



## Subcutaneous transplantation promotes organ formation of the fetal rat urogenital sinus



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### ABSTRACT

The aim of this study is to develop a novel experimental model of the subcutaneous transplantation of fetal urogenital sinus (UGS) into normal and castrated adult male rats for the pathophysiological investigation of the normal and developing prostate. Fetal UGS obtained from 20-day-old male rat embryos was subcutaneously transplanted into 7-week-old normal and castrated male rats. We observed the growth pattern, histopathological characteristics and immunohistochemical localization of cytokeratin 5 (CK 5), cytokeratin 8 (CK 8) and androgen receptor (AR) in the transplanted tissues. Almost all of the transplanted UGS organs gradually increased in weight over time in the non-castrated recipient animals, and the histopathological observations and immunohistochemical analysis of CK 5 and CK 8 revealed that the morphological changes in the tissues were in accordance with the features of normal prostate development. The histological characteristics included glandular epithelial dominant and stromal dominant area, with an increase in the glandular epithelial dominant areas over time and resemblance among a portion of the transplanted tissues within a certain period during the developmental course to the histopathology of human benign prostatic hyperplasia (BPH). The effects of androgens and resemblance in the immunohistochemical localization pattern changes in AR to that observed in the normal differentiating rat prostate were also noted. We conclude that the subcutaneous space provides an adequate microenvironment for UGS growth.

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### Introduction

The prostate is a male accessory sex gland found only in mammals whose development depends on the actions of androgens. In male rodent embryos, the urogenital sinus (UGS) develops into the prostate and prostatic urethra during the late embryonic period. The prostatic bud arising from the UGS proliferates into the peri-urethral mesenchyme, which later differentiates into the prostatic stroma.

Renal subcapsular transplantation of the fetal UGS is the common experimental model of prostate development. Especially, this model plays an important role in prostate developmental experiment of genetically modified animals (Kurita et al., 2004; Doles

et al., 2006). Some studies have reported that the transplantation of rodent fetal UGS into the subcapsular space of the adult prostate results in tissue differentiation, leading to prostatic hyperplasia (Chung et al., 1984a,b; Mori et al., 2009). Hence, the prostatic subcapsular transplantation of UGS has been determined to be a suitable histopathological experimental model of benign prostatic hyperplasia (BPH). However, there are some disadvantages in these model animals. For example, the surgical technique for renal or prostatic subcapsular transplantation is very delicate, and the recipient animal undergoes severe surgical stress. Therefore, it is desirable to develop a new transplantation method in order to simplify the transplantation procedure and reduce operative stress for experimental animals. Recently, it has been reported that bone marrow-derived cells in titanium mesh cages transplanted subcutaneously play a role in bone formation (Tanoue et al., 2012), thus indicating that the subcutaneous space is a potential candidate as a suitable microenvironment for the growth of immature organs. Previous reports have described the injection of cultured prostatic or UGS cells into the subcutaneous space (Gao et al., 2001; Lawson

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et al., 2006; Garraway et al., 2010). However, there have so far been no reports of fetal UGS being directly transplanted into the subcutaneous space.

In the present study, we attempted to establish the subcutaneous transplantation of UGS as an experimental model and observed the histopathological and immunohistochemical characteristics of the transplanted UGS tissue in order to evaluate the growth patterns and histopathological changes that occur during the development of UGS implanted into the subcutaneous space.

## Materials and methods

### Animals

Twenty-four 7-week-old male and pregnant (with 18-day embryos) as well as 15-week-old male Sprague-Dawley rats were obtained from JAPAN SLC, Inc. (Hamamatsu, JAPAN). Each rat was housed separately under sterile conditions with free access to food and water. Two days later, the animals were used as either donors or recipients for the experimental surgery. Twenty-four 7-week-old male rats were used as recipients of transplantation, 12 of which were castrated at the time of transplantation and assigned to the castrated group; the remaining 12 were assigned to the non-castrated group. The prostate in the 15-week-old male rats was used as a control tissue for the histopathological and immunohistochemical analyses. The animal experiments were approved by the Institutional Animal Care Committee of Kurume University, Fukuoka, Japan.

### Antibodies

A rabbit polyclonal antibody raised against cytokeratin 5 (CK 5) (ab53121) and the androgen receptor (AR) (ab74272) and a mouse monoclonal antibody raised against cytokeratin 8 (CK 8) (ab182875) were purchased from Abcam, Ltd. (Cambridge, UK) and used as the primary antibodies. As secondary antibodies, a goat anti-rabbit secondary antibody conjugated with both peroxidase and Alexa Fluor 568 (Invitrogen, Carlsbad, CA, USA) was used for CK 5 and AR and a goat anti-mouse antibody conjugated with both peroxidase and Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) used for CK 8. The antibodies used in the present study are summarized in Table 1.

### Transplantation of the UGS into the subcutaneous pocket in the male rats

For the surgical procedure, the rats were anesthetized with the inhalation of 10% diethyl ether and intraperitoneal injection of 30 mg/kg of pentobarbital (Somnopenyl; Kyoritsu Seiyaku, Inc., Tokyo, Japan). The undifferentiated lower urinary tract, composed of the undifferentiated bladder, prostate, seminal vesicles, Wolfian duct and urethra, was obtained from 20-day-old male embryos (E-20), and the undifferentiated bladder, seminal vesicles, Wolfian duct and distal urethra were removed using a fine steel blade and forceps under a dissecting microscope, as previously described (Cunha and Donjacour, 1987). The trimmed UGS was washed and kept in phosphate-buffered saline (PBS) (pH 7.4) until use. The abdominal wall skin of the anesthetized male rats was incised, and subcutaneous tissue pockets were formed using fine forceps. The UGS was placed in the pocket under a microscope, which was then closed using subcutaneous fascia sutures. Each recipient animal received four UGS transplants in separate pockets. Each step of the procedure is shown in Fig. 1A–C.

