Asian Journal of Andrology (2018) 20, 414–416
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LETTER TO THE EDITOR

Reproduction

Production of germline chimeric quails following spermatogonial cell transplantation in busulfan-treated testis

Young Min Kim[1], Jin Se Park[1], Jong Won Yoon[1], Hee Jung Choi[1], Kyung Je Park[1], Tamao Ono[2,3], Jae Yong Han[1,3]

Asian Journal of Andrology (2018) **20**, 414–416; doi: 10.4103/aja.aja_79_17; published online: 6 February 2018

Dear Editor,

The merits of using quail as an avian experimental model include high egg production, low maintenance cost, small body size, and short generation period (approximately 6–8 weeks). Indeed, these characteristics make quail an ideal species for many biological areas including transgenic research.[1] Methods of primordial germ cell (PGC)-mediated germline chimera production are considered very reliable and have been used in avian transgenesis. The production of germline chimeras for transgenic research mediated by PGCs is the most prominent system used to develop avian models, especially in the chicken.[2] Several studies have also reported successful production of germline chimeric quails by transferring gonad-derived PGCs, and the method allows the production of diverse transgenic quails.[3,4] However, compared with those for chickens, the methods for long-term culture and *in vitro* manipulation of quail PGCs are still limited for their full use.

The transplantation of male germ cells including spermatogonia and spermatogonial stem cells (SSCs) is an efficient method to study spermatogenesis and the control of male fertility. Recently, we reported successful cultivation of quail SSCs for certain periods by optimizing the culture conditions.[5] Because the primary cells are hard for the delivery of genes, we attempted short-term cultivation of SSCs to reduce the stiffness of the cells and make it easier to introduce exogenous genes. These results to allow male germ cell transplantation could be another method to produce quail germline chimeras and a suitable route to quail transgenesis. In this regard, we produced here germline chimeric quail by transplantation of testicular cells (TCs) and cultured SSCs into recipient testis as an alternative way for producing germline chimeric quails.

To prepare TCs, we obtained about 2×10^7 testicular cells from a piece (approximately 1 cm^3) of one wild-type plumage (WP) strain quail (d^+/d^+). Subsequently, TCs were seeded on Datura stramonium agglutinin (DSA)-coated plates with a sequential enzymatic digestion method used in previous studies (**Figure 1a**).[5] One day after seeding, the adherent cells were mechanically removed and transferred to a culture containing mitotically inactivated mouse embryonic fibroblasts (MEFs) as the feeder cells. The SSCs were stably maintained and grew for 20 days (until the fourth passage) in the presence of MEFs (**Figure 1b**). The expression of representative pluripotent markers, namely POU domain class 5 (*POUV*) and Nanog homeobox (*NANOG*), was detected both in TCs and cultured SSCs, but not in Sertoli cells (STCs) or quail embryonic fibroblasts (QEFs). Germ cell markers deleted in azoospermia such as *DAZL* and DEAD-box helicase 4 (*DDX4* or *VASA*) were also strongly expressed in TCs and SSCs, but not in STCs or QEFs (**Figure 1c**).

Busulfan, an antispermatogonial alkylating agent, significantly reduces the chicken testicular weight and number of spermatozoa.[6] In quails, a single dose of busulfan (40 mg kg^{-1}, 30 mg kg^{-1} or 20 mg kg^{-1} per intraperitoneal injection) significantly reduces the production rate of fertile eggs.[7] From the previous data,[7,8] we used the optimal concentration of busulfan (40 mg kg^{-1}) in the present study to deplete the endogenous germ cells of the quail testis without causing lethality of busulfan-treated quails. Dominant black (D) strain male quails received a single intraperitoneal injection of busulfan. After 2 weeks of busulfan treatment, the VASA (a germ cell specific marker)-positive cells of the testes were greatly reduced in number in the recipients compared with the nontreated controls (**Figure 1d** and **1e**). The testicular weight of busulfan-treated recipients (0.4 ± 0.1 g) was also significantly lower than that of the wild-type control (3.0 ± 0.4 g) ($P < 0.001$, **Figure 1f**).

