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Germ cell transplantation in infertility mouse

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This work investigated the spermatogenesis in an infertility BALB/c-nu mouse model by reinfusing germline stem cells into seminiferous tubules. Donor germ cells were isolated from male FVB/NJ-GFP transgenic mice. Seminiferous tubule microinjection was applied to achieve intratubular germ cell transfer. The germ cells were injected into exposed testes of the infertility mice. We used green fluorescence and DNA analysis of donor cells from GFP transgenic mice as genetic marker. The natural mating and Southern blot methods were applied to analyze the effect of sperm cell transplantation and the sperm function after seminiferous tubule microinjection. The spermatogenesis was morphologically observed from the seminiferous tubules in 41/60 (68.33%) of the injected recipient mice using allogeneic donor cells. In the colonized testes, matured spermatozoa were seen in the lumen of the seminiferous tubules. In this research, BALB/c-nu infertility mouse model, the recipient animal, was used to avoid immunological rejection of donor cells, and germ cell transplantation was applied to overcome infertility caused by busulfan treatment. These results demonstrate that this technique of germ cell transplantation is of great use. Germ cell transplantation could be potentially valuable to oncological patients.

germ cell, transplantation, microinjection, infertility, GFP

Spermatogenesis is found to be a great systemized one that maintains all the adult life of the male to produce virtually unlimited numbers of spermatozoa. The basic principle of spermatogenesis is that spermatogonial stem cells have strong ability to self-renew and may bring distinguished posterity that finally form spermatozoa[1−4].

Spermatogonia cells transplantation has been developed to establish donor-derived spermatogenesis through introduction of stem spermatogonia into a recipient testis. It was reported[5] that transplantation of testicular germ cells from fertile donor mice into the testes of infertile recipient mice resulted in donor-derived spermatogenesis by the recipient animal. Also, other observations[6−8] indicated that the donor haplotype could be passed on to the offspring. In mice, spermatogenesis was reestablished following spermatogonial transplantation into an infertile male, and up to 40% of the progeny that resulted from mating of the recipient male carried the donor-derived transgene[5]. The technique of spermatogonial transplantation has been applied successfully for nearly 12 years in other many animals such as rats[9−11], pigs[12] and cynomolgus monkeys[13]. To date, many researches have confirmed that this technique is fit to study basal aspects of germ cell function and spermatogenesis[14−17]. It has

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also been applied to effectively produce transgenic animals with higher efficiency and lower expense[18−21].

Three routes for spermatogonia cells transplantation has been developed, namely microinjection into superficial seminiferous tubules——the initial one for transplantation, the efferent ducts microinjection and the rete testis microinjection. They were shown to be equally efficient[22,23]. The donor cell preparations can be composed of single cells, small fragments of all testicular germ and somatic cell types[5,6,24] in germ cell transplantation experiments. The donor germ cells can be either freshly prepared before transplantation or stored in long-term culture systems[7] or by cryopreservation[25].

In order to explore and develop the technique of spermatogonial transplantation in our lab, the route of seminiferous tubules microinjection was used to transfer germ cell into testes of infertility mouse model. The transgenic mouse, FVB/NJ-GFP (green fluorescent protein), was selected as donor animal because its green fluorescent makes it possible to track donor cell originality using fluorescent marker. And natural mating method was used to analyze the effect of sperm cell transplantation and the sperm function after seminiferous tubules microinjection. To further confirm the genotypes of the progeny, Southern blot analysis was applied to analyze the DNA from progeny resulting from the mating of normal females with recipient males transplanted with donor germ cells.

1 Materials and methods

1.1 Experimental animals

In the first series of experiments, the recipient testes of BALB/c-nu mice (n=60) were obtained from infertility mice (provided by Dr. Ma Lianghong) produced by busulfan treatment (40 mg/kg) after they have reached sexual maturity. Donor testis cells were collected freshly from testis of 10-d-old FVB/NJ-GFP mice. FVB/NJ-GFP mice (n=10) were obtained from Shanghai Laboratory Animals Center of the Chinese Academy of Sciences. Animals were handled and treated according to the guidelines of the Animal Care and Use Committee at Shanghai Laboratory Animals Center of Chinese Academy of Sciences.