### Extirpation of the transplanted UGS

At two, four and eight weeks after transplantation, the animals were sacrificed under deep anesthesia with 10% diethyl ether inhalation and the intraperitoneal injection of 30 mg/kg of pentobarbital. The transplanted UGS tissues were subsequently extirpated with the surrounding connective tissue after being fixed with 4% paraformaldehyde and then weighed. The tissue weight was measured by two experimenters three times for each sample and averaged. The tissues were then further processed for the histological analysis.

### Fixation and embedding of the transplanted UGS

For immunohistochemistry, the removed transplanted UGS tissues and normal rat ventral prostate were immediately frozen in OCT compound (Sakura Finetek, Torrance, CA, USA). The tissue blocks were stored at  $-80^{\circ}\text{C}$  until use. For the histological examination, the removed transplanted tissues were fixed for 3 h in 4% paraformaldehyde and washed with PBS (pH 7.4) three times for 5 min each. The tissues were embedded in paraffin blocks following dehydration.

### Histology

Sections of the paraffin-embedded specimens were cut at a thickness of 3 to 5  $\mu\text{m}$ , adhered to charged slides and stained with hematoxylin and eosin (HE) for the histological examination.

### Immunohistochemistry

Frozen sections were cut at 3 to 5  $\mu\text{m}$  in thickness, adhered to charged slides, and air dried for approximately 10 min. The slides were then washed with PBS for 5 min, and the sections were fixed with acetone at  $-20^{\circ}\text{C}$  for 30 min. Subsequently, incubation with 3% Normal Goat Serum and 0.05% Triton-X100 in PBS was applied for 30 min at room temperature. The sections were then incubated overnight at  $4^{\circ}\text{C}$  with AR, CK 5 and CK 8 antibodies, followed by 1.5 h of incubation with the secondary antibodies. The dilution values for the primary and secondary antibodies are summarized in Table 1. The sections were counterstained with 4',6-diamino-2-phenylindole (DAPI; Wako, Kumamoto, Japan) at a 1:2000 dilution in order to define the nuclei, and Permafluor (Thermo Fisher Scientific, Fremont, CA, USA)-mounted sections were observed on a confocal laser scanning microscope (Fluo View<sup>®</sup> FV1000-D; Olympus, Tokyo, Japan).

### Calculation of the proportion of glandular epithelial dominant areas

An analysis of the proportion of glandular epithelial dominant areas in the growing UGS tissue in the non-castrated group was performed at two, four and eight weeks after transplantation using a computerized image analysis software program (Image J, National Institutes of Health). The analysis was performed using the major axis sections of six different transplant samples obtained at each time point after transplantation. The proportion of glandular epithelial dominant areas was calculated by two experimenters three times for each section, and the results were averaged.

### Statistics

All data were analyzed using the JMP, version 11 software program (SAS institute Inc., Cary, NC, USA). Differences in the survival rate of the transplanted UGS tissue between the groups at each time point were cross tabulated using the  $\chi$ -squared test. The weights

**Table 1**  
Antibodies used for immunohistochemistry.

Antigen	Primary antibody	Secondary antibody	Dilution
AR	Anti-AR, rabbit, polyclonal (Abcam, Cambridge, UK)	Anti-rabbit IgG, goat 1:500 (Invitrogen, Carlsbad, USA)	1:200
CK 5	Anti-CK 5, rabbit, polyclonal (Abcam, Cambridge, UK)	Anti-rabbit IgG, goat 1:1000 (Invitrogen, Carlsbad, USA)	1:200
CK 8	Anti-CK 8, mouse, monoclonal (Abcam, Cambridge, UK)	Anti-mouse IgG, goat 1:100 (Invitrogen, Carlsbad, USA)	1:25

and proportions of the glandular epithelial dominant areas in the transplanted UGS tissues are expressed as the means and standard error of the means. A comparison of each time point was performed using the Kruskal–Wallis test with a post-hoc Wilcoxon rank-sum test. Differences with a  $p < 0.05$  were considered to be significant.

## Results

### Transplanted tissue analysis

Nodular enlargement was detected in the transplanted areas of the abdominal wall (Fig. 1D). At two and four weeks after subcutaneous transplantation in the non-castration group, all specimens were found to have survived. At eight weeks, however, one of the 16 specimens had disappeared during the incubation period. In the castrated group, the number of palpable transplanted UGS tissues reduced as time passed after the transplantation.

