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# FACS selection of valuable mutant mouse round spermatids and strain rescue via round spermatid injection

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## Summary

Round spermatid injection (ROSI) into mammalian oocytes can result in the development of viable embryos and offspring. One current limitation to this technique is the identification of suitable round spermatids. In the current paper, round spermatids were selected from testicular cells with phase contrast microscopy (PCM) and fluorescence-activated cell sorting (FACS), and ROSI was performed in two strains of mice. The rates of fertilization, embryonic development and offspring achieved were the same in all strains. Significantly, round spermatids selected by PCM and FACS were effectively used to rescue the infertile Pten-null mouse. The current results indicate that FACS selection of round spermatids can not only provide high-purity and viable round spermatids for use in ROSI, but also has no harmful effects on the developmental capacity of subsequently fertilized embryos. It was concluded that round spermatids selected by FACS are useful for mouse strain rederivation and rescue of infertile males; ROSI should be considered as a powerful addition to the armamentarium of assisted reproduction techniques applicable in the mouse.

Keywords: Mouse, FACS, ROSI, Round spermatids, Strain rescue

## Introduction

Previous studies (Ogura *et al.*, 1994; Kimura & Yanagimachi, 1995a) have shown that mouse oocytes fused or injected with round spermatids were able to develop into normal offspring. This technique is referred to as round spermatid injection (ROSI). To date, viable offspring have been obtained after ROSI in several species such as mouse (Ogura *et al.*, 1994), rabbit (Sofikitis *et al.*, 1994), rat (Hirabayashi *et al.*, 2002) and humans (Tesarik *et al.*, 1995). This technique has been demonstrated to be a powerful tool for examining the developmental potential of spermatids in mutants that lack the ability to become fertilized (Romero *et al.*, 2011; Sharan *et al.*, 2004; Meng *et al.*, 2002). Furthermore, it can also be used to rescue mutant lines of mice that have become infertile with age. The current authors believe that under circumstances in which other assisted reproduction techniques (ART), such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), are unsuccessful, ROSI may be the last resort.

Despite the potential of ROSI as a useful ART, there are some limitations. For example, spermatogenic cell populations are frequently contaminated by somatic cells that are present in germ cell suspensions recovered after the dissection and enzymatic

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digestion of seminiferous tubules. To counteract this problem, various procedures have been developed for isolating purified populations of germ cells from the testis in humans and rodents, including: unit-gravity sedimentation (Wykes & Krawetz, 2003; La Salle *et al.*, 2009); centrifugal elutriation (Barchi *et al.*, 2009); separation using immuno-panning (Pelengaris & Moore, 1995); removal of contaminating somatic cells through adhesion to plastic dishes coated with lectin (Morena *et al.*, 1996); and fluorescence-activated cell sorting (FACS; Lassalle *et al.*, 1999; Chang *et al.*, 2011; Getun *et al.*, 2011). The use of FACS in particular is able to provide large numbers of high-purity and viable round spermatids, which are essential for the success of ROSI.

The purpose of the current study was to isolate relatively pure populations of viable round spermatids from mouse testicular cell populations by phase contrast microscopy (PCM) or FACS, and use the selected round spermatids for ROSI to 'rescue' (i.e. produce live-born offspring using spermatids from mutant male mice) two genetically altered mouse lines for which live-born offspring could not be derived using natural mating, IVF or ICSI. It was found that besides PCM, FACS technology could also be successfully utilized to derive live-born mice with high effectiveness and reliability, therefore ROSI should be considered a powerful addition to the armamentarium of ART applicable in the mouse, especially when the fertility of males is affected by age or genetic mutation.

## Materials and methods

### Reagents and media

Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from the Tianjin Animal Hormone Factory (Tianjin City, China) and the Second Hormone Factory of Ningbo City (Ningbo City, China), respectively. All other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated. The potassium simplex optimization medium with amino acids (KSOMaa) used for culturing oocytes and embryos before and after ROSI was purchased from Specialty Medium (Phillipsburg, NJ, USA). The medium used for ROSI was HEPES-buffered CZB medium (Kimura & Yanagimachi, 1995a) and 10% polyvinyl alcohol (PVA; 360 kDa) in HEPES-buffered CZB medium.

