Autologous and homologous transplantation of bovine spermatogonial stem cells

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The aim of this study was to develop a method for spermatogonial stem cell transplantation into the bovine testis. Five-month-old Holstein–Friesian calves were used and half of the calves were hemicastrated to allow autologous transplantation and the other half were used for homologous transplantation. Approximately 20 g of each testis was used for cell isolation. On average 10^6 cells per gram of testis containing about 70% type A spermatogonia were isolated. The cells were frozen in liquid nitrogen until transplantation. Testes were irradiated locally with 10–14 Gy of X-rays to deplete endogenous spermatogenesis. At 2 months after irradiation, cells (approximately 10×10^6) were injected into the rete testis through a long injection needle (18 gauge), using ultrasonography and an ultrasound contrast solution. At 2.5 months after transplantation, calves were castrated and samples of testes were taken for histological examination. After 2.5 months in the irradiated

non-transplanted control testes, only 45% of the tubules contained type A spermatogonia. However, after autologous spermatogonial transplantation, > 80% of the tubule cross-sections contained type A spermatogonia. In addition, only 20% of the tubules of the control testes contained spermatocytes and, except for a few tubules (5%) with round spermatids, no more advanced germ cells were found. After autologous spermatogonial transplantation, about 60% of the tubules contained spermatocytes; 30% contained spermatids and in about 15% of tubules spermatozoa were found. No improvement in spermatogonial repopulation was found after homologous transplantation. The results of this study demonstrate, for the first time, successful autologous transplantation of bovine spermatogonial stem cells resulting in a complete regeneration of spermatogenesis.

Introduction

Spermatogenesis is a highly organized process which is initiated and maintained by continuous proliferation and differentiation of spermatogonial stem cells. In many non-primate mammals, including rodents and sheep, the so-called A single (A_s) spermatogonia are the stem cells of spermatogenesis (Huckins, 1971; Lok *et al.*, 1982; de Rooij and Grootegoed, 1998). After a number of spermatogonial divisions, these cells give rise to spermatocytes, which after the meiotic divisions produce spermatids that develop into spermatozoa.

Spermatogonial stem cell transplantation in rodents offers unique strategies for the analysis of germ cell development. After the original breakthrough in rodents (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994), various modifications and many new approaches

have been described in different species. This technique was used to investigate the behaviour of spermatogonia from a variety of species, including rats (Clouthier *et al.*, 1996), hamsters (Ogawa *et al.*, 1999a), rabbits and dogs (Dobrinski *et al.*, 1999), large domestic species (Dobrinski *et al.*, 2000; Izadyar *et al.*, 2002a), primates (Nagano *et al.*, 2001) and humans (Nagano *et al.*, 2002), in recipient mouse testes. These studies consistently showed that the success of heterologous transplantation is dependent on the phylogenetic distance between the donor and the recipient species. As in cell suspensions, spermatogonial stem cells cannot be morphologically distinguished from other type A spermatogonia, and as only the stem cells are able to colonize and repopulate the testes, transplantation has become a functional assay for spermatogonial stem cell activity (Shinohara *et al.*, 2000a). This assay was also used to study surface markers on spermatogonial stem cells (Shinohara *et al.*, 2000b) and to determine genes (Boettger-Tong *et al.*, 2000)

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and factors involved in regulation of proliferation and differentiation of spermatogonial stem cells (Ohta *et al.*, 2000, 2001).

It also appeared that removal of endogenous stem cells from the recipient testis is important for spermatogonial transplantation (Ogawa *et al.*, 1999b). Endogenous spermatogenesis was blocked in the host testis mainly by using cytotoxic treatment with busulfan. In addition, in most of the studies, immunodeficient mice were used as recipients to avoid rejection of transplanted spermatogonia. It is clear that immunodeficiency is essential for heterologous transplantation (from one species to another); however, it is not clear to what extent immunodeficiency is critical for homologous transplantation. It has been shown that homologous transplantation of spermatogonia in pigs and goats is as efficient as autologous transplantation in terms of colonization (Honaramooz *et al.*, 2002; 2003). However, complete regeneration of spermatogenesis was not observed in pigs after autologous or homologous transplantation.