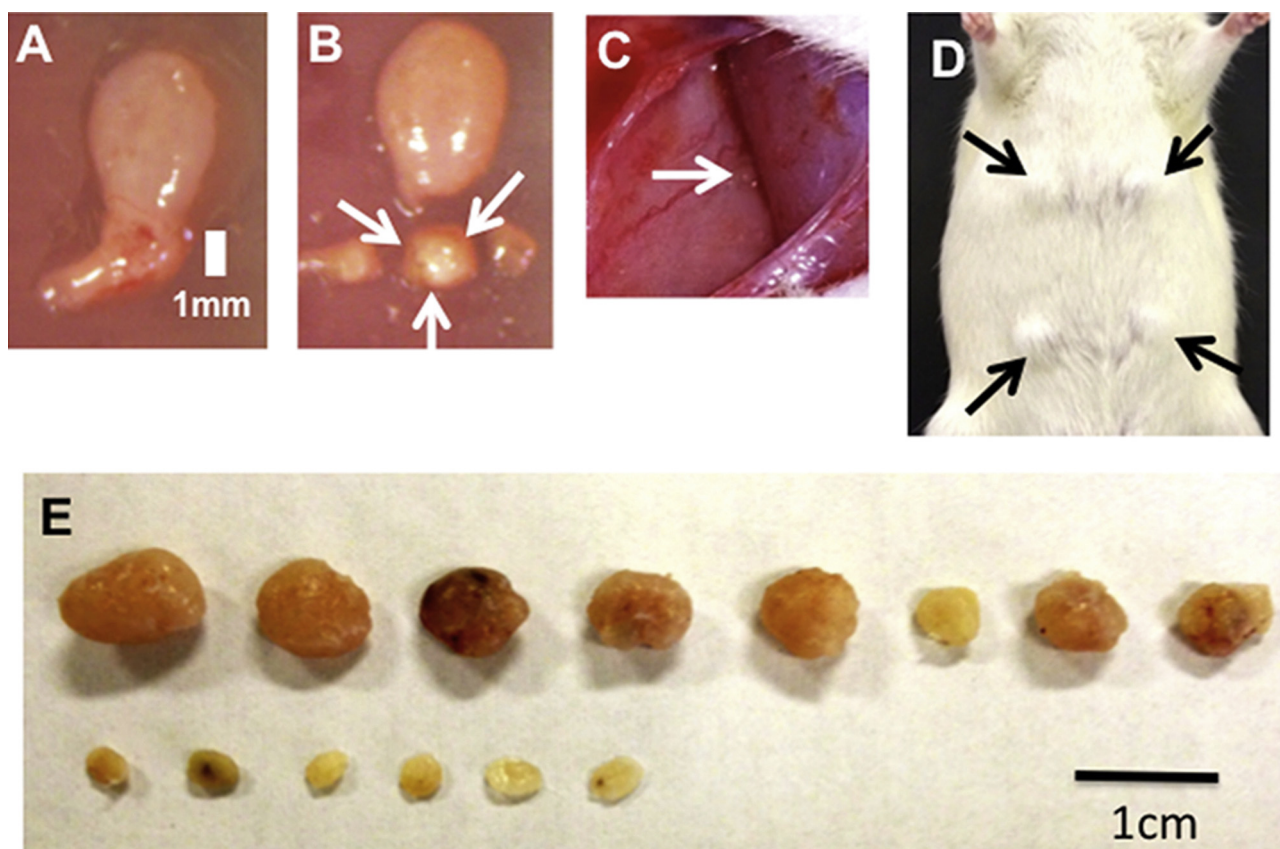
Upon resection, we found that the growing UGS adhered to the surrounding tissue and therefore had to be removed carefully. The tissues removed in the non-castrated group were larger than those removed in the castrated group (Fig. 1E). The growing UGS tissue had a soft texture, and the accumulation of secreted material

inside a glandular structure was prominent at eight weeks after transplantation in the non-castrated group.

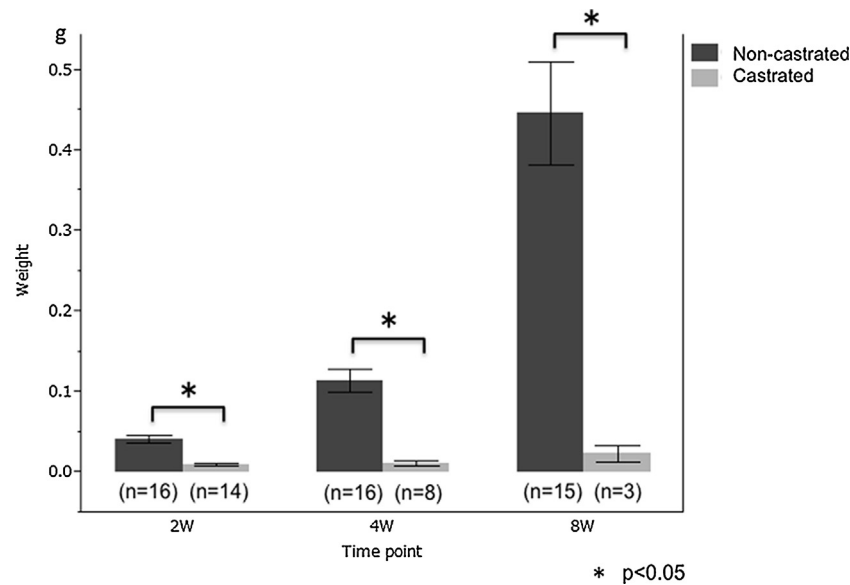
The transplanted UGS tissues removed from the non-castrated group increased in weight over time (Fig. 2). The average weight of the growing UGS tissue in the non-castrated group at two, four and eight weeks after transplantation was  $41.3 \pm 4.9$ ,  $114.6 \pm 14.9$  and  $446.4 \pm 64.1$  mg, respectively. On the other hand, the weights of the transplanted tissues in the castrated group at two, four and eight weeks after transplantation were  $10.2 \pm 1.7$ ,  $11.3 \pm 3.0$  and  $23.6 \pm 18.8$  mg, respectively.

### Histopathological analysis

In the histopathological analysis of the transplanted UGS tissues using HE staining at two and four weeks after transplantation, two distinct areas were recognized in the non-castrated group: an glandular epithelial dominant areas and a stromal dominant areas (Fig. 3A and B). On the other hand, at eight weeks after transplantation, almost all fields were occupied by tubular glandular tissue, and the tubular glands had expanded, exhibiting abundant levels of secretion in comparison with that observed following two and four weeks after transplantation (short-term) cultivation (Fig. 3C). At two and four weeks after transplantation, the histopathological



