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## Abstract

Transplantation of male germ line cells into sterilized recipients has been used in mammals for conventional breeding as well as for transgenesis. We have previously adapted this approach for the domestic chicken and we present now an improvement of the germ cell transplantation technique by using an enriched subpopulation of c-Kit-positive spermatogonia as donor cells. Dispersed c-Kit positive testicular cells from 16 to 17 week-old pubertal donors were transplanted by injection directly into the testes of recipient males sterilized by repeated gamma irradiation. We describe the repopulation of the recipient's testes with c-Kit positive donor testicular cells, which resulted in the production of functional heterologous spermatozoa.

Using manual semen collection, the first sperm production in the recipient males was observed about nine weeks after the transplantation. The full reproduction cycle was accomplished by artificial insemination of hens and hatching of chickens.
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## 1. Introduction

Transplantation of male germ line cells from a donor animal to the testes of an infertile or sterilized recipient was first developed in rodents [1]. This technique has now been used in a number of animal species, including domestic mammals [2], fish [3], and chicken [4,5]. Donor germ cells repopulate the spermiogenic epithelium in recipient's testis and produce fertile donor-derived sperm, such that the recipient male can distribute the genetic material of the germ cell donor. Transplantation of

male germ cells is a uniquely valuable approach for the study of spermatogenesis, preservation of animal genetic resources and transgenesis [1].

Spermatogenesis is characterized by sequential steps of cell proliferation and differentiation resulting in the continuous production of spermatozoa [6]. The principal role in this system is played by the spermatogonial stem cells, which have the unique potential for both self-renewal and gradual differentiation of daughter cells towards spermatozoa [7–9]. In mammals, particularly in mouse and rat, there is relatively extensive knowledge of the molecular and biochemical characteristics of spermatogonial stem cells. The knowledge of proper markers in this cell population made possible their isolation and increased the efficiency of male germ line transplantation

[10]. In contrast, our understanding of spermatogenesis in chicken is much poorer and transcriptomic, proteomic as well as cytochemical characterizations of testicular cells are urgently needed for the progress of male germ line transplantation. Morphological and histochemical identification of stem spermatogonial populations in post-hatch individuals has been successfully performed only in fixed preparations of testicular tissues from quail and turkey [11–13] but remains to be established in fresh preparations. DNA content can be used as another marker for flow-cytometry sorting of various subpopulations of testicular cells in chicken [14].

In the absence of proper marker proteins specific for chicken spermatogonial stem cells, we can get some insight from the analogy with mouse. Here, the stem cell marker gene *Stra8* has been identified among genes expressed at an early stage of spermatogenesis [15]. Additional observations by the same authors indicated the genes *Dazl*, c-Kit, *Hsp70-2* and *Crem τ* as markers expressed prior to the first meiotic division. Particularly, *Dazl* and c-Kit genes are expressed only in the side population (SP) and tetraploid cells whereas transcription of *Hsp70-2* and *Crem τ* genes occurs at or after the spermatocyte stage [16]. Barocca et al [10] described that c-Kit-positive spermatogonial progenitors represent the majority of SP in adult mice and, although committed to differentiate, retain at least low potential to recover spermatogenesis after transplantation into germ cell-depleted recipient testes. On the other hand these authors showed the highest regenerative potential for SP fraction of undifferentiated spermatogonial cells expressing *α*-6 integrin and with none or low expression of c-Kit. In the present study, we assessed the suitability of c-Kit for the selection of donor testicular cells able to colonize sterilized recipient testes in our original and well established system of male germ line transplantation in chicken [4,17].

## 2. Materials and methods

### 2.1. Experimental animals and irradiation treatment

A total of seven pubertal, 16–17 week-old inbred Barred Leghorn (BL; genotype ii, Ee, B/b) fowl males were used as donors. The seven recipient males were 26 week-old (adult) White Leghorns (WL; genotype II). Two males having the same genetic origin as recipients were kept as positive controls while three other WL males of the same origin were subjected to the same treatment as the recipient males but kept as negative controls (described hereafter). All birds used in these experiments were obtained from the Experimental An-

imal Farm of the Institute of Molecular Genetics, Prague, Czech Republic. All experiments were performed in accordance with Czech legal requirements for animal handling and welfare.