1.2 Donor cell preparation

Before microinjection, suspension of testicular cells were prepared by sequential digestion as previously described[26]. The testes capsule was removed, and then the tissue was minced using fine scissors. Digestion of the tissues was firstly performed using collagenase type I (1 mg/mL; Sigma, Steinheim, Germany), DNase (5 mg/mL) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) supplemented with antibiotics (Gibco, Paisley, UK) for 5 min at 37℃ in a shaking water bath (90 r/min). The tissue fragments were aspirated 6 times through a 10-mL pipette using an automatic pipettor. The interstitial cells were discarded with the supernatant after sedimentation for 2 min. The separation step was repeated after addition of fresh DMEM. A second digestion using collagenase type IA (Sigma), DNase I (Sigma) and hyaluronidase type II (0.5 mg/mL) (Sigma) was performed for 20 min. Every 5 min, cells were aspirated through a 10 mL pipette using an automatic pipettor. The cells were pelleted by centrifugation (5 min at 500 g) and resuspended in phosphate-buffered saline (PBS, 50 mmol/L, 150 mmol/L NaCl, pH 7.2). Filtered trypan blue (4%) was added to the cell suspension to visualize solution flow in the seminiferous tubule and count the number of dead cells in the injection suspension.

1.3 Cell transplantation procedure

To transfer male germ cells from donor testes into a recipient testis, we applied the Jiang and Short's technique to microinject cells directly into individual seminiferous tubules. Before surgery, feed and water were withheld overnight. The recipient mouse was anesthetized, and the testis was exteriorized through a midline abdominal incision, immobilized and oriented to align a group of tubules with the injection pipette. The pipette size (inner diameter: 0.75 mm, outer diameter: 1.0 mm, Sutter Instrument Company, USA) and construction were similar to those described by Ali et al.[12], Jiang and Short[10]; Ogawa et al.[9,11], and Schlatt et al.[13], Under a special dissecting microscope (Nikon SMZ1000, Nikon Corporation, Japan), the tunica was reflected off the testis to expose the seminiferous tubules, and at the same time, the pictures of microinjection process were taken by the JVC TK-C1481EG Digital Camera System (Nikon Corporation, Japan). The injection system was connected to a tube, and the pressure in the injection tubing was raised by pipetting until the cell suspension flowed into the tubule; the flow was monitored by observing the color change. Between 75% and 90% of the surface tubules were routinely filled with the cell solution. After cell transplantation, testes were returned to the scrotum,

the scrotal skin was closed, and the animals were allowed to recover.

1.4 Analysis of the recipient mice

The recipient males were maintained for at least 50 d following microinjection of donor germ cells before analysis. The testes of recipient mice were dissected following transplantation and examined to assess the extent of intratubular infusion. To examine the presence and distribution of the transplanted donor cells, samples of seminiferous tubules were dispersed using fine forceps and the presence of sperms was detected with a scanning electron microscope (SEM; JEOL, JSM-6700F, Japan). In addition, the sperm suspension was obtained from the corresponding caudal epididymis and vas deferens, and then spermatogenesis was also observed in the presence of fluorescent sperm using a fluorescent microscope (Nikon X-Cite 120, Japan).

1.5 Southern blot analysis

To further confirm the genotypes of the progeny, we analyzed the DNA from weanling mice resulting from the mating of normal BALB/c-nu females with BALB/c-nu recipient males transplanted with FVB/NJ-GFP testis cells. Genomic DNA was extracted from tail samples from each offspring by phenol/chloroform extraction, followed by ethanol precipitation. Thirty micrograms of DNA genome were digested by restriction enzyme Pvu II, then separated on a 1.0% agarose gel. DNA was transferred to Hybond$^+$ nylon film, then DNA hybridization was performed according to the protocol of ECLTM Direct Nucleic Acid Labeling and Detection System (QIAGEN, Germany). The fragment of the GFP cDNA (about 700 bp) was used as a hybridization probe.