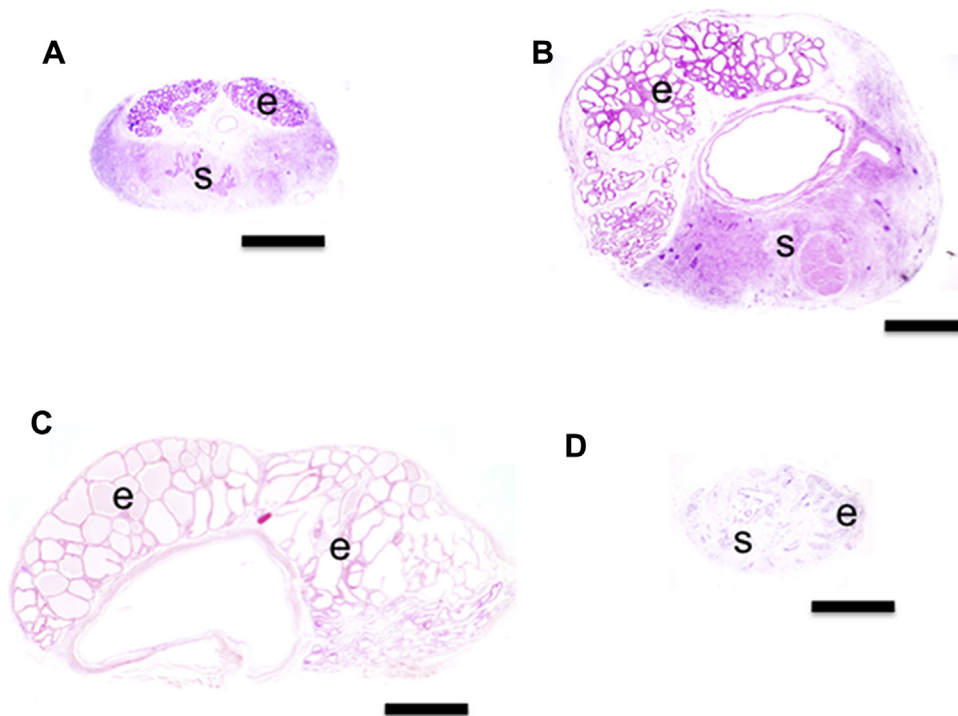
**Fig. 1.** Gross appearance of the experimental specimens. (A) Microscopic findings of the extracted lower urinary tract in the 20-day-old male rat embryo. (B) Trimming observations of the UGS (surrounding the white arrow). (C) The transplanted UGS exhibited wide spreading in the host rats subcutaneously. External appearance of the host rat abdominal specimens four weeks after transplantation. (D) Swelling of the abdominal wall (E) following extraction.



**Fig. 2.** Weights of the surviving UGS tissues at two, four and eight weeks after transplantation. The values represent the average  $\pm$  SEM (mg) transplanted UGS weight at each time point. The asterisk indicates the results of Wilcoxon's rank-sum test at a significance level of  $p < 0.05$ .

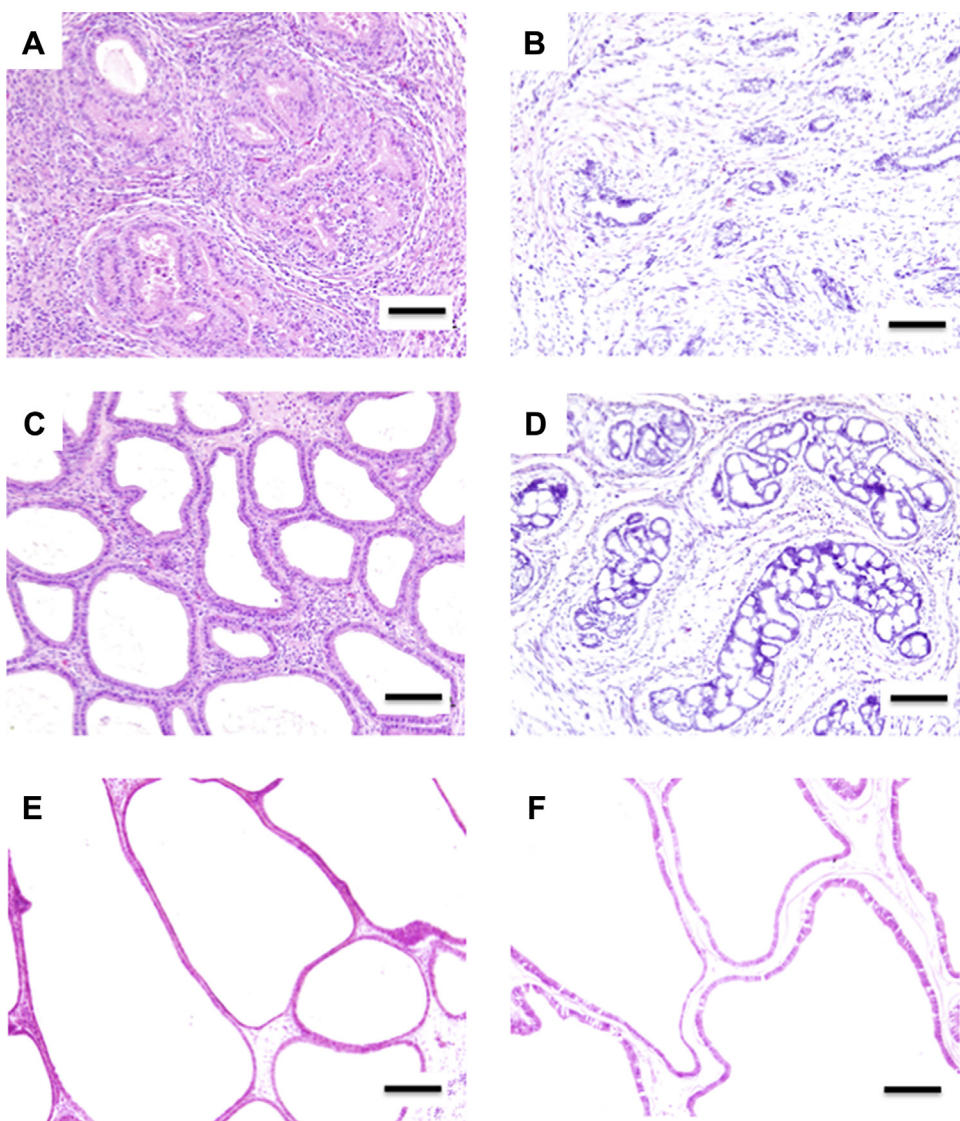
characteristics of the UGS tissue transplanted in the stromal dominant area included scattered tubular glands surrounded by a thick layer of flattered stromal cells together with a number of inflammatory cells in some portions of the region. The tubular glands and stromal cells formed histological nodules constructed of epithelia and stroma, although the proportion of each component varied (Fig. 4A). Similar findings of stromal dominant areas were observed in the periurethral region. Mature and differentiated tubular glands were noted in the epithelial dominant areas, while the number of flattered stromal cells surrounding the tubular glands was lower