All Barred Leghorn hens (BL; genotype ii, ee, B/-) and males were kept in individual pens (4200 cm$^2$) under standard husbandry conditions with photoperiod 12L:12D. Feed and water were provided *ad libitum*. Incubations were performed in a forced air incubator (BIOS MIDI, Czech Republic) adjusted for fowl egg incubation conditions.

Theratron T1000 radiation treatment unit (Theratronics International Ltd., Kanata, Ontario, Canada) was used to irradiate WL testes according to the previously described protocol [17]. Briefly, each male was subjected to five irradiations (8 Gy each) repeated at 3–4 day intervals.

### 2.2. Chicken germ line cell transplantation model

Phenotypic identification of one-day-old chicks issued from ova fertilized by heterologous sperm was made possible by using feather markers [4]. c-Kit-positive testicular cells from BL donors possessed barred phenotype due to homozygous recessive (ii) alleles at the I (white) locus while the WL sterilized males were homozygous dominant (II) at the I (white) locus. The dominant I allele of this locus originates the white phenotype of WL fowl males through the epistatic inhibition of the E allele encoding eumelanin. The specific e allele ('recessive black') in the used chicken lines confers a similar phenotype as the E allele. The dominant allele bar (B) is incompletely dominant in BL hens and is localized on the Z chromosome. It is phenotypically expressed as striping with broad stripes in cockerels (BB) or narrow stripes in cockerels (Bb) and narrow stripes in hens (B), or black color in hens (b-). Hence, the phenotype in the F1 progeny after AI of the BL hens with semen of the recipient WL males was used to evaluate the success of the transplantation of c-Kit-positive testicular cells. A black or barred phenotype in the F1 progeny indicated successful transplantation and restoration of normal spermatogenesis from donor (BL) germ cells, while the presence of white chicks in the F1 progeny indicated incomplete sterilization and persistence of WL germ cells.

### 2.3. Preparation and selection of c-Kit-positive donor germ cells

All donor males were sacrificed by decapitation and their testes quickly removed and washed in phosphate buffered saline (PBS). Testicular cells were isolated

from the cockerel testes by performing a two-step enzymatic digestion of testicular tissues in order to remove interstitial cells. Briefly, the *tunica albuginea* was removed and the seminiferous tubules dissociated by enzymatic digestion in 100 U/ml Type I collagenase (BIOCHROM AG, Germany) for 50 minutes at 34 °C in PBS, pH 7.2, supplemented with 1,2 mM MgSO4, 1,3 mM CaCl$_2$, 6,6 mM sodium pyruvate, and glutamine. The resulting suspension was filtered to remove cell clumps. After washing in PBS, the cell pellet was resuspended in PBS and the dispersed cells were washed twice in PBS for 5 minutes each wash. Mouse anti-chicken c-Kit conjugated with biotin diluted 1:40 was applied and after 30 min at 4 °C, all c-Kit-positive cells were selected using magnetic activated cell sorting (MACS) according to Dynal (Norway) instructions. The c-Kit-positive cells obtained in this way were subsequently used in germ line transplantation experiments.