### Animal husbandry and mice

All experimental procedures reported here were conducted at the Department of Laboratory Animal Sciences, Shanghai Jiao Tong University School of Medicine (China). The research activity performed was approved and monitored by the Animal Care and Use Committees of Shanghai Jiao Tong University School of Medicine, and all mice were cared for following the guidelines of *Use of Animals of Department of Laboratory Animal Sciences*, Shanghai Jiao Tong University School of Medicine. All mice in this project were housed singly or in groups in ventilated cages within a dedicated barrier facility under specific pathogen-free (SPF) conditions.

The following strains of mice were used as spermatid donors: transgenic EGFP and Pten-null mutant ($Pten^{-/-}$) mice. The EGFP male mouse had been bred repeatedly, but was unable to produce offspring due to its extreme age, resulting in a poor physiological state. This sub-infertile mouse was used to collect round spermatids as a control. The $Pten^{-/-}$ male was infertile due to the dysfunction of spermiogenesis, despite showing signs of libido and the detection of vaginal plug in females after overnight mating. Seven- to 10-week-old female BDF1 mice were used as oocyte donors. Eight- to 12-week-old female ICR mice were made pseudopregnant for use as foster mothers by mating with verified-sterile vasectomized male mice the day before surgical embryo transfer.

### Preparation of round spermatids for PCM selection

$Pten^{-/-}$ and the control EGFP male mice were euthanized, and the caudae epididymides were removed using a pair of fine scissors. To collect spermatogenic cells for ROSI, the seminiferous tubules of the testes were minced as described previously (Kimura & Yanagimachi, 1995a), except that the cells were suspended in HEPES-buffered CZB medium.

### Isolation of haploid cells from the $Pten^{-/-}$ male mouse by FACS

The testes from the $Pten^{-/-}$ male mouse were cut into pieces after removing the tunica albuginea, and the testicular fragments were immersed in phosphate-buffered saline (PBS) and rocked to flush out Sertoli cells near the lumen. Cells were then collected and stained with Hoechst 33342 stain (5 μg/ml). After 90 min incubation, cells were resuspended in ice-cold PBS with 10% fetal bovine serum (FBS) and 2 μg/ml propidium iodide to identify living versus dead cells. All solutions from the incubation step onward contained verapamil (50 μM/ml), to decrease the speed of dye extrusion. Finally, FACS was carried out using the FACS Aria II with an ultraviolet (UV) laser (BD Biosciences, San Jose, CA, USA).

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### Oocyte preparation

Female mice were super-ovulated by intraperitoneal injection with 5–7 IU PMSG, followed by 5–7 IU of hCG 48 h later. At 13–14 h after hCG injection, oocytes were collected from the oviduct into HCZB medium, and cumulus cells were removed by treatment with bovine testis hyaluronidase (300 U/ml; Sigma Chemical Co.) for 3–5 min. Oocytes were then washed three times in KSOMaa and were kept at 37°C in a 5% $CO_2$ atmosphere until ROSI, which was performed within 3 h of oocyte collection.

### ROSI and oocyte activation

A 1-μl aliquot of the spermatogenic cell suspension obtained as described above was mixed with approximately 10 μl of HEPES-CZB medium that contained 12% (w/v) polyvinylpyrrolidone (PVP; 360 kDa) in a micromanipulation chamber. Round spermatid injection was carried as described previously by Kimura & Yanagimachi, (1995a), except that the current experiments were performed at room temperature. Briefly, a single spermatid was drawn repeatedly in and out of an injection pipette, until its plasma membrane was completely broken and the nucleus became almost completely separated from the cytoplasm. The nucleus was injected into an oocyte using an injection pipette (internal diameter 5–8 μm) with the aid of a piezo-driven micromanipulation system (Primetech, Ibaraki, Japan). The injected oocytes were left for 10 min on the stage of the microscope at room temperature (24–26°C), after which time they were transferred to KSOMaa medium at 37°C in 5% $CO_2$ in air. Finally, the injected oocytes were activated in $Ca^{2+}$-free CZB medium that contained 5 mM $SrCl_2$ for 20 min (Bos-Mikich *et al.*, 1995).

### Embryo transfer

Pseudopregnant ICR mice were used as recipients of ROSI embryos. Specifically, blastocysts were transferred to a day 2.5 pseudopregnant mouse that had been previously mated with a vasectomized male. Six to 10 embryos were transferred into each oviduct. At day 18.5 of gestation, the offspring were born naturally or delivered by Caesarean section.