than that observed in the stromal dominant areas (Fig. 4C). In the stromal dominant areas in the short-term cultivated (two and four weeks after transplantation) tissues, the histopathological findings showed some resemblance to the pathology of human BPH, with lymphocyte invasion in the stroma (Fig. 5A), nodular formation in the stroma (Fig. 5B) and nodular formation in both the epithelia and stroma (Fig. 5C). Such nodules were also found in the periurethral region (Fig. 5D). In addition, the morphology of some portions of glandular epithelial dominant areas at eight weeks after transplantation was similar to that of the normally developed ventral



**Fig. 3.** Low-power histopathological features of the transplanted UGS. (A and B) In the non-castrated group, glandular epithelial dominant areas (e) and stromal dominant areas (s) appeared at two and four weeks after transplantation. (C) At eight weeks after transplantation, almost all areas were occupied by epithelial tubular glandular tissue. (D) Epithelial and stromal areas were noted in the castrated group. Scale bar = 1 mm.





**Fig. 4.** High-power features of the transplanted UGS. (A and B) Stromal dominant areas were observed at four weeks after transplantation in the non-castrated and castrated groups. (C and D) Epithelial dominant areas appeared at four weeks after transplantation in both the non-castrated and castrated groups. (E) Areas of glandular epithelial dominant areas appeared at eight weeks after transplantation in the non-castrated group. (F) Normal rat ventral prostate tissue (15 weeks of age). Scale bar = 100  $\mu$ m.

prostatic lobes (Fig. 4E and F). However, the stromal region in the transplanted UGS was invaded by more lymphocytes than the normal prostatic stroma.

In the castrated group, faint staining was prominent, and the cell density was sparse in comparison with that observed in the non-castrated group (Fig. 3D). The stroma contained various kinds of cells, including smooth muscle cells, fibroblasts, lymphocytes and blood vessel cells (Fig. 4B). Undeveloped residual small tubular glands with thin surrounding smooth muscle cells elongated to the stroma were also detected (Fig. 4D).

#### Immunohistochemical analysis

In both the normal ventral prostate and transplanted UGS tissues, the luminal epithelial cells were positive for CK 8 and the basal epithelial cells were positive for CK 5 (Fig. 6A–F).

In both the transplanted UGS tissues in the non-castrated group and the normal ventral prostate tissues, almost all of the nuclei in the epithelial cells were positive for AR immunoreactivity (IR). At two and four weeks after transplantation, AR-IR-positive and -negative nuclei were intermixed in the stromal region (Fig. 7A–F).

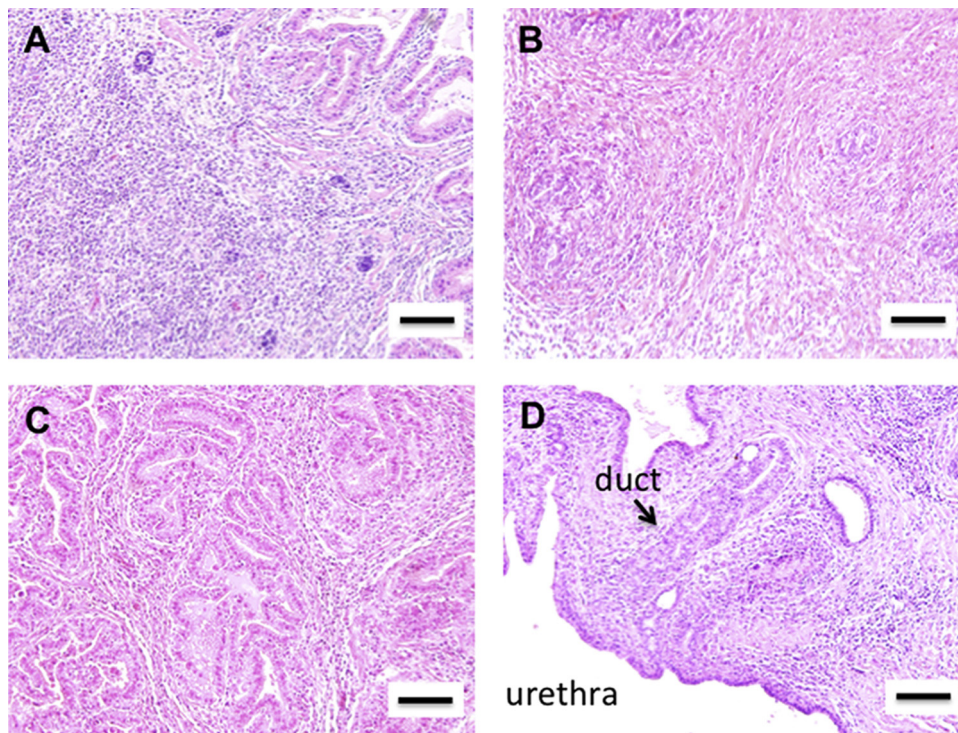
However, the number of AR-IR-positive cells in the stromal region after transplantation was diminished at four weeks in comparison with that observed at two weeks after transplantation. On the other hand, almost all of the nuclei in the stromal cells were AR-IR-negative at eight weeks after transplantation and in the normal ventral prostate tissues (Fig. 7G–L).