Creemers *et al.* (2002) developed a method for depletion of endogenous spermatogenesis within recipient mouse testes using local fractionated X-irradiation. This method resulted in > 95% of empty tubules after 1 month of irradiation without any apparent effect on the somatic Sertoli cells. These mice were later used as recipients in a variety of transplantation studies and could be colonized by mouse (Creemers *et al.*, 2002), rat (van Pelt *et al.*, 2002) and bovine (Izadyar *et al.*, 2002a) spermatogonia.

An ultrasound-guided injection of testis has been described by Schlatt *et al.* (1999). In the present study, a more efficient protocol was developed for injecting the rete testis in bulls using an ultrasound contrast solution. In addition, purified bovine type A spermatogonia were transplanted into the testes of recipient calves of which endogenous spermatogenesis was depleted by local X-irradiation. The efficiency of transplantation was assessed after autologous and homologous attempts using histological and immunohistological studies.

Materials and Methods

Anaesthesia and castration procedures

The experimental protocol of this study followed the Guidelines for Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of the Utrecht University. Fourteen Holstein–Friesian calves of 5 months old were used in the present study. Half of the calves were hemicastrated to allow autologous transplantation and the other half were used for homologous transplantation. For all animals, food was withheld for 18 h before anaesthesia, but water was always available. Before premedication with detomidine (Domosedan®, Pfizer Animal Health B.V., Capelle a/d IJssel, 10 µg kg⁻¹, i.v.) an 8 cm 12G Teflon catheter (Intraflon 2®, Vygon Nederland B.V., Veenendaal) was inserted into

the jugular vein. As soon as premedication took effect, anaesthesia was induced with midazolam (0.06 mg kg⁻¹) and ketamine (Narketan®, Chassot, Hasselt, 2.2 mg kg⁻¹). Maintenance of anaesthesia was achieved by infusion of a combination of guaiphenesin (Cujatal®, Eurovet, Bladel, 100.0 mg ml⁻¹), ketamine (2.0 mg ml⁻¹) and detomidine (0.02 mg ml⁻¹) ('triple-drip-infusion'). The initial infusion rate was 0.02 ml kg⁻¹ min⁻¹, which was adjusted on the basis of clinical signs. Simultaneously, a Ringer infusion was administered (0.05 ml kg⁻¹ min⁻¹). Supplementary oxygen was administered through a small nose tube. At the end of anaesthesia, detomidine effects were antagonized by atipamezole (Antisedan®, Pfizer Animal Health B.V., 10 µg kg⁻¹, i.v.). This protocol was used for each anaesthesia in the whole experiment. The average time of anaesthesia needed for hemicastration, radiation therapy, transplantation and the final castration or hemicastration was 10, 20, 15 and 12 min, respectively. For castration, the animal was positioned in lateral recumbency. For hemicastration, a scalpel blade was used to make an incision in the lateral scrotal wall in a vertical direction. When both testes had to be removed, the distal part of the scrotum was removed. In both techniques, the spermatic cord was ligated with USP 2 polyglactin 910 (Vicryl®, Ethicon, Brussels) and subsequently sharply transected. Skin wounds were left open to heal by second intention. After castration, flunixin meglumine (Finadyne®, Schering-Plough B.V., Maarsse, 1.1 mg kg⁻¹, i.v.) and procaine penicillin-G (Depocilline®, Mycofarm Nederland B.V., de Bilt, 20 000 IU kg⁻¹) were administered i.m. All recoveries from anaesthesia and surgery were smooth and without any complications.

Preparation of donor cells and cryopreservation

After hemicastration, the testes were transferred on ice to the laboratory. A sample was taken for protein extraction and histological examination, and approximately 20 g of each testis was used for cell isolation, as described by Izadyar *et al.* (2002a). On average 10⁶ cells per gram of testis containing about 70% of type A spermatogonia were isolated. Viability of cells after isolation was always > 90%. The cells were mixed with the freezing medium containing 10% (v/v) dimethyl sulphoxide and 10% (v/v) fetal calf serum supplemented with 0.07 mol sucrose l⁻¹, which has been shown to be beneficial for cryopreservation of bovine type A spermatogonia (Izadyar *et al.*, 2002b), and frozen in liquid nitrogen until transplantation. On the day of transplantation, cells were thawed at 38°C for 30 s and, after viability assessment, were resuspended in 3 ml minimum essential medium containing 1% BSA and transferred on ice to the farm for transplantation. With this protocol, 70% of cells survived cryopreservation and could be used for transplantation.