2 Results

2.1 Seminiferous tubules microinjection

In the process of microinjection, seminiferous tubules in mouse testis are found not highly convoluted (Figure 1(a)), and most of them are accessible through the surface of the testis. After microinjection, about 90%—95% seminiferous tubules were turned into blue (Figure 1(b)) because of the trypan blue solution. The extent of filling the seminiferous tubules injected with is shown in Figure 2.

2.2 Analysis of recipient mice after injected

Donor cells isolated from males FVB/NJ-GFP were microinjected into 60 BALB/c-nu infertility mouse models, and spermatogenesis was observed from the seminiferous tubules in 41/60 (68.33%) of the injected mice. The testes of BALB/c-nu infertility mice before microinjected did not undergo any matured stages of spermatogenesis (Figure 3(a)), but in the colonized testes, mature spermatozoa were seen in the lumen of the seminiferous tubules (Figure 3(b)). Moreover, the sperms shown by GFP fluorescence (Figure 4) were observed in the sperm suspension obtained from the BALB/c-nu recipient male transplanted with FVB/NJ-GFP germ cells. These results confirmed that the donor-derived spermatogenic cells could be survival in the testis of BALB/c-nu infertility mice models and maybe promote spermatogenic ability of the infertility models successfully.

2.3 Southern blot of progeny

We got the progeny (n=22) from BALB/c-nu recipient mice injected with FVB/NJ-GFP donor testis cells and

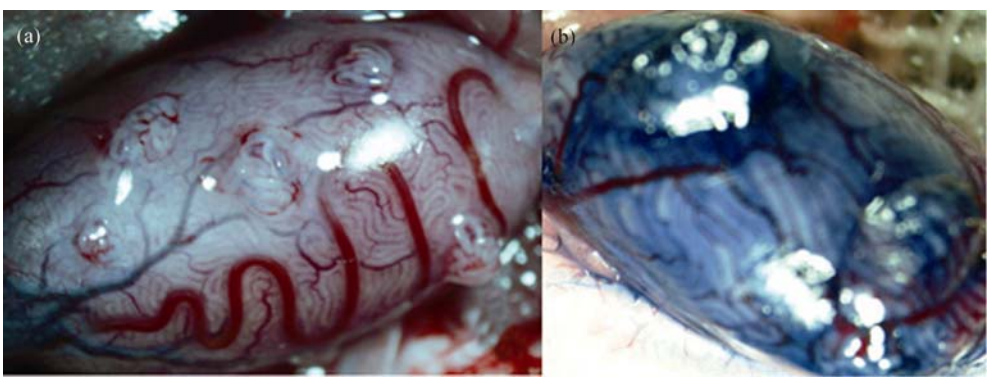

Figure 1 The contrast of uninjected and injected mouse testis. (a) The uninjected testis, seminiferous tubules in mouse testis are not highly convoluted as well; (b) the injected testis, about 90%—95% seminiferous tubules were turned into blue.