### Statistics

The rates of surviving oocytes, embryonic development and pups born were analysed using a chi-squared test with SPSS software (SPSS Inc., Chicago, IL, USA). Significant differences were accepted at *P*-values $<0.05$.

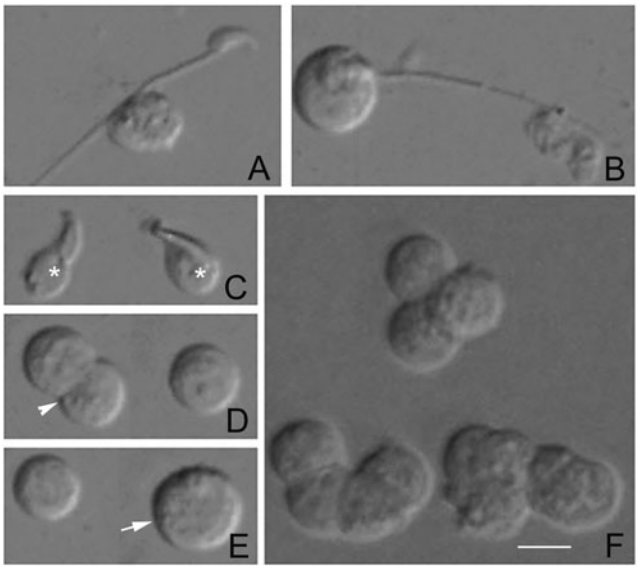


**Figure 1** Accurate selection of round spermatids using phase contrast microscopy (PCM) and fluorescence-activated cell sorting (FACS). Representative PCM images of mouse spermatogenic cells are shown, as selected by PCM only (*A–E*), or FACS (*F*). Mature spermatozoon (*A*); maturing spermatozoon (*B*); elongating spermatids (*C*); round spermatid and dividing round spermatids (*D*); primary pachytene spermatocyte (*E*); round spermatids selected by FACS (*F*). Scale bar = 10 μm. *: acrosome; arrowhead: dividing round spermatids; arrow: primary pachytene spermatocyte.

## Results

### Enrichment of spermatogenic cells by FACS

Spermatogenic cell suspensions were collected from male mice. These cell suspensions contained spermatozoa, elongating and round spermatids and primary pachytene spermatocytes (Fig. 1*A–E*).

In the current study, attempts were made to collect round spermatids from the adult $Pten^{-/-}$ mouse testis by the FACS technique. The results for mouse spermatogenic cells showed the distribution histograms of Hoechst 33342-stained testicular germ cells based on DNA flow cytometry (Fig. 2). Testicular cells were fractioned into five populations (P1–P5 in Fig. 2*A*,*C*) including predicted round spermatid population (P5). The FACS experiments revealed that about 15% of the cells were round spermatids in the total cell population of interest (Fig. 2*C*).

Processing by FACS was used to facilitate the collection of round spermatids. Approximately 99% of cells were identified morphologically as round spermatids in the P5 fraction (Fig. 1*F*), demonstrating that round spermatids can be enriched efficiently from testicular cells by FACS based on the side scatter (SSC) and forward scatter (FSC) phenotypes.