### 2.4. Flow cytometry analysis and description of c-Kit positive testicular cells in cell mixture

Single cell suspensions of testes tissue were labeled with monoclonal antibody anti-c-Kit conjugated with phycoerythrin (Southern Biotech, Birmingham, AB). The identification of c-Kit-positive testicular cells was performed on the basis of their DNA content using testicular cell suspensions stained with 5 $\mu$M H33342 diluted in PBS for 90 min at 37 °C. The analyses of cell cycle in these cells were performed from cell suspensions stained for 30 min with 10 $\mu$M H33342 diluted in PBS at room temperature and 5$\mu$g/ml propidium iodide (PI) was used as a counter stain to exclude non-viable cells (red fluorescence of their nuclei). Flow cytometry analyses were performed with a FACSVantage SE flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with the two-stream argon–helium laser (Coherent Enterprise EE, Orsay, France). The various categories of cells stained by H33342 were excited by a UV laser adjusted to 50 mV using a combination of 485 nm long-pass and 505 nm short-pass filters in front of the first detector. Cell populations stained with PI were detected with a 682 nm/22 nm bandpass filter in front of the second detector. Fluorescence intensity was measured as a linear scale. Some cell types display shift of fluorescence into red light after staining with H33342. Dead cells were not visible because they were outside the frame of the diagram. Control of the elimination of

dead cells was made possible by using PI staining (excitation wavelength: 488 nm).

### 2.5. Transplantation of c-Kit positive cells

Approximately 300 $\mu$L of the dispersed c-Kit positive testicular cells with cell densities varying from $10^5$ to $10^6$ cells/mL were administered into each of the testis of the seven anaesthetized recipient WL males. Three additional WL males subjected to the same sterilization treatment were kept as negative controls. Recipient males were anesthetized with an intramuscular injection of a mixed preparation of 15 mg/kg ketamine (Narkamon, Spofa, Czech Republic) and 4 mg/kg xylazine (Rometar, Spofa, Czech Republic). c-Kit-positive cells were then applied through the *tunica albuginea* at four to five different locations randomly distributed among each testis.

### 2.6. Assessment of the sperm output

Ejaculates from transplanted and control males were collected once a week for 13 weeks using the conventional abdominal massage technique [18]. To determine the number of sperm per ejaculate, sperm concentration was estimated with a prescaled centrifuge [19] and the volume with a scale (nearest mg). Weekly semen output (WSO, [20]) per male was estimated by addition of the number of ejaculated spermatozoa per week.

### 2.7. Artificial insemination (AI) with semen from transplanted males

Outbred BL hens were AI with semen collected from WL recipient males (2) in which semen production resumed after transplantation. Six hens (three per cockerel) were inseminated twice a week with individual ejaculates from transplanted males producing spermatozoa and their eggs (32 eggs per hen) in 4 batches were incubated under standard conditions. Intra-vaginal inseminations were performed with $2 \times 10^5$ spermatozoa per hen diluted in 100 $\mu$L BPSE [21] in duration of seven weeks. The percentage of hatched eggs was determined from the number of eggs incubated divided by the total number of eggs incubated.

### 2.8. Statistical analysis

Test of equality of the linear regression line between two males that ejaculated heterologous populations of sperm was done by Chow test and compared by F distribution. (Statistica 7.1, StatSoft).
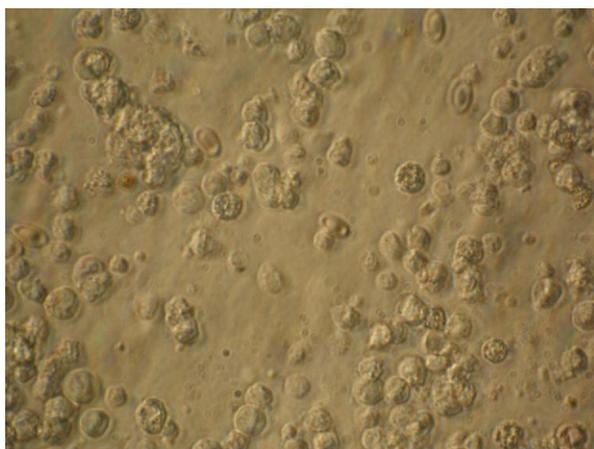
Fig. 1. Clumps of c-Kit-positive cells selected from testicular cells of a 16–17 week-old cockerel after magnetic cell sorting (original magnification × 400).

