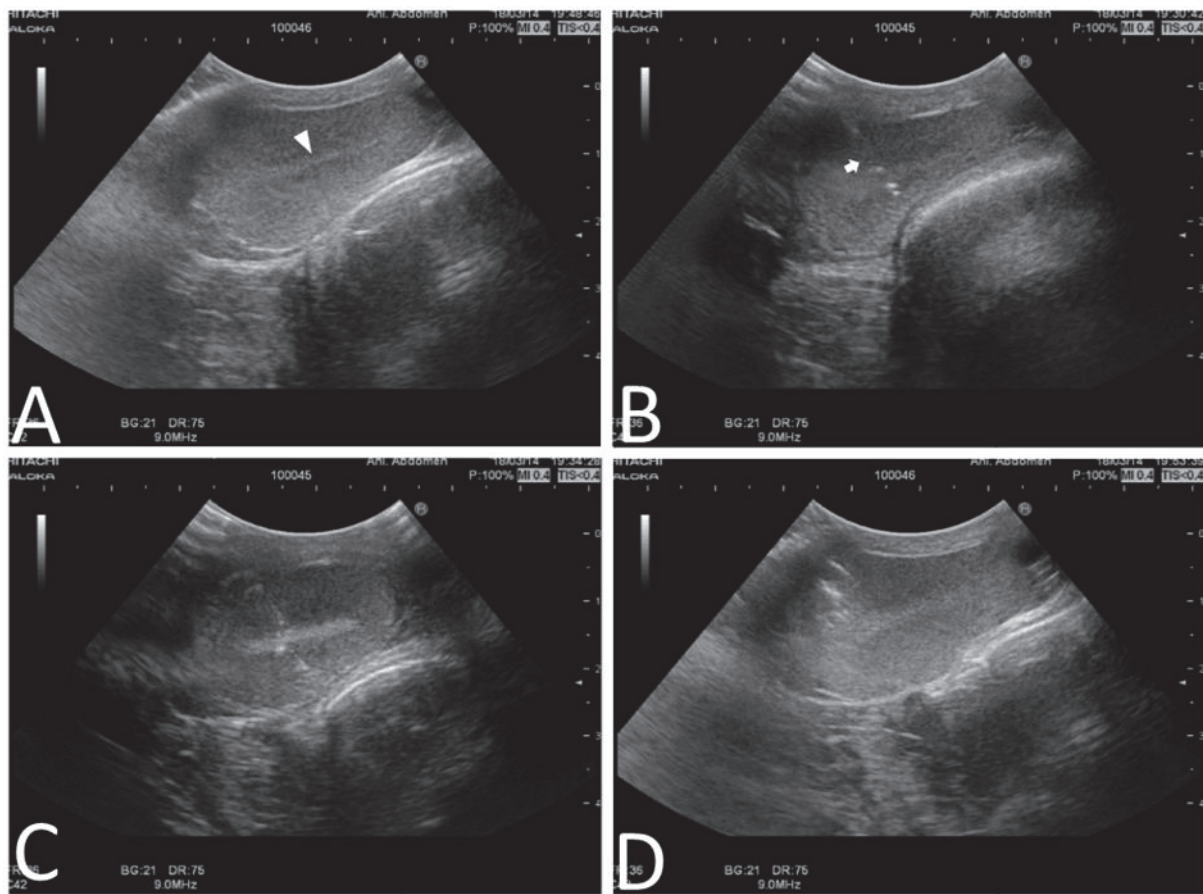


Figure 3-2. Injection of the piglet rete testis using ultrasonography.

Note the presence of rete testis in the middle of the testis (arrowhead) (A). A long injection needle inserted into the rete testis (arrow) (B). After the transplant, changes in the opacity of the rete testis and testis parenchyma were observed (C-D).



Figure 3-3. Semen collection from transplanted microminipig.

Ejaculates from transplanted boar was collected. The animal showed interest in mounting sows in heat during all the semen collection training.

General Discussion

In the chapter I of this study, I characterized the microminipig SSC populations, with the objective of determining the optimal age of donor SSCs isolation for the autologous transplantation. The results of this chapter showed that 30 days or younger age was the appropriate age for isolation donor cells to perform the SSC transplantation in microminipigs. In chapter II, I determined a suitable radiation dose to create a biological niche in the testes of pre-weaning microminipigs. The results showed that a 6-Gy dose to 30-days-old microminipig testes was suitable to create a biological niche as a recipient for SSC transplantation. In chapter III, I performed the autologous transplantation of testicular cells in one pre-weaning microminipig using the conditions studied in the first two chapters. From three months after the transplantation, I collected the ejaculates, and evaluated sperm quality.

Major protocol for the autologous transplantation in microminipigs was established and provided a step closer to reality to establish a new transgenic technique via the autologous SSC transplantation. Some more steps, such as development of an efficient transfection technique to the SSCs, for establishment of the transgenic technique are remained to be completed. A transgenic technique via the autologous SSC transplantation has advantages; as the method requires 5-6 months for sexual maturity of the transplanted pig, it may be possible to shorten a duration of transgenic animal production; it is one-male completed method, and therefore it can reduce a use of animal; compare to transgenic techniques dealing with zygotes or embryos, the procedure is easy and done using popular equipment for veterinarians.

Pigs are similar physiologically and anatomically to humans, and therefore use of pigs will increase for translational research. For the research, uncomplicated technique is required for transgenic pig production. The autologous transplantation in microminipigs is a suitable method that can generate a large number of human pathology model pigs. For example, because a human

hereditary disease often has a low incidence or a high death rate in early age, studying about these diseases is difficult. But these diseases can be constantly studied by using microminipigs models in laboratory. Moreover, a large number of human tissue and organs for xenotransplantation can be provided from transgenic microminipigs [25, 33, 47, 52].

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