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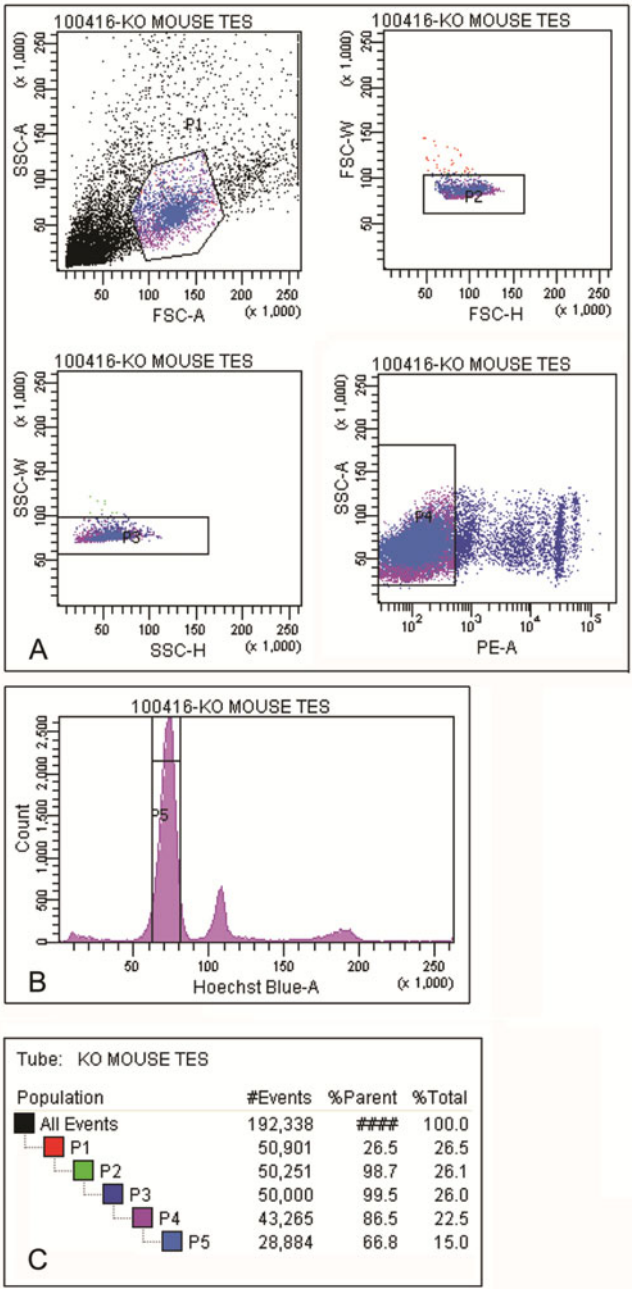


**Figure 2** Fluorescence-activated cell sorting (FACS)-based selection of round spermatids isolated from a Pten$^{-/-}$ mouse. Representative FACS data for the selection of haploid round spermatids. (*A*) P1, P2, P3 and P4 gates around the haploid cell population of interest in the forward scatter (FSC) versus side scatter (SSC) plots. P1 represents total haploid cell population of interest; P2 and P3 represent P1 population without adhesive cells; P4 represents P3 population without dead cells. (*B*) FACS histogram plots of pure round spermatids. (*C*) Distribution proportion of cell populations. P5 representing pure haploid cells was about 15% among all cells.

### ROSI and embryo transfer

The numbers of oocytes injected, survival rate (number of oocytes surviving injection), fertilization rate (number of embryos surviving injection that developed to zygote stages), embryonic developmental rates, total number of pregnant foster mothers and birth rate (number of pups born) are presented in Table 1. For all strains of mice studied, approximately 96.88–98.67% of injected oocytes survived the ROSI procedure. Of these cells, 98.18–100% of surviving embryos were artificially activated, 98.39–100% of which developed to 2-cell embryos, and 50.82–52.05% developed into blastocysts.

For conventional PCM-selected ROSI-derived embryos, two out of two recipients became pregnant using round spermatids from EGFP males, and one of two for Pten$^{-/-}$ mice. These pregnancies yielded 17 and 11 pups from EGFP and Pten$^{-/-}$ male mice respectively. The birth rate was 44.74% and 35.48% for EGFP and Pten$^{-/-}$ mice respectively. For FACS-selected ROSI-derived embryos from Pten$^{-/-}$ mouse, both recipient females became pregnant and gave birth to 12 pups (42.86% birth rate). There was no significant difference in birth rate between all strains studied, or the selection method used.

## Discussion

Advances in reproductive techniques have allowed infertile males to gain fertility by micro-injection of spermatozoa and pre-spermatozoal cells into oocytes (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993; Ogura *et al.*, 2001; Yanagimachi, 2001). The nuclei of round spermatids are able to participate in normal embryonic development, which therefore suggests that genomic and epigenetic elements needed for embryonic development are already established in round spermatids. The present study succeeded in rescuing two strains of sub-infertile or infertile mice with the help of conventional ROSI and validated the feasibility of this approach.