## 3. Results

### 3.1. Sterilization of recipient testes and their re-population with labeled donor dispersed testicular cells

Within one month after the sterilization of recipient males no semen was expressed by manual semen collection. At that point the recipient males were ready for transplantation of donor c-Kit positive testicular cells (Fig. 1).

### 3.2. DNA content of c-Kit positive cells

The flow cytometric analyses showed that the used isolation procedure resulted in 2.16% c-Kit positive cells in the isolated testicular cells from 16 to17 week-old cockerels (Fig. 2A). In separate analyses, based on Hoechst 33342 staining of nuclei, the percentages of the c-Kit positive donor testicular cells with haploid, diploid, and tetraploid DNA content was 7.8%, 51.8% and 28.3%, respectively (Fig. 2B).

### 3.3. Restoration of spermatogenesis in recipient testes

Five weeks after transplantation, higher numbers of spermatozoa were observed in the ejaculates of two WL males out of the seven WL recipients. Sperm output from recipient males started on week 5 after transplantation and then gradually increased up to week 13, reaching an average of $350 \times 10^6$ sperm/wk (see Fig. 3A, B), i.e. 15 times less compared to average cockerel semen week output. Males A and B differed in the number of ejaculated spermatozoa produced after transplantation. The three age-matched sterilized but non-transplanted WL males (negative control) failed to produce semen after attempts at manual semen collection.

### 3.4. Hatched chicks

Two out of seven recipients transplanted with dispersed testicular cells from pubertal donors successfully produced progeny expressing donor cell phenotypes (Table 1). Six hens were inseminated with semen collected from these two recipients in course of 7 weeks and egg lay resulted in totally 195 eggs hatched in four batches.
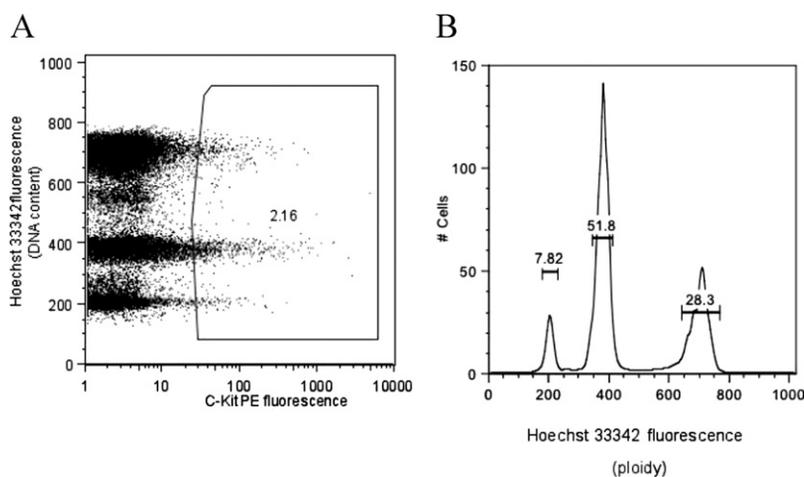