To produce germline chimeric quails using spermatogonial cells, 3×10^6 non-cultured WP quail TCs and 14-day cultured SSCs labeled with PKH26 red fluorescence dye (Sigma-Aldrich, St. Louis, MO, USA) were transplanted into four *D* strain quail testes (two quails each for TC transplantation and SSC transplantation) 2 weeks after busulfan treatment (**Table 1**). To confirm the localization of spermatogonial cells in the transplanted testes, 20-μm-thick cryosections from one *D* strain recipient testis after 24 h of cell transplantation were examined in a fluorescence microscope (SMZ1000; Nikon Corporation, Tokyo, Japan). As a result, the transplanted TCs were identified in the inner spaces of the seminiferous tubules of the recipient testes, confirming the localization of the implanted cells (**Figure 1g**). According to a previous study in the quail, the fertility is increased to about 60% after about 45 days of 40 mg kg^{-1} busulfan treatment.[7] Therefore, subsequent testcross analyses were performed after 1 month from TC/SSC transplantation. The results showed that germline transmission had occurred in two of three recipients. Regarding the phenotypic characteristics, the hybrids (D/d^+) had dark brown feathers, whereas the donor (d^+/d^+)-derived progenies

[1]Department of Agricultural Biotechnology, Research Institute of Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea; [2]Division of Animal Science, Faculty of Agriculture, Shinshu University, Minamiminowa, Nagano 399-4598, Japan; [3]Institute for Biomedical Sciences, Shinshu University, Minamiminowa, Nagano 399-4598, Japan.
Correspondence: Dr. JY Han (jaehan@snu.ac.kr)
Received: 08 September 2017; Accepted: 14 December 2017