#### Proportion of glandular epithelial dominant areas in the growing UGS

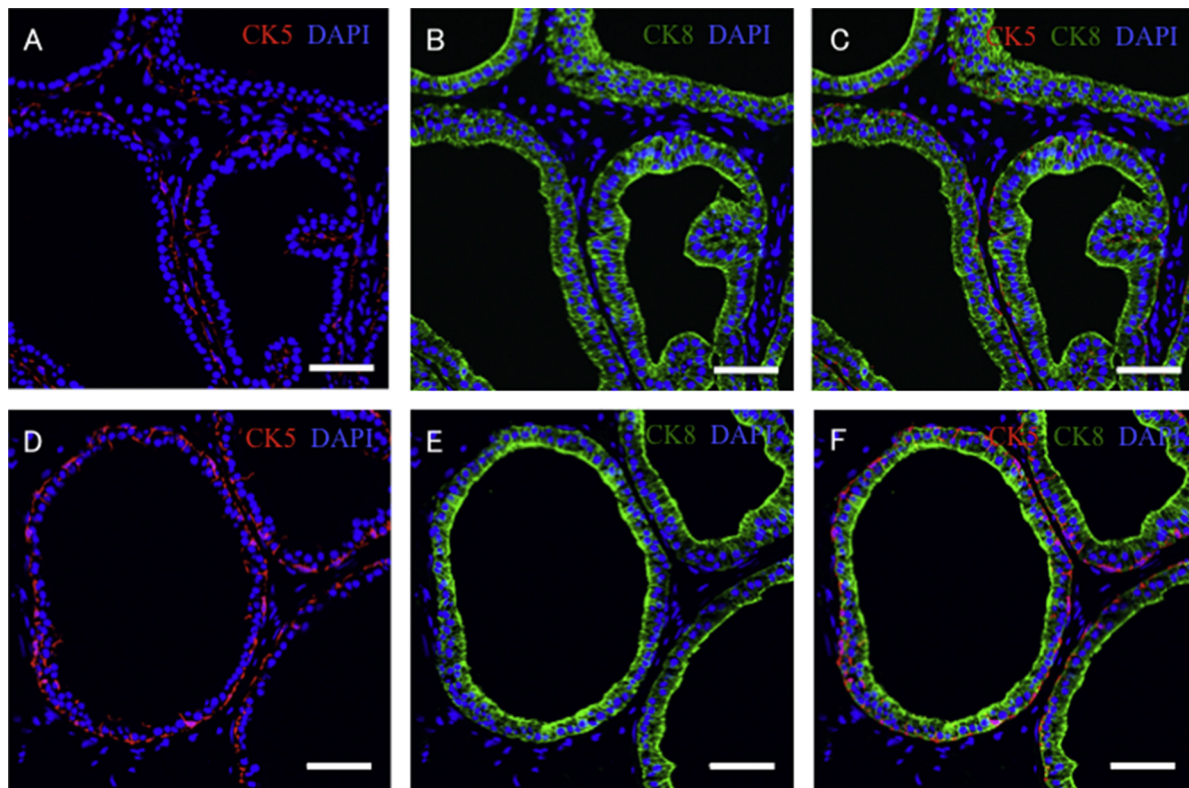
The proportion of glandular epithelial dominant areas increased over time after transplantation (Fig. 8). The average proportion of the epithelial dominant areas in the UGS tissue in the non-castrated group at two, four and eight weeks after transplantation was  $18.8 \pm 2.5\%$ ,  $29.0 \pm 6.0\%$  and  $74.3 \pm 5.2\%$ , respectively.

#### Discussion

In the present study, we, for the first time, succeeded in achieving the subcutaneous cultivation of transplanted UGS in non-castrated male rats. The histological findings of the differentiating