Fig. 2. Flow cytometric analysis of c-Kit positive pubertal cockerel testicular cells stained with Hoechst 33342 and anti-c-Kit conjugated with phycoerythrin (c-Kit PE) (A) and percentages of the c-Kit positive dispersed donor testicular cells with haploid, diploid, and tetraploid DNA content (B).
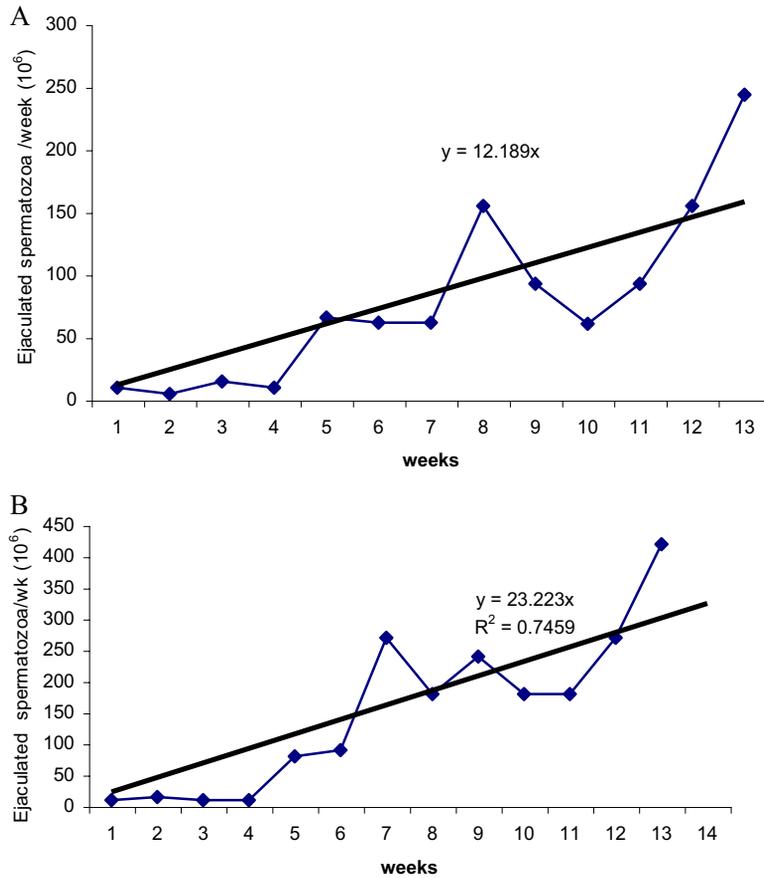
Fig. 3. Time course of the weekly sperm output in two (A and B) WL recipient chicken males transplanted with dispersed c-Kit positive testicular cells from BL donors. Zero represents first day after transplantation.

Of the 110 hatched eggs from hens inseminated with semen from these two males, 26 chicks (24%) expressed the following phenotypes: barred cockerels (ii, Ee, B/b or Ee, B/B or ee, B/B or ee, B/b genotype), black hens (ii, Ee, b- or ee, b-), or barred hens (Ee, B- or ee, B-). The remaining chicks (76%) from these two males were white chickens (Ii) apparently suggesting that donor cells apparently did not fully outnumber the background of White genotype stem cells, which still remained intact after incomplete sterilization (Table 2).

Table 1
Frequency of sperm recovery and percentage of males with achieved fertility in fowl males primarily transplanted with dispersed testicular cells from pubertal or adult donors.

| Age of BL donors (weeks) | No. (%) of WL recipients producing progeny carrying donor genes/total number of transplanted cockerels |
|---|---|
| 16–17 | 2/7 (29)[x] |

## 4. Discussion

In the present study, we have shown that dispersed c-Kit positive testicular cells from 16 to 17 week-old cockerels have the capacity of successful re-colonization of the seminiferous epithelium, resumption of spermatogenesis, and the production of viable and functional spermatozoa after transplantation into sterilized recipient testes. When inseminated into hens, sperm from two of the seven recipients produced chicks expressing the donor's haplotype, thus reproducing previous work in the mouse [22,23] and chicken [4]. It was apparent that c-Kit positive testicular cells from 17 week old cockerels were suitable as donor cells for transfer to recipient males. What was not so apparent was that the percentage of c-Kit positive cells from the younger males (2.16%) was nearly twice as high as that recovered from the adult males (1.27%) in our previous results. We were able to identify c-Kit-positive testicular cells in all main parts of the cell cycle, i.e. we

Table 2
Hatchability of eggs after the insemination with semen of WL recipients producing progeny carrying donor genes.