The correct identification of round spermatids represents a technical difficulty that limits the widespread use of ROSI. Several authors have succinctly described the morphology and size of live mouse round spermatids (Kimura & Yanagimachi, 1995a,b; Sasagawa & Yanagimachi, 1997; Sasagawa *et al.*, 1998). They are approximately 10 μm in diameter (range, 9-–11 μm) and round in shape with a smooth outline. The round nucleus contains a centrally located nucleolus. These characteristics mean that round spermatids can be easily distinguished from other cell types in testicular biopsies. The current results show that round spermatids can be identified using PCM, and

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**Table 1** Embryonic development and reproductive success of ROSI in two mouse strains using PCM- and FACS-based selection methods

| Strains | Selection methods | Oocytes *n* | Surviving oocytes (%) | Zygotes *n* (%) | 2-cell embryos *n* (%) | Blastocysts *n* (%) | Recipients (pregnant) *n* | Pups born *n* (%) |
|---|---|---|---|---|---|---|---|---|
| EGFP | PCM | 75 | 74 (98.67)[a] | 73 (98.65)[a] | 73 (100)[a] | 38 (52.05)[a] | 2 (2) | 17 (44.74)[a] |
| Pten KO | PCM | 64 | 62 (96.88)[a] | 62 (100)[a] | 61 (98.39)[a] | 31 (50.82)[a] | 2 (1) | 11 (35.48)[a] |
| Pten KO | FACS | 56 | 55 (98.21)[a] | 54 (98.18)[a] | 54 (100)[a] | 28 (51.85)[a] | 2 (2) | 12 (42.86)[a] |

[a]Values with different superscripts within columns are significantly different at $P < 0.05$ (chi-square test).
FACS: fluorescence-activated cell sorter; PCM: phase contrast microscope.

that their morphology and size are significantly different from other spermatogenic cells, such as spermatozoon, elongating spermatids, and primary pachytene spermatocytes.

In order to simplify and accurately select round spermatids, other researchers have successfully taken advantage of the polarization of round spermatid mitochondria and used MitoTracker to select live round spermatids (Sutovsky *et al.*, 1999; Hikichi *et al.*, 2005). This staining technique has no harmful effects on embryonic development, leading to the recommendation that this approach be used for research and infertility treatment. This approach does have its limitations however, it is technically difficult for researchers and technicians with limited experience, and may result in the selection of cells that are not round spermatids. Indeed, there is limited information available regarding the morphological features of live round spermatids to allow for their accurate selection for injection into oocytes. In order to overcome this limitation, Lassalle *et al*. (1999) suggested that viable round spermatids could be isolated from testis by FACS. The experience with this technique in the current paper shows that FACS sorted cells were almost all round spermatids, and that more than 99% of sorted round spermatids remained viable. Indeed, round spermatids were easily recognizable in the enriched spermatogenic cell populations analysed by FACS. Sorted cells were homogeneous in size (approximately 11 μm) and had the cytological characteristics of round spermatids.

In the present study, Hoechst 33342 was used to stain cell nuclei and it was clearly demonstrated that this refined method allows one to easily visualize, identify, and purify round spermatids. Parrilla *et al*. (2004) demonstrated that Hoechst 33342 staining does not induce genotoxic effects in flow-sorted boar spermatozoa and in our procedure Hoechst 33342 was at a relatively low concentration. The membranes of all living cells have selective permeability. Propidium iodide (PI) is membrane-impermeant and is generally excluded from viable cells, therefore PI could only adhere passively to the live cells and the side effects should be minimal. Verapamil is used in cell biology as an inhibitor of drug efflux pump proteins such as P-glycoprotein and it can decrease the speed of dye (Hoechst 33342) extrusion before/in sorting (Goodell, 2005). The biological half-life of verapamil is also short (2.8–7.4 h) and thus would not influence the following work. Moreover, whether Hoechst 33342, PI or verapamil was used, they all could be removed through medium washing after sorting.

In addition, it was found that all the cells collected by FACS in the current study were almost the same size and had centrally located nucleoli. It was also shown that round spermatids from $Pten^{-/-}$ male mice injected into mature metaphase II (MII) stage oocytes were able to support embryonic development, with a satisfactory survival, fertilization and developmental rate, as well as the birth of healthy offspring. These data clearly show that FACS-sorted round spermatids can be effectively used for ROSI to rescue some infertile mouse strains. However, FACS selection of round spermatids for ROSI inevitably has the following two drawbacks: it must be based on the existing FACS platforms, otherwise the cost will be very high; the cell number for FACS should be in a larger quantity ($>10^6$), therefore it is not suitable for human ROSI.

In conclusion, FACS selection of round spermatids can not only provide high-purity and viable round spermatids for ART use, but also has no harmful effects on the developmental capacity of subsequently fertilized embryos. Therefore, round spermatids selected by FACS can be used for rederivation or rescue of infertile male mice, thus facilitating development biology research and ART. Lastly, ROSI should be considered a powerful addition to the armamentarium of ART applicable in the mouse.

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