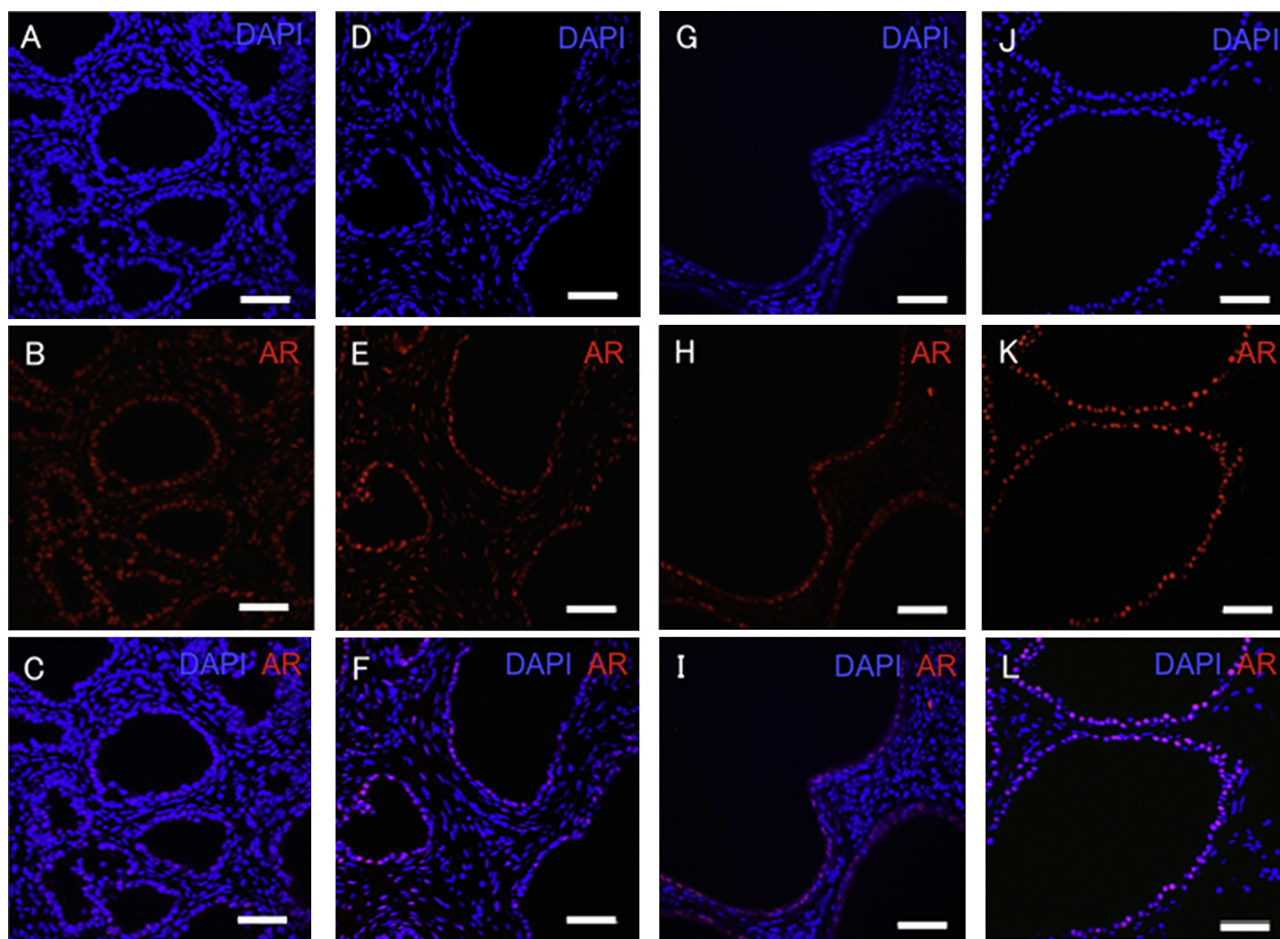


**Fig. 5.** High-power histopathological findings four weeks after transplantation in the non-castrated group. (A) Inflammatory cells in the stroma. (B) Stromal nodules. (C) Nodular formation composed of epithelia and stroma. (D) Nodules in the periurethral area. Scale bar = 100  $\mu$ m.



**Fig. 6.** Immunohistochemical findings for CK 5 and CK 8. (A–C) At eight weeks after transplantation in the non-castrated groups, the luminal epithelial cells were positive for CK 8-IR and the basal epithelial cells were positive for CK 5-IR. (D–F) In the normal ventral prostate, the luminal epithelial cells were positive for CK 8-IR and the basal epithelial cells were positive for CK 5-IR. Scale bar = 50  $\mu$ m.





**Fig. 7.** Immunohistochemical findings for AR. (A–F) At two and four weeks after transplantation in the non-castrated group, almost all of the nuclei in the epithelial cells were positive for AR-IR, and AR-IR-positive and -negative nuclei were intermixed in the stromal area. (G–I) At eight weeks after transplantation in the non-castrated group, almost all of the nuclei in the epithelial cells were AR-IR-positive, whereas the number of AR-IR-positive nuclei in the stromal cells was decreased in comparison with that observed in the short-term cultivated tissues. (J–L) In the normal ventral prostate, the distribution of AR-IR-positive and -negative nuclei in the epithelial and stromal cells was similar to that observed in the transplanted UGS at eight weeks after transplantation. Scale bar = 50  $\mu$ m.

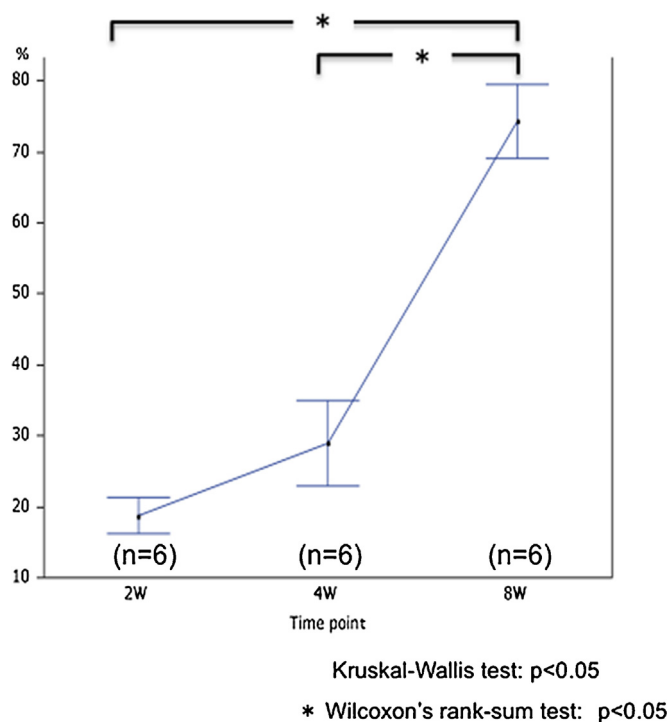
pattern were compatible to those of normal development. In the normal prostate tissues, the epithelial cells were classified into two major types: luminal and basal epithelial cells. The luminal epithelial cells were found to be immune-positive for CK 8, but not CK 5. In contrast, the basal epithelial cells were immune-positive for CK 5, not CK 8. In the present experiment, the transplanted UGS tissues exhibited the same epithelial IR pattern as the normal prostate tissue, indicating that the transplanted UGS tissues had characteristics similar to those of normal prostate tissue.

Our results further indicate that the subcutaneous space provides an adequate microenvironment for the growth of transplanted UGS tissue. The advantages of subcutaneous transplantation include: (1) the operative technique is easier to perform than subcapsular transplantation of the kidney and prostate, (2) the surgical invasion of subcutaneous transplantation is much lower than that of open surgery for renal subcapsular transplantation, (3) the growth of the transplanted UGS tissue can be assessed by observing the external appearance or palpating the location through the skin, (4) prostate growth can be measured noninvasively and repeatedly on the same transplanted tissue over time using a caliper, (5) the number of experimental animals can be reduced because multiple UGS tissues can be transplanted in the skin and (6) fluorescent markers can be visualized under the skin compared to the renal capsule if the transplanted tissues are labeled. Furthermore, the value of this experimental model is not

that it is preferable to *in situ* prostate development, rather it provides a means to study the prostate development when it is not possible *in situ* (e.g., after the pharmacological treatment of UGS in organ culture or to rescue the development when the newborn rats would otherwise die or have compromised androgen signaling).