| Age of BL donors (weeks) | No. of incubated eggs | No. of fertile eggs (%) | No. of hatched eggs from fertile eggs (%) | No. of barred hatched chickens (%) | No. of white hatched chickens (%) |
|---|---|---|---|---|---|
| 16–17 | 195 | 146 (74.8) | 110 (75.3) | 26 (24) | 84 (76) |

described the highest amount (51.8%) in diploid cell subpopulation, 28.3% in tetraploid cell subpopulation and 7.8% in haploid cell subpopulation. In comparison with testicular cells without the selection for c-Kit [14], we found a slight increase in the fraction of diploid cells, whereas the fraction of haploid cells was reduced to 7.8%. Presence of haploid cells can be due to random admixture of spermatids into the subpopulation of c-kit-selected cells.

There is the possibility of nonspecific binding of anti c-Kit antibody on the surface of some haploid cells. However, these cells were not included in the process of renewal spermatogenesis.

F1 progeny resulting from the AI of BL hens with semen collected from transplanted WL males revealed that only 24% of the chicks possessed barred or black phenotypes and the remaining 76% of the hatch were white chick (Ii). This means that sterilization of the recipient males had apparently been incomplete although the control, non-transplantated animals did not restore spermiogenesis and the transplanted animals were negative as well at the time immediately before transplantation when examined by the control of ejaculates. Not all transplanted recipients restored spermatogenesis suggesting that the transfer process was either inefficient or the seeded donor cells failed to colonize the recipients seminiferous epithelium. The fact that five complete sterilized recipient cockerels failed to resume spermiogenesis, while two apparently incompletely sterilized cockerels resume spermiogenesis, may, if not coincidence, suggests that milder irradiation may have been more suitable for colonization of transplanted spermatogonia and that completely sterilized testes were also damaged during the process of sterilization. For the efficient repopulation of spermiogenic epithelium, however, a specific subpopulation of c-Kit-positive cells must be present in the mix of transplanted cells and this selection for c-Kit positivity could then be used for enrichment of the mixture.

We also experienced technical problems with the precise orientation of the gamma radiation on the abdominal testes during the sterilization process. This erratic irradiation probably allowed some spermatogonia in the recipient's seminiferous epithelium to survive to produce sperm concurrently with the transferred c-Kit positive cells. This supports our previous observations [17] where we confirmed that repeated exposure of mature fowl testes to gamma irradiation resulted only in a partial elimination of germ cell populations and that seminiferous tubules exposed to such radiations kept in a pre-functional state. We also revealed for the first time in chicken that c-Kit positive testicular cells transferred into recipient testes maintain their potential to restore spermatogenesis in heterologous testes, which is similar to that previously performed in the mouse [10].

The recovery of functional heterologous spermatozoa was observed in two out of seven recipients produced semen and is a consequence of transferring donor c-Kit positive testicular cells rather than the unselected dispersed donor testes cells used previously [17]. Given that the spermatogonia stem cell is most likely a c-Kit-positive cell, we assume that a larger percentage of the donor cells transferred into the recipient testes were spermatogonia, thus also increasing chances for a successful seed of the sterilized seminiferous epithelium.

Unfortunately, there are no reliable morphological criteria or readily identifiable surface markers to differentiate the spermatogonial stem cell from the other spermatogonial subtype. Consequently, the precise number of stem spermatogonia transferred into each male recipient is not known. However, we suspect that a significant percentage of the transferred cells did not seed seminiferous tubules but were lost to the interstitial spaces. Ogawa et al [23,24] noted that the number of colonies re-colonizing seminiferous tubules was indicative of the number of stem spermatogonia transferred to the recipient in mice.

In conclusion, about 24% (26 chicks) of the fertilized eggs from hens inseminated with heterologous semen were fertilized with sperm derived from the donor c-Kit-positive cell population. When optimized, this approach could be an alternative to PGC manipulation and gene transfer in avian species. Furthermore, this technique can also be used as a mean to restore endangered or lost lines of birds, thus opening new approaches to preservation of genetic resources in avian species.

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