Preparation of recipient bulls

Two groups of recipients were used. One group was hemicastrated and received their own cells (autologous) and the other group which retained two testes received cells from other calves (homologous). In the case of autologous transplantation, a period of 2 weeks was allowed for the animals to recover and heal from hemicastration before irradiation. For irradiation, calves were anaesthetized as described above and maintained in the lateral position. The testes with 2 cm build-up material were irradiated with single graded doses of 10–14 Gy using 6 MV X-rays; the dose rate was approximately 3 Gy min⁻¹ (Philips SL25 linear accelerator, Eindhoven).

Rete testis injection and transplantation

For the development of the rete testis injection procedure, testes from twelve calves between the ages of 5 and 12 months were collected from the abattoir. Three injection methods were used and for each method eight testes were injected. The first method was an injection without use of ultrasonography (blind injection). A long injection needle (18 gauge) was inserted along the midline of the testis through the epididymis. Subsequently, a syringe was connected to the needle and a blue dye was injected slowly (1 ml min⁻¹) allowing the injection site and the efficiency of each injection to be traced. The second method used an ultrasound guided injection and a blue dye was injected in the testes. A 7.5 mHz transducer (Piomedicals) was used and an image from three perpendicular levels was taken to find the mediastinum and to insert the needle in that area; however, it was not possible to guide the needle into the rete testes using ultrasonography alone. Therefore, a contrast solution Levovist (Shering, Berlin) was used. Levovist is a powder which mainly (99%) contains galactose and when mixed with water makes micro-bubbles of 2–3 µm which are stable for 30 min. Studies in human medicine have shown that this solution can be injected safely into various tissues and that it produces a very good contrast in ultrasonography (Almeida *et al.*, 2002; Hansberg *et al.*, 2002). Two recommended concentrations (200 and 400 mg ml⁻¹) were tested. In addition, the required volume to fill the testis was determined. The efficiency of Levovist in ultrasonography of a living calf was tested by injecting only one testis as the other testis was used as the control. In addition, for determination of the toxicity of Levovist for donor cells, different concentrations (200 and 400 mg ml⁻¹) of Levovist were added to purified type A spermatogonia and the viability of these cells was assessed after 6 and 24 h of culture.

For transplantation at 2 months after irradiation, calves were anaesthetized as described above and under aseptic conditions a long injection needle (18 gauge) was inserted into the mediastinum using ultrasonography. The needle was guided in the rete testes by injecting

1 ml Levovist as described above and cells (5–10 × 10⁶) were injected into the rete testis with the velocity of 1 ml min⁻¹. Six calves received their own cells (autologous) and another six calves received cells from other calves (homologous) in one testis and the other testis was used as control. In addition, two other calves received only medium (sham transplantation).

Evaluation of recipient testes after transplantation

At 2.5 months after transplantation, calves were castrated and testes were weighed. On account of the large size of the testes in this species and to include the whole testes for a reliable comparison between the animals, each testis was cut in eight parts and from each part some samples were fixed in Bouin's solution, preserved in 70% (v/v) ethanol and then embedded in paraffin wax for histological or immunohistological studies. For histological staining, sections of 5 µm in thickness were mounted on Tespa-coated glass slides. The paraffin wax was removed and after rehydration through a graded series of alcohol the sections were stained with periodic acid–Schiff and haematoxylin, and then dehydrated and mounted under a coverslip with Pertex (Cell Path, Compulink). The sections were analysed under a light microscope and images were taken with a digital camera (Nikon, Tokyo). For immunohistochemical staining of *Dolichos biflorus* agglutinin (DBA), sections of 7 µm in thickness were used, the sections were deparaffinized, rehydrated and were transferred into 0.1 mol PBS l⁻¹. The lectin DBA (E.Y. laboratories, San Mateo, CA) was used, as described by Ertl and Wrobel (1992), to identify type A spermatogonia.

Statistical analysis

The results are presented as means ± SEM unless otherwise indicated. Statistical analysis was performed by two-sample *t* test and the difference was considered significant at *P* < 0.05.