During the process of rat prostatic development in the embryonic period, epithelial budding from the UGS begins in the small ventral prostate buds only at E-18. In the UGS at E-20 (the grafts used in the present study), ductal budding from the UGS occurs in the ventral, dorsolateral and anterior prostatic lobes (Marker et al., 2003; Timms, 2008; Timms and Hofkamp, 2011). Therefore, the UGS tissues used in this experiment contained three types of developing prostatic lobes, which possibly originated from diverse placodes of three different lobes. In this study, we observed a few histologically different types of tubular glands in the transplanted UGS (data not shown). However, it is highly possible that the developing anterior prostatic lobe was partially removed with the seminal vesicle at the time of trimming. Trimming was a very important technique in this experiment because the transplantation of incompletely trimmed UGS tissue into the subcutaneous space may result in a portion of the transplanted tissues differentiating to seminal vesicles and the urethra, as previously described (Mori et al., 2009).

Our histopathological examinations revealed that the morphological characteristics of the transplanted UGS tissue can



**Fig. 8.** Proportion of epithelial dominant areas in the surviving UGS tissues at two, four and eight weeks after transplantation. The values represent the average  $\pm$  SEM (%) proportion of glandular epithelial dominant areas in the transplanted UGS tissues at each time point. The asterisk indicates the results of the *post-hoc* Wilcoxon rank-sum test at a significance level of  $p < 0.05$ .

be classified into two tissue patterns depending on the region observed during short-term cultivation: glandular epithelial dominant and stromal dominant area. We consider that the formation of glandular epithelial dominant areas may be a sequel to the expansion and differentiation of the outgrowth of prostatic lobes in the transplanted UGS. On the other hand, the stromal dominant areas exhibit more immature features and may provide space for new epithelial outgrowth from the developing urethra, as observed in normal fetal ductal growth. As a result, glandular epithelial dominant areas spread into the stromal region over time, and the tubular glands gradually expand to become the dominant tissue pattern at eight weeks after transplantation. In the present study, the proportion of glandular epithelial dominant areas in the growing UGS tissue increased over time after transplantation in the non-castrated group.

A previous study (Mori et al., 2009) reported that the transplantation of fetal UGS into the adult male prostatic subcapsular space results in a stromal hyperplastic phenotype similar to that of BPH. Other studies (McNeal, 1990; McNeal, 1978) have also documented that the newly formed structure of ductal–acinar organization observed in the BPH tissues of elderly males is similar to that seen in particular stages of prostatic development in the embryo. Therefore, it has been postulated that the pathogenesis of BPH involves the embryonic reawakening of the transitional zone, causing glandular budding and new ductal branching. In this study, we observed that the fetal UGS grew and changed in shape sequentially in the subcutaneous position in the non-castrated host animals. In addition, we noted portions of the tissue in the stromal dominant areas at particular stages during the course of development of the transplanted tissues with features that resembled the histopathology of human BPH. These findings may be interpreted to indicate that transplanted UGS differentiates under the subcutaneous microenvironment, as it does in the process of normal prostatic development in embryonic and newborn animals.

It is well established that androgens are involved in the development and physiological function of male accessory sex organs, including the prostate. Castration inhibited the expansion and differentiation of the UGS under the skin in the present experiment. This finding clearly highlights the blood-borne androgen dependency of prostate development, even in the subcutaneously transplanted UGS.

The actions of androgens are mediated by the AR, which belongs to the superfamily of ligand-responsive transcription regulators. In the rat prostate, the distribution of AR in glandular epithelial and stromal cells changes as the organ develops (Sar et al., 1990; Cooke et al., 1991; Pelletier et al., 2000; Yamashita, 2004). In previous reports, the distribution patterns of AR during normal prostatic development have shown that a number of initially AR-positive cells located in the mesenchyme until approximately four days postnatally subsequently exhibit a decreased expression of AR-IR, and, in turn, the epithelial cells begin to strongly express AR-IR after six days (Cooke et al., 1991; Yamashita, 2004). In addition, some prostatic developmental studies have demonstrated that the AR-positive urogenital mesenchyme (UGM) plays an important role in the development of the prostate gland (Cunha and Lung, 1978; Cunha et al., 2004) and that the UGM has the ability to induce glandular outgrowth of the urogenital epithelia and prostatic glandular budding, even in the adult bladder transitional epithelia (Cunha et al., 1983; Neubauer et al., 1983). We observed similar changes in the distribution pattern of AR in the transplanted tissues in this study. These similarities support the above interpretation of the induction of epithelial tissue growth by mesenchymal cells, even in the subcutaneous space. We thus conclude that epithelial–mesenchymal interactions play an important role in prostate morphogenesis, even in the subcutaneous microenvironment.

In the present study, we also observed lymphocytic invasion in the stroma, indicating an immunological reaction. Despite the detection of this immune response, the transplanted UGS tissues were able to grow and differentiate in the non-castrated group. The transplanted UGS may have encountered mild rejection, as late embryonic organs display antigenicity (Foglia et al., 1986). In this respect, athymic rats may be better recipients for such transplantation experiments.

## Conclusions

In the present study, we, for the first time, succeeded in achieving the subcutaneous cultivation of transplanted UGS in non-castrated male rats. Our results demonstrate that the subcutaneous space provides an adequate microenvironment for growth of the UGS and that androgens are indispensable for this process. Furthermore, the observations of the entire course of ectopic growth of the UGS revealed that the histopathological characteristics and immunolocalization of AR resemble those observed during normal prostate development, while the histopathology of the stromal dominant areas that appear at certain developmental stages in the transplanted tissues resembles that of human BPH. Therefore, the subcutaneous transplantation and cultivation of the UGS provides a useful model of prostate development.

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