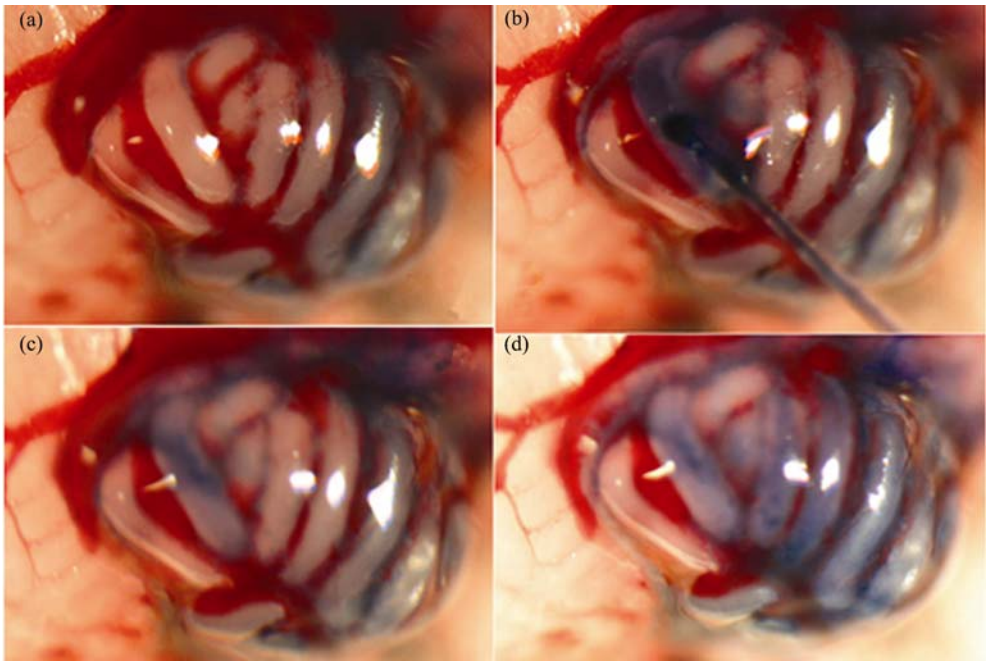

Figure 2 The example of microinjection pattern. (a) The uninjected seminiferous tubules; (b) the injected seminiferous tubules; (c) the injected seminiferous tubules after the injection pipette were pulled out immediately; (d) the injected seminiferous tubules after the injection pipette were pulled out for several seconds.

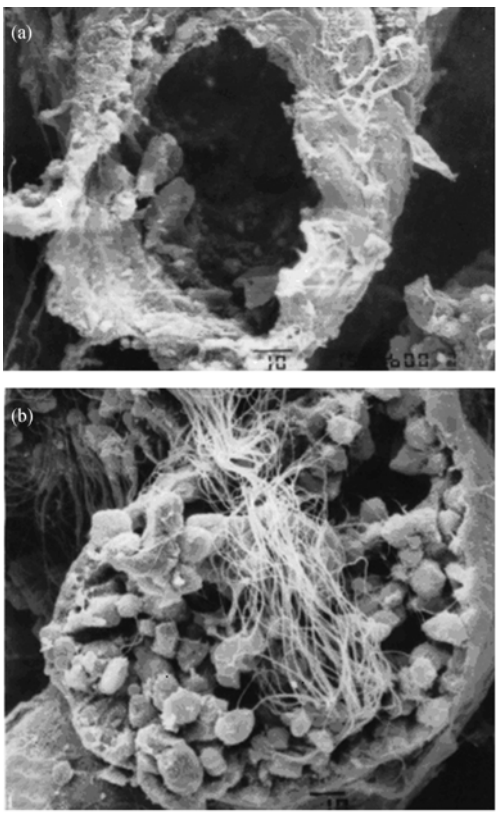

Figure 3 Electron micrograph of receipt mice testis before and after seminiferous tubule microinjection (SEM×1500). (a) The testis of infertility model mice before microinjection, few germ cells were seen in seminiferous tubule; (b) the testis of infertility model mice after microinjection, many germ cells were seen in seminiferous tubule.

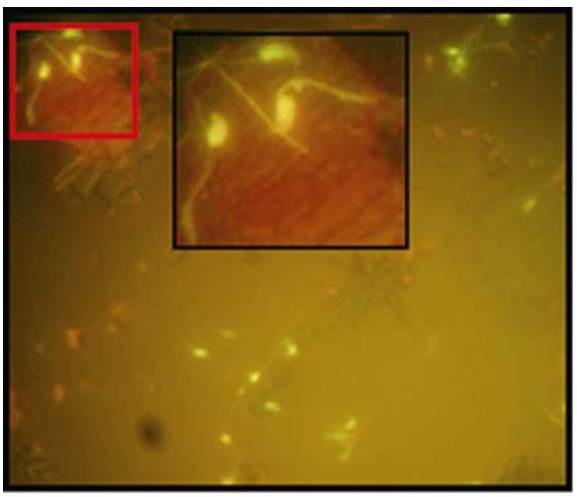

Figure 4 The detection of sperms with GFP fluorescence isolated from the BALB/c-nu recipient male transplanted with FVB/NJ-GFP germ cells under the fluorescent microscope. Enlargement of sperms (indicated by arrow) was shown. The sperms were intact with special falciform head and long tail.

examined the DNA from these progenies by Southern blot analysis. The result showed that DNA from five progeny generated a band of about 700 bp size (Figure 5), suggesting that the germ cells from FVB/NJ-GFP mice were developed and matured in testes of the BALB/c-nu infertility mice model, and the matured sperm could be functional in this infertility model.