Results

Development of a rete testes injection method

From the eight testes used for injection without ultrasound guidance, only one was injected properly. Application of ultrasonography markedly improved the injection efficiency and four of the eight testes were injected properly. Although, ultrasonography helped to locate the mediastinum and to insert the needle in this area, it was not possible to guide the needle into the rete testes; therefore, the contrast solution Levovist was used. Two recommended concentrations (200 and 400 mg ml⁻¹) were tested and both concentrations resulted in a good contrast in the testes; therefore, 200 mg ml⁻¹ was used for the present study. Injection of Levovist into the testis

Fig. 1. Injection of the bovine rete testes using ultrasonography and contrast solution Levovist. (a) Note the presence of a longitudinal mediastinum in the middle of the testis. (b) A long injection needle inserted from the epididymis towards the mediastinum. (c,d) Immediately after injection, a uniform distribution of the contrast solution along the rete testes was observed.

gave a clear contrast at the injection site and when the needle was placed in the rete testes, a longitudinal distribution of the contrast along the rete testes and even in the seminiferous tubules could be seen (Fig. 1). Application of Levovist further enhanced the injection efficiency and in this case six of the eight testes were injected properly. In general, injection in larger testes was easier than in smaller ones.

A volume of 3.5 ml of the cell suspension was found to be enough to fill the testis of a 5-month-old calf. After the injection procedure was optimized using testes obtained from an abattoir, the efficiency was tested in the testis of a live calf. The distribution of the contrast solution in testes of live calves was similar to that in testes collected from the abattoir. In addition, no harmful effect of Levovist was observed on testis morphology or function, as found by repeated ultrasonography after injection and subsequent histological examination. Moreover, no harmful effect of Levovist on the viability of purified type A spermatogonia was found during a 24 h incubation (Table 1). No difference was found in the success of injection between the groups.

Analysis of recipient testes after transplantation

Several parameters have been used to determine the efficiency of transplantation. First, testicular mass was assessed. Testes from 5-month-old calves weighed 34.5 ± 3.8 g. After irradiation and 2.5 months after transplantation, the calves were 10 months old and the average testis mass of each animal ($n=2$) in the sham group was 41.2 g. An almost threefold increase in testis mass

Table 1. The effect of Levovist on the viability of a pure population of bovine type A spermatogonia after isolation and after 24 h of culture

Levovist (mg ml ⁻¹)	Living cells at time 0 h (%)	Living cells after 24 h (%)
0	79.5 ± 3.5	69.2 ± 2.9
200	82.5 ± 4.1	67.4 ± 3.8
400	83.3 ± 3.2	68.8 ± 3.5

Type A spermatogonia were isolated from 5-month-old calf testis and cultured as described by Izadyar *et al.* (2003). Sperm viability was assessed by a live and dead kit (Molecular Probes). This experiment consisted of three replicates.

was observed after autologous transplantation (106.7 ± 13.5); however, after homologous transplantation testis mass (47.7 ± 10.6 g) was very similar to that of the non-transplanted control testis (Fig. 2). In addition, a clear difference in testis histology between the non-transplanted and the transplanted group was observed only after autologous transplantation. Spermatogenesis was restored in >80% of the calves after autologous transplantation, whereas only 20% of the calves showed a regeneration in spermatogenesis after homologous transplantation (Table 2). Histological examination showed that at 5 months of age almost all the tubule cross-sections contain type A spermatogonia. After hemicastration, irradiation and sham transplantation, >60% of tubules contained no germ cells and in tubules that did contain germ cells the number of type A spermatogonia was clearly decreased at 5 months after irradiation. In addition, only 20% of the tubules of the sham group contained

Table 2. Efficiency of autologous or homologous transplantation of bovine type A spermatogonia

Type of transplantation	Number of calves	Calves showing enhanced colonization (%)	Calves showing enhanced spermatogenesis (%)
Autologous	6	5/6 (83.3)	5/6 (83.3)
Homologous	5	1/5 (20.0)	1/5 (20.0)

The results are from two separate experiments. At 2.5 months after transplantation, the colonization efficiency was assessed by *Dolichos biflorus* agglutinin staining and spermatogenesis was evaluated by histological examination.

Fig. 2. Bovine testes mass before irradiation and 2.5 months after sham or spermatogonial transplantation. The results are the pool of two separate experiments. The values in the columns represent the number of testes examined. *Note that a significant ($P < 0.001$) difference was observed only after autologous transplantation.