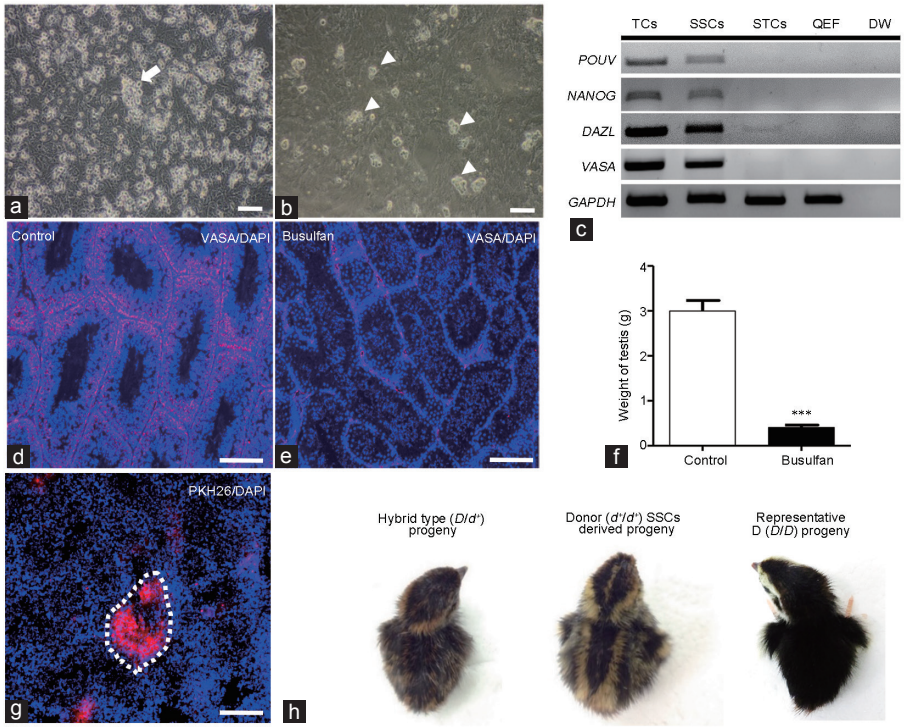

Figure 1: Production of germline chimeric quail and donor-derived offspring by TC/SSC transplantation. (**a**) Morphology of TCs one day after seeding. White arrow indicated primary TCs. (**b**) Morphology of colonized quail SSCs (20 days after culture, passage 4) on MEF feeder layers. White arrowhead indicated clustered SSCs. Scale bars = 200 μm in **a** and **b**. (**c**) RT-PCR analysis of *POUV*, *NANOG*, *DAZL*, and *VASA* in quail SSCs cultured for 20 days. Quail TCs were used as the positive control, and STCs and QEFs were used as the negative controls. Immunohistochemistry of VASA in adult testes (**d**) of control and (**e**) after busulfan treatment. Scale bars = 100 μm in **d** and **e**. (**f**) The weight of the testes in 2 weeks after busulfan treatment with control testes ($^{***}P < 0.001$, $n = 3$). (**g**) Localization of TCs (d^+/d^+) in the seminiferous tubule (white dotted line) after injection into busulfan-treated testis (*D/D*). Scale bar = 100 μm. (**h**) Donor-derived progenies (d^+/d^+) from strain D quail recipients containing donor cells (*D/D* and d^+/d^+) mated with WP quail (d^+/d^+). Hybrid progeny (*D/d*$^+$) and strain D progeny (*D/D*) are presented as controls. *DAZL*: deleted in azoospermia like; D quail: dominant black quail; DW: distilled water; *NANOG*: Nanog homeobox; MEF: mitotically inactivated mouse embryonic fibroblast feeder cells; *POUV*: POU domain class 5; QEF: quail embryonic fibroblast cells; RT-PCR: reverse transcription-polymerase chain reaction; SSCs: spermatogonial stem cells; STCs: Sertoli cells; TCs: testicular cells; WP quail: wild-type plumage quail; *VASA*: DEAD-box helicase 4 (*DDX4* or *VASA*).

Table 1: Birth of germline chimeras produced by transfer of whole testicular cells or quail spermatogonial stem cells

Recipient ID	*Type of donor cells*	*Transplanted cells (n)*	*Incubated eggs (n)*	*Hatched quails (n)*	*Donor-derived quail (n)*	*Donor-derived quail (produced/total, %)**
TC #1	Whole testicular	3×10^6	100	61	7	11.5
SSC #1	14-day cultured SSCs #1	3×10^6	82	64	0	0
SSC #2	14-day cultured SSCs #2	3×10^6	54	42	7	16.7

*Percentage of hatchlings shown to be of the donor-derived WP (d^+/d^+) genotype. TC: testicular cell; SSCs: spermatogonial stem cells; WP: wild-type plumage

had yellow and black stripes (**Figure 1h**). As shown in **Table 1**, 61 progenies were hatched from one recipient transplanted with TCs, and seven of them were identified as donor-derived progenies (11.5% germline transmission efficiency). In the two recipients of SSCs, 64 progenies were produced from recipient quail SSC #1, but no donor-derived progenies were identified. On the other hand, in recipient quail SSC #2, 42 progenies were produced, and seven of them were identified as donor-derived progenies (16.7% germline transmission efficiency) (**Table 1**). These germline transmission efficiencies are slightly lower than the chicken germline chimera achieved by testicular cell transplantation into the gamma-ray-irradiated testis[9] but higher than the chicken germline chimera achieved by spermatogonial cell transplantation into the chicken testis without sterilization.[10] Our results showed that the one SSC transplant could not produce donor-derived progenies. Since busulfan-induced infertility is highest after approximately 20 days and the effect can last up to 50 days in quails,[7] we presumed that residual busulfan may inhibit donor cell's (SSCs #1) localization or spermatogenesis in the recipient testis.

In conclusion, our study demonstrates that quail germline chimeras can be produced by simple transplantation of spermatogonial cells with busulfan-mediated endogenous germ cell reduction. We are the first to use this strategy to produce donor-derived progeny using adult quail germ cells. Compared with the PGC-mediated method, this strategy is simple and leads to rapid generation of quail germline chimeras. This will lead to production of transgenic models from adult germ cells and, through the production of germline chimeras, help in efforts to conserve avian specie.

AUTHOR CONTRIBUTIONS

JYH participated in the study design and coordination. YMK participated in the design of the study, carried out the experiments and statistical analysis, and wrote the first draft of the manuscript. JSP, JWY, HJC, and KJP were involved in data interpretation. TO participated in

writing the final version of the manuscript. All authors have read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) Grant 2015R1A3A2033826 (Ministry of Science, Information and Communication Technology, and Future Planning; MSIP) and the Cooperative Research Program for Agriculture Science and Technology Development (Project PJ012866012017) from the Korean Rural Development Administration. This research was also supported by the International Research & Development Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning of Korea (NRF-2016K1A3A1A21005676).

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