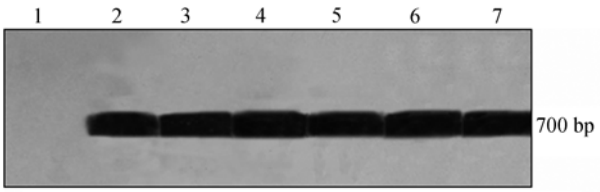

Figure 5 Southern blot analysis of the progeny from the mating of normal BALB/c-nu females with BALB/c-nu recipient males transplanted with FVB/NJ-GFP testis cells. The positive samples can come out the 700 bp band after the DNA hybridization. Lane 1, Negative control; lanes 2—6, positive sample; lane 7, positive control.

3 Discussion

In the previous studies, syngeneic strains of mice were always used as donor and recipient animals[5,13,27,28], and some immunologically compromised recipients also have been used for germ cell transplantation between different species[8,9,11,14,24,27,28]. Since BALB/c-nu mouse is one of the strains homozygous for the nude spontaneous mutation (*Foxn1*^{nu}, formerly *Hfh11*^{nu}), they are characterized by abnormal hair growth and defective development of the thymic epithelium. They are immunologically compromised mice and can be used for many immunology and inflammation researches such as research on immunodeficiency[29−34].

In this study, BALB/c-nu infertility mouse model was used to avoid immunological rejection of donor cells, and germ cell transplantation was applied to overcome infertility caused by busulfan treatment. Generally, assessment of the presence and proportion of donor-derived sperm production should require a permanent or a long-term, traceable labeling system for donor cells. Moreover, many labeling systems for donor cells were reported before, such as fluorescent markers and PKH26-labeled cells that were detectable up to 4 month after transplantation in the host, rat brain[35]. Here, we used green fluorescence and DNA analysis of donor cells from GFP transgenic mice as genetic marker. These results confirm that this technique of germ cell transplantation established in our lab might be of great use. The high level of fertility achieved here was unexpected, but the GFP positive progeny (22.72% (5/22)) was not achieved hopefully. A possible explanation is that here some tubules could be repopulated by surviving endogenous stem cells when there was busulfan leakage at the time of injection.

In recent years, the spermatogonial transplantation technique has made much headway, and will soon find uses in some cases of human infertility. The technical feasibility of homologous male germ cell transfer has been reported in primate testis[13] and in many other animals[5,9−11,24,27,28]. Studies on rat and hamster[8,9,12] demonstrated that xenogeneic spermatogenesis following germ cell transplantation will come true and may not be difficult because of the immunological differences. Since autologous transfer of spermatogonial stem cells could be of potential clinical use for curing patients undergoing radiation or chemotherapy that will destroy their endogenous spermatogenesis. In many other situations, xenogeneic transplantation of spermatogonia[9,12,24,27,28] could provide a new therapeutic approach. Whatever the procedure used or the reasons for germ cell transplantation, a healthy microenvironment in recipient testes is important for successful colonization. In the future, we should pay more attention to the improvement of donor cell-derived colonization of recipient testes, such as treatment of recipient mice and rat with the gonadotropin-releasing hormone agonist leuprolide[9,12,36]. Similar methods should be applied in other recipient species.

In conclusion, our studies indicate that the microinjected infertility mouse model could produce offspring from donor cell-derived spermatozoa. The ability to achieve an effective level of seminiferous tubule colonization and produce effective donor cell-derived progeny has facilitated a wide range of experimental approaches using spermatogonial transplantation. This would mean so much for males who have never been fertile or have been infertile for a long period. The differentiated or even matured cells generated from remaining spermatogonial stem cells could be useful for assisted reproduction. Furthermore, transplantation of stem cells from an infertile testis to a normal testis could give a preferable way to avoid competition between endogenous and exogenous spermatogenesis, and provide a necessary environment for stem cells to occur.

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