Fig. 3. Germ cells present in calf testes 2.5 months after sham or spermatogonial stem cell transplantation. The results are the mean $\pm$ SEM of two separate experiments. A significant ($P < 0.01$) difference was observed only after autologous transplantation (asterisks). ■: spermatogonia; □: spermatocytes; ▨: spermatids; ▩: spermatozoa.

Fig. 4. Bovine testes histology before irradiation and 2.5 months after sham or spermatogonial stem cell transplantation. (a) Testis histology before irradiation. (b) The same section as in (a) at a higher magnification. Note type A spermatogonia is the predominant type of germ cells in the testis (arrows). (c). Tubule cross-section of a calf 2.5 months after sham transplantation. (d) The same section as in (c) at a higher magnification. Note that after irradiation, the population of spermatogonia has decreased markedly and many tubules contained only Sertoli cells (arrows). (e) Tubular cross section of a calf 2.5 months after autologous spermatogonial transplantation. Note that most of the tubules show spermatogenesis and more advanced germ cells were found. (f) The same section as in (e) at a higher magnification. Scale bars represent 50 μm .

spermatocytes and no more advanced germ cells were found. Testes of calves that were not hemicastrated but were irradiated but not transplanted (control group) also showed the same germ cell distribution. However, after autologous spermatogonial transplantation, $83 \pm 3.5\%$ of the tubule cross-sections contained type A spermatogonia, $> 60\%$ of the tubules contained spermatocytes, 30%

contained spermatids and spermatozoa were found in about 15% of tubules (Figs 3 and 4). Furthermore, both the percentage of tubules containing type A spermatogonia and the number of type A spermatogonia per tubule cross-section increased after autologous transplantation, but not after homologous transplantation (Figs 5 and 6).

Fig. 5. Proportion of bovine type A spermatogonia as detected by *Dolichos biflorus* agglutinin staining. Data are presented as the number of type A spermatogonia per tubule cross-section. The results are the mean $\pm$ SEM of two separate experiments. The values in the columns represent the number of testes examined. Note a significant ($P < 0.01$) increase was observed only after autologous transplantation.

Discussion

The present study demonstrates successful transplantation of bovine type A spermatogonia in recipient bulls resulting in full spermatogenesis after autologous transplantation. For successful transplantation of spermatogonial stem cells, three factors are important: (i) the donor cell suspension should contain as many spermatogonial stem cells as possible; (ii) recipient testes should contain as few endogenous spermatogenic cells as possible, while containing healthy somatic cells to support the transplanted spermatogonia; and (iii) an efficient transplantation procedure to insert the donor cells into the seminiferous tubules should be available. The present study used purified type A spermatogonia from 5-month-old calves as donor cells. At this age, the testes predominantly contain type A spermatogonia consisting of stem cells and early spermatogonial types of cell which are actively proliferating (Izadyar *et al.*, 2002a). Our previous studies showed that these cells have a high stem cell activity as $> 65\%$ of tubules of recipient mouse testes could be colonized after several heterologous transplantation experiments. In addition, results of a study by Izadyar *et al.* (2003) showed extensive colony formation by these cells during a long-term culture. It was proposed that the donor cell suspensions used in our experiments contained relatively large numbers of spermatogonial stem cells compared with previous studies (Schlatt *et al.*, 1999) in which a mixed germ cell population was used. Furthermore, in pigs (Honaramooz *et al.*, 2002), donor cells were obtained from very young

piglets (1–10 weeks) and because spermatogonia, for the first time, could be seen in the pig testis at 2 months of age (Hughes and Varley, 1980), many of the donor pigs may have contained only gonocytes and no spermatogonia. In addition, the cryopreservation method used by Izadyar *et al.* (2002b) was developed especially for type A spermatogonia and allowed survival of more cells after thawing compared with the conventional cryopreservation protocols used in those studies.

The results of the present study show that although autologous transplantation was successful, homologous transplantation was not effective. This finding is in contrast to observations in pigs and goats in which autologous and homologous transplantation brought about a similar repopulation of the recipient testes (Honaramooz *et al.*, 2002, 2003). However, in these studies younger recipients were used than in the present study. The low efficiency of homologous transplantation in cows is most likely due to immunorejection, as in two calves a clear cellular infiltration was observed after homologous transplantation (data not shown). The calves used in the present study may already have been too old to allow successful homologous transplantation. The developmental stage of the recipient testis and in particular the formation of blood–testis barrier seems to have a critical role in homologous transplantation efficiency. It has been shown that homologous transplantation of mouse spermatogonia into the testes of adult recipient mice causes immunorejection, but when immature mice were used as recipients a complete restoration of spermatogenesis was achieved (Shinohara *et al.*, 2003).

Fig. 6. Immunodetection of bovine type A spermatogonia using *Dolichos biflorus* agglutinin (DBA) staining before irradiation and 2.5 months after sham or spermatogonial transplantation. (a) Note the presence of DBA-positive cells in all tubules before irradiation (arrows shown in (b)). (b) The same section as in (a) at a higher magnification. (c) Tubule cross-section of a calf 2.5 months after sham transplantation. Note that after irradiation, few DBA positive cells are present in some tubules (arrows) and a majority of the tubules show only Sertoli cells. (d) The same section as in (c) at a higher magnification. (e) Tubule cross-section of a calf 2.5 months after autologous spermatogonial transplantation. Note the remarkable increase in number of DBA-positive cells. (f) The same section as in (e) at a higher magnification. Scale bars represent 50 μm .

Another important consideration in transplantation experiments is the depletion of endogenous spermatogenesis in the seminiferous tubules of the recipients. Local fractionated X-irradiation (1.5 and 12.0 Gy with a 24 h interval) was used to develop a protocol for the mouse testis that eliminates endogenous spermatogenesis in

> 95% of the seminiferous tubules (Creemers *et al.*, 2002). As fractionated irradiation was not practical with calves, a single X-irradiation dose of 10–14 Gy was used. After these doses, about 60% of tubules were empty at 5 months after irradiation. At that time, the calves were 10 months old and the average testis mass of Holstein

calves at this age is about 175 g (Amann, 1983). The weight of non-transplanted testes was very low and did not differ from that of testes at 5 months age. Only a small percentage of the tubules contained spermatocytes and very few spermatids were found. It is possible that because of extensive cell death among the endogenous stem cells after irradiation, these cells underwent a long period of self renewing divisions before producing differentiating germ cells as seen in the mouse (Van Beek *et al.*, 1990).

However, a marked increase in testis mass was observed after autologous transplantation. As after hemicastration the weight of the other testis increases to compensate for this (Putra and Blackshaw, 1982; Jenkins and Waites, 1983; Lunstra *et al.*, 2003), the enlargement of the testis after autologous transplantation might be due to the hemicastration. However, because calves that had been hemicastrated and irradiated but did not receive spermatogonia did not have enlarged testes (44 and 52 g), this possibility seems unlikely. Interestingly, after autologous transplantation a substantial proportion of seminiferous tubules contained spermatids and spermatozoa. This finding indicates that after transplantation the donor stem cells relatively quickly restored the formation of differentiating germ cells within the recipient testes and also that the Sertoli cells in the irradiated recipient testes remained functional after the irradiation procedure and supported proliferation and differentiation of transplanted spermatogonial stem cells up to the final stages of spermatogenesis. Autologous spermatogonial transplantation in monkeys, in which the recipient testes had been prepared with X-ray irradiation, also resulted in a restoration of spermatogenesis (Schlatt *et al.*, 2002).

Finally, it was noted that ultrasonography is essential for a successful rete testis injection in the bovine testis, which is in agreement with observations in large animals (Schlatt *et al.*, 1999; Honaramooz *et al.*, 2002). In addition, the results of the present study show that the application of contrast solution during ultrasonography of the testis considerably enhances the efficiency of the rete testis injection in this species. As this compound has no adverse effect on the viability of spermatogonia and has no obvious long-term effect on testis function, it can be safely used for spermatogonial transplantation protocols in large animals.

In summary, a successful spermatogonial stem cell transplantation procedure for domestic species was developed leading to a full spermatogenesis in the recipient testis. Optimization of the transplantation procedure using an ultrasound contrast solution, the source of the donor cells and the depletion of the recipient testes were essential factors in the effectiveness of the transplantation. The inefficiency of homologous transplantation, which seems to be due to immunological rejection, needs to be studied in more detail. The results may have some industrial implications like preservation and replic-

ation of germ line in valuable males as well as generation of transgenic animals for pharmaceutical